Monophasic *Salmonella* Typhimurium in Australian pigs

by

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Summary

Salmonella enterica enterica 1,4,[5],12:i:- colonization in Australian pig herds was investigated. The research considered: the distribution of *S*. 1,4,[5],12:i:- in the Australian pig industry; dynamics of colonization in herds; diversity in the Australian porcine population; comparison of study strains with related domestic serovars and strains reported internationally; antimicrobial resistance characteristics and determinants; and implications for optimal typing and surveillance.

In total 773 faecal samples were collected from Australian pig herds in cross-sectional (16 herds) and longitudinal (five herds) observational epidemiological studies. Samples were cultured and where *Salmonella* was confirmed multiple colonies were collected, 2326 isolates in total. Representative isolates were characterized by serotyping, phage typing, antimicrobial susceptibility testing and multiple-locus variable-number tandem-repeat analysis (MLVA). In addition, the genomes of a sample of the study collection isolates were sequenced.

The results indicated that *S*. 1,4,[5],12:i:- has spread rapidly through the Australian pig industry. Persistent *S*. 1,4,[5],12:i:- shedding and considerable escalation among weaners was observed in the sampled herds. High levels of shedding were also observed among finisher pigs, indicating a possible pathway into the human food chain.

Low *S*. 1,4,[5],12:i:- phenotypic and MLVA profile diversity was observed, suggesting the Australian porcine *S*. 1,4,[5],12:i:- population is closely related. Comparative genomic studies demonstrated that the *S*. 1,4,[5],12:i:- had undergone clonal expansion, consistent with the population having emerged from a single event. The characteristics of the study *S*. 1,4,[5],12:i:- strains closely resembled those of the European clone strains, supporting the hypothesis that *S*. 1,4,[5],12:i:- was recently introduced to Australia from overseas. In spite of the close relatedness of the study strains, phylogenetic analyses readily differentiated *S*. 1,4,[5],12:i:- strains on the basis of source.

This study found very little resistance to critical antimicrobials for the treatment of human salmonellosis. *Salmonella* resistance types varied considerably between herds and were serovar associated within herds. The majority of *S*. 1,4,[5],12:i:- were multidrug resistant, whereas the majority of non-*S*. 1,4,[5],12:i:- serovars were pansusceptible. The variation in resistance types between contemporary serovars within herds indicated that antimicrobial use on farm was not driving selection for *Salmonella* resistance types. However, selection pressure for resistance types appeared to vary between herds. In some herds resistance diminished over time due and gene loss was identified. In other herds, there were indications of horizontal resistance gene acquisition among some of the more resistant strains. The most common resistance genes identified among the study *S*. 1,4,[5],12:i:- isolates also matched reports from overseas.

Phage typing proved to be of limited value in differentiating Australian porcine *S.* 1,4,[5],12:i:- strains but MLVA proved promising for surveillance and broader epidemiological purposes. However, these studies further illustrated the value of comparative genomics for surveillance, source attribution and broader epidemiological purposes.

This research has generated original insights into the epidemiology of *S.* 1,4,[5],12:i:- in pig herds. The findings have implications for pig industry and public health risk mitigation and risk management.

Declaration

I declare that:

(i) This thesis comprises only original work towards the degree of Doctor of Philosophy

(ii) Due acknowledgement has been made in the text to all other material used and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged; and

(iii) The thesis is fewer than the maximum word limit in length, exclusive of tables, maps, bibliographies and appendices, as approved by the Research Higher Degrees Committee.

Thomas R.D. Weaver

Preface

Due to logistical challenges, approximately 10% of the samples included in the cross-sectional study results were collected on behalf of the author by the consulting veterinarian.

Salmonella detection, confirmation and isolation were conducted by technicians at the South Australian Research and Development Institute (SARDI). The author processed several batches of samples to better understand the process.

All *Salmonella* characterization—serotyping, phage typing, antimicrobial susceptibility testing, multiple-locus variable-number tandem-repeat analysis and genome sequencing—was conducted by technicians at the Microbiological Diagnostic Unit Public Health Laboratory (MDU PHL), University of Melbourne. The author followed several batches of isolates through characterization to better understand the process. The author also characterized 20 duplicate isolates using multiple-locus variable-number tandem-repeat analysis at SARDI, the results of which were discarded due to the differences in the SARDI and MDU PHL platforms.

Part of the work has been published in the following paper, in which the co-authors provided inputs into the study design, aforementioned laboratory work and in reviewing the paper:

Weaver, T., Valcanis, M., Mercoulia, K., Sait, M., Tuke, J., Kiermeier, A., Hogg, G., Pointon, A., Hamilton, D., Billman-Jacobe, H., 2017. Longitudinal study of *Salmonella* 1,4,[5],12:i:- shedding in five Australian pig herds. Prev. Vet. Med. 136, 19-28.

Parts of this work were presented in the Australasian Pig Science Association Conference (APSA) Manipulating Pig Production XIV and presented at the APSA Conference in 2013, with the following citation, as above, the co-authors contributed to the study design, aforementioned laboratory work and in reviewing the paper:

Weaver, T., Hogg, G., Dimovski, K., Valcanis, M., Kiermeier, A., Billman-Jacobe, H. & Hamilton, D. (2013) 'The Potential Value of MLVA to Porcine Salmonella Surveillance in Australia', Manipulating Pig Production XIV, Australasian Pig Science Association (Inc.), Australia

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Of course, this study would never have been completed without the insight, time and openness of the veterinary practitioners and Australian pig producers who have supported this research. I owe

them all a great deal and can only hope this research contributes in some small way to the ongoing success of the industry.

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Finally, I wish to thank my extraordinary wife, Jade, and our soon to be born son, without whose love, support and patience this work would never have been completed. Taking on this research meant considerable changes to our lives and at various occasions I have no doubt I have been a less than ideal husband. I cannot thank you enough.

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Abbreviations

A	ampicillin
ALOP	Appropriate Level of Protection
APIQ	Australian Pork Industry Quality Assurance Programme
APL	Australian Pork Limited
AUD	Australian dollar
a_W	water activity
C	chloramphenicol
CDC	Centers for Disease Control and Prevention
Cf	ceftiofur
cfu	colony forming units
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
Cp	ciprofloxacin
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DFAT	Australian Government Department of Foreign Affairs and Trade
DNA	deoxyribonucleic acid
DT	definitive phage type
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
ESAM	*Escherichia coli* and *Salmonella* Monitoring Programme
EU	European Union
EUR	euros (currency)
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
FSO	Food safety objective
FSIS	Food Safety and Inspection service

G	gentamicin
GHP	Good Hygiene Practices
HACCP	Hazard Analysis and Critical Control Points
IMVS	Institute of Medical and Veterinary Science
ISO	International Standards Organization
K	kanamycin
MAT	moving annual total
MDU PHL	Microbiological Diagnostic Unit Public Health Laboratory
MLST	multi-locus sequence typing
MLVA	multiple-locus variable number tandem repeat analysis
MRCA	most recent common ancestor
MST	minimum spanning tree
NEPSS	Australian National Enteric Pathogens Surveillance Scheme
PCR	polymerase chain reaction
PCV2	porcine circovirus type 2
PFGE	pulsed field gel electrophoresis
PT	phage type
P_T	Estimated true prevalence
QAP	quadratic assignment procedure
RNA	ribonucleic acid
S	streptomycin
SARDI	South Australian Research and Development Institute
SLV	single locus variant
SNP	single nucleotide polymorphism
Sp	spectinomycin
SPS	sanitary and phytosanitary
ST	multiple locus sequence type
STTR	*Salmonella* Typhimurium tandem repeat locus
Su	sulphathiazole

SW	slaughterweight
T	tetracycline
Tm	trimethoprim
USDA	United States Department of Agriculture
VLA	Veterinary Laboratories Agency (now Animal and Plant Health Agency)
VNTR	variable number tandem repeat
WHO	World Health Organization
WTO	World Trade Organization
µl	microliter

Chapter 1 - Risk profile of Australian porcine *Salmonella* 1,4,[5],12:i:-: a review of the literature and available data

Preface

This chapter is presented in the form of risk profile of *Salmonella* spp. with special focus on *S. enterica* (I) serovar 1,4,[5],12:i:-. Initially a brief introduction to risk profiling is presented. The hazard is then identified and characterized in terms of bacteriology, pathogenesis, and epidemiology—on-farm and on product, and an overview of outbreaks and current trends in human salmonellosis globally and within Australia is presented. An assessment of exposure is then presented, describing and discussing the current industry controls and best estimates of prevalence in stock, on carcass and on/in products. A preliminary risk characterization in then proffered. The chapter concludes with identification of the major knowledge gaps identified in the risk profile, which inform the questions addressed by the research conducted as described and discussed in the remainder of this thesis.

1.1. Introduction

1.1.1. Risk-based approaches

Salmonella spp. are among the most important and well described foodborne hazards; *Salmonella* have been known as a cause of human illness for over 125 years (CDC, 2016c). European *Salmonella* monitoring and control programmes have long recognized a direct relationship between hazard status on-farm and product contamination, establishing that effective risk mitigation strategies in primary production can reduce food safety risk for consumers (Berends et al., 1997; Mousing et al., 1997a; Dahl and Sørensen, 2001; Alban and Stärk, 2005; Alban et al., 2012; De Busser et al., 2013; Andres and Davies, 2015; Snary et al., 2016). The purpose of this study was to assess herd and human health risk associated with *Salmonella enterica* subsp. *enterica* ser. 1,4,[5],12:i:- in Australian pig herds to inform risk management strategies and practice.

The Australian pig industry commenced the establishment of an industry-wide strategy for managing *Salmonella* risks in 1997, chiefly inspired by the efforts of Danish counterparts (Mousing et al., 1997a). This occurred against the backdrop of the 1995 World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), which established risk-based approaches to the facilitation of trade in food and agricultural products (FAO/WHO, 1995, 2012).

Food safety risk management has evolved from end-product control to whole chain control approaches—from early heat treatment methods, through the establishment of Codex Alimentarius, to increasing legislative enforcement of Hazard Analysis and Critical Control Points (HACCP) and quantitative risk assessment methods. Food Safety Objectives (FSO) towards Appropriate Level of Protection (ALOP) now typically establish standards for microbial levels and processes in food chains while the International Standards Organization provides the current international standards for food safety management systems (ISO, 2005; Zweitering, 2013; Doménech and Martorell, 2016).

In managing foodborne risks clarity of purpose has proven essential. In this vein, the FAO/WHO (2013) defined a hazard as '[a] biological, chemical or physical agent in food, or condition of food, with the potential to cause an adverse health effect.' For these purposes risk is defined as a combination of the likelihood of an adverse health effect on pigs and/or humans and the severity of the effect as a consequence of the hazard (Manning and Soon, 2013).

Risk analysis refers to the development of an understanding of risk in a given context (Manning and Soon, 2013). Risk analysis informs risk evaluation and, thereby, decision-making processes in relation to specified risks (ISO, 2009). Manning and Soon (2013) identify three components of risk analysis, based on Codex Alimentarius: risk assessment (science of understanding hazards, likelihood and consequences), risk management (policymaking in response to risk, controls) and risk communication (information exchange between stakeholders). The ISO (2009) identifies four components within a risk assessment: hazard identification, hazard characterisation, appraisal of exposure and risk characterisation. For the purposes of this research an additional section 'risk management information' is included in the risk profile, identifying current and potential on-farm approaches to hazard control. Detailed definition of international standards in risk management is provided in ISO (2009); Leitch (2010) and Knight (2010) provide critical discussion of these standards. This study applies a similar risk-based approach to that advocated by the FAO/WHO (2012). FAO/WHO (2012) defines a risk profile as:

> '… a description of a food safety problem and its context that presents in a concise form, the current state of knowledge related to a food safety issue, describes potential MRM [microbiological risk

management] options that have been identified to date, when any, and the food safety policy context that will influence further possible actions.'
(FAO/WHO, 2012)

Food safety risk is commonly assessed using a combination of qualitative and quantitative methods. Risk quantification typically entails either a structured sampling frame and chemical or biological laboratory detection methods to establish presence/absence of the hazard. Risk quantification may also include enumeration of hazard(s) and/or statistical methods, primarily Baysian, that are employed to model risk and deliver quantitative outputs (Manning and Soon, 2013). Additionally, risk assessment may include value-based judgements, which ideally account for less measurable elements of risk, such as risk perception among identified stakeholders and/or other socio-economic, cultural and political considerations. Quantitative microbiological assessments are well described by Hoornstra and Notermans (2001) and EFSA (2010d). Comparative evaluation and risk ranking and examples of statistical methods applied to *Salmonella* spp. are presented by Hald et al. (2004), Hald et al. (2007) and David et al. (2013), and more generally Vose (2008). Stärk et al. (2006) presents an excellent review of risk-based surveillance approaches.

This chapter commences with definition of the hazard, followed by characterization of the hazard, assessment of hazard occurance and detailing of risk management strategies that have shown demonstrable potential. This approach has been adapted by On et al. (2010), Pointon et al. (2006) and Pointon and Horchner (2010), and further adapted to this purpose. The risk profile is based on secondary data and literature review with the addition of quantative risk assessement elements where feasible.

1.2. Hazard identification

Hazard:
Non-typhoidal *Salmonella enterica* (I) *enterica*, with special focus on the serovar *S. enterica* (I) ser. 1,4,[5],12:i:-

Food vehicles:
Pork and pig-derived products

1.2.1. Scope

This study profiles the food safety risks posed by porcine *Salmonella enterica* subspecies *enterica* 1,4,[5],12:i:- (*S.* 1,4,[5],12:i:-), also known as monophasic *Salmonella* Typhimurium, based on a review of the literature. Where data specific to *S.* 1,4,[5],12:i:- was not available comparable data from *S.*

Typhimurium is used. Where no specific information relating to Group B serovars was available, information relating to generic *Salmonella* is presented.

1.3. Hazard characterisation

1.3.1. *Salmonella enterica enterica*

Salmonellae are a family of Gram negative, non-spore forming, rod-shaped bacteria of the family *Enterobacteriaceae*. *Salmonellae* are usually motile by way of peritrichous flagella (Hocking, 2003). Over 2600 *Salmonella* serovars have been identified, the majority of which are serovars of *Salmonella enterica enterica* (Grimont and Weill, 2007). Numerous *Salmonella enterica enterica* serovars are known to have caused human disease.

1.3.2. *Salmonella enterica enterica* 1,4,[5],12:i:-

Salmonella 1,4,[5],12:i:-, a Typhimurium-like strain, is an emergent non-host specific, multi-drug resistant, non-typhoidal *Salmonella* serovar of global public health importance that appears to exhibit similar pathogenicity and virulence to *S*. Typhimurium in humans (Echeita et al., 1999; Jones et al., 2008; EFSA, 2010b; Lucarelli et al., 2010). The somatic and first phase flagellar antigenic structure of *S*. 4,[5],12:i:- is that of *S*. Typhimurium, but the phase-2 H antigen is not expressed. Since the serovar's general recognition in the mid-1990s it has risen to international prominence due to increasing isolation and implication in human disease (Hopkins et al., 2010; CDC, 2013b; Davies, 2013). The serovar is now reported among the top ten *Salmonella* serovars isolated from humans in the US and several EU countries, and is frequently reported in Asian and Latin American countries (Tavechio et al., 2004; Switt et al., 2009; Tavechio et al., 2009; EFSA, 2010b; Hauser et al., 2010; Ido et al., 2011; Ido et al., 2014). Multiple high profile human outbreaks of *S*. 1,4,[5],12:i:- have occurred in recent years (Mossong et al., 2007; CDC, 2008, 2011a; Gossner et al., 2012; Guillier et al., 2013; Nguyen, 2013; CDC, 2015b). There is strong evidence of close relatedness between *S*. 4,[5],12:i:- and *S*. Typhimurium (Echeita et al., 2001; Laorden et al., 2010; Trüpschuch et al., 2010a; García et al., 2013) from initial studies employing DNA microarray-based typing (Garaizar et al., 2002) and later sequence-based studies (Soyer et al., 2009b; Hauser et al., 2010; García et al., 2013; Petrovska et al., 2016).

Salmonella 1,4,[5],12:i:- does not appear to have a single reservoir species (Switt et al., 2009; Gosling et al., 2011; García et al., 2013), however, it is commonly isolated from pigs and pork products and research and source attribution investigations have linked the pig industry to human *S*. 1,4,[5],12:i:- cases on numerous occasions (Barone et al., 2007; Dionisi et al., 2009; Bone et al., 2010; Davies et al., 2011; García et al., 2011; Hopkins et al., 2012; García et al., 2014; Gomes-Neves et al., 2014).

The serovar can be pathogenic to pigs as demonstrated by high rates of detection among passive laboratory submissions in response to clinical disease overseas (Mueller-Doblies et al., 2013).

Although monophasic *S*. Typhimurium-like strains have been reported for some time, for example in Portugal (1986-87) and Thailand (1993), the first characterisations of the *S*. 1,4,[5],12:i:- clades currently prominent internationally were from pigs in Spain in 1997 (Switt et al., 2009; EFSA, 2010b). These organisms were found to be missing the *fljB* structural gene, which confers expression of the phase-2 flagellar antigen in *S*. Typhimurium (Hauser et al., 2010). In *S*. Typhimurium the *fljAB* operon is involved in expression of both the first and second phase flagellar antigens. The operon encodes a negative regulator of the *fliC* gene, relevant to expression of the first-phase antigen, and the *fljB* gene relevant to phase-2 antigen expression (EFSA, 2010b). Various deletions and mutations can affect the *fljAB* operon, and, hence, the expression of flagellar antigens (García et al., 2013). The *fljAB* operon includes three consecutive genes *fljA*, *fljB*_1, x (x may be various antigenic markers) and a DNA invertase *hin*. The structural gene *fljB* accounts for expression of the H2 antigen, which is regulated by *fljA* and *hin,* without this pathway only one protein is expressed at any one time, therefore, no phase variation of the flagellar antigen occurs (EFSA, 2010b; Hauser et al., 2010; Bugarel et al., 2013).

Although the absense of *fljB* can explain the monophasic phenotype (García et al., 2013), Hauser et al. (2010) found that nine isolates of the 148 monophasic *Salmonella* strains they tested were PCR positive for all three genes that consitute the *fljAB* operon, and additional PCRs showed seven positives for the complete *fliB* gene. The Hauser et al. (2010) results, show that mutations in or around the gene may explain the monophasic phenotype, also postulated by Bugarel et al. (2013), among others (Boland et al., 2015). This is supported by other reports of anomolous *fljB*-positive 'monophasic' strains in a small percentage of *S*. 1,4,[5]:i- isolates, whereby the gene is present but appears to not express, for example Lailler et al. (2013) found 0.03% of 654 serovar *S*. 1,4,[5]:i:- they tested were *fljB*-positive. Studies have shown that the transposition of the insertion element IS*26* may be involved in the deletion of the *fljAB* operon and surrounding genes, thereby likely playing a role in the monophasic phenotype *S*. 4,[5],12:i:- (García et al., 2013; Boland et al., 2015).

García et al. (2013) note that *S*. 1, 4,[5],12:i:- is not a unique clonal group, which suggests that a number of separate and unrelated emergence events may have occurred. This assertion is supported by the results of García et al. (2013) which show a number of genetic differences between the two clonal lines of *S*. 4,[5],12:i:- in Europe: the Spanish clone (first reported in 1997, predominantly phage type (PT) U302) and the European clone (common since 2000, typically described by PT193 and PT120). García et al. (2013) suggest this genetic variation could be used as markers in epidemiological surveillance and investigations. Additional clones also appear to have emerged, in particular the US clone that is typically less resistant than the clones identified in Europe (Soyer et al., 2009a). Both the Spanish and US clones are missing the *fljA* and *fljB* genes; while the *hin* gene is

detected in US clone only (Bugarel et al., 2013).

The Spanish clone typically has plasmid-mediated antimicrobial resistances while the European clone is associated with resistance genes located in a chromosomal region (García et al., 2011; García et al., 2013; García et al., 2016; Petrovska et al., 2016). The European clone *S*. 1,4,[5],12:i:- typically exhibits resistance to the antibiotic group: ampicillin (A), streptomycin (S), sulphathiozole (Su) and tetracyclines (T), encoded by the bla_{TEM}, *strA-strB*, *sul2* and *tet*(B), respectively (Bugarel et al., 2013). These organisms may also be resistant to spectinomycin (Sp) (García et al., 2013). In addition, the European clone typically carries the recently described *Salmonella* genomic island 2 (Lucarelli et al., 2010). Whereas Spanish clone strains describe ASSuT resistance and, in addition, are commonly resistant to chloramphenicol (C), gentamicin (G) and trimethoprim (Tm), typically encoded by *flo*R or *cmlA, aac(3)-IV*, and *dfrA12*. Additional Spanish clone resistance genes reported include *aadA2, aadA1, sul1, sul3, tet*(A)(EFSA, 2010b; Hopkins et al., 2010; García et al., 2013).

Davies (2013) noted the timing of the emergence of *S*. 1,4,[5],12:i:- coincided with regulated reductions in use of antibiotics as growth promoters in animal production and the increased use of zinc oxide and other metal oxides in feeds, particularly in weaned pig diets, to reduce control enteric problems and improve pig performances. Davies (2013) sites the two genomic islands coding for the typical ASSuT resistance phenotype and resistance to heavy metals in the PT193 strains; suggesting this change in diet may have selected for the monophasic strains. This assertion is supported by research, such as Campos et al. (2016) who found the presence of the metal tolerance genes *pcoD*, *silA*, *merA* and *terF* among Portuguese *S*. 1,4,[5],12:i:- isolates of porcine origin, and Petrovska et al. (2016) who identified clusters of genes associated with heavy metal tolerance on a novel genomic isoland among epidemic *S*. 1,4,[5],12:i:- strains in their study.

Davies (2013) also speculated that the lack of expression of the phase 2 flagellar antigen may allow the strain to partially evade host cytokine responses (Crayford et al., 2011). The apparent increasing proportion of the strains not expressing the O:5 antigen (also known as variant Copenhagen, particularly in the US literature) may also be associated with slowing the host immune response (Davies, 2013).

1.3.3. Growth, survival and inactivation

Salmonella multiplication can occur in colonized stock and on carcasses and products (Wray and Wray, 2000; Torrence and Isaacson, 2003; Bell and Kyriakides, 2008; On et al., 2010). *Salmonella* serovars vary somewhat in their specific tolerances to stressors, and the nature of the material in which they reside can have a considerable impact on their inactivation (Burns et al., 2016). Temperature ranges at which *Salmonella* growth may occur vary between strains, however, *Salmonella* multiplication typically ceases outside the range seven to 46-47°C, with optimum growth rates at

around 35 to 37°C (Hocking, 2003; Juneja et al., 2007; Juneja et al., 2009; On et al., 2010). *Salmonella* growth will occur between pH 4.0 and 9.5, but is optimal at pH 6.5 to 7.5 (Hocking, 2003), *S. enterica* are more sensitive to low pH than *E. coli* and *Shigella* spp. . Minimum water activity (a_w) for growth for most *Salmonellae* is approximately 0.94, with optimum a_W of approximately 0.99 . *Salmonellae* are highly resilient to most storage conditions, though the bacteria may not multiply. *Salmonella* can survive for 10-12 weeks in water and long periods in organic matter, viability after many months reported in faeces, soil and on pasture (Hocking, 2003). Survival time on clean surfaces is considerably shorter (EFSA, 2010d).

Salmonella inactivation can occur through physical processes such as heat treatments, hot water, irradiation, ultrasonic energy, pulsed electric fields, oscilating magnetic field pulses, high pressure, high intensity visible light, ultraviolet light and microwaves (Wray and Wray, 2000; Torrence and Isaacson, 2003; Bell and Kyriakides, 2008; Hamilton et al., 2010; On et al., 2010). Freezing is not an effective method of inactivating *Salmonella*, particularly when the organism is resident in organic matter and/or in low a_W products, though they may not multiply. Thermal inactivation varies dependent on the organisms' thermal history, previous heating to sublethal temperatures increases heat tolerance and the composition of the food in which the *Salmonellae* is resident affects survival rates; higher fat content produces a protective effect, lower water activity conditions increase heat resistance, and lower pH reduces heat tolerance (Juneja and Eblen, 2000; Bell and Kyriakides, 2008; On et al., 2010; Gurman et al., 2015; Gurman et al., 2016). Chemical inactivation methods include pH treatments such as organic acids, chlorine, organic preservatives, oxidising agents, various transdermal compounds, nontoxic antimicrobial peptides (e.g. nisin-based formulations) and acidified sodium chlorite (Sanova®) (Bell and Kyriakides, 2008; Hamilton et al., 2010).

1.3.4. Detection and quantification

The current Australian standard for detection of *Salmonella* is AS 5013.10-2009 (AS5013.10-2009, 2014), which officially replaced ISO 6579:2002 (ISO, 2002). However, ISO 6579 is also endorsed as an appropriate culture method for *Salmonella* in Australia. This method employs pre-enrichment in buffered peptone water and culture on modified semisolid Rappaport-Vassiliadis and confirmation on xylose lysine deoxycholate plates. However, various methods, both enriched and non-enriched, may be applied to culture *Salmonella*. Various agar-based plating media may be used, such as: MacConkey (slightly selective); *Salmonella-Shigella*, desoxycholate citrate, hexctoen enteric, xylose lysene desoxycholate (moderately selective); and bismuth sulphite, brilliant green, brilliant green sulphonamide (highly selective and differential) (Hocking, 2003).

There are numerous rapid *Salmonella* detection methods which include: fluorescent antibody stains, enzyme immunoassay, enrichment serology, immuno-sensors, fluorogenic staining, bacteriophage methods, hydrophobic grid membrane filtration, electrical measurements of metabolic byproducts,

shortened liquid enrichment, geneprobes, and polymerase chain reaction (PCR)-based methods (Hocking, 2003).

Serological methods, indicating exposure, are primarily specialized *Salmonella* enzyme-linked immunosorbent assay (ELISA) kits produced by a number of commercial diagnostic test manufacturers such as Qiagen® and Prionics®. Generally, these tests are not serovar specific although they may be biased towards detection of specific to *S. enterica* (I) groups—antibodies typically bind to specific somatic antigens, so that the test plate may be coated with specific inactivated *Salmonella* antigens, as in the Danish mix-ELISA that favours detection of *S.* Typhimurium (Nielsen et al., 1995; Hamilton et al., 2005).

Salmonella counts are primarily conducted using most probable number dilution estimation methods. Modified semisolid Rappaport-Vassiliadis agar plates may also be used for estimations. Nowadays, PCR-based quantification methods are also common (Malorny et al., 2008b; Pires et al., 2013b).

1.3.5. *Salmonella* typing

Two *Salmonella* reference laboratories currently operate in Australia: the Institute of Medical and Veterinary Science (IMVS), SA Pathology, Adelaide, South Australia, and the Microbiological Diagnostics Unit, University of Melbourne, Melbourne, Victoria. Serotyping is the main method of differentiating *Salmonellae* and a range of phenotypic and molecular subtyping methods can be used to further differentiate strains below the level of serovar. The main typing methods are briefly described and discussed below.

Phenotyping

Serotyping

Numerous changes to *Salmonella* serovar and subtype naming have occurred since White's system, published in 1929 and later adapted by Kauffmann, was accepted by the International Association of Microbiologists in 1934 (Hocking, 2003). The accepted modern seroformulae is presented by Grimont and Weill (2007), coding somatic (O) and flagellar (H) antigen expression using the White-Kauffmann-le Minot scheme (Wray and Wray, 2000; Hocking, 2003; Grimont and Weill, 2007; Bell and Kyriakides, 2008). The standard ISO/TR 6579-3 'guidance document' describes the White-Kauffmann-Le Minor scheme and known serovars (Grimont and Weill, 2007).

Serotyping employs slide agglutination with a panel of specific sera to identify the antigens present, informing serovar identification using the Kauffmann-White-Le Minor nomenclature (Van Belkum et al., 2007). A panel of sera are used to determine the basis of the lipopolysaccharide, or somatic, (O) antigens, and flagellar protein (H) antigens. The K antigens, occurring as capsules around the

cells, are not significant for *Salmonella* (Hocking, 2003; EFSA, 2010b). At present 46 somatic antigens and 114 flagellar antigens are currently used for *Salmonella* serotyping (Ranieri et al., 2013). The somatic antigen expression are coded for by the flippase *wzc* and polymerase *wzy* genes (Ranieri et al., 2013). The H antigens may be apparent in two phases, H1 and H2 antigens, encoded by *fliC* (phase-1 flagellin) and *fljB* (phase-2 flagellin). *Salmonella* may be biphasic motile and non-motile (or specific and non-specific), monophasic, or have no H antigens. Reversible H antigens can also occur. Monophasic *Salmonella* do not express one of either the phase-1 or the phase-2 flagellar antigens (Hopkins et al., 2010). Strains that do not express phase-1 or both H antigens are uncommon (EFSA, 2010b).

Serotyping of *Salmonella* has a long history and continues to be widely used as the first approach to differentiating *Salmonellae*. Herikstad et al. (2002) argue that serotyping should continue to be promoted in national *Salmonella* surveillance schemes to aid targeting of prevention efforts and to ensure global comparability. Serotyping provides a useful starting point in discriminating *Salmonella* spp, however, its utility is limited by its low disciminatory power relative to other typing methods and that only a relatively small number of serovars are of importance to animal and public health. Moreover, seroptyping cannot be used for phylogenetic analyses (Barco et al., 2013; Ranieri et al., 2013). Although serotyping methods are well established and are undemanding from a technical perspective, the method is time consuming and expensive taking at least three days and requiring maintainenance of over 250 typing sera and 350 different antigens. Furthermore, accurate reading of plates requires experience and can lead to variability between laboratories and individual technicians.

Phage typing

Bacteriophage typing, or phage typing, is the traditional method of further discriminating within *Salmonella* serovars (Best et al., 2007). The technique employs a standardized set of serovar specific bacteriophages, which have been developed for a number of important serovars including *S*. Typhimurium, *S*. Enteritidis and *S*. Bovismorbificans (Olsen et al., 1993). The resultant pattern of reactivity—lysing—is then compared with known phage types (Anderson et al., 1977).

Although relatively inexpensive phage typing requires maintenance of the appropriate phages and considerable technical expertise in interpretation, which can make reproducibility and comparison between laboratories challenging (Ross and Heuzenroeder, 2005; Boxrud et al., 2007; Barco et al., 2013). Baggesen et al. (2010) provide an illuminating example of the influence of different interpretation of lysis patterns in different laboratories and the subsequent assignment of different phage types to identical strains. This study demonstrates the need to further standardize the technique, even after years of employment, if it is to remain relevant. Phage typing is of limited value for investigation of more common phage types, such as *S*. Typhimurium definitive phage type

(DT) 104 and phage type (PT) 193, where the lack of discriminatory power can make it impossible to differentiate epidemiologically linked or independent strains (Lindstedt et al., 2004). Furthermore, phage conversion, which may occur through temperate phage expression, gene mutations and gain or loss of plasmids, can occur both within outbreaks and in laboratories at uncertain rates (Olsen et al., 1993; Cho et al., 2008; Hopkins et al., 2012; Barco et al., 2013). Numerous studies have demonstrated the role of plasmids in the loss of sensitivity to specific phages, thereby causing phage conversion, within *S*. Typhimurium and *S*. Enteritidis lineages (Anderson et al., 1973; Platt et al., 1987; Rankin and Platt, 1995; Brown et al., 1999).

In time, comparative genomic approaches, in particular single nucleotide polymorphism (SNP) typing, will likely replace phage typing of *S*. Typhimurium (Pang et al., 2012). However, Baggesen et al. (2010) argue that in spite of the upsurge in new subtyping techniques, primarily molecular and sequence-based, phage typing will likely remain useful in the short-term due to the techniques relative rapidity and cost effectiveness, and, perhaps less convincingly, the techniques discriminatory power and reproducibility.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing may be used as a means of differentiating *Salmonella*, however, there are a number of important drawbacks to using antibiogram results as a typing technique for epidemiological purposes, certainly without application of other typing methods.

Salmonella colonies may be tested for antimicrobial resistance phenotypes using a number of standardized antimicrobial panels, breakpoints and testing methods—most commonly disk diffusion and dilution methods. Like other phenotype-based methods of differentiation comparability is hampered by issues of harmonization between laboratories. For antimicrobial resistance phenotypes variability is particularly acute between public and animal health laboratories, where the compounds employed in panels and breakpoints may vary (Silley et al., 2011). Furthermore, recording of resistance phenotypes may vary considerably depending on the specific mechanism and the length of time in, and method of, storage. For example, some resistance phenotypes may be conferred by multiple resistance genes potentially located in different parts of the bacterial genome with variable levels of stability and, due to fitness costs of carrying resistance genes without selection pressure, gene loss can occur in storage (Le Minor, 1988; Barco et al., 2013). Some resistance types and genes are highly stable, typically resistance genes located on the chromosome, however, others, such as genes located on genomic islands, integrons or plasmids, may be more variable and have the potential to be transferred horizontally between strains (Miriagou et al., 2006; Barco et al., 2013).

Other phenotyping methods

Other less commonly used phenotyping methods include examination of morphology, biochemical identification methods, bacteriocin typing and biotyping (Olsen et al., 1992; Wray and Wray, 2000; Hocking, 2003). These techniques suffer from one of a number of limitations relating to costs, technical burden, reliability, comparability, discriminatory power and usefulness for epidemiological purposes. Hence, these techniques are not commonly employed by current *Salmonella* spp. surveillance systems.

Molecular typing/genotyping

Molecular typing methods have advanced significantly over the past 20 or so years. A plethora of gel electrophoresis-based, fluorescence, DNA hybridization and sequence-based methods are now used to differentiate *Salmonella* strains for epidemiological purposes (Liebana, 2002; Best et al., 2007; Van Belkum et al., 2007; Levin, 2009; Li et al., 2009b; Levin, 2010).

Three main types of molecular typing methods have been employed for *Salmonella* typing: deoxyribonucleic acid (DNA) banding pattern-based methods, DNA sequencing-based methods, and array-based methods (Li et al., 2009b; Barco et al., 2013). DNA banding pattern methods employ amplification of DNA and/or DNA cleavage using restriction enzymes and sizing of fragments to differentiate strains (Wray and Wray, 2000; Hocking, 2003; Harbottle et al., 2006; Van Belkum et al., 2007; Kirchner et al., 2011; Achtman et al., 2012; Fabre et al., 2012b; Zou et al., 2012; Arguello et al., 2013b). DNA sequencing methods assess polymorphisms in the nucleotide sequences at specific targets (Kotetishvili et al., 2002; Achtman et al., 2012). Array-based methods employ arrays of DNA probes to differentiate strains (Li et al., 2009b).

DNA banding pattern-based methods

Pulsed field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a highly discriminatory DNA fingerprinting technique that can be used on a wide array of bacteria. PFGE was adapted to *Salmonella* in the 1990s and has been the most widely used molecular subtyping method for *Salmonella* internationally. PFGE is considered the 'gold standard' for subtyping *Salmonella* (Call et al., 2008; Wattiau et al., 2011a; Bopp et al., 2016). The PFGE techique involves restriction pattern analysis of DNA digested with restriction enzymes. In conducting PFGE a small number of high molecular-weight restriction fragments are generated in an agarose DNA suspension using restriction enzymes, which cleave at infrequent but specific restriction sites. The agarose-DNA 'plugs' are then loaded into an agarose gel and undego electrophoresis using a pulsed electric field, varying in direction and duration in accordance with an established programme, to determine the sizes of the fragments (Olsen et al., 1994; Gautom, 1997; Birren and Lai, 2012). The resulting electrophoretic patterns may then be compared to records in databases such as the CDC's PulseNet for purposes of epidemiological

investigation (CDC, 2016b).

Salmonella PFGE profile strains have high correlation with epidemiological relatedness and PFGE produces a stable and reproducible restriction pattern. The technique is more discriminatory than phenotyping and other subtyping methods such as ribotyping and multilocus sequence typing (MLST) for *Salmonella* (see below). The standardization of PFGE methods via the CDC's PulseNet protocols and PulseNet database has improved comparability between laboratories. A strong correlation between PFGE profile and serovar has been described allowing inference of serovar by comparison with the PulseNet database and other PFGE profile databases (Kérouanton et al., 2007; Scallan et al., 2011; Zou et al., 2012). Given the required capacity and the time and costs associated with conventional serotyping, Bopp et al. (2016) make a case for using PFGE results to determine serovar in the majority of cases where PFGE is already being applied routinely, as in the US.

However, PFGE has a number of significant drawbacks. Conducting PFGE requires a high level of technical expertise and in spite of improvements in speed, such as the accelerated method proposed by Gautom (1997) and now widely used, it remains time consuming and labour-intensive (Wattiau et al., 2011b; Fabre et al., 2012a). It is not possible to automate PFGE and comparison of strains is relatively difficult for untrained personnel. Moreover, in spite of the considerable efforts of PulseNet, among others, to standardize PFGE and improve cross-laboratory comparability, issues remain. There can be variability in the resultant DNA restriction patterns between technicians. It is also possible for bands of the same size to come from different parts of the chromosome and for changes at one restriction site to result in multiple bands (Li et al., 2009b). Furthermore, the PulseNet database now has a very large number of clustered PFGE profile records exhibiting only minor differences, which may increase the likelihood of drawing unreliable and/or subjective associations (Wattiau et al., 2011b). A further limitation of PFGE typing is that it does not discriminate between all unrelated isolates; for example, some important strains, such as *S.* Typhimurium DT104, have dominant PFGE profiles that could lead to linking of epidemiologically unrelated organisms on the basis of PFGE profiles (Barco et al., 2013).

Although PFGE remains the gold standard for molecular typing of *Salmonella,* due to technical and comparability advantages MLVA is now widely used in place of PFGE, particularly outside the US. Moreover, with the rapid advances in whole genome sequencing it is likely that the predominance of PFGE will continue to diminish.

Multiple-locus VNTR analysis (MLVA)

The MLVA methodology was first used by Keim et al. (2000) to subtype *Bacillus anthracis*, and has since been adapted to a number of other bacteria including *Yersinia pestis*, *Mycobacterium tuberculosis,*

E. coli O157 and *Salmonella* (Van Belkum et al., 1998; Noller et al., 2006; Vogler et al., 2006; Best et al., 2007). To date MLVA has only been validated for *S*. Enteritidis and *S*. Typhimurium. MLVA involves a multiplex polymerase chain reaction targeting variable number tandem repeat sequences at defined loci within the bacterial genome. At present the most commonly employed MLVA method for *Salmonella* is that described by Lindstedt et al. (2004), with a number of later studies and authorities advocating and use of the protocol (Best et al., 2007; Lindstedt et al., 2007; Torpdahl et al., 2007; PulseNet, 2009b, a; ECDC, 2011; Lindstedt et al., 2013). The Lindstedt et al. (2004) protocol targets five loci: STTR9, STTR5, STTR6, STTR10pl (located on the virulence plasmid pSLT) and STTR3 (a locus with two fragment sizes of 27bp and 33bp). Two additional loci are included by the CDC and affiliated typing laboratories in the US. Strain MLVA profiles are expressed as a string of numbers representing the number of variable number tandem repeat (VNTR) units at each specified locus, aiding comparison of strains between laboratories and jurisdictions (Gilbert, 2008; Larsson et al., 2009).

The Lindstedt et al. (2004) VNTR loci were selected as they are not thought to be under selective pressure, making them 'neutral and effective for molecular typing' (Best et al., 2007). Chiou et al. (2010) compared the advantages and disadvantages of various loci selections and found that four or five highly polymorphic loci were sufficient to replace PFGE in epidemiological inverstigations and surveillance of *S*. Typhimurium. However, the authors found that 16 VNTR loci could aid phylogenetic studies in the determination of clonal groups (Chiou et al., 2010).

The techniques MLVA and PFGE have similar levels of discriminatory power, for example Barco et al. (2014) showed that PFGE and MLVA differentiated outbreak strains to a similar degree, producing the same subtyping picture in relation to correlating strains. However, correlation between phage type, PFGE, single nucleotide polymorphisms and MLVA is imperfect, supporting the continued application of multiple characterization methods in epidemiological investigations of *Salmonella* (Hopkins et al., 2012; Barco et al., 2013; Brown et al., 2013). For example, Hopkins et al. (2007) have shown considerable differences in PFGE and MLVA descriptions of genomic diversity within the same *Salmonella* population.

The MLVA technique has proven useful in *Salmonella* surveillance and research of on-farm colonization and contamination in supply chains. Exampoes include the Kirchner et al. (2011) study of *S*. Typhimurium from three British pig supply chains and the Arguello et al. (2013b) study of *Salmonella* in Danish pig supply show that MLVA can be used to monitor *Salmonella* infection within populations and supply chains.

Applying MLVA provides a number of advantages over PFGE. The technique is relatively quick, inexpensive, robust, and easily automated (Fabre et al., 2012a). Kurosawa et al. (2012) demonstrated

that MLVA is easier, quicker and more efficient than PFGE to implement. The results of MLVA are considerably easier to compare between laboratories and internationally, although MLVA does employ capillary electrophoresis and therefore there may be some inaccuracy/variability in the sizing of fragments between laboratories as with PFGE, which could affect comparability (Hopkins et al., 2011). MLVA can also differentiate clonal isolates, for example lineages within *S*. Typhimurium DT104 strains, which PFGE may fail to achieve (Fabre et al., 2012a). Paranthaman et al. (2013) strongly advocate MLVA notably for the techniques ability to discriminate within a single phage group. Eyre et al. (2013) found MLVA to have similar power when compared with whole genome sequencing of *Clostridium difficile*, in terms of discrimination and for use in epidemiological investigations of pathogen transmission.

However, the high polymorphism observed at some loci, notably STTR5, STTR6 and STTR10, where present, indicate that clustering of closely related single locus variants at these loci is warranted. The approach to clustering requires further consideration and consensus to aid identification of outbreak strains and improve the utility of MLVA for in epidemiological investigations (Boxrud et al., 2007; Malorny et al., 2008a; Hopkins et al., 2011; Dimovski et al., 2014). Cadel-Six et al. (2013) note questions remain in terms of the instability of VNTR loci, an issue that has been discussed further in other studies (Hopkins et al., 2007; Barua et al., 2013; Wuyts et al., 2013; Dimovski et al., 2014). The lack of stability of some loci, notably STTR5 and STTR6, may be too high to provide indications of reliable phylogenetic relationships among closely related strains, furthermore, changes in VNTR copy numbers may occur at these loci during an outbreak hampering identification of an outbreak strain (Li et al., 2009b; Fabre et al., 2012a; Dimovski et al., 2014). Dimovski et al. (2014) recommended that single locus variants (SLVs) with copy number variation of 1-2 be considered clonal, Niemann et al. (2015b) adopted a similar approach in which they collapsed single or double locus variants with low copy number variation at loci STTR5 and STTR6 into groups. However, other studies have shown greater stability. In an *in vitro* study of 670 samples and 8 strains of *S*. 1,4,[5],12:i:- (differing in PFGE and MLVA patterns), Cadel-Six et al. (2013) found very low VNTR locus variation (VF 1.87%) when subjecting isolates in various media with thermal shocks—3 days at -20°C then immediately after 15 mins at 56°C; stress was verified by counts before and after to ensure the temperature abuse had affected bacterial growth. Cadel-Six et al. (2013) found that all variations occurred at STTR5 and STTR6, echoing findings from other *S*. Typhimuirum MLVA studies cited previously; these loci are recognized as being more polymorphic than other loci used in *Salmonella* MLVA. A further possible disadvantage of MLVA by comparison with PFGE is the limited availability of historical data (Barco et al., 2014).

Other DNA banding pattern-based methods

Plasmid profiling and plasmid restriction profiling involves approximation of plasmid molecular mass by electrophoresing plasmid DNA and comparison with plasmids of known molecular weight

(Holmberg et al., 1984; Millemann et al., 1995). The restriction fragment length polymorphism (RFLP) technique uses DNA digestion and gel electrophoresis followed by Southern blotting with labelled probes to size restriction fragements. Ribotyping involves restriction enzyme digests of the DNA being hybridized with RNA probes, restriction enzyme cleaving of the DNA and observation of rDNA restriction patterns upon electrophoresing of products (Grimont and Grimont, 1986; Stull et al., 1988; Esteban et al., 1993; Olsen et al., 1994; Liebana et al., 2001; Bailey et al., 2002; Capita et al., 2007; Guard et al., 2012; Barco et al., 2013). The techniques RFLP and ribotyping are relatively cheap, although more expensive in terms of capital investment when ribotyping is automated. The main drawbacks of these techniques are that they require large amounts of DNA and are time and labour intensive (Li et al., 2009b). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based approaches involves PCR amplification of CRISPR loci and sequencing of purified amplicons. (Fabre et al., 2012a; DiMarzio et al., 2013). There are also various other PCR-based methods involving the targeting of a wide variety of specific genes or loci and analysis of restriction patterns. For example, repetitive extragenic palindromic sequence-based PCR (rep-PCR), which Wise et al. (2009) found showed considerable promise as a relatively rapid method of determining the serovar. Chenu et al. (2012) applied the DiversiLab® system to Australian *Salmonellae* and found similar results, concluding that the system was relaiable and cost and time effective, putatively determining the serovar within several hours. Other PCR-based methods include random amplified polymorphic DNA (RAPD), PCR-RFLP, denatured gel electrophoresis and amplified fragment length polymorphism (AFLP), among others (Hadrys et al., 1992; Olsen et al., 1994; Millemann et al., 1995; Van Belkum et al., 2007; Li et al., 2009b; Prendergast et al., 2013; Turki et al., 2014). As with all PCR-based assays, contamination and generation of artifacts can be an issue, and they require the use of multiple controls to verify the reliability of results.

DNA sequencing-based methods

Multilocus sequence typing (MLST)

The typing technique MLST involves comparing the sequences of specific housekeeping genes to identify allelic variation; as such the sequence type may be inferred from whole genome sequence data (Kotetishvili et al., 2002; Achtman et al., 2012). As MLST targets housekeeping genes, which are constrained due to their function and the essential proteins that they encode, MLST has relatively low discriminatory power, approximately that of serotyping; in fact, some authors have suggested that MLST may one day replace serotyping (Harbottle et al., 2006; Achtman et al., 2012). The technique is therefore better suited to bacteria that undergo high rates of recombination or longer-term studies of bacterial population structure in organisms like *Salmonella*.

Whole genome sequencing

Whole genome mapping, single nucleotide polymorphism (SNP) analyses, genotype clustering, and genome wide association studies involve the sequencing of nucleotides in the genome and

bioinformatic analyses identifying core and/or accessory genome polymorphisms and/or genes and/or other genomic features (Li et al., 2009b; Okoro et al., 2012a; Okoro et al., 2012b; Eyre et al., 2013; Hawkey et al., 2013; Mather et al., 2013; Miller, 2013; Dimovski et al., 2014; Perreault et al., 2014). The rapid decline in costs and increase in access to, and speed of, whole genome sequencing has made this a viable approach to investigating bacterial genetics and evolution and in surveillance and epidemiological research, such as the presence of resistance genes and identification of outbreak strains. A range of benchtop sequencing machines, such as the Illumina, Roche and Ion Torrent, producing single and paired-end reads at various levels of speed and quality, have allowed in-house sequencing capacity, or made fee-for-service sequencing readily available, to many laboratories. Platforms producing long reads, produced by companies such as PacBio and Illumina, are also increasingly widely available. The resulting sequence data can then be cleaned and analysed in a wide variety of ways using an extensive array of programs that employ a variety of algorithms, depending on the purpose of the research. For comparative purposes, extensive quantities of genetic data are freely available through databases such as GenBank. Whole genome sequencing is being employed routinely in outbreak investigations where the resources are available.

In the conduct of whole genome sequencing, genomic DNA is extracted from the isolates under investigation and prepared as a unique library, typically using commercially available kits. The libraries are then sequenced resulting in the raw sequence read data. Short sequences are then assembled into contiguous assemblies of overlapping sequence reads without the use of a reference genome, typically conducted using de Buijin graphs for short read sequences in an assembler such as Velvet (Compeau et al., 2011; Edwards and Holt, 2013). Velvet is applied via a two-stage process of converting sequence reads to k-mers then applying de Bruijin graphs to assemble contigs (Zerbino and Birney, 2008). Determining the best k-mer length and assessing expected coverage of the genome, the length of the insert sizes in paired-end read libraries, and the minimum read depth cut-off are necessary to optimize the assembly. Contigs can then be arranged relative to a reference genome, preferably the most closely related bacteria with a fully mapped genome, using a tool such as MUMmer or Mauve, among numerous others (Kurtz et al., 2004; Rissman et al., 2009; Darling et al., 2010). For the purposes of *S*. Typhimurium or *S*. 1,4,[5],12:i:- in Australia, Hawkey et al. (2013) demonstrated that *S*. Typhimurium SL1344 (phage type definitive type 44) is an appropriate reference. The genome can then be annotated to identify the genes present using a variety of annotation tools (Edwards and Holt, 2013).

The reads aligned to the reference, generating pileups most commonly using SAMtools, can then be analysed to identify sites of single nucleotide polymorphisms (SNPs) (Li et al., 2009a). Depending on the aims of the research, the sequence data can then be parsed to remove 'noise' in various ways in order to discern the underlying signals of vertical inheritance and generate a robust ancestral phylogenetic tree. This requires comparison of the core of the genomes under study, in other words

the parts of the genome shared by the genomes under investigation. To identify the core genome, repeat sequences, insertion sequences and prophages are filtered and excluded (Edwards and Holt, 2013; Edwards et al., 2015). Further filtering is then conducted to identify predicted SNPs generated by recombination events, identified by establishing a cutoff for the number of SNPs in a given length of sequence, using a program such as Gubbins (Croucher et al., 2015). The resulting phylogenetic data can then be represented as a phylogenetic tree on the basis of core genome SNPs. Maximum likelihood methods are generally preferred, using a tool such as RAxML, though trees may also be generated using other methods such as neighbor joining and unweighted pair group method with arithmetic mean (UPGMA) (Stamatakis, 2014). To study the accessory genes in the pan-genome, the core genome identified previously can then be filtered out, using a tool such as ROARY, to compare the areas of greater variability in the collection under investigation (Page et al., 2015).

A further array of tools, such as SRST2, can then be used to search the core and/or accessory genome for features such as resistance genes, MLST genes, markers for phage types or genes known to confer specific phenotypes such as monophasism, against a variety of databases or specific sequences of interest (Gupta et al., 2014b; Inouye et al., 2014).

Analysis of whole genome sequence data has enormous potential for studies of the evolution of bacterial populations and their phylogenetic relationships, with subsequent uses in surveillance and epidemiological investigations. Several recent studies have shown the potential uses of whole genome sequencing, in concert with traditional epidemiological data, for outbreak investigations and source attribution (Okoro et al., 2012b; Hawkey et al., 2013; Mather et al., 2013; Leekitcharoenphon et al., 2014). The main limitations at present relate to effective storage and analysis of the large datasets produced by whole genome sequencing. The available software for analysis is largely open source and constantly being updated. Being open source the underlying code is typically readily available to analysts, however, although improving rapidly, bioinformatic analyses generally require considerable computing power and technical expertise to be conducted reliably.

Array-based methods

Methods of typing bacteria using array-based typing methods primarily employ arrays of DNA probes. With these approaches, DNA probes of known sequences attached to a surface are used to identify the presence of target free nucleic acids (Garaizar et al., 2002; Cai et al., 2005; Li et al., 2009b; García et al., 2013). The DNA probes are typically fluorescently labeled and the hybridized targets are identified and their abundance are measured, usually by luminescence. The main classes of array-based methods are macro-array and micro-array, so named for the number of oligonucleotides arrayed on the substrate. Macro-arrays are cheaper to run than micro-arrays but are less discriminatory (Li et al., 2009b).

1.3.6. Disease characteristics: enteropathogenic salmonellosis

In most cases the symptoms of human salmonellosis are diarrhoea, fever and abdominal cramps, which occur eight to 72 hours post-infection (Hocking, 2003; Bell and Kyriakides, 2008; CDC, 2016c). The illness usually lasts one to seven days and most people recover without treatment (Hocking, 2003; Bell and Kyriakides, 2008; On et al., 2010; CDC, 2016c). However, in some cases hospitalization may become necessary, particularly among infants, the elderly and persons with compromised immune systems. In these extreme cases the infection can become systemic as bacteria enter the bloodstream and then infect other sites. In such cases death may occur unless the patient is treated quickly with appropriate antimicrobials (CDC, 2016c). The highest shedding of organisms occurs at the onset of clinical signs and decreases over time (Hocking, 2003). Shedding duration varies with the individual host and strain of *Salmonella*, but is typically around five weeks from the onset of clinical signs (Hocking, 2003). Few persons become carriers of *Salmonella*, Hocking (2003) estimated less than 1%.

Though uncommon long-term *S. enterica* Group B infection associated sequelae do occur. Reported long-term sequelae include: septicaemia, which may last up to one year post infection but more typically 6 months or less, which usually occurs 3-4 weeks post-enteritis (Hocking, 2003; On et al., 2010); reactive arthritis or Reiter's syndrome, caused by an autoimmune response, that may be syndromic in conjunction with conjunctivitis and urethritis or cervicitis in men or women, respectively, and various other manifestations (Dworkin et al., 2001; Hocking, 2003; Bell and Kyriakides, 2008); and irritable bowel syndrome (Gradel et al., 2009; Havelaar et al., 2012).

Salmonella isolations from humans typically describe seasonal patterns peaking in the summer in both northern and southern hemispheres. Australia and New Zealand typically experience a peak in late summer, around March, with considerably lower numbers of isolations in winter. This may be related to eating habits, such as barbeques, at which relatively higher risk products are consumed and the effectiveness of the cooking step in deactivating *Salmonella* bacteria may be less reliable (On et al., 2010).

There are numerous *Salmonella* serovars associated with pigs that are also of particular public health interest, these include: *S*. Typhimurium, *S*. 1,4,[5],12:i:-, *S*. Derby, *S*. Heidelberg, *S*. Worthington and *S*. Infantis (Harris, 2016a). The most clinically and economically important *Salmonella* serovar to the pig industry internationally is *S*. Choleraesuis, a pig host specific serovar associated with high rates of morbidity and considerable mortality. *Salmonella* Choleraesuis has not been reported in recent Australian passive surveillance data (NEPSS, 2014), however, the serovar has been isolated in the past (Beh, 1971). In Australia clinical salmonellosis in pigs has historically been most commonly associated with *S*. Typhimurium (Hamilton et al., 2015).

Enteropathogenic salmonellosis in pigs causes similar symptoms to those observed in humans and other animals, typified by scouring and/or thriftiness. Acute enteric and systemic disease, generally associated with a virulent strain and/or impaired immunity, can lead to mortality, particularly among young pigs. Gross caecal and ileal lesions may be observed among confirmed cases during post-mortem. The disease may affect pigs of any age (Harris, 2016b). Pigs can become asymptomatic carriers with infection in the blood and lymph nodes. *Salmonella* colonisation can be difficult to detect in older live animals due to little or no clinical manifestations and low and/or intermittent shedding (Kirchner et al., 2012a; Pires et al., 2013a).

1.3.7. Aetiology and pathogenesis

Non-typhoidal salmonellosis is caused by infection with an organism of the genus *Salmonella* other than *S*. Typhi. The disease is clinically characterised by enteritis or septicaemia/typhoid in systemic cases (Gruenberg, 2016). The illness causes inflammation and necrosis in the intestines. The main route of *Salmonella* transmission is faecal-oral, although the upper respirtory tract may also present a route of infection (Fedorka-Cray et al., 1995). The initial colonisation of the intestines is followed by invasion of enterocytes of the small and/or large intestines. The *Salmonella* bacteria adhere to the intestinal wall and employ type III secretion systems in two discrete stages injecting various toxins, the bacterium is enveloped during this process and replicates within the enterocyte cell (Wray and Wray, 2000; Van der Heijden and Finlay, 2012). Invasion of the epithelial cells stimulates the release of proinflammatory cytokines, the inflammatory response causes diarrhoea and may cause ulceration (Giannella, 1996). After initial colonisation, the bacteria can spread to the lymph nodes and other organs causing systemic disease (Giannella, 1996; Ball et al., 2011).

1.3.8. Host specificity

Salmonellae vary in their degree of host specificity (Uzzau et al., 2000; Foley et al., 2008; Foley et al., 2013); *S*. Choleraesuis is pig specific, while others such as *S*. Enteritidis and *S*. Typhimurium may colonise a variety of host species. Most non-typhoidal *Salmonellae* of importance to public health have a wide host-range, but disease caused by serovars with a narrower host-range tends to be more severe with a greater likleihood of invasive disease (Vugia et al., 2004; Foley et al., 2013). The serovar *S*. 1,4,[5],12:i:- is not host specific and has been isolated from humans, poultry and bovines, among other species, but is most closely associated with pigs in Europe (Hauser et al., 2010). It has been postulated that the serovar emerged in pigs and the majority of isolates internationally have come from pigs (Hauser et al., 2010; Petrovska et al., 2016).

1.3.9. Pathogenicity and virulence

Specific serovars may exhibit different pathogenicity levels in different host species (Kingsley and Bäumler, 2000). *Salmonella* Typhimurium and *S.* 1,4,[5],12:i:- are thought to be closely related and commonly harbor the same or similar pathogenicity gene repertoires indicating that the serovars are similarly pathogenic, which is reflected in the relatively high rate of isolation of these serovars in passive surveillance systems (Hauser et al., 2010). In human hosts the serovar *S.* 1,4,[5],12:i:- is pathogenic and virulent and has been associated with septicaemia. In an outbreak in New York City in 1998 some 70% of cases led to hospitalization (Agasan et al., 2002a), though considerably lower hospitalization rates are normally reported. There is little evidence of significant variation in pathogenicity or virulence between serovar subtypes (Wray and Wray, 2000; Torrence and Isaacson, 2003; Bell and Kyriakides, 2008; On et al., 2010). *Salmonella* are thought to have a large number of virulence factors, few of which have been fully characterized (Fedorka-Cray et al., 2000). *Salmonella* virulence is thought to be expressed through an array of genes located in 12 key pathogenicity islands (Morgan, 2007; Foley et al., 2013). Virulence relating to invasiveness, such as encoding of type II and type III secretion systems, and resistance to immune responses are known to include complex regulons, or systems of genes in various locations on the chromosome and plasmids (Miller et al., 1989; Shea et al., 1996). Though not universally present, virulence plasmids, such as pSLT, which was mapped in the *S.* Typhimurium LT2 genome, are known to play an important role in pathogenesis, particularly in relation to bacterial multiplication in the reticulo-endothelial system of the host (Gulig and Curtiss, 1987; McClelland et al., 2001; Rotger and Casadesús, 2010).

1.3.10. Immune response

The initial human host reaction to non-typhoidal *Salmonella* gastro enteric colonization is the production of inflammatory cytokines, followed by the development of specific immunity through the production of specific T- and B-cells, establishing strong acquired immunity in the host post-infection (Mastroeni et al., 2001). It has been postulated that the *S.* 1,4,[5],12:i:- non-expression of the phase 2 flagellar antigen may be a mechanism by which the bacteria can at least partially evade the initial cytokine response of the immune system (Crayford et al., 2011). This has been posited as a possible reason for the recent rise of *S.* 1,4,[5],12:i:- isolation from both humans and production animals, particularly European pigs (Davies, 2013).

1.3.11. Susceptible human populations

There is no evidence of gender bias or genetic predisposition to salmonellosis in humans. The incidence and severity of salmonellosis is highest in the elderly, children and persons suffering from reduced immunological responses (On et al., 2010). The highest notification rate for salmonellosis

are among children aged 0-4, exhibiting a rate three times that of 5-14 year olds and five times that of persons aged above 14 years . Food preparation customs may increase the risk of salmonellosis, for example consumption of raw ground pork dishes in Southeast Asia, Belgian raw boar meat and anecdotal evidence of increasing consumption of rare pork dishes in Australia likely present higher risks from pig products.

1.3.12. Dose response

Variation in dose response is primarily associated with: age and health of the consumer (elderly, young and immunocompromised are more susceptible), mode of consumption (food vehicle characteristics), and characteristics of the strain of *Salmonella*. Estimates of the required challenge doses have ranged from a few cells to 10^5-10^9 colony forming units (cfu). For ethical reasons there have been no recent dose-response studies in humans, however, a 'volunteer' study—involving prison inmates conducted in the early 20th century—found 10^5 cfu were required (Hocking, 2003). Although there have been reports that 100 and 1000 bacteria may present a strong enough challenge to cause disease (Wray and Wray, 2000; Torrence and Isaacson, 2003; Bell and Kyriakides, 2008; On et al., 2010), having conducted a thorough review of the literature Kothary and Babu (2001) estimated that the median infective dose (ID_{50}) is more likely around 10,000 cfu. The *Salmonella* infectious dose varies between strains and is lower for the young, elderly and immunocompromised (Hocking, 2003). However, more recent counts on implicated food vehicles post outbreaks indicate as few as 1-10 cfu may cause illness, particularly in food vehicles with high fat content, which appears to offer some protection to the organisms as they move through the digestive tract (Hocking, 2003; On et al., 2010). Bell and Kyriakides (2008) assert that high numbers, estimated at greater than 10 000 cfu, are normally required for *S. enterica* (I) strains to cause gastroenteritis, but that in high fat foods less than 100 cfu may prove sufficient. Various models have been used to estimate dose response for *Salmonella*, including the United States Department of Agriculture (USDA)-Food Safety and Inspection service (FSIS)-Food and Drug Administration (FDA) *Salmonella* Enteritidis model, the Health Canada *Salmonella* Enteritidis model, and a beta-Poisson model. While each of these models have drawbacks, the outputs show that at low attack rates the probability of disease is low but present; therefore, if products with low concentrations are widely consumed some cases will likely occur (FAO, 2016).

1.3.13. *Salmonella* ecology in pig herds

Salmonella, more specifically *S.* Typhimurium, has been shown to persist on livestock farms for extended periods. For example, McLaren and Wray (1991) found specific *S.* Typhimurium strains persisted among calves for up to two years. Similarly, Sandvang et al. (2000) found the same *S.* Typhimurium PFGE pulsotypes among pigs sampled 20 months after the first isolation.

The introduction of infected animals has long been recognized as an important mode of *Salmonella* transmission between livestock herds (Wray et al., 1990; Wray et al., 1991; Evans and Davies, 1996; Langvad et al., 2006). A number of other routes of transmission between herds are also possible, including vectors such as wild animals, pets and people, insects and dust acting as mechanical vectors, and fomites such as vehicles or equipment (Fedorka-Cray et al., 1994; Fedorka-Cray et al., 2000). For instance, a study conducted by Langvad et al. (2006) found that routes other than live animal movement, such as people equipment and other physical sources played a significant role in the spread of *S*. Typhimurium DT104 between cattle and pig herds in Denmark.

It is also well recognized that *Salmonella* can enter pig herds through feed components, including coarse grains, oilseeds, meat/blood/meat and bone meals, fishmeal and other protein cakes and meals (Harris et al., 1997; Funk and Gebreyes, 2004). In order for feed to infect pigs on-farm, feed components may arrive at mills contaminated and dilution or kill steps, such as rendering, in the milling process fail. Feed may also be contaminated post-inactivation steps at the mill via resident or transient populations and feed may become contaminated on farm via fomites or, commonly, rodents, birds and cats (Burns et al., 2016).

The feed ingredients, acidity and physical structure of feed can also affect *Salmonella* prevalence within herds (Funk and Gebreyes, 2004). There is considerable epidemiological evidence that pigs fed on fine ground feeds with low roughage content are at greater risk of *Salmonella* colonization, likely due to the associated reductions in gut length and the lower pH gradient within the gastrointestinal tract of animals fed diets with these characteristics (Bush et al., 1999; Funk and Gebreyes, 2004; Lo Fo Wong et al., 2004). *Salmonella* may also be introduced and spread within herds through vectors, mechanically or through colonization and shedding, such as new stock, wild animals, pets, arthropods and humans (Funk and Gebreyes, 2004; Ball et al., 2011).

Salmonella are typically transmitted between pigs within herds via the faecal-oral route (Fedorka-Cray et al., 1994). *Salmonella* can remain viable for extended periods in organic matter outside the host, presenting a risk of transmission via the environment and/or fomites or mechanical vectors (Kirchner et al., 2012b). Vertical transmission is also thought to be important for maintaining the bacteria (Funk and Gebreyes, 2004; Ball et al., 2011). Studies of poultry and mice have shown aerosolized transmission of *Salmonella*, and aerosol transmission of *S*. Typhimurium between weaners at close proximity has been reported (Oliveira et al., 2006; Ball et al., 2011). Pigs can be colonized via the lungs, studies such as Lo Fo Wong et al. (2004) found that snout-to-snout contact between cohorts was a risk factor which may indicate aerosol transmission over short distances and/or via contact/faecal-oral routes (Fedorka-Cray et al., 2000).

Clinical signs are generally most apparent in weaners between 4 and 10 weeks of age, which may relate to protection from maternal antibodies via colostrum while suckling (Beloeil et al., 2003;

Funk and Gebreyes, 2004; Ball et al., 2011; Wales et al., 2011). Responses in older animals are typically sub-clinical and sporadic *Salmonella* shedding can make detection difficult. Lower *Salmonella* shedding rates among sows and suckling piglets observed in this study have been reported extensively by other studies (Funk et al., 2001; Kranker et al., 2003; Nollet et al., 2005a; Rajić et al., 2005; Pires et al., 2013a; Pires et al., 2014). However, colonization of young piglets via the sow has been demonstrated. Longitudinal studies of farrow-to-finish herds conducted by Nollet et al. (2005a); Nollet et al. (2005b) found exposure of young pigs could occur via the sow post-partum or via some alternate source(s) in the rearing environment. Kranker et al. (2003) and Vigo et al. (2009b) suggest that sows may play a more important role in transmitting *Salmonella* to suckling piglets than is typically recognised. A farm transmission model using data from the European Union (EU) developed by Hill et al. (2015) concluded that if more than 10% of sows were colonised by *Salmonella* they would account for the majority of *Salmonella* transmission within the herd; below 10% sow prevalence feed became the dominant contributor to slaughter pig *Salmonella* status. Sows may become reservoirs of disease by shedding slowly for extended periods with consequent effects on pathogen cycling within a herd. Other studies have also demonstrated the nucleus and multiplier herd *Salmonella* status, and number of supplier herds, to be risk factors for grow-out herd *Salmonella* colonisation (Kranker et al., 2001; Lo Fo Wong et al., 2004; Wales et al., 2009).

Escalation in shedding is correlated with immune suppression, which can coincide with malnutrition, relate to production phase, genetic predisposition or environmental factors such as season, temperature and humidity, or could relate to infection with other pathogens (Funk and Gebreyes, 2004). Causality of clinical salmonellosis in pigs may be complex, Takada-Iwao et al. (2011) conducted a case control study that found porcine circovirus type 2 (PCV2) associated disease increased the likelihood and severity of clinical *S*. Choleraesuis among infected pigs. Dual infected pigs exhibited significantly greater signs of morbidity including higher shedding, reduced weight gain and lung lesions. Stocking density and other management factors have been shown to affect the rate of *Salmonella* transmission (Funk and Gebreyes, 2004).

Pig stress is associated with increased *Salmonella* shedding, corroborating observed increases in shedding and prevalence during and after transport and lairage (EFSA, 2010d; Ball et al., 2011). Shedding increases significantly when pigs endure the stress of live transport and lairage, increasing transmission rates. Transport and lairage pens may also become 'dirty' with resident populations of *Salmonella* infecting and reinfecting cohorts of pigs, if cleaning and disinfection is suboptimal (EFSA, 2010d; Ball et al., 2011). For obvious reasons, finisher animals present the highest risk of transmission from pigs to humans in pig-derived food product supply chains.

1.3.14. Characteristics of food vehicles

Salmonella can contaminate any organic product. A wide range of food products including processed and fresh vegetables, nuts, chocolate and meat and animal derived products such as milk and eggs have been implicated in salmonellosis outbreaks (Hocking, 2003). *Salmonella* presence on or within food products may originate in production or have been transferred to the product postharvest. The organisms may be present in numbers capable of causing disease in products where kill steps and/or dilution step have failed or cross contamination has occurred post-critical control points. High fat and high protein foods present greater risk for transmission of *Salmonella* (Bell and Kyriakides, 2008). *Salmonella* may display higher heat tolerance in products with low water activity, while, in low pH environments heat tolerance is reduced (Bell and Kyriakides, 2008).

1.3.15. Pork and pig derived products as food vehicles

Pig-derived products are the second most important source of *Salmonella* in the human population (Andres and Davies, 2015). While cross-contamination with transient and/or resident strains at abattoirs and during processing is known to occur, it has long been recognized that herd colonization, and hazard presence at finish, presents a pathway for *Salmonella* into abattoirs, boning rooms and end products (Berends et al., 1997; Bolton et al., 2013). Pig derived products have been implicated in, or suspected in, Australian human salmonellosis cases, including *S*. 1,4,[5],12:i:-, both as the immediate food vehicle and/or as the probable ultimate source of the hazard (OzFoodNet Working Group, 2012b).

Although higher rates of colonization among slaughter pigs might increase risk on product, Swanenburg et al. (2001a); Swanenburg et al. (2001b) found that the level of *Salmonella* colonization within herds had no bearing on carcass contamination, only *Salmonella*-free status. Assuming the risk of contamination in processing is managed effectively, the *Salmonella* status of herds may be the most important information for effective process control and verification systems in abattoirs and boning rooms to mitigate pork related *Salmonella* food safety risks.

Salmonellae are primarily invaders of the enterocytes, which may, in cases of systemic infection, also enter the lymphatic system, lungs and other organs . Therefore, some offal, primairily intestines, may be contaminated prior to slaughter. Organisms may also be present on the skin of animals before entering the slaughter floor and there may be resident populations on equipment used in slaughtering and processing (Van Hoek et al., 2011). Evisceration, trimming, boning and cutting present opportunities for cross contamination of products if the organism is present and control steps, such as washes and heat treatment(s), are not employed effectively prior to human consumption. Furthermore, skin and muscle may be contaminated during bung removal, evisceration, hanging, boning and further processing through direct exposure or via fomites

(Berends et al., 1997). During slaughtering and primary processing ruptured intestines and fomites such as surfaces, bung droppers, knives and other utensils are potential means of contaminating tissue and cross contaminating carcasses with *Salmonella* organisms from the gastro-intestinal tract and lymph nodes (Berends et al., 1997). High throughput slaughtering and/or processing and value adding, such as small goods manufacturers, increase the risk of high case number salmonellosis outbreaks in human populations.

If *Salmonella* is present, poor cold chain management from slaughter/processing to retail risks multiplication of the organisms on or within the product. Further, inadequate cooking or recontamination post-cooking can lead to human colonization if the organism is present.

The nature of the product will also affect the likelihood of disease transmission. Processed products, such as pork mince, and products which do not require cooking, such as some fermented sausages, can present higher risks. In processed products the hazard may be distributed through the product if critical control points have failed. Furthermore, higher fat content and distribution of fat common in comminuted products, such as sausages and pork mince, can also increase the risk associated with these products (Bell and Kyriakides, 2008; On et al., 2010). Primal cuts are more likley to be contaminated superficially, which facilitates destruction of the organisms through cooking.

The role of needle tenderising has not been adequately investigated for pork products but some studies have demonstrated that by breaking the surface of primal products bacteria may be internalized in the product without adequeate critical control points (Graumann and Holley, 2007). This would likely reduce the effectiveness of inactivation steps and increase the importance of consumer terminal steps i.e. heat treatment via cooking. Moisture infusion typically employs injection of (sterile) saline, the effects of rupturing the product surface and on product structure may increase the risk of the hazard penetrating the product (Gill et al., 2008).

Salmonella has been isolated from a wide range of pig-derived products (Table 1-1).

Table 1-1. Pork products implicated in *Salmonella* transmission to human consumers.

Pork products				**Examples**
Muscle–cuts	Chilled	-	-	Pork cuts e.g. loin chops
	Frozen	-	-	Pork cuts e.g. loin chops
	Needle tenderized			Pork cuts e.g. loin chops
Muscle–mince	Chilled	-	-	Pork mince, lean pork mince
	Frozen	-	-	Pork mince, lean pork mince
Muscle–joints	Chilled	-	-	Shoulder
(bone in)	Frozen	-	-	Shoulder
	Needle tenderized	-	-	Shoulder
	Cured chilled	-	-	Leg ham

	Cured frozen	-	-	Leg ham
Offal	Chilled	-	-	Liver, kidney, heart, tripe
	Frozen	-	-	Liver, kidney, heart, tripe
Processed	Fermented	Dry	Cooked	-
			Uncooked	Salamis e.g. Hungarian, Varzi
		Semi-dry	Cooked	Figatelli, summer sausages
			Uncooked	Mettwurst, summer sausages
		Moist	Cooked	Cervelas a l'ail, saucisson brioche
			Uncooked	French chipolatas
	Unfermented	-	-	Toulouse, Lincolnshire sausages
	Cured	Dry	Uncooked	Parma, Serrano, Virginia hams
		Dry-wet	Uncooked	Cured/smoked pork loin, Breakfast ham
	Dried	Dry	Cooked	Pork floss

Adapted from Ockerman and Basu (2007) and Heinz and Hautzinger (2007).

1.3.16. Consumer terminal step

If *Salmonella* is present and viable in the food product then failure to reach necessary cooking temperatures to kill organisms risks *Salmonella* colonisation of the consumer. Temperature abuse may increase the risk of consumer colonization, this includes breakdowns in cold chains, inadequate cooking time and/or temperatures and inadequate hot holding. Consumer cooking of pork products typically meets the temperatures and durations required to inactivate *Salmonella* spp. Reduced cooking times will have greater effects on higher risk products, potentially minced fresh pork for use in burgers and needle tenderized cuts (Gurman et al., 2015; Gurman et al., 2016).

Minced products, such as mince/ground meat and sausages, potentially present a higher risk if consumer terminal steps fail to control the hazard, due to the comminuted nature of the product, which does not have an intact surface barrier, may include contributions from multiple carcasses, increased potential for cross contamination via fomites, the homogenous nature of the product and, often, higher fat (Giovannini et al., 2004). Poor consumer hygiene practices in food preparation can lead to cross contamination of cooked or ready-to-eat product and, such as cooked meat or salads, from *Salmonella* contaminated products, via fomites such as food preparation utensils and surfaces.

Many fermented and cured products that are ready-to-eat, such as salamis and some hams, present potential for higher risk, thereby typically relying on Good Hygiene Practices and processing controls to meet performance and food safety objectives. As with pork mince, comminuted products increase the potential for contamination due to potential contributions from multiple

carcasses, via fomites in processing, the breached surface barrier and homogenous, typically high fat, content of the product. Failure of controls in these products could lead to wide contamination of a product batch with the potential to cause a widespread outbreak, as occurred during the frozen pot pie outbreak in the US (CDC, 2008).

1.3.17. Significance of non-foodborne transmission

Foodborne transmission is the most significant route of *Salmonella* infection in the human population. However, horizontal transmission through faecal-oral routes can occur, typically between persons living in close proximity and associated with suboptimal hygiene. Nosocomial outbreaks have been widely reported (Chalker and Blaser, 1988). *Salmonella* may be contracted from hand to mouth having been contaminated from the envrionment. It is likely persons working with livestock risk infection through faecal-oral routes due to poor hand hygiene. Similarly, pet reptiles have been implicated in human salmonellosis, typically with serovars rarely observed in food animals and chains (Mermin et al., 2004) and petting zoos for children have been identified as sources of infection (McMillian et al., 2007). While typically foodborne, persons contracting salmonellosis overseas are generally considered as a separate subset of cases due to the likelihood of being exposed to different risk factors.

1.4. Preliminary exposure assessment

1.4.1. *Salmonella* and *S.* 1,4,[5],12:i:- in the global context

Non-typhoidal *Salmonella* are estimated to cause 93.8 million cases of gastroenteritis worldwide per year (Majowicz et al., 2010; Ranieri et al., 2013). Pork and pork products are among the most common sources of foodborne salmonellosis outbreaks globally . It has been estimated that over five million human salmonellosis cases occur per year in Europe and over 53 million cases in East Asia alone (Majowicz et al., 2010). Despite underreporting, over 100,000 cases of salmonellosis in humans are reported annually in the EU and it is estimated that the disease costs the EU EUR 3 billion per year (EFSA, 2016). In the US *Salmonella* is the second most commonly reported agent in foodborne illness and is estimated to cause around one million illnesses each year including approximately 23,000 hospitalisations and 450 deaths (Agasan et al., 2002b; CDC, 2011b, 2013b, 2016c).

In much of the world the poultry associated serovar *S.* Enteritidis and *S.* Typhimurium (generally including *S.* 1,4,[5],12:i:-) are the most common serovars isolated from cases of human salmonellosis and non-human sources (Galanis et al., 2006; Hendriksen et al., 2011; WHO, 2016);

these serovars account for approximately 60% and over 20% of human cases in the EU, respectively .

The rapid international rise of *S.* 1,4,[5],12:i:- isolations since the mid-1990s, in spite of the likelihood of high levels of underreporting of *S.* 1,4,[5],12:i:- due to misclassification as conventional *S.* Typhimurium (Switt et al., 2009), prompted Hopkins et al. (2010) and Davies (2013) to describe the serovar, and phage type 193 more specifically, as a new pandemic *Salmonella* strain. The rise in isolation of *S.* 1,4,[5],12:i:-, exemplified by the rate of isolation per 100,000 in the US (Figure 1-1), is reminiscent of *S.* Typhimurium DT104 in the 1990s-2000s, a multi-drug resistant 'pandemic strain' that caused significant public health concern and livestock industry losses globally (Threlfall, 2000). *Salmonella* 1,4,[5],12:i:- has been isolated in dozens of countries in multiple regions (Table 1-2), and is now reported among the top ten serovars isolated from humans globally. The serovar *S.* 1,4,[5],12:i:- has been reported as the the fourth most prevalent *Salmonella* serovar in European finished pigs (EFSA, 2010a; Hopkins et al., 2010; Morris Jr et al., 2011). Data from the European Surveillance System (TESSy) show *S.* 1,4,[5],12:i:- accounted for 4.6%, 7.2% and 8.6% of salmonellosis cases reported to in the EU in 2011, 2012 and 2013, respectively, and was the third most common serovar identified in humans over the same period (EFSA, 2015; Cito et al., 2016). The serovar has also been identified as the causative agent in a number of high profile human disease outbreaks (Table 1-3). (Echeita et al., 1999; Hopkins et al., 2010; CDC, 2013a).

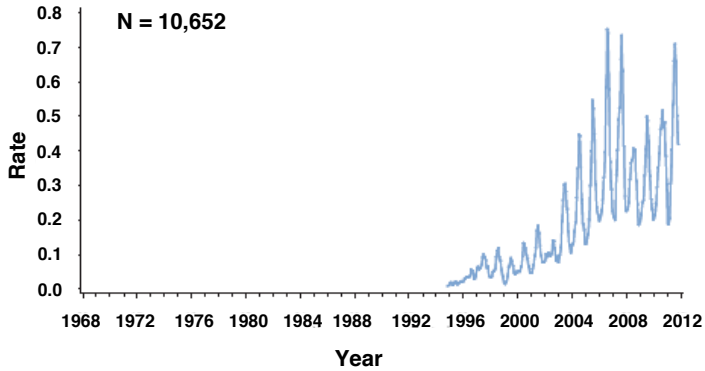

Figure 1-1. US rate of reported *S.* 1,4,[5],12:i:- isolates per 100,000 population. Three-month moving average, by month and year, 1968-2011. Adapted from CDC (2013a).

Table 1-2. Reports of human isolations of *S.* 1,4,[5],12:i:- internationally.

Country	Detail	References
Barbados	Isolated from humans and animals (2006 data).	Moreno Switt et al. (2009)
Brazil	Human salmonellosis associated with septicaemia.	Vieira-Pinto et al. (2012); Hopkins et al. (2010); Morris Jr et al. (2011); Hopkins et al. (2012)
Canada	Reported human isolation (2004 data). Fifth most	Moreno Switt et al. (2009); Mulvey et al.

	commonly isolated serovar from human cases.	(2013a)
Chile	No details available.	Moreno Switt et al. (2009)
Costa Rica	No details available.	Moreno Switt et al. (2009)
Denmark	Estimated 14 percent of cases from pork and pork products.	Baggesen et al. (1996); Mousing et al. (1997a); Mousing et al. (1997b); Nielsen and Mousing (1997); Stege et al. (2001); Benschop et al. (2008)
France	Increasing isolations from humans from 11th to third 2005-2010. Accounted for 15% of all human strains collected in 2010.	Lailler et al. (2013)
Germany	Second most prevalent serovar isolated from humans, in 2008 42% of all *S. enterica* (I) Group B isolates from human cases; second most common serovar isolated from pigs, third from cattle. Pork products were implicated as the food vehicle in 48% of human cases.	Friedrich et al. (2010); Hauser et al. (2010)
Greece	First reported in 2007, third most isolated serovar by 2011.	Mandilara et al. (2013)
Italy	Third most commonly isolated serovar from humans in the years 2004-2008, second most common in 2009	Lucarelli et al. (2010); Cito et al. (2016)
Korea, Republic of	Prevalence study found third most prevalent serovar, 5% found in a sample of 44 organic and conventional farms in 2012-2013.	Tamang et al. (2015)
Netherlands	First human reports in 2004 grown; risen to third most isolated serovar from human salmonellosis cases. Fourth most commonly isolated serovar from pigs in the period 2004-2008, rose to second in 2009.	Van Der Wolf et al. (2001b); Van Der Wolf et al. (2001c); Van der Wolf et al. (2001d); Wong et al. (2004)
Portugal	First reported in the late 1980s, from chicken carcass, may or may not relate to emergence in the 1990s. Now among the most frequently isolates serovars	Machado and Bernardo (1990); Vieira-Pinto et al. (2012); Marçal et al. (2015)
Spain	Top 5 *Salmonella* serovars isolated from human salmonellosis cases	Echeita et al. (1999); De La Torre et al. (2003a)
Taiwan	Confirmed presence in swine	Chiu et al. (2006)
Thailand	Among the top five serovars isolated from human salmonellosis cases, associated with septicaemia. The most frequently isolated serovar in swine, and the second most common serovar in pork products.	Boonmar et al. (1998); Bangtrakulnonth et al. (2004); Amavisit et al. (2005); Pornruangwong et al. (2008); Huoy et al. (2014)
UK	Over 30% increase in number of cases from 47 in 2005 to 151 cases in 2009	Hopkins et al. (2012); Vieira-Pinto et al. (2012); Petrovska et al. (2016)
USA	From 2002 isolation has increased consistently, now firmly among the top six serovars isolated from human salmonellosis cases	CDC (2011b, 2013b); Jackson et al. (2013); CDC (2016a)

Table 1-3. Notable international human *S.* 1,4,[5],12:i:- salmonellosis outbreaks 1998-2016

Country	Year	Food vehicle	Purveyor/ venue	Location	Detail	References
USA	2015-2016	Whole hog BBQ (implicated)		Washington, surrounds	188 people ill from *S.* 1,4,[5],12:i:- . Contaminated pork produced by Kapowsin Meats implicated. A further 15 cases linked to the same product at a 'Good Vibe Tribe Luau' in July 2016	CDC (2015a, 2016a); Larsen (2016)
Italy	2013-2014	Unknown		Abruzzo region	206 cases, 98 confirmed, 101 probable, seven possible. Source was not determined but a possible link to contaminated irrigation systems.	Cito et al. (2016)
USA	2013	Cooked chorizo (confirmed)	Restaurant	Las Vegas, Nevada	Firefly in Las Vegas (n=294, probable + confirmed, at 24/05/2013 73 confirmed). Source: cooked chorizo had confirmed outbreak strain (culture and PFGE)	Nguyen (2013)
France	2012-2013	Various meat	Local butcher	Paris	30 confirmed, four possible, six probable, serovars *S. enterica* (I) ser. 1,4,[5],12:i:- and S. Typhimurium CT51	Bassi (2013)
France	2011	Dried pork sausage (suspected)	Supermarket	National	337 cases identified nationwide.	Gossner et al. (2012)
USA	2010	Alfalfa sprouts (implicated)		Multistate	140 cases, 26 states and District of Colombia, 24% of persons for whom information available were hospitalized (PFGE was used to discriminate, the pattern was a common US pattern so some cases may not have been linked to this outbreak).	CDC (2011a)
USA	2007	Frozen pot pies (implicated)	Home cooked	Multistate	401 cases in 41 states linked on the basis of the identified outbreak PFGE pattern, 32% of persons for whom information available were hospitalized.	CDC (2008)
Germany	2006-	various		Nationwide	Diffuse outbreak with high	Trüpschuch et

					rates of hospitalization.	al. (2010b)
Luxem-bourg	2006	Local pork (implicated)		National	Two major outbreaks, 133 confirmed human cases, 24 hospitalizations (quoted as 21% in other sources), 1 death. High proportion of elderly.	Mossong et al. (2007); Vieira-Pinto et al. (2012); Hopkins et al. (2010)
USA	2004	-	-	California	-	Moreno Switt et al. (2009)
USA	1998	Unknown	Dinner reception	New York, New York	86 persons, 70% of persons who sought medical attention were hospitalized, *S.* 1,4,[5],12:i:- isolated from individuals stool during the outbreak, no specific food vehicle was implicated.	Agasan et al. (2002b); Moreno Switt et al. (2009)

There are indications that *S.* 1,4,[5],12:i:- may have fitness advantages over *S.* Typhimurium and could be displacing the biphasic serovar; for example, in Germany *S.* 1,4,[5],12:i:- isolation and decreasing *S.* Typhimurium isolation from humans have been reported in recent years (Figure 1-2)(Lailler et al., 2013; Simon et al., 2013). The decline in *S.* Typhimurium isolations may reflect the unexplained decline in *S.* Typhimurium DT104 isolations (Davies, 2013).

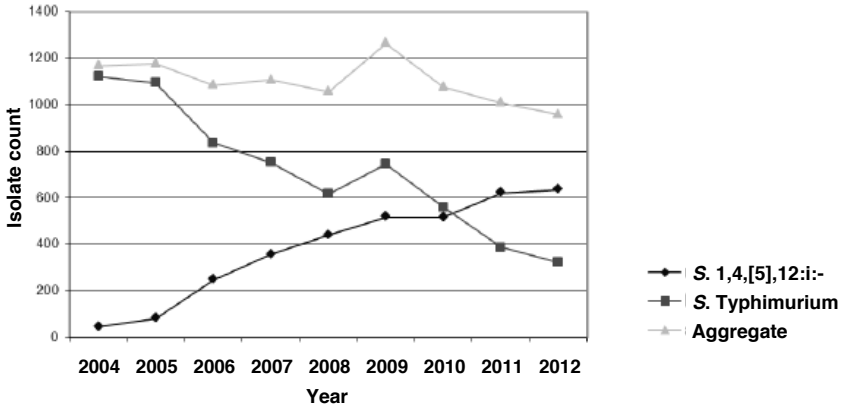

Figure 1-2. Human *S.* Typhimurium (biphasic) and *S.* 1,4,[5],12:i:- isolates sent to the German National Reference Centre (NRC) from 2004 to 2012. Adapted from Simon et al. (2013), data from RKI SalmoDB database.

1.4.2. *Salmonella* and *S.* 1,4,[5],12:i:- in Australia

Salmonella spp. is the second most notified foodborne pathogen from humans in Australia (OzFoodNet Working Group, 2012b). There were approximately 16,000 reports of human salmonellosis cases in Australia in 2014 (DOH, 2015). However, *Salmonella* is notoriously underreported. Estimated rates of underreporting vary considerably, Paranthaman et al. (2013) and

Kirk et al. (2014) estimated that one in four *Salmonella* cases were reported and that approximately 40,000 Australian human cases occurred in 2010. Others have estimated underreporting in Australia to be as high as seven to 38-fold for bacteria causing non-bloody diarrhoea (Sumner et al., 2000; Hall et al., 2008). Ford et al. (2014) estimated that salmonellosis was the cause of irritable bowel syndrome in 3500 cases and reactive arthritis in a further 3250 instances in Australia in 2010. Acknowledging estimated underreporting, Plass et al. (2014) estimated the public health burden of salmonellosis in Germany to be in the range 18.0-29.5 disability adjusted life years/100,000 population/year. Assuming the Australian situation is broadly similar to the German case, using DOH (2015) figures and accounting for underreporting conservatively, as per Paranthaman et al. (2013) and Kirk et al. (2014), this would equate to a cost of approximately 4,080-6,690 disability adjusted life years/year in Australia.

Salmonella Typhimurium is consistently the most commonly isolated serovar from human salmonellosis cases in Australia (Sumner et al., 2004). *Salmonella* Typhimurium and *S*. 1,4,[5],12:i:- combined are the most commonly isolated serovars from human cases in Australia, accounting for 44% in 2010 (OzFoodNet Working Group, 2012b). Australia is currently considered free of *S*. Enteritidis, unlike most of the world where it is commonly the most isolated serovar from humans. *Salmonella* Enteritidis is regularly isolated from cases suspected of being acquired overseas, and has occasionally been isolated from poultry related samples, such as from chicken litter and meat (SA Pathology, 2014). The majority of Australian *S*. Typhimurium outbreaks have implicated the poultry industry as the ultimate source. However, *S*. Typhimurium has also been the agent in the majority of Australian *Salmonella* outbreaks implicating pork or pork products (Table 1-4). However, historically, the Australian pig industry has maintained relatively low *S*. Typhimurium prevalence in comparison with pigs in Europe and the US (Funk et al., 2001; Beloeil et al., 2003; Hamilton et al., 2015). Carcass surveillance data from the *Escherichia coli* and *Salmonella* Monitoring Programme (ESAM) identified *S*. Typhimurium in only 5% of *Salmonella* positive carcasses between 2000 and 2006 (AQIS, 2003, 2007).

Although *S*. 1,4,[5],12:i:- emerged internationally in the early to mid-1990s, it appears to have emerged more recently in Australia. Isolation of *S*. 1,4,[5],12:i:- has been reported from Australian domestic sources with increasing frequency since 2008 (Figure 1-4) (OzFoodNet Working Group, 2012d; NEPSS, 2014). Since 2011 there have been several Australian *S*. 1,4,[5],12:i:- outbreaks (Table 1-5), and numerous sporadic cases. Australian passive *Salmonella* surveillance data from live animals and humans suggests that Australian pig *S*. 1,4,[5],12:i:- prevalence may be increasing and may have surpassed *S*. Typhimurium in primary production (NEPSS, 2014). Prior to 2011, *S*. 1,4,[5],12:i:- isolates from Australian pigs were predominantly untypable or PT120, however, since 2012 phage type 193 has been reported with increasing frequency (Figure 1-3) (SA Pathology, 2013b; NEPSS, 2014; SA Pathology, 2014).

Table 1-4. Reported pork and pork-associated outbreaks of foodborne illness in the food service sector where the hazard was identified in Australia 1995-2012.

Year	*Salmonella* serovar	Food vehicle (suspected or implicated)	Cases
2011	1,4,[5],12:i:- PT193	Home-made pork salami	4
2011	Typhimurium 135	Spit roast pig	5
2010	Typhimurium 204	Barbequed pork	4
2009	Anatum	Uncooked pork sausage	5
2008	Johannesburg	Roast pork	14
2007	Oslo	Roast pork	3
2006	Bovismorbificans PT11	Capocollo (cured pork)	13
2006	Typhimurium PT170 var	Suspected pork in plum sauce, fried ice cream	2
2005	Typhimurium	Suspected roast pork	20
2004	Typhimurium RDNC, PT170	Roast Pork	5
2003	Typhimurium PT170	Spit roast pork	27
2003	Typhimurium PT170	Spit roast pork	12
2003	Typhimurium PT135	Pork rolls	213
2003	Typhimurium U307	Suspected roast pork	21
2003	4,12:d:-	Pork	4
2002	Typhimurium	Sliced ham	29
1997	Typhimurium PT1	Pork rolls	862
1997	Typhimurium PT9	Pork rolls	150
1995	Typhimurium PT9	Roast pork	22

Sources: Food Science Australia (2002), OzFoodNet Working Group (2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011a, 2011b, 2012b, 2012a, 2012d, 2012c, 2012e, 2012f, 2012g, 2013, 2015a, 2015b).

Table 1-5. Reports of Australian human *S.* 1,4,[5],12:i:- outbreaks.

Year	Location	Number of cases	Detail	References
2014	South Australia	8	Fair/festival, no vehicle implicated	OzFoodNet Working Group (2015b)
2013	Nationwide	54	Sporadic cases, no vehicle implicated	SA Pathology (2014)
2013	South Australia	5	Nosocomial, suspected person to person contact	SA Pathology (2013a)
2011	South Australia	4	Home-made pork salami. One hospitalization.	OzFoodNet Working Group (2012b)

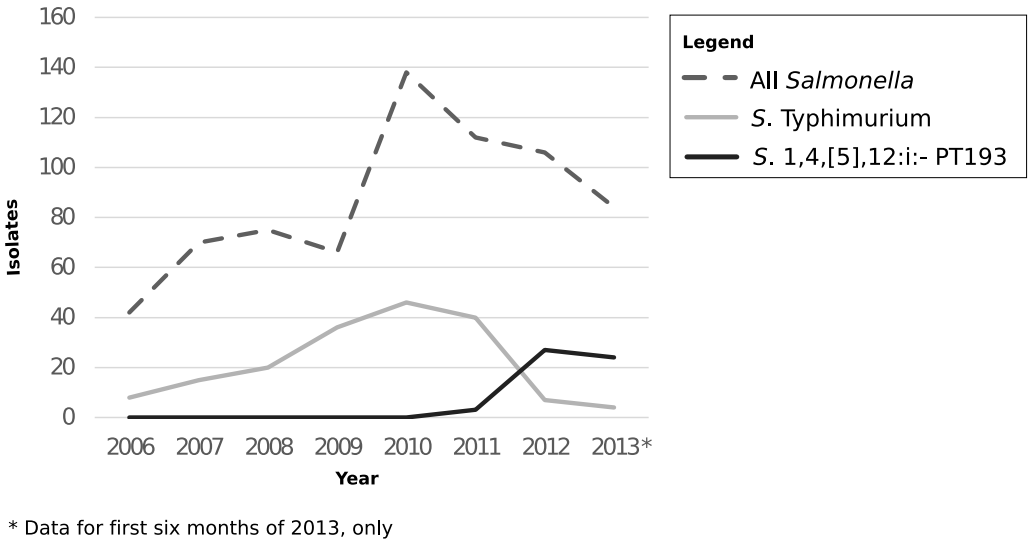

Figure 1-3. *Salmonella* spp. *S.* Typhimurium and *S.* 1,4,[5],12:i:- PT193 isolations from Australian pigs by year 2005-2012. Produced from NEPSS (2013) data.

There are indications that *Salmonella* isolations from Australian pigs may mirror reports of *S.* 1,4,[5],12:i:- displacing *S.* Typhimurium elsewhere (Figures 1-2 and 1-3)(Lailler et al., 2013; SA Pathology, 2013b; Simon et al., 2013). However, these data are obtained from passive surveillance and are therefore indicative but do not relate to a population and are prone to bias; special surveys, such as sampling conducted by this research, have been omitted from the results presented. The apparent surge in *S.* 1,4,[5],12:i:- isolations is likely enhanced by increasing recognition of the serovar and/or changes in testing and reporting protocols. It is also likely that some *S.* 1,4,[5],12:i:- were misclassified due to limited awareness of the serovar in laboratories and the need to test for flagellar antigen phase change and associated additional time and laboratory resources (Switt et al., 2009). Australian passive surveillance has been hampered by increases in the cost of *Salmonella* typing in the late-2000s and early 2010s, which affected the rate of submission of *Salmonella* isolates for further characterization.

Although the first Australian reference to domestic *S.* 1,4,[5],12:i:- PT193 isolation was made in 2011 (OzFoodNet Working Group, 2012b, d), unpublished data indicates that the first Australian *S.* 1,4,[5],12:i:- isolation may have been sourced from a suspected overseas acquired human case in 2007 (NEPSS, 2014). The first reported detection of a domestically acquired *S.* 1,4,[5],12:i:- case was canine, from a sample collected in 2009 (NEPSS, 2014). The first domestically acquired human case and isolation from a domestic food production animal (bovine) occurred in 2010 (NEPSS, 2014). The first domestic pig isolation was detected in 2011. By 2014 *S.* 1,4,[5],12:i:- had been isolated from Australian live cattle, beef, live poultry, poultry meat, live pigs, pork and pork

products, live sheep, live alpaca, horses, cats and dogs, and an echidna, as well as from feed products including milled feed, blood, meat and meat and bone meals (NEPSS, 2014).

It is interesting that *S*. 1,4,[5],12:i:- has emerged in Australia, given the enforcement of strict quarantine restrictions to minimize the risk of introductions and that the somewhat similar pandemic *Salmonella* Typhimurium DT104 was not isolated from domestic Australian sources (Hamilton et al., 2015). However, the nature of emergence of *S*. 1,4,[5],12:i:- in Australia remains unknown. The serovar could have emerged from circulating biphasic *S*. Typhimurium strain(s) in Australia or via introduction through pigs, pig-derived products, feed components, migratory birds, or humans—such as farm personnel who have spent time overseas, among other possible sources. Alternatively the serovar could have entered via pork products, however, while pork imports have increased over the past 20 years, Australia remains stringent in the nature of imports only allowing processed boned-out products or products that are processed upon entry to Australia making these products highly unlikely sources (APL, 2011).

1.4.3. Australian public perception of food safety risks

Food safety is gaining increasing traction among the major Australian food retailers, this is likely due to a combination of increasing consumer awareness of foodborne hazards and retailers seeking differentiation or marketing parity with competitors. Consumers and retailers perceive pork and pork products, among others, as relatively high risk for *Salmonella* and other foodborne pathogens; a perception which the industry is trying to change given evidence of relatively low prevalence of *Salmonella* serovars of importance to public health (Galanis et al., 2006; Hamilton et al., 2015).

Australian consumer and retailer perception of risk in relation to specific serovars is limited. The serovar *S*. 1,4,[5],12:i:- is a relatively recent addition to international food safety agendas and is not generally recognized by livestock industry stakeholders nor consumers in Australia. However, as described, *S*. 1,4,[5],12:i:- has been isolated from human cases with increasing frequency in recent years, which may raise concerns among public health officials and lead to reevaluation of current surveillance systems and regulations. Increased public health concern might also affect major retailer terms of supply. Changes in regulations and the possibility of future product recalls would increase costs of pig production in Australia, which, as a negative externality, the industry would most likely bear. The industry recognizes the importance of mitigating and managing risk of future outbreaks, to maintain consumer trust and competitiveness and to limit the real or perceived need for additional regulation.

1.4.4. Australian *Salmonella* controls employed on-farm

The majority of Australian commercial herds employ all-in, all-out systems for grow-out pigs as a cornerstone of disease management, although it is not uncommon for some batch mixing to occur. Various antimicrobials are permitted for use at subtherapeutic levels for growth promotion, and an expanded set for treatment of disease. Metal oxides and other microingredients are included in most commercial pig rations. Dietary organic acids, such as acetic, formic, propionic and butyric acids, have variously been shown to promote growth and aid management of undesirable bacteria in weaned pigs (Partanen and Mroz, 1999; Van der Wolf et al., 2001a; De Busser et al., 2011; Arguello et al., 2013a). The use of organic acids as an alternative to prophylactic antimicrobials and/or mineral oxides in weaned pig diets has increased with growing concerns over the development of antimicrobial resistance and the build-up of metals, such as copper and zinc oxides, in the environment (Stensland et al., 2015). Five of the 16 herds sampled in the course of this study supplemented feed or water with organic acids. Various prebiotics and probiotics are used in some herds, though not primarily to control *Salmonella*. Vaccination for *Salmonella* it is extremely rare. Depopulation-repopulation are employed relatively commonly, often the 'Swiss depop-repop' method, to combat embedded disease problems, typically in response to dysentery or mycoplasma issues. Some farms use meal feeds and low grind pellets, which are believed to have a protective effect in relation to *Salmonella*, though they are not specifically employed for this purpose (Mikkelsen et al., 2004; Wilhelm et al., 2012; Lebel et al., 2013).

By and large, Australian herds enjoy the natural biosecurity advantages of a relatively dry climate and, often, relatively large distances to the nearest neighbouring pig herd. However, these conditions vary considerably between regions, the southeast being relatively wetter and herds are more concentrated. The majority of commercial herds employ strong biosecurity measures in terms of controlling on and off-farm traffic and enforcing minimum periods between farm visits. Cleaning and disinfection practices vary between herds, the majority employing pressure cleaning and a disinfection agent between cohorts. However, much of the industry houses pigs in conventional buildings—typically employing concrete and/or slatted flooring and concrete or barred pen partitions—that are relatively dated, presenting a challenge in terms of maintaining hygiene.

1.4.5. Australian pig industry *Salmonella* surveillance and controls

Swill feeding is banned in Australian pig production. The industry also implemented new rendering standards in 2007 with the objective of improving hygiene and increasing product safety (CSIRO, 2007). The industry advocates Good Agricultural Practices in production through the food safety quality assurance programme, an element of the Australian Pork Industry Quality Assurance

Programme (APIQ). This includes the recommendation to withhold feed for six to 24 hours before slaughter, in part to mitigate the risk of *Salmonella* carcass contamination by reducing bacterial build up and the likelihood of rupture of the gastrointestinal tract during evisceration.

The application of Hazard Analysis and Critical Control Points (HACCP) was mandated in Australian meat processing in the late 1990s. The ESAM surveillance system monitors *Salmonella* on carcasses, with swabbing of one in 5000 pig carcasses, primarily for the purposes of export market access (AQIS, 2003, 2007). The industry also devotes considerable resources to research projects on *Salmonella* and other food safety issues. The processing industry employs Good Management Practices and is moving towards more holistic process control approaches that routinely monitor hazards in boning rooms, in addition to current routine carcass sampling through ESAM.

At present Australian regulations and retailer requirements in relation to *Salmonella* surveillance and control in pork supply are relatively lenient in comparison with some other jurisdictions. For example Denmark, where herd status and logistic slaughtering—strategic slaughtering of finished batches in accordance with assessed risk—are employed (Alban et al., 2012).

Australian food safety standards are developed and administered by the bilateral Food Standards Australia New Zealand (FSANZ). The authority builds on the 'Food Standards Australia New Zealand Act 1991' in maintaining the 'Australian New Zealand Food Standards Code'. The standards adopt a risk-based whole chain approach to establishing standards and monitoring of food safety in primary production and processing. This includes on farm and post-farmgate quality assurance monitoring and verification addressing industry foodborne hazards, such as *Salmonella* in pigs and pork.

1.4.6. *Salmonella* spp. in the Australian pig and pork supply chain

Salmonella prevalence among Australian pig herds has not been reliably estimated (Hamilton et al., 2006). *Salmonella* detection is commonplace, however, *S.* Typhimurium prevalence among Australian pigs has, historically, appeared low relative to European and US pigs (Hamilton et al., 2015). In a study to investigate variation in *Salmonella* ecology within herds Hamilton et al. (2004) used the 'Australian' mix-ELISA among 23 study herds selected on a variety of risk factors and found an average 35% seroprevalence, with considerable variation, three to 78%.

At present no *S.* 1,4,[5],12:i:- prevalence in Australian pig derived products are available. The scope of Australian pig *S.* 1,4,[5],12:i:- herd colonization is unknown. However, as shown previously, passive surveillance data indicates that the number of infected pigs and herds may be higher than *S.* Typhimurium and appears to be increasing (NEPSS, 2011, 2013; SA Pathology, 2013a, b; NEPSS, 2014; SA Pathology, 2014). In addition, industry stakeholders have reported identifying the serovar with increasing frequency, often in connection with clinical disease among weaners (personal

communication with consulting veterinarians and primary laboratories). Identification of *S.* 1,4,[5],12:i:- from post-mortems after sudden death in young weaners and in association with morbidity among growers have been reported in multiple herds.

1.4.7. Pig carcass and product exposure to *Salmonella* in Australia

A number of Australian studies of *Salmonella* presence at slaughter and through processing were conducted in the early to mid-1990s and found up to 10% of carcasses were contaminated, although there was considerable variability between abattoirs (Widders et al., 1996). However, Australian abattoirs and processing have since improved hygiene considerably and now implement critical control points and GMP. Several national baseline surveys of foodborne hazards associated with Australian pork products have been conducted since, their key findings are considered below (Coates, 1997; Hamilton et al., 2007; Hamilton et al., 2008; Hamilton, 2011).

In terms of Australian pork products, though somewhat dated, in a study comparing Australian pig postmortem inspection procedure methods Hamilton et al. (2002) found *Salmonella* spp. retail cut prevalence of 1.4% (4/296). A later study that sampled raw pork sausages from around Australia found 17.2% (5/29) and 5.7% (5/87) of butcher shop and supermarket samples, respectively, positive for *Salmonella* (Figure 1-4)(Hamilton et al., 2008; Hamilton, 2011). The higher numbers of detections from butchers' sausages most likely reflects the use of trim, as opposed to low value primal cuts in supermarket sausages. A related study of the same year found higher rates of *Salmonella* detection from offal and sow meat in processing. Hamilton et al. (2008) found *Salmonella* present in 17% (21/127), 22% (64/125) and 38% (48/125) of hearts, livers and tongues, respectively, which could be associated with contamination during evisceration. The same authors also found sow meat *Salmonella* prevalence was also relatively high, which they suggested may relate to the added difficulty of dressing sows (Hamilton et al., 2007; Hamilton et al., 2008). Hamilton (2011) found relatively low *Salmonella* prevalence in minced pork. The authors suggest that this may relate to the use of shoulders in Australian pork mince production and the high hygiene standards employed in the large-scale facilities producing these products (Hamilton, 2011). Pointon et al. (2000) found submaxillary lymph node *Salmonella* spp. prevalence in slaughtered pigs to be 1.4% in their control group (no grossly detectable abnormalities) of 500 carcasses. In this study, *Salmonella* spp. was detected only once in other lymph nodes tested from 400 controls which include portal (1/100), lumbar (0/100), iliac (0/100) and superficial inguinal (0/100) (no samples were taken from mesenteric lymph nodes because they are discarded after inspection) (Pointon et al., 2000). On the basis of these studies *Salmonella* spp. prevalence in Australian pork products overall is likely to be <3%, which compares favourably with rival producers, such as the EU where *Salmonella* has been detected in lymph nodes on up to 29% of carcasses, 9% of samples collected from primary processing and 6% from retail products (EFSA, 2008; Snary et al., 2016).

The results of Australian carcass sampling show the presence of *S*. Typhimurium has been low and stable since 2000. The first isolation of *S*. Typhimurium PT193 occurred in 2007, while *S*.

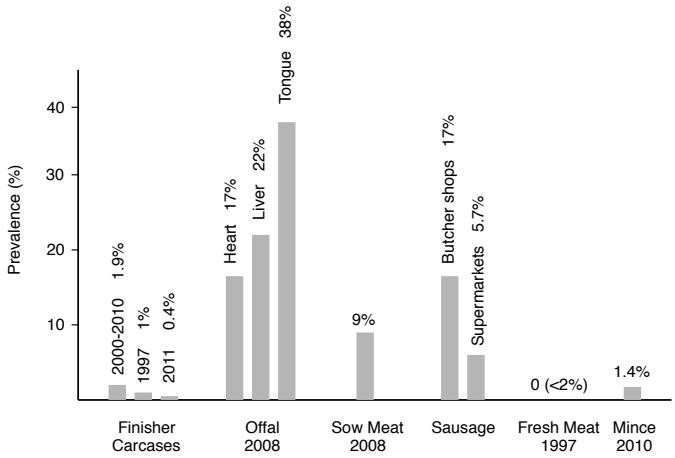

Figure 1-4. *Salmonella* prevalence estimates from Australian pork products. Adapted from Hamilton (2011).

1,4,[5],12:i:- was first identified in 2009 (Table 1-6).

Table 1-6. Pork carcass regulatory monitoring results for *Salmonella* spp., *S.* Typhimurium, *S.* Typhimurium PT193 and *S.* 1,4,[5],12:i:-

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Salmonella spp.	7	28	18	32	23	15	13	14	18	17	8	19	23	15
*S.*Typhimurium[a]	1	1	1		3	1		1	3		1	2	1	2
S. Typhimurium PT193								1				2		
S. 1,4,[5],12:i:-										2		1		1
Other serovars[b]	6	27	17	32	20	14	13	12	15	15	7	14	22	12
No. of samples	586	1023	1147	1315	1284	1031	1066	1018	1051	987	891	1074	1025	858

[a] Excluding *Salmonella* Typhimurium PT193 and monophasic *Salmonella* spp.

[b] *S.* London, *S.* Derby and *S.* Infantis were the serovars most commonly isolated over this period (in descending order).

Source: A. Pointon, personal communication, data from the Australian Department of Agriculture, Canberra.

1.4.8. Australian consumer exposure

Australians are estimated to consume approximately 22kg of pork and pork derived products per person per year (APL, 2014). Australian shelf life standards are three to five days for raw chilled pork at 5°C, six months for frozen pork portions at -12°C, 10 months at -18°C and two to three months for frozen pork mince (PrimeSafe, 2016). Pork is typically well cooked by Australian consumers due, primarily, to effective historic risk communication campaigns. However, the industry is currently promoting shorter cooking times due to the negative effects of overcooking (at high temperatures) on eating quality. The increased risk associated with reduced cooking times has not yet been quantified.

1.5. Preliminary risk characterisation

The risk characterization approach used here is an adaptation of the FAO/WHO (2003) and ICMSF (2002) approach and methods. This section presents a qualitative discussion of the potential *S*. 1,4,[5],12:i:- risk associated pork products in Australia, with brief reasoning and caveats for the rating ascribed.

Process for Risk Rating of Hazard

Hazard Severity:

IA Severe hazard for general population: life threatening or substantial chronic sequelae or long duration

IB Severe for restricted populations: life threatening, in this case to immunocompromised, infants and the elderly.

II Serious, incapacitating but not life threatening; sequelae infrequent; moderate duration

III Moderate, not usually life threatening; no sequelae; normally short duration; symptoms are self-limiting; can be severe discomfort

Key considerations in risk characterization

Occurrence risk:

This considers the estimated prevalence of the hazard and is classified as low, medium or high. Where no Australian data is available and no Australian outbreaks have been recorded with a given food and a specific pathogen, but an overseas epidemiological link has been established, the likelihood is rated as low.

Growth:

This provides an indication of whether growth of a hazard in the product is required to cause disease.

Processing effect:

This considers if the production, processing or handling of the food may increase, decrease or not affect the hazard.

Consumer terminal step:

This considers whether the terminal step is likely to increase or reduce the likelihood of the exposure. This considers consumer steps such as cooking is applied to the product.

Epidemiological link:

This assesses whether there is documented evidence of food poisoning incidents with this hazard-product combination.

Comments:

This considers other factors that may affect the risk rating of the product.

Risk rating:

This is assessed as Low, Medium or High. The greatest emphasis is placed on the severity and occurrence risk, and to a lesser extent the likelihood of failure of the consumer terminal step, if there is a consumer terminal step, and the chance of growth of the hazard in products.

Results

The rationale for the values ascribed to each factor considered within the following risk rating table (Table 1-7) is based on the evidence provided in the preceding sections of this document.

Table 1-7. Summary for the rationale relating to each risk rating factor considered.

Hazard	***S.* 1,4,[5],12:i:-** Where specific data relating to *S.* 1,4,[5],12:i:- was unavailable *S.* Typhimurium or *Salmonella* spp. data is used.
Severity	**Moderate** for healthy consumers (III). **Severe** for immunocompromised populations, the elderly and infants, in such cases the illness can be life threatening; long-term sequelae are infrequent but may be costly (IB).
Occurrence risk	**Low** There are no on-farm prevalence figures for *S.* 1,4,[5],12:i:- in Australian pigs, on-farm or at finish, nor for pig derived products available. There has been little research conducted internationally on the ecology of the hazard in pig populations and the possible hazard burden among finished pigs. *Salmonella* spp. prevalence in Australian pork products is estimated to be <3% (Pointon et al., 2000; Hamilton et al., 2002; Hamilton et al., 2011). Hazard levels in imported products likely vary with product and origin, but are most likely low as Australia does not allow fresh imports. Ready-to-eat products with moderate pH likely present as the highest risk to consumers, relatively.
Growth	**Medium in chilled products** **Low in frozen products** Growth in product significantly increases the risk of disease in the consumer due to the *Salmonella* dose-response relationship. If temperatures below approximately 7°C are maintained the hazard will generally not multiply. The likelihood of temperature abuse above 7°C prior to consumption is very low in frozen products and marginally more likely in chilled products. There is little concrete data available on the risk of growth in/on Australian product, the risk is assumed to be relatively low.

Processing effect	**Low for primal derived products** **Low for joints e.g. bone in leg of ham** **Risk is increased for minced and needle tenderized products** **Risk is increased for offal** **Risk is increased cooked and uncooked processed products, such as smallgoods** Slaughter and processor hygiene will affect the likelihood of contamination of product. Critical points include evisceration and potential for cross contamination between carcasses directly or via fomites such as hands, aprons, knives, bung droppers, chains and surfaces. Australian abattoir hygiene is generally of a high standard. Carcass decontamination steps should inactivate the majority of organisms. While no specific data has been identified to date, needle tenderizing may increase the risk of bacteria penetrating primal cut products by breaching the product surface barrier. Needle tenderizing also presents a possible means of fomite mediated transfer from contaminated to uncontaminated products, if previous control steps have not been effective and hygiene levels have not been maintained. Mincing increases the risk of presence of the hazard in the product, as contributions from multiple carcasses may occur, the surface barrier is no longer intact and the hazard may be distributed throughout the product. Furthermore, the nature of the product may be conducive to organism survival and, subsequently, in the consumer's gastrointestinal tract—a more homogenous product with higher fat content—further increasing the importance of effective consumer terminal steps. Processing of offal is likely to increase risk due to the potential of faecal contamination during evisceration, the possibility of offal contamination from lymph nodes, where the hazard had colonized this system, and that tripe and other gastrointestinal tract products are included in this product group. Fermentation and curing has little effect on *Salmonella* growth unless a pH of below 4.0 is achieved and maintained. Uncooked fermented or cured products may present a heightened risk as many are consumed without cooking by the consumer. Chilling to below approximately 7°C will halt hazard replication but is an ineffective method of inactivation.
Consumer terminal step	**The risk is reduced for all products that are in the large majority of instances cooked by the consumer before consumption.** **The risk is not reduced for ready-to-eat products.** Adequate cooking temperatures inactivate the hazard. However, the

	specific temperatures will depend on the product composition and structure and the hazards thermal history. For example, high fat minced products present a greater risk and require higher cooking temperatures and/or duration for inactivation. Primal cuts without further processing present relatively low risk as the hazard is typically only present superficially and the consumer, typically, cooks the product prior to consumption. Minced products have higher risk of contamination due to the likely presence of contributions from multiple carcasses and higher risk due to the potential of the hazard being distributed throughout the product. This likely necessitates reaching and maintaining internal temperatures for inactivation prior to consumption. For example, chilled mince is more likely to be used in burgers, which present a higher risk method of consumption than slow cooked recipes due to the method of consumer preparation and risk of internal temperatures not reaching levels that inactivate the hazard. Ready-to-eat products that do not require cooking present a heightened risk if controls fail, as they are typically not cooked by the consumer. Additionally, these products typically incorporate meat from multiple carcasses and have high fat content relatively homogenously distributed.
Epidemiological link	**There are epidemiological links for all products** There is epidemiological evidence of *S.* 1,4,[5],12:i:- illness implicating consumption of specific pork and other pig derived products in Australia and extensive international epidemiological evidence of human *S.* 1,4,[5],12:i:- infection implicating pig derived products.
Relative risk ratings and general comments	**Fresh/chilled/frozen primals: low risk** Fresh and chilled primal products, such as cuts and joints, are considered low risk due to relatively low likelihood of contamination when compared with other cuts and products, the intact surface barrier, and the likely effectiveness of consumer terminal steps. Frozen products are considered low risk as the likelihood of product temperature exceeding 7°C in the supply chain is considerably lower than among chilled, cured or fermented products. **Offal: medium risk** Offal is higher risk than primals due to the higher likelihood of contamination during evisceration and the unlikely possibility of animals suffering systemic infection entering the abattoir. However, these products are consumed in low volumes in Australia and in the large majority of cases the consumer terminal step will destroy *Salmonella* organisms that may be present. **Uncooked processed products: high risk** More heavily processed uncooked products, primarily comminuted (mince, fermented, unfermented) or needle tenderized products, are

considered high risk due to the increased likelihood of direct or fomite mediated cross-contamination and increased risk of control step failures. Comminuted products also increase risk due to the likely contributions of multiple carcasses in individual products, and as the risk of cross contamination is such that control failures could lead to widespread distribution of contaminated products and large scale outbreaks, as occurred with frozen pot pies in the US. Depending on the specifications of the product, risk is further increased by the breach of the product surface barrier, more homogenous distribution of the hazard through the product, products providing greater protection to the hazard—such as high fat content, and potential for no or inadequate consumer cooking i.e. failure to reach/maintain necessary internal temperatures for inactivation.

Cooked processed products: high risk
Products that are processed and cooked are considered to be of higher risk as they are often ready-to-eat, thus removing the cooking step by the consumer. However, effective application of GMP and HACCP in Australian processers minimizes the risk.

Processed products encompass a wide range of products, some of which are consumed predominantly by culture-based subpopulations, therefore, the risk associated with specific products may be broad or narrowly focused within the Australian population.

1.6. Major knowledge gaps and research questions

The food safety and production risks associated with *S*. 1,4,[5],12:i:- have not yet been described. In order to assess and manage food safety risk associated with Australian porcine *S*. 1,4,[5],12:i:- a better understanding of the occurrence, nature of colonization and potential risk of pig and consumer exposure is required. Consumer exposure risk is dependent on hazard levels on product, which, working backwards, ultimately derives from hazard levels in processing and boning rooms, at abattoirs, and in finishers and herds. It is well recognized that carcass contamination is affected by the presence of the hazard in finished pigs at slaughter, in lairage and in transport, which is ultimately determined by hazard levels in primary production (Mousing et al., 1997a; Dahl and Sørensen, 2001; Alban and Stärk, 2005; Hauser et al., 2010; Alban et al., 2012; De Busser et al., 2013; Andres and Davies, 2015; Snary et al., 2016). Therefore, while critical control points in the postharvest chain are essential, a clearer picture of the scope and nature of the serovar's colonization within the Australian herd is needed to inform occurrence risk, risk characterization and ultimately to design risk management strategies that mitigate risk within herds.

How widespread has *S.* 1,4,[5],12:i:- become in the Australian industry?

Although *S.* 1,4,[5],12:i:- colonization of an Australian pig herd has been confirmed, it is not known whether the serovar is confined to specific geographic regions or types of production system within the Australian industry. This would affect level of food safety risk associated with particular producers and supply chains, which could aid the industry in developing optimally targeted interventions to mitigate *S.* 1,4,[5],12:i:- associated risk. Furthermore, *S.* 1,4,[5],12:i:- can be pathogenic and virulent in pigs. Identifying the types of herd colonized could inform Australian producers and veterinary practitioners in terms of diagnosis and management of clinical disease on farm.

Identifying common factors between *S.* 1,4,[5],12:i:- colonized herds could aid generation of hypotheses on possible methods of introduction to herds and risk factors for disease and maintenance of colonization within herds. This could lead to identification of potential pathways, important nodes and different management practices that with further investigation could offer insights into the transmission of *S.* 1,4,[5],12:i:- and other infectious agents within the industry. This would have implications for biosecurity and broader animal health regimens currently employed on farms and in the industry as a whole.

What happens in *S.* 1,4,[5],12:i:- colonized herds over time?

Salmonella shedding within herds is complex and, as pigs can shed the bacteria intermittently, can vary considerably between points in time (Funk et al., 2001; Pires et al., 2013a). Funk et al. (2001) showed that point estimates of *Salmonella* prevalence and the serovars present may not be reliable means of establishing *Salmonella* status in pig herds. To determine the likely hazard burden at the farm gate a more nuanced description of *S.* 1,4,[5],12:i:- and contemporary *Salmonella* spp. shedding among grow-out pigs is required. Hamilton et al. (2015) demonstrated persistent shedding of *S.* 1,4,[5],12:i:- among grower pigs in a single herd in which *S.* 1,4,[5],12:i:- appeared to be the only serovar present. Critically, this study demonstrated a potential pathway into the human food chain. Investigation of *S.* 1,4,[5],12:i:- shedding dynamics and apparent hazard load at finish in other herds and contexts, such as in herds harbouring multiple *Salmonella* serovars, is needed to better understand the ecology of *S.* 1,4,[5],12:i:- within pig herds. Furthermore, Hamilton et al. (2015) sampled grow-out pigs exclusively. Investigating extent, persistence and characteristics of *S.* 1,4,[5],12:i- and contemporary serovar shedding from sows through to finish could provide insights into hazard maintenance and cycling within herds with implications for targeting control measures.

The ecology of *S.* 1,4,[5],12:i:- within herds is not well understood. Identifying possible modes of cycling, maintenance and transmission between individuals, cohorts and production stages could aid identification of possible risk factors for colonization of *S.* 1,4,[5],12:i:- and related *Salmonella* serovars. Hypothesis generation in relation to dynamics of colonization and shedding within herds

will contribute to the epidemiological literature in relation to *Salmonella* in livestock production systems and will indicate possible on-farm risk management strategies.

How diverse is the Australian porcine *S.* 1,4,[5],12:i:- population?

To address the question of origin it is necessary to assess whether the *S.* 1,4,[5],12:i:- population currently circulating in Australia is most likely a single clone or comprised of multiple clones. It is not known if the serovar emerged via a single event or has emerged on multiple occasions. Identification of multiple clones or a predicted most recent common ancestor that corresponds to a realistic timeline of introduction would provide evidence of emergence via a single event or multiple events. The recent publication of a core genome SNP-based ancestral phylogeny of British and Italian epidemic *S.* 1,4,[5],12:i:- sequences from human, animal and product sources provides strong indications that the strains investigated were part of the same clade undergoing clonal expansion (Petrovska et al., 2016). Investigating the relatedness of the Australian porcine *S.* 1,4,[5],12:i:- population could have implication for national, industry and herd biosecurity.

In addition, determining the levels of relatedness among isolates may provide indications of the nature of the organisms' spread within the industry. This could provide identifications of possible routes of transmission and herd-level risk factors for *S.* 1,4,[5],12:i:- and comparable infectious agents. This could have implications for industry and herd level biosecurity.

Furthermore, with the rapid rise of *S.* 1,4,[5],12:i:- isolations internationally, from both pigs and humans, and indications of similar trends in Australia, there is a need for further investigation of the phenotypes and genetic characterstics of the serovar. The current literature includes some tentative speculation on the rise of *S.* 1,4,[5],12:i:- in Europe and the possible bases for the apparent selective or competitive advantages of the organism over *S.* Typhimurium. However, the biological and epidemiological nature of, and reasons for, the apparent rise of the serovar remain uncertain.

How does the Australian S. 1,4,[5],12:i:- population compare to *S.* 1,4,[5],12:i:- populations reported internationally?

The origins of *S.* 1,4,[5],12:i:- in Australia are unknown. It is possible that the serovar emerged in Australia independently from an *S.* Typhimurium strain or strains circulating domestically, as appears to have occurred in Europe (García et al., 2013). Alternatively, the serovar may have been introduced to Australia from overseas. Comparing the characteristics of Australian isolates with domestic *S.* Typhimurium isolates and *S.* 1,4,[5],12:i:- isolates reported overseas could provide indications of whether the serovar emerged in Australia independently or was introduced from overseas.

What are the resistance characteristics and determinants of Australian porcine S. 1,4,[5],12:i:-?

Antimicrobial usage and resistance are major concerns for public health authorities globally and consumer awareness of these issues is an increasingly important factor in consumer decision-making. Inevitably these issues will increasingly affect retailer demands and production practices. Multidrug resistance among *S*. 1,4,[5],12:i:- strains is commonly reported internationally (Moreno Switt et al., 2009; García et al., 2016; Petrovska et al., 2016). The Australian *S*. 1,4,[5],12:i:- population appears to describe comparable resistance types (NEPSS, 2014; Hamilton et al., 2015). Further information on the levels and dynamics of *S*. 1,4,[5],12:i:- and other *Salmonella* serovar antimicrobial resistance phenotypes is needed to inform animal and public health. Determining the *S*. 1,4,[5],12:i:- resistance mechanisms will also allow further comparison with overseas reports, providing further indications for hypothesis generation in relation to the serovar's emergence within Australia. Furthermore, comparing herd management practices with *Salmonella* spp. resistance phenotypes and gene repertoires through cross-sectional studies and over time could provide important information for veterinary practitioners, producers and regulators.

What are the optimal typing methods for *S.* 1,4,[5],12:i:-?

A cornerstone of effective *Salmonella* surveillance is the reliable, comparable and meaningful typing and subtyping of strains. At present traditional phenotyping methods for *Salmonella* continue to be employed alongside a plethora or molecular methods. The molecular typing method MLVA is now widely employed globally and in Australia for *S*. Typhimurium and *S*. 1,4,[5],12:i:- and has surpassed PFGE as the most widely reported method outside the US. However, while MLVA presents a number of logistical and analytical benefits the stability of profiles and the meaning of similarity and/or variation at specific loci, when to cluster and when to differentiate, remains uncertain. Furthermore, the rapid decline in costs of whole genome sequencing and comparative genomic studies suggest that sequencing will eventually overtake other typing approaches. Given the costs of typing and recent changes in Australian cost recovery for such services, identifying the optimal 'package' of typing methods for epidemiological studies and risk analysis and cost efficiency would be of value to public health, animal health and industry.

The genetic bases of key *S*. 1,4,[5],12:i:- phenotypes have been discussed in the literature, and hypotheses proffered, however, consensus has not yet been reached. For example, the basis for monophasism has been linked uncertainly to the presence and expression of various functional genes; the relationship between monophasic expression and R-type remains unclear; anecdotal evidence suggests *S*. 1,4,[5],12:i:- exhibits greater persistence and extent within colonized herds, but, again, this has not been effectively documented. Comparative research on genetic fingerprinting techniques, such as MLVA, in conjunction with whole genome sequencing and analyses could offer further insights phenotypes and the nature of *S*. 1,4,[5],12:i:- colonization. Furthermore, these analyses can also aid in the evaluation of molecular fingerprinting techniques, such as MLVA, as tools for pathogen surveillance and epidemiological investigations.

Chapter 2 - Materials and Methods

2.1. Introduction

This chapter details the materials and methods employed in the study. The chapter includes descriptions of the design and sampling methodology used in each study, the *Salmonella* detection and characterization methods employed and the statistical, including bioinformatic, methods employed in analyzing the resulting data.

2.2. Occurrence and diversity study sampling

A cross-sectional observational study design was employed to investigate occurrence and diversity of *S*. 1,4,[5],12:i:- and *Salmonella* spp. shedding among Australian pig herds. The study initially sought to sample ten pig herds that approximated the geographic and production system diversity employed in Australian commercial pig production systems operating in a Mediterranean-like climate. The study was subsequently expanded to include a further six herds.

Herd selection. Herds were selected to maximise diversity of Australian commercial pig production in terms of location, scale, management, production system and inputs. The herds were selected on the basis of: confirmed or suspected colonization with *S*. 1,4,[5],12:i:- or *S*. Typhimurium, based on veterinary records and evidence of clinical enteritis indicative of salmonellosis in the preceding 12 months; representativeness of major pig producing regions in Australia operating in a Mediterranean-like climate; approximate representativeness of scale and animal husbandry systems employed in Australian commercial pig production; willingness to participate and accessibility. The clinical condition of salmonellosis in pigs was defined as: scouring and/or thriftiness in weaners, growers or finishers, death or signs of morbidity, and subsequent confirmation of the presence of *Salmonella* through culture and confirmation; upon post mortem lesions in the colon and/or ileum may or may not have been present. The 16 herds were located in four Australian states—New South Wales (4), South Australia (5), Western Australia (3) and Victoria (5). Herd sow numbers ranged from 100 head to >800 head.[1] The sampled herds employed a range of conventional, deep-bedding, outdoor and mixed production systems, they employed a variety of reproductive, nutritional and animal health management practices and sourced inputs from a range of different suppliers. Among the herd sampled, 15/16 were selected on the basis of previous isolation of *S*. 1,4,[5],12:i:-; the exception was Herd 16, which was selected on the basis of previous *S*. Typhimurium isolation. Herd 16 was sampled speculatively to see if *S*.

[1] Due to the structure of the Australian pig industry, the number of sows has not been specified from herds to maintain confidentiality.

1,4,[5],12:i:- isolates might also be present, which may have provided indications of their relatedness.

The sources of all isolates were profiled in detail through observation and/or discussions with management and/or clinicians. Further detail on the herds in provided in Chapter 3.

Sampling design. An opportunistic study design was employed to maximize coverage and representativeness of herds and the *S*. 1,4,[5],12:i:- and contemporary serovar populations in the Australian industry. Faecal samples were collected in the period December 2012 to March 2014. Sampling focused on terminal line cohorts. Weaners were prioritized as practitioners observed clinical signs of salmonellosis most commonly in this age group. Finisher sampling were also prioritized as a potential route of the hazard into the human food chain. However, the study design was flexible and, where possible, sampling sought to represent the herd. Other age groups sampled included: replacement gilts, gestating sows, lactating sows and litters, boars and growers (Table 2-1). When sow crates were sampled they were treated as a single unit, as it was assumed that individual sows and litters would shed similar *Salmonella* populations; contributions from both sow and suckling pigs were collected from five to six non-sequential crates.

Table 2-1. Cross-sectional study sampling design.

Sample source population	**Definition**	**Sample type (contributing pats per sample)**[a]	**Sampling method**	**Sample number per source** (approximate number of pigs represented)
Weaners	Wean (3 to 4 weeks) to 10 weeks of age	Pooled (5-6) faecal	Fresh from floor	5-6 (25-30), opportunistic
Finishers	15 weeks to finish (22 to 24 weeks)	Pooled (5-6) faecal	Fresh from floor	5-6 (25-30), opportunistic
Other pigs	Dry sows, lactating Sows/suckling pigs, replacement gilts, growers 10-15wks, boars	Pooled (5-6) faecal	Fresh from floor	Opportunistic
Total samples per sampling occasion per herd (total pigs represented per sampling occasion)				8-20 (40-120)

[a] individual samples were collected in from one herd, Herd 9.

Sampling methods. In total 243 faecal samples were collected. All samples were pooled samples with the exception of individual samples from one herd (Herd 9), which were collected by a collaborator. Where pooled samples were collected portions of five to six undisturbed faecal pats, each weighing approximately 5g, were collected from a single pen floor and aggregated (pooled) in

a sterile 120ml pot. Where pigs were housed in large groups, pats were collected from distant areas within the pen/latrine area. In some cases, conventionally housed weaners occupied rooms that were partitioned into smaller groups of 10 to 30 weaners, in which case the entire room was defined as a pen and a sample was collected from five or six of the partitioned groups. It has been shown the faecal weight influences the results of culture for *Salmonella* with a sample weight of >25 g being required to ensure a relative sensitivity of 75% (Funk et al., 2000b). Hence, sample weights of >25g were desired and largely achieved, with occasional exceptions among samples from young weaners. The author conducted the sampling when logistically feasible (14/16 herds), in which case 14-20 pooled faecal samples were collected, otherwise the samples were collected by the consulting veterinarian, in which case sample numbers ranged from eight to 20. The number of occupied pens at the time of the visit and logistical considerations determined the number of samples collected. All samples were de-identified and immediately stored in iceboxes at approximately 4°C.

2.3. Herd 4 case study sampling

A prospective longitudinal observational study was designed to investigate *Salmonella* shedding among grow-out pigs in a farrow-to-finish pig herd.

Herd selection. The herd was selected on the basis of results of the previous cross-sectional sampling. The herd had been selected for participation in the cross-sectional study on the basis of previous laboratory results identifying *S.* 1,4,[5],12:i:-. The consulting veterinarian, who had collected samples following an acute and extensive outbreak of clinical enteritis, provided the preliminary laboratory confirmation. The other criteria considered during the selection process were: herd size, rearing system, accessibility, willingness of the ownership to participate, and willingness of the consulting veterinarian to participate. Further detail of the herd is provided in Chapter 3 and Annex 1.

Sampling methods. The study was conducted in close consultation with the pig specialist veterinary practitioner who consulted to the herd. Sampling occasions were coordinated with the veterinarian's regular visits. Ten to 20 individual faecal samples were collected from a cross section of weaners, growers and finishers (3-4 weeks to 22 weeks) at approximately two-month intervals. The first sampling was reported in the cross-sectional study (Chapter 4), in which pooled samples were collected. In total 56 samples were collected. The majority of samples weighed between 5.7g and 25.8g, with the exception of five samples collected from diarrhoea in young weaner pens (3-7 weeks) that weighed less than 5g. The author collected the second batch of samples while visiting the herd, on the other sampling occasions the consulting veterinarian collected the samples. The number of samples collected by the veterinarian varied due the time available during the consultations.

2.4. Longitudinal study of five herds sampling

The study employed a prospective longitudinal observational study design to investigate *Salmonella* shedding in five farrow-to-finish pig herds located in two southern states of Australia. Samples were collected at three-month intervals in 2014 and 2015. *Salmonella* 1,4,[5],12:i:- had been detected in each herd on at least one occasion prior to the first sampling of this study.

Herd selection. Herds were selected to provide comparable case studies of Australian commercial pig herds. Herds were defined as a single flow of pigs from farrow to finish, irrespective of business ownership or number of sites. The sow herd sizes ranged from 400 to 600 head, approximating the mean parent herd size in Australian commercial production. The herds were geographically isolated and had no live animal linkages. Each herd employed their own transport to deliver pigs to slaughter; products were destined for both domestic and export markets.

Three herds employed multi-site production systems and two employed single-site operations. One of the multi-site herds (Herd 3) was owned and operated by two separate enterprises, operating as farrow-to-wean and wean-to-finish businesses, in a sole supplier-client arrangement resembling a contract grower. The herds were selected on the basis of: laboratory confirmed presence of *S*. 1,4,[5],12:i:-; an appropriate production system i.e. 400-600 sow farrow to finish with no external sources of terminal stock and high heath status—defined as less than 3% average post-wean mortality; approximate representativeness of Australian conventional enclosed sheds and deep bedding systems, willingness to participate and accessibility. Each herd was profiled in detail at the first visit and the profiles were updated on subsequent visits. Further detail on the sampled herds is provided in Chapter 6 and Annex 1.

Sampling design. The five herds were sampled at three-monthly intervals over one year to monitor persistence over an extended time period. Herds were stratified by age group (Table 2-2). On each sampling occasion five pooled faecal samples (six pats per sample) were collected from gestating sows, sows and litters, weaners and finisher stock, respectively. Dry sows were defined as gestating sows—empty sows and sows in-pig prior to the move to the farrowing house; weaners were 3-4 weeks to 10 weeks old; finishers were 15 weeks to finish (22-24 weeks); samples from farrowing crates included faeces of lactating sows and suckling piglets. The collection of 20 pooled samples per herd sampling occasion, representing approximately six pigs per sample (n=120), was designed to maximize the likelihood of *Salmonella* detection, providing 95% confidence of detecting *Salmonella* or *S*. 1,4,[5],12:i:- in at least one sample if herd shedding prevalence was above approximately 8%, respectively, assuming perfect test sensitivity (Cannon and Roe, 1982; Humphry et al., 2004). Likewise, collection of five pooled samples per production stage per sampling occasion (n=30) provided 95% confidence of detecting at least one *Salmonella* positive per sampling event per production stage at a minimum production stage shedding prevalence of approximately 10%,

assuming perfect test sensitivity, or approximately 25% per sampling occasion with test sensitivity of 75% (Funk et al., 2000b).

Table 2-2. Longitudinal study sampling design.

Sample stock	Sample type (pats per pool)	Sampling method	Sample number (n pigs)
Dry sows	Pooled (6) faecal	Fresh from floor	5(30)[2]
Lactating sows/suckling pigs	Pooled (6) faecal	Fresh from floor	5(30)
Weaners (period of highest clinical signs; 5-8 wks)	Pooled (6) faecal	Fresh from floor	5(30)
Finishers/replacements (>15 wks; selected gilts >15 wks premating)	Pooled (6) faecal	Fresh from floor	5(30)
Total samples/sample batch/herd (total pigs represented/batch)			20 (120)

Sampling methods. Portions of six undisturbed faecal pats, each weighing approximately 5g, were sampled from a single pen floor and aggregated (pooled) in a sterile 120ml pot. The samples were de-identified and stored in iceboxes at approximately 4°C. As described previously, sample weights of at least 25g were desired, to provide relative sensitivity of detection by bacterial culture of approximately 75% (Funk et al., 2000b), and were largely achieved; there were occasional exceptions among samples from young weaners. If a herd employed less than five pens to house a single production stage at the time of sampling (i.e. employing large pens housing high numbers of animals, at least 200 head) a second pooled sample was collected from a distant location in the pen housing the largest population, to ensure five pooled samples were collected per sampling occasion. Sow crates were treated as a single unit, contributions from both sow and suckling pigs were collected from six non-sequential crates. It was assumed that individual sows and litters would shed similar *Salmonella* populations. Farrowing shed sampling was designed to maximize the likelihood of detection and representation of the *Salmonella* population diversity present as a potentially important linkage, and point of transmission, between breeder and finisher animals. A *Salmonella* or *S.* 1,4,[5],12:i:- detection from a sample equated to at least one pig in the sampled pen shedding *Salmonella* or *S.* 1,4,[5],12:i:-, with the exception of farrowing shed samples in which case at least one crate could be deemed positive.

[2] Collection of five pooled samples, representing six pats per sample (approximately six pigs), per stratum (n=30) n=30 for each strata, or age-group, providing 95% confidence of detecting one positive at a minimum prevalence of 10%, assuming 100% test sensitivity or shedding prevalence of approximately 25% assuming 69% test sensitivity (Funk et al., 2000b).

2.5. Sample submissions

In each of the studies the chilled samples (stored in insulated ice boxes with ice packs; ice packs were changed three times per day) were transported to the Food Safety and Innovation Microbiological Laboratory, South Australian Research and Development Institute (SARDI), South Australia, Australia. The chilled samples were received at the SARDI laboratory within four days of sample collection, within 48 hours among samples collected in the longitudinal study, and were immediately refrigerated at 4ºC upon arrival. Although the time between sample collection and processing at the SARDI laboratory was minimized to the extent possible it was longer than preferred; unfortunately, this was unavoidable for logistical reasons that related to the candidate collecting the majority of the samples personally, the large distances between herds and, in a small number of cases, reliance on veterinary practitioners sending the samples in a timely manner—samples submitted by collaborating veterinarians were also kept chilled with ice packs and delivered to the SARDI within two to four days. O'Carroll et al. (1999) demonstrated that although faecal sample processing on the day of collection is preferable for the detection of Salmonella spp., if stored at 4ºC a delay of up to six days did not result in a meaningful loss of test sensitivity. Of course, the suboptimal time between sample collection and processing and testing at the laboratory must be considered when interpreting the results and findings of the studies described.

2.6. Isolate selection for the phylogenetic study

The sequenced strains were selected to provide maximum diversity of the *S*. 1,4,[5],12:i:- study collection with additional comparator biphasic *S*. Typhimurium and non-motile *S*. Typhimurium strains. The strains were selected on the basis of representativeness of strain characteristics: MLVA profile, phage type, antimicrobial resistance phenotype, and serovar; and on the basis of source attributes: herd profile, year of isolation and pig production stage. In addition to the isolates from the cross-sectional and longitudinal studies, select isolates from the Hamilton et al. (2015) study, Herd 11, and from pig carcasses in two abattoirs were included. The carcass samples were collected by belly strip excision from carcasses sampled on two days, one day at each of the two abattoirs. The majority of the isolates (n=56) were from primary production, derived from seven pig herds with no direct live animal linkages and located in three Australian states. Four isolates were from pig carcass samples from two abattoirs. Among the isolates sequenced, 53 were serotyped as *S*. 1,4,[5],12:i:-, five were serotyped biphasic *S*. Typhimurium, and two were identified as non-motile *S*. Typhimurium. The selected isolates were described by 19 MLVA profiles. Five isolates could not be phage typed and two isolates from the earlier study were not tested (for reasons unknown). In total 13 different antimicrobial resistance phenotypes were observed, ranging from fully sensitive to resistance to eight antimicrobials. The *S*. Typhimurium isolates increased the diversity of phage types, they included isolates characterized as PT170, PT126 and pool O negative PT193.

2.7. Isolates included in the antimicrobial resistance study

The study included all isolates tested for antimicrobial susceptibility from the cross-sectional study and the two longitudinal studies. The data on resistance genes was derived from the strains sequenced in the phylogenetic study.

2.8. *Salmonella* detection

Culture methods followed the Australian Standard methods (AS5013.10-2009, 2014). Faecal samples were homogenized using a stomacher then the entire sample up to 25g was added to Buffered Peptone Water at a ratio of 1:10 for pre-enrichment—a non-selective medium to aid recovery of *Salmonellae* before selective enrichment and isolation. All samples were then incubated overnight at 37°C. The selective enrichment step was then performed, aliquots of 0.1ml of the pre-enrichment incubated Buffered Peptone Water suspension were inoculated into modified semi-solid Rappaport-Vassiliadis medium (Micro Media, MOE, Victoria) and were then incubated overnight at 42°C +/- 1°C and observed for the typical halos indicating motile bacteria. Sub-cultures were then taken from the outside edge of the halo and plated on Xylose Lysine Desoxycholate agar plates (Micro Media, MOE, Victoria) and incubated at 37°C for 24 hrs. Three typical colonies from each Xylose Lysine Desoxycholate plate were subcultured for purity onto Cystine Lactose Electrolyte-Deficient agar with Andrade's agar and confirmed by latex agglutination using Serobact™ *Salmonella*. Colonies that were negative for latex agglutination were checked by biochemistry using MICROBACT™ 24E (ThermoFisher Scientific Thebarton, South Australia).

Where *Salmonella* was confirmed, multiple colonies were picked from each plate—the number varied depending on the study, to maximize the likelihood of detecting *Salmonella* diversity within pens, age groups and the herd. Colony picks were transferred to individual nutrient agar slopes, which were then transported overnight to the Microbiological Diagnostic Unit, University of Melbourne, Victoria, Australia, for further characterization.

2.9. *Salmonella* characterization

All *Salmonella* characterization was conducted by MDU PHL technicians, the author followed several batches of isolates through characterization to better understand the process. The author also characterized 20 duplicate isolates using MLVA at SARDI, the results of which were discarded due to the differences in the SARDI and MDU PHL platforms.

2.9.1. Serotyping

Isolates were serotyped by agglutination with specific O and H antigen antisera in accordance with the conventions of the Kauffmann-White-Le Minor scheme (Grimont and Weill, 2007). Upon confirmation of *Salmonella* spp., purified colonies were tested against a pool of O-antisera followed by the H-antisera; colonies were mixed with a drop of the specific antisera on a glass slide. The results were then read by eye, positive results indicated by agglutination in the suspension. If one H-phase was identified a phase inversion was conducted to repress the dominant/first H-phase and the sample was retested for agglutination with antisera to determine presence/absence of the second H-phase.

Due to resource constraints, not all isolates were fully serotyped (Figure 2-1). Test decision making was made on the basis that the study was primarily interested in *S*. 1,4,[5],12:i:-. The first isolate from each sample was serotyped and if this isolate was found to conform with *S*. 1,4,[5],12:i:- then the isolate was further characterized, and the remaining isolates from that sample were stored without further characterization. If the first isolate was not *S*. 1,4,[5],12:i:- the four to nine remaining colonies were partially serotyped. If the partially serotyped isolates indicated *S*. 1,4,[5],12:i:- or another serovar that differed from the first isolate a representative of each was fully serotyped. The equivalence of the geometric testing regimen employed and a binomial testing approach is demonstrated in the statistical methods section of this chapter.

2.9.2. Phage typing

All isolates confirmed to be *S*. 1,4,[5],12:i:- or *S*. Typhimurium were phage typed in accordance with Anderson et al. (1977) and Rabsch (2007). Once purity was confirmed, a culture was incubated at 37°C in enrichment broth. The culture was then inoculated by flooding onto a marked plate, excess was removed and the plates were dried. A multi-pointed inoculator was then employed to apply the 34 typing phages in the specified order. The plate was dried and incubated at 37°C for 5-18 hours. The degree of lysis—the grade of lysis, number of plaques and size of plaques—was then read at low magnification (10x) through the bottom of the plate. The lysing pattern was then compared with known patterns to designate if the isolate conformed to a provisional phage type (PT) or definitive phage type (DT). Strains describing an unrecognized lysis pattern were designated 'react but did not conform' (RDNC). Strains that did not react to any the typing phages are described as 'untypable'.

2.9.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted at the MDU PHL using the methods described in Commons et al. (2012) and in detail in CLSI (2011). Representatives of all serovars identified

among the colony picks from individual samples were tested for antimicrobial sensitivity by agar dilution, the test results were determined by interpreting the breakpoint concentrations for resistance described in CLSI (2011)(Table 2-3). The veterinary standards were not used because testing was performed at a Public Health Laboratory and the same antimicrobial susceptibility testing methodology is applied to human and non-human samples. The method was introduced for epidemiological purposes and the antimicrobials in the panel are relevant for human therapy at CLSI breakpoint concentrations for resistance. Moreover, ascertaining resistance to the ampicillin-streptomycin-sulphathiozole-tetracycline quartet is important in relation to the global epidemiology and spread of *S*. 1,4,[5],12:i:-.

The method employed was as follows: dried agar plates containing the specified antimicrobials at known concentrations were inoculated with pure culture using a multi-point inoculator. The spots of inoculum were allowed to dry then plates were inverted prior to incubation at 37°C for 16-20 hours. The inoculation-suspension was incubated on non-selective media under the same conditions as a purity control. Having confirmed purity, the results were read. For sulphathiozole and trimethoprim susceptibility was recorded as a growth reduction of 80-90%, due to the action of these antimicrobials. Resistance to nalidixic acid was used as a substitute for fluoroquinolone, by convention. This is because there have been reports of patients infected with nalidixic acid resistant strains experiencing poor clinical response rates to ciprofloxacin in spite of susceptibility testing indicating that the strains were susceptible to ciprofloxacin using breakpoints for *Enterobacteriaceae* (Rowe et al., 1997). The abbreviations used throughout this thesis to report antimicrobial resistance phenotypes are those most frequently used in the published literature to describe S. 1,4,[5],12:i:- antimicrobial resistance phenotypes (Switt et al., 2009; Lucarelli et al., 2011; García et al., 2016)(Table 2-3).

Table 2-3. Antimicrobial resistance phenotypes of S. 1,4,[5],12:i:- isolates by herd.

Antimicrobial Agent	**Abbreviation**[a]	**Resistance breakpoint**[b] **(mg/L)**
Ampicillin	A	≥ 16
Cefotaxime	Cf	≥ 1
Chloramphenicol	C	≥ 16
Ciprofloxacin	Cp	≥ 2
Gentamicin	G	≥ 8
Kanamycin	K	≥ 32
Nalidixic acid	Na	≥ 16
Spectinomycin	Sp	≥ 50
Streptomycin	S	≥ 32
Sulphathiozole	Su	≥ 512
Tetracycline	T	≥ 8
Trimethoprim	Tm	≥ 8

[a] The abbreviations employed are those most frequently used in the published literature to describe *S*. 1,4,[5],12:i:- antimicrobial resistance phenotypes (Switt et al., 2009; Lucarelli et al., 2011; García et al., 2016).
[b] Breakpoints in accordance with CLSI (2011) and Commons et al. (2012).

2.9.4. MLVA typing

The MLVA was performed in accordance with Lindstedt et al. (2004) and Larsson et al. (2009) and analysed using GeneMapper software (Applied Biosystems®). Isolates serotyped as *S.* 1,4,[5],12:i:- and *S.* Typhimurium were sub cultured onto a nutrient agar plate and incubated at 37°C for 18-24 hours. Approximately five pure isolated colonies (approximately half a 1µl loop-full) were picked off the plate and emulsified. Isolate DNA was extracted using a QIAextractor instrument with a DX reagent kit (Qiagen®), in accordance with manufacturer instructions. A quantity of 5µl of DNA was added to a PCR mix containing one Qiagen multiplex PCR master mix and the oligonucleotide forward and reverse primers for the five loci: STTR6-FAM (0.05µM), STTR6-R (0.05µM), STTR3-F-NED (0.2µM), STTR3-R (0.4µM) STTR9-F FAM (0.025µM), STTR9-R (0.05µM), STTR5-F NED (0.05µM), STTR5-R (0.1µM), STTR10PL-F VIC (0.05µM) and STTR10PL-R (0.1µM) in a final volume of 25µl (Table 2-4). The PCR cycling was performed on the Veriti 96 well thermocycler (Applied Biosystems®) with 15 minutes at 95°C then 25 cycles of 94°C for 30 seconds, 63°C for 90 seconds and 72°C for 90 seconds. After the final cycle the solutions were maintained at 72°C for 10 minutes. The PCR reactions were then diluted at 1:10 in ddH_2O and 1µl of the diluted PCR product added with 0.4µl Geneflo-625-ROX size marker (CHIMERx®), and 12µl of Hi-di formamide. Samples were denatured for 5 minutes at 95C, cooled to 4°C and placed on the 3130xl genetic analyser (Applied Biosystems®) for fragment analysis. The strain VNTR copy numbers were then assigned resulting in MLVA profiles, which are presented in the Australian nomenclature (Gilbert, 2008); the equivalent MLVA profile in the European nomenclature are presented in Table 2-5.

Table 2-4. Primer used for *S.* 1,4,[5],12:i:- and *S.* Typhimurium MLVA typing.

Primer	Dye sequence (5' – 3')
STTR3-F NED	NED-CCCCCTAAGCCCGATAATGG
STTR5-F NED	NED-ATGGCGAGGCGAGCAGCAGT
STTR6-F FAM	6FAM-TCGGGCATGCGTTGAAA
STTR9-F FAM	6FAM-AGAGGCGCTGCGATTGACGATA
STTR10pl-F VIC	VIC-CGGGCGCGGCTGGAGTATTTG
STTR3-R	TGACGCCGTTGCTGAAGGTAATAA
STTR5-R	GGTCAGGCCGAATAGCAGGAT
STTR6-R	CTGGTGGGGAGAATGACTGG
STTR9-R	CATTTTCCACAGCGGCAGTTTTTC
STTR10pl-R	GAAGGGGCCGGGCAGAGACAGC

Table 2-5. Equivalent MLVA profiles in the Australian and European nomenclatures.

Australian MLVA profile	Equivalent European MLVA profile
04-15-11-00-490	02-06-05-00-02
04-15-12-00-490	02-06-06-00-02
04-16-11-00-490	02-07-05-00-02
04-16-12-00-490	02-07-06-00-02
04-16-13-00-490	02-07-07-00-02
04-16-14-00-490	02-07-08-00-02
04-17-11-00-490	02-08-05-00-02
04-16-10-00-490	02-07-04-00-02
04-15-13-00-490	02-06-07-00-02
04-26-11-00-490	02-22-05-00-02
04-12-11-00-490	02-03-05-00-02
04-16-06-00-490	02-07-02-00-02
04-18-11-00-490	02-12-05-00-02
04-14-11-00-490	02-05-05-00-02
04-14-13-00-490	02-05-07-00-02
04-15-10-00-490	02-06-05-00-02
04-14-14-00-490	02-05-08-00-02
04-14-12-00-490	02-05-06-00-02
04-17-10-00-490	02-08-04-00-02
04-14-09-00-490	02-05-20-00-02

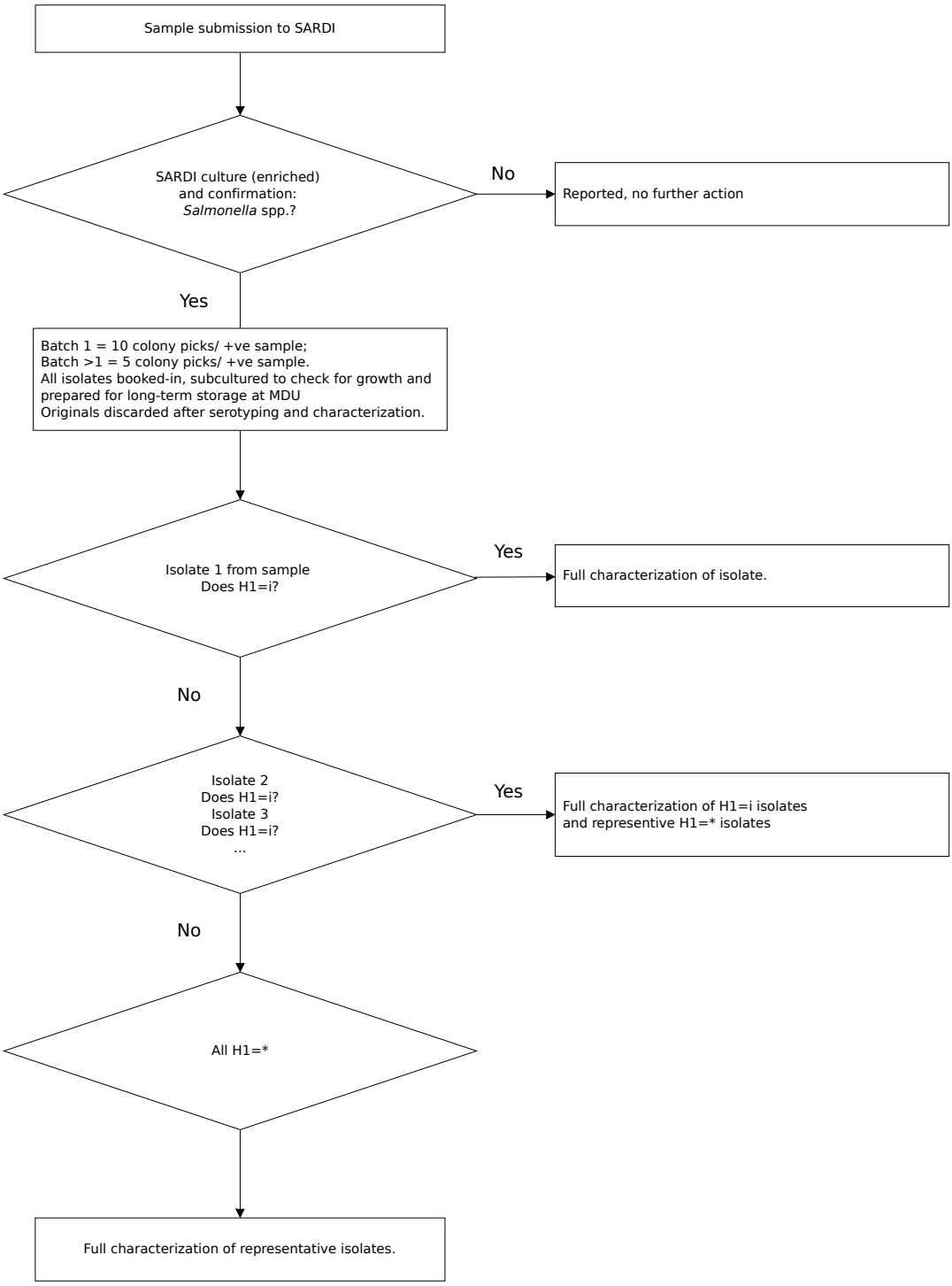

Figure 2-1. Laboratory decision-making process from sample submission to storage.

2.9.5. Sequencing

Genomic DNA from pure isolates was extracted using the JANUS Chemagic automated workstation (PerkinElmer®) with the Chemagic Viral DNA/RNA kit (PerkinElmer®). Unique dual indexed libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina®). Libraries were sequenced on the Illumina NextSeq® 500 with 150-cycle paired end chemistry as described by the manufacturer's protocols.

2.10. Statistical methods

The author conducted all statistical analyses unless otherwise stated. Data were collated in Excel (Microsoft Excel, 2011, Microsoft Corporation, Redmond, WA, USA). Data cleaning, exploration and descriptive statistical analyses were conducted in R (R Core Team, 2016). Other software used in conducting analyses is cited in the following section.

2.10.1. Diversity indices

Estimation of *Salmonella* serovar diversity by herd was conducted by calculating Shannon (H) and inverse Simpson (D) diversity indices (Simpson, 1949; Shannon and Weaver, 1963; Hurlbert, 1971).

2.10.2. Equivalence of the geometric testing regimen with a binomial testing regimen

A geometric regimen was investigated and employed in characterizing *Salmonella* spp. colonies, due to resource constraints and the high numbers of colonies picked per positive sample (5-10). A geometric laboratory testing protocol was proved to be equivalent, in terms of serovar representativeness, to a binomial testing protocol; this was confirmed by simulation (J. Tuke, personal communication).

To demonstrate equivalence of the testing approaches, if the probability of a positive test result for a serovar is p, for binomial testing a fixed number of tests, n, are conducted and if one or more tests are positive then we conclude a positive overall test result. Whereas for geometric testing, tests are performed sequentially and testing concludes upon a positive test result, therefore, if n tests are concluded and no positive result is obtained then the overall result is negative. To determine if the methods are equivalent for the same number of total or possible tests, n, the probability, p, of a positive overall result is the same.

To test equivalence both testing regimens were modelled.

For binomial testing, it was assumed that the total number of positive tests, *X*, could be modelled by a binomial distribution

$$X \sim B(n, p)$$

The probability of an overall positive test result, *P*, is the probability of one or more positive tests

Equation 1.

$$P = P\ (X\ \geq 1) = \sum_{i=1}^{N} P(X = i) = \sum_{i=1}^{N} \binom{N}{i} p^i (1-p)^{N-i}$$

For geometric testing, the total number of positive test, Y, has a geometric distribution, so that the probability that Y = y is:

$$P(Y = y) = (1-p)^{y-1} p$$

Therefore, the probability of an overall positive test result, *P*, is the probability of a positive test result in the first *n* tests.

Equation 2.

$$P = P(Y \leq N) = \sum_{i=1}^{N} P(Y = i) = \sum_{i=1}^{N} (1-p)^{i-1} p$$

By assessing the probability of an overall negative result to demonstrate equivalence between Equations 1 and 2:

For the binomial methodology:

$$P(X = 0) = \binom{N}{0} p^0 (1-p)^N = (1-p)^N$$

For the geometric testing regimen:

$$P(Y \geq N+1) = \sum_{i=N+1}^{\infty} (1-p)^i\, p$$

If we take $j = i - N$

$$= \sum_{j=i}^{\infty} (1-p)^N (1-p)^j p$$

$$= (1-p)^N \sum_{j=1}^{\infty} (1-p)^j p$$

$$= (1-p)^N$$

Therefore, the probability of a negative overall test result in the same, and therefore the probability of a positive test result must also be the same.

2.10.3. eBURST analyses

Minimum spanning trees were drawn using eBURST analysis based on the rules defined by Feil et al. (2004) and PHYLOViZ software was used for representation (Francisco et al., 2009; Francisco et al., 2012). Standard eBURST distance measures were applied as Dimovski et al. (2014) found this method to be most reliable in describing relationships between MLVA profiles.

2.10.4. Network analyses

Edge and node lists were prepared and manipulated using the R package 'igraph'. Three network graphs were constructed: (1) a directed, unweighted 'physical' network of herds in which in which *S*. 1,4,[5],12:i:- was detected, 'physical' edges were identified through herd profiles and related to breeder herd, feed mill and abattoir; (2) a directed, unweighted 'physical' network of all herds sampled, using the same definition of physical links; and (3) an undirected, unweighted 'transmission' network of herds in which *S*. 1,4,[5],12:i:- was detected where herds with a shared MLVA profile were connected by an edge (VanderWaal et al., 2014).

Gilt and feed supply were included as known transmission pathways for *Salmonella* spp. (Davies et al., 2000; Zheng et al., 2007; Papadopoulou et al., 2009; Wales et al., 2009; EFSA, 2010b). Abattoirs were included as stock transporting vehicles visit herds and abattoirs regularly presenting a possible mode of transmission. Other possible transmission pathways, such as rodents, bird access to animal housing, stock persons, salespersons, consulting veterinarian and the sampler were not considered due to the large distances between herds, lack of information or for ethical reasons. Edges were identified through herd profiles and considered the five years preceding sampling. Node degree, average path length, betweenness centrality, closeness centrality and eigenvector centrality were calculated (Farine and Whitehead, 2015). These metrics are correlated, therefore, it was decided to present only degree and betweenness centrality, as these metrics are the most intuitive (Rothenberg et al., 1995; VanderWaal et al., 2014). Degree refers to the number of edges at each node, indicating the number of herds sharing the MLVA profile or nodes with physical connections to other nodes among the sampled herd networks. Betweenness refers the number of shortest paths to which the node contributes, it provides an indication of key 'linking' nodes between communities within a

network. Betweenness centrality was calculated using the algorithm presented by Brandes (2001). To identify community structure, or clusters, within networks a modularity algorithm was applied. Modularity was calculated using the algorithm in Blondel et al. (2008). The software package Gephi 0.9.1 was used to visualize physical networks between herds, initially the Fruchterman-Reingold force-directed layout algorithm was applied to 'untangle' the networks, followed by the Force Atlas layout algorithm, which generated the final networks presented (Bastian et al., 2009). The Gephi package was also used to conduct statistical analyses on the network data.

In an attempt to generate hypotheses, univariate analyses of possible predictor variables and response variables were conducted. Fisher exact tests were applied as an initial test of whether identified physical links might be associated with strain isolation from sampled herds. The response variables were detection/no detection of *S*. 1,4,[5],12:i:- and the detection/no detection of shared *S*. 1,4,[5],12:i:- MLVA profiles. The predictor variables, applied in both cases, were: genetic supplier, feed supplier (mill), abattoir, if herds shared any edge, and whether herds were members of the same community. Two-by-two contingency tables for each predictor and response combination were created and Fisher exact tests were conducted using the 'stats' package in R (R Core Team, 2016).

The possibility of predictor networks being associated with response networks was further investigated using a quadratic assignment procedure (QAP). Edge and node lists were prepared and manipulated using the R package 'igraph' and the analysis was carried out in the package 'sna' (Butts, 2008; R Core Team, 2016). The models were applied with Dekker's 'semi-partialling plus' procedure for permutations (Dekker et al., 2003; Dekker et al., 2007). The subjects were defined as pairs of farms taken together. Again, dependent variables were defined as detection/no detection of *S*. 1,4,[5],12:i:- and detection/no detection of the same *S*. 1,4,[5],12:i:- MLVA profile. A success was defined as *S*. 1,4,[5],12:i:- isolated from both herds in the pair or the same *S*. 1,4,[5],12:i:- MLVA profile having been isolated from both herds. All other possibilities, i.e. *S*. 1,4,[5],12:i:- or specific *S*. 1,4,[5],12:i:- MLVA profile isolated in neither of the herds in the pair or *S*. 1,4,[5],12:i:- or specific *S*. 1,4,[5],12:i:- MLVA profile not isolated from one herd in the pair, were defined as failures. This caused some unavoidable loss of information. Predictor variables were defined as: shortest path length via all physical connections (directed and undirected), genetic supplier, feed supplier (mill), abattoir and membership of the same community. Univariate models were constructed for each predictor variable in relation to each of the two dependent variables to determine if predictor variables were significant. Chi-squared associated p-values were calculated to test if the models were a good fit. The intention was then to run multivariate models incorporating all significant predictor variables and remove predictors using a step-up approach and a likelihood ratio test to test for a significant difference in the models. However, the only significant association was between predictor directed shortest path length and response detection/no detection of *S*. 1,4,[5],12:i:- and

the models were badly overfitted. This was due to the paucity of network graph structure in the data. Multivariate QAP analyses were, therefore, deemed unreliable and inappropriate for this dataset.

2.10.5. Prevalence estimation

Estimation of true prevalence for pooled samples

To account for imperfect test sensitivity in the detection of *Salmonella* and *S*. 1,4,[5],12:i:-, and the influence of pooling samples and unknown external variables, a Bayesian approach was used to estimate true prevalence for each herd, and each age group and sampling occasion within herds (Boelaert et al., 2000; Branscum et al., 2005; Speybroeck et al., 2012). The posterior estimate of 68.8% was used as the expert 'best guess' for *Salmonella* culture test sensitivity for pooled pig faecal samples (Se_p) for defining the beta distribution, on the basis of Funk et al. (2000b), and an Se_p lower uncertainty limit of 50%, a conservative value based on the lowest value, 56.6%, also calculated by Funk et al. (2000b). Near perfect test specificity was assumed, given the organisms were cultured and characterized extensively (Funk et al., 2000b). The pooled sensitivity (Se_p) was a function of the estimated sensitivity for single samples (Se), the size of the pools (k) and the true prevalence of infection (P_T):

Equation 3.

$$Se_p = 1 - [(1 - Se)^{kP_T} \times Sp^{k(1-P_T)}] \qquad \text{(Speybroeck et al., 2012)}$$

Likewise, pooled specificity, Sp_p, was a function of the specificity of single samples, Sp, and pool size, k:

Equation 4.

$$Sp_p = Sp^k \qquad \text{(Speybroeck et al., 2012)}$$

The expression for apparent prevalence, P_A, was then:

Equation 5.

$$P_A = P_T \times Se_p + (1 - P_T)(1 - Sp_p) \qquad \text{(Speybroeck et al., 2012)}$$

A Markov chain Monte Carlo sampling method was used to calculate Se_p, Sp_p and P_T estimates. An uninformed prior distribution was used for true prevalence and Bayesian inferences, based on the

joint posterior distribution, were approximated using JAGS (Just Another Gibbs Sampler) (Plummer, 2003). The model was run for 110,000 iterations, discarding the first 10,000 iterations as burn-in. The R packages 'rjags' and 'prevalence', available through The Comprehensive R Archive Network (https://cran.r-project.org), were used to conduct the analyses.

Estimation of true prevalence for individual samples

As above, to account for imperfect test sensitivity in the detection of *Salmonella* spp. and individual serovars, a Bayesian approach was used to estimate true prevalence on the basis of apparent prevalence at each sampling occasion (Speybroeck et al., 2013). A posterior estimate of 68.8% for *Salmonella* culture test sensitivity was used, the expert 'best guess' for pig faecal samples (*Se*), on the basis of Funk et al. (2000b). In defining the beta distribution; a *Se* lower uncertainty limit of 50% was used; a conservative value based on the lowest value, 56.6%, calculated by Funk et al. (2000b). Test specificity was assumed to be near perfect, as the bacteria was identified and characterized extensively (Funk et al., 2000b). A Markov chain Monte Carlo sampling was used to calculate sensitivity, specificity, and true prevalence estimates. An uninformed prior distribution was used for true prevalence and Bayesian inferences based on the joint posterior distribution were approximated using JAGS(Plummer, 2003). The model was run for 110,000 iterations, discarding the first 10,000 iterations as burn-in. Again, the R packages 'rjags' and 'prevalence', were used to conduct the analyses.

2.10.6. Bioinformatic analyses

Assembly and mapping of reads

The Illumina reads were assembled and mapped using the RedDog v1beta.2 pipeline applying SPAdes 3.6.2 for assembly (Bankevich et al., 2012; Edwards et al., 2015). The mean depth, mean reads and total reads per isolate are provided in Chapter 7: Supplementary Table 7-2. The reads were aligned to the closest available reference, *S*. Typhimurium SL1344 (DT44), with Bowtie2 2.2.3 and/or Burrows-Wheeler Aligner (BWA) 0.6.2 (Li and Durbin, 2009; Langmead and Salzberg, 2012). The references used were chromosome: *S*. Typhimurium SL1344 (NC_016810.1); plasmid 1, pSLT (NC_017720.1); plasmid 2, pCol1B9 (NC_017718.1) and plasmid 3, pRSF1010 (NC_017719.1). Sequence reads mapped to an average 97.9% of the reference genome, with a mean depth of 77.9-fold across all isolates. Pileups were generated and single nucleotide polymorphisms (SNPs) were identified and filtered using SAMtools 1.1 (Li et al., 2009a). Raw SNP calls were filtered for quality (phred score ≥20), depth (≥10x) and homozygosity (Holt et al., 2012). In order to identify the underlying signals of vertical inheritance repeat sequences, insertion sequences and prophages were filtered and excluded from phylogenetic analyses. Gubbins 1.4.7 was then employed to remove SNPs predicted to have been introduced via recombination (Croucher et al., 2015). The finalized maximum likelihood phylogenetic tree was inferred using RAxML v8.1.15

to analyse the concatenated alignment of SNP alleles using a general time-reversible (GTR) substitution model with γ correction of nucleotide substitution, with 10 replicate runs and 1,000 bootstraps (Stamatakis, 2014). The resulting tree was visualized in FigTree v1.4.2.

Parsing of sequence data

Where the serovar was contested—two of the strains phenotyped biphasic *S*. Typhimurium were missing the virulence plasmid pSLT, indicating they may have been true monophasic strains—the SeqSero tool was employed to confirm the serovar using the raw reads for matches to serovar determinants: *rfb* gene cluster, *fliC* and *fljB* alleles (Zhang et al., 2015). To further confirm these results, an SRST2 v0.1.8 comparison of the *fljAB* region—specifically STM2760, STM2762, STM2766, *fljB*, *fljA* and *hin*—of the two genomes with *S*. Typhimurium LT2 (NC_003197.2) was conducted by Yuhong Liu (Inouye et al., 2014).

The software SRST2 v0.1.8 was used to infer multiple locus sequence types (MLST) (Inouye et al., 2014). The MLST type was based on allele combinations for *aroC*, *dna*N, *hemD*, *hisD*, *purE*, *sucA* and *thrA* through comparison with the pubMLST database entries for *Salmonella enterica* (WWW http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

An SRST2 v0.1.8 comparison was also employed to search the sequence reads for matches to resistance genes in the ARG-Annot antimicrobial resistance gene database (Gupta et al., 2014b; Inouye et al., 2014). The ancestral tree and resistance genes and alleles were presented by adapting the plotTree.R code developed by Dr Kat Holt (https://github.com/katholt/plotTree)(R Core Team, 2016).

Chapter 3 - Descriptions of the herds sampled

3.1. Introduction

This chapter presents details of the herds sampled in the cross-sectional and longitudinal components of the study. The key features of all herds are presented and the known commonalities in terms of gilt supply, feed supply and abattoirs are described. The five herds that were sampled in longitudinal studies are described in greater detail in Annex 1. Herd features that could allow identification, such as location, sow herd numbers and consulting veterinarian, have been described ambiguously so as to maintain anonymity. All animal health products employed by the herds sampled were permitted for use in pigs under Australian regulations, however, specific details have not been described as permission to publish was not obtained from producers or consulting veterinarians.

In total 17 herds are described in this chapter. Of these herds 16 were sampled during this study and one, Herd 11, was sampled in a previous Australian Pork Limited funded study described briefly in Hamilton et al. (2015). The investigators engaged in this study kindly allowed access to their results for comparative purposes, the herd's profile, connections within the industry and published results are presented, with appropriate citation, as a selection of Herd 11 isolates were included in the comparative genomic studies reported in this thesis. Where Herd 11 is included in tables it is highlighted in grey.

3.2. The cross-sectional study herds

The majority of Australian commercial herds are farrow-to-finish; however, some specialized producers of weaned piglets and grower-finishers do exist. Of the 16 herds sampled in this study only Herd 3 met this description, the breeder and grower herds were owned and operated by independent enterprises that interacted in a sole supplier-client relationship. Herd 3 was treated as a single farrow-to-finish system for the purposes of this study. The other 15 herds were single or multi-site farrow-to-finish systems under single ownership (Table 3-1).

Table 3-1. Summary of herd profiles.[3]

		Variable	Herd																	No. of herds
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Salmonella isolation	Isolated	*S.* 1,4,[5],12:i:-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓							11
		*S.*Typhimurium																✓		1
		Other *Salmonella* serovars	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓				✓	✓	✓	15
	Previous isolation	*S.* 1,4,[5],12:i:-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	16
		*S.*Typhimurium																✓		1
	Clinical signs	Recent outbreak of acute enteritis		✓	✓	✓	✓			✓	✓	✓		✓			✓	✓	✓	11
		Chronic low-grade enteritis	✓		✓			✓	✓				✓	✓	✓	✓	✓			10
Herd summary	Ownership	Independent farrow-to-finish	✓	✓	✓	✓	✓	✓		✓		✓	✓		✓	✓			✓	12
		Opportunistic breeder	✓			✓		✓												2
		Wean-grower			✓															1
		Large integrator							✓		✓			✓			✓	✓		5
	Sow numbers	100-400								✓										1
		401-800	✓	✓	✓	✓	✓	✓					✓		✓	✓			✓	10
		>800							✓		✓	✓		✓			✓	✓		6
	Site(s)	Single site				✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓		✓	12
		Multisite	✓	✓	✓									✓				✓		5
	Clustering	Single site				✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓		✓	12
		<50km separation	✓	✓										✓						3
		>50km separation			✓													✓		2
	Grower housing	Conventional				✓		✓										✓		3
		Deep bedding		✓	✓					✓										3
		Mixed	✓				✓					✓	✓	✓	✓	✓	✓		✓	9
		Outdoor							✓		✓									2
	Gestating sow housing	Group housing	✓	✓		✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	15
		Mixed (stalls and group housing)			✓								✓							2
	Nearest pig farm	<10km	✓														✓			2
		11-50km		✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓		✓	✓	14
		>50km								✓										1
	Live gilts brought on	Closed herd (>4 year)	✓																	1
		Occasional imports (<quarterly)								✓				✓			✓			3
		Regular import (>quarterly)		✓	✓	✓	✓	✓	✓		✓	✓	✓		✓	✓		✓	✓	13
	Gilt source	NA (closed herd)	✓														✓			2
		Single source (>5 years)				✓	✓	✓	✓	✓	✓	✓	✓		✓	✓		✓	✓	12
		Multiple sources		✓	✓															2
	Feed supply	Home mixed								✓							✓			2
		Commercial	✓	✓		✓	✓	✓	✓		✓	✓	✓	✓	✓	✓		✓	✓	14
		Both			✓															1
	Batching	All-in, all-out (approx.)	✓	✓		✓	✓		✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	13
		Continuous			✓			✓		✓										4
	Organic acids	Yes	✓		✓	✓		✓									✓			5
		No		✓			✓		✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	12

[3] Herd 11 was sampled in a previous APL funded study, reported in Hamilton et al (2015)

3.2.1. Identified connections between herds

The sampled herds were located in geographically distant areas and generally employed recommended biosecurity protocols, such as control of traffic, quarantine of new stock, cleaning and disinfection procedures and rodent control. The herds had minimal linkages in terms of live animal movement between enterprises. The only pig movement between sampled herds in the preceding five years was from Herd 1 to Herd 8. Herd 8 had a longstanding relationship with Herd 1, receiving 10 gilts at approximately six-month intervals.

The Australian industry is relatively small and concentrated, producing approximately 2-3 million pigs per year as compared to over 200 million domesticated animals in Australia (APL, 2011). Due to the size of the industry, many Australian herds' share suppliers and service providers. Australia has few nucleus and multiplier herds, the industry in dominated by three main commercial suppliers of gilts, supplying approximately 60%, 20% and 10% of non-vertically integrated herds; approximately 10% are supplied by ad hoc/opportunistic breeders of mixed genetics. Although animal-to-animal contact is generally recognized as highest risk for transmission, it is well-known that *Salmonella* can be transmitted via various other pathways (Funk and Gebreyes, 2004; Lurette et al., 2011). The Australian pig industry is also supplied by a limited number of companies and mills producing pig feed. In addition, only a small number of Australian abattoirs are able to export, these abattoirs are supplied by the majority of Australian commercial producers.

Network analyses of the sampled herds provides an indication of the close connectedness of the Australian commercial pig industry. Description of directed networks where edges were drawn between nodes sharing gilt supply, feed supplier and/or abattoir demonstrate the close connectedness in the Australian industry (Figures 3-1 and 3-2). In the combined network (Figure 3-2) the average path length was 1.27, average degree, 3.21, and modularity, 0.56; four communities were identified. In both cases communities were found to approximate geographic location.

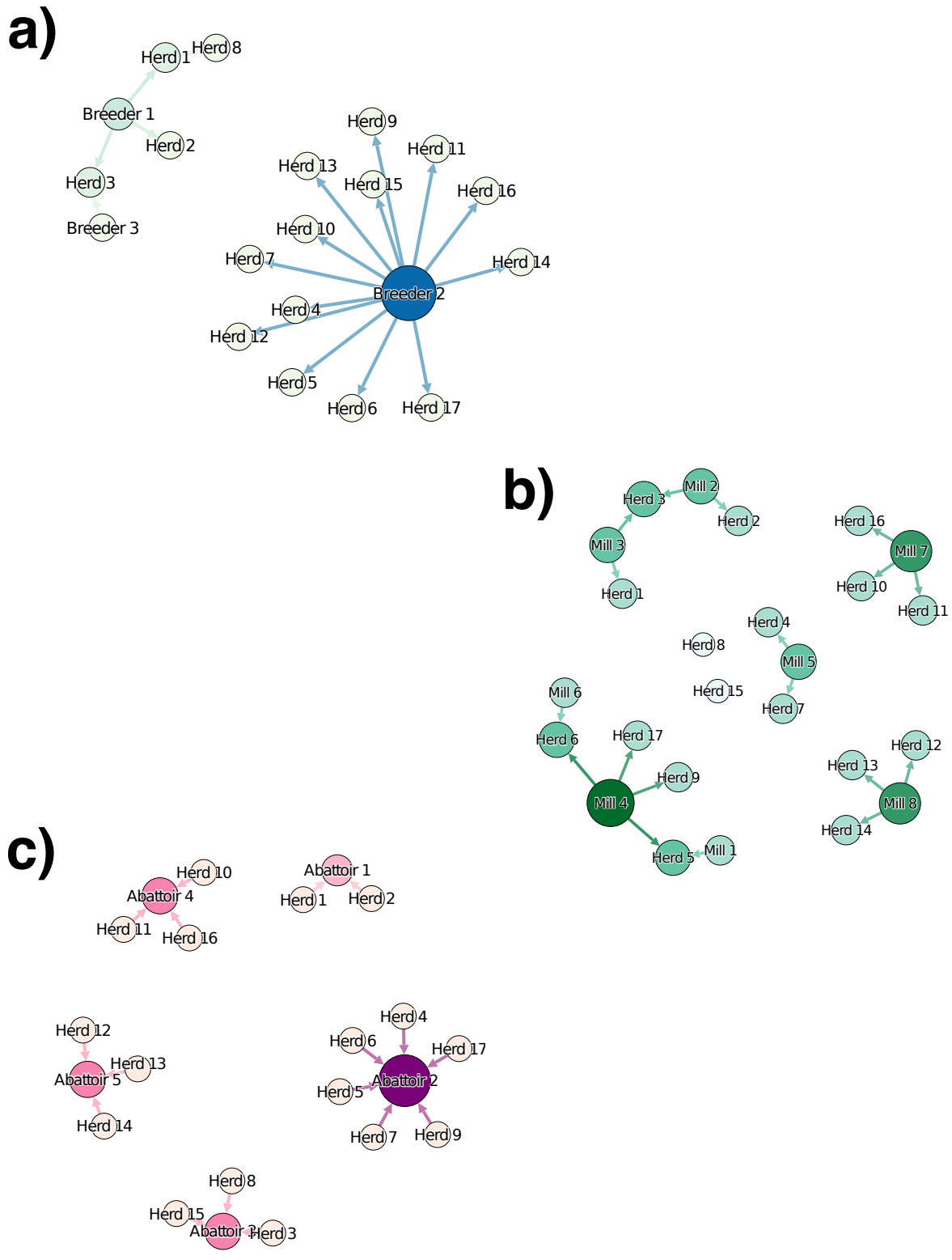

Figure 3-1. Directed physical network graphs of known physical linkages between herds, genetic supply, feed supply and abattoirs. Nodes are coloured and scaled by number of degrees (light to dark). a) genetic supplier b) feed mill c) abattoir. Note that the location of nodes is arbitrary, it does not reflect physical locations of actors. Variation in the length of edges is purely for the purposes of representation.

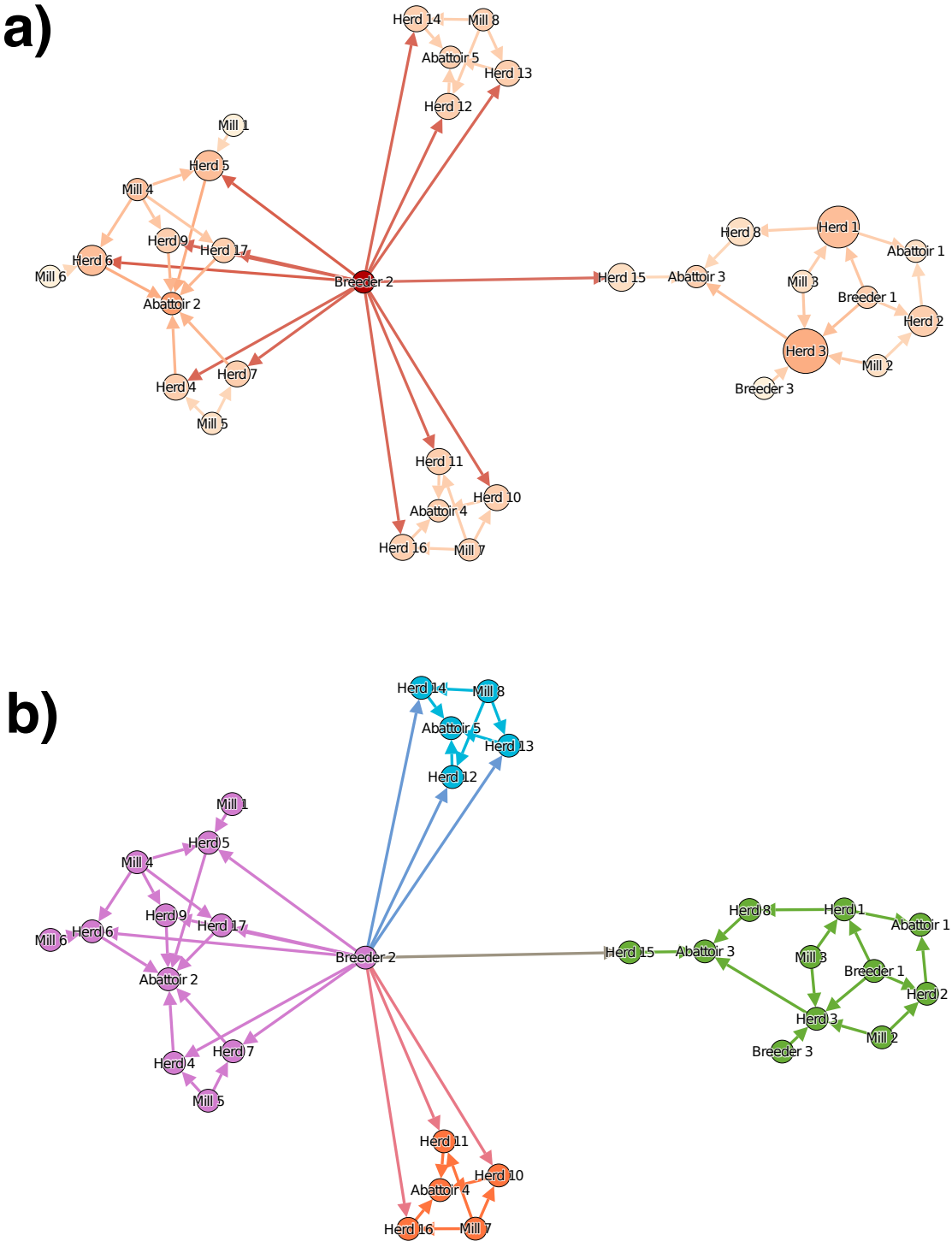

Figure 3-2. Directed networks of physical links between all sampled herds. a) Nodes are scaled by betweenness centrality and coloured by degree (light to dark). b) coloured by community. Note that the location of nodes is arbitrary, it does not reflect physical locations of actors.

3.3. Longitudinally sampled herds

The Herds sampled longitudinally were ostensibly unconnected. No known live animal movement between the herds had occurred for at least ten years in the case of these herds. A brief summary of the herds sampled longitudinally is provided in Table 3-2. Extended profiles of each of the five herds are provided in Annex 1. The profiles include detailed descriptions of the enterprise and the herd's management, including diagrams depicting animal movement within the herds, and detailed description of animal health status, recent animal health events and control measures employed.

Table 3-2. Summary of variation between longitudinally sampled herds.

Factor	Herd 1	Herd 2	Herd 3	Herd 4	Herd 5
Ownership	Independent	Independent	Two independently owned and managed sites	Independent, opportunistic breeder for a genetic supplier	Independent
Main outputs	Market weight hogs, gilts	Market weight hogs	Growers and market weight hogs by site, respectively	Market weight hogs, gilts	Market weight hogs
Live animal imports	Closed herd >4 year	Regular delivery of gilts 30hd/year; 350 in 2 years including study period (herd expansion)	Regular delivery of gilts 120-150hd/year	Regular delivery of gilts 30hd/year	Regular delivery of gilts 120-150hd/year
Live animal source	Closed herd	Changed supplier just prior to joining study	Single long-term supplier	Single long-term supplier	Single long-term supplier
Number of sites	Multisite	Multisite	Multisite	Single site	Single site
Site concentration	<50km	<50km	>100km	-	-
Husbandry system	Farrow to finish. Mixed conventional and deep bedding. Group housed sows farrowing crates. All in all out.	Farrow to finish Mixed conventional and deep bedding. Mixed group housed sows and individual stalls, farrowing crates. All in all out.	Farrow to wean, wean to finish. Mixed conventional and deep bedding. Mixed group housed sows and individual stalls, farrowing crates. All-in, all-out.	Farrow to finish. Conventional. Group housed sows, farrowing crates. Weaner mixing 8-12 weeks.	Farrow to finish. Mixed conventional and deep bedding. Group housed sows, farrowing crates. All-in, all-out.
Average wean age and frequency	21 days (weekly weaning)	21 days (monthly weaning)	24.5 days (monthly weaning)	23 days (weekly weaning)	24.5 days (weekly weaning)
Feed supplier	Supplier A	Supplier A	Supplier B and home mixed	Suppler B	Supplier B and C
Feed types	Pellets (low grind, regular)	Pellets	Pellets, mash	Pellets	Pellets
Organic acid use	Selko pH (Nutreco®)	None	Selko pH (Nutreco®)	Selko pH (Nutreco®)	None
Water source	River, diluted with mains	Mains	Mains	River/reservoir	River/reservoir
Abattoir	Abattoir A	Abattoir A	Abattoir B	Abattoir C	Abattoir C

Chapter 4 - Occurrence and diversity of *Salmonella* 1,4,[5],12:i:- and contemporary serovars in Australian pig production

Preface

The research that informed this chapter sought to address the questions of: how widespread has *S*. 1,4,[5],12:i:- become in the Australian industry? Discussion of the characteristics of the sampled herds, the *Salmonella* populations within herds, and indications of the level of phenotypic and *S*. 1,4,[5],12:i:- MLVA profile diversity are presented.

Risk profiling established that Australian pork supply could be an ultimate source of *S*. 1,4,[5],12:i:- Australian food supply. However, it was not known how widespread *S*. 1,4,[5],12:i:- colonization had become within the Australian industry, whether had spread widely or was confined to specific locations or production systems. To investigate further a cross-sectional observational study was designed. Previous studies indicated that *S*. Typhimurium prevalence among Australian pigs was low relative to other pig producing countries, given the similarities between these serovars it was thought this might also be the case for *S*. 1,4,[5],12:i:- (Coates, 1997; Hamilton, 2011; Hamilton et al., 2015). A randomized study design, to determine herd prevalence, was not feasible due to the logistical challenges of sampling pig herds in Australia and resource constraints. Moreover, the study also aimed to describe *S*. 1,4,[5],12:i:- diversity in Australian pigs and randomized herd selection increased the likelihood of low numbers of detections.

Initially inquiries were made to Australian pig specialized veterinary practices/veterinarians, many of which kindly provided laboratory reports and/or access to their records. All data was deidentified by the veterinary practices prior to viewing. A list of herds with possible *S*. 1,4,[5],12:i:- colonization was constructed and an approximately representative cross-section of Australian commercial producers were selected. Requests to sample were made through the consulting veterinarians.

The following chapter presents the findings of the cross-sectional study in terms of *Salmonella* spp. occurrence and phenotypic diversity and *S*. 1,4,[5],12:i:- MLVA profile diversity.

4.1. Introduction

Historically Australian pigs have had low estimated *S*. Typhimurium prevalence relative to European herds (Hamilton et al., 2015). However, Australian passive surveillance data suggested that *S*. 1,4,[5],12:i:- prevalence had been increasing, to the point where it may have surpassed *S*. Typhimurium in primary production (NEPSS, 2011, 2013, 2014). The presence of *S*. 1,4,[5],12:i:- in an Australian pig herd had been confirmed (Hamilton et al., 2015), in this herd *S*. 1,4,[5],12:i:- was overwhelmingly predominant among the isolates they characterized—it may have been the only serovar present within the herd they sampled. All the *S*. 1,4,[5],12:i:- isolates characterized in the Hamilton et al. (2015) study herd were phage type (PT) 193, however, greater MLVA profile diversity was present.

It was unknown whether *S*. 1,4,[5],12:i:- was associated with particular geographic areas or types of pig production system. The level of phenotypic and MLVA profile diversity within the Australian *S*. 1,4,[5],12:i:- population was unknown. Comparisons of phage typing and MLVA in terms of differentiating *S*. 1,4,[5],12:i:- strains was also uncertain. Being a relatively new approach to *Salmonella* typing, particularly in relation to Australian *S*. 1,4,[5],12:i:-, few studies had considered the utility of MLVA in comparison with phage typing (Dimovski et al., 2014; Petrovska et al., 2016). This was particularly significant in the Australian context, where *Salmonella* typing was no longer conducted routinely. The low importance attributed to *Salmonella* in the Australian pig industry and the rise in typing costs had led to the perception that expensive characterization was of limited value to on-farm control strategies, which discouraged cost coverage by producers and veterinary practitioners.

The objectives of this study were to: determine if *S*. 1,4,[5],12:i:- could be isolated from multiple, widely dispersed, representative Australian commercial pig production enterprises; to determine if *S*. 1,4,[5],12:i:- coexisted with other *Salmonella* serovars in Australian herds; to describe *S*. 1,4,[5],12:i:- diversity observed among the isolates; and to compare the utility of phenotyping and MLVA in differentiating Australian porcine *S*. 1,4,[5],12:i:- strains.

4.2. Results

The herds sampled represented an approximate cross-section of the Australian commercial pig industry (Chapter 3, Table 3-1). The herds in which *S*. 1,4,[5],12:i:- was detected were located in each of the four states from which herds were sampled. The herd sizes ranged from 100 sows to considerably more than 800 sows. The herds included conventional rearing systems, deep bedded housing, mixed systems and outdoor production. The herds employed a variety of feeding systems and a range of animal health measures to control enteric problems and improve pig performance.

4.2.1. Serovar diversity

In total *Salmonella* spp. was detected in 35.8% (87/243) of faecal samples from 13 of the 16 herds sampled. In total 861 colonies were isolated from the 87 *Salmonella* spp. positive samples, of which 109/861 were fully serotyped and a further 414 partially serotyped and found not to correspond to *Salmonella* (I) Group B. In the 13 herds in which *Salmonella* spp. was detected between one and six serovars were identified (Table 4-1). Herd level diversity ranged from no diversity to a Shannon's (H) Diversity index of 1.52 (D=3.56).

The most widespread serovar was *S*. 1,4,[5],12:i:-, identified in 10 herds. The other serovars identified were: *S*. London, *S*. Derby and *S*. Infantis, three herds each; *S*. Adelaide and *S*. Bovismorbificans detected in two herds; *S*. Ohio (and *S*. Ohio var 14), *S*. Agona, *S*. Hofit, *S*. Muenchen, *S*. Rissen, *S*. Worthington and *S*. Oranienburg, identified among isolates sourced from one herd each. Six of the 10 herds in which *S*. 1,4,[5],12:i:- was detected harboured multiple serovars.

Table 4-1. Serovars identified among isolates from the first sampling of each herd. The table lists the date of sampling, number of samples collected and number of samples in which *Salmonella* spp. was detected, the serovars identified and number of isolates of each, and herd *Salmonella* diversity indices, where appropriate.

Herd	Sampling date (M/D/Y)	*Salmonella* positive samples	Serovars identified	No. of isolates	Shannon (H) index	Simpson (D) index
1	7/11/13	2/15	*S*. 1,4,[5],12:i:-	2	-	-
2	1/10/14	13/20	*S*. 1,4,[5],12:i:-	1	1.52	3.56
			S. Hofit	1		
			S. Ohio	8		
			S. Ohio var 14+	1		
			S. Rissen	8		
			S. Worthington	1		
3	3/12/14	6/20	*S*. 1,4,[5],12:i:-	3	1.31	3.52
			S. Agona	1		
			S. Oranienburg	2		
4	1/10/14	13/14	*S*. 1,4,[5],12:i:-	10	1.43	3.17
			S. Adelaide	2		
			S. Bovismorbificans	2		
			S. Derby	1		
			S. London	1		
5	8/8/13	3/13	*S*. 1,4,[5],12:i:-	3	-	-
6	6/20/13	4/18	*S*. 1,4,[5],12:i:-	3	0.90	2.17
			S. Infantis	1		
7	12/1/12	13/18	*S*. 1,4,[5],12:i:-	10	1.06	2.79
			S. Adelaide	7		
8	11/20/13	5/18	*S*. 1,4,[5],12:i:-	5	-	-
9	3/27/13	3/8	*S*. 1,4,[5],12:i:-	2	0.72	1.67
			S. Infantis	1		
10	5/2/13	4/11	*S*. 1,4,[5],12:i:-	2	1.14	2.67
			S. Infantis	6		
			S. Muenchen	1		
11[a]						
12	10/30/13	0/20	-	-	-	-
13	10/30/13	0/20	-	-	-	-
14	5/19/13	0/20	-	-	-	-
15	5/27/13	7/14	*S*. Bovismorbificans	2	1.01	2.12
			S. Derby	4		
			S. London	2		
16	11/24/13	9/18	*S*. Typhimurium	10	-	-
17	8/27/13	5/14	*S*. Derby	3	0.75	1.72
			S. London	3		

[a] The Hamilton et al. (2015) study herd was designated Herd 11. The results from this herd were not included in the cross-sectional study presented here.

4.2.2. *Salmonella* 1,4,[5],12:i:- diversity

All of the *S*. 1,4,[5],12:i:- isolates were phage type PT193.

In total nine *S*. 1,4,[5],12:i:- MLVA profiles were identified, one of which exhibited minor variation at locus STTR3 (VNTR copy number 492) and was considered a probable laboratory artifact and aggregated for the purposes of analysis. Herd level MLVA profile diversity was low, ranging from no diversity to a Shannon's (H) diversity index of 0.93 (Table 4-2). The most widespread MLVA profile was 04-15-11-00-490, identified among isolates from 5/10 herds. Profiles 04-15-12-00-490 and 04-14-09-00-490 were identified in two herds, and the remaining types were found in individual herds only. All eight MLVA profiles were closely related as represented in the minimum spanning tree of the MLVA profiles identified, drawn using eBURST analysis, in which all the *S*. 1,4,[5],12:i:- isolates clustered closely with the exception of MLVA Profile F: 04-16-10-00-490 (Figure 4-1)(Francisco et al., 2009; Francisco et al., 2012). The MLVA profiles exhibited variation at the STTR5 and STTR6 loci only. All isolates were MLVA single locus variants (SLVs), with the exception of MLVA profile 04-16-10-00-490 (Profile F), a double locus variant with minor copy number variation at STTR5 and STTR6. Locus STTR5 VNTR copy numbers ranged from 14 to 16, STTR6 copy numbers ranged from 9 to 13. One to two *S*. 1,4,[5],12:i:- MLVA profiles were detected in each herd in which the serovar was isolated.

Table 4-2. Diversity of MLVA profiles among S. 1,4,[5],12:i:- isolates.[a]

	Herd (no. isolates, n)											
MLVA profile	**1** (2)	**2** (1)	**3** (3)	**4** (10)	**5** (3)	**6** (3)	**7** (12)	**8** (5)	**9** (2)	**10** (2)	**No.** (10)	**% Total isolates** (43)
04-14-09-00-490					1			2			2	7.0
04-14-11-00-490						1					1	2.3
04-14-12-00-490								3			1	7.0
04-14-13-00-490	2										1	4.7
04-15-11-00-490		1		10	2	2			2		5	39.5
04-15-12-00-490			3				8				2	25.6
04-15-13-00-490							4				1	9.3
04-16-10-00-490										2	1	4.7
No. MLVA profiles	1	1	1	1	2	2	2	2	1	1		
Shannon (H) index	-	-	-	-	0.90	0.93	0.47	0.45	-	-		
Simpson (D) index	-	-	-	-	2.13	1.98	2.47	2.19	-	-		

[a] VNTR copy number of 00 represents no amplification of the PCR product at that locus.

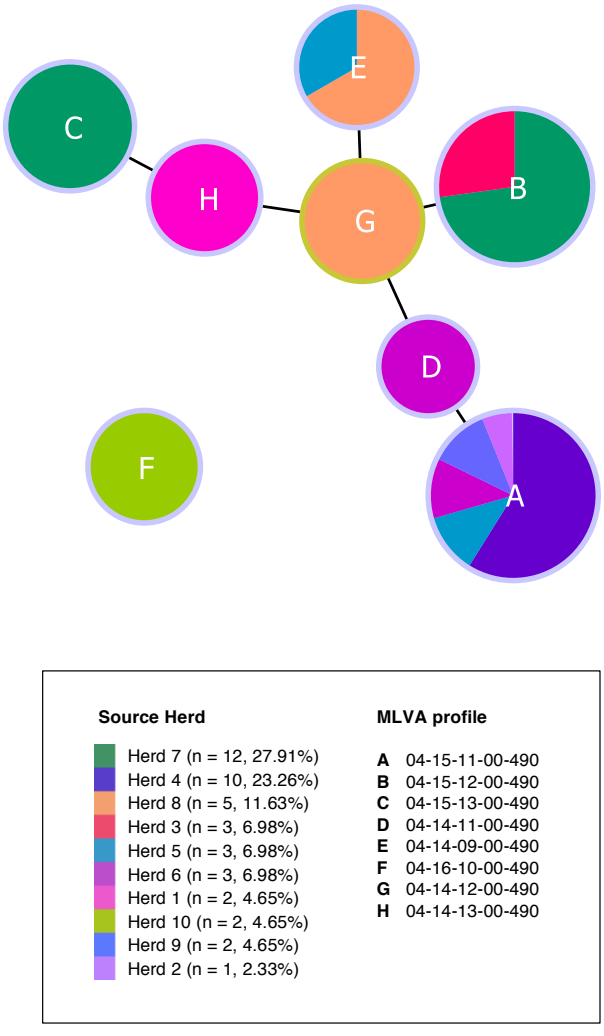

Figure 4-1. Minimum spanning trees of *S.* 1,4,[5],12:i:- MLVA profiles by herd. Created using eBURST analysis with standard goeBURST distance measures, visualized in PHYLOViZ. Edge = 1 SLV. The inferred founder profiles are designated by a gold outline. The coloured pie segments identify which herds the MLVA profile was found in, the node size (non-linear) indicates relative number of herds.

4.2.3. Herd connections

Herds sharing an MLVA profile are represented as an undirected network graph in which an edge identifies the presence of a shared MLVA profile in two herds (Figure 4-2). Physical connections between herds in which *S*. 1,4,[5],12:i:- was detected are represented as a directed network graph (Figure 4-3); the average path length in the directed graph of connections was 1.32, the average degree per node was 2.88 and the network modularity was 0.55, three communities identified within the network

The herds in which *S*. 1,4,[5],12:i:- was detected had received gilts from three breeders, eight feed mills—two herds produced feed themselves, and sold market weight pigs to five abattoirs. Geographic location was, unsurprisingly, closely associated with supplying feed mill and abattoir. Furthermore, although eight mills supplied feed to the sampled herds, some key ingredients, such as fishmeals and blood/meatmeals, are sourced from a limited number of suppliers nationally; information on the specific suppliers and sources of these ingredients was not available. All herds practiced self-replenishment, however, with a single exception, all received gilts from one of two major genetic suppliers.

The undirected *S*. 1,4,[5],12:i:- MLVA profile network bears little resemblance to the physical connections between herds described.

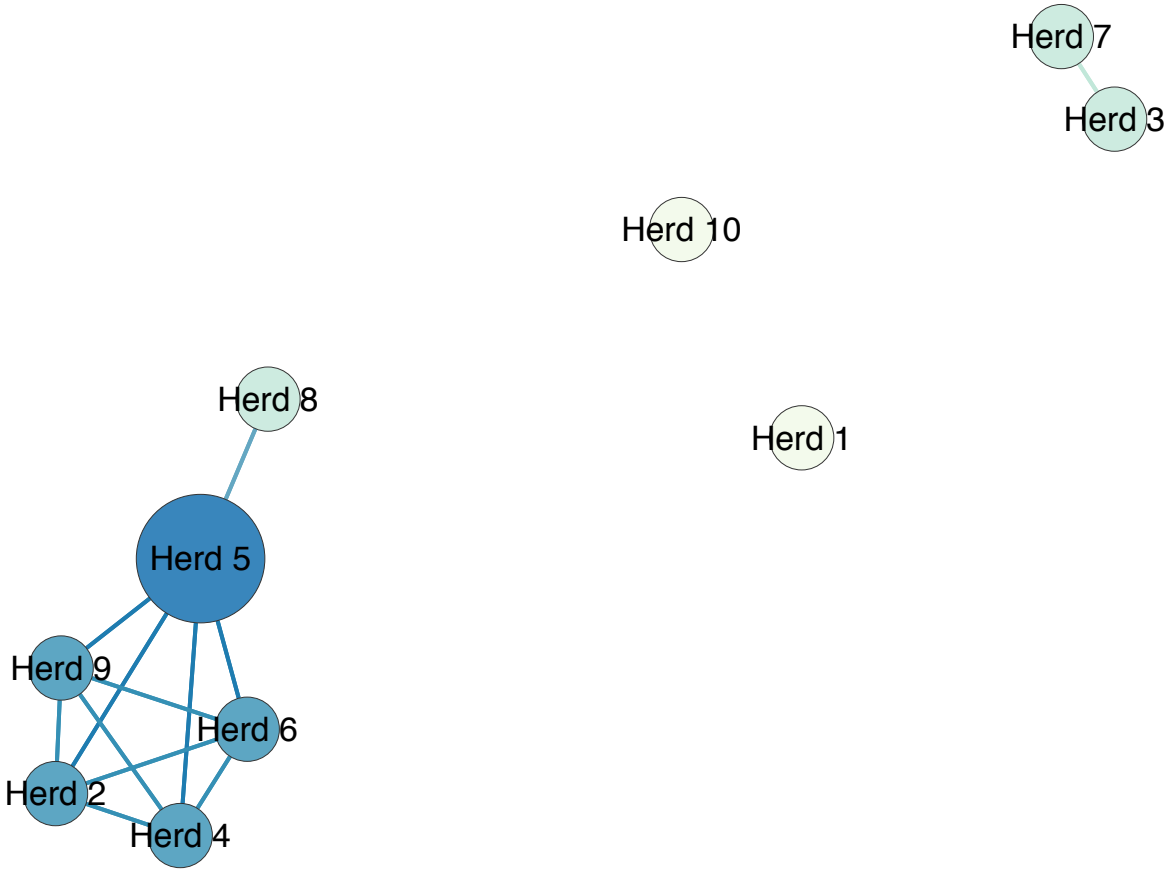

Figure 4-2. Undirected network of shared MLVA profiles and sampled herds. Edges are drawn where two herds shared the same S. 1,4,[5],12:i:- MLVA profile. Nodes are scaled by betweenness centrality, coloured by degree (light to dark). Note that the location of nodes is arbitrary, it does not reflect physical locations of actors.

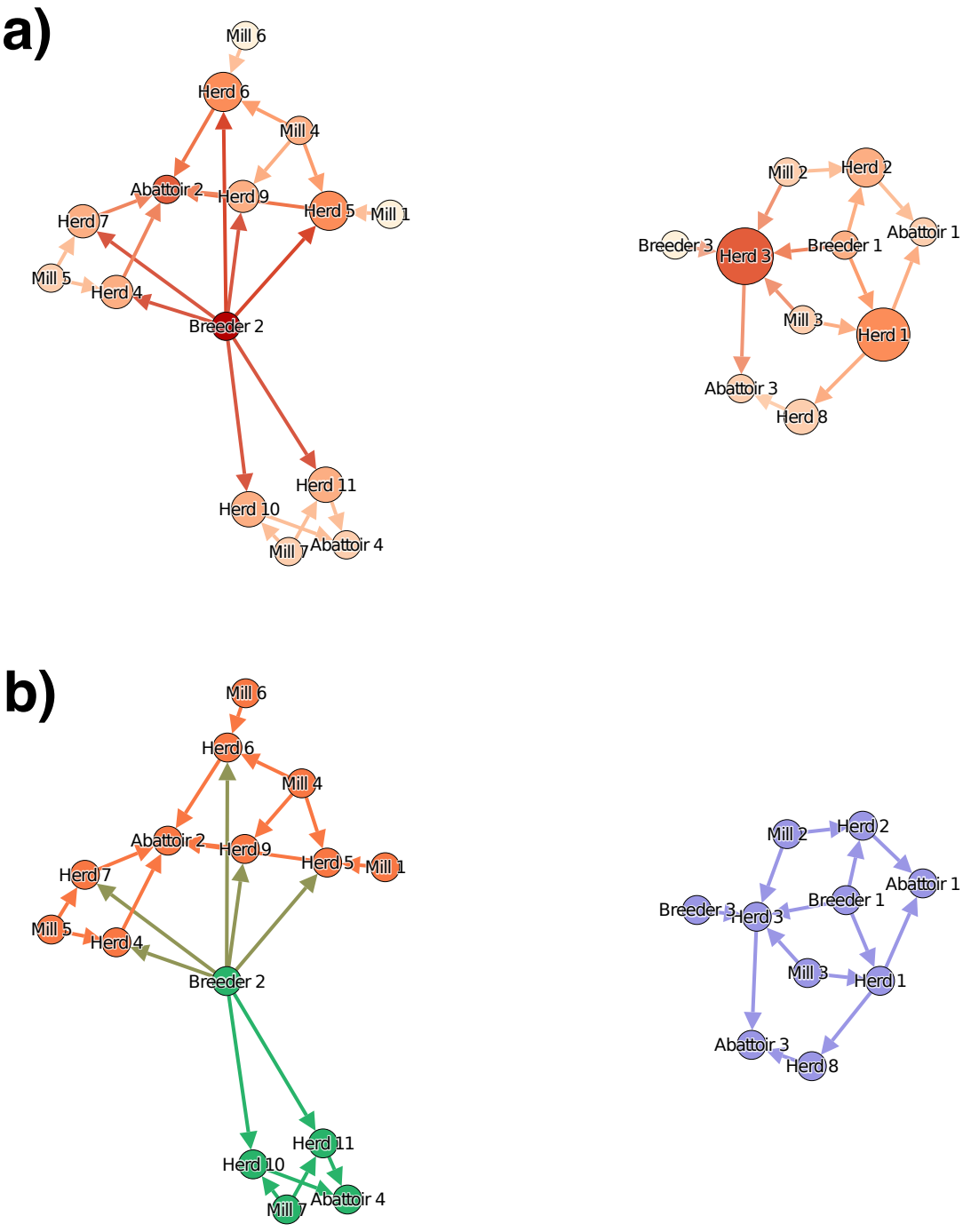

Figure 4-3. Directed network of physical links between herds where *S.* 1,4,[5],12:i:- was detected. Figure a) Nodes are scaled by betweenness centrality and coloured by degree (light to dark). b) coloured by community. Note that the location of nodes is arbitrary, it does not reflect physical locations of actors.

4.3. Discussion

The isolation of *S*. 1,4,[5],12:i:- from Herds 1 to 10 confirms that the serovar has colonized a diverse selection of Australian pig herds. These findings indicate that *S*. 1,4,[5],12:i:- has become established in pig production systems that broadly represent the Australian commercial pig industry. The detection of *S*. 1,4,[5],12:i:- from a diverse selection of Australian pig herds is consistent with reports of increasing *S*. 1,4,[5],12:i:- isolation in Australian passive surveillance (NEPSS, 2011, 2013, 2014). Moreover, isolation from this spectrum of herds with direct linkages via live animal movement suggests that the serovar may have become widespread within the industry. This mirrors reports of increasing isolation of *S*. 1,4,[5],12:i:- overseas (Switt et al., 2009; Hopkins et al., 2010; CDC, 2013a; Davies, 2013; Simon et al., 2013). These findings suggest that *S*. 1,4,[5],12:i:- could, or may already have become, more widespread than *S*. Typhimurium. This could relate to serovar's ability to become established within herds and/or the pathways through which the serovar is being transmitted. The serovar *S*. 1,4,[5],12:i:- may be transmitted through a pathway not typically associated with the spread of *S*. Typhimurium strains in Australia. The serovar could also have some form of advantage through changing management in relation to gut performance, such as changes in antimicrobial usage and increasing employment of organic acids and/or heavy metal compounds in pig feeds, as has been suggested by Davies (2013).

The observed *Salmonella* serovar diversity varied considerably between herds. *Salmonella* 1,4,[5],12:i:- was the only serovar isolated from samples collected from three of the 16 herds, indicating that *S*. 1,4,[5],12:i:- was the predominant *Salmonella* serovar being shed at the time of sampling and could have been the sole *Salmonella* serovar present in some or all three of these herds. Multiple serovars were identified in 9/16 herds sampled, echoing numerous studies that have found multiple serovars in individual herds (Funk et al., 2001; Gebreyes et al., 2004; Rajić et al., 2005; Miller et al., 2011; Mueller-Doblies et al., 2013; Pires et al., 2014; Niemann et al., 2015a). However, caution is needed when interpreting results of cross-sectional sample collection in relation to *Salmonella*. Studies conducted by Davies et al. (1999), Funk et al. (2001) and Pires et al. (2013a), among others, demonstrate that point sampling may present a distorted impression of the *Salmonella* serovars present within herds. *Salmonella* shedding is typically intermittent, which may result in false negatives and misclassification of herd *Salmonella*-status or a false impression of the or the characteristics of the *Salmonellae* present within a herd. To improve the reliability of findings pooled sampling was employed—with the exception of Herd 9 samples, from which individual samples were collected by the collaborating veterinarian—to increase the likelihood of detecting *Salmonella* shedding within cohorts given intermittent shedding by individuals. Studies have demonstrated that pooled faecal sampling improves efficiency of detection for *Salmonella* dramatically, Arnold and Cook (2009) demonstrated efficiency gains of nearly 100% in terms of detection when collecting pooled faecal samples as opposed to individual rectal samples from pigs. Furthermore, colonization

of individual pigs with multiple serovars may also occur, therefore isolation of a single serovar from a pig may not present the full picture of the animal's *Salmonella* burden. During this study, it was common to detect multiple serovars among isolates from a single pooled sample, demonstrating that the characterization of multiple colonies per positive sample presented a more complete picture of *Salmonella* shedding in the herds sampled.

Salmonella 1,4,[5],12:i:- was isolated with at least one other contemporary serovar in 6/10 herds. This finding differs from the results of the Hamilton et al. (2015) study but resembles reports elsewhere (Niemann et al., 2015a). Hamilton et al. (2015) found that among the 346 *Salmonella* isolates from 71 samples collected from grow-out pigs all were *S*. 1,4,[5],12:i:- with the exception of 11 non-motile *S*. Typhimurium (*S*. 1,4,[5],12:-:-) isolates and one *S*. rough:i:- isolate. Whereas Niemann et al. (2015a) found *S*. 1,4,[5],12:i:- and *S*. Derby coexisting in German pig herds. It appears likely that the herd studied by Hamilton et al. (2015) was *Salmonella*-free prior to the introduction of *S*. 1,4,[5],12:i:- and that no other serovars became established over the study period. This may also have been the case in the three study herds in which *S*. 1,4,[5],12:i:- was the only serovar isolated. This could indicate that the herds were *Salmonella*-free prior to the introduction of *S*. 1,4,[5],12:i:-. This suggests that *S*. 1,4,[5],12:i:- may have been introduced to these herds via a different transmission pathway to other *Salmonella* serovars. Identifying possible pathways would contribute to controlling spread of *S*. 1,4,[5],12:i:-. Alternatively, some component of the herd-pathogen-environment context in these herds favoured the serovar or disadvantaged other serovars, allowing *S*. 1,4,[5],12:i:- to become established as the sole or predominant serovar within these herds. It is also possible that *S*. 1,4,[5],12:i:- may have enjoyed some form of competitive advantage over other serovars in the herd and come to predominate the *Salmonella* population over time. In which case herds harbouring *S*. 1,4,[5],12:i:- among other serovars could come to be dominated by *S*. 1,4,[5],12:i:-. Variation in virulence and shedding rates among different *Salmonella* serovars is well recognized (Fedorka-Cray et al., 2000). However, the presence of other serovars, and/or the composition of the wider microbiome, could present greater competition for *S*. 1,4,[5],12:i:-, leading to a stable *Salmonella* spp. population comprised of multiple serovars. Competitive exclusion between *Salmonella* and other microflora and competition between *Salmonella* serovars has been described (Mead, 2000; Rabsch et al., 2000). At the macro-level Rabsch et al. (2000) found retrospective epidemiological data from Germany indicated that the eradication of *S*. Gallinarum created a vacant niche for *S*. Enteritidis in poultry, resulting in an observed rise in human *S*. Enteritidis cases. The effects of the presence of contemporary *Salmonella* serovars on shedding of *S*. 1,4,[5],12:i:- populations within herds over time may warrant further investigation.

All 43 *S*. 1,4,[5],12:i:- isolates in the collection were identified as PT193, mirroring the Hamilton et al. (2015) study which also found PT193 was ubiquitous among their *S*. 1,4,[5],12:i:- isolates. This contrasts with other studies outside Australia that have reported isolation of *S*. 1,4,[5],12:i:- phage

types U311, DT195, DT104b, DT120, DTU302, and DT138, among others (Hopkins et al., 2010; García et al., 2013; Arguello et al., 2014; Andrés-Barranco et al., 2016). Phage type 193 has been widely reported internationally and has been described as a possible 'pandemic strain' (Hopkins et al., 2010; Davies, 2013). Given the herds sampled were broadly representative of the Australian industry, this suggests that a related *S*. 1,4,[5],12:i:- population may have emerged from a single point and spread widely within the industry.

Two major *S*. 1,4,[5],12:i:- clones have been reported extensively in Europe and Asia, the so-called 'Spanish' and the 'European' clones (EFSA, 2010b). The more recent 'European clone' is typically characterized by PT193 and PT120, while the earlier 'Spanish clone' is associated with U302 (de la Torre et al., 2003b; Petrovska et al., 2016). Among these isolates neither PT120 nor PT U302 were identified. Australian public records include PT120 isolates, however, there are no public records of U302 in Australia (NEPSS, 2011, 2013, 2014)—national reference laboratories do have the capacity to identify U302. Therefore, in terms of phage type, the collection appears to more closely resemble the 'European' clone. However, as Pang et al. (2012) note, evolutionary relationships between *S*. Typhimurium phage types are not well described. Moreover, the same phage type can comprise multiple lineages, for example Markogiannakis et al. (2000) identified six clones among *S*. Typhimurium DT104 isolates, and Liebana et al. (2002) employed multiple genotyping methods to demonstrate that multiple clones were present among groups of *S*. Typhimurium isolates describing distinct phage types. Furthermore, it is widely believed that *S*. 1,4,[5],12:i:- emerged in Europe, North America, and possibly elsewhere, from *S*. Typhimurium on a number of occasions leading to the establishment of multiple *S*. 1,4,[5],12:i:- clones (Soyer et al., 2009a; Hopkins et al., 2010; García et al., 2013). This most likely occurred via a, or multiple, deletion(s) and/or mutation(s) of the *fljAB* operon causing loss of expression of the second phase flagellar antigen. The Australian porcine *S*. 1,4,[5],12:i:- could have emerged from a domestic *S*. Typhimurium PT193 or related phage type through a similar but independent event. Therefore, while consistent with a recent common ancestor, the identification of a single phage type among the study isolates does not conclusively demonstrate that the study isolates are clonal. Further investigation of the relatedness of the study isolates would likely to provide greater insight into the probable timeframe and mode of *S*. 1,4,[5],12:i:- emergence in Australia. This could have implications for both herd, industry and national biosecurity.

Although MLVA subtyping proved to be considerably more discriminatory than phage typing when applied to the study *S*. 1,4,[5],12:i:- isolates, all the *S*. 1,4,[5],12:i:- isolates were MLVA single locus variants (SLVs), with a single minor exception. Dimovski et al. (2014) found that an MLVA profile MSTs generated using the standard eBURST algorithm, without weighting for locus mutation rates at STTR5 and STTR6 or the inclusion of Euclidean distance measures, accurately depicted relatedness between *Salmonella* Group B strains. The minimum spanning tree (MST) presented here,

generated in the manner recommended by Dimovski et al. (2014), shows the close relatedness of the study *S*. 1,4,[5],12:i:- isolates. The Hamilton et al. (2015) study found a greater number of MLVA profiles among *S*. 1,4,[5],12:i:- isolates, 13 profiles, but similarly all profiles were SLVs at loci STTR5 or STTR6. These results provide further evidence suggesting that the *S*. 1,4,[5],12:i:- population circulating within the Australian industry could be highly related.

The herds sampled appeared to harbor relatively distinct *S*. 1,4,[5],12:i:- MLVA profiles in spite of the apparent close relatedness of the study *S*. 1,4,[5],12:i:- isolates. Although three *S*. 1,4,[5],12:i:- MLVA profiles, 04-15-11-00-490, 04-15-12-00-490 and 04-14-09-00-490, were identified among isolates from multiple herds, MLVA proved considerably more effective than phage typing in distinguishing strains from different herds. Given the apparent high relatedness of the *S*. 1,4,[5],12:i:- MLVA profiles observed among the study isolates, the minor MLVA variation at STTR5 and STTR6 between herds may indicate minor variation between the strains introduced to herds or gains and losses of VNTR units while cycling within the herds. These findings provide empirical evidence of the considerable discriminatory power of MLVA in differentiating *S*. 1,4,[5],12:i:-, supporting use of the technique in outbreak strain identification and potentially in source attribution investigations. However, MLVA loci STTR5 and STTR6 are recognized as being more variable than the other MLVA loci employed in the Lindstedt et al. (2004) method (Wuyts et al., 2013; Dimovski et al., 2014). The stability of the STTR5 and STTR6 loci and the phylogenetic meaning of minor variation in MLVA profiles remain uncertain (Hopkins et al., 2007; Barua et al., 2013; Wuyts et al., 2013; Dimovski et al., 2014). Dimovski et al. (2014) were able to demonstrate relative stability of strain MLVA profiles by passaging strains *in vitro* for approximately 28,600 generations and *in vivo* in mice. Further investigation of the stability of MLVA profiles in the 'real-world' environment of pig herds over time would provide further evidence of the value and/or limitations of MLVA and the meaning of variation for epidemiological purposes.

This study found MLVA to be considerably more discriminatory than phage typing, providing additional empirical support for use of the technique in surveillance. This study has also established an extensive national pig *S*. 1,4,[5],12:i:- strain collection, these isolates present a powerful opportunity to test and optimize current and proposed *Salmonella* characterization methods. Further investigation of these isolates using available characterization tools can inform best-practices in Australian *Salmonella* monitoring to the ultimate benefit of both animal and human health practitioners and authorities.

These results suggest that *S*. 1,4,[5],12:i:- emerged recently in the Australian pig herd, possibly from a single event, and spread widely within the industry. The dataset was not suitable for testing hypotheses in relation to the ultimate source and pathways of introduction of *S*. 1,4,[5],12:i:- to the sampled herds. However, it was apparent that, despite the diversity of herds selected in this study, the Australian industry is closely connected, highlighting the potential for an infectious agent to

spread quickly with in the industry. The network graphs presented showed that sampled herds were connected when gilt supply, feed mill and abattoir were considered. To further test the association between these connections, and other possible routes of transmission, and *S*. 1,4,[5],12:i:- detection herds would need to be randomly selected, unfortunately this was beyond the scope of this study.

The interconnectedness within the Australian commercial pig industry and apparent spread of *S*. 1,4,[5],12:i:- described underlines the importance of strong herd-level biosecurity to reduce the risk of pathogens being introduced to herds (Niemann et al., 2015a). Identifying potential *S*. 1,4,[5],12:i:- transmission pathways, such shared suppliers and/or service providers, and developing a greater understanding of the ecology of the bacteria within herds is likely prove informative in the development of risk management strategies for controlling *S*. 1,4,[5],12:i:- and emerging pathogens of importance to both pig performances and food safety.

4.4. Conclusion

The identification of *S*. 1,4,[5],12:i:- in the Australian pig herds demonstrates that pandemic *Salmonella* strains can emerge within the Australian industry. Moreover, these findings suggest that *S*. 1,4,[5],12:i:- has spread rapidly through the industry. Despite the disparate nature of the herds sampled, the structure of the Australian commercial pig industry is such that most commercial herds are tangibly connected in some way. Further exploration of connections between seemingly unrelated herds may provide insights into the means by which *S*. 1,4,[5],12:i:- spread between herds. These results serve as a reminder of the risks associated with the emergence of a pathogen within a closely connected industry and the importance of maintaining strong herd-level biosecurity measures. These findings also indicate that the Australian porcine *S*. 1,4,[5],12:i:- population may be closely related. This suggests that the serovar may have emerged recently in Australian pigs, possibly from a single event such as a mutation of a domestic *S*. Typhimurium strain or a breakdown in national biosecurity. Finally, these results provide evidence of the considerable discriminatory power of MLVA in relation to the serovar *S*. 1,4,[5],12:i:-, even among apparently closely related strains, indicating that use of the technique in routine use in animal and public health surveillance systems may be warranted. However, further investigation of the stability of MLVA profiles in herds over time is needed to determine the meaning of minor profile variation for the purposes of epidemiological investigations in the farm setting. Effective surveillance of *Salmonella* serovars, such as *S*. 1,4,[5],12:i:-, and other infectious agents is essential to ensure food products are safe and that the industry remains competitive in both domestic and export markets. Given the high costs associated with typing pathogens it is essential that methods are employed strategically. Monitoring *S*. 1,4,[5],12:i:- shedding within Australian herds over time and comparison of these isolates with *S*. 1,4,[5],12:i:- isolates from humans and other sources is likely to inform animal health management and Australian human source attribution investigations.

Chapter 5 - A case study of *Salmonella* 1,4,[5],12:i:- and contemporary serovar shedding among grow-out pigs in an Australian herd

Preface

Cross-sectional sampling can present a misleading picture of *Salmonella* colonization within pig herds, due to the often intermittent nature of shedding among pigs, as has been shown in studies conducted by Davies et al. (1999), Funk et al. (2001) and Pires et al. (2013a), among others. Hamilton et al. (2015) monitored *S.* 1,4,[5],12:i:- shedding in a single Australian herd and found very little *Salmonella* serovar diversity. Having isolated *S.* 1,4,[5],12:i:- with contemporary *Salmonella* serovars in herds approximating the diversity of Australian commercial pig production, a longitudinal study of *Salmonella* shedding was designed to monitor *Salmonella* shedding in herds in which *S.* 1,4,[5],12:i:- appeared to coexist with other *Salmonella* serovars. The study targeted grower-finishers as a potential route into the human food chain. Three herds sampled in the cross-sectional study (Chapter 4) in which *S.* 1,4,[5],12:i:- shedding was detected from grow-out pigs among other serovars were selected.

The herds employed the same consulting veterinarian. Given the logistics of sampling, the veterinarian kindly offered to collect some of the samples on behalf of the study. One herd was an outdoor operation that the veterinarian only visited at six-month intervals and the herd was sampled on one further occasion. Of the two remaining herds one was sampled on three occasions, unfortunately, however, the author was unable to make arrangements to visit this herd and so the herd was not fully profiled. The third herd, Herd 4, was sampled on four occasions, and the author visited the herd numerous times during this study and a subsequent longitudinal study in which the whole herd was sampled (Chapter 6).

This chapter presents the results of longitudinal sampling of Herd 4 grow-out pigs. A detailed description of the herd is provided in the preceding Annex 1. The results that informed this chapter generated the hypotheses that were subsequently addressed in the longitudinal study of *Salmonella* shedding in five herds, the results and discussion of which are presented in the following Chapter 6.

5.1. Introduction

The cross-sectional study, described in Chapter 4, determined that *S*. 1,4,[5],12:i:- had colonised a range of pig herds located across the major pig producing regions of Australia. *Salmonella* shedding within herds is complex and varies over time, as pigs shed the bacteria intermittently (Funk et al., 2001; Pires et al., 2013a). Funk et al. (2001) showed that point estimates of *Salmonella* prevalence and the serovars present may not be a reliable means of establishing *Salmonella* status in pig herds. A prospective longitudinal case study was designed to provide a more nuanced description of *S*. 1,4,[5],12:i:- and contemporary *Salmonella* spp. shedding among grow-out pigs

The study was designed to provide indications of the nature and characteristics of *S*. 1,4,[5],12:i:- and contemporary serovar shedding among grow-out pigs within a 'typical' conventionally housed Australian commercial pig herd. The objective of the study was to monitor *S*. 1,4,[5],12:i:- and other serovars shed by the herd over an extended period of time and to generate further hypotheses in relation to the dynamics of shedding and colonization and potential approaches to control of the serovar. Herd 4 was selected for the case study on the basis of previous results and willingness to participate.

5.2. Results

Salmonella was confirmed in 50/56 samples collected. In total 268 colonies were isolated, 56 isolates were fully serotyped and characterized; a further 51 isolates were partially serotyped and found not to conform with *Salmonella* Group B. The serovar *S*. 1,4,[5],12:i:- was detected among isolates from 41 samples and the majority of the isolates characterized were *S*. 1,4,[5],12:i:- (41/56). Four other serovars were also identified: *S*. London (6), *S*. Adelaide (4), *S*. Bovismorbificans (4) and *S*. Derby (1). In addition to the pig faecal samples, two pooled samples of rodent faeces were collected, *Salmonella* was detected in both samples, *S*. 1,4,[5],12:i:- was isolated from one sample and *S*. London was detected in the other.

Salmonella 1,4,[5],12:i:- was detected in 58.3% to 100% of samples per sampling occasion, while the other serovars were detected considerably less frequently, up to 20% of samples per sampling occasion (Table 5-1, Figure 5-1). This was reflected in the mean true prevalence estimates (P_T) of serovar shedding: above 86.3% (95% confidence interval [CI] 43.7-98.7%) for *S*. 1,4,[5],12:i:- and below 28.5% (95%CI 11.6-64.6%) for all other serovars.

The *S*. 1,4,[5],12:i:- isolates were PT193 (36) or PT 120 (5) (Table 5-2, Figure 5-2). The *S*. 1,4,[5],12:i:- MLVA profile 04-15-11-00-490 was the only MLVA profile identified in the first three

sample batches. One isolate with the SLV MLVA profile 04-15-11-00-490 was identified among samples collected on the final sampling occasion.

Table 5-1. Herd 4 estimated true shedding prevalence for *Salmonella* spp and individual serovars shedding by sampling occasion.

Sampling occasion	Date of sampling (M/D/Y)	*Salmonella* detections/ Sample	Serovars (number of samples)	Proportion of samples (%)	Mean estimated true prevalence of pigs shedding serovar (P_T, %)	Mode estimated true prevalence of pigs shedding serovar (P_T, %)	95% confidence interval
1	1/11/2013	13/14	*Salmonella* spp.	92.9	92.3	98.9	74.1, 99.8
			S. 1,4,[5],12:i:- (10)	71.4	85.3	97.4	58.7, 99.4
			S. Adelaide (2)	14.3	27.8	21.9	6.0, 61.5
			S. Bovismorbificans (2)	14.3	27.7	21.0	5.9, 61.8
			S. Derby (1)	7.1	18.4	10.1	2.0, 48.7
			S. London (1)	7.1	18.4	10.8	2.0, 48.3
2	3/12/2013	18/20	*Salmonella* spp.	90.0	93.6	99.1	78.4, 99.8
			S. 1,4,[5],12:i:- (14)	58.3	86.8	97.9	63.3, 99.5
			S. Adelaide (1)	5.0	13.3	6.6	1.3, 36.3
			S. London (4)	20.0	33.8	29.7	11.6, 64.7
3	5/20/2013	9/12	*Salmonella* spp.	75.0	85.9	97.6	58.8, 99.5
			S. 1,4,[5],12:i:- (7)	70.0	76.5	84.3	43.6, 98.7
			S. Adelaide (1)	8.3	21.1	11.7	2.4, 54.9
			S. Bovismorbificans (2)	16.7	31.8	23.8	6.9, 69.4
			S. London (1)	8.3	21.2	11.6	2.4, 55.0
4	7/2/2013	10/10	*Salmonella* spp.	100	91.7	98.8	76.1, 100.0
			S. 1,4,[5],12:i:- (10)	100	91.7	98.9	76.3, 100.0

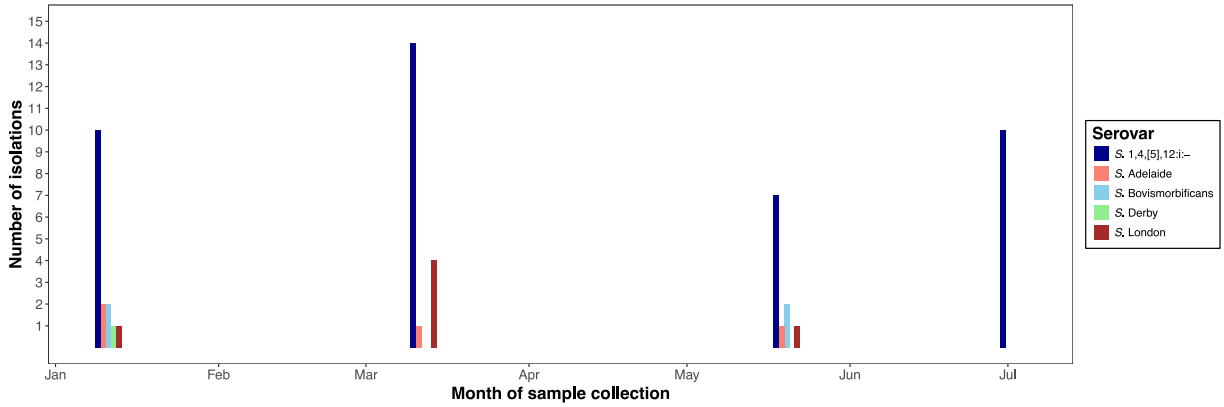

Figure 5-1. Serovar isolations from Herd 4 over time.

Table 5-2. Herd 4 *S.* 1,4,[5],12:i:- phage type and MLVA profiles by sampling occasion.

Sampling occasion	Number of *S.* 1,4,[5],12:i:- isolates	Phage type (n)	MLVA profile (n)
1	10	193	04-15-11-00-490 (10)
2	14	193 (11), 120 (3)	04-15-11-00-490 (14)
3	7	193 (5), 120 (2)	04-15-11-00-490 (7)
4	10	193 (10)	04-15-11-00-490 (9), 04-14-11-00-490 (1)

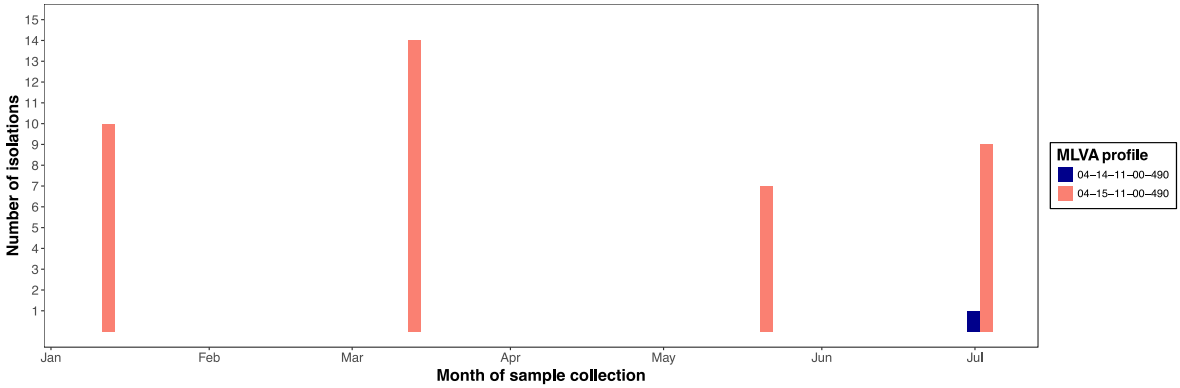

Figure 5-2. *S.* 1,4,[5],12:i:- MLVA profile isolations over time.

5.3. Discussion

Multiple serovars were detected in each of the first three sampling occasions. The shedding of *S.* 1,4,[5],12:i:- and other serovars contrasted with the observations of Hamilton et al. (2015) who found virtually all isolates were *S.* 1,4,[5],12:i:- in a similarly designed longitudinal study of grow-out pigs in one Australian herd. In contrast, grow-out pigs in Herd 4 appear to have been routinely exposed to multiple serovars showing that the herd maintained a diverse *Salmonella* population. Other studies have found *S.* 1,4,[5],12:i:- and other serovars coexisting in pig herds, such as Niemann et al. (2015a) who identified both *S.* 1,4,[5],12:i:- and *S.* Derby among isolates collected through a similar longitudinal study of five pig herds in Germany. The presence of *S.* 1,4,[5],12:i:- and other serovars indicates an introduction of mixed infection or multiple introductions potentially through different pathways and/or from different sources.

Salmonella 1,4,[5],12:i:- was detected in high numbers of samples on each sampling occasion, whereas, the other serovars detected, *S.* Adelaide, *S.* London and *S.* Bovismorbificans, were detected less frequently and were not detected on all sampling occasions. This variability could relate to the prevalence of each serovar among grow-out pigs or the rate and consistency at which colonized pigs shed specific serovars. Intermittent *Salmonella* shedding among pigs has been well

described by other studies (Davies et al., 1999; Funk et al., 2001; Ivanek et al., 2012; Pires et al., 2013a). For example, longitudinal studies of grow-out pig cohorts by Kranker et al. (2003) describe the intermittent nature of *Salmonella* shedding and the considerable variability in the onset and duration of *S*. Typhimurium specific shedding among grow-out pigs in three Danish herds. While Scherer et al. (2008) found pigs inoculated with *S*. Typhimurium DT104 shed the organism at high levels for two-weeks post inoculation before shedding intermittently. It is possible that *S*. 1,4,[5],12:i:- colonization induced consistently high levels of shedding in individuals, perhaps even inducing 'super shedding' in some animals leading to high rates of detection. While *Salmonella* 'super shedders' have been demonstrated in other species, this phenomenon has not yet been described in pigs (Berriman et al., 2013a; Berriman et al., 2013b). However, this study was not designed to assess individual shedding rates or durations. Nevertheless, the higher levels of *S*. 1,4,[5],12:i:- detection relative to detection of other serovars observed in this study indicate a higher likelihood of grow-out pig exposure to the serovar. Further investigation of the nature of *S*. 1,4,[5],12:i:- shedding in individuals in relation to maintenance of colonization within cohorts could inform approaches to controlling the serovar.

Grow-out pigs have been well-described as a potential route of *Salmonella* into the human food chain (Berends et al., 1997; Mousing et al., 1997a; Dahl and Sørensen, 2001; Alban and Stärk, 2005; Alban et al., 2012; De Busser et al., 2013; Andres and Davies, 2015; Snary et al., 2016). These results show *S*. 1,4,[5],12:i:- colonization in Australian grow-out pigs from weaning through to finish, demonstrating a potential pathway into the domestic food chain. These findings indicate that weaner cohorts were exposed to *S*. 1,4,[5],12:i:- routinely, mirroring the findings of Hamilton et al. (2015) study. Further investigation of when grow-out pigs become colonized within the herd could indicate the method of exposure and would be informative for control strategies.

The route through which weaner cohorts in this herd were exposed to the *S*. 1,4,[5],12:i:- and other serovars remains uncertain. The *Salmonellae* could have been circulating throughout the herd, involving other production stages such as the sow herd, or may have involved other forms of maintenance and transmission, such as the resident rodent population or the farm environment. It is possible that suckling pigs were being exposed to *S*. 1,4,[5],12:i:- via the sow during or post farrowing and colonization subsequently persisted in the weaned cohort. Some research has suggested that vertical transmission between sow and progeny plays a minor role in transmission of *Salmonella*, such as studies by Dahl et al. (1997) and Fedorka-Cray et al. (1997) which demonstrated control of *Salmonella* infection by strategic movement of weaned piglets. However, Kranker et al. (2001) showed that seropositivity among sows was significantly associated with isolation of *S*. Typhimurium from nursing piglets. Furthermore, longitudinal studies conducted by Nollet et al. (2005a); Nollet et al. (2005b) found highly similar strains among both sows and grow-out pigs and evidence of sows maintaining *Salmonella* populations within herds. Alternatively rodents could have

played a role in maintaining the herd's *S.* 1,4,[5],12:i:- population. Rodents were an ongoing problem in the herd despite control efforts. Isolation of *S.* 1,4,[5],12:i:- from rodent faeces samples indicates that rodents were shedding the serovar. However, the directionality of transmission is unknown. Meerburg and Kijlstra (2007) present a review of the literature demonstrating the potential of rodent populations in agro-ecological settings to transmit *Salmonella* to food animals. Among others, Vico et al. (2011) found that lack on rodent control, as a proxy for rodent problems, presented a risk factor in their study of *Salmonella* colonization of pigs from 80 Spanish herds. Exposure could also have occurred via the environment or fomites, however, the serovar was routinely detected among piglets immediately after weaning in the weaner rooms, which were modern and hygienic with strong enforcement of cleaning and disinfection protocols. Investigation of *S.* 1,4,[5],12:i:- shedding among other production stages in herds with endemic colonization would provide indications as to how weaner cohorts are routinely exposed and the serovar is maintained.

Weaner cohorts underwent a particularly challenging period prior to entering the grower shed, designated Weaner Shed 2 (Annex 1). Weaner Shed 2 was a building of poor quality, and consequently low hygiene, in which stocking density was particularly high. Funk et al. (2000a) among others have found that high stocking rates, likely associated with stress induced shedding and increased pig-to-pig contact, increase the risk of *Salmonella* transmission (Lo Fo Wong et al., 2002; Funk and Gebreyes, 2004). Furthermore, pigs in Weaner Shed 2 had snout-to-snout contact with pigs from other cohorts through barred partitions. Wilkins et al. (2010) and Lo Fo Wong et al. (2004) found snout-to-snout contact between cohorts to be a significant risk factor for *Salmonella* prevalence, OR 2.2 and OR 1.7, respectively, for pigs testing seropositive for *Salmonella*. It is likely that Weaner Shed 2 was a point of exposure or re-exposure to *S.* 1,4,[5],12:i:- between cohorts, however, the serovar was identified among samples from weaned pigs prior to entering the shed. Sampling of weaner-grower cohorts before, during and after Weaner Shed 2 would likely indicate whether this suspected point of transmission and/or escalation affected prevalence and the dynamics of colonization and shedding.

The observation of two *S.* 1,4,[5],12:i:- phage types, PT193 and PT120, could indicate two lineages were coexisting within the herd. However, phage types PT193 and PT120 are closely related (Hopkins et al., 2010; Petrovska et al., 2016). The observed variation in phage type could have been associated with minor genetic changes in the strains while circulating within the herd that conferred resistance or lack thereof to specific phages in the typing panel, rather than two distinct lineages cohabiting within the herd. Sequencing the genomes of comparable strains would likely prove informative and provide further evidence of the level of relatedness of the *S.* 1,4,[5],12:i:- population observed in this herd.

The negligible *S*. 1,4,[5],12:i:- MLVA profile diversity in this herd contrasts with the Hamilton et al. (2015) study in which 13 profiles were identified. The large majority of *S*. 1,4,[5],12:i:- isolates were characterized as MLVA profile 04-15-11-00-490 (41/42 isolates tested). This MLVA profile was also identified in other herds sampled in the cross-sectional study (Chapter 4) and was identified among isolates in the Hamilton et al. (2015) study (D. Hamilton, personal communication). In the Hamilton et al. (2015) study the MLVA profile 04-15-11-00-490 and an SLV were the only profiles identified among isolates from each of the five sampling occasions. It is likely that the other MLVA profile identified among the Herd 4 isolates, 04-14-11-00-490, was derived from 04-15-11-00-490, the strain have lost a VNTR unit at the relatively unstable STTR5 locus (Wuyts et al., 2013; Dimovski et al., 2014). The MLVA profiles observed suggest that a closely related *S*. 1,4,[5],12:i:- population was circulating within Herd 4, however, independent introductions cannot be completely ruled out. The other Herd 4 *S*. 1,4,[5],12:i:- MLVA profile, 04-14-11-00-490, was not identified among isolates in the Hamilton et al. (2015) study and was only detected in one other herd, Herd 6, sampled in the cross-sectional study. Herd 6 had no direct live animal link to Herd 4, but both herds received gilts from the same nucleus herd.

The identification of apparently closely related *S*. 1,4,[5],12:i:- strains throughout the growing period suggests the serovar persisted within cohorts. Persistence could have occurred through individual carriage, pig-to-pig transmission from colonized to naïve animals or re-colonization of pigs, repeated exposure of cohorts to a strains resident within the herd—possibly from other cohorts, sows, feed, the environment, fomites or vectors, or via a combination of routes (Davies et al., 1997; Kranker et al., 2003). A cohort study could shed more light on the dynamics of colonization within cohorts.

The presence of an apparently highly-related *S*. 1,4,[5],12:i:- population could indicate a single introduction to Herd 4 or multiple introductions from a stable source population likely through a single pathway, whereas multiple distinct lineages could suggest introduction from different sources. The serovar *S*. 1,4,[5],12:i:- is believed to have emerged on a number of separate occasions (Soyer et al., 2009a; Hauser et al., 2010), most likely from *S*. Typhimurium strains losing the *fljAB* operon conferring the second phase flagellar antigen. During this study, no *S*. Typhimurium isolates were identified. Given *S*. Typhimurium can persist in pig herds (Sandvang et al., 2000; Wales et al., 2009), that multiple colony picks were serotyped, and that other serovars were also identified among the study isolates, it is likely *S*. Typhimurium would have been identified if the serovar were present. On this basis it is unlikely that the *S*. 1,4,[5],12:i:- population present in Herd 4 emerged independently within the herd from an *S*. Typhimurium. This suggests that the serovar was introduced to the herd via a point source. A point source introduction could have occurred through an isolated or uncommon breach in the herd's biosecurity, or via a pathway that was only

contaminated for a limited period. However, repeat introduction from a highly related and stable source population cannot be ruled out.

This study was unable to provide indications of how *S*. 1,4,[5],12:i:- may have been introduced to Herd 4, assuming the serovar was introduced. There are numerous studies demonstrating the role of purchased pigs, or supply from multiple herds, in determining herd *Salmonella* status (Berends et al., 1996b; Lo Fo Wong et al., 2004; Zheng et al., 2007). This has resulted in a general consensus that *Salmonella* infection in breeder herds is correlated with risk among downstream herds. Given the same MLVA profiles have been identified among other herds, including a herd that received gilts from the same nucleus herd, sampling the nucleus herd directly or new gilts prior to introduction could indicate a possible route of *S*. 1,4,[5],12:i:- introduction. Feed is also well recognized as a potential route of *Salmonella* introduction (Berends et al., 1996a; Lo Fo Wong et al., 2002; Funk and Gebreyes, 2004). The serovar *S*. 1,4,[5],12:i:- has been isolated from feed components in Australia, suggesting it could have been introduced through this pathway (NEPSS, 2011, 2013, 2014). Other known pathways that warrant consideration include fomites, such as transport and other visiting vehicles, and vectors such as humans, rodents, birds and other wildlife, via infection or mechanical carriage (Funk and Gebreyes, 2004).

The farm management had effectively controlled clinical disease among weaners prior to the study, which was associated with isolation of *S*. 1,4,[5],12:i:-. Control of clinical disease was attributed to the inclusion of organic acids in the weaner diet by the management and consulting veterinarian. This mirrors the findings of Creus et al. (2007) who observed significant reductions in *Salmonella* prevalence among pigs fed an acidified diet by comparison with the control group in their study. However, these results demonstrate that the control of clinical disease did not indicate control of *Salmonella* colonisation within the herd. In fact, detection of *Salmonella*, *S*. 1,4,[5],12:i:- more particularly, remained high among grow-out pig cohorts throughout the study; *S*. 1,4,[5],12:i:- was detected in 75-100% of samples across the four sampling occasions. The presence of *Salmonella* serovars of importance to human health, such as *S*. 1,4,[5],12:i:-, at finish risks escalation in transport and lairage and an increased likelihood of the hazard being present in or on products (Berends et al., 1996a; Hurd et al., 2001). This demonstrates that clinically healthy market-destined pigs could still present an *S*. 1,4,[5],12:i:- food safety risk. Further investigation of the ecology of *S*. 1,4,[5],12:i:- in Australian pig herds is needed to develop effective controls that manage both clinical disease on farm and food safety risk post-farmgate.

5.4. Conclusion

The study found *S*. 1,4,[5],12:i:- coexisting with other serovars. The serovar was detected considerably more frequently than other serovars, indicating higher *S*. 1,4,[5],12:i:- prevalence

and/or higher shedding rates among cohorts of weaner-grower pigs. The results demonstrated that cohorts of grow-out pigs were routinely exposed to *S*. 1,4,[5],12:i:- and colonization with the serovar appeared to persist through to finish. The *S*. 1,4,[5],12:i:- isolate page types and MLVA profiles described low diversity, suggesting that a closely related population was being circulating within the herd's weaner-finisher pigs. The high levels of detection of *S*. 1,4,[5],12:i:- among market-destined pigs indicated a risk of product contamination with the hazard. The addition of organic acids to weaner-grower diets appeared to be effective in controlling clinical salmonellosis, however, *Salmonella* continued to be isolated from grow-out pigs. Determining how *S*. 1,4,[5],12:i:- is maintained within herds could inform approaches to controlling the serovar on-farm and managing associated food safety risks. As pressure increases to reduce the use of antimicrobials on farm, developing effective strategies for managing pig health and controlling food safety pathogens such as *S*. 1,4,[5],12:i:- will become increasingly important.

Chapter 6 - Longitudinal study of *Salmonella* 1,4,[5],12:i:- shedding in five Australian pig herds

Preface

The results of the Herd 4 case study (Chapter 5) indicated that a closely related *S.* 1,4,[5],12:i:- population was being shed persistently by grow-out pigs in the herd. This suggested that new cohorts of grow-out pigs were routinely being exposed to *S.* 1,4,[5],12:i:-, among other *Salmonella* serovars, raising the question of how the *Salmonellae* populations were being maintained within the herd. Furthermore, the studies presented in earlier chapters suggested that MLVA could be useful for epidemiological investigations. The cross-sectional study indicated herd populations might be differentiated on the basis of MLVA typing, and the case study showed a high level of stability of MLVA profiles among *S.* 1,4,[5],12:i:- isolates from grow-out pigs in a single herd over an extended period. Describing the within-herd shedding of *S.* 1,4,[5],12:i- and contemporary *Salmonella* serovars could inform strategies for control of clinical disease and management of hazard levels in finished pigs.

Building on the findings of the preceding chapters, this chapter describes and discusses *Salmonella* shedding in five case study herds over a period of 12-months. This study followed the Herd 4 case study presented in the previous chapter (Chapter 5).

A manuscript was developed on the basis of this chapter and published in the journal Preventive Veterinary Medicine, the details of the article follow.

Weaver, T., Valcanis, M., Mercoulia, K., Sait, M., Tuke, J., Kiermeier, A., Hogg, G., Pointon, A., Hamilton, D., Billman-Jacobe, H., 2017. Longitudinal study of *Salmonella* 1,4,[5],12:i:- shedding in five Australian pig herds. Prev. Vet. Med. 136, 19-28.

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6.1. Introduction

A prospective observational longitudinal study was designed to further investigate *S*. 1,4,[5],12:i:- and contemporary serovar shedding in colonized herds, the dynamics of MLVA profiles over time, and the implications of this in terms of herd health and food safety. Five farrow-to-finish herds employing conventional housing and/or deep bedding systems were selected for sampling. No live animal movement had occurred between the herds prior to or during the study.

The aim of the study was to generate hypotheses on *Salmonella* transmission and the dynamics of shedding and colonization within and between herds and to provide evidence of persistence or changes in *S*. 1,4,[5],12:i:- MLVA profiles over time. The objectives of the study were to: monitor rates of detection of *Salmonella* and *S*. 1,4,[5],12:i:- shedding within herds and between production stages within herds, indicative of extent and persistence of colonization; describe *Salmonella* serovar and subtype populations shed; and monitor *S*. 1,4,[5],12:i:- MLVA profiles over an extended period.

6.2. Results

6.2.1. *Salmonella* spp. detections and *S*. 1,4,[5],12:i:- phage types

The key characteristics of the five study herds are summarized in Chapter 3, Table 3-2 and a detailed profile of each herd are provided in Annex 1.

Salmonella spp. was detected and confirmed in 171/400 pooled samples collected, therefore, *Salmonella* was present in approximately 224 samples assuming test sensitivity of 69% (Funk et al., 2000b). An aggregate estimated true shedding prevalence (P_T) of 15.4% (95%CI 12.3-22.2). In total 960 *Salmonella* spp. isolates were further characterized, of which 181 were fully serotyped and 18 serovars were identified. A further 375 isolates were partially serotyped and found not to be *S*. 1,4,[5],12:i:-.

Salmonella 1,4,[5],12:i:- was detected in 95/400 samples (P_T 9.7%; 95%CI 7.3-14.0) across this study. All *S*. 1,4,[5],12:i:- isolates were PT193, with the exception of one PT6 isolate and five isolates that did not react when exposed to the phages, and were therefore deemed untypable. A single *S*. Typhimurium isolate was identified from Herd 1 samples, which was untypable.

6.2.2. *Salmonella* spp. and *S*. 1,4,[5],12:i:- detections by herd, production stage and sampling occasion

The results showed variation in *Salmonella* shedding by herd, substantially higher shedding among weaners and, to a lesser extent, finishers, and the extended persistence of both *Salmonella* spp. and

S. 1,4,[5],12:i:- shedding in four of the five herds. The detection of *Salmonella* spp., *S.* 1,4,[5],12:i:- and estimates of the proportion of specified populations shedding *Salmonella* spp. and *S.* 1,4,[5],12:i:- are provided by herd and production stage and herd and sampling occasion in Tables 6-2 and 6-3 and Figures 6-1 and 6-2.

The number of *Salmonella* detections in each herd ranged from 17/80 in Herd 5 (P_T 8.9%) to 46/80 (P_T 20.6%) in Herd 4 (Table 6-2). *Salmonella* 1,4,[5],12:i:- detections from each herd ranged from 11/80 (P_T 6.8%) in Herd 2 to 28/80 (P_T 13.0%) in Herd 4.

Salmonella detection rates were much higher among weaner and finisher samples than among samples from sows and farrowing crates (Figure 6-1a). *Salmonella* 1,4,[5],12:i:- was detected in a very high proportion of *Salmonella* positive samples from weaner and finisher pigs, 84.3% (86/102), and a considerably lower proportion of *Salmonella* positive sow and farrowing shed samples, 20.9% (9/43).

Herd *Salmonella* detections from weaners ranged from 9/20 to 17/20 (P_T 16.1-36.0%) and for *S.* 1,4,[5],12:i:-from 5/20 to 17/20 (P_T 10.3-34.8%) (Table 6-1). Shedding rates appeared to be lower among finishers, 3/20 to 17/20 (P_T 6.9%-35.8%) for *Salmonella* and 3/20 to 8/20 (P_T 7.0-14.8%) for *S.* 1,4,[5],12:i:-, respectively. *Salmonella* detections from farrowing crate samples ranged from 1/20 in Herd 3 to 9/20 in Herd 4 (P_T 3.0-16.4%); *S.* 1,4,[5],12:i:- detections from none to three (P_T 0.4-7.2%). Gestating sows shedding detections were: 0/20 to 11/20 (P_T 0.4-19.6%) for *Salmonella* and 0/20 to 2/20 (P_T 0.4-5.2%) for *S.* 1,4,[5],12:i:-.

Salmonella and *S.* 1,4,[5],12:i:- shedding persisted in four of the five herds (Figure 6-1b). In two herds, Herds 2 and 3, the proportion of *S.* 1,4,[5],12:i:- detections relative to other serovars increased over time. *Salmonella* detections by sampling occasion in Herd 1 ranged from 6/20-16/20 (P_T 11.6-31.5%), Herd 2: 7/20-13/20 (P_T 13.0-23.8%), Herd 3: 5/20-9/20 (P_T 10.3-16.7%), Herd 4: 9/20-13/20 (P_T 16.3-23.6%), and Herd 5: 0/20-8/20 (P_T 0.4-13.0%) (Table 6-2). Detections of *S.* 1,4,[5],12:i:- shedding for Herd 1 ranged from 4/20-6/20 (P_T 8.8-11.6%), Herd 2: 1/20-5/20 (P_T 3.0-10.0%), Herd 3: 3/20-8/20 (P_T 6.8-14.6%), Herd 4: 6/20-9/20 (P_T 11.6-16.3%), and Herd 5: 0/20-7/20 (P_T 0.4-13.0%). A possible seasonal or climate-related effect was observed—relatively higher numbers of detections on cooler, wetter sampling occasions—however, results of mixed effects modeling were not robust due to the low number of herds in the study and a one year sampling period (data not presented).

Table 6-1. Herd *Salmonella* spp. and *S.* 1,4,[5],12:i:- detections and estimated true prevalence (mode) of pigs shedding *Salmonella* spp. and *S.* 1,4,[5],12:i:- stratified by production stage. Aggregated results of the four sampling occasions per herd. The for estimated true prevalence 95% confidence intervals are provided in parentheses.

Herd	Production stage	Number of pools (n=20) with *Salmonella* detected; herd total (n=80)	Estimated mean true prevalence (P_T mode, %) of pigs shedding *Salmonella* (95% confidence interval)	Number of pools (n=20) with *S.* 1,4,[5],12:i:- detected; herd total (n=80)	Estimated true prevalence (P_T mode, %) of pigs shedding *S.* 1,4,[5],12:i:- (95% confidence interval)	% Pools *S.* 1,4,[5],12:i:- present among pools *Salmonella* detected
1	Farrowing	4	8.5 (3.7, 17.1)	0	0.4 (0.0, 6.2)	0.0
	Weaners	14	25.8 (17.2, 47.1)	14	25.7 (17.3, 47.1)	93.3
	Finishers	17	35.8 (24.1, 72.2)	6	11.5 (6.4, 21.5)	35.3
	Dry sows	4	8.5 (3.6, 17.1)	0	0.4 (0.0, 6.2)	0.0
	Herd total	**39**	**17.4 (13.1, 26.6)**	**20**	**10.1 (6.9, 15.7)**	**51.3**
2	Farrowing	4	8.6 (3.6, 17.1)	1	3.4 (0.3, 10.0)	20.0
	Weaners	17	36.0 (24.1, 72.1)	5	10.3 (5.1, 19.3)	29.4
	Finishers	16	31.9 (21.5, 61.6)	3	7.0 (2.0, 14.9)	18.8
	Dry sows	5	10.1 (5.0, 19.2)	2	5.2 (0.8, 12.4)	40.0
	Herd total	**42**	**18.7 (14.1, 28.7)**	**11**	**6.8 (3.2, 10.9)**	**26.2**
3	Farrowing	1	3.0 (0.3, 10.0)	1	3.4 (0.3, 10.0)	100.0
	Weaners	17	35.0 (24.1, 71.6)	17	34.8 (24.1, 71.4)	100.0
	Finishers	9	16.6 (10.1, 28.8)	5	10.2 (5.1, 19.3)	55.6
	Dry sows	0	0.4 (0.0, 6.1)	0	0.4 (0.0, 6.2)	0.0
	Herd total	**27**	**12.6 (9.1, 19.5)**	**23**	**11.1 (7.8, 17.4)**	**85.2**
4	Farrowing	9	16.4 (10.0, 28.5)	3	7.2 (2.0, 14.9)	50
	Weaners	17	35.3 (24.1, 71.9)	16	31.5 (21.4, 61.1)	94.4
	Finishers	9	16.5 (10.1, 28.9)	8	14.8 (8.8, 26.3)	88.9
	Dry sows	11	19.6 (12.7, 34.7)	1	3.2 (0.3, 10.0)	9.1
	Herd	**46**	**20.6 (15.6, 31.5)**	**28**	**13.0 (9.4, 20.0)**	**60.9**
5	Farrowing	1	3.3 (0.3, 10.0)	1	3.3 (0.3, 10.0)	100.0
	Weaners	9	16.1 (10.1, 28.7)	9	16.6 (10.0, 28.8)	100.0
	Finishers	3	6.9 (2.0, 14.8)	3	7.2 (2.0, 14.9)	100.0
	Dry sows	4	8.5 (3.6, 17.1)	0	0.4 (0.0, 6.2)	0.0
	Herd total	**17**	**8.9 (5.8, 14.1)**	**13**	**7.3 (4.2, 12.0)**	**76.5**

Table 6-2. Herd *Salmonella* spp. and *S.* 1,4,[5],12:i:- detections and estimated true prevalence of pigs shedding *Salmonella* spp. and *S.* 1,4,[5],12:i:- by sampling occasion. Aggregated results of the four production stages sampled per herd. The 95% confidence intervals for estimated true prevalence of pigs shedding the specified organisms are provided in parentheses.

Herd	Sampling occasion	Season[a]	Number of pools (n=20) with *Salmonella* detected	Estimated true prevalence (P_T mode, %) of pigs shedding *Salmonella* (95% confidence interval)	Number of pools (n=20) with *S.* 1,4,[5],12:i:- detected	Estimated true prevalence (P_T mode, %) of pigs shedding *S.* 1,4,[5],12:i:- (95% confidence interval)	% Pools *S.* 1,4,[5],12:i:- present among pools *Salmonella* detected
1	A	Sp	6	11.6 (6.4, 21.6)	5	10.2 (5.1, 19.3)	83.3
	B	Su	7	14.4 (7.6, 23.7)	4	8.8 (3.7, 17.1)	57.1
	C	A	16	31.5 (21.4, 61.2)	6	11.6 (6.4, 21.5)	37.5
	D	W	10	18.0 (11.3, 31.5)	5	9.9 (5.1, 19.4)	50.0
2	A	Su	13	23.8 (15.6, 42.2)	1	3.1 (0.3, 10.0)	7.7
	B	A	11	20.0 (12.7, 34.9)	1	3.0 (0.3, 10.0)	9.1
	C	W	11	20.0 (12.6, 34.6)	4	8.8 (3.6, 17.0)	36.4
	D	Sp	7	13.0 (7.6, 23.8)	5	10.0 (5.1, 19.3)	71.4
3	A	A	6	11.4 (6.4, 21.5)	3	6.8 (2.0, 14.9)	50
	B	W	9	16.7 (10.0, 28.8)	8	14.6 (8.8, 26.3)	88.9
	C	Sp	7	13.0 (7.6, 23.9)	7	13.1 (7.6, 23.9)	100.0
	D	Su	5	10.3 (5.1, 19.3)	5	10.2 (5.1, 19.3)	100.0
4	A	Sp	11	19.8 (12.6, 34.6)	9	16.3 (10.0, 28.7)	81.8
	B	Su	13	23.6 (15.6, 42.2)	6	11.7 (6.3, 21.6)	46.2
	C	A	9	16.3 (10.0, 28.8)	6	11.6 (6.4, 21.6)	66.7
	D	W	13	23.2 (15.6, 41.8)	7	13.0 (7.6, 23.7)	53.8
5	A	A	2	5.3 (0.8, 12.6)	0	0.4 (0.0, 6.2)	0
	B	W	8	14.8 (8.8, 26.2)	7	13.0 (7.6, 23.9)	87.5
	C	Sp	7	13.1 (7.6, 23.9)	6	11.7 (6.4, 21.4)	85.7
	D	Su	0	0.4 (0.0, 6.2)	0	0.4 (0.0, 6.2)	0

[a] Sp = Spring, Su = Summer, A = Autumn, W = Winter; defined by the median temperature and rainfall in the month prior to sampling (source: nearest Bureau of Meteorology station http://www.bom.gov.au) and time of year.

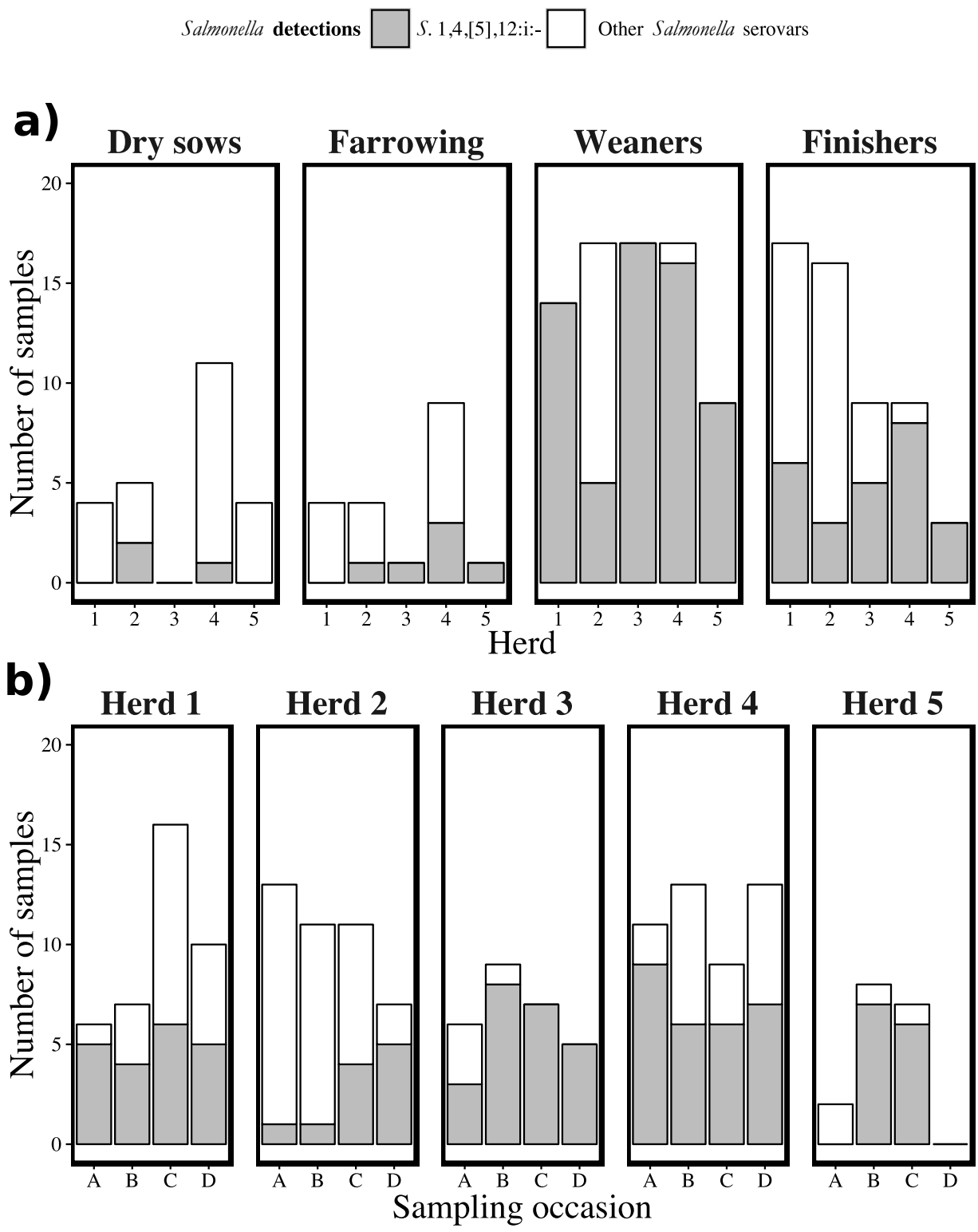

Figure 6-1. Number of pools (pooled samples, each consisting of contributions from six individual faecal pats) in which *S.* 1,4,[5],12:i:- and other *Salmonella* serovars were detected. Figure a) production stage (facet) and Herd 1-5 (x-axis). b) herd (facet) and sampling occasion A-D (x-axis). Total 80 pools per herd; 20 pools per production stage/sampling occasion per herd.

6.2.3. *Salmonella* serovar distribution

Serotyping results indicated relatively diverse *Salmonella* populations were present in each of the five herds. Herd *Salmonella* populations varied in number of serovars present (four to eight) and relative rates of serovar detections (Table 6-3). At least one *S.* 1,4,[5],12:i:- isolate was identified in 55.6% (95/171) of *Salmonella* positive samples. A single isolate was serotyped as *S.* Typhimurium (subsequently found to be a true *S.* 1,4,[5],12:i:- strain, described in Chapter 7). Four isolates were inconclusive upon serotyping; these isolates were sub-cultured and on each occasion it was determined that the colonies consisted of two distinct serovars. Partial serotyping biased against *S.* 1,4,[5],12:i:- isolations relative to other serovars, as isolate serotyping was concluded upon identification of the serovar within a group of sample isolates. However, the representativeness of the population using this testing protocol (geometric) was proven to be equivalent to a binomial approach i.e. testing all isolates submitted.

Table 6-3. *Salmonella* serovar detection frequency and estimated diversity by herd.

Serovar	Herd					Total
	1	2	3	4	5	
S. 1,4,[5],12:i:-	**20**	**11**	**23**	**30**	**13**	**97**
S. Adelaide	5			8		13
S. Agona			1			1
S. Anatum[a]					2	2
S. Bovismorbificans	1	3		2		6
S. Give	1					1
S. Havana					1	1
S. Hofit		1				1
S. London				10		10
S. Mbandaka	1	1	1			3
S. Ohio[b]		10			1	11
S. Oranienburg			2			2
S. Rissen		22				22
S. Typhimurium	1					1
S. Worthington	12	2				14
S. I rough:z_{10}:e,n,x	1					1
Number of serovars	8	7	4	4	5	18
Shannon (H) index	1.49	1.60	0.84	1.24	1.15	1.76
Simpson (D) index	3.22	3.91	1.65	2.69	2.42	3.32

[a] Includes an *S.* Anatum var 15+ isolate
[b] Includes *S.* Ohio var 14+

6.2.4. *Salmonella* 1,4,[5],12:i:- MLVA profile distribution

Molecular typing using MLVA indicated low diversity but distinguishable *S.* 1,4,[5],12:i:- populations present in each of the five herds (Table 6-4). Twelve MLVA profiles were identified among the study *S.* 1,4,[5],12:i:- isolates. *Salmonella* 1,4,[5],12:i:- MLVA profile VNTR copy number

variation was found at loci STTR5 and STTR6 only; all study isolates were identified by VNTR copy numbers 04, 00 (no PCR product, locus not present) and 490 at loci STTR9, STTR10 and STTR3, respectively, applying the Australian nomenclature (Gilbert, 2008). The sole isolate serotyped as biphasic *S.* Typhimurium was also typed using MLVA and had an MLVA profile identified among study *S.* 1,4,[5],12:i:- isolates. The most frequently isolated *S.* 1,4,[5],12:i:- MLVA profiles for STTR5 and STTR6 were (in parentheses: number of herds; percentage of study isolates): 15-11 (4; 35.6%), 14-13 (1; 16.7%) and 15-12 (3; 14.4%).

Table 6-4. *Salmonella* 1,4,[5],12:i:- MLVA profile detections in the five herds and estimated diversity by herd.

MLVA profile	Herd					Total
	1	2	3	4	5	
04-14-11-00-490				1		1
04-14-12-00-490	2					2
04-14-13-00-490	16					16
04-14-14-00-490	2					2
04-15-10-00-490		3		4		7
04-15-11-00-490		1	1	22	10	33
04-15-12-00-490			10	2	3	15
04-15-13-00-490			12			12
04-16-10-00-490		1		1		2
04-16-11-00-490		1				1
04-16-12-00-490		1				1
04-17-10-00-490		4				4
Number of MLVA profiles	3	6	3	5	2	12
Shannon (H) index	0.80	1.78	1.10	1.16	0.98	1.96
Simpson (D) index	1.66	5.12	2.66	2.21	2.42	5.24

6.2.5. *Salmonella* spp. and *S.* 1,4,[5],12:i:- distribution and shedding dynamics by herd

Herd 1.[4] The herd was managed as a multi-site operation employing isolated breeder and grow-out sites. Herd sow numbers remained stable throughout the study. Low level scouring was observed among weaners during sampling, however, no outbreak of clinical salmonellosis occurred during the study period.

Salmonella detections varied considerably between sampling occasions, however, *S.* 1,4,[5],12:i:- detections were consistent, ranging from four to six detections per sampling occasion (Figure 6-1). Eight *Salmonella* serovars were identified. *Salmonella* 1,4,[5],12:i:- and *S.* Worthington were the predominant serovars identified; *S.* 1,4,[5],12:i:- was found among isolates from the majority of

[4] Detailed herd profiles, including management practices, pig movements within herds, animal health history and animal health protocols are provided in Annex 1.

Salmonella positive samples (Figure 6-2). *Salmonella* 1,4,[5],12:i:- shedding persisted throughout the study, whereas shedding of other serovars appeared to be less consistent. All *S*. 1,4,[5],12:i:- isolates were PT193 except two untypable isolates. Three closely related *S*. 1,4,[5],12:i:- MLVA profiles were identified with variation at STTR6 only. The single *S*. Typhimurium colony isolated in the study was from Herd 1 and had an MLVA profile identified among *S*. 1,4,[5],12:i:- PT193 isolates from Herd 1, 04-14-13-00-490; the isolate was PT untypable. It was later determined that the S. Typhimurium isolate was in fact a true *S*. 1,4,[5],12:i:- (Chapter 7).

Herd 2. The herd was a multi-site operation under single ownership and management employing a grow-out site and, two farrowing sites on sampling occasions A, B and C; the sow herd was moved to a single new farrowing site between sampling occasions C and D. The sow herd was expanding throughout the study period, through increased import of gilts and self-replacement. Prior to the first sampling the herd suffered a laboratory confirmed outbreak of salmonellosis, *S*. 1,4,[5],12:i:- was identified among samples collected by the consulting veterinarian affecting approximately 80 grow-out pigs in a single cohort. The consulting veterinarian associated the clinical outbreak with a PCV2 outbreak within the herd. The herd suffered a localised mouse plague around sampling occasion B that was quickly followed by a major outbreak of leptospirosis that affected approximately 50% of the sow herd between sampling occasions C and D. Biosecurity and cleaning and disinfection standards were high initially and stringency was noticeably increased in response to the leptospirosis outbreak.

Eight *Salmonella* serovars were identified among Herd 2 samples during this study, *S*. Ohio and *S*. Rissen were the most frequently identified, followed by *S*. 1,4,[5],12:i:-. A decreasing trend in *Salmonella* detections was observed over the course of the study, however, *S*. 1,4,[5],12:i:- detections increased as the study progressed. All *S*. 1,4,[5],12:i:- isolates tested were PT193 except two untypable isolates from sampling occasions C and D and one phage type 6 isolate from sampling occasion D. Five relatively diverse *S*. 1,4,[5],12:i:- MLVA profiles were identified, with variation at both STTR5 and STTR6.

Herd 3. The herd consisted of two independently owned and managed sites: a farrow to wean enterprise and a grow-out enterprise. Sow numbers remained stable throughout the study. A severe outbreak of confirmed salmonellosis in weaners, in which approximately 100 pigs died, occurred at the breeder site prior to the study. *Salmonella* 1,4,[5],12:i:- was identified among samples collected by the consulting veterinarian. No major health problems were observed or reported at the breeder site during the study. Hygiene levels were low at the grow-out site and ongoing respiratory issues were observed and reported by the producer. The farm manager at the breeder site changed between sampling occasions B and C which led to noticeable improvements in hygiene standards. Herd 3 was the only herd in which sow drinking water was supplemented with organic acids (Selko-pH®).

Four serovars were identified among *Salmonella* isolates from Herd 3, *S*. 1,4,[5],12:i:- appeared to be the predominant serovar during the study. *Salmonella* 1,4,[5],12:i:- persisted among weaners but was identified among finisher isolates in sampling occasions B to D. All *S*. 1,4,[5],12:i:- were PT193, three closely related MLVA profiles were identified with variation at STTR6 only.

Herd 4. The herd was housed on a single site. Sow numbers remained stable throughout the study. Some scouring was observed among weaners during sampling visits, but no other health problems were noted. Hygiene was variable; all weaner-grower cohorts come into close contact in a low hygiene shed from 8 to 12 weeks of age, and a rodent problem persisted throughout the study period. The herd experienced a major outbreak of laboratory confirmed *S*. 1,4,[5],12:i:- salmonellosis approximately 12 months prior to the study.

Four *Salmonella* serovars were identified over the course of the study, *S*. 1,4,[5],12:i:- and *S*. London were most commonly identified. *Salmonella* 1,4,[5],12:i:- detections persisted throughout the study. All *S*. 1,4,[5],12:i:- isolates tested were PT193. Five apparently closely related *S*. 1,4,[5],12:i:- MLVA profiles were identified, exhibiting variation at both STTR5 and STTR6.

Herd 5. The herd was managed on a single site. Sow numbers remained stable during the study period. Limited scouring was observed among weaners, there were no notable disease outbreaks during the period of observation.

Five *Salmonella* serovars were identified, *S*. 1,4,[5],12:i:- was the predominant serovar. *Salmonella* was not detected among samples from sampling occasion D and *S*. 1,4,[5],12:i:- was not detected on sampling occasions A nor D. All *S*. 1,4,[5],12:i:- were PT193 except a single untypable isolate from sampling occasion B. Two similar *S*. 1,4,[5],12:i:- MLVA profiles with minor variation at STTR6 were identified.

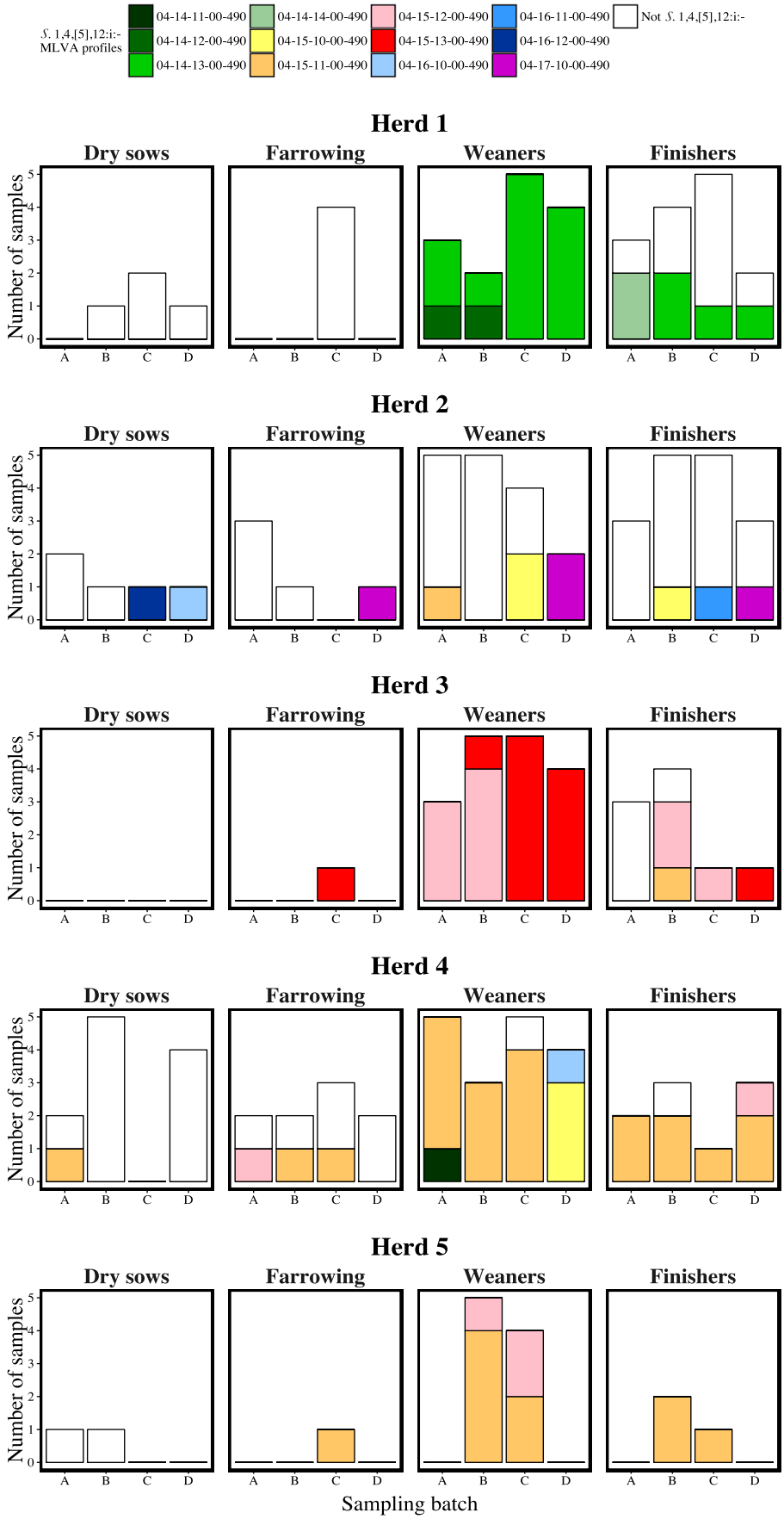

Figure 6-2. *Salmonella* spp. and *S.* 1,4,[5],12:i:- MLVA profiles detections by herd, production stage and sampling occasion. Rows show results by herd, columns by production stage and sampling occasion (x-axis). The bar fill identifies the *S.*1,4,[5],12:i:- MLVA profile per *S.*1,4,[5],12:i:- positive sample; white fill indicates samples in which the serovar *S.* 1,4,[5],12:i:- was not detected.

6.3. Discussion

Many studies have investigated *Salmonella* shedding in pig herds (Funk et al., 2001; Rajić et al., 2005; Pires et al., 2013a; Pires et al., 2014). *Salmonella* 1,4,[5],12:i:- emergence, distribution and persistence within pig production systems have also been explored in Europe (Davies et al., 2011; Niemann et al., 2015a). This exploratory study sought to monitor and compare the persistence and characteristics of emergent *S.* 1,4,[5],12:i:- and contemporary *Salmonella* serovar shedding over time in five independent farrow-to-finish herds operating in the context of the Australian pig industry.

A variety of *Salmonella* serovars were isolated from each of the herds monitored. In each herd, specific serovars were identified with greater frequency than others. In four of the five herds *S.* 1,4,[5],12:i:- was identified among isolates from the majority of *Salmonella* positive samples. *Salmonella* 1,4,[5],12:i:- shedding persisted within herds and specific age groups over extended periods. *Salmonella* and *S.* 1,4,[5],12:i:- detections were considerably higher among terminal animal samples, particularly weaners, in comparison with older animals. Frequent *S.* 1,4,[5],12:i:- detections among finishers demonstrates that primary pig production could be a potential hazard in Australian food supply. The findings indicate that colonized herds may maintain considerable hazard load to slaughter with implications for food safety risk management. The *S.* 1,4,[5],12:i:- strains identified described low MLVA profile diversity and limited variation over the extended period of the study. Given these results were obtained from five independent herds, this suggests that MLVA may have the necessary balance of discriminatory power and stability to be of value in *S.* 1,4,[5],12:i:- outbreak strain identification and source attribution investigations. The low diversity observed among the *S.* 1,4,[5],12:i:- strains, obtained from ostensibly unconnected herds, suggests that the strains were closely related, consistent with recent emergence from a point source.

6.3.1. Findings and implications for each herd

Herd 1. *Salmonella* was detected in the majority of samples sourced from terminal stock, indicating high *Salmonella* shedding prevalence among pigs destined for market. A diverse *Salmonella* population was identified in this herd. Persistent *S.* 1,4,[5],12:i:- shedding, but inconsistent shedding of *S.* Worthington and other serovars, suggests that the prevalence of colonization with the serovar was higher or that there was a stronger association between *S.* 1,4,[5],12:i:- colonization and shedding than that of other serovars. *Salmonella* 1,4,[5],12:i:- was detected among isolates from all *Salmonella* positive samples from weaners, indicating high serovar prevalence among weaners and/or a high rate of shedding among colonized weaners relative to other serovars. *Salmonella* 1,4,[5],12:i:- was only identified among isolates from terminal stock samples suggesting that colonization was limited to these age groups. However, detections from breeder samples were low across the study herds, and may be a factor of lower shedding rates among older animals and/or

test sensitivity in samples from breeder animals given higher roughage diets in this herd (Annex 1)—breeder animals are known to shed at lower rates and/or intermittently (Funk et al., 2001; Vigo et al., 2009a; Wales et al., 2009).

Three *S.* 1,4,[5],12:i:- MLVA profiles were identified, none of which were identified among isolates from the other herds. A high proportion of *S.* 1,4,[5],12:i:- isolates were profile 04-14-13-00-490, which persisted throughout. Herd 1 was the only herd in which neither MLVA profile 04-15-11-00-490 nor profile 04-15-12-00-490 were identified. An earlier *S.* 1,4,[5],12:i:- introduction and subsequent genetic drift within the herd's unique host-environment-agent context could explain the highly related but relatively distinct *S.* 1,4,[5],12:i:- population observed, Herd 1 was the most isolated herd having not introduced live pigs for more than four years prior to the study.

Herd 2. A high proportion of samples from Herd 2 were positive for *Salmonella*, particularly among terminal stock samples, indicating high *Salmonella* shedding prevalence. A diverse resident *Salmonella* population was observed. Increased biosecurity and hygiene mid-way through the study may have been associated with the decline in *Salmonella* detection. *Salmonella* 1,4,[5],12:i:- was detected in a relatively small proportion of *Salmonella* positive samples, but detections persisted and despite diminishing *Salmonella* detections, increased considerably as the study progressed. The results from sampling occasion D indicate that *S.* 1,4,[5],12:i:- may have become the predominant serovar shed in the breeder herd and among weaners. *Salmonella* 1,4,[5],12:i:- was detected in all production stages, however, it was detected with the greatest frequency in weaner samples indicating a higher proportion of weaners colonized than other age groups or more widespread shedding among this age group. The cause of the contemporary drop in *Salmonella* spp. detections and rise in *S.* 1,4,[5],12:i:- isolations is unknown, but could have been associated with increased herd hygiene, perhaps providing the serovar with a selective advantage over contemporaries. This possibility is discussed further below.

Six *S.* 1,4,[5],12:i:- MLVA profiles were identified, indicating relatively diverse *S.* 1,4,[5],12:i:- population was present in this herd. No predominant *S.* 1,4,[5],12:i:- MLVA profile was observed. However, the relatively distinct MLVA profile 04-17-10-00-490 was first detected on sampling occasion D and identified among isolates from four samples, which may have presaged a newly predominant strain.

Herd 3. Herd level *Salmonella* shedding was relatively low, when compared with other study herds, however, weaner sample detection frequency was high, mirroring other herds. *Salmonella* 1,4,[5],12:i:- appears to have been the predominant serovar during the study, the serovar persisted throughout and was identified in an increasing proportion of isolates as the study progressed. *Salmonella* 1,4,[5],12:i:- was identified in every *Salmonella* positive weaner sample and contributed to the majority of *Salmonella* positive finisher samples. In contrast, *S.* 1,4,[5],12:i:- was not detected

amongst dry sow samples and in only a single farrowing shed sample, indicating lower colonization and shedding prevalence. The herd's use of organic acids in sow water, the only herd employing this strategy, could have affected *Salmonella* shedding levels among sows; discussed further below.

Herd 3 *S*. 1,4,[5],12:i:- MLVA profile diversity was low. The most common MLVA profiles identified among weaners were also identified among finishers and the single positive sample from the farrowing shed, indicating persistence via carriage and/or transmission between age groups. Herd 3 results indicate a strain of *S*. 1,4,[5],12:i:- described by MLVA profile 04-15-13-00-490 may have been more competitive than strains 04-15-11-00-490 and 04-15-12-00-490; the change in predominant MLVA profile also coincided with improved hygiene levels in the herd, as in Herd 2, which may inform further studies; also discussed further below.

Herd 4. Herd 4 exhibited the highest *Salmonella* detection frequency among the study herds but the serovar diversity was relatively low. The results indicated a persistent, stable, embedded *Salmonella* population, particularly apparent among terminal stock. *Salmonella* 1,4,[5],12:i:- was detected with high frequency and was the predominant serovar identified within the herd. Herd 4 had endured a substantial outbreak of laboratory confirmed salmonellosis among grower stock six months prior to the study. The outbreak was successfully controlled, which was attributed to the inclusion of organic acids in the diet and during sampling there was little evidence of enteritis, supported by reports of the consulting veterinarian and farm staff. However, *S*. 1,4,[5],12:i:- was isolated persistently, adding further evidence to that described in Chapter 5 that limited clinical signs and *S*. 1,4,[5],12:i:- presence within a herd may not be correlated.

Salmonella 1,4,[5],12:i:- was identified among isolates from all four production stages in Herd 4, however, the serovar was predominant among weaner and finisher samples only. All Herd 4 *S*. 1,4,[5],12:i:- colonies isolated during this study were PT193.

A high proportion of *S*. 1,4,[5],12:i:- isolates were identified by MLVA profile 04-15-11-00-490—a relatively common profile identified among both human and non-human derived *S*. 1,4,[5],12:i:- isolates in Australia (NEPSS, 2011, 2013, 2014). However, the final sampling occasion (D) was the first in which this profile was not detected among weaners in Herd 4 and two MLVA profiles not previously isolated from this herd were identified. This may have signaled a change in the predominant *S*. 1,4,[5],12:i:- MLVA profile(s) within the herd.

Herd 5. The results obtained from this herd differ from the observed detection patterns in other herds. *Salmonella* was detected infrequently, indicating low colonization levels and/or effective control of bacterial shedding in the herd. Unlike the other herds examined, *Salmonella* and *S*. 1,4,[5],12:i:- detection did not persist; *Salmonella* was not detected from sampling D, and *S*. 1,4,[5],12:i:- was not detected in samples from either sampling occasion A or D. These findings could indicate variability in challenges to pigs, strain pathogenicity, or persistence of *Salmonella* and

S. 1,4,[5],12:i:- in this herd. However, *Salmonella* detection was high amongst weaner samples in samples B and C, mirroring observations in other herds. *Salmonella* 1,4,[5],12:i:- was predominant, identified in all *Salmonella* positive samples collected from weaners, finishers and the farrowing shed. The two *S*. 1,4,[5],12:i:- MLVA profiles identified among isolates from this herd were the most widespread profiles identified in the five study herds.

There were no obvious management or animal health changes during the period of observation of Herd 5. Among other explanations, the unusual detection patterns in this herd could be associated with low herd prevalence, limitations of the sampling approach and methods, or seasonal affects. The herd employed deep straw bedding more extensively than other herds—under gestating sows and all terminal pigs post weaning. A higher roughage diet could have reduced faecal culture sensitivity, due to higher faecal roughage content diluting samples, and/or altered digestive conditions reducing shedding. Mikkelsen et al. (2004) found that feeding coarse ground meals provided a strong protective effect in relation to *S*. Typhimurium DT12, possibly related to slower passage and the nature of the organic acids in the gut, in particular undissociated lactic acid. Other *Salmonella* studies have also described the apparent protective effect of low pH in feed (van Winsen et al., 2001). It is conceivable that the higher roughage diet of pigs housed on straw deep bedding might produce a similar effect. Alternatively, the wider use of deep bedding systems may have facilitated more effective cleaning between cohorts than conventional flooring in old sheds. The season during which samples were collected (defined by median temperature, rainfall and time of year in the month prior to sampling) was recorded at each study sampling. Summer and spring (the hottest and driest seasons in all herds) may have been a predictor for lower *Salmonella* and *S*. 1,4,[5],12:i:- detections. This was examined at herd and age group levels. However, due to the low number of herds, numerous potential confounders, and data collection over a single year, the study did not have sufficient statistical power to present results of logistic regression analyses incorporating season.

6.3.2. Findings and implications across the herds

In each herd sampled the rate of detection of *Salmonella* per sampling occasion fluctuated only moderately over the study period, with the exception of Herd 5 in which no *Salmonella* were detected on the final sampling occasion. Multiple *Salmonella* serovars were detected in each of the study herds, as has commonly been reported elsewhere (Funk et al., 2001; Gebreyes et al., 2004; Rajić et al., 2005; Mueller-Doblies et al., 2013; Pires et al., 2014). Including S. 1,4,[5],12:i:-, at least four serovars were detected in each herd, a relatively high number of serovars in comparison with other studies (Rajić et al., 2005; Hamilton et al., 2015). Each herd appears to have maintained a distinct *Salmonella* population and the results indicated variation in the number, frequency and proportion of specific serovars shed. The variation in herd serovar diversity and the specific

serovars detected in each herd suggests that each herd's resident *Salmonella* population may have derived from a different source and been introduced via different pathways. The identification of *S*. 1,4,[5],12:i:- and multiple contemporary serovars differs from the findings of Hamilton et al. (2015) but resembles findings elsewhere (Niemann et al., 2015a). This suggests that the Hamilton et al. (2015) herd was *Salmonella*-free prior to the introduction of *S*. 1,4,[5],12:i:- and that no other serovars became established during their study, whereas other serovars had been introduced to these herds and they may have maintained a resident *Salmonella* population prior to the introduction of *S*. 1,4,[5],12:i:-. These observations also suggests that *Salmonella* was introduced to these study herds via a pathway, or pathways, that were either not present or were not contaminated with *Salmonella* in the case of the Hamilton et al. (2015) study herd. Although the Hamilton et al. (2015) herd was a grow-out site, receiving weaned pigs, operating in a distant location there were no obvious management explanations for the difference in resident *Salmonella* populations detected (D. Hamilton, personal communication).

Although other *Salmonella* serovars were detected in this study, only one biphasic *S*. Typhimurium was identified among 181 isolates fully serotyped. Moreover, 365 isolates were partially serotyped and the first phase flagellar antigen (H1) ≠ i, indicating no serovar *S*. Typhimurium was present among the wider collection of isolates. The sole *S*. Typhimurium isolate from Herd 1 was described by an MLVA profile also identified among *S*. 1,4,[5],12:i:- isolates from the same herd, indicating this isolate may also be closely related. Due to the similarity in all other characteristics the isolate was sequenced and the genome was searched for the *fljAB* region, which was found to be missing, indicating that the isolate should be classified monophasic (data not shown, Chapter 7).

Almost all of the *S*. 1,4,[5],12:i:- isolated were PT193 (89/95) and the level of *S*. 1,4,[5],12:i:- MLVA profile diversity per herd was low (H=0.80-1.16), with the exception of Herd 2 (H=1.78). In total 12 MLVA profiles were identified. Multiple *S*. 1,4,[5],12:i:- MLVA profiles were identified in each of the herds, ranging from two to six profiles per herd. Minor VNTR copy number variations were identified at the relatively polymorphic STTR5 and STTR6 loci, only, the majority of which were SLVs, indicating highly related populations within each herd. In Herds 1, 3 and 5, a single SLV cluster was identified, while the herd with the greatest MLVA profile diversity, Herd 2, described only minor variation at the two most unstabl loci. These findings are consistent with stable populations experiencing minor genetic changes over the period of observation and suggest a single lineage may have persisted in each herd during the study period. The *S*. 1,4,[5],12:i:- populations in each of the five herds were distinguishable, mirroring recent findings in Germany (Niemann et al., 2015a). Variation at loci STTR5 and STTR6 is relatively common and most authors suggest that isolates with single locus variations at these loci be considered related (Hopkins et al., 2007; Larsson et al., 2009; Dimovski et al., 2014). In a recent study Niemann et al. (2015a) considered strains with single or double locus variants at STTR5 and STTR6 to be clonal variants. If, among these results,

STTR5 and/or STTR6 single or double locus variants with maximum VNTR copy number differences of ≤2 are clustered, then the number of *S*. 1,4,[5],12:i:- MLVA types in each farm collapses to one. Herd 1 (MLVA 04-14-(12/13/14)-00-490) was distinct from Herd 3 and 5, which had closely related MLVA profiles 04-15-(11/12/13)-00-490; each exhibited STTR6 variation, only. Additional variation at locus STTR5 was observed in isolates from Herds 2 and 4, herds with higher *S*. 1,4,[5],12:i:- MLVA profile diversity. In Herd 2 *S*. 1,4,[5],12:i:- MLVA profiles 04-(15/16/17)-(10/11/12)-00-490; in Herd 4 *S*. 1,4,[5],12:i:- MLVA profiles 04-(14/15/16)-(10/11/12)-00-490. In Herd 2 high levels of detection of *Salmonella* serovars other than *S*. 1,4,[5],12:i:- and a diverse serovar population were also observed (H=1.60). However, in Herd 4, levels of detection of other serovars and *S*. 1,4,[5],12:i:- were higher but serovar diversity was lower relative to Herd 2. This could indicate that *S*. 1,4,[5],12:i:- and other *Salmonella* serovars were introduced to Herd 2 from multiple sources and/or on multiple occasions, perhaps unlike the other herds. Inter-serovar interactions and interactions with other constituents of the gut microbiota—within an individual, pen or herd—could have affected *S*. 1,4,[5],12:i:- prevalence, shedding and observations of clinical disease (Fedorka-Cray et al., 1999; Mead, 2000).

That each herd appears to have maintained a stable, highly related *S*. 1,4,[5],12:i:- population suggests that the serovar was either introduced from a single point source and subsequently became established in each of the five herds or that multiple introductions from a stable source population occurred. Identification of the likely pathways through which *S*. 1,4,[5],12:i:- is or was transmitted between herds would have considerable implications for the Australian pig industry and merits further investigation. Identifying and eliminating these pathways would aid control of the spread of this serovar, other *Salmonella* serovars, and potentially other unrelated pathogens within the industry.

The *S*. 1,4,[5],12:i:- populations observed suggest allelic drift among resident populations, which may or may not be associated with strain selection. Variation in *S*. 1,4,[5],12:i:- MLVA profiles, or lack thereof, did not correspond to phenotypic characteristics that were tested for (Chapter 8). Specific MLVA profiles could be associated with strain competitiveness, however, at present any relationship between MLVA loci copy number and phenotype is unknown. Historically the importance of VNTRs were often dismissed, however, a wide range of roles are now recognized, such as in determining gene expression (Van Belkum et al., 2001; Van Belkum, 2007; Gemayel et al., 2010).

Further genotyping of *S*. 1,4,[5],12:i:- by full genome sequencing and subsequent analyses would likely establish the level of core genome relatedness among apparently similar and different *S*. 1,4,[5],12:i:- MLVA profiles within and between study herds. This could inform discussion of *S*. 1,4,[5],12:i:- population dynamics within herds and wider systems and aid in the establishment of practical criteria for differentiating organisms for the purposes of epidemiological investigations.

The results of analyzing the sequences of a selection of these isolates are presented in subsequent chapters.

Salmonella 1,4,[5],12:i:- detection was frequent and remained stable over the course of the study in Herds 1 and 4, while identification of the serovar per positive sample increased in Herds 2 and 3 as the study progressed. At least one isolate was identified as *S.* 1,4,[5],12:i:- in more than 50% of *Salmonella* positive samples in Herds 1, 3, 4 and 5. These results indicate that the serovar was endemic and colonization was extensive in each of these herds during the sampling period. The results from Herds' 2 and 3 indicate that the serovar was being shed more commonly than other serovars and prevalence may have been increasing and as the study progressed. These results demonstrate persistence of the serovar within these herds over an extended period and are consistent with speculation that *S.* 1,4,[5],12:i:- enjoys some form of competitive advantage over other serovars (Gebreyes et al., 2011; Davies, 2013; Simon et al., 2013).

Salmonella 1,4,[5],12:i:- had been isolated from each of the herds prior to the study. In each case samples were collected in response to outbreaks of scouring amongst grow-out pigs. The outbreaks varied in scale and acuteness but in each herd the outbreak was controlled and only mild scouring among weaned pigs was observed during the sampling period. It is likely that shedding rates increased during the outbreaks, however, it was interesting to note that high detection rates were recorded even after a considerable period of time had elapsed since the disease outbreak had subsided, particularly among grow-out pigs. The clinical disease outbreaks could have coincided with the introduction of the serovar to each herd or may have been associated with other host or environmental factors, such as other disease issues affecting the herds. This suggests that further research into the rates of *S.* 1,4,[5],12:i:- shedding during and post outbreaks would be informative. Furthermore, the effects of *S.* 1,4,[5],12:i:- exposure to naïve pigs on pig health and subsequent shedding among might provide an indication of whether clinical disease outbreaks tend to coincide with introduction, which might help to identify possible introduction pathways.

Salmonella 1,4,[5],12:i:- was identified among isolates sourced from weaners and finishers in all five study herds. In each herd higher *S.* 1,4,[5],12:i:- shedding among weaners and, to a lesser extent, finisher pigs was routinely observed demonstrating persistent challenges and shedding among terminal pigs. Aggregating results from the five herds, *S.* 1,4,[5],12:i:- was detected in 43% (86/200) of terminal stock samples (average P_T15.5%; 95%CI 12.1-22.9%), but only 4.5% (9/200) sow and farrowing shed sample (average P_T3.1%; 95%CI 0.3-5.3%). The serovar may have been maintained within cohorts through carriage and/or ongoing challenges and transmission between and within cohorts. These findings mirror observation and veterinary reports of clinical salmonellosis among younger pigs in these herds prior to and during the study. The considerably higher *S.* 1,4,[5],12:i:- detections and estimated shedding prevalence observed among terminal stock indicates either greater exposure to *S.* 1,4,[5],12:i:- among these animals or an enhanced ability to colonize younger

pigs or greater capacity to cause shedding among pigs in this age group, or a combination of these factors. Higher rates of *Salmonella* detection among grow-out pigs relative to sows has been well described, for example by Kranker et al. (2003) in their culture and serological study of three Danish herds. However, Davies et al. (1998) describe considerable shedding, particularly of exotic serovars, among sows in US herds. The Davies et al. (1998) study found only *S*. Typhimurium among growing pigs, indicating the serovar's propensity to cause shedding among swine of these age groups. These observations indicate that *S*. 1,4,[5],12:i:- possesses a similar propensity to cause shedding among grow-out pigs, which is unsurprising given the close relatedness of the serovars and reports of the presence of the same virulence factors (Zamperini et al., 2007).

In each of the five herds *S*. 1,4,[5],12:i:- MLVA profiles identified in weaner-derived samples were also identified in finisher samples. This provides further evidence of persistence within cohorts as observed in the case study of Herd 4 grow-out pigs (Chapter 5), indicating hazard burden at slaughter with consequent implications for food safety risk management. This study was not designed to describe the nature of persistence, the bacteria may be persisting via carriage and/or repeat exposure and/or exposure to 'herd' strains from other sources, such as other pigs within the herd, the environment or (mechanical) vectors. Further investigation would require more frequent sampling in a cohort study. In addition, isolates obtained from samples from farrowing sheds and/or gestating sows in Herds 2, 3, 4 and 5 described MLVA profiles that were also identified among weaners and finishers in the same herd consistent with transmission between breeding and terminal animals within herds. Three of the four herds in which common strains were isolated from both weaners and breeders, Herds 2, 3 and 5, practiced all-in, all-out batch systems as a cornerstone of their disease management practices. Davies et al. (1997) found that practicing all-in, all-out management did not reduce *Salmonella* prevalence among grower cohorts when compared to continuous systems. These findings also suggest that maintaining batch integrity may not be effective in the control of *S*. 1,4,[5],12:i:- exposure and colonization of pig cohorts.

The very high *S*. 1,4,[5],12:i:- detection rates among weaner samples in each herd over multiple sampling occasions—over the course of the study more than 30 cohorts of growing pigs were sampled within each herd—demonstrates routine exposure of cohorts of terminal line pigs. The apparent relatedness of *S*. 1,4,[5],12:i:- strains isolated indicates that cohorts were routinely exposed to a persistent population. It is possible that sows played a role in maintaining *S*. 1,4,[5],12:i:- within these herds despite low *S*. 1,4,[5],12:i:- detection rates. Exposure of young stock could have occurred via the sow post-partum or at some point in the rearing environment with carriage and or subsequent pig-to-pig transmission among contemporaries and/or different cohorts. Colonization of suckling pigs in the rearing environment has been demonstrated in longitudinal studies of farrow-to-finish herds conducted by Nollet et al. (2005a); Nollet et al. (2005b). Studies have shown that sporadic *Salmonella* shedding by sows and suckling piglets can make detection challenging, but

colonization of young piglets is well-described (Davies et al., 1999; Funk et al., 2001; Kranker et al., 2003; Vigo et al., 2009a; Davies et al., 2011). Hill et al. (2015) developed a farm transmission model using data from the EU and concluded that if sow *Salmonella* prevalence was greater than 10% sows would account for the majority of *Salmonella* transmission within the herd, below this prevalence feed became the dominant contributor to slaughter pig *Salmonella* status. Hill et al. (2015) noted the high variability in individual shedding of organisms, and thereby exposure of pigs within herds, therefore, to predict a high incidence within a cohort sow shedding must be at very high numbers. Sporadic shedding rates may account for the low sow *Salmonella* detections in this study, potentially masking relatively higher prevalence of colonization and their importance in maintaining *S.* 1,4,[5],12:i:- colonization within herds and exposing younger pigs to the serovar. Alternatively, new cohorts could have been challenged via the environment—such as penfloors, fomites—such as boots and equipment, or via vectors—such as birds, rodents, flies and cats (Barber et al., 2002). However, this study was not designed to assess risk factors or indications of routes and directionality of transmission within herds.

The lower *Salmonella* shedding rates among sows and suckling piglets observed in this study have been reported extensively by other studies (Funk et al., 2001; Kranker et al., 2003; Nollet et al., 2005a; Rajić et al., 2005; Pires et al., 2013a; Pires et al., 2014). These results show considerably greater escalation in *S.* 1,4,[5],12:i:- specific shedding among weaners and, to a lesser extent, finishers, than was observed among other serovars and older pigs. Variation in the numbers of *S.* 1,4,[5],12:i:- detections by production stage could be due to variation in *S.* 1,4,[5],12:i:- prevalence, which would likely be associated with with higher shedding rates and/or longer duration of shedding leading to greater exposure and detection (Pires et al., 2014). Many studies have shown variation in shedding rates between serovars, these findings indicate that *S.* 1,4,[5],12:i:- may cause high shedding rates amongst colonized pigs, particularly among younger animals (van Winsen et al., 2001; Österberg et al., 2010; Ivanek et al., 2012; Pires et al., 2014). These results support calls for further investigation of serovar specific shedding and the development of control strategies targeted at serovars particular importance to animal and human health, such as *S.* 1,4,[5],12:i:- (Clothier et al., 2010; Hauser et al., 2010; Correia-Gomes et al., 2012; Keelara et al., 2013; Pires et al., 2014).

Repeated and widespread isolation of identical and very similar *S.* 1,4,[5],12:i:- MLVA profiles in this study to reports available Australian surveillance data indicate a closely related *S.* 1,4,[5],12:i:- population may be circulating within the Australian pig industry, and perhaps more widely within Australian domestic animal industries. The most widespread *S.* 1,4,[5],12:i:- MLVA profiles, 04-15-11-00-490 and 04-15-12-00-490, were identified in four and three herds, respectively and both have been identified from both human and non-porcine animals in Australian passive surveillance data (NEPSS, 2011, 2013, 2014). Although seven *S.* 1,4,[5],12:i:- MLVA profiles found among the study

isolates had not previously been associated with pigs or pork in Australia these MLVA profiles were also very similar to other profiles (SLVs) from both porcine and other sources identified in Australian databases (NEPSS, 2011, 2013, 2014). This suggests that the Australian *S*. 1,4,[5],12:i:- population might be clonal. A single *S*. 1,4,[5],12:i:- clone in Australia would differ from reports elsewhere, particularly Europe, where multiple *S*. 1,4,[5],12:i:- clones have been identified (Moreno Switt et al., 2009; EFSA, 2010a; Hopkins et al., 2010). A recent phylogenetic study of epidemic *S*. 1,4,[5],12:i:- strains by Petrovska et al. (2016) found a distinct clone emerged in multiple species. A point emergence of a strain in Australia could lead to a similarly clonal population to the clade of epidemic strains described by Petrovska et al. (2016). The recent identification of *S*. 1,4,[5],12:i:- in Australia, the apparent relatedness of the population observed in this study, and the similarities of strains reported by national passive surveillance systems suggest that the organism emerged recently and possibly during a single event (NEPSS, 2014). The timeframe and nature of emergence, via parallel evolution or introduction, requires further investigation of the relatedness of Australian *S*. 1,4,[5],12:i:- strains and comparison with other domestic Group B *Salmonella* serovars and strains reported or having originated overseas. Whole genome sequencing and comparative genomic studies will contribute to this discussion.

The Australian pig industry enjoys the natural biosecurity advantages of operating within an island continent, further enhanced by stringent quarantine restrictions and industry specific protections such as the prohibition of live pig and fresh pork imports. The pandemic *S*. Typhimurium DT104 has never been isolated in Australian livestock, suggesting that national biosecurity measures were operating with high efficacy. Yet this study describes an emergent infectious agent that appears to have spread to widely dispersed pig herds with no live animal connections or direct animal transportation links and little overlap in terms of feed supplying mills. These findings indicate that the risk of spread of infectious agents within the industry may be considerable, reaffirming the importance of effective herd level biosecurity and the need to investigate the means and pathways by which *S*. 1,4,[5],12:i:- has spread, such as via breeding stock, feed, people or wildlife. This would inform strategies to control the spread of *S*. 1,4,[5],12:i:- and related infectious agents among Australian pigs and other food animals.

These findings reiterate the importance of further investigation into the efficacy and economic efficiency of control measures, such as dietary supplementation with organic acids, to inform expenditure on *Salmonella* control strategies within herds and the industry as a whole. Further investigation of measures that target control of *Salmonella* at the herd level, such as measures that address *Salmonella* status among sows as well as market destined pigs, warrants further consideration.

6.3.3. Public health implications

The potential pathways by which *Salmonella* in primary pig production may reach end-consumers have been well described (Berends et al., 1997; Mousing et al., 1997a; Dahl and Sørensen, 2001; Alban and Stärk, 2005; Alban et al., 2012; De Busser et al., 2013; Andres and Davies, 2015; Snary et al., 2016). Persistently high levels of *S*. 1,4,[5],12:i:- shedding were observed among terminal pigs to market weight in the five independent herds studied, thereby identifying a potential hazard source in the Australian food chain. The implication of domestic pork products in human *S*. 1,4,[5],12:i:- cases and the isolation of *S*. 1,4,[5],12:i:- strains with MLVA profiles that have also been identified among isolates from pig carcasses and domestic human salmonellosis during this study cases indicates a potential risk pathway to human consumers (NEPSS, 2011; OzFoodNet Working Group, 2012b; NEPSS, 2013; SA Pathology, 2013a, b; NEPSS, 2014; SA Pathology, 2014).

Observed variation in *S*. 1,4,[5],12:i:- detections from finishers between herds could be reflected in herd associated food safety risk. However, studies conducted by Swanenburg et al. (2001a); Swanenburg et al. (2001b) found that, though many factors may affect *Salmonella* hazard load on carcass, the level of *Salmonella* colonization within herds had no bearing on carcass contamination. Consequently, determining the status of herds may be the most important information from primary production. This information can inform effective process control and verification systems during slaughter and in pork boning rooms that mitigate *Salmonella* or *S*. 1,4,[5],12:i:- associated food safety risk. At present Australia does not monitor herd *Salmonella* status let alone specific serovar status, and this study showed that observation of clinical disease or lack thereof was a poor indicator of hazard status within herds. Therefore, pigs harbouring these pathogens may not be identified pre-slaughter, increasing the potential risk of the hazard reaching consumers via pork products. Although Swanenburg et al. (2001a); Swanenburg et al. (2001b) demonstrated that status is the most important on-farm parameter for potential contamination of carcasses, there may still be merit in enumerating *S*. 1,4,[5],12:i:- contamination in finished pigs from the farmgate through lairage, slaughter and processing to end-product. It seems plausible that higher rates on on-farm detection and, thereby, estimated prevalence might be associated with increased risk of product contamination. This could inform risk management option evaluation on-farm and provide evidence for or against increased investment in *Salmonella* surveillance systems, from both pig performance and food safety perspectives.

Salmonella 1,4,[5],12:i:- MLVA profile stability was greater than anticipated over the extended period of the study. Previous research conducted by Dimovski et al. (2014), in which *S*. Typhimurium was passaged *in vitro* and *in vivo* to assess the stability of MLVA loci, argued for clustering SLVs differing at one of loci STTR5, STTR6 or STTR10. These results provide empirical evidence of similar levels of stability from a field setting, supporting the clustering of MLVA SLVs with VNTR copy number

changes of ≤2. These findings support use of MLVA in outbreak strain identification. Although there were indications of herd associated MLVA profile clusters common MLVA profiles were identified, which have also been reported from other Australian food animals, further comparison of isolates from pig herds and other sources is needed to determine the value of MLVA for source attribution.

6.3.4. Implications for risk mitigation and risk management

Most previously published Australian porcine *Salmonella* studies have employed serological sampling methods in a cross sectional design and/or have focused beyond the farmgate (Hamilton et al., 2000; Hamilton et al., 2003; Hamilton et al., 2005), with the exception of this study's precursor (Hamilton et al., 2015). This study reiterates the value of collecting faecal samples longitudinally and extensively characterizing multiple isolates to effectively describe *Salmonella* populations and population dynamics within animal production systems (Funk et al., 2001; Wong et al., 2004).

Collecting pooled samples reduced the costs associated with sampling and bacterial culture; *Salmonella* shedding prevalence across the study herds was estimated to be 15.4% (95%CI 12.3-22.2%), however, by using a pooled sampling approach *Salmonella* was detected in 42.8% (171/400) of samples. The high rates of *Salmonella* and *S.* 1,4,[5],12:i:- detection and estimated proportion of pigs shedding among terminal stock, particularly weaners, indicate that surveillance, researchers and herd health efforts would obtain efficiency benefits from targeting these production stages for sampling. Furthermore, these results demonstrate that typing a single isolate from a culture positive sample does not provide adequate information to accurately describe the *Salmonella* population within a pen, production stage or herd. Throughout this study, it was common to identify multiple serovars and multiple phage types and MLVA profiles of individual serovars among isolates sourced from a single pen sample. This has implications for laboratory investigations for study designs and practitioners whom typically have only single colony picks from individual samples typed—a single colony pick from an individual sample collected on an isolated occasion could give a false impression of the *Salmonella* population in the target population.

Phage typing proved to be of little value in differentiating *S.* 1,4,[5],12:i:- in this study as virtually all *S.* 1,4,[5],12:i:- isolates were identified as PT193. Available Australian surveillance data indicates that this is likely to be the case across Australian food industries (NEPSS, 2011, 2013, 2014). This is unlike *Salmonella* studies conducted elsewhere that have found the application of both phage typing and MLVA in tandem advantageous (Prendergast et al., 2011). In this study MLVA proved to be substantially more discriminatory than phage typing for *S.* 1,4,[5],12:i:- and specific MLVA profiles were identified over multiple sampling occasions, indicating adequate stability to be of value for epidemiological studies and outbreak strain identification. However, the current uncertainty in relation to the clustering of MLVA profiles hampers interpretation (Barco et al., 2013). These

findings support the recommendations of Dimovski et al. (2014), whereby small VNTR copy number changes at loci STTR5 and STTR6 should be clustered. The increasing application of comparative genomic studies will provide further insights into the meaning of MLVA profile variation or lack thereof, and best approaches to applying and interpreting MLVA profiles for epidemiological purposes.

The relatively low *S*. 1,4,[5],12:i:- phage type and MLVA profile diversity found within the study herds is consistent with a single point source introduction to each herd or multiple point source introductions from a stable *S*. 1,4,[5],12:i:- source population. The study herds form components of unconnected and stable market chains, include closed herds and herds sourcing replacements from a variety of suppliers, employ effective traffic control, are geographically isolated and employ a range of input suppliers and service providers. However, there are a limited number of genetic suppliers in Australia and few nucleus herds. For example, the same genetic company supplied Herds 2 and 3, while a separate breeder supplied both Herds 4 and 5. The serovar *S*. 1,4,[5],12:i:- has also been isolated from animal feed samples in Australia (NEPSS, 2014). Several herds shared feed supply companies, however, only Herds 1 and 2 sourced feed from the same mill. The *S*. 1,4,[5],12:i:- MLVA profiles identified among Herd 1 and 2 isolates differed but only to the extent observed across all five herds. None of the herds used the same company or vehicles to move stock though they did share some specific service providers and each of the herds was to degree accessible to birds and other potential vectors. Given no live animal movements between the herds occurred, possible transmission pathways between herds might include breeder herds, feed, humans, vehicles and wildlife as other potential pathways of introduction. Investigation of possible routes of introduction could consider sampling live replacements before they enter the herd, human workers, service providers or visitors, vectors such as rodents and birds and widely distributed feed components such as protein meals.

In two of the case study herds, Herds 2 and 3, improved hygiene coincided with increased *S*. 1,4,[5],12:i:- detection frequency among *Salmonella* positive samples. Causality is unknown, however, it is possible that the *S*. 1,4,[5],12:i:- were more tolerant of the altered conditions. Although development of bacterial resistance to most disinfectants is considered unlikely, as many disinfectants inactivate multiple targets and would therefore typically require multiple mutations, *Salmonella* resistance can occur through the development of physiological and intrinsic defences, such as biofilms and the oxidative stress or SOS response (McDonnell and Russell, 1999; Braoudaki and Hilton, 2004; Karatzas et al., 2007). McDonnell and Russell (1999) describe the SOS response in *Salmonella*, which entails the production of neutralizing enzymes and repair of damage to the bacterial DNA, increasing tolerance to peroxides. *Salmonella* tolerance to disinfectants, biocides, heavy metals and antimicrobials are linked and complex, and are likely affected by levels and time of exposure (Wales and Davies, 2015). Investigating *S*. 1,4,[5],12:i:- resistance to

commonly used on-farm disinfectants in *vitro* and in Australian herds and the development of resistance, at different concentrations, lengths of contact time and via various modes of application, may prove informative.

At present, *Salmonella* relevant control measures used in the Australian pig industry, such as dietary organic acids, typically target terminal stock, where clinical signs are most commonly observed. Though this strategy appeared to be effective in the control of clinical disease in these herds, continued detection of *Salmonella* at high rates showed that substantial sub-clinical colonization remained. Furthermore, these results did not discount the possible role of the breeder herd in exposure of young pigs and indicated that batch management may not be effective in controlling *S.* 1,4,[5],12:i:- transmission. A return to investigating and trialing controls targeted at sows, as advocated by Kranker et al. (2001), could provide efficacy and efficiency benefits by reducing herd prevalence, batch-to-batch transmission, and associated morbidity and hazard load among market-destined animals. Further investigation of other proposed and trialed on-farm *Salmonella* controls, such as efficient antibiotic use, vaccination, competitive exclusion and segregated weaning, in relation to *S.* 1,4,[5],12:i:- also warrants further investigation.

6.4. Conclusion

This study again demonstrated the substantial contemporary *Salmonella* serovar diversity that can exist within pig herds. The study showed *S.* 1,4,[5],12:i:- persistance over extended periods and that the serovar may enjoy a fitness advantage over other *Salmonella* serovars in some contexts. The study demonstrated substantial escalation of *S.* 1,4,[5],12:i:-shedding among weaner pigs and persistence through to slaughter, which suggests that market weight pigs present a risk of hazard entry into the human food chain and has implications for surveillance and study designs. The application of MLVA proved informative and suggested that each of these herds maintained a closely related and stable *S.* 1,4,[5],12:i:- population during the study period, which may have been introduced through a single point source introduction. Findings suggest that with appropriate interpretation MLVA would be valuable for outbreak strain investigation and broader epidemiological purposes. Further investigation of the efficacy and economic efficiency of potential *Salmonella* spp. controls is needed to optimize food safety and animal health risk management approaches, particularly given the current pressure to minimize antimicrobial use in animal production.

Chapter 7 - Evidence of clonal expansion among Australian porcine *Salmonella* 1,4,[5],12:i:- isolates

Preface

The phenotype and MLVA characteristics of the *S.* 1,4,[5],12:i:- isolates collected during the preceding cross-sectional diversity study and the two longitudinal studies suggested that the Australian pig *S.* 1,4,[5],12:i:- population was closely related. If the *S.* 1,4,[5],12:i:- collection was closely related it could describe clonal expansion. Given the study isolates were collected from a wide spectrum of herds and over an extended period of time, clonal expansion would indicate that the Australian pig *S.* 1,4,[5],12:i:- population emerged via a single event and had then spread throughout the industry. This would have considerable implications for biosecurity within the industry and in terms of national biosecurity.

Furthermore, the phylogenetic meaning, if any, of variation in MLVA profiles among *S.* 1,4,[5],12:i:- isolates was not known. Sequencing and comparing a selection of isolates with the same MLVA profiles and various levels of difference could provide further insights into the potential uses and value of the technique for epidemiological investigations.

To further investigate relatedness among the isolates a comparative genomic study proposal was developed. This chapter describes the results of the phylogenetic study of a selection of *Salmonella* Group B isolates.

7.1. Introduction

The serovar *S.* 1,4,[5],12:i:- is believed to have emerged as a number of clones from *S.* Typhimurium through independent events (Soyer et al., 2009a; Hopkins et al., 2010; Ido et al., 2014). The 'Spanish clone' emerged rapidly in the mid 1990s, characterized by phage type U302 and high prevalence of isolates exhibiting multidrug resistance; there are reports of Spanish clone isolates expressing resistance to seven antimicrobial agents—ampicillin (A), cloramphenicol (C), gentamicin (G), streptomycin (S), sulphonamides/sulphathiazole (Su), tetracycline (T) and trimethoprim (Tm) (Petrovska et al., 2016). Since the early 2000s many European countries have reported increasing incidence of *S.* 1,4,[5],12:i:- strains of phage type DT193 or DT120 that are

predominantly characterized by the ASSuT resistance phenotype and have been termed the 'European clone' (EFSA, 2010c). In addition, less resistant, often pansusceptible, *S*. 1,4,[5],12:i:- lineages have emerged in the US (Soyer et al., 2009a). Recent publication of a sequence based phylogeny of British and Italian epidemic *S*. 1,4,[5],12:i:- sequences from human, live animal and product sources indicated the strains were part of the same clade undergoing clonal expansion (Petrovska et al., 2016).

Through the studies described previously, this research established a large, unique and well-characterized collection of Australian porcine *S*. 1,4,[5],12:i:- strains. The *S*. 1,4,[5],12:i:- collection had low phage type diversity; PT193 was highly predominant, and multiple PT120 isolates were identified among early isolates. The MLVA profile diversity was relatively low, providing a further indication that the strains were closely related. On the basis of phenotypic and MLVA characteristics, it was hypothesized that the Australian porcine *S*. 1,4,[5],12:i:- population was clonal and undergoing expansion from a recent common ancestor, mirroring the findings of Petrovska et al. (2016).

Previous observations of *S*. 1,4,[5],12:i:- characteristics also indicated that fairly stable populations were circulating within each of the herds sampled. This suggested that *S*. 1,4,[5],12:i:- lineages might be differentiated by source. This could have considerable implications for improving source attribution investigation and outbreak responses, and thereby inform public health risk management.

To investigate further the whole genomes of a selection of *Salmonella* Group B isolates, predominantly *S*. 1,4,[5],12:i:- isolates, in the study collection were sequenced, and the phylogenetic and epidemiological relationships investigated. The objectives of this study were to sequence and analyse a selection of porcine *S*. 1,4,[5],12:i:- and potentially closely related comparator isolates from a range of Australian sources, representing the diversity of the wider study isolate collection, to determine: if the selected porcine *S*. 1,4,[5],12:i:- could be readily differentiated from *S*. Typhimurium isolates; if the *S*. 1,4,[5],12:i:- strains appeared to be part of a single expanding clonal complex or if evidence of multiple clones could be identified; if herds/sources could be differentiated on the basis of the *S*. 1,4,[5],12:i:- populations they harboured; and how the core SNP ancestral phylogeny of *S*. 1,4,[5],12:i:- isolates compared to the apparent relatedness/unrelatedness of the same isolates based on MLVA typing.

7.2. Results

7.2.1. Phage typing, MLST and MLVA

Phage typing. *Salmonella* 1,4,[5],12:i:- PT193 was highly predominant in the study isolate collection (97.9%; 505/516) (Table 7-1). Among the sequenced *S*. 1,4,[5],12:i:- isolates five were untypable, not typed or identified as PT120 (isolates TW-STm43, TW-STm4, TW-STm54, TW-STm33 and TW-STm32). The remaining 49 *S*. 1,4,[5],12:i:- isolates were PT193. The two isolates phenotyped as biphasic *S*. Typhimurium isolates (TW-STm55, TW-STm56) were not phage typed. Examination of the genomic sequences revealed that each of these seven strains contained the *thrW* PT193 genomic island and would therefore be expected to be phenotypically PT193.

Multiple locus sequence typing. The MLST was based on allele combinations for *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA* through comparison with the pubMLST database entries for *Salmonella enterica* (WWW http://mlst.warwick.ac.uk/mlst/dbs/Senterica). The *S*. 1,4,[5],12:i:- isolates were all multiple locus sequence type (ST) 34. The biphasic *S*. Typhimurium isolates were all ST 19.

MLVA typing. The sequenced isolates included strains with 22 MLVA profiles, three of which were only identified among *S*. Typhimurium isolates and were considerably different to the profiles identified among the *S*. 1,4,[5],12:i:- isolates sequenced. The 19 MLVA profiles identified among *S*. 1,4,[5],12:i:- isolates were all SLVs at the relatively highly polymorphic STTR5 and STTR6 loci (Figure 7-1). The only isolates in which amplification at the STTR10 locus was observed were three of the *S*. Typhimurium isolates (Table 7-1).

7.2.2. Whole genome sequencing

Genes. Using the RedDog pipeline approximately 4620 genes mapped to the reference *S*. Typhimurium SL1344 (NC_016810.1) (a summary report of the sequencing run is provided in Supplementary Table 7-2). Among the *S*. 1,4,[5],12:i:- isolates 4588 genes were identified. An estimated 4312 genes with >95% coverage were found in all the *S*. 1,4,[5],12:i:- strains analysed.

Phylogeny. The *S*. 1,4,[5],12:i:- isolates formed a tight clade with ≤30 core SNPs to a predicted most recent common ancestor (MRCA) (Figure 7-2). The four true biphasic *S*. Typhimurium strains formed a clearly defined outgroup, separated from the *S*. 1,4,[5],12:i:- clade by ≥541 SNPs pairwise, and were designated as such in subsequent analyses. The *S*. Typhimurium isolates TW-STm58 and TW-STm57 were sourced from samples collected from the same herd and were closely related (11 SNPs, pairwise), but were considerably different to the other *S*. Typhimurium isolates, ≥123 SNPs pairwise.

Two basal clades were identified within the *S*. 1,4,[5],12:i:- clade, designated Clades I and II. Clade I consisted of Herd 11 isolates collected in 2011, ≤11 SNPs MRCA. Clade II encompassed the remainder of the *S*. 1,4,[5],12:[i]:- isolates, ≤22 SNPs MRCA. Clade II included isolates from five herds and one abattoir, from samples collected over the period 2013-2015. The two clades were separated by ≥17 SNPs MRCA (≥36 SNPs pairwise). Six subclades, or lineages, were apparent within Clade II, labeled subclades II.a. to II.e, which correlated with the strain source. Each subclade contained ≤12 SNPs MRCA (≤19 SNPs between pairs). There was strong bootstrap support for the differentiation clades and subclades (≥90%).

Plasmids. The *Salmonella* virulence plasmid pSLT was detected in three of the four *S*. Typhimurium isolates sequenced (TW-STm58, TW-STm45, TW-STm46; 100% match) and none of the other strains; none of the *S*. 1,4,[5],12:i:- had pSLT (Table 7-1). The highest pSLT percentage coverage among *S*. 1,4,[5],12:i:- was 14.69% in the two isolates from Abattoir 2 (TW-STm45, TW-STm46). The absence of the plasmid in the *S*. 1,4,[5],12:i:- isolates explains the absence of MLVA locus STTR10 in the collection; locus STTR10 is located on pSLT. The pCol1B9 plasmid was absent in 28 of 60 isolates. Only three *S*. 1,4,[5],12:i:- isolates had >90% coverage of pCol1B9 (TW-STm34, TW-STm10, Herd 4; and TW-STm22, Herd 3). The pRSF1010 plasmid was completely absent in 18 isolates and poorly covered in the remainder. The four isolates with the greatest coverage of pRSF1010 had between 83-86% match, all of which were *S*. 1,4,[5],12:i:- isolates sourced from Herd 11, which was sampled in 2011 (TW-STm3, TW-STm15, TW-STm17).

Two strains identified as biphasic in the laboratory, and thus initially serotyped *S*. Typhimurium (TW-STm54 and TW-STm60), lacked the *Salmonella* virulence plasmid pSLT. To confirm or refute their biphasic status, an SRST2 comparison of the *fljAB* region of these two genomes with reference to the *S*. Typhimurium LT2 *fljAB* genomic region was conducted by Yuhong Liu. The analysis showed that TW-STm54 did not have STM2760, STM2762, STM2766, *fljB*, *fljA* and *hin* genes and, therefore, should be identified as monophasic (Arguello et al., 2014), whereas TW-STm60 had all the genes and was, therefore, a true biphasic *S*. Typhimurium. These results were confirmed with the SeqSero tool using the raw reads (Zhang et al., 2015). The two isolates phenotyped as non-motile *S*. Typhimurium were both found to have *fliC*, which encodes the phase 1 flagellin protein FliC, and were therefore genetically serovar *S*. 1,4,[5],12:i:-, this was reflected in their tight clustering with other *S*. 1,4,[5],12:i:- isolates from the same herd in the ancestral phylogeny (Figure 7-2) (De Vries et al., 1998; Ido et al., 2014). Numerous other mutations or deletions could explain the lack of motility observed among these isolates, Bogomolnaya et al. (2014) found 130 mutations in the *S*. Typhimurium genome could influence the motility of the organism.

Table 7-1. Sequenced isolate characteristics. All isolates were serovar *S.* 1,4,[5],12:i:- with six exceptions, as identified.

Sample origin	Production stage	Isolate	Phage type	MLVA profile[a]	MLST	Coverage (%) pRSF1010	Coverage (%) pCol1B9	Coverage (%) pSLT
Herd 1	Weaner	TW-STm31	193	04-14-13-00-490	34	54.91	0	0.16
	Weaner	TW-STm13	193	04-14-13-00-490	34	54.91	0.41	0.18
	Weaner	TW-STm14	193	04-14-12-00-490	NF	83.63	0.1	0.23
	Finisher	TW-STm1	193	04-14-14-00-490	34*	54.91	0	0.28
	Weaner	TW-STm15	193	04-14-12-00-490	34	85.24	0.09	0
	Weaner	TW-STm16	193	04-14-13-00-490	34	77.37	0.09	0
	Finisher	TW-STm2	193	04-14-13-00-490	34	54.91	0	0
	Finisher	TW-STm3	193	04-14-13-00-490	34	85.19	0.09	0
	Weaner	TW-STm17	193	04-14-13-00-490	34	84.81	0.1	0
	Weaner	TW-STm54	untypable	04-14-13-00-490	34	54.91	0.09	0
	Finisher	TW-STm4	untypable	04-14-13-00-490	34	54.91	0.1	0
	Weaner	TW-STm18	193	04-14-13-00-490	34	54.91	0.1	0.12
Herd 3	Weaner	TW-STm19	193	04-15-12-00-490	34	54.91	0.22	0.16
	Weaner	TW-STm20	193	04-15-12-00-490	34	54.91	0.22	0.12
	Weaner	TW-STm21	193	04-15-13-00-490	34	54.91	0.22	0
	Finisher	TW-STm5	193	04-15-11-00-490	34	54.94	0.28	0
	Finisher	TW-STm6	193	04-15-12-00-490	34	54.91	0.22	0.21
	Farrowing	TW-STm39	193	04-15-13-00-490	34	55.74	0.22	0
	Weaner	TW-STm22	193	04-15-13-00-490	34	54.91	93.38	0.77
	Finisher	TW-STm7	193	04-15-12-00-490	34	54.91	0.22	0.29
	Weaner	TW-STm23	193	04-15-13-00-490	34	20.49	0.22	0
	Weaner	TW-STm24	193	04-15-13-00-490	34	54.91	0.22	0.12
	Finisher	TW-STm8	193	04-15-13-00-490	34	54.91	0.22	0
Herd 4	Finisher	TW-STm32	120	04-15-11-00-490	34	4.47	33.57	0.31
	Weaner	TW-STm33	120	04-15-11-00-490	34	4.47	33.66	0.44
	Weaner	TW-STm34	193	04-15-11-00-490	34	0	92.27	1.14
	Finisher	TW-STm35	193	04-15-11-00-490	34	0	0	0.1
	Weaner	TW-STm36	193	04-15-11-00-490	34	54.91	33.27	0.23
	Weaner	TW-STm37	193	04-15-11-00-490	34	54.91	34.11	0.32
	Gestating sow	TW-STm40	193	04-15-11-00-490	34	0	0	0.16
	Farrowing	TW-STm41	193	04-15-12-00-490	34	0	0	0
	Finisher	TW-STm9	193	04-15-11-00-490	NF	0	0	0
	Weaner	TW-STm25	193	04-14-11-00-490	34	0	0	0
	Weaner	TW-STm26	193	04-15-11-00-490	34	0	0	0.19
	Farrowing	TW-STm38	193	04-15-11-00-490	34	0	0	0.19
	Weaner	TW-STm27	193	04-15-11-00-490	34	0	0	0.12
	Finisher	TW-STm10	193	04-15-11-00-490	34	0	92.19	1.42
	Farrowing	TW-STm42	193	04-15-11-00-490	34	0	0	0
	Weaner	TW-STm28	193	04-15-11-00-490	34	0	0	0
	Finisher	TW-STm11	193	04-15-11-00-490	34	0	0	0
	Weaner	TW-STm29	193	04-15-10-00-490	34	0	0	0
	Weaner	TW-STm30	193	04-16-10-00-490	34	0	0	0
	Finisher	TW-STm12	193	04-15-12-00-490	34	0	0	0.12
Herd 5	Weaner	TW-STm43	untypable	04-15-11-00-490	34	54.91	0	0.12
Herd 6	Weaner	TW-STm44	193	04-14-11-00-490	NF	54.91	0	0.1
Herd 11	Weaner	TW-STm48	193	04-15-12-00-490	34	0	0	0.18
	Weaner	TW-STm47	193	04-15-11-00-490	34*	54.91	0	0
	Weaner	TW-STm49	193	04-17-11-00-490	34	54.91	0	0
	Weaner	TW-STm50	193	04-17-11-00-490	34	54.91	0	0.18
	Weaner	TW-STm55[b]	not tested	04-16-11-00-490	34	54.91	0.35	0.16
	Weaner	TW-STm56[b]	not tested	04-15-11-00-490	34	54.91	0	0
	Weaner	TW-STm51	193	04-16-06-00-490	34	54.91	0	0
	Weaner	TW-STm52	193	04-12-11-00-490	34	54.91	0	0
	Grower (11-15wk)	TW-STm53	193	04-26-11-00-490	34	54.91	0	0.16
Herd 16	Grower (11-15wk)	TW-STm57[c]	untypable	05-14-12-12-490	19	57.69	89.68	100
	Finisher	TW-STm58[c]	untypable	05-14-12-12-490	19	57.86	11.21	100
Abattoir1	Carcass	TW-STm59[c]	126	05-17-10-13-490	19	39.69	84.35	100
	Carcass	TW-STm60[c]	170	04-16-14-00-518	19	0	0	0
Abattoir2	Carcass	TW-STm45	193	04-15-07-00-490	NF	54.91	3.14	14.69
	Carcass	TW-STm46	193	04-15-08-00-490	34	54.91	3.45	14.55
Diversity			6	19	5	0-85.24	0-93.38	0-100

* mismatch against *hisD* (1 SNP)
NF *aroC* 482, allele combination was not found
[a] Presented in the Australian nomenclature (Gilbert, 2008)
[b] Phenotyped *S.* 1,4,[5],12:-:- (non-motile *S.* Typhimurium), subsequently shown to be *S.* 1,4,[5],12:i:-
[c] *S.* 1,4,[5],12:i:1,2 (*S.* Typhimurium; biphasic)

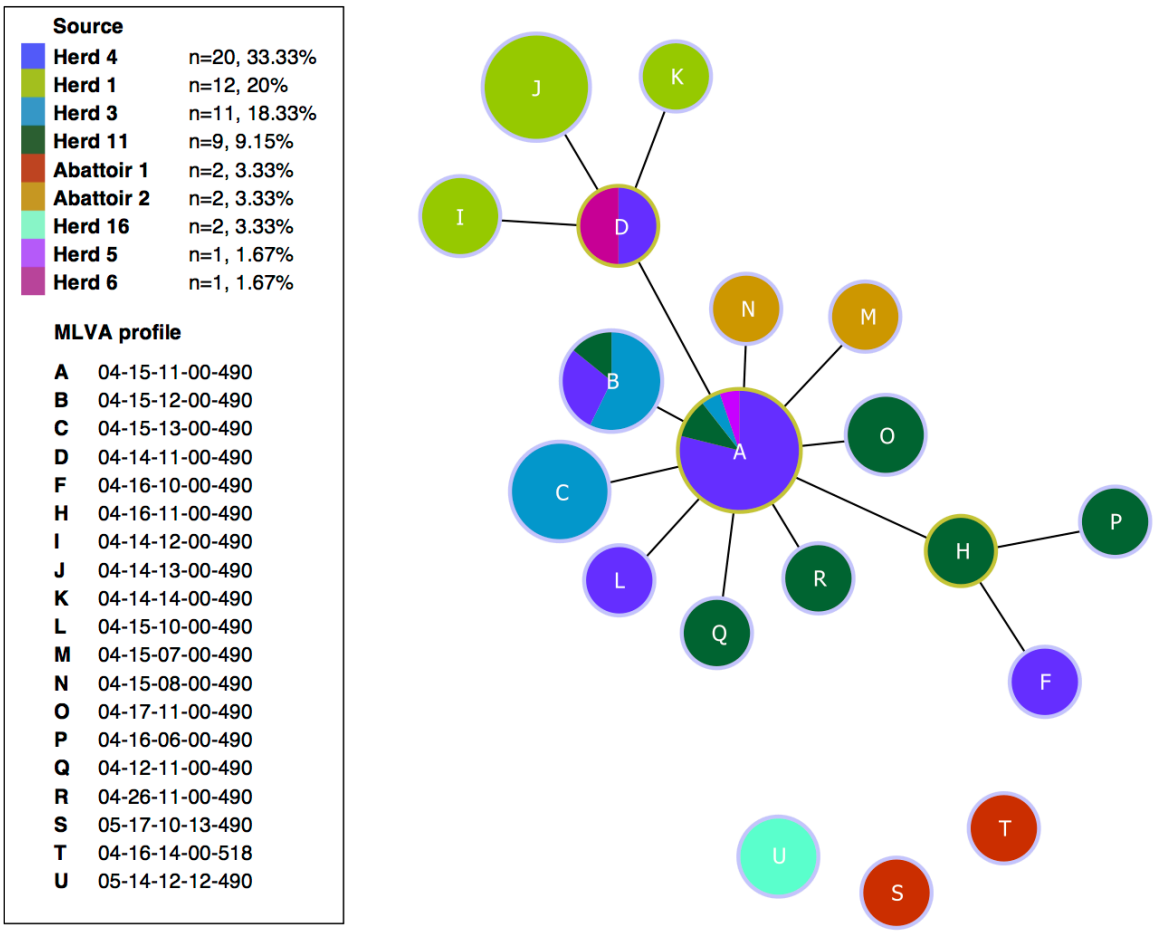

Figure 7-1. Minimum spanning trees of MLVA profiles. Minimum spanning tree of study MLVA profiles by herd created using eBURST analysis with standard goeBURST distance measures, visualized in PHYLOViZ. Edge = 1 SLV. The inferred founder profiles are designated by a gold outline. The coloured pie segments identify the sources of the isolates, the node size (non-linear) indicates relative number of isolates.

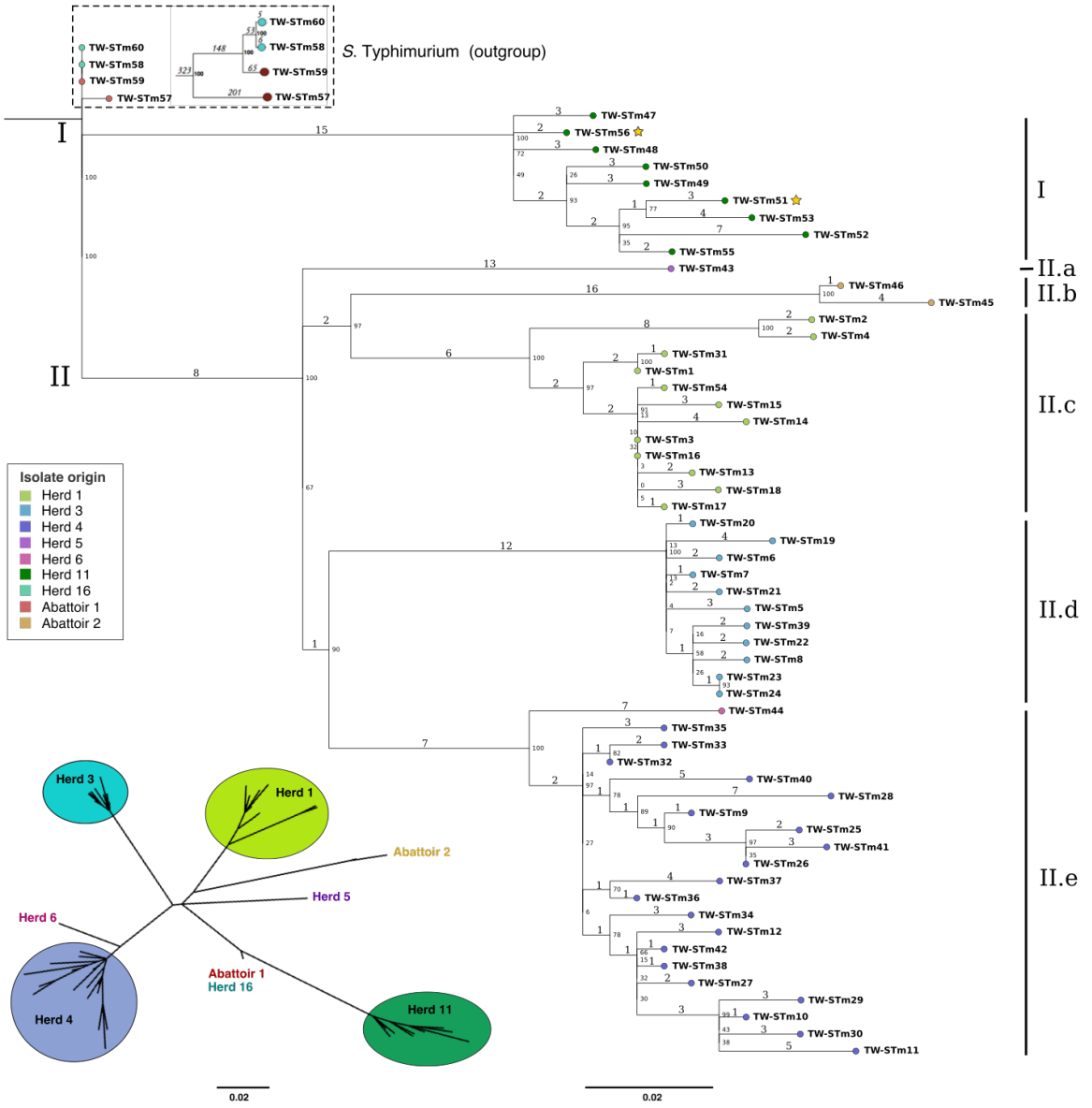

Figure 7-2. Phylogenetic tree of sequenced *Salmonella enterica* (I) Group B genomes. Maximum likelihood tree constructed on base substitutions identified by mapping to the reference strain *S.* Typhimurium SL1344; SNPs found in phage, insertion, repeat regions, or predicted to be caused by recombination were removed prior to tree construction. The reference was removed to view the branch topologies of the study isolates. The tree is rooted on the *S.* Typhimurium isolates, an outgroup. Tips are coloured by source and labeled with the isolate identification number. Gold stars indicate isolates phenotyped as non-motile *S.* Typhimurium, subsequently shown to be *S.* 1,4,[5],12:i:-. The two *S.* 1,4,[5],12:i:- basal clades are labelled I and II at the bipartitions to the left of the tree. Subclades, defined as isolates with ≤12 SNPs to a predicted most recent common ancestor, are identified by II.a to II.e and solid lines, to the right of the tree. Branches are labeled with number of base substitutions. Bootstrap support figures for each bipartition are indicated at the end node (%). Inset: unrooted tree shows clades by sample source, constructed with prior identification of the outgroup.

7.3. Discussion

The sequenced *S*. 1,4,[5],12:i:- isolates formed a distinct clade with only ≤30 core SNPs MRCA indicating they were members of a single clone. As Van Belkum et al. (2007) note the use of terminology in bacterial typing is not always consistent and can cause confusion. The term 'clone', in particular, is often used ambiguously in the literature. For this reason, Van Belkum et al. (2007) proposed the following definition of a clone:

> 'Bacterial isolates that, although they may have been cultured independently from different sources in different locations and perhaps at different times, still have so many identical phenotypic and genotypic traits that the most likely explanation for this identity is a common origin within a relevant time span.' (Van Belkum et al., 2007).

The study isolates meet these criteria given the very low number of core genome SNPs within the collection. However, the Van Belkum et al. (2007) definition still requires considerable interpretation on the part of the researcher and reader. Petrovska et al. (2016) defined the isolates in their study of the genomes of UK and Italian epidemic *S*. 1,4,[5],12:i:- strains from 2005-2012 with a maximum root-to-tip distance of ≈70 SNPs as a clonally expanding clade. This is considerably more SNPs than were observed among the isolates sequenced in this study. The assertion that the study isolates are clonal also compares with Hawkey et al. (2013) who described ten *S*. Typhimurium isolates with <23 core genome SNPs, pairwise, as a single clone.

The *S*. 1,4,[5],12:i:- basal clades, I and II, had 100% bootstrap support and clearly differentiated isolates by time of sample collection and source(s). Clade I encompassed only isolates from Herd 11, all collected in 2011, whereas the remaining isolates, all collected in 2013-2014, were located in Basal Clade II. Year of isolation and source herd were confounding variables. However, it was interesting to note that the SNP difference to a MRCA was lower among the Herd 11 isolates collected in 2011 than among the later isolates collected from multiple herds in 2013-2014. The greater variation in the later samples would be consistent with clonal expansion from a single point of emergence in the recent past.

These findings indicate that the *S*. 1,4,[5],12:i:- strains isolated in this study likely emerged in Australian pigs via a single event that occurred relatively recently. Given the diversity of herds from which the isolates were sourced the results also suggest that the *S*. 1,4,[5],12:i:- strains currently circulating in the Australian pig population, and possibly other industries, emerged through a single recent event. Mutation rates for *S*. Typhimurium have been estimated 1-5 SNPs per year, a reasonable proxy for *S*. 1,4,[5],12:i:- given the serovars' close relatedness (Okoro et al., 2012a; Hawkey et al., 2013; Mather et al., 2013; Hayden et al., 2016; Petrovska et al., 2016). This implies that a most recent common ancestor of the sequenced *S*. 1,4,[5],12:i:- isolates likely existed

approximately six to 30 years ago. This timeframe for emergence in Australia is eminently plausible given the earliest records of domestic *S*. 1,4,[5],12:i:- isolation in Australia and the likelihood of misclassification and underreporting prior to wider recognition of the serovar in typing laboratories (OzFoodNet Working Group, 2012c; OzFoodNet, 2015).

The *S*. 1,4,[5],12:i:- basal clade II was further divided into five tight subclades. The subclades accurately differentiated herds with the exception of the single isolate from Herd 6, which loosely clustered with isolates from Herd 4 (Subclade II.e). The identification of subclades corresponding to herds indicates that the *S*. 1,4,[5],12:i:- population in each source herd had diverged, albeit minutely, from the strains colonising other herds. This suggests that the *S*. 1,4,[5],12:i:- populations colonizing each of the herds were stable and had persisted within the herds for some time, supporting MLVA findings presented earlier in this thesis (Chapter 5).

The Herd 6 isolate TW-STm44 was separated from the other members of the subclade, isolates from Herd 4, by ≥10 SNPs pairwise demonstrating that it was somewhat distinct, which is clearly visible in the maximum likelihood phylogenetic SNP tree of all sequenced isolates. The isolates from the earliest samples collected from Herd 4, TW-STm37 and TW-STm36, were collected in January 2013 and had an eight SNP difference, pairwise, to the Herd 6 strain, indicating that they descended from an MRCA 5-8 years ago, assuming an evolutionary rate of 1-1.5 SNPs/year (Hawkey et al., 2013). This is also consistent with the likely time of *S*. 1,4,[5],12:i:- emergence in Australian pigs. Interestingly, Herds 4 and 6 shared a supplier of gilts. However, Herd 5 (Subclade II.a) also sourced gilts from the same supplier and yet the sole Herd 5 isolate (TW-STm43) had a 24 to 33 SNP difference, pairwise, when compared with isolates in Subclade IIe. This provides further support for investigation of gilts brought into farms and gilt supplier herds as a potential source of *S*. 1,4,[5],12:i:- introduction, as recommended in the previous chapter (Chapter 6).

Despite the tight clustering within herds (≤12 SNPs MRCA), possible divergence within herds can also be identified within the phylogeny. Two or more possible lineages could be identified among the Herd 1 and Herd 4 subclades—Subclades II.c and II.e, respectively. Isolates TW-STm4 and TW-STm2 (4 SNPs pairwise) show signs of divergence from the other Herd 1 isolates sequenced (≥14 SNPs pairwise), and the bipartition was strongly supported (100% bootstrap values). Among Herd 4 strains divergence of two lineages was also apparent. In the ancestral tree presented Herd 4 strains TW-STm34 to TW-STm11, collected in 2014 with the exception of TW-STm34, appear to have diverged from the other strains that were collected in 2013, with the exception of TW-STm28. By comparison, Herd 3 (Subclade II.d) shows no indication of divergence with ≤4 SNPs MRCA separating all the sequenced strains from this herd—the Herd 3 isolates are essentially indistinguishable (Hawkey et al., 2013). This could indicate that Herd 3 was colonized more recently than Herds 1 and 4, and the resident *S*. 1,4,[5],12:i:- population has not had time to diverge. Alternatively, this may relate to purifying selection occurring in response to selective

pressure within the Herd 3 *S*. 1,4,[5],12:i:- population. It is notable that the possible lineages identified within Herds 1 and 4 were contemporary. Having established that *S*. 1,4,[5],12:i:- can persist for extended periods within herds it would be interesting to continue monitoring populations within a selection of herds to see if further divergence occurs or if the isolates remain tightly clustered as a single lineage predominates. This would also present an opportunity to further investigate the rate of SNP generation among *S*. 1,4,[5],12:i:- strains in the 'real world' context of commercial pig operations.

It was not possible to differentiate isolates from different production stages within herds. This variable lacked sensitivity due to the very low levels of *S*. 1,4,[5],12:i:- detection among sows. The only sequenced isolates from gestating sows and farrowing sheds were from Herds 3 and 4. However, in each case these isolates clustered closely with isolates sourced from grow-out pigs. This is consistent with indications that *S*. 1,4,[5],12:i:- populations were cycling throughout these herds, rather than being confined to specific age groups or cohorts (Chapter 6). Niemann et al. (2015a) have also described *S*. 1,4,[5],12:i:- strains cycling throughout herds in their longitudinal study of German pig herds. Herd 3 did not practice all-in, all-out management of grow-out pigs, whereas Herd 4 did. These findings are consistent with speculation that all-in, all-out management of grow-out cohorts may not be effective in protecting new batches from being challenged by *S*. 1,4,[5],12:i:-, reported in the preceding chapter.

The two *S*. 1,4,[5],12:i:- isolates obtained from carcass samples (abattoir 2, Subclade II.b) were closely related (5 SNPs pairwise) and clustered centrally within Basal Clade II. The strains were isolated from samples collected on the same day. Unfortunately, more detailed epidemiological information on the carcasses sampled was not available. It is possible that pig or carcass contamination occurred at the abattoir—in lairage, during slaughter or on chains—and that a clonal lineage was circulating within the abattoir. However, the two sequenced isolates were the only *S*. 1,4,[5],12:i:- isolates among several hundred samples collected at the abattoir that day. It is perhaps more likely that the samples were collected from nearby carcasses that originated in the same herd, the *S*. 1,4,[5],12:i:- having been brought in with the live animals. The central clustering of these isolates within Clade II provides further evidence that a clonal *S*. 1,4,[5],12:i:- population is circulating within the Australian pig industry.

Two of the *S*. Typhimurium isolates were sourced from the same herd (Herd 16). The isolates were sourced from samples collected from different age groups but were closely related, 11 SNPs pairwise, mirroring observations of the *S*. 1,4,[5],12:i:- strains. This suggests that the grow-out pigs in Herd 16 harboured an embedded *S*. Typhimurium population, as has been reported elsewhere (Funk et al., 2001). In contrast, the two *S*. Typhimurium isolates sourced from carcass samples at an abattoir were readily differentiated from the two isolates from primary production (≥65 SNPs MRCA) and each other (≥213 SNPs MRCA). *Salmonella* Typhimurium has been present in Australia

much longer than *S*. 1,4,[5],12:i:-, the population is therefore likely to contain greater diversity. Although only four *S*. Typhimurium isolates were sequenced, this diversity appears to be reflected in the greater number of core SNP differences between the isolates included in this study.

The virulence plasmid pSLT was not present in any of the *S*. 1,4,[5],12:i:- isolates. Furthermore, no amplification was found at the locus STTR10 used in MLVA typing in any of the *S*. 1,4,[5],12:i:- isolates in the full study collection, indicating that the plasmid may not be present in the wider Australian *S*. 1,4,[5],12:i:- population. While the sporadic loss of pSLT has been reported (Petrovska et al., 2016), that none of the sequenced *S*. 1,4,[5],12:i:- isolates had the plasmid and that MLVA results indicated the plasmid may not have be present in the wider study collection suggests that the Australian *S*. 1,4,[5],12:i:- population may have emerged without pSLT.

High matches to pRSF1010, associated with resistance to S and Su, with very high read depth (5-10 times the chromosomal read depth) were found for three *S*. 1,4,[5],12:i:- isolates from Herd 1 (TW-STm15, TW-STm3, TW-STm17; 85% match). Partial matches to the plasmid pRSF1010was observed widely among the isolates (Scholz et al., 1989). Twenty-nine isolates had a partial match to pRSF1010 of exactly 54.1% at a low read depth, providing yet more evidence of shared heritage among the isolates.

The colicin plasmid, pCol1B9, was only present in two unrelated *S*. Typhimurium isolates (TW-STm57, TW-STm59; 89% match) and three *S*. 1,4,[5],12:i:- isolates (TW-STm22, TW-STm34, TW-STm10; 92-93% match). The read depth through each of these matches was 1.9 to 3.1 times the chromosomal read depth suggesting that the sequences were extra chromosomal on a low copy number plasmid.

The majority of isolates from Herd 4 did not harbour pSLT, RSF1010-like or pCol1B9-like plasmids and only a small number of isolates had any match any of the plasmids; the three *S*. 1,4,[5],12:i:- isolates with high matches to pCol1B9 were from Herds 3 and 4. The plasmid pCol1B9 is a plasmid that can be horizontally transferred between commensal bacteria inhabiting the gut, acquisition might explain the presence of the plasmid in these strains (Stecher et al., 2012; Nedialkova et al., 2014). The plasmid pCol1B9 confers production of the bacteriocin colicin 1B, which is known to provide considerable benefit to *S*. Typhimurium over competing *Escherichia coli* in the digestive system of mammals (Nedialkova et al., 2016). Although no other isolates were found to have the plasmid, it would be interesting to continue monitoring *S*. 1,4,[5],12:i:- in this herd to see if the presence of the pCol1B9 plasmid is selected for in future.

Results of MLST and phage typing were of limited value in differentiating *S*. 1,4,[5],12:i:- isolates in the study collection. The low phage type diversity and strong predominance of PT193 among the wider *S*. 1,4,[5],12:i:- study collection differs from reports elsewhere which identified greater variation in phage types (de la Torre et al., 2003b; Petrovska et al., 2016). Although PT193 has been

described as polyphyletic, the strains sequenced in this study all clustered closely in the same clade (Petrovska et al., 2016). The two PT120 isolates sequenced in this collection were from Herd 4 and clustered closely with the PT193 strains from the same herd. This differs from earlier European reports that have identified PT193 and PT120 as two distinct lineages (Hauser et al., 2010), but appears similar to a more recent report which found the two phage types did cluster in some cases (Petrovska et al., 2016).

Application of MLVA typing was considerably more effective at differentiating *S*. 1,4,[5],12:i:- strains than phage typing or MLST and the preceding longitudinal study showed that MLVA profiles can persist within herds for extended periods. Moreover, despite the majority of *S*. 1,4,[5],12:i:- isolates being MLVA SLVs, *S*. 1,4,[5],12:i:- MLVA profile clusters did appear to be associated with individual herds, as depicted in the minimum spanning tree. The *S*. 1,4,[5],12:i:- MLVA profiles identified in each herd could be collapsed into SLV clusters with 1-2 VNTR copy number differences at the STTR5 and STTR6 loci, with the exception of Herd 11 isolates. However, as anticipated, core genome sequence analysis provided much greater resolution. The MLVA profile clusters observed were not necessarily unique to herds, unlike the subclades derived from phylogenetic analysis of core genome SNPs. These findings support the use of MLVA in outbreak strain identification, and demonstrate that the method could be informative for source attribution investigations, but also show that analysis of whole genome sequences and comparative genomic studies of core genome SNPs is more discriminatory and reliable in terms of identification of ultimate source.

7.4. Implications

Salmonella 1,4,[5],12:i:- appears to have emerged on multiple occasions internationally, and has likely spread across country borders, as occurred with pandemic *S*. Typhimurium DT104 (Leekitcharoenphon et al., 2016). However, *S*. Typhimurium DT104 was never isolated from domestically acquired cases in Australia. *Salmonella* 1,4,[5],12:i:- has emerged and now appears to be widespread within the Australian pig industry (Chapter 3)(Hamilton et al., 2015). The results of this comparative genomic study strongly suggest that the Australian porcine *S*. 1,4,[5],12:i:- population is clonal. Unfortunately, this study was unable to directly compare the sequences of these strains with strains from Australian human isolates, however, on the basis of published data they share many of the same phenotypic and genotypic characteristics (NEPSS, 2013, 2014). Phylogenetic analyses of core genome SNPs showed that the *S*. Typhimurium isolates sequenced, admittedly a small sample, were quite distinct from the *S*. 1,4,[5],12:i:- isolates. These results are consistent with the serovar being introduced to Australian pigs from overseas and subsequent spread within the industry. However, domestic emergence cannot be ruled out given the biased selection of strains and the herds sampled and the small number of *S*. Typhimurium isolates sequenced in this study. A

further comparison of Australian *S*. 1,4,[5],12:i:- strains with domestic *S*. Typhimurium strains and strains from overseas is needed. Determining whether the serovar was introduced and, if it indeed was, then how this occurred could help to improve national and industry biosecurity strategies and protocols.

These findings support the continued application of MLVA for epidemiological purposes. Although analysis of core SNPs provided greater resolution, MLVA typing was considerably more discriminatory that phage typing and MLST and MLVA profiles persisted within herds. Observations of herd associated MLVA profiles were reflected in the phylogenetic analyses presented here, showing that herds did harbour closely related *S*. 1,4,[5],12:i:- lineages. At this point MLVA remains cheaper and the outputs considerably easier to store, analyse and compare than whole genome sequence data, due to the vast differences in data volumes, computational requirements and necessary analytical expertise. Establishing a standard method of clustering similar MLVA profiles, particularly for outbreak strain identification, such as the method proposed by Dimovski et al. (2014) and supported by findings presented earlier in this thesis, will increase the utility of MLVA.

These findings further demonstrate the accuracy of comparative genomics for differentiating subpopulations of bacterial pathogens, even within a highly clonal population. The core SNP phylogeny presented provides further evidence of the potential of comparative genomic studies, in parallel with epidemiological data, for outbreak investigation and public health inquiries relating to *Salmonella* and comparable pathogens (Okoro et al., 2012b; Hawkey et al., 2013; Mather et al., 2013; Leekitcharoenphon et al., 2014). Further sequencing of pathogens and application of comparative genomic approaches will no doubt continue to provide insights into the ecology and epidemiology of infectious agents and the dynamics of infectious diseases of importance to both animal and human health. As the costs of sequencing continue to diminish and the ability to store, process and analyze large quantities of sequence data efficiently increases, it is anticipated that these approaches will become the primary method for outbreak investigation and source attribution. Routine sequencing will quickly establish a widely accessible library of local and international strains, which will increase the scope of phylogenetic studies and aid interpretation. Comparative genomic studies will provide new insights into pathogen populations and their dynamics in hosts and their environments, which will benefit industry and public health-led surveillance and risk mitigation and management decision-making.

7.5. Conclusion

This study found a clonal *S*. 1,4,[5],12:i:- population, indicating that the serovar emerged in the Australian pig herd from a single event. Given the striking similarities between the study *S*.

1,4,[5],12:i:- strains and those that have been reported outside Australia it is likely the serovar was introduced from overseas. Despite the clonal nature of the *S*. 1,4,[5],12:i:- population, the sources of samples were clearly distinguishable on the basis of core SNP differences. These results demonstrated the usefulness of MLVA as a typing method for differentiating *S*. 1,4,[5],12:i:- strains. However, these findings also provide yet more evidence of the enormous potential of whole genome sequencing and comparative genomic studies for epidemiological and risk management purposes.

Supplementary Table 7-2. Summary report of all replicons in the reference file.

Isolate	Cover %[a]	Mean depth[b]	Reads mapped (%)[c]	Total reads[d]	Mean Q[e]
TW-STm1	97.9	79.8	94.7	3024082	32.8
TW-STm2	97.8	85	94.7	3071976	32.6
TW-STm3	97.9	74.3	88.7	2892292	32.5
TW-STm4	97.9	81.7	84.8	3304910	32.6
TW-STm5	97.9	93.6	93.3	3481742	32.7
TW-STm6	97.9	71.4	86.2	2856108	32.5
TW-STm7	97.9	72	85.5	2926246	32.7
TW-STm8	97.8	76	86.9	3030340	32.6
TW-STm9	97.9	67.8	94.7	2495924	31.1
TW-STm10	97.9	86.2	90.6	3291458	32.6
TW-STm11	97.9	77.9	94.3	2832992	32.6
TW-STm12	97.9	84.4	94.4	3114112	32.6
TW-STm13	97.9	94.4	88.5	3694108	32.7
TW-STm14	97.9	83.5	86	3446030	32.8
TW-STm15	97.9	75.8	83.1	3209860	32.7
TW-STm16	97.9	80.1	89.4	3156348	32.8
TW-STm17	97.9	77.4	87.1	3061164	32.7
TW-STm18	97.9	98.3	90.1	3866988	32.7
TW-STm19	97.9	75.1	88.5	2909882	32.6
TW-STm20	97.9	75.7	87.2	2990516	32.6
TW-STm21	97.9	93.3	88	3662864	32.6
TW-STm22	97.9	68.7	81.3	2944606	32.7
TW-STm23	97.9	73.5	87.4	2927542	32.6
TW-STm24	97.9	71.7	86.9	2856070	32.7
TW-STm25	97.9	90.3	95	3311116	32.7
TW-STm26	97.9	88.1	94.9	3353076	32.8
TW-STm27	97.9	78.9	94.5	2959202	32.8
TW-STm28	97.9	70.8	94.3	2598252	32.6
TW-STm29	97.9	87	94.3	3180194	32.6
TW-STm30	97.9	92.7	94.5	3406036	32.6
TW-STm31	97.9	81.1	93.9	2997144	31
TW-STm32	97.9	63.2	89.5	2429828	31.5
TW-STm33	97.9	79.1	88.7	3253726	31.4
TW-STm34	97.9	65.8	90.7	2505898	31.7
TW-STm35	97.9	81.8	94.9	3076584	31.3
TW-STm36	97.9	50	88.7	1914050	31.5
TW-STm37	97.9	58.7	86.8	2345564	31.7
TW-STm38	97.9	93.5	94.5	3499908	32.7
TW-STm39	97.9	74.3	86.2	3059518	32.8
TW-STm40	97.9	78.5	94.8	2916534	31.1
TW-STm41	97.9	73.6	94.8	2727754	31.2
TW-STm42	97.9	99.6	94.5	3699420	32.9
TW-STm43	97.9	89.4	94.8	3275522	32.7
TW-STm44	97.9	69.2	84.8	2857008	31.1
TW-STm45	97.9	90.5	90.8	3430704	32.7
TW-STm46	97.9	90.1	90	3487644	32.6
TW-STm47	97.9	73.9	95.1	2635738	31.5
TW-STm48	97.9	83.1	95.6	2980944	31.6
TW-STm49	97.8	67	93.9	2425466	31.6
TW-STm50	97.9	62.3	94.9	2236954	31.6
TW-STm51	97.9	63.5	93.6	2344608	31.7
TW-STm52	97.5	75	94.9	2722200	31.7
TW-STm53	97.9	72.4	95.3	2650850	31.7
TW-STm54	97.9	71.7	90.4	2693790	32.5
TW-STm55	97.9	68.3	95.1	2473866	31.6
TW-STm56	97.9	66.9	94.9	2463804	31.8
TW-STm57	98.8	72.7	84	3047978	32.7
TW-STm58	98.8	74.9	88.2	2973744	32.7
TW-STm59	98.6	67.1	85.9	2754724	32.6
TW-STm60	98.5	89.3	90.4	3478540	32.7
Mean	97.9	77.9	90.8	2986934	32.3
Median	97.9	75.9	90.7	2985730	32.6

[a] percentage of bases of the reference with at least one read mapped
[b] average depth of reads for bases with at least one read
[c] percentage of the total reads mapped to each replicon
[d] total reads (mapped and unmapped)
[e] average quality scores for the read set

Chapter 8 - Observations of Australian porcine *Salmonella* antimicrobial resistance phenotypes and *S*. 1,4,[5],12:i:- resistance gene repertoires

8.1. Preface

Antimicrobial susceptibility phenotype testing was conducted on isolates that were representative of each *Salmonella* serovar and phage type, where appropriate, isolated from each sample collected in the cross-sectional study, the longitudinal case study of grow-out pigs in Herd 4 and the longitudinal study of five herds. The study designs are described in Chapter 2, and the results of each of the three studies are described in Chapters 3, 4 and 5, respectively. This chapter describes the antimicrobial resistance phenotypes observed among *Salmonella* isolates in the various study collections and the antimicrobial resistance genes identified within the sequenced *Salmonella* Group B serovar genomes in relation to herd origin and the ancestral phylogenetic tree described in the previous chapter (Chapter 7).

8.2. Introduction

Increasing bacterial antimicrobial resistance is a cause for concern for animal and human health worldwide (Cohen, 1992; Neu, 1992; Livermore, 2002; Tenover, 2006; Holmes et al., 2016). *Salmonella* spp. resistance to multiple antimicrobial agents, in numerous combinations, is well-described (Cohen and Tauxe, 1986; Su et al., 2004). Managing multidrug resistant *Salmonella* strains capable of causing serious human and animal illness, strains resistant to critical antimicrobials for the treatment of salmonellosis, and minimizing the development of resistance pose considerable challenges to animal production and human health. *Salmonella* Group B strains are commonly virulent and multidrug resistant, typified by the penta-resistant pandemic strain *S*. Typhimurium DT104, prominent in the 1990s and 2000s (Glynn et al., 1998; Poppe et al., 1998; Threlfall, 2000). *Salmonella* Typhimurium DT104 typically possess resistance to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamides/sulphathiazole (Su) and tetracycline (T) abbreviated ACSSuT. These resistances are typically encoded by $bla_{\text{CARB-2}}$, *cmlA*, *aadA2*, *sul1* and *tetA*, respectively, on the chromosome and involving integrons (Briggs and Fratamico, 1999). Like *S*. Typhimurium DT104,

the serovar *S.* 1,4,[5],12:i:- is commonly multidrug resistant, typically possessing to ampicillin (A), streptomycin (S), sulphonamides/sulphathiazole (Su) and tetracycline (T), abbreviated ASSuT, reports of *S.* 1,4,[5],12:i:- strains possessing extended resistance types in addition to the typical ASSuT quartet are also common (Echeita et al., 1999; Echeita et al., 2001; Switt et al., 2009; Hauser et al., 2010; Lucarelli et al., 2010; Davies, 2013; Gallati et al., 2013; García et al., 2013). Unlike *S.* Typhimurium DT104, *S.* 1,4,[5],12:i:- ASSuT resistance is typically encoded by $bla_{\text{TEM-1}}$, *strA*, *strB*, *sul2* and *tetB*. The genes can be found on the chromosome or may be mediated by plasmids, depending on the clone (García et al., 2011; García et al., 2013; García et al., 2016; Petrovska et al., 2016).

The predominant *S.* 1,4,[5],12:i:- clones are differentiated by their phenotypes and genotypes (EFSA, 2010b). The 'Spanish clone', which emerged in the late 1990s, typically has plasmid mediated resistance to ASSuT and often additional resistances, most commonly to C, co-trimoxazole, gentamicin (G) and trimethoprim (Tm) (Barone et al., 2007; Hauser et al., 2010; Laorden et al., 2010; Lucarelli et al., 2010; García et al., 2013; Andrés-Barranco et al., 2016; García et al., 2016). The 'European clone', which emerged in the 2000s, typically exhibits resistance to the ASSuT quartet with resistance genes located on the chromosome. The predominant phage type among the study isolates, PT193, is associated with the European clone, whereas the Spanish clone has typically been characterized by phage type U302 (EFSA, 2010b; Petrovska et al., 2016). A third clone, the 'US clone' that has been reported most commonly in North America, is characterized by lower levels of resistance and is often pansusceptible; for example a Canadian study conducted by Mulvey et al. (2013b) found 60% of *S.* 1,4,[5],12:i:- isolates were pansusceptible.

Petrovska et al. (2016) found a 15,726bp deletion of the *fljAB* region (located between STM2759 and *iroB*) and a 27,473bp insertion had occurred in the same location of the chromosome in the epidemic *S.* 1,4,[5],12:i:- clade they identified. Petrovska et al. (2016) found that the insertion sequence included the markers for the ASSuT resistance phenotype, specifically the bla_{TEM}, *strA*, *strB*, *sul2* and *tetB* gene cassette. The insertion sequence was not present in isolates from outside the epidemic clade isolates in their study, indicating that the insertion event occurred during the clonal expansion of *S.* 1,4,[5],12:i:-.

The isolation of multidrug resistant *Salmonella* strains from Australian animal production has been reported since at least as early as the 1970s (Murray et al., 1986). A recent study by Abraham et al. (2014) found relatively low resistance among *Salmonella* spp. isolates from New South Wales, Australia, relative to reports overseas. Among the Abraham et al. (2014) study isolates sourced from clinically infected food animals, over 66% were susceptible to the full study panel of 18 antimicrobials and less than 9% were resistant to four or more compounds. Smith et al. (2016) described antimicrobial resistance phenotypes and genes identified among commensal *E. coli* in Australian finisher pigs, finding resistance to registered agents was relatively common but observing

very low levels of resistance to critical antimicrobials for human health. However, the resistance phenotypes and determinants of *S.* 1,4,[5],12:i:- in Australia have not yet been reported or discussed. The studies reported in this thesis indicate that *S.* 1,4,[5],12:i:- has become widespread in the Australian pig industry, meanwhile the serovar has been implicated in a considerable number of human salmonellosis cases (SA Pathology, 2014; OzFoodNet Working Group, 2015a).

The NEPSS passive surveillance database contains records of 372 *S.* 1,4,[5],12:i:- PT193 strains isolated from domestic human sources between the years 2010 and 2014 (NEPSS, 2014). In total 73.7% (274/372) of strains in the database were resistant to ASSuT only and 86.8% (323/372) were resistant to ASSuT or ASSuT in combination with other resistances. Bearing in mind the passive nature of submission of the entries found in the NEPSS database, the most common *S.* 1,4,[5],12:i:- PT193 isolates from domestic human cases recorded in the NEPSS database up to December 2014 were T (96.8%), followed by A (92.2%), S (90.6%) and Su (89.0%) (Table 8.1). Disaggregating by year, the predominance of the ASSuT resistance type and the emergence of additional resistances is apparent in the NEPSS data (Figure 8-1). In 2010, the majority of the 28 isolates were resistant to ASSuT or a combination thereof; in later years ASSuT resistance remained common but extended resistance types, ASSuT+, began to emerge.

Table 8-1. Antimicrobial resistance phenotypes of Australian *S.* 1,4,[5],12:i:- PT193 strains isolated from domestic human sources. Data sourced from NEPSS (2014).

Antimicrobial agent	Abbrev.	Resistance breakpoint[a] (mg/L)	No. *S.* 1,4,[5],12:i:- PT193 resistant (% isolates exhibiting resistance) n = 372
Full sensitivity	-	-	4 (1.1)
Ampicillin	A	≥ 16	344 (92.2)
Cefotaxime	Cf	≥ 1	10 (2.7)
Chloramphenicol	C	≥ 16	18 (4.8)
Ciprofloxacin	Cp	≥ 2	0 (0)
Gentamicin	G	≥ 8	7 (1.9)
Kanamycin	K	≥ 32	32 (8.6)
Nalidixic Acid	Na	≥ 16	3 (0.8)
Spectinomycin	Sp	≥ 50	10 (2.7)
Streptomycin	S	≥ 32	337 (90.6)
Sulphathiazole	Su	≥ 512	331 (89.0)
Tetracycline	T	≥ 8	360 (96.8)
Trimethoprim	Tm	≥ 8	39 (10.5)

[a] Breakpoints in accordance with CLSI (2011) and Commons et al. (2012).

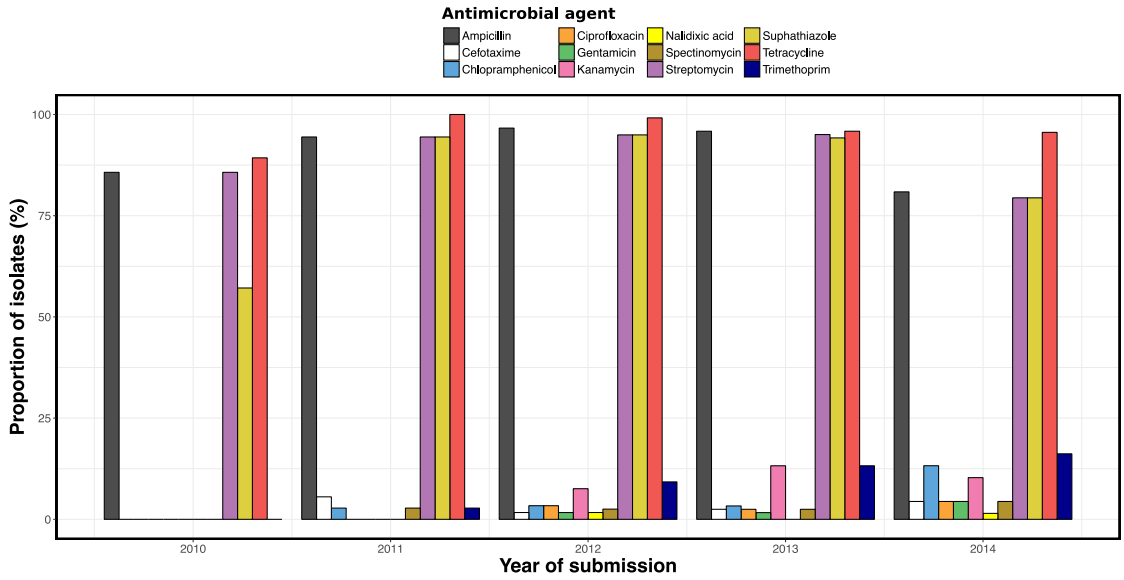

Figure 8-1. Proportions of human *S.* 1,4,[5],12:i:- PT193 isolates in the NEPSS database phenotypically resistant to specified antimicrobials. The number of isolates in the database for 2010, 2011, 2012, 2013 and 2014 were 28, 36, 119, 121 and 68, respectively. Data sourced from NEPSS (2014).

Describing the antimicrobial resistance phenotypes and possible mechanisms for resistance among Australian porcine *S.* 1,4,[5],12:i:- isolates is important for the pig industry and animal and public health more generally. The objectives of this study were to: describe the antimicrobial resistance phenotypes and trends over time among the study *Salmonella* spp. isolate collection; describe the resistance genes present in the *Salmonella* Group B strains sequenced; identify patterns in antimicrobial resistance types and genes in relation to epidemiological and phylogenetic relationships between strains; and to compare the study *S.* 1,4,[5],12:i:- strain resistance phenotypes to those reported in the NEPSS database and with the *S.* 1,4,[5],12:i:- resistance phenotypes and genes reported outside Australia.

8.3. Results

8.3.1. Antimicrobial resistance phenotypes among the cross-sectional study isolates

Among the cross-sectional study collection isolates, *S.* 1,4,[5],12:i:- was detected in 10/16 herds (excluding Herd 11) and resulted in 76 fully serotyped *S.* 1,4,[5],12:i:- isolates sourced from 34 samples. Isolates with identical phage type, antimicrobial resistance phenotype and MLVA profile from the same sample were assumed to be duplicates and removed from the analysis; 41 *S.* 1,4,[5],12:i:- isolates remained and were included in the following results. Isolates of non-*S.* 1,4,[5],12:i:- serovars were also tested by antibiogram for comparative purposes—these serovars were either contemporary to *S.* 1,4,[5],12:i:- (11 herds) or were from herds where *Salmonella* was

detected but *S*. 1,4,[5],12:i:- was not identified (three herds); in total 67 isolates describing other serovars were tested.

The most common *S*. 1,4,[5],12:i:- resistance was to T,[5] 95.1% of isolates (39/41), followed by A 78.0% (32/41), S 53.7% (22/41) and Su 51.3% (20/39) (Table 8-2). Multidrug resistant strains were defined as strains that were phenotypically resistant to three or more classes of antimicrobial, in accordance with the widely accepted definition of Magiorakos et al. (2012) and Parry and Threlfall (2008). Multidrug resistant *S*. 1,4,[5],12:i:- colonies were isolated in all but one herd in which the serovar was found (9/10) (Table 8-3). Almost half of the of study *S*. 1,4,[5],12:i:- isolates were multidrug resistant, 48.8% (20/41). Resistance to ASSuT was the most common *S*. 1,4,[5],12:i:- resistance phenotype, 22.0% (9/41), identified among isolates from 5/10 herds. Resistance to ASSuT in combination with other resistances were identified in 43.9% (18/41) of isolates in 9/10 herds. The majority of the other serovars tested were less resistant than the *S*. 1,4,[5],12:i:- isolates, ranging from full sensitivity to resistance to AT, with the exception of *S*. Ohio, *S*. Ohio var 14+ and *S*. Rissen from Herd 2, *S*. Bovismorbificans from Herd 15 and *S*. Typhimurium isolates from Herd 16, which were highly multidrug resistant (Table 8-3).

Table 8-2. Antimicrobial resistance phenotypes of *S.* 1,4,[5],12:i:- isolates from the cross-sectional study by herd.

Antimicrobial Agent	Resistance breakpoint (mg/L)[a]	Herd (number of isolates, n)												
		1	2	3	4	5	6	7	8	9	10	11[b]	No. herds	% Total[c]
		(2)	(1)	(3)	(10)	(3)	(3)	(10)	(5)	(2)	(2)	(11)	(10)	(41)
Ampicillin	≥ 16	2	1	3	2	3	3	10	4	2	2	11	10	78.0
Cefotaxime	≥ 1													-
Chloramphenicol	≥ 16	2											1	4.9
Ciprofloxacin	≥ 2													-
Gentamicin	≥ 8													-
Kanamycin	≥ 32			3			2						2	12.2
Nalidixic acid	≥ 16													-
Spectinomycin	≥ 50			3			1						2	9.8
Streptomycin	≥ 32	2	1	3	2	3	3	2	4		2	11	9	53.7
Sulphathiazole	≥ 512	2	1	3	2	3	1	2	4		2	11	9	48.8
Tetracycline	≥ 8	2	1	3	10	3	3	10	3	2	2	11	10	95.1
Trimethoprim	≥ 8			3	5		1	1					4	24.4

[a] Breakpoints in accordance with CLSI (2011) and Commons et al. (2012).
[b] Herd 11 excluded from totals, isolates derived from the Hamilton et al. (2015) study.
[c] Number of resistant isolates/total isolates presented as a percentage.

[5] The abbreviations used throughout this thesis to report antimicrobial resistance phenotypes are those most frequently used in the published literature to describe *S*. 1,4,[5],12:i:- antimicrobial resistance phenotypes (Switt et al., 2009; Lucarelli et al., 2011; García et al., 2016) (Table 2-3).

Table 8-3. Serovars and antimicrobial resistance phenotypes identified among isolates from the cross-sectional study. Presented: date of sampling, number of samples collected and number of samples in which *Salmonella* spp. was detected, the serovars identified and number of isolates of each, and antimicrobial resistance phenotype.

Herd	Sampling date (D/M/Y)	*Salmonella* positive samples	Serovars identified	No. of isolates	Antimicrobial resistance phenotypes (No. of isolates)
1	11/07/13	2/15	*S.* 1,4,[5],12:i:-	2	ACSSuT (2)
2	10/01/14	13/20	*S.* 1,4,[5],12:i:-	1	ASSuT (1)
			S. Hofit	1	Sensitive (1)
			S. Ohio	8	AGSSpSuTTm (8)
			S. Ohio var 14+	1	AGSSpSuTTm (1)
			S. Rissen	8	ASpSuTm (6), AGSSpSuTTm (1)
			S. Worthington	1	Sensitive (3)
3	12/03/14	6/20	*S.* 1,4,[5],12:i:-	3	AKSSpSuTTm (3)
			S. Agona	1	Sensitive (1)
			S. Oranienburg	2	Sensitive (2)
4	10/01/14	13/14	*S.* 1,4,[5],12:i:-	10	T (5), TTm (3), ASSuTTm (2), AT (1)
			S. Adelaide	2	Sensitive (2)
			S. Bovismorbificans	2	Sensitive (2)
			S. Derby	1	T (1)
			S. London	1	Sensitive (1)
5	08/08/13	3/13	*S.* 1,4,[5],12:i:-	3	ASSuT (3)
6	20/06/13	4/18	*S.* 1,4,[5],12:i:-	3	AKSSpSuTTm (1), AKST (1), AST (1)
			S. Infantis	1	A (1)
7	01/12/12	13/18	*S.* 1,4,[5],12:i:-	10	AT (9), ASSuTTm (1), ASSuT (1)
			S. Adelaide	7	Sensitive (7)
8	20/11/13	5/18	*S.* 1,4,[5],12:i:-	5	ASSuT (2), ASSu (2), T (1)
9	27/03/13	3/8[b]	*S.* 1,4,[5],12:i:-	2	AT (2)
			S. Infantis	1	Sensitive (1)
10	02/05/13	4/11	*S.* 1,4,[5],12:i:-	2	ASSuT (2)
			S. Infantis	5	Sensitive (5)
			S. Muenchen	1	Sensitive (1)
11[a]	06/02/11	7/18	*S.* 1,4,[5],12:i:-	11	ASSuT (11)
12	30/10/13	0/20	-	-	-
13	30/10/13	0/20	-	-	-
14	19/05/13	0/20	-	-	-
15	27/05/13	7/14	*S.* Bovismorbificans	2	ACCfGSSpSuTTm (1), ACSSpSuTTm (1)
			S. Derby	4	Sensitive (4)
			S. London	2	AT (2)
16	24/11/13	9/18	*S.* Typhimurium	10	ASSpSuTTm (6), AGSSpSuTTm (3), ACSSpSuTTm (1)
17	27/08/13	5/14	*S.* Derby	3	AT (3)
			S. London	3	Sensitive (3)

[a] Herd 11 isolates from an earlier study reported in Hamilton et al. (2015).

8.3.2. Antimicrobial resistance phenotypes among isolates from the herds sampled longitudinally

In total 188 isolates from herds sampled longitudinally were tested for antimicrobial sensitivity; 98 of the isolates tested were serovar *S*. 1,4,[5],12:i:-, 90 were other *Salmonella* serovars (Table 8.4). *Salmonella* spp. resistances ranged from fully sensitive to resistance to nine antimicrobials in 14 combinations. *Salmonella* spp. resistance phenotypes were herd associated and persisted over the 12-24-month sampling period with the exception of Herd 5 (Figure 8-2). The Group B serovars were the most resistant serovars isolated from herds sampled longitudinally, with the exception of Herd 2. Highly resistant *S*. Ohio and *S*. Rissen strains were isolated from Herd 2, exhibiting resistance to seven and five antimicrobials, respectively.

The *S*. 1,4,[5],12:i:- isolates ranged from T resistance, only, to resistance to up to eight antimicrobials in seven combinations. The majority, 69.4% (68/98), of *S*. 1,4,[5],12:i:- isolates were multidrug resistant. Resistance to ASSuT was the most widespread *S*. 1,4,[5],12:i:- resistance type, observed among isolates from 3/5 herds and 31.6% (31/98) of individual isolates. In total 65.3% (64/98) of the *S*. 1,4,[5],12:i:- isolates expressed resistance to the ASSuT group in combination with resistance to other antimicrobial agents. The other antimicrobials to which *S*. 1,4,[5],12:i:- isolates were phenotypically resistant were C, K, Sp and Tm.

Table 8-4. Longitudinal study *Salmonella* antimicrobial resistance phenotypes by serovar and sampling occasion. Listed in order of most resistant to least resistant.

Herd	Sampling occasion			
	A	**B**	**C**	**D**
1	*S.* 1,4,[5],12:i:- ACSSuT(3) ASSuT(2) *S.* Worthington A (1)	*S.* 1,4,[5],12:i:- ACSSuT (3) ASSuT (2) *S.* Adelaide AT (2) *S.* Bovismorbificans Sensitive (1) *S.* Mbandaka Sensitive (1) *S.* Worthington A (1) SI rough:z10:e,n,x Sensitive (1)	*S.* 1,4,[5],12:i:- ASTCSu (5) ASSuT (1) *S.* Adelaide AT (2) *S.* Worthington A (3) Sensitive (5)	*S.* 1,4,[5],12:i:- ACSSuT (3) ASSuT (2) *S.* Adelaide AT (1) *S.* Give A (1) *S.* Worthington Sensitive (1)
2	*S.* 1,4,[5],12:i:- ASSuT (1) *S.* Hofit Sensitive (1) *S.* Ohio AGSSuSpTTm (8) *S.* Rissen AGSSpSuTTm (1) ASSpSuTTm (7) *S.* Worthington Sensitive (1)	*S.* 1,4,[5],12:i:- ASSuT (1) *S.* Mbandaka Sensitive (1) *S.* Ohio AGSSpSuTTm (1) *S.* Rissen ASSpSuTTm (7) ATTm (1)	*S.* 1,4,[5],12:i:- ASSuT (4) *S.* Bovismorbificans AT (2) Sensitive (1) *S.* Rissen ASSpSuTTm (4) ASpTTm (1)	*S.* 1,4,[5],12:i:- ASSuT (5) *S.* Rissen ASSpSuTTm (2)
3	*S.* 1,4,[5],12:i:- AKSSpSuTTm (3) *S.* Agona Sensitive (1) S. Oranienburg Sensitive (2)	*S.* 1,4,[5],12:i:- ACKSSpSuTTm (8) *S.* Mbandaka Sensitive (1)	*S.* 1,4,[5],12:i:- AKSSpSuTTm (7)	*S.* 1,4,[5],12:i:- ACKSSpSuTTm (4) ACKSpSuTTm (1)
4	*S.* 1,4,[5],12:i:- T (10) *S.* London Sensitive (3)	*S.* 1,4,[5],12:i:- T (7) *S.* Adelaide Sensitive (6) *S.* Bovismorbificans Sensitive (1)	*S.* 1,4,[5],12:i:- T (6) S. Adelaide Sensitive (1) *S.* Bovismorbificans Sensitive (1) *S.* London Sensitive (1)	*S.* 1,4,[5],12:i:- T (7) *S.* Adelaide Sensitive (1) *S.* London Sensitive (5)
5	*S.* Havana Sensitive (1)	*S.* 1,4,[5],12:i:- ASSuT (7) *S.* Anatum Sensitive (1) *S.* Anatum var 15+ A (1)	*S.* 1,4,[5],12:i:- ASSuT (6) *S.* Ohio Sensitive (1)	

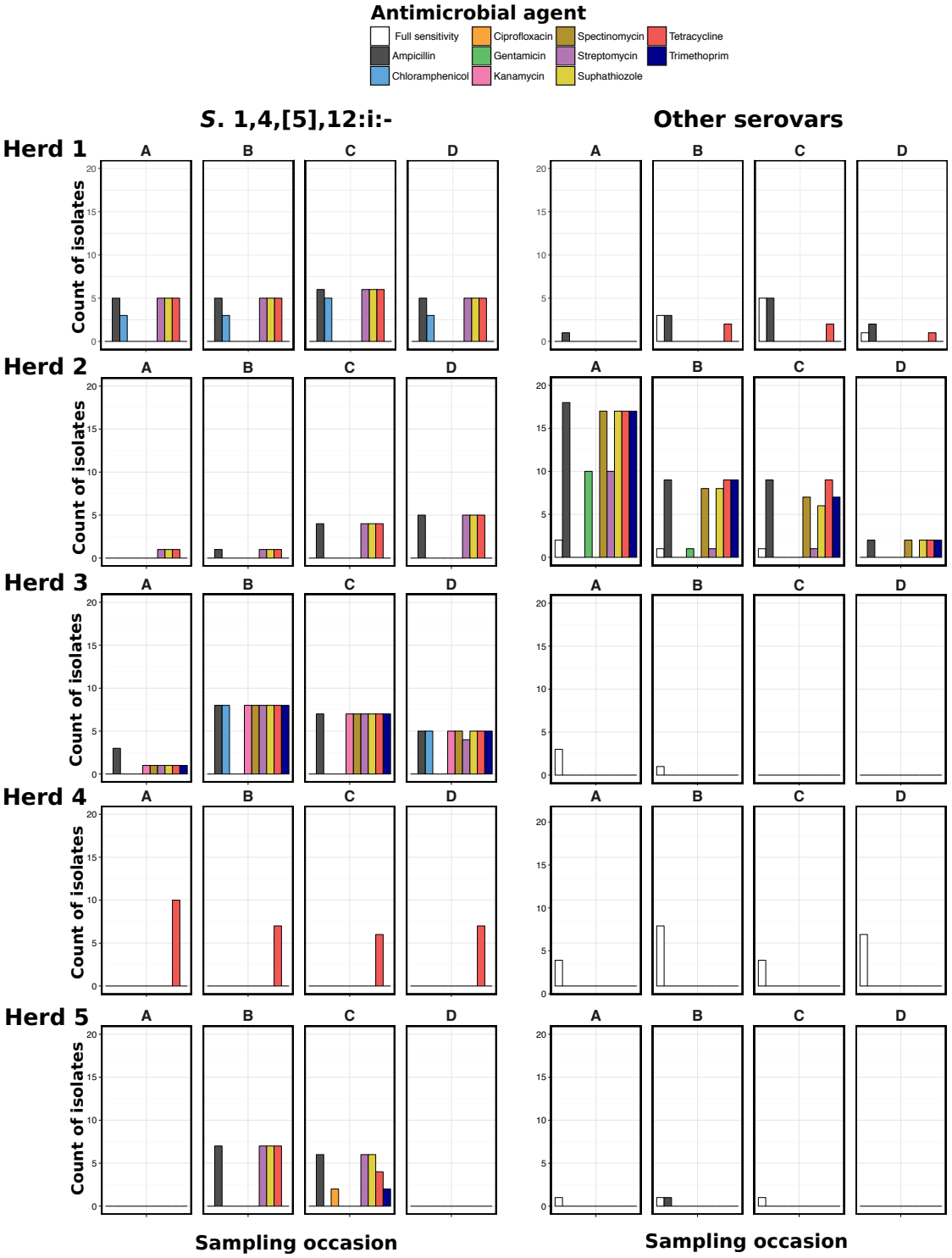

Figure 8-2. *S.* 1,4,[5],12:i:-and other *Salmonella* serovar antimicrobial resistance phenotypes by sampling occasion. *Salmonella* 1,4,[5],12:i:- (left) and other serovar (right) antimicrobial resistance phenotypes by herd (row) and sampling occasion (column). The bar fill identifies specific compounds to which isolates were resistant. For the purposes of this figure, Cp resistance equates to intermediate resistance ($\geq$ 0.06 mg/L and $\leq$ 2 mg/L), in accordance with Commons et al. (2012).

Herd 1. *Salmonella* 1,4,[5],12:i:- isolates were resistant to ASSuT, with additional resistance to C found in 68.2% of isolates tested. Resistance types identified were consistent over the 12-month period of sampling. *Salmonella* Worthington isolates were fully sensitive with a single exception, an isolate expressing A resistance, only. A single AT resistant *S*. Adelaide isolate was the only other resistant strain isolated from Herd 1.

Herd 2. All *S*. 1,4,[5],12:i:- isolates were resistant to the typical ASSuT quartet. The other serovars isolated from Herd 2 samples were highly resistant. *Salmonella* Ohio, isolated on the first and second sampling occasions, described the extensive resistance type AGSSpSuTTm. *Salmonella* Rissen isolates ranged from ATTm resistance to a single AGSSpSuTTm resistant isolate from the first sampling occasion. The majority (86.3%) of *S*. Rissen isolates were resistant to ASSpSuTTm. The only other resistant isolate was an AT resistant *S*. Bovismorbificans isolate.

Herd 3. Among Herd 3 isolates, *S*. 1,4,[5],12:i:- strains were highly resistant while other serovars identified were fully sensitive. All *S*. 1,4,[5],12:i:- isolates were resistant to AKSpSuTTm, in addition 95.7% were resistant to S and 56.5% to C.

Herd 4. Herd 4 grow-out pigs were sampled longitudinally as part of the initial case study (Chapter 5) and the whole herd was then sampled in the longitudinal study of five herds (Chapter 6). The only multidrug resistant isolates were *S*. 1,4,[5],12:i:- isolates; 8/41 *S*. 1,4,[5],12:i:- isolates ranging from resistance type ATTm to type ASSuTTm from samples collected in the initial case study of grow-out pigs (Table 8-5, Figure 8-3). All the Herd 4 *S*. 1,4,[5],12:i:- isolates were resistant to T. During the whole herd study resistance to T was the only resistance identified among *S*. 1,4,[5],12:i:- isolates. The other serovars identified were fully sensitive with the exceptions of the single *S*. Derby isolate that was T resistant and an (1/15) A resistant *S*. London isolate.

Table 8-5. Phenotype and antimicrobial resistance phenotypes of the *Salmonellae* isolated during the Herd 4 case study of grow-out pigs. Listed in order of most resistant to least resistant.

	Sampling occasion			
	1	**2**	**3**	**4**
Serovars and antimicrobial resistance types (n)	*S.* 1,4,[5],12:i:- ASSuTTm(2) TTm(3) T(5) *S.* Adelaide Sensitive (2) *S.* Bovismorbificans Sensitive (2) *S.* Derby T (1) *S.* London Sensitive (1)	*S.* 1,4,[5],12:i:- ATTm (4) AT (4) T (6) *S.* Adelaide Sensitive (1) *S.* London A (1) Sensitive (3)	*S.* 1,4,[5],12:i:- ATTm (2) AT (1) T (4) *S.* Adelaide Sensitive (1) *S.* Bovismorbificans Sensitive (2) *S.* London Sensitive (1)	*S.* 1,4,[5],12:i:- AT (1) T (9)

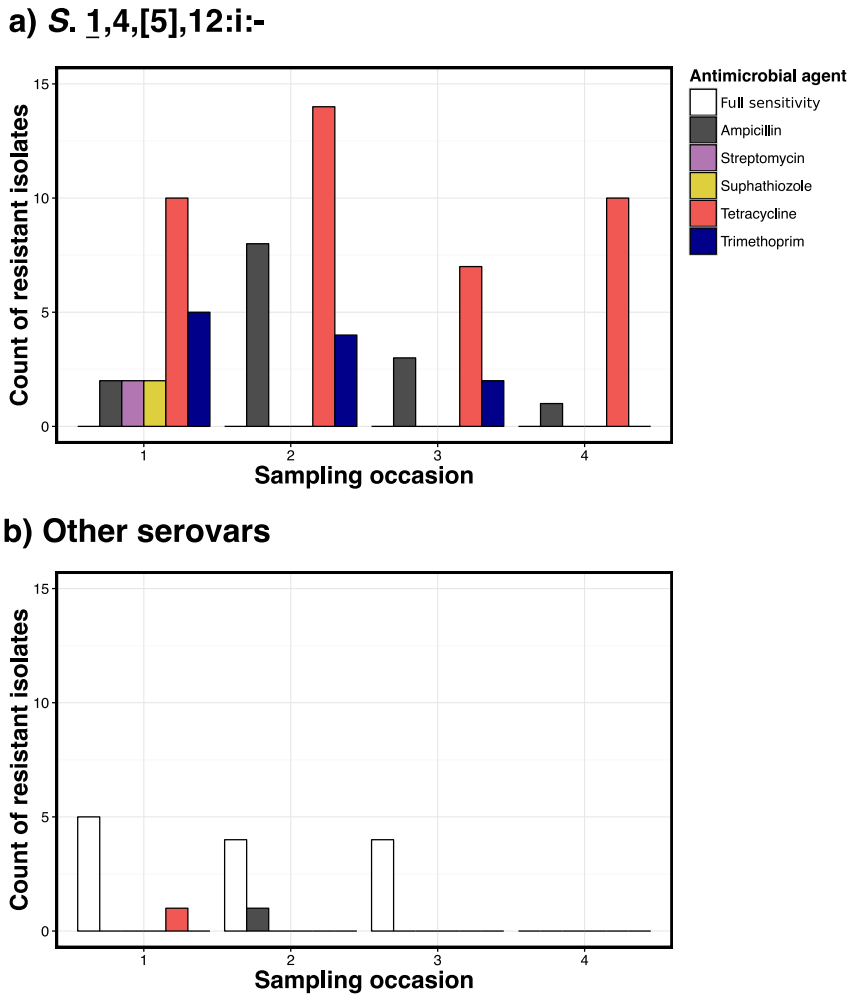

Figure 8-3. Herd 4 grow-out pig case study *S.* 1,4,[5],12:i:- and other serovar antimicrobial resistance phenotypes over time. Figure a) *S.* 1,4,[5],12:i:- resistance types; b) non-*S.* 1,4,[5],12:i:- resistance types.

Herd 5. All Herd 5 *S.* 1,4,[5],12:i:- isolates tested were resistant to ASSuT. The other serovars identified were fully sensitive, with the exception of a single A resistant *S.* Anatum var 15+ isolate.

8.3.3. Antimicrobial resistance phenotypes among all study isolates tested

In total 381 *Salmonella* isolates were tested for antimicrobial sensitivity. *Salmonella* spp. resistances identified ranged from full sensitivity to resistance to nine antimicrobials in 23 combinations (Table 8-6). The most resistant isolate in the collection was an *S*. Bovismorbificans isolate from herd 15 that exhibited resistance to nine compounds. This was also the only isolate that was resistant to cefotaxime (Cf) in the study collection, a 3rd generation cephalosporin of importance in the treatment of human salmonellosis. The *S*. 1,4,[5],12:i:- isolates exhibited up to eight resistances and 14 antimicrobial resistance types were identified. The most widespread and frequently identified *S*. 1,4,[5],12:i:- resistance type was ASSuT (Tables 8-6 and 8-7). The *S*. 1,4,[5],12:i:- isolates were almost all resistant to T (99.1%) (Table 8-7). None of the *S*. 1,4,[5],12:i:- isolates were pansusceptible. The *S*. Typhimurium isolates were all multidrug resistant; to five to seven antimicrobials. The *S*. Adelaide, *S*. Agona, *S*. Hofit, *S*. Muenchen, *S*. Oranienburg, *S*. Worthington isolates were exclusively pansusceptible. The *S*. London resistances ranged from fully sensitive to resistance to AT, *S*. Bovismorbificans from sensitive to resistance to ACCfGSSuSpTTm, and *S*. Derby from sensitive to resistance to AT. Among the non- *S*. 1,4,[5],12:i:- serovars, 57.7% (94/163 isolates) were pansusceptible; pansusceptible strains were detected in 11 herds (Table 8-7).

Table 8-6. *Salmonella* serovars antimicrobial resistance phenotypes.

Serovar	Resistance type	No. herds (isolates)
S. 1,4,[5],12:i:-	ASSuT	7 (83)
S. 1,4,[5],12:i:-	AT	3 (21)
S. 1,4,[5],12:i:-	T	2 (54)
S. 1,4,[5],12:i:-	AKSSpSuTTm	2 (12)
S. 1,4,[5],12:i:-	ASSuTTm	2 (3)
S. 1,4,[5],12:i:-	ACSSuT	1 (15)
S. 1,4,[5],12:i:-	ACKSSpSuTTm	1 (12)
S. 1,4,[5],12:i:-	ATTm	1 (6)
S. 1,4,[5],12:i:-	ACSSpSuTTm	1 (4)
S. 1,4,[5],12:i:-	TTm	1 (3)
S. 1,4,[5],12:i:-	ASSu	1 (2)
S. 1,4,[5],12:i:-	AST	1 (1)
S. 1,4,[5],12:i:-	AKST	1 (1)
S. 1,4,[5],12:i:-	ACKSpSuTTm	1 (1)
S. Typhimurium	ASSpSuTTm	1 (6)
S. Typhimurium	AGSSpSuTTm	1 (3)
S. Typhimurium	ACSSuT	1 (1)
S. Typhimurium	ACSSpSuTm	1 (1)
S. Adelaide	Sensitive	2 (31)
S. Adelaide	AT	1 (5)
S. Agona	Sensitive	1 (1)
S. Anatum	Sensitive	1 (1)
S. Anatum var 15+	A	1 (1)
S. Bovismorbificans	Sensitive	3 (8)
S. Bovismorbificans	AT	1 (2)
S. Bovismorbificans	A	1 (1)
S. Bovismorbificans	ACSSpSuTTm	1 (1)
S. Bovismorbificans	ASCCfGSpSuTTm	1 (1)
S. Derby	AT	2 (7)
S. Derby	T	1 (1)
S. Give	A	1 (1)
S. Havana	Sensitive	1 (1)
S. Hofit	Sensitive	1 (1)
S. Infantis	Sensitive	4 (15)
S. Infantis	A	1 (1)
S. London	Sensitive	3 (19)
S. London	A	1 (1)
S. Mbandaka	Sensitive	3 (3)
S. Muenchen	Sensitive	1 (1)
S. Ohio	Sensitive	1 (1)
S. Ohio (incl. var 14+)	AGSSpSuTTm	1 (7)
S. Oranienburg	Sensitive	1 (2)
S. Rissen	Sensitive	1 (1)
S. Rissen	ASuSpTTm	1 (19)
S. Rissen	AGSSpSuTTm	1 (1)
S. Rissen	ATTm	1 (1)
S. Rissen	ASpTTm	1 (1)
S. Worthington	Sensitive	2 (7)
S. Worthington	A	1 (5)

Table 8-7. Proportion of *Salmonella* spp. isolates sourced from primary production resistant to specified antimicrobial agent.

Antimicrobial agent	Resistance breakpoint[a] (mg/L)	No. herds *Salmonella* resistant (% isolates; no. isolates) n = 381	No. herds *S.* 1,4,[5],12:i:- resistant (% isolates; no. isolates) n = 218	No. herds other serovars resistant (% isolates; no. isolates) n = 163
Full sensitivity	-	11 (24.7; 94)	0 (0; 0)	11 (57.7; 94)
Ampicillin	≥ 16	14 (60.1; 229)	11 (73.9; 161)	8 (41.7; 68)
Cefotaxime	≥ 1	1 (0.3; 1)	0 (0; 0)	1 (0.6; 1)
Chloramphenicol	≥ 16	5 (9.4; 36)	3 (14.7; 32)	3 (2.5; 4)
Ciprofloxacin	≥ 2	0 (0; 0)	0 (0; 0)	0 (0; 0)
Gentamicin	≥ 8	3 (3.9; 14)	0 (0; 0)	3 (8.6; 14)
Kanamycin	≥ 32	2 (6.6; 25)	2 (11.5; 25)	0 (0; 0)
Nalidixic Acid	≥ 16	0 (0; 0)	0 (0; 0)	0 (0; 0)
Spectinomycin	≥ 50	5 (18.6; 71)	2 (13.3; 29)	3 (25.8; 42)
Streptomycin	≥ 32	12 (40.9; 156)	10 (61.0; 133)	4 (14.1; 23)
Sulphathiazole	≥ 512	12 (45.7; 174)	10 (60.6; 132)	4 (25.8; 42)
Tetracycline	≥ 8	14 (72.2; 275)	11 (99.1; 216)	6 (36.2; 59)
Trimethoprim	≥ 8	7 (22.0; 84)	4 (18.9; 41)	3 (26.4; 43)

[a] Breakpoints in accordance with Commons et al. (2012) and CLSI (2011).

8.3.4. Antimicrobial resistance genes among the *Salmonella* Group B isolates sequenced

The SRST2 program was used to parse the sequence read data from the selected isolates, described in the previous chapter, against the ARG-ANNOT resistance gene database with a minimum match set at 90% (Gupta et al., 2014a; Inouye et al., 2014). The lowest coverage of a resistance gene meeting the criteria was 99.2%, demonstrating very high matches for the resistance genes identified.

The aminoglycoside acetyltransferase gene *aac6*-Iaa (NC_003197), present in all *Salmonella* spp., is presented for completeness, however, the gene is 'cryptic', or silent, and does not confer resistance to aminoglycosides in *Salmonella* (Salipante et al., 2003; Salipante and Hall, 2003). For clarity, *tet*R, which is associated with regulation of numerous tetracycline resistance genes, is not included in the following figures and discussion (identified in Supplementary Table 8-8 for reference).

Discrepancies between the observed resistance phenotype and genes identified in the sequences using SRST2 were identified and were removed from the analysis and discussion. The reasons for the differences could relate to loss of genes in storage and/or the cutoffs used in the antibiogram. Genotype instability and/or selection for gene loss while in the laboratory is perhaps the most likely conclusion, given some isolates spent upwards of 12-months on a storage medium between the time of phenotyping and sequencing. Supporting this assertion, in a study *E. coli* mutations in

storage Snyder et al. (2012) found the alternative sigma factor *rpoS*, a gene also present in *Salmonella* that is associated with virulence and controls a complex regulon with involvement in stress responses, were likely to occur under the stress of low nutrient storage media.

Resistance genes associated with the main antimicrobial classes used in the treatment of human salmonellosis, fluoroquinolones and cephalosporins, were not identified among the sequenced isolates. However, it should be noted that the Cf resistant *S*. Bovismorbificans was not sequenced.

The only isolates with the *aac(3)-IVa* gene (X01385), conferring resistance to G and tobramycin, were three *S*. Typhimurium isolates one of which was phenotyped as G resistant (Figure 8-4). One *S*. Typhimurium isolate (TW-STm57) had full coverage of *ermA* (X03216) that confers resistance to macrolides, a phenotype not tested for.

The most commonly identified resistance gene among the *S*. 1,4,[5],12:i:- isolates sequenced was *tetB* (AB089594), which was present in all except two isolates and found in isolates from all herds (Figure 8-4). None of the four *S*. Typhimurium isolates had *tetB*, however, three carried *tetA* (JX424423) (Supplementary Table 8-8). The genes $bla_{\text{TEM-ID}}$ (AB700703), *sul2* (EU360945), *strA* (AB366441) and *strB* (FJ474091) were the only other genes identified in at least one isolate from each herd, and were also present in the three S resistant *S*. Typhimurium isolates.

Herd 1 *S*. 1,4,[5],12:i:- isolates possessed the typical genes associated with the ASSuT resistance type. However, they were also predominantly C resistant which was reflected in identification of the *floR* gene (AKLJ01000508) in the sequence data for these strains. This gene encodes co-resistance to florfenicol and chloramphenicol.

Herd 3 *S*. 1,4,[5],12:i:- isolates were the most resistant of this serovar in the study collection, each were resistant to seven or eight antimicrobials. Among the sequenced isolates, Herd 3 strains were the only strains in which K resistance and the only *S*. 1,4,[5],12:i:- isolates resistant to Sp. These resistances were encoded by *aphA2* (X57709) and *aadA1* (JQ690540), respectively, genes that were only identified among isolates from this herd. Herd 3 isolates were also the only *S*. 1,4,[5],12:i:- strains in which *aph(3)-Ia* (V00359), *dfrA* (Z21672) and *sul3* (HQ875016) were present, and the only *S*. 1,4,[5],12:i:- isolates in which *cmlA1* and *sul1* (AF071413) were present. Among the Herd 3 isolates sequenced, 11/12 had *tetA* in addition to *tetB*. The *dfrA* gene was present among Herd 3 isolates, conferring resistance to Tm. This Tm resistance gene differed from isolates from Herd 4 *S*. 1,4,[5],12:i:- isolates and the Tm resistant *S*. Typhimurium isolates, in which genes *dfrA5* (X12868) and *dfrA10* (L06418), and *dfrA5*, were identified, respectively.

Herd 4 *Salmonella* isolates harboured considerably less resistant *S*. 1,4,[5],12:i:- strains than the other herds in the study, which was reflected in the resistance genes identified. All Herd 4 isolates were T resistant and the *tetB* gene was identified in all but one strain, an isolate in which no other genes

known to confer T resistance were identified. Multidrug resistance among Herd 4 isolates was only identified in isolates from samples collected before February 2014. The most resistant Herd 4 strain, with the phenotype ASSuTTm, was also the oldest Herd 4 strain sequenced, sourced from a sample collected in 2013. This isolate and one other isolate with the resistance phenotype TTm had the typical resistance genes present in ASSuT resistant *S*. 1,4,[5],12:i:- strains reported elsewhere: *bla*$_{\text{TEM-1D}}$, *strA-strB*, *sul2* and *tetB*. In addition, the gene *dfrA5* was present in these two isolates, which were also the only Herd 4 isolates in which *tetA*, in addition to *tetB,* was present.

The single Herd 6 isolate sequenced (TW-STm44) was the only isolate with *aph(3)*-Ia, and the only *S*. 1,4,[5],12:i:- in which *sul1* was present. The remaining isolates from Herds 5 and 11 and Abattoir 2 were resistant to the typical ASSuT combination, reflected in the presence of *strA*, *strB*, *sul2*, *bla*$_{\text{TEM-1D}}$ and *tetB*.

No resistance genes were identified in the only pansusceptible isolate sequenced, an *S*. Typhimurium isolate. The three other *S*. Typhimurium isolates were multidrug resistant, which was reflected in their resistomes in which eight to 15 resistance genes were identified. The three multidrug resistant *S*. Typhimurium isolates each contained resistance gene repertoires that included a number of genes identified among *S*. 1,4,[5],12:i:- isolates. However, five resistance genes not found among the sequenced *S*. 1,4,[5],12:i:- isolates were also identified in these isolates: *aac(3)-IVa, aph(4)-Ia* (V01499)*, dfrA10, ermA* and *bla* $_{\text{OXA-7}}$ (HQ386834). Among the *S*. Typhimurium isolates from Herd 16 the *ermA* gene, which may confer macrolide-lincosamide-streptogramin B resistance, was identified in the C resistant *S*. Typhimurium isolate but not in the other *S*. Typhimurium isolate. Resistance to macrolide-lincosamide-streptogramin B was not tested for in the antibiogram. Although both Herd 16 *S*. Typhimurium isolates were resistant to S, and both harboured genes conferring resistance to aminoglycosides (*aac(3)-IVa*, *aadA1* and *aph(4)-Ia*), only one was phenotypically resistant to G.

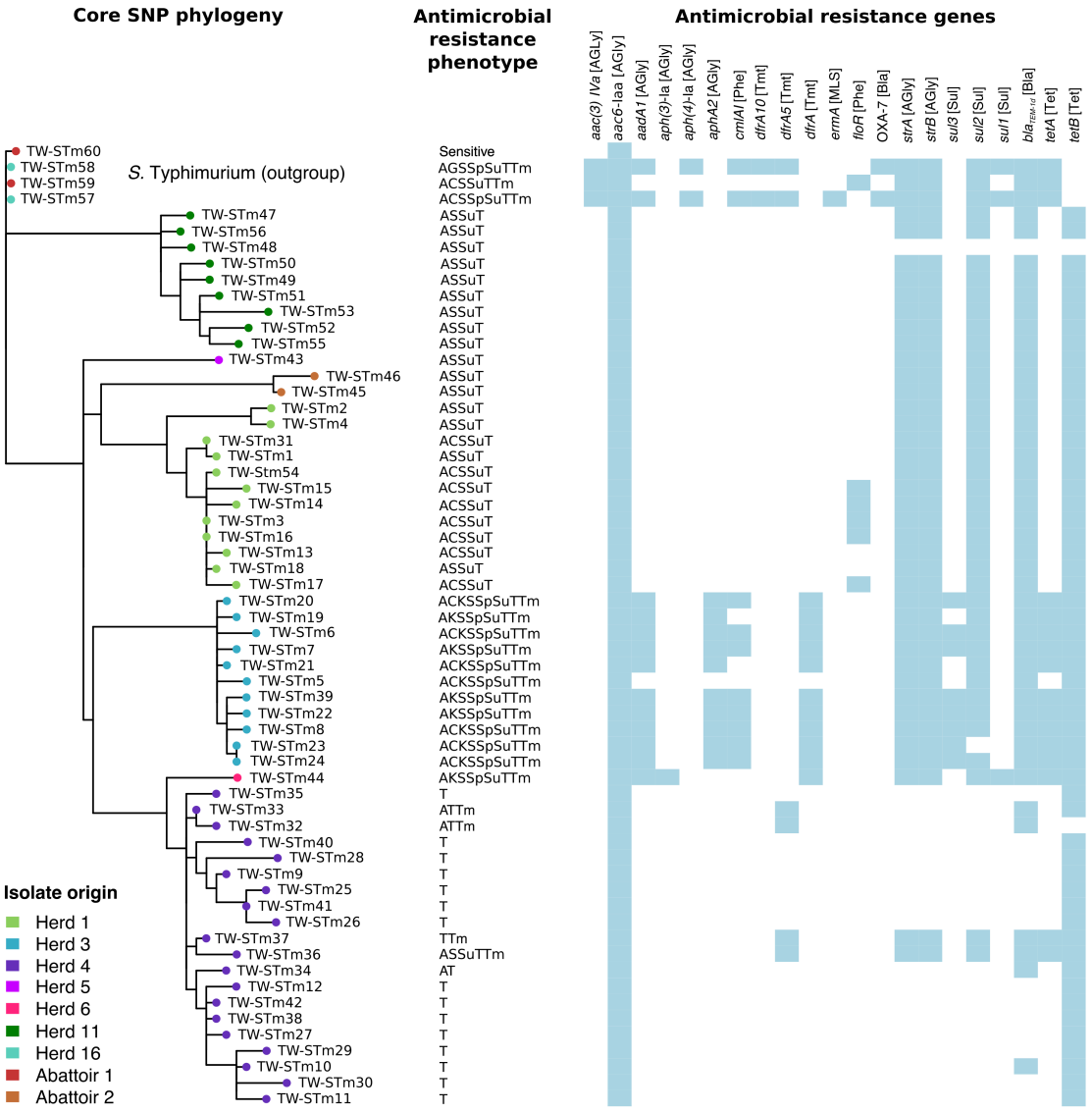

Figure 8-4. Phylogenetic tree, antimicrobial resistance phenotypes and resistance genes identified in the sequenced isolates. Maximum likelihood phylogenetic tree with tips coloured by isolate origin (as described in Figure 7-2) aligned with the isolate phenotype and heatmap showing presence/absence of resistance genes.

As described previously, samples were collected from Herd 4 at two-three month intervals for over 18-months. The genomes of 20 representative Herd 4 strains were sequenced. Herd 4 suffered a widespread outbreak of acute enteritis among grow-out pigs prior to the study, during which *S.* 1,4,[5],12:i:- was confirmed. The clinical problems had been effectively controlled by the time of sampling, which the management attributed to the addition of dietary organic acids. Despite control of clinical disease, *S.* 1,4,[5],12:i:- continued to be isolated. In Chapters 5 and 6 escalation of *S.* 1,4,[5],12:i:- shedding among Herd 4 weaners and considerable shedding among finishers was shown; using a Bayesian approach the mean estimated true shedding prevalence over the last four sampling occasions in weaners was 31.5% (95%CI 21.4-61.1%) and finishers, 14.8% (95%CI 8.8-26.3%).

Five *S.* 1,4,[5],12:i:- MLVA profiles were found in Herd 4, all of which were single locus variants with a single copy number change at the unstable STTR5 or STTR6 loci (Figure 8-5). The closest relative of the sequenced Herd 4 isolates was from Herd 6 (TW-STm44) had MLVA profile D; an MLVA profile that was also identified among Herd 4 strains.

The Herd 4 and Herd 6 strains were very closely related, ≤10 SNPs MRCA; there were ≤ 7 SNPs to an MRCA between TW-STm44 and the nearest Herd 4 strain (TW-STm33), 10 SNPs pairwise (Figure 8-5). The chromosomal STM2759-*iroB* region (site of the *fljAB* deletion) of the Herd 4 and Herd 6 isolates were mapped (Figure 8-6). The *bla-strAB-sul2-tet* locus was present in the STM2759-*iroB* region of the Herd 6 strain (TW-STm44) and the two oldest strains from Herd 4 (TW-STm36 and TW-STm37). However, a deletion of the bla_{TEM}-*strAB-sul2* genes in the STM2759-*iroB* region had occurred in the other strains; the TW-STm10 and TW-STm34 *bla* gene was in a different location, indicating acquisition and the location of the *bla* gene in strains TW-STm32 and TW-STm33 was uncertain, as the genes were flanked by repeating regions identical to the repeating regions found in the ASSuT locus (Figure 8-6). The *tetB* was located outside the lost region and retained by the majority of strains despite no direct selective pressure for tetracycline resistance in the herd, with the exception of TW-STm32 that had an even larger deletion in the region, which also included loss of the *tetB* in this location.

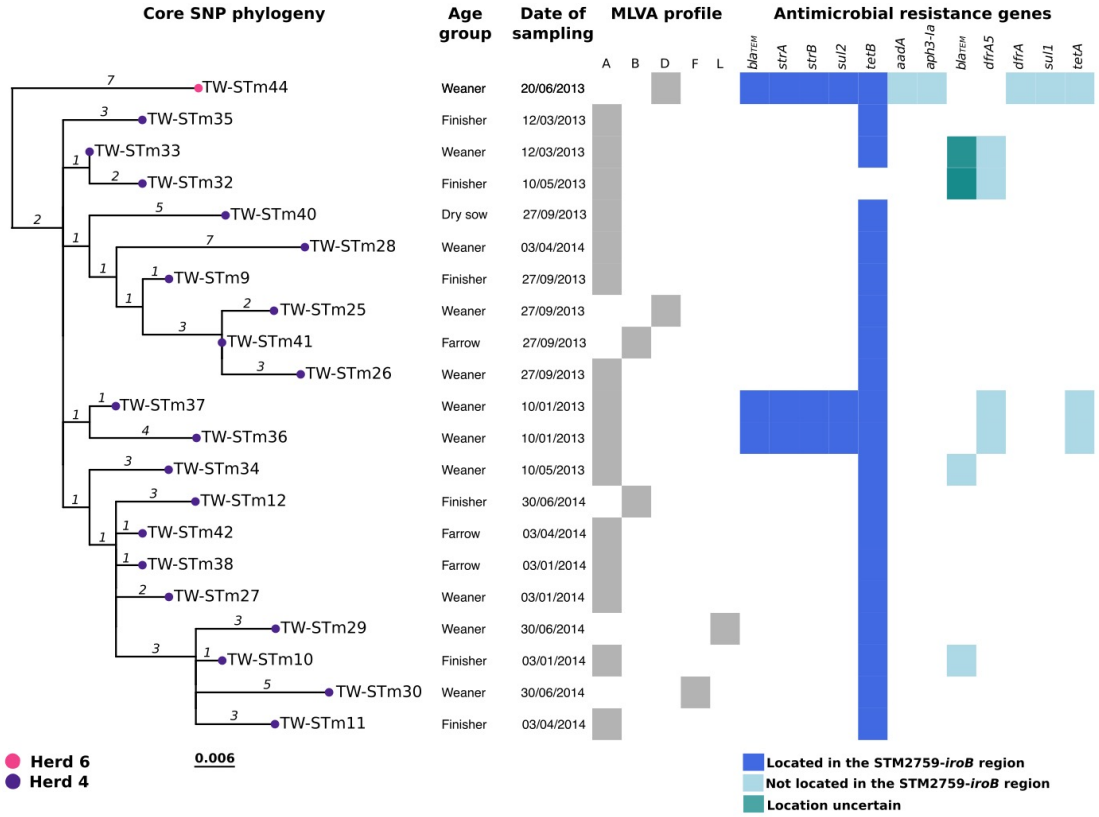

Figure 8-5. Maximum likelihood phylogeny annotated with sample origin, age group, sampling date, MLVA profile, antimicrobial resistance genes. Number of SNPs per branch indicated in italics. MLVA profiles: A=04-15-11-00-490, B=04-15-12-00-490, D=04-14-11-00-490, F=04-16-10-00-490, L=04-15-10-00-490; grey block in the heatmap identifies the strain MLVA profile.

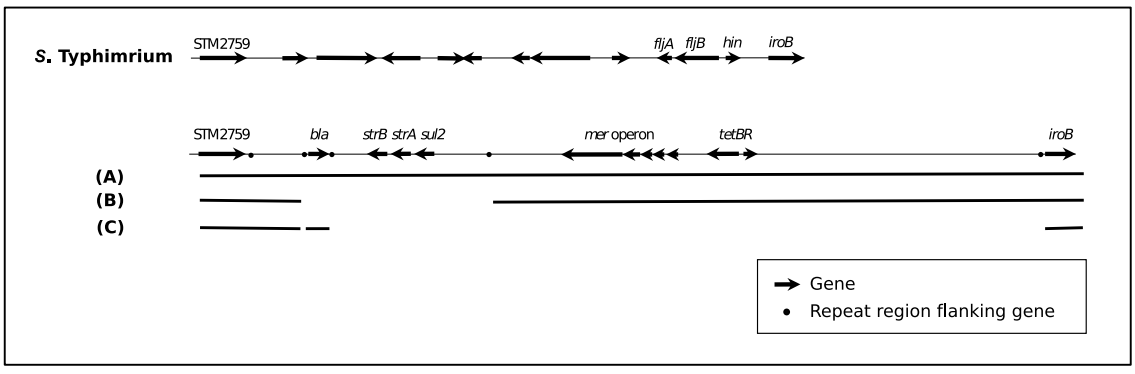

Figure 8-6. Comparison of the *bla-strAB-sul2-tet/fljAB* (STM2759…*iroB*) region in wild type *S.* Typhimurium, the typical European clone *S.* 1,4,[5],12:i:- and Herd 4 and 6 study strains. Strain TW_STm44 (ASSuTTmKSp) and the oldest Herd 4 strains, TW-STm37 and TW-STm36, had the *bla-strAB-sul2-tet/fljAB* (A). Whereas the *bla-strAB-sul2* region was absent in the remaining Herd 4 strains (B). The strain TW-STm32 was missing a larger region which included the *tetB* gene (C), explaining the lack of T resistance described by this isolate.

8.4. Discussion

There was no indication of increasing antimicrobial resistance among the *Salmonella* strains isolated in these studies. Diminishing resistance was observed among serovars in some herds, notably among the Herd 4 *S*. 1,4,[5],12:i:- isolates. Resistance to the key drugs used to treat human salmonellosis—Cp (fluoroquinolones), ceftiofur and cefotaxime (cephalosporins)—was not identified among the study isolates, with the exception of a Cf resistant *S*. Bovismorbificans. These isolates were not sequenced, however, fluoroquinolone resistance is usually a result of alterations in the target enzymes (typically DNA gyrase in Gram-negative bacteria) and Cf resistance in the *S*. Bovismorbificans isolate would likely be encoded by the $bla_{\text{CMY-2}}$ gene (Giles et al., 2004; Jacoby, 2005). Only 3.9% of *Salmonella* isolates were resistant to G, none of which were *S*. 1,4,[5],12:i:- isolates, despite resistance to G having been reported amongst *S*. 1,4,[5],12:i:- isolates in Europe (Echeita et al., 1999; Mossong et al., 2007; EFSA, 2010b; Hopkins et al., 2010). Australia is the only country never to have allowed the use of fluoroquinolones and G in food animal production and only one third generation cephalosporin, ceftiofur, is registered for use in cattle only, although 'off-label' use of ceftiofur in other animals is permitted (Abraham et al., 2014; APVMA, 2014). This may explain the very low levels of resistance to these compounds observed among the study isolates. These findings mirror other Australian reports, which have found little or no resistance to these compounds among *Enterobacteriaceae* (Barton et al., 2003; Abraham et al., 2014; Smith et al., 2016).

Salmonella serovars isolated in the study could be readily distinguished on the basis of resistance phenotypes. This has been reported previously by Gebreyes et al. (2004) who found a strong correlation between *Salmonella* serovar and resistance type within pig herds. In the cross-sectional study multidrug resistant *S*. 1,4,[5],12:i:- strains were isolated from all but one herd in which the serovar was found, and across the whole study collection only 54/218 *S*. 1,4,[5],12:i:- isolates were T monoresistant, all but one of which (53/54) were from Herd 4. In contrast, the majority of the non-*S*. 1,4,[5],12:i:- serovars were pansusceptible or resistant to T and/or A only, with the exception of the other serovars isolated from Herd 2. The low levels of resistance observed among the non-*S*. 1,4,[5],12:i:- strains in the majority of herds indicates that the *S*. 1,4,[5],12:i:- resistance genes were not being transferred horizontally between different serovars, as expected given the ASSuT gene cluster is typically located on the chromosome (Petrovska et al., 2016)—discussed further below. The high proportion of multidrug resistant *S*. 1,4,[5],12:i:- isolates but low rates among other serovars in the study collection echoes reports in Australia which have found higher levels of resistance among Group B serovars (Barton, 2010; Abraham et al., 2014).

The resistance genes identified among the study *S*. 1,4,[5],12:i:- isolates have been reported elsewhere and have previously been identified among *Salmonella* spp. isolates from animal

production in Australia. Resistance to A, C, S and T has been reported in the Australian food animal *Salmonella* population for some time (Murray et al., 1986; Abraham et al., 2014). In a survey of antimicrobial resistance phenotypes of *Salmonella* from Australian food animals between 1975 and 1982 Murray et al. (1986) identified T resistance as the most common resistance type (29%) among porcine sourced isolates, followed by S, A, and C, at low levels. The resistance genes associated with the most widespread (seven herds) and common (81 isolates) *S*. 1,4,[5],12:i:- resistance type ASSuT mirror reports elsewhere, they were: *bla*$_{\text{TEM-1}}$ (β-lactamase), *strA-strB* (aminoglycoside phosphotransferase), *sul2* (dihydropteroate synthase), and *tetB* (efflux pump) (EFSA, 2010b; Hauser et al., 2010; Hopkins et al., 2010; Lucarelli et al., 2010; García et al., 2016). Among the *S*. 1,4,[5],12:i:- isolates sequenced, this gene cassette was identified in at least one sequence from each of the seven *S*. 1,4,[5],12:i:- sources. The other resistance genes identified at lower frequencies among the *S*. 1,4,[5],12:i:- strains sequenced—*tetA, aph(3)-Ia*, *sul1*, *sul3, aadA1*, *flo*R, *cmlA1, dfrA* and *dfrA5*—have also been reported elsewhere (Hauser et al., 2010; Hopkins et al., 2010; Mulvey et al., 2013b). Abraham et al. (2014) found *bla*$_{\text{TEM-1}}$, *sul2* and *tetB* to be the most common resistance genes among *Salmonella enterica* isolates from Australian food production animals. The Abraham et al. (2014) study also identified *cmlA1* among their study strains, but did not detect *flo*R; they did not test for *strA* or *strB*. The serovar *S*. 1,4,[5],12:i:- was not reported among the isolates analysed in the Abraham et al. (2014) study. The presence/absence of genes known to encode resistance among the *S*. 1,4,[5],12:i:- isolates sequenced approximated the source of the isolates and the core SNP phylogeny presented in Chapter 7.

The study *S*. 1,4,[5],12:i:- strains were shown to be closely related (Chapter 8) and their characteristics most closely resemble the *S*. 1,4,[5],12:i:- 'European clone'. The *S*. 1,4,[5],12:i:- isolates in the study collection were almost exclusively PT193 or PT120, ST34 and were missing the *S*. Typhimurium virulence plasmid pSLT, which are typical characteristics of the European clone (Table 8-1) (García et al., 2014). Furthermore, the study *S*. 1,4,[5],12:i:- strains appear to be very similar to the European epidemic clade described by Petrovska et al. (2016). Like the study isolates, the Petrovska et al. (2016) subclade A strains included pig sourced isolates, were predominantly PT193 and were all ST34. Moreover, the ASSuT phenotype observed among the majority of the study *S*. 1,4,[5],12:i:- colonies mirrors the phenotypic characteristics of the epidemic clade identified by Petrovska et al. (2016). This suggests that the same insertion, including the antimicrobial resistance gene cassette described by Petrovska et al. (2016), was also present in the study isolates. European clone *S*. 1,4,[5],12:i:- typically harbour ASSuT resistance encoded by the genes *bla*$_{\text{TEM}}$, *strA* and *strB*, *sul2* and *tetB,* respectively, in the chromosomal region where the deletion of the *fljAB* operon occurred (STM2579-*iroB* region) (Petrovska et al., 2016). Genome mapping showed that this gene cassette was present in the majority of the study strains, although there was also evidence of gene loss and acquisition, as was found among Herd 4 and Herd 6 strains in this study. Although a small proportion of the Petrovska et al. (2016) *S*. 1,4,[5],12:i:- isolates had the pSLT (13%) none

of the study isolates did. However, it is known that this plasmid can be lost and in the Australian case the *S*. 1,4,[5],12:i:- strain introduced may not have had the plasmid.

Following the ASSuT resistance type, the most widespread and frequently isolated antimicrobial resistance types among the *S*. 1,4,[5],12:i:- isolates were AT (three herds; 21 isolates) and T (two herds; 54 isolates). These narrower resistance types are most likely descendants of the typical ASSuT type, reflecting loss of genes from the ASSuT insertion region on the chromosome.

Among the study isolates the typical resistance types were stable over extended periods in most cases, however, as described, loss of resistance to the ASSu trio did occur. Herds 4 and 11 provide interesting cases. *Salmonella* 1,4,[5],12:i:- was extremely dominant among Herd 11 isolates and all expressed resistance to ASSuT. This is unsurprising given the inserted ASSuT resistance gene cluster is typically located on the chromosome and would therefore be expected to be fairly stable. The Herd 11 *S*. 1,4,[5],12:i:- population maintained the ASSuT resistance type over an extended period, indicating there was little fitness advantage in losing these resistance genes. In contrast, Herd 4 harboured multiple *Salmonella* serovars and the spectrum of resistances declined over the course of the study. Among *S*. 1,4,[5],12:i:- isolates from the final year of Herd 4 sampling T resistance was the only resistance type observed. The Herd 4 *S*. 1,4,[5],12:i:- population appears to lost the other four resistance markers found in the Herd 11 population, *strA*, *strB*, *sul2* and bla_{TEM}, but retained *tetB* in the majority of cases. This suggests that the *bla-strAB-sul2* genes did not offer any selective advantage under Herd 4 conditions. However, Herd 4 isolates show persistence of the *tetB* gene and T resistant strains. The ancestral SNP tree showed that Herd 11 and Herd 4 were more deeply branched than isolates from other herds. This suggests that the strain may have been circulating within these herds for a longer period than in other study herds. In turn this could indicate selective pressure for the resistance types observed in each herd or other factors maintaining the ASSuT quartet in Herd 11 while the ASSu trio were lost in Herd 4. As reviewed by Andersson and Hughes (2010), there is considerable evidence of the fitness costs associated with bacteria carrying resistance genes, therefore, without selection pressure resistance will tend to be lost over time. For example, through passage experiments on wild-type *S*. Newport strains to evaluate persistence of $bla_{\text{CMY-2}}$ plasmids, Subbiah et al. (2011) demonstrated that after an extended time period without selection pressure sensitive, plasmid-free strains came to dominate the population. Andersson and Hughes (2010) note that the rate of loss of resistance, typically months or years in a given community, will vary in relation to a number of factors including co-selection.

It is interesting to compare the Herd 4 strains and their closest relatives. The Herd 4 strains and the Herd 6 strain descended from a very recent predicted common ancestor that most likely existed only 5-8 years before samples were collected. Given the oldest Herd 4 strains and the Herd 6 strain (TW-STm44) had the ASSuT resistance genes located in the typical region of the chromosome, the ancestor of the Herd 4 and Herd 6 strains and must have had the *bla-strAB-sul2-tet* locus. Stepping

further back in the phylogeny (Figure 8-4 and Chapter 7, Figure7-2), Herd 3 isolates also descended from a recent common ancestor shared by Herd 4 and Herd 6 strains. However, only Herd 4 lacked the *bla-strAB-sul2* locus. This indicates strongly that the missing locus in the Herd 4 strains was due to gene loss.

García et al. (2016) and Petrovska et al. (2016) describe deletion of the *fljAB* region and its replacement with antibiotic resistance genes in the European clone of *S*. 1,4,[5],12:i:-. It has been speculated that *S*. 1,4,[5],12:i:- arose in pigs and spread to other hosts such as cattle and humans (Petrovska et al., 2016). This raises the question of whether antibiotic use in pigs is selecting for these strains or if they have other advantages in pigs. The apparent long term persistence of *S*. 1,4,[5],12:i:- without the *bla-strAB-sul2* genes in Herd 4 suggests that use of those antibiotics is not a selective pressure. Although we cannot discount that retention of *tetB* may reflect past use of tetracycline, this antimicrobial was not used during the 18-month period of study and yet the strain persisted when it could have been displaced by other circulating serovars. Speculatively, it may be that T resistance among *S*. 1,4,[5],12:i:- should be considered part of the normal flora.

Interestingly, the reduction in antimicrobial resistance within Herd 4 coincided with a management change that may have been influenced selection pressure for *Salmonellae* in the herd. Having endured a widespread outbreak of clinical enteritis among weaner pigs prior to the first sampling, the farm management opted to supplement the weaner diet with organic acids, which have been demonstrated to reduce *Salmonella* shedding in pigs (Van der Wolf et al., 2001a). The effects of disinfectants, biocides and heavy metals on selection and co-selection for antimicrobial resistance are known to be complex and likely affected by level and length of exposure, which may also be the case with increased exposure to particular organic acids (Wales and Davies, 2015). The change of management may have reduced selection pressure for the wider cluster of resistance genes leading to their gradual loss over the course of the study. Testing *S*. 1,4,[5],12:i:- isolate and contemporary serovars for tolerance to organic acids and searching for markers for tolerance to acidity could prove informative for understanding selection or co-selection of specific traits, which may inform the design of control strategies and therefore warrants further investigation.

Although the majority of the herds sampled maintained *S*. 1,4,[5],12:i:- populations describing low levels of resistance, ASSuT only, a smaller but significant group of *S*. 1,4,[5],12:i:- isolates had extended resistance phenotypes. These isolates appear to have been restricted to certain herds. The isolates from Herd 3, which was sampled on multiple occasions, had an extended spectrum of antimicrobial resistance genes that persisted. The Herd 3 strain sequences showed very shallow branching in their core SNP-based phylogeny. This suggests that the extended resistance spectrum likely conferred some form of fitness advantage and may have been was selected for. Conversely, four fully sensitive *S*. Agona and *S*. Oranienburg were identified among the other serovars isolated from Herd 3, but only among strains from the first two sampling occasions. This suggests that

these serovars were not selected for, which is consistent with selection or co-selection for more resistant strains. The rearing conditions in Herd 3 may have contributed to the expanded spectrum of resistances observed among isolates from this herd. Herd 3 did not employ effective all-in, all-out management of pig cohorts. General hygiene levels in Herd 3 were low at the grow-out site; very limited cleaning was employed between batches, bedding was not removed and no disinfectants were employed. It was also notable that grow-out shelters were open and accessible to birds and rodents and considerable evidence of both was observed during sampling visits. Water at both the breeder and grow out site had been supplemented with organic acids for a number of years, however, at the breeder site this was only to sows, acids did not reach weaner water. Low-level enteric ailments were reported and observed throughout the terminal herd during the study period and ongoing respiratory issues were apparent at the grow-out site throughout the study period. Due to relatively low hygiene levels, particularly at the grow-out site, a more diverse population of commensal bacteria may have been present enabling acquisition of resistance genes and/or the management may have employed different or higher levels of sub-therapeutic and/or therapeutic antimicrobials to combat health or productivity issues, which could have affected selection for more resistant strains.

The high levels of T resistance observed among *S.* 1,4,[5],12:i:- isolates have been reported elsewhere, however, the virtual ubiquity of T resistance among the study isolates (99.1%) distinguishes the study collection (Gebreyes et al., 2004; Arguello et al., 2014). Selective pressure for T resistance likely relates to animal management and/or animal health protocols employed in the herds, this pressure may be acting directly or by processes of co-selection. The use of tetracyclines could provide selective pressure for tetracycline resistance and/or the extended cluster of ASSuT genes identified frequently in among the study isolates. Velge et al. (2005) have discussed the likelihood of co-selection of linked gene clusters in relation to *S.* Typhimurium DT104 and this study has shown that the ASSuT gene cassette was clustered in a defined region of the chromosome in the study isolates sequenced (Wedel et al., 2005; Garrido et al., 2014). The Australian pig industry uses tetracyclines in considerable volumes, primarily therapeutically and administered through feed. The most recently published figures estimate that the industry used an estimated 40 tonnes (active constituent) of tetracyclines in 2009-2010 (APVMA, 2014). It was notable that among Herd 3 strains (11/12) and, to a lesser extent, Herd 4 strains (2/20) *tetA* was identified in addition to *tetB*. Although the presence of multiple *tet* genes in a single organism is more typically associated with Gram-positive bacteria, this is understood to increase resistance to tetracyclines, which may explain these observations (Chopra and Roberts, 2001).

Ampicillin resistant *S.* 1,4,[5],12:i:- isolates were identified in each of the herds in which the serovar was detected (10; 78% of cross-sectional study isolates). Resistance to β–lactams is widely reported, an intensive study of five pig herds in the US conducted by Gebreyes et al. (2004) found 42% of

Salmonella spp. were resistant to A, while Arguello et al. (2014) reported that 88.3% of Danish *S*. 1,4,[5],12:i:- isolates were A resistant. Resistance to A was conferred by the TEM family of genes in all of the A resistant study isolates sequenced, including the *S*. Typhimurium strains; neither the PSE, CTX-M nor the CMY families of β-lactamase genes were identified, unlike reports from Europe, Thailand and North America (Kiratisin et al., 2007; Hauser et al., 2010; Gallati et al., 2013; Mulvey et al., 2013b). This is of course consistent with the clonal nature of the study *S*. 1,4,[5],12:i:- population.

Resistance to C was identified among *Salmonella* spp. isolates in five herds and among *S*. 1,4,[5],12:i:- isolates in three herds, including longitudinal study Herds 1 and 3. Chloramphenicol use has not been permitted in Australian animal production since the mid-1980s (NHMRC, 1984), indicating that resistance to this compound was not selected for directly. However, Murray et al. (1986) identified low levels of C resistance among porcine sourced isolates and C resistance among Australian *Salmonellae* has been reported since at least the late 1970s. Interestingly, *flo*R encoded C resistance in Herd 1 while *cmlA1* was present in Herd 3 isolates. The gene *flo*R is known to impart cross-resistance to both C and florfenicol (Smith et al., 2016). In Australia florfenicol may be administered to livestock, which could explain observation of C resistance among the *S*. 1,4,[5],12:i:- isolates, although co-selection may be equally likely. The *flo*R and *cmlA1* genes have been reported among *S*. 1,4,[5],12:i:- strains elsewhere (Mulvey et al., 2013b; García et al., 2014). Mulvey et al. (2013b) identified both C resistance genes among the *S*. 1,4,[5],12:i:- isolates in their study, however, they only identified *cmlA1* in ST19 isolates; all the study *S*. 1,4,[5],12:i:- isolates were ST34. Given the close relatedness of the *S*. 1,4,[5],12:i:- isolates in the study collection, the presence of different accessory genes associated with C resistance indicates that the genes were most likely acquired horizontally.

Among human sourced *S*. 1,4,[5],12:i:- PT193 strains described in NEPSS passive surveillance data, from approximately the same period (2010-2014), resistance to the ASSuT group was highly predominant mirroring observations among pig-sourced isolates in these studies. Given these findings it stands to reason that resistance to ASSuT among the wider Australian *S*. 1,4,[5],12:i:- population also reflects the insertion at the site of the *fljAB* deletion on the bacterial chromosome reported here and by Petrovska et al. (2016). Moreover, as observed among these isolates, the increasing observation of additional *S*. 1,4,[5],12:i:- antimicrobial resistances beyond ASSuT may be due to horizontal acquisition unrelated to the chromosomal insertion containing the ASSuT resistance gene cassette. However, this could also be an artefact of the passive nature of the NEPSS data and the likelihood of misclassification of early *S*. 1,4,[5],12:i:- isolates.

8.4.5. Implications for the pig industry, food safety and national biosecurity

Antimicrobial resistance to compounds approved for use in treatment and prophylaxis in Australia identified among *Salmonella* isolates in this study were T, S, Sp, Su and Tm (Shaban et al., 2014). The high level of *S*. 1,4,[5],12:i:- resistance to the ASSuT group, and less frequently Sp and Tm, demonstrates that alternative approaches to the management of subclinical *S*. 1,4,[5],12:i:- colonization and disease within Australian pig herds are needed to effectively manage this serovar. Moreover, the use of these compounds may be associated with selection for these resistance types within herds, which could explain the rise of *S*. 1,4,[5],12:i:- detections in passive surveillance data over recent years and the predominance of the serovar in some of the herds studied.

Although direct selection for some resistance types could occur through on-farm antimicrobial usage, the identification of C and G resistance among *Salmonella* spp. strains demonstrates that selection may not be directly associated with use of specific compounds in primary production. Some of the antimicrobial resistance traits and genes have, most likely, been co-selected for along with clusters of linked genes, while others may have been acquired horizontally from other organisms. The selection for antimicrobial resistance genes in *Salmonella*, and other bacteria, can be complex and warrants further investigation in the Australian context, in both laboratory and on-farm settings.

The antimicrobial resistance phenotypes and genes and molecular and genomic characteristics of the study *S*. 1,4,[5],12:i:- collection strongly suggest that they share a common ancestor with the European clone, most likely a member of the epidemic clone identified by Petrovska et al. (2016). This supports speculation that the serovar was introduced to Australia in the recent past. In conjunction with other typing methods, comparison of antimicrobial resistance types and determinants among Australian porcine *S*. 1,4,[5],12:i:- isolates and *S*. 1,4,[5],12:i:- isolates sourced from other animal and human populations overseas may shed further light on the serovar's emergence in Australia. This information would be informative for herd, industry and national biosecurity and have implications for risk mitigation and management in public health circles.

On the basis of available passive surveillance data for Australian domestically acquired human *S*. 1,4,[5],12:i:- isolates the strains isolated from human cases describe similar antimicrobial resistance phenotypes to the strains isolated in this study. However, comparison with other potential hazard sources, such as other food and non-food animal populations and non-animal food sources, have not been made. Moreover, directionality of transmission/contamination between humans and pigs and other sources, if it occurs, is unknown. Further investigation of non-porcine Australian *S*. 1,4,[5],12:i:- populations is needed to determine the likely ultimate source(s) of human infection, as well as to inform surveillance and risk management initiatives.

Antibiogram typing is limited by the number of compounds included in the panel, the cutoff values employed and the potential for variability in the reading of plates. In contrast, by allowing rapid parsing of sequence data for matches to all known resistance genes in frequently updated and globally managed databases, the application of whole genome sequencing and genomic analyses provides considerably wider scope for identifying potential resistances. This, in turn, provides greater insight into the resistance mechanisms potentially conferring phenotypes and considerably greater resolution for epidemiological investigations. In this study parsing strain sequence data for known resistance genes identified genes with the potential to confer resistance to antimicrobials not tested for with the standard CLSI (2011) methodology. Furthermore, it was common to identify multiple genes associated with resistance to specific classes of antimicrobial in isolate genomes. Further investigation of the specific genes identified through employment of deep sequencing methods will provide further insights into the mechanisms through which organisms can acquire and lose resistance and the potential sources of selection and co-selection pressure. This information is valuable for the monitoring and management of drug resistant bacteria, can provide considerable insights into the mechanisms through which resistance may be acquired, and will be informative for epidemiological studies relating to the origins and ecology of organisms.

This study focused on *Salmonella* spp. in isolation, however, there is much that could be learned by investigating wider microbial communities within herds. Of course, the horizontal transfer of resistance genes between commensal bacteria is well-established (Ochman et al., 2000). Sequencing samples of the wider microbiome and examining the pan-resistome will provide a more nuanced picture of resistance within herds. Moreover, studies of the microbiome can provide a sharper picture of resistance within particular environments with considerable implications for understanding the development and maintenance of resistance in pathogenic and coexisting microbial populations. As the gut microbiome of pigs has been shown to change considerably with age, collecting pooled faecal samples from individual pigs, pen floors or effluent streams representing different age groups presents an interesting starting point (Kim, 2011). Swabbing/boot swabbing of floors and surfaces and aerosol sampling in sheds are alternative sampling methods that may provide further insights into resistance among microbes in pig herds beyond the gut microbiome.

As Barton (2010) advocates, it is essential that the monitoring of antimicrobial resistance phenotypes and determinants among *Salmonellae* in Australia be improved, particularly, but not exclusively, for compounds currently administered in the treatment of humans and animals.

8.5. Conclusions

The herds sampled harboured identifiable antimicrobial *Salmonella* spp. resistance types that were serovar associated and tended to persist or, in some cases, diminish over time. The *S*. 1,4,[5],12:i:- isolates from these studies were all resistant to at least one antimicrobial, were commonly multidrug resistant, and were often the most resistant strains isolated in a herd. The study findings do not suggest that Australian *S*. 1,4,[5],12:i:- pig production practices contributed to the emergence of multidrug resistant strains, however, differences between on-farm and/or within pig environments could have led to preferential selection or co-selection for specific resistance types, or lack thereof, which warrants the attention from the industry. The *S*. 1,4,[5],12:i:- antimicrobial resistance phenotypes and genotypes identified were very similar to those of the European clone and, although it is possible that the same deletion-insertion occurred in Australia, these findings, in the context of other available data on the serovar, provide yet more evidence that the study *S*. 1,4,[5],12:i:- isolates share recent ancestry with overseas *S*. 1,4,[5],12:i:- strains.

Supplementary Table 8-8. Presence/absence of resistance genes and alleles. Depth refers to the read depth at that location. The maxMAF column provides the highest minor allele frequency of variants across the gene. Continues on following page.

Sample	Depth	maxMAF	*aac3-IVa*	*aadA*	*aph(3'')-Ia*	*aph4-Ia*	*aphA2*	*cmlA*	*dfrA10*	*dfrA5*	*dfrA*	*ermA*	*floR*
TW-STm48	56.75	0.09	-	-	-	-	-	-	-	-	-	-	-
TW-STm47	40.31	0.12	-	-	-	-	-	-	-	-	-	-	-
TW-STm49	38.18	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm50	30.52	0.20	-	-	-	-	-	-	-	-	-	-	-
TW-STm55	42.23	0.13	-	-	-	-	-	-	-	-	-	-	-
TW-STm56	39.09	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm51	33.40	0.13	-	-	-	-	-	-	-	-	-	-	-
TW-STm52	41.11	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm53	48.64	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm37	30.49	0.12	-	-	-	-	-	-	-	324	-	-	-
TW-STm36	23.34	0.20	-	-	-	-	-	-	-	324	-	-	-
TW-STm34	44.28	0.12	-	-	-	-	-	-	-	-	-	-	-
TW-STm32	30.74	0.13	-	-	-	-	-	-	-	324	-	-	-
TW-STm33	51.52	0.19	-	-	-	-	-	-	-	324	-	-	-
TW-STm35	60.66	0.18	-	-	-	-	-	-	-	-	-	-	-
TW-STm44	44.55	0.15	-	2 1605*	1218*	-	-	-	-	-	12 1089	-	-
TW-STm31	45.17	0.15	-	-	-	-	-	-	-	-	-	-	-
TW-STm40	47.14	0.13	-	-	-	-	-	-	-	-	-	-	-
TW-STm41	43.70	0.14	-	-	-	-	-	-	-	-	-	-	-
TW-STm9	34.84	0.16	-	-	-	-	-	-	-	-	-	-	-
TW-STm25	49.34	0.07	-	-	-	-	-	-	-	-	-	-	-
TW-STm26	55.26	0.10	-	-	-	-	-	-	-	-	-	-	-
TW-STm13	55.83	0.08	-	-	-	-	-	-	-	-	-	-	-
TW-STm14	48.54	0.09	-	-	-	-	-	-	-	-	-	-	1212*
TW-STm1	43.12	0.09	-	-	-	-	-	-	-	-	-	-	-
TW-STm57	40.16	0.11	1489*	1-pm1597*	-	1061	-	5 1538	613	324	-	857*	-
TW-STm58	40.77	0.09	1489*	1-pm1597*	-	1061	-	5 1538	613	324	-	-	-
TW-STm38	49.92	0.10	-	-	-	-	-	-	-	-	-	-	-
TW-STm27	43.69	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm10	47.51	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm15	44.42	0.07	-	-	-	-	-	-	-	-	-	-	1212*
TW-STm16	44.65	0.08	-	-	-	-	-	-	-	-	-	-	1212*?
TW-STm2	42.27	0.12	-	-	-	-	-	-	-	-	-	-	-
TW-STm3	39.69	0.09	-	-	-	-	-	-	-	-	-	-	1212*
TW-STm45	48.90	0.10	-	-	-	-	-	-	-	-	-	-	-
TW-STm46	45.73	0.09	-	-	-	-	-	-	-	-	-	-	-
TW-STm59	35.68	0.13	1489*	-	-	-	-	-	-	-	-	-	1212*
TW-STm60	53.05	0.10	-	-	-	-	-	-	-	-	-	-	-
TW-STm19	36.24	0.08	-	2 1605*	-	-	1492*	-	-	-	12 1089	-	-
TW-STm43	48.33	0.10	-	-	-	-	-	-	-	-	-	-	-
TW-STm42	62.24	0.06	-	-	-	-	-	-	-	-	-	-	-
TW-STm28	37.16	0.08	-	-	-	-	-	-	-	-	-	-	-
TW-STm11	40.02	0.09	-	-	-	-	-	-	-	-	-	-	-
TW-STm17	43.39	0.08	-	-	-	-	-	-	-	-	-	-	1212*
TW-STm54	36.94	0.12	-	-	-	-	-	-	-	-	-	-	-
TW-STm4	41.49	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm20	41.71	0.14	-	2 1605*	-	-	1492*	1 1537*	-	-	12 1089	-	-
TW-STm21	49.08	0.09	-	2 1605*	-	-	1492*	-	-	-	12 1089	-	-
TW-STm5	49.33	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm6	39.42	0.16	-	2 1605	-	-	1492*	1 1537*	-	-	12 1089	-	-
TW-STm29	44.81	0.08	-	-	-	-	-	-	-	-	-	-	-
TW-STm30	53.72	0.11	-	-	-	-	-	-	-	-	-	-	-
TW-STm12	45.84	0.13	-	-	-	-	-	-	-	-	-	-	-
TW-STm18	61.80	0.07	-	-	-	-	-	-	-	-	-	-	-
TW-STm39	40.95	0.08	-	2 1605	-	-	1492*	1 1537*	-	-	12 1089	-	-
TW-STm22	37.20	0.10	-	2 1605	-	-	1492*	1 1537*	-	-	12 1089	-	-
TW-STm7	41.68	0.10	-	2 1605	-	-	1492*	1 1537*	-	-	12 1089	-	-
TW-STm23	41.19	0.07	-	2 1605	-	-	1492*	1 1537*	-	-	12 1089	-	-
TW-STm24	40.31	0.10	-	2 1605	-	-	1492*	1 1537*	-	-	12 1089	-	-
TW-STm8	37.51	0.11	-	2 1605	-	-	1492*	1 1537*	-	-	12 1089	-	-

– gene not present (minimum coverage 90%)
* indicates a mismatch

Sample	Depth	maxMAF	OXA-7	*strA*	*strB*	*sul3*	*sul2*	*sul1*	*bla*$_{\text{TEM-1D}}$	*tetA*	*tetB*	*tetR*
TW-STm48	56.75	0.09	-	-	-	-	-	-	-	-	-	-
TW-STm47	40.31	0.12	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm49	38.18	0.11	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm50	30.52	0.2	-	1501*	1614	-	1219*	-	30_907*	-	1213*	-
TW-STm55	42.23	0.13	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm56	39.09	0.11	-	1501*	1614	-	1219*	-	176_1018*	-	1213*	-
TW-STm51	33.4	0.13	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm52	41.11	0.11	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm53	48.64	0.11	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm37	30.49	0.12	-	1501*	1614	-	1219*	-	198_1035*	1545	1213*	1473
TW-STm36	23.34	0.2	-	1501*	1614	-	1219*	-	198_1035*	1545	1213*	1473
TW-STm34	44.28	0.12	-	-	-	-	-	-	198_1035*	-	1213*	-
TW-STm32	30.74	0.13	-	-	-	-	-	-	198_1035*	-	-	-
TW-STm33	51.52	0.19	-	-	-	-	-	-	198_1035*	-	1213*	-
TW-STm35	60.66	0.18	-	-	-	-	-	-	-	-	1213*	-
TW-STm44	44.55	0.15	-	1501	1614	-	1219*	1616	104_955*	1545	1213*	1473
TW-STm31	45.17	0.15	-	1501*	1614	-	1219*	-	30_907*	-	1213*	-
TW-STm40	47.14	0.13	-	-	-	-	-	-	-	-	1213*	-
TW-STm41	43.7	0.14	-	-	-	-	-	-	-	-	1213*	-
TW-STm9	34.84	0.16	-	-	-	-	-	-	-	-	1213*	-
TW-STm25	49.34	0.07	-	-	-	-	-	-	-	-	1213*	-
TW-STm26	55.26	0.1	-	-	-	-	-	-	-	-	1213*	-
TW-STm13	55.83	0.08	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm14	48.54	0.09	-	1501*	1614	-	1219*	-	33_908*	-	1213*	-
TW-STm1	43.12	0.09	-	1501*	1614	-	1219*	-	33_908*	-	1213*	-
TW-STm57	40.16	0.11	10_1515	1501*	1614	-	1219*	1616	198_1035*	1545*	-	1473
TW-STm58	40.77	0.09	10_1515	1501*	1614	-	1219*	1616	198_1035*	1545*	-	1473
TW-STm38	49.92	0.1	-	-	-	-	-	-	-	-	1213*	-
TW-STm27	43.69	0.11	-	-	-	-	-	-	-	-	1213*	-
TW-STm10	47.51	0.11	-	-	-	-	-	-	198_1035*	-	1213*	-
TW-STm15	44.42	0.07	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm16	44.65	0.08	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm2	42.27	0.12	-	1501*	1614	-	1219*	-	30_907*	-	1213*	-
TW-STm3	39.69	0.09	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm45	48.9	0.1	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm46	45.73	0.09	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm59	35.68	0.13	-	1501	1614	-	1219*	-	198_1035*	1545*	-	1473
TW-STm60	53.05	0.1	-	-	-	-	-	-	-	-	-	-
TW-STm19	36.24	0.08	-	1501	1614	-	1219*	-	198_1035*	1545	1213*	1473
TW-STm43	48.33	0.1	-	1501*	1614	-	1219*	-	30_907*	-	1213*	-
TW-STm42	62.24	0.06	-	-	-	-	-	-	-	-	1213*	-
TW-STm28	37.16	0.08	-	-	-	-	-	-	-	-	1213*	-
TW-STm11	40.02	0.09	-	-	-	-	-	-	-	-	1213*	-
TW-STm17	43.39	0.08	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm54	36.94	0.12	-	1501*	1614	-	1219*	-	148_997*	-	1213*	-
TW-STm4	41.49	0.11	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm20	41.71	0.14	-	1501	1614	1498	1219*	-	198_1035*	1545	1213*	1473*
TW-STm21	49.08	0.09	-	1501*	1614	-	1219*	-	198_1035*	1545	1213*	1473*
TW-STm5	49.33	0.11	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm6	39.42	0.16	-	1501	1614	1498	1219*	-	198_1035*	1545	1213*	1473
TW-STm29	44.81	0.08	-	-	-	-	-	-	-	-	1213*	-
TW-STm30	53.72	0.11	-	-	-	-	-	-	-	-	1213*	-
TW-STm12	45.84	0.13	-	-	-	-	-	-	-	-	1213*	-
TW-STm18	61.8	0.07	-	1501*	1614	-	1219*	-	198_1035*	-	1213*	-
TW-STm39	40.95	0.08	-	1501	1614	1498	1219*	-	198_1035*	1545	1213*	1473
TW-STm22	37.2	0.1	-	1501	1614	1498	1219*	-	198_1035*	1545	1213*	1473
TW-STm7	41.68	0.1	-	1501	1614	1498	1219*	-	198_1035*	1545	1213*	1473*
TW-STm23	41.19	0.07	-	1501	1614	1498	-	-	198_1035*	1545	1213*	1473
TW-STm24	40.31	0.1	-	1501	1614	1498	1219*	-	198_1035*	1545	1213*	1473
TW-STm8	37.51	0.11	-	1501	1614	1498	1219*	-	198_1035*	1545	1213*	1473*

– gene not present (minimum coverage 90%)
* indicates a mismatch

Chapter 9 - General discussion, conclusions and further work

9.1. Introduction

This study was conceived in the context of early isolations of the *Salmonella* serovar *S*. 1,4,[5],12:i:- from human salmonellosis cases in Australia. Internationally *S*. 1,4,[5],12:i:- has emerged over the past two decades and risen to prominence due to its pandemic status, frequent isolation from human salmonellosis cases, apparent pathogenicity and virulence, and typical multidrug resistance. Although a non-host specific serovar, *S*. 1,4,[5],12:i:- has most commonly been associated with pigs and pork. Unlike *S*. Typhimurium DT104, *S*. 1,4,[5],12:i:- had been identified among domestically acquired infections in Australia (OzFoodNet Working Group, 2012d; NEPSS, 2014). Furthermore, a study confirmed the presence of the serovar in an Australian pig herd, indicating that domestically produced pig products could present a possible immediate and/or ultimate source of human *S*. 1,4,[5],12:i:- salmonellosis (Hamilton et al., 2015). In this context, further investigation of *S*. 1,4,[5],12:i:- in Australian pigs was needed to better inform consumer health, the industry and to contribute to the global literature on *S*. 1,4,[5],12:i:- and related pathogens.

The studies reported in this thesis contribute new knowledge on the dynamics of *S*. 1,4,[5],12:i:- colonization within pig herds and the characteristics of the *S*. 1,4,[5],12:i:- populations currently circulating in Australian pigs. The research sought to explore the distribution, persistence and diversity of *S*. 1,4,[5],12:i:- colonization among Australian commercial pig herds and compare Australian *S*. 1,4,[5],12:i:- phenotypes and genotypes with those reported overseas. The studies were designed to contribute to the global *Salmonella* literature and inform industry and public health risk mitigation and risk management strategies.

The main research questions addressed in these studies were: How widespread has *S*. 1,4,[5],12:i:- become in the Australian pig industry? What happens in *S*. 1,4,[5],12:i:- colonized herds over time? How diverse is the Australian *S*. 1,4,[5],12:i:- population? How does the Australian *S*. 1,4,[5],12:i:- population compare to *S*. 1,4,[5],12:i:- populations reported internationally? What are the resistance characteristics and determinants of Australian porcine *S*. 1,4,[5],12:i:-? What are the optimal typing methods for *S*. 1,4,[5],12:i:-?

9.2. Summary of key findings

This study found that *S*. 1,4,[5],12:i:- has colonized pig production enterprises employing a broad range of production methods located across the major pig producing regions of Southern Australia. This indicates that the serovar is now widespread in the Australian industry and contrasts with the low historical prevalence estimates for *S*. Typhimurium in the Australian pig industry (Hamilton et al., 2015).

This study found that *S*. 1,4,[5],12:i:- persisted for extended periods in five herds monitored longitudinally. This is consistent with previous research demonstrating persistence of individual *Salmonella* serovars within pig herds (Funk et al., 2001; Van Der Wolf et al., 2001c; Nollet et al., 2005b), and similar to recent reports of *S*. 1,4,[5],12:i:- shedding in pig production clusters in Germany (Niemann et al., 2015a). A considerable escalation in detections of *Salmonella* was detected in weaners, also reflecting studies of other *Salmonella* serovars (Kranker et al., 2003). This research found particularly high rates of *S*. 1,4,[5],12:i:- detection in grow-out pigs relative to contemporary serovars, whereas, *S*. 1,4,[5],12:i:- and contemporary serovar shedding among sows was more balanced. This suggests that *S*. 1,4,[5],12:i:- has a particularly high propensity to cause shedding among grow out pigs. *Salmonella* 1,4,[5],12:i:- shedding rates in comparison with other serovars have not been described previously, however, apparent variation in shedding rates between serovars has been reported elsewhere (van Winsen et al., 2001; Österberg et al., 2010; Ivanek et al., 2012; Pires et al., 2014). The study also observed *S*. 1,4,[5],12:i:- strains described by the same phenotypes and MLVA profiles among both weaner and finisher animal samples. This indicates that colonization and/or shedding peaked among weaners and then persisted through to finish. Moreover, this demonstrates that pig farms may be a potential source of the hazard in the human food chain and reaffirms the importance of effective control measures on farm, at slaughter and in pork boning rooms for managing food safety risks associated with the pathogen (Swanenburg et al., 2001a; Swanenburg et al., 2001b). Despite low *S*. 1,4,[5],12:i:- detection among samples from sows, strains with the same phenotypes and MLVA profiles were identified among grow-out pigs and sows in the same herd. Moreover, comparative genomic studies showed that sows and grow-out pigs in the same herd were shedding *S*. 1,4,[5],12:i:- strains that were essentially indistinguishable. In relation to other *Salmonella* serovars Kranker et al. (2003), among others, suggest that sows could play an important role in maintaining herd colonization, these results indicate that this could also be the case for *S*. 1,4,[5],12:i:- in the Australian herds studied (Nollet et al., 2005a; Nollet et al., 2005b; Hill et al., 2015).

Phage typing proved to be of limited epidemiological value in relation to *S*. 1,4,[5],12:i:- in Australia. However, these studies provided empirical support for the use of MLVA in relation to *S*. 1,4,[5],12:i:- and related serovars. These results indicate that MLVA could offer the appropriate

balance of discriminatory power and stability to be useful for outbreak strain identification and, potentially, for source attribution investigations. As a typing methodology, the potential value and interpretation of *Salmonella* MLVA profiles remains somewhat uncertain (Hopkins et al., 2007; Barua et al., 2013; Wuyts et al., 2013; Dimovski et al., 2014). Previous studies have employed *in vitro* passaging and *in vivo* methods in a laboratory setting to assess the stability of MLVA profiles among *Salmonellae* and the interpretation of results (Wuyts et al., 2013; Dimovski et al., 2014). Among others, Dimovski et al. (2014) found that loci STTR5 and STT6 were unstable relative to other loci employed in the standard Lindstedt et al. (2004) approach and recommended clustering profiles with variations of one or two VNTR copy numbers at these loci. This study found variation in *S.* 1,4,[5],12:i:- MLVA profiles at these loci only, however, MLVA profiles were associated with herds and profiles were surprisingly stable within herds over extended periods. These results provide empirical evidence from an on-farm setting supporting clustering MLVA with ≤2 VNTR copy number differences at loci STTR5 and STTR6 when interpreting *S.* 1,4,[5],12:i:- MLVA results as recommended by Dimovski et al. (2014).

Phylogenetic analysis of the study *S.* 1,4,[5],12:i:- core genome SNPs readily differentiated strains on the basis of source herd or abattoir. These analyses also provided strong evidence that the Australian porcine *S.* 1,4,[5],12:i:- population is highly related and undergoing clonal expansion. Prior to this research the only published *S.* 1,4,[5],12:i:- phylogeny was reported by Petrovska et al. (2016), which described an expanding epidemic clone of *S.* 1,4,[5],12:i:- strains from animal and human sources in Italy and the United Kingdom. The findings of this study provide evidence that Australian porcine *S.* 1,4,[5],12:i:- strains are even more closely related that the strains analysed by Petrovska et al. (2016) and are undergoing a similar clonal expansion, indicating that the current Australian *S.* 1,4,[5],12:i:- population emerged and possibly spread from a single event or limited source.

This study provided empirical evidence to support the hypothesis that *S.* 1,4,[5],12:i:- was introduced to Australia from overseas. The phage types, MLVA types, antimicrobial resistance phenotypes and antimicrobial resistance genes identified among the study *S.* 1,4,[5],12:i:- strains were typical of *S.* 1,4,[5],12:i:- characteristics reported overseas, particularly strains of the European clone commonly reported in Europe and Asia (Soyer et al., 2009a; Switt et al., 2009; Hauser et al., 2010; García et al., 2013; Petrovska et al., 2016). If *S.* 1,4,[5],12:i:- was introduced to Australia, which never occurred in the case of the comparable pandemic *S.* Typhimurium DT104, this may have implications for national and industry biosecurity.

This study found very little resistance to antimicrobials considered critical in the treatment of human salmonellosis. *Salmonella* resistance types varied considerably between herds and were serovar associated within herds. The majority of *S.* 1,4,[5],12:i:- were resistant to the ASSuT quartet or variations thereof. The majority of non-*S.* 1,4,[5],12:i:- serovars were pansusceptible and only a

small proportion of non-*S*. 1,4,[5],12:i:- serovars were multidrug resistant, which were limited to specific herds. The variation in resistance types between contemporary serovars within herds suggests that on farm use of antimicrobials was not driving selection for *Salmonella* resistance types. However, there were indications of differing selection pressures for resistance types in individual herds. In some herds resistance levels diminished over time, consistent with gene loss due to the fitness cost associated with maintaining resistance genes with a lack of selection pressure (Andersson and Hughes, 2010). The most common resistance genes identified among the study *S*. 1,4,[5],12:i:- isolates mirrored reports of the ASSuT gene cassette that Petrovska et al. (2016) found was located on an insertion sequence at the site of the *fljAB* operon deletion. Parsing sequence data and mapping the STM2759-*iroB* region—site of the *fljAB* deletion and insertion of the ASSuT resistance genes among the Petrovska et al. (2016) isolates—of the chromosome of lower resistance study isolates sequenced showed that a deletion had occurred causing loss of part or all of this resistance gene cassette. The identification of *S*. 1,4,[5],12:i:- strains with extended resistance types in some herds was reflected in the identification of additional genes and indicated horizontal acquisition via mobile genetic elements. All resistance genes identified among the study *S*. 1,4,[5],12:i:- strains had been reported in other Australia studies of *Salmonella* and commensal *Enterobacteriaceae* (Abraham et al., 2014; Smith et al., 2016). Resistance to some compounds not used in Australian pigs was identified, it is possible that co-selection may account for this such as the use of florfenicol may have selected for chloramphenicol resistance (Smith et al., 2016).

9.3. Limitations of the study

This study was primarily concerned with *S*. 1,4,[5],12:i:-. At the time the study was designed very little was known about the presence of *S*. 1,4,[5],12:i:- in Australia, let alone in Australian pig herds; the first published record of *S*. 1,4,[5],12:i:- isolation from a human domestically acquired case occurred in 2012 (OzFoodNet Working Group, 2012d). Furthermore, previous research and passive surveillance data indicated that *S*. Typhimurium prevalence in Australian pigs has been low, historically, suggesting this might also be the case for *S*. 1,4,[5],12:i:- give the similarities of these serovars (NEPSS, 2014; Hamilton et al., 2015). In this context, and given the logistical challenges of sampling pig herds in Australia—the large distances between herds and biosecurity protocols requiring minimum time off farm—the selection of herds was non-random and biased in favour of finding *S*. 1,4,[5],12:i:-.

A cohort longitudinal study design would have allowed further investigation of risk factors, and possible modes of transmission within herds, associated with *S*. 1,4,[5],12:i:-. However, the logistical challenges of sampling widely dispersed herds at the shorter intervals and the additional costs associated with a cohort study design made this approach unfeasible. Furthermore, an extensive literature on *Salmonella*, and *Salmonella* Group B serovar, risk factors in pigs already exists (Wales et

al.; Bush et al., 1999; Funk et al., 2000a; Hamilton et al., 2000; Belœil et al., 2004; Funk and Gebreyes, 2004; Lo Fo Wong et al., 2004; Nollet et al., 2004).

The study results did suggest that *Salmonella* and *S.* 1,4,[5],12:i:- shedding may have been higher in wetter, colder conditions, as has been described previously, for example in Davies et al. (1999). Ideally the longitudinal studies would have been extended to allow seasonal variation in shedding to be taken into account. This would likely be informative for risk management strategies on farm and process control efforts post-harvest. Unfortunately, it was not possible to extend the studies due to time and resource constraints.

The use of a geometric testing regimen and a binomial testing regimen for the characterization of isolates from *Salmonella* positive samples was proven to be equivalent in terms of the number of *S.* 1,4,[5],12:i:- positive samples and estimating true prevalence estimates. However, this approach may have reduced the level of diversity observed among *S.* 1,4,[5],12:i:- strains as further characterization of *S.* 1,4,[5],12:i:- strains from the same sample was not conducted. However, the effects of this bias were likely minimal given the low diversity observed within herds over the course of the longitudinal studies conducted.

The time isolates spent in storage between phenotyping and sequencing varied, typically 12 to 24 months and in some cases approaching five years. Authors such as Snyder et al. (2012) have demonstrated that gene loss can occur in storage. Gene loss in storage after antimicrobial sensitivity phenotyping could have accounted for the small number of discrepancies observed between resistance phenotypes and gene repertoires in some strains. This was unavoidable in the context of the study as funding for sequencing was only obtained in the latter stages of the project.

9.4. Implications of findings

These studies demonstrate the value of intensive sampling and longitudinal study designs in describing *Salmonella* populations on farms. As this study, among others, has shown, *Salmonella* populations and subpopulations within pig herds are often diverse, and *Salmonella* shedding may be intermittent (Pires et al., 2013a; Niemann et al., 2015a). In the course of these studies, it was common to find multiple serovars and subtypes circulating within age groups and herds. These studies also showed that the collection of pooled samples and characterization of multiple colony picks per positive sample can provide efficiency and efficacy benefits when describing a resident *Salmonella* population within a pig population. Moreover, different serovars and subtypes were often described by different characteristics of practical importance, most notably in relation to antimicrobial sensitivity. At present, it is common for veterinary practitioners to collect individual samples in small numbers and on a single occasion, typically from post-mortems or where there is evidence of clinical disease, and to characterize a single colony pick per *Salmonella* positive sample.

This sampling approach risks presenting a misleading impression of the *Salmonella* population present within an age group or herd, which could lead to suboptimal treatment and control measures.

This research indicates that *S*. 1,4,[5],12:i:- is now widespread in the Australian industry and may now be more prevalent than *S*. Typhimurium. This suggests that *S*. 1,4,[5],12:i:- may now present a greater food safety risk than *S*. Typhimurium, the most commonly isolated *Salmonella* serovar from Australian human salmonellosis cases. Therefore, this study provides empirical support for the establishment of more rigorous surveillance of *S*. 1,4,[5],12:i:- and other *Salmonella* serovars of importance to human health in food animals and post-harvest.

The results of this research indicate that a clonal *S*. 1,4,[5],12:i:- population has spread quickly through the Australian herd. However, the manner in which the serovar has spread to and between farms remains unknown. This serves to reiterate the importance of applying rigorous biosecurity measures on farm for the management of *S*. 1,4,[5],12:i:- and other pathogens.

These findings are consistent with *S*. 1,4,[5],12:i:- having been introduced to Australia from overseas, most likely within the last ten years. This implies that a breakdown in national and industry biosecurity has occurred. Investigating how this occurred could inform risk management in relation to the future emergence of pandemic *Salmonella* serovars and other microbes of animal and public health importance.

This study indicates that selection for antimicrobial resistance may not relate directly to on-farm use of antimicrobials, at subtherapeutic or treatment levels. Furthermore, the study identified very low levels of resistance to antimicrobial agents considered critical for the treatment of human salmonellosis. However, given rising global concern surrounding antimicrobial resistance and the variation in specific resistance types observed between herds in this study, monitoring *Salmonella* and other bacterial resistance phenotypes and determinants is necessary for public health and to inform and protect the industry (Barton, 2010).

Sequencing proved to be more reliable than conventional serotyping. Two of the isolates sequenced were phenotyped as biphasic *S*. Typhimurium strains but were without the *fljAB* operon and were, therefore, true monophasics. Furthermore, sequencing provided considerable information on strain antimicrobial resistance determinants presenting a more detailed and informative picture of strain resistance than phenotyping by conventional antibiogram. At present, it would be judicious to continue storing cultured *Salmonella* isolates, given the wealth of information that is, and may become, available from viable colonies. However, given the high and rising costs of serotyping and phage typing in Australia, which combined are now considerably more expensive to conduct than sequencing on a per strain basis, and that phenotyping can be simply and reliably achieved from

sequence data using tools like SeqSero and SRST2, routine sequencing of *Salmonella* isolates may be more cost effective and informative than phenotyping (Inouye et al., 2014; Zhang et al., 2015).

The direct relationship between *Salmonella* status on farm and risk to consumers is well documented (Berends et al., 1997; Mousing et al., 1997a; Dahl and Sørensen, 2001; Alban and Stärk, 2005; Alban et al., 2012; De Busser et al., 2013; Andres and Davies, 2015; Snary et al., 2016). The high rates of *S.* 1,4,[5],12:i:- shedding among Australian market-destined pigs observed in the study results demonstrates that pigs on farm could play the role of ultimate source in some cases of Australian human salmonellosis. A future foodborne *S.* 1,4,[5],12:i:- outbreak linked to Australian pigs would most likely prove costly to the industry, in terms of consumer perceptions of Australian pork products and in terms of the possibility of subsequent regulatory changes and adjusted requirements from public health authorities, domestic retailers and export markets. These results provide further support for improved surveillance and the development of effective and efficient risk mitigation and risk management strategies for *S.* 1,4,[5],12:i:- and related foodborne pathogens on farm, on carcass, in boning rooms and on products.

9.5. Areas for future research

This study has shown that *S.* 1,4,[5],12:i:- has spread quickly to ostensibly unconnected herds throughout the industry indicating that it has spread relatively rapidly through the Australian commercial pig herd. It is therefore likely that the mode or modes of *S.* 1,4,[5],12:i:- transmission include routes that connect the industry as a whole. Although various connections between herds are apparent the specific mode or modes of transmission are unknown and warrants further investigation. Identifying how *S.* 1,4,[5],12:i:- has spread among Australian pig herds would be of considerable value to the industry in developing control strategies for *S.* 1,4,[5],12:i:- and other pathogens. Many studies have identified modes of transmission for *Salmonella* including various vectors and fomites, such as live animals, feed, people, vehicles and wild animals, their possible role in *S.* 1,4,[5],12:i:- transmission should be considered (Funk and Gebreyes, 2004; Lo Fo Wong et al., 2004). Investigating industry bottlenecks such as gilt supply, feed components, key nodes—such as abattoirs, and service providers who regularly visit multiple farms would likely prove informative.

These studies suggested that *S.* 1,4,[5],12:i:- could have greater tolerance for certain disinfectants and biocides employed in Australian pig farming than other serovars, however, this was not tested for in this research. Various studies have found that the *Salmonella* may become more tolerant to some compounds through mechanisms such as the development of biofilms and oxidative stress responses (McDonnell and Russell, 1999; Braoudaki and Hilton, 2004; Karatzas et al., 2007; Wales and Davies, 2015), however, the links and selective effects of differing exposure to specific biocides, heavy metal salts and antimicrobials, for example, are undoubtedly complex (Wales and

Davies, 2015). Investigating Australian *S.* 1,4,[5],12:i:- phenotypic tolerance to the various disinfectants, biocides and heavy metals used in the industry and the determinants of tolerance, if it exists, would inform the design of *S.* 1,4,[5],12:i:- control strategies on farm.

This study generated a unique collection of Australian *S.* 1,4,[5],12:i:- isolates from a broad cross-section of the industry and found them to be highly related. Moreover, in terms of both phenotypes and genotypes these isolates bear close resemblance to *S.* 1,4,[5],12:i:- isolates reported overseas, particularly in Europe and Asia. These findings also indicate that a similar pattern of emergence to that reported by Petrovska et al. (2016), who found that the majority of Italian and British epidemic *S.* 1,4,[5],12:i:- isolates formed a closely related clade that indicated clonal expansion. Conducting comparative genomic studies of *S.* 1,4,[5],12:i:- isolate genomes from the study collection and *S.* 1,4,[5],12:i:- strains isolated overseas would provide further indications of the phylogeny of *S.* 1,4,[5],12:i:- internationally, indicate the timing of Australian emergence, and may provide further evidence of an *S.* 1,4,[5],12:i:- introduction to Australia. This information would contribute to the global literature on pandemic *Salmonella* strains and could inform Australian biosecurity strategies.

Available Australian data shows that *S.* 1,4,[5],12:i:- strains describing the same phenotypes and MLVA profiles have been isolated from non-porcine sources in Australia, notably cattle and people (OzFoodNet Working Group, 2012d; NEPSS, 2014). Petrovska et al. (2016) found evidence supporting epidemiologic findings that a clade of *S.* 1,4,[5],12:i:- strains likely originated in pigs and was later transmitted to cattle. Conducting comparative genomic studies on *S.* 1,4,[5],12:i:- strains from this study and *S.* 1,4,[5],12:i:- strains sourced from other Australian animals could indicate the existence of a single Australian *S.* 1,4,[5],12:i:- population or several *S.* 1,4,[5],12:i:- subpopulations circulating in different host species. Determining the relatedness of the wider Australian *S.* 1,4,[5],12:i:- population would inform source attribution investigations, could indicate if *S.* 1,4,[5],12:i:- has or is being transmitted between species domestically, and would provide additional data points for phylogenetic and microevolution studies of *S.* 1,4,[5],12:i:-. Furthermore, this may provide indications as to whether *S.* 1,4,[5],12:i:- emerged in Australia from pigs, cattle, people or other animal populations such as birds, which may also inform biosecurity strategies.

The study focused on the epidemiology of Australian porcine *S.* 1,4,[5],12:i:-, however, the work generated a well-characterized library of other *Salmonella* serovars circulating in Australian pig herds during the study period. The results showed that antimicrobial resistance phenotypes and genes varied considerably between *Salmonella* subpopulations, in different herds and between serovars within individual herds. This likely relates to selection pressures within individual herds and/or the presence of resistance genes on mobile elements within resident commensal microbiomes. Further investigation of the non-*S.* 1,4,[5],12:i:- strains in the collection could provide insights into the spread of *Salmonella* between herds and the dynamics of *Salmonella* colonization within herds. Moreover, identifying resistance and other phenotype determinants among contemporary *Salmonella*

serovars could help to explain variation between contemporary serovars within herds and provide indications of gene loss and acquisition between *Salmonellae* in herds. Furthermore, examining the pan-resistome in pig herds, beyond *Salmonella*, through sampling and sequencing the microbiomes of individual pigs, pig cohorts and the farm environment is likely to be informative. Studies of the pan-microbial resistome could inform antimicrobial usage recommendations and future regulation, generate alternative control strategies, and provide further information of the nature and dynamics of the development, maintenance and spread of antimicrobial resistance among microbial populations residing in pigs.

The isolation of *S.* 1,4,[5],12:i:- from human salmonellosis cases and the implication of Australian pork products suggests pigs could be a source of the hazard in food supply. The study found considerable levels of *S.* 1,4,[5],12:i:- shedding among finisher pigs, presenting a potential source of the hazard in the food chain. Furthermore, *S.* 1,4,[5],12:i:- has been isolated from Australian pig carcasses in abattoirs, examples of which were found to be closely related to the isolates sourced from farm samples, and strains from human cases and pig products describing very similar characteristics to the study strains have been identified in Australian passive surveillance data (NEPSS, 2014). Further research into the risk pathways through which Australian pork consumers might be exposed to *S.* 1,4,[5],12:i:- is warranted. Kirchner *et al.* (2011), among other studies internationally, employed repeat sampling and multiple typing methods in a study of *Salmonella* presence in pig finishers through to carcass in the UK and were able to differentiate farm aquired strains from and abattoir aquired strains. On this basis the authors argued for the importance of 'integrated control strategies along the pork food chain' to mitigate *Salmonella* food safety risk. Further investigation of *S.* 1,4,[5],12:i:- hazard loads in lairage and contamination levels on carcasses, in boning rooms and on products, and comparison of farm *S.* 1,4,[5],12:i:- populations with abattoir populations, is needed to assess food safety risks associated with farm *S.* 1,4,[5],12:i:- colonization and to develop optimal risk management strategies that adequately protect consumers and the industry.

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Annex 1: Longitudinal herd profiles

Herd 1

Management

The enterprise was comprised of two sites located approximately 20km apart; a farrow to grower site (to 11-12 weeks) and a grow-out site. The herd produced finished hogs and acted as an occasional gilt supplier to several clients. The sow herd numbered in the range 400-600 head.[6] The herd was relatively closed, having not regularly brought in breeder animals since the 1970s, with the exception of a small introduction of duroc genetics in the early 1990s (under the previous management) and two batches of 40 gilts bought from a single supplier in 2010 and mid-2013. Semen was sourced from own boars at an independent boar stud.

Herd 1 employed pelleted feed throughout the herd supplied from a single mill. Dry sows were fed on a 'no-grind' pellet. Drinking water was sourced from a nearby river. The herd employed on-site effluent ponds and straw composting of culls.

Dry sows were group housed in conventional sheds on concrete and slatted floors before service and during early gestation, then moved to a straw-based deep-bedded shed for late gestation. Half a dozen boars were maintained in the service shed, which were moved to pens near sows post weaning to promote oestrus. Sows were then moved to crates for farrowing and lactation. Farrowing crates were housed in either one of four smaller rooms—holding 20 crates each, or a larger farrowing room—housing 60 crates. The herd employed continuous mating. Average born alive was approximately 10.5; the herd markedly improved reproductive performance over the previous five years, raising pigs/sow/year from approximately 17 to over 20. The improved reproductive performance was broadly attributed to improved genetic and sow management and the inclusion of a nutritional supplement in gilt and sow diets (SALMATE®), to which the management attributed increased conception rates and litter sizes and reduced returns to service. However, farrowing rates remained suboptimal at 65-70%, which the management was seeking to address. Weekly weaning was employed at an average age of approximately 21 days, around 5kg in weight; approximately 200 head per batch. Piglets received a milk supplement (Veanavite®) in creep. Piglets were weaned into one of four weaner rooms housing eight pens on slatted floors with possible contact with neighbouring pens through slatted partitions; each pen held 20-30 weaned piglets. At age 7-8 weeks weaners were moved to conventional pens with slatted floors in groups of 20-30 head, again with possible snout-to-snout contact with neighbouring pens through slatted partitions. Growers were moved to the grow-out site at 11-12 weeks; at which point two batches

[6] Due to the structure of the Australian pig industry a more precise number of sows present has not been provided to protect the producer's confidentiality.

were combined, approximately 400 per batch. The aggregated batch was transported to the grow-out site using a stock truck owned by the enterprise, where the integrity of the larger batch was maintained to finish. The grow-out site employed approximately 75% deep bedding systems. Grower-finishers were housed on deep bedding from arrival at 11-12 weeks to 17-18 weeks before being moved to conventional concrete and slatted floored finisher shed with adjoining loading pens to finish at 20-22 weeks, at approximately 100kgLW. The business employed the same stock truck used to transport growers to transport finished hogs to a slaughtering facility located approximately 75km from the grow-out site. The abattoir served both domestic and export markets.

The flows of pigs within the farm are depicted as a flow diagram (Figure 0-1).

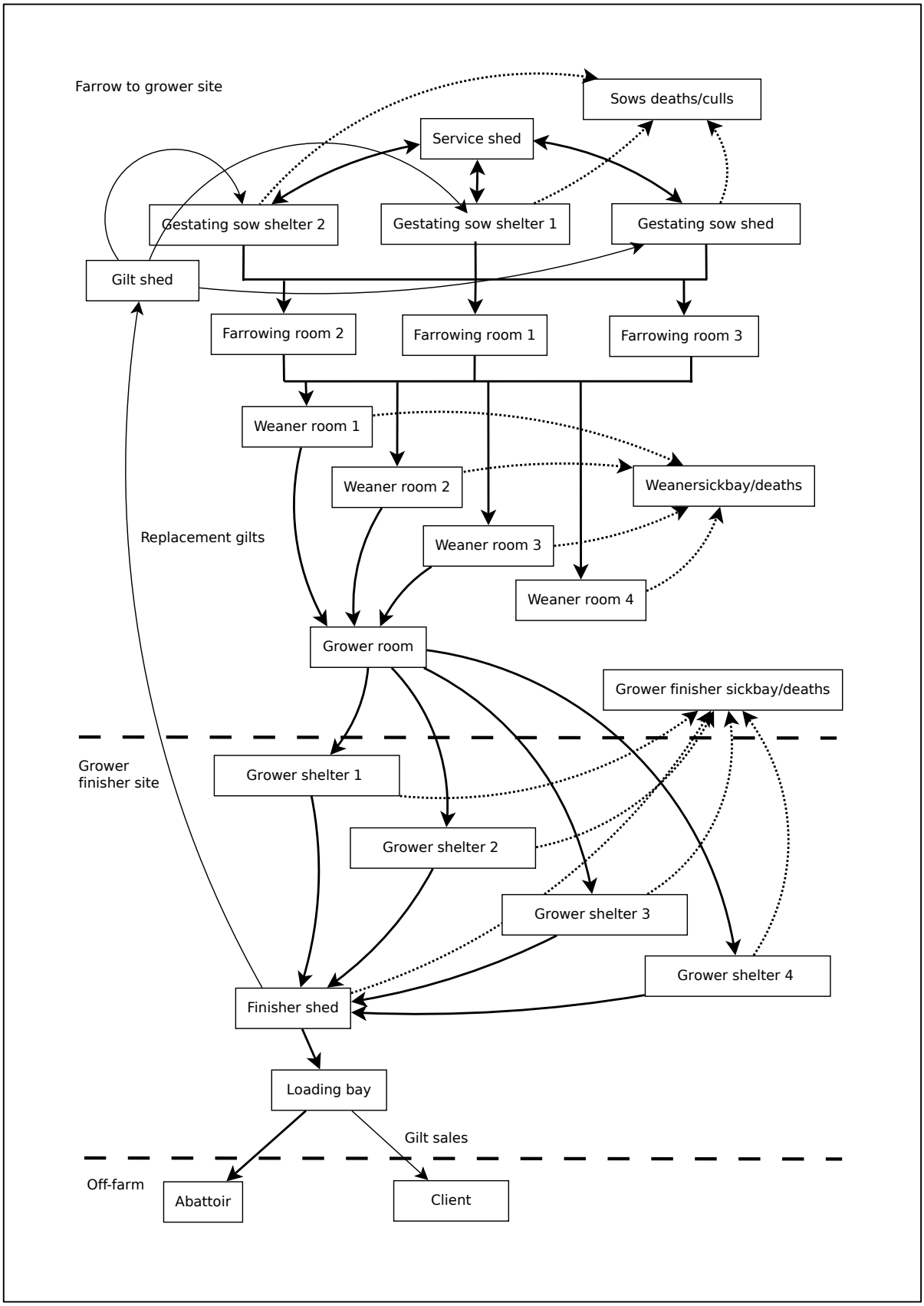

Figure 0-1. Flow diagram of animal movements within Herd 1. Thick links indicate the majority of the batch, finer links indicate gilts for replacement or sale, dashed links indicate movement of sick pigs, deaths or sow culls, as indicated. Larger dashed lines indicate different production sites.

Animal health

The herd operated at a high health status, as defined by the study (<3% pre-wean mortality per annum). They did not have *Mycoplasma* or dysentery and minimal porcine circovirus type 2 (PCV2) associated disease problems. During sampling scouring was observed in farrowing sheds and among weaners, *Salmonella* had been isolated on several occasions prior to the study. The main health concerns during the period of study related to sow lameness, salmonellosis, *Actinobacillus pleuropneumoniae* (APP) and Glasser's disease. The breeder site did not suffer from APP, which appeared to be isolated to the grow-out site. The herd had endured an outbreak of streptococcal meningitis among finishers in mid-2014, resulting in 30 deaths. Since mid-2014 the herd was vaccinated against PCV2 (CircoFLEX®); prior to the introduction of the PCV2 vaccine the herd was vaccinated against *Haemophilus parasuis* and *Mycoplasma hyopneumoniae* (RespiSure-ONE®), which had been removed in mid-2014. Previously the herd had also been vaccinated against *Lawsonia intracellularis* (Enterosol®), however this ceased several months prior to the study commencing sampling. Organic acids (Selko®-pH) were provided in water to piglets for three weeks from weaning, primarily to control enteric problems.

Traffic control at both Herd 1 production sites was effective, with ample signage and gates prohibiting unsolicited entry. The sites were located some distance from main thoroughfares with minimal passing traffic (the grow out site was particularly isolated, some 200m off a small dirt road surrounded by cropping areas). Boot and clothing changes were mandatory at each site, with visitors required to change when moving between sites. The herd employed a minimum 48 hours off-farm policy for visitor entry. Both production sites were within 20kms of several other pig herds. The herd exhibited mixed hygiene levels. The deep bedding and conventional dry sow sheds, service pens and grow-out shelters were relatively hygienic; it was clear that straw was effectively removed, surfaces cleaned (high pressure hosed) and disinfected (FarmFluid S™) and straw replaced between batches. The smaller farrowing and weaner rooms were in reasonably good condition, with good surfaces, clean and protected from weather and birds, these pens were also well cleaned with high pressure hosing and disinfected (FarmFluid S™) between batches. The larger farrowing shed and weaner-grower pens had relatively low hygiene standards, crates and pens were pressure hosed and disinfected (FarmFluid S™) between batches but the buildings remained heavily soiled due to the age of the buildings and quality of surfaces. Furthermore, these rooms were poorly protected from the environment and birds and evidence of rodents was apparent despite rodent control measures being in place (baiting). The bulk of scouring was observed in the less hygienic production areas.

Herd 2

Management

Herd 2 was independently managed as a multisite farrow to finish operation with sow numbers in the range 400-600 head. The herd produced pigs for slaughter only. The production sites were located within 50km of one another. At the initiation of the study the herd operated two approximately equal size sow units located within 10km of one another, piglets were weaned into a grow-out unit within 50km of the sow sites. During the course of the study the business acquired a new farrowing site. All sows were relocated to the newly acquired farrowing site between longitudinal sampling batches C and D, the final dry sow and farrowing samples were, therefore, collected from the new site. As previously, suckling pigs continued to be weaned into the grow-out unit, batch D terminal stock samples were collected there as in previous batches.

The enterprise typically employed self-replenishment (F1s) with an additional 30 gilts/year sourced from a single commercial genetic supplier breeder herd. The herd genetic supplier had been changed approximately 12 months before the first batch of samples was collected thus during the period of sampling the sow herd was of mixed genetics. The herd was being rebuilt and expanded following a reduction in the early 2010s due to financial constraints. The total sow herd increased by approximately 30% over the course of the study. The expansion was being achieved through self-replenishment and the import of new genetics, approximately 350 gilts over two years (including the period of the study). Semen was supplied from own boars housed at an independently owned and managed boar stud.

All pigs were fed on commercial pelleted complete feed from a single supplier. Mains water was employed at all sites. Losses and culls were composted a considerable distance from sheds.

Dry sows were housed in sow stalls around service then moved to group housing, approximately 50 sows per pen, before moving to crates for farrowing. The group sow housing was conventional with concrete and slatted floors. The herd maintained six boars to stimulate oestrus. Farrowing and lactation was batched and managed in conventional farrowing crates. Weaning age averaged approximately 21 days, at approximately 4.8-5.2kg. The herd had averaged over 22 pigs/sow/year, but this dropped to under 20 pigs/sow/year during the study, which may have been due to the stressors associated with relocation and a substantial disease outbreak affecting reproductive performance (described below). Piglets were weaned monthly in batches of approximately 800 head into four straw-based deep-bedded semi-circular barns, commonly referred to as 'ecoshelters' in Australia, at the grow-out site. Batches were split into four shelters, approximately 200 head per shelter. At 14 weeks growers were then moved to larger shelters where they remained to finish. Finished hogs were typically around 100-110kgLW at slaughter at 20-24 weeks of age. Hogs were delivered to an abattoir located approximately 30km from the herd's grow-out site by the

enterprise's own truck twice per week in consignments of approximately 100 head. The abattoir served both domestic and export markets.

The flows of pigs within the farm are depicted as a flow diagram (Figure 0-2).

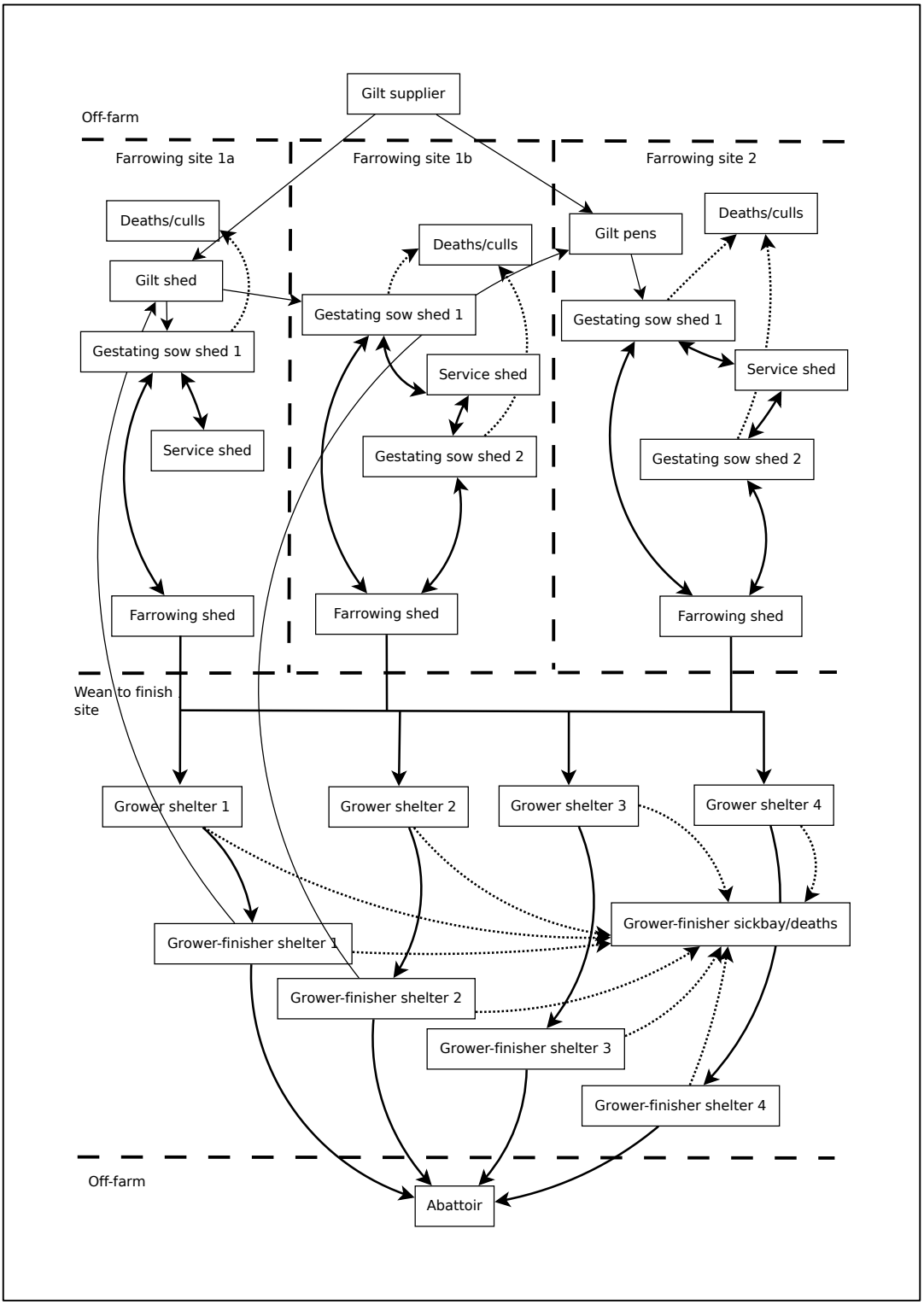

Figure 0-2. Flow diagram of animal movements within Herd 2. Farrowing sites 1a and 1b were employed up to sampling occasion C, Farrowing site 2 was employed at the time of sampling D. Thick links indicate the majority of the batch, finer links indicate gilts for replacement or sale, dashed links indicate movement of sick pigs, deaths or sow culls, as indicated. Larger dashed lines indicate different production sites.

Animal health

The herd operated at high health with low average mortality rates (<3% annual average) among pre-weaned piglets. The herd had suffered from *Campylobacter* and *Mycoplasma* problems, which were brought under control after the change of genetic suppliers 12 months prior to the first sampling. Low-level respiratory problems were observed among growers and finishers during the study. Despite vaccination PCV2 associated disease was a persistent problem. *Salmonella* spp. were isolated from faecal samples collected in mid to late 2013 during an acute outbreak of scouring, deep coughing and ill thrift among approximately 80 piglets. The affected pigs were approximately 10kg light at slaughter; the owner speculated that salmonellosis might have been associated with a PCV2 outbreak. A rodent population explosion at the grow-out site occurred in May-June 2014 which had subsided by July, an event that occurs periodically. The management responded with a large-scale baiting programme. A major herd-wide outbreak of leptospirosis occurred in June-July 2014. The disease broke out in replacement gilts, then housed at the grow-out site prior to transport to the newly acquired breeder site. It was suspected that the gilt replacements introduced the disease to the wider sow herd at the new breeder site. The epidemic appeared to have peaked in October, with approximately 50% of breeding animals affected. In response biosecurity protocols were further strengthened and animals exhibiting clinical signs were segregated.

The herd did not supplement feed or water with organic acids. Electrolytes were provided in the farrowing sheds. The farm vaccinated against PCV2 (CircoFLEX®) and swine respiratory disease (RespiSure-ONE®).

All Herd 2 sites controlled traffic effectively, and were away from main roads. Boot and clothing changes were mandatory with strong cleaning and disinfection (Virkon S®) when moving between sites. All sites employed a minimum 48 hours off-farm policy for entry, but there was free movement between the farm sites. Several other pig farms were located within approximately 20km of the production sites; there were no direct linkages in terms of staff or stock. The enterprise employed strong hygiene and biosecurity protocols, which were clearly observed during the study period. The grow-out site and the new farrowing site had relatively new shelters in excellent condition. The management employed quality pressure hosing of all shelters between animals/batches and disinfection (rotated Virkon S® or Pink Panther®; previously the enterprise had used FarmFluid S®). However, as described, the herd suffered a localised mouse plague in mid-2014. Biosecurity protocols were further strengthened between batches C and D in response to the ongoing leptospirosis outbreak. The farrowing sheds were clean and well protected from birds, and the new site was particularly secure in terms of both birds and rodents. The new farrowing shed had climate control—the only farrowing shed in the study with this capacity. However, dry sows were accessible to birds and rodents.

Herd 3

Management

The enterprise was comprised of two independently owned and managed sites approximately 100km apart: a farrow to wean enterprise and a wean to finish enterprise. The farrow-to-wean unit managed a sow herd in the range 400-600 head producing weaned pigs for fattening. The units operated in a sole supplier-client relationship during the period of the study, an arrangement that had been in place for over two years prior to the study—the breeder site did not supply any other grow-out units, and the grow-out did not source weaned pigs from any other supplier. Self-replenishment supplied the bulk of the breeding herd's gilts with an additional 120-150 gilts/year sourced from a single commercial genetic supplier herd. The replacement supplier had been changed in mid-2013, prior to the first sampling, during the sampling period the stock were of mixed genetics. The breeding herd owner intended to replace all old stock with the new genetics over a period of 3-4 years. Semen was supplied from own boars housed at an independently owned and managed boar stud. The owner of the breeder herd also raised sheep on an adjacent property and owned another sheep farm, approximately 100km distant. Only the owner engaged with both the sheep and pig enterprises, however, the pig and sheep operations shared access roads.

Both sites used mains to supply drinking water. Both sites employed on-site effluent ponds and straw composting of losses and culls.

At the breeding site dry sows were housed in groups on straw-based deep bedding during late gestation and in concrete and slatted floored stalls prior to service and in early gestation. The service shed housed a couple of boars used to stimulate oestrus. Farrowing and lactation was batched and managed in conventional farrowing crates. Average born alive was approximately 10.5. Piglets were weaned monthly in batches of 800-900 head, at approximately 25 days and around 5kg in weight. Approximately 600 were weaned directly into straw-based deep-bedded pens, lower weight piglets were selected off and placed into conventional concrete floored pens of 20-30head for one to two weeks before being moved to an equivalent deep bedded pen. All pens had concrete partitions of height 1m. Weaned piglets were managed in five pens of approximately 170 head per pen for 4-5 weeks before being transported to the grow-out operation using the enterprise's own truck.

Sows were fed on home mixed feed, while weaners received a complete commercial pelleted feed; protein feed components and pellets were each supplied from a single source, a different source in each case. The farm manager at the breeder operation was replaced between sampling batches B and C. After the change in farm manager, notable management changes occurred, including instigating substantially improved hygiene throughout the breeder herd, described below.

The Herd 3 wean-to-finish operation received batches of 800-900 growers delivered at approximately eight weeks. Upon arrival the batch was split into two batches and housed on deep bedding in poor condition. All-in, all-out batches were maintained, as pigs were not moved again until finish. Of the sheds two were conventional buildings and four were ecoshelters. Pigs were fed on home-mixed rations (pellets and mash) produced on site. A weekly delivery of 100-200 finished pigs were transported to a slaughtering facility approximately 250kms distant from the production site aged 19-24 weeks and weighing approximately 85-105kgLW. The slaughtering facility served both domestic and export markets.

The flows of pigs within the farm are depicted as a flow diagram (Figure 0-3).

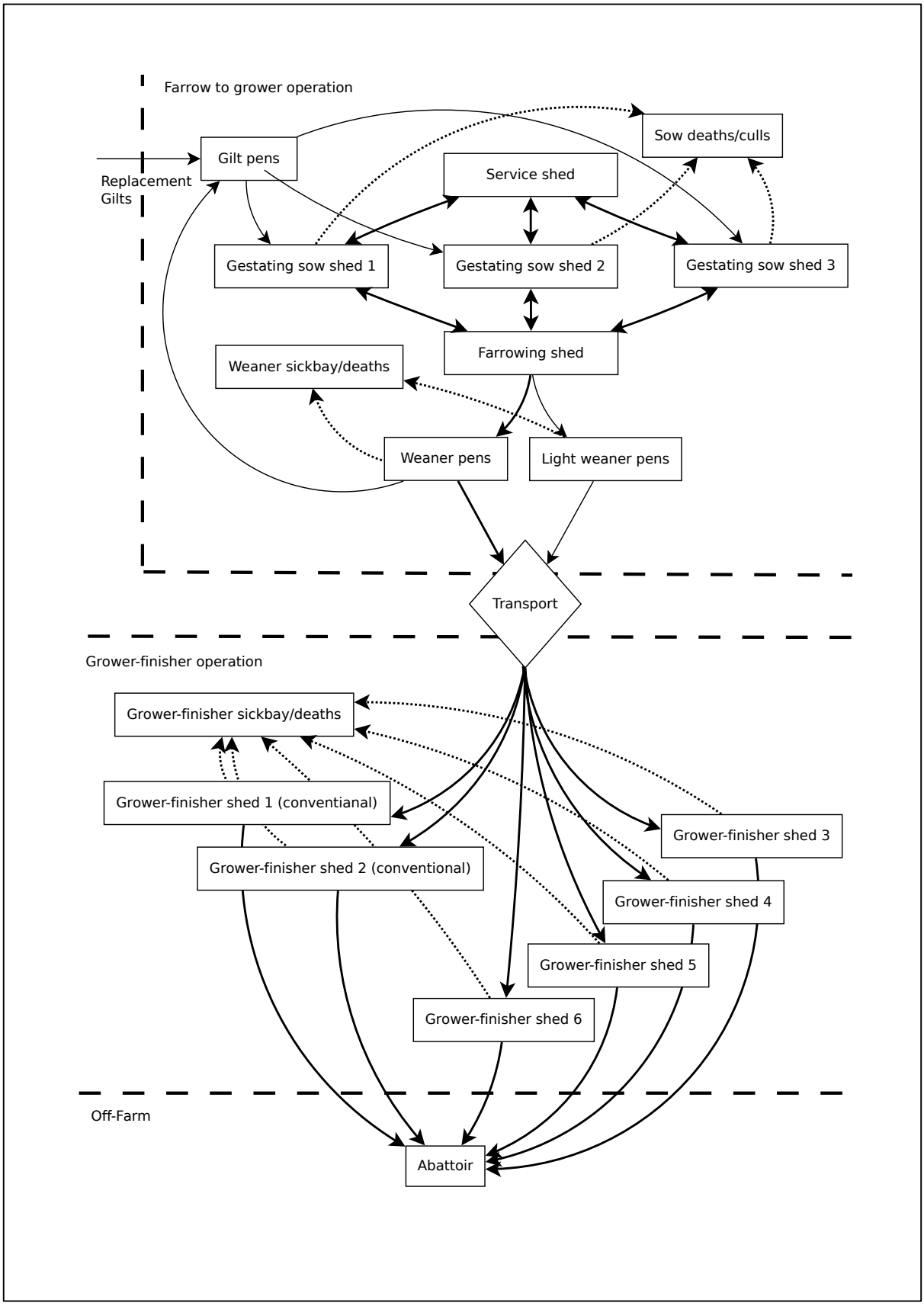

Figure 0-3. Flow diagram of animal movements within Herd 3. Thick links indicate the majority of the batch, finer links indicate gilts for replacement or sale, dashed links indicate movement of sick pigs, deaths or sow culls, as indicated. Larger dashed lines indicate different production sites.

Animal health

The breeder herd had operated at a high health status with low average mortality rates (<3% annual average) among pre-weaned piglets for a number of years, however, a disease outbreak early in the study caused a spike in weaner losses. The breeder site did not have *Mycoplasma*, dysentery and PCV2 associated disease problems; however, PCV2 vaccination was reinstated during the study period as a precaution and in response of a disease outbreak among weaners. All new stock and grower pigs were vaccinated against *Mycoplasma pneumoniae* (RespiSure-ONE®) and PCV2 (CircoFLEX®). During sampling occasions C and D sows were also vaccinated against *Pasteurella* three weeks prior to farrowing. Prior to the study the herd had experienced frequent scouring in farrowing sheds and among weaners and *Salmonella* had been isolated on several occasions. A severe outbreak of enteritis among weaners at the breeder site in which approximately 100 pigs died occurred in mid-2014. *Salmonella* was isolated from samples sourced from affected pigs during the outbreak. The management responded to the outbreak supplementing the weaner water supply with organic acids (Selko®-pH) and reintroducing the PCV2 vaccine (CircoFLEX®), having not administered the vaccine for approximately two years. Sow water had been supplemented with acids for a number of years, however, weaners had not previously received the supplement. The water at the grow-out site had also been supplemented with acids for several years prior to the study. Low-level enteric problems were reported and observed throughout the terminal herd during the study period. Ongoing acute respiratory issues were apparent at the grow-out site throughout the study period.

The breeder site operated at a reasonable standard of hygiene at the commencement of the study. Cleaning included both low and high pressure hosing and the disinfectant FarmFluid S™ was employed. Hygiene levels improved markedly with introduction of the new farm manager, between sampling batches B and C. The new management instigated high pressure hosing in all rearing areas with increased frequency and quality of application, the disinfectant was changed to chlorhexidine (HiBiTane®). Notably herd performance records indicated substantial improvements in reproductive performance and weaning rates during the six months the herd was sampled after the change in management—the owner, as opposed to the farm manager himself, presented this information. Farrowing sheds were enclosed, however, dry sow, grower and finisher sheds were accessible to birds and rodents. No indications of rodent problems were observed at the breeding site.

General hygiene levels were low at the grow-out site. Very limited cleaning was employed between cohorts of pigs, bedding was not removed and no disinfectants were employed. All shelters were openly accessible to birds and rodents, evidence of rodents was very apparent.

Traffic was controlled effectively at both sites. However, boot changes were only mandatory at the breeding site. The breeding site was relatively near several other pig herds (<20km) but was

approximately 30kms from the nearest main road. The grow-out site was closer to a main thoroughfare, but did not have much passing traffic. There were no stock person or animal linkages between the breeding herd and neighbouring herds. A minimum 24 hours off-farm policy for visitors was enforced at both sites, an exception was made for sample collection, which was conducted on a single day.

Herd 4

Management

The enterprise was a farrow-to-finish operation located on a single site managing a sow herd in the range 400-600 head. The herd produced finished hogs and also acted as an opportunistic breeder for a genetic supplier. All pigs were fed on complete commercial pelleted feed sourced from a single supplier. Drinking water was obtained from an open reservoir on the property. The herd employed on-site effluent ponds, with separate road access, and straw composting of culls.

The owners also raised a flock of approximately 6,000 ewes primarily for meat on the hill blocks surrounding the pig facility. The sheep did not have any contact with the pig facilities, however, stock trucks used the same access road. The pig farm staff did not handle sheep.

The sow herd was primarily self-replenished with occasional import of gilts, approximately 20-40 gilts once per year, from a single supplier nucleus herd for over 10 years—they also bred gilts for sale to other clients of the supplier on occasion. The gilts were quarantined for a minimum three weeks before being introduced. Semen was procured from the same genetic supplier who supplied the gilts. All gestating sows were housed in groups in one of two conventional sheds on concrete and slatted floors. Mating was batched, and the herd maintained teaser boars in the service shed. Farrowing and lactation was managed in conventional crates in a clean conventionally designed farrowing shed with mechanical convection ventilation and manual hosing with water employed in hot conditions. Crates were pressure cleaned and disinfected (Farm Fluid S®) prior to the arrival of sows. Piglets were provided with fresh creep feed daily from eight days of age. Piglets were weaned weekly in batches of approximately 190 head at, an average weight of 5.2kg at an average 23 days of age; average weaned rates were between 9.7 and 10.4, over the period of sample collection. Batches were weaned into one of four enclosed conventional rooms. The rooms were pressure cleaned and disinfected (Farm Fluid S®) prior to arrival of the batch, however, little or no vacant period was employed due to the limited availability of space. The rooms each contained six mesh-floored pens into which each batch was divided; approximately 30 head per pen. Metal grill dividers, allowing snout-to-snout contact, separated the pens within rooms. Each batch remained in a single room for four to five weeks. At approximately eight weeks of age growing pigs were moved to a weaner shed that was subdivided into pens of approximately 90 head, i.e. three weaner room pens per weaner shed pen. The shed was of conventional design and of low quality and hygiene. The stocking

density was high. The pigs had snout-to-snout contact with neighbouring pens through metal bar partitions. The pigs remained in these pens for approximately four weeks. At 12 weeks pigs were moved to a grower shed, again with metal bar partitions, in which the pen cohorts from the previous shed were maintained. After a further two weeks, at approximately 14 weeks of age, the pigs were moved to the one of two grower sheds with concrete and slatted flooring that were divided into concrete walled pens of 10-20 head per pen where they remained for a further three to five weeks. The pigs were then moved to the finishing shed, which contained the same style of concrete pens. Finally, the pigs were moved to the loading bay for transport. The farm used their own transport to move 100-200 finished pigs per week aged 21-25 weeks weighing 85-100kgLW to a slaughtering facility approximately 300kms distant. The abattoir supplied both domestic and export markets. The farm selected and marked potential gilts for replacement at weaning, they were then draughted off from the finishing shed.

Although in theory all-in, all-out batches were maintained, due to the nature of the buildings there was close contact between batches as certain points during rearing, particularly in Weaner Shed 2, where some pigs were held back or moved forward on occasion.

The flows of pigs within the farm are depicted as a flow diagram (Figure 0-4).

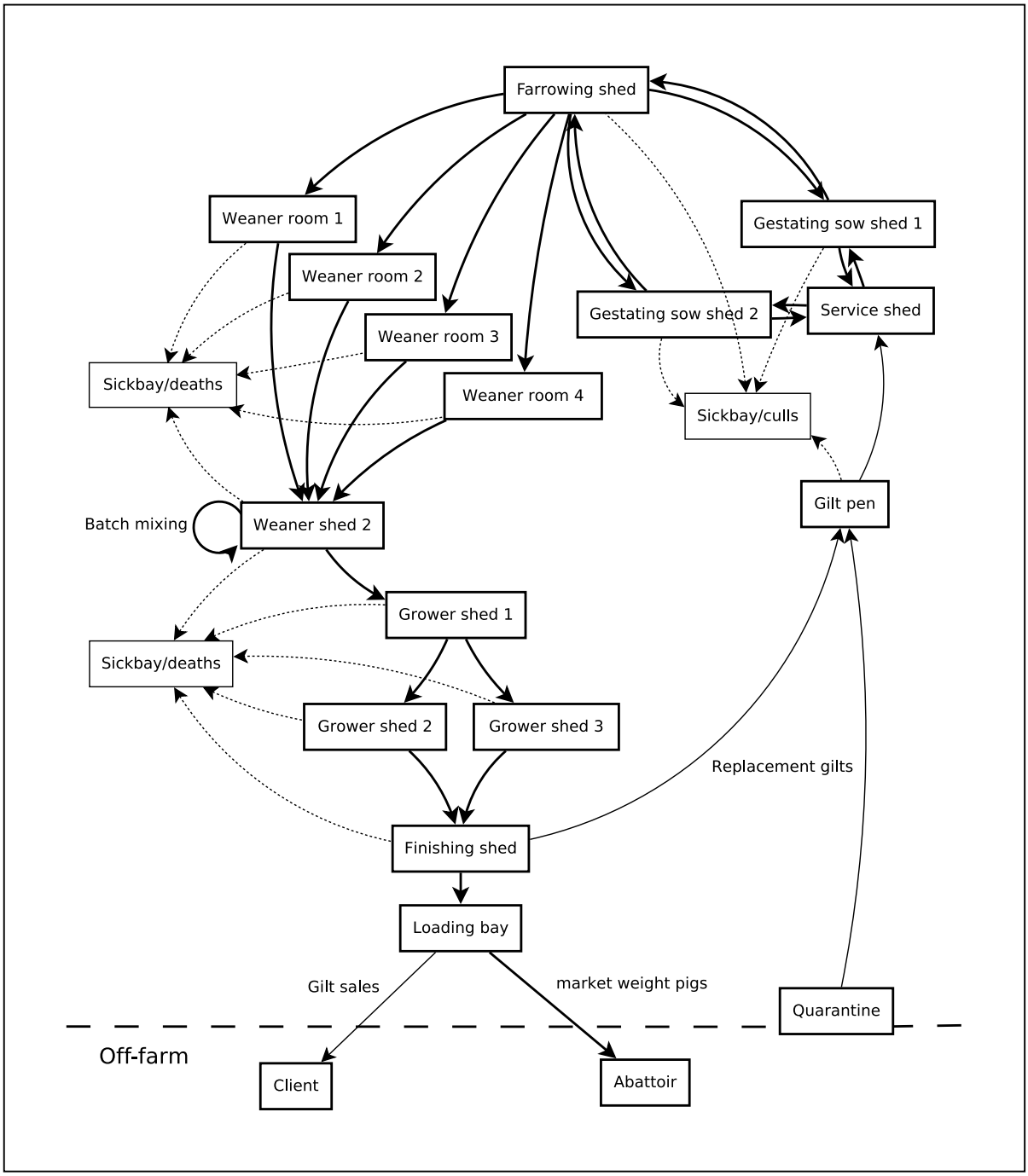

Figure 0-4. Flow diagram of animal movements within Herd 4. Thick links indicate the majority of the batch, finer links indicate gilts for replacement or sale, dashed links indicate movement of sick pigs, deaths or sow culls, as indicated. Larger dashed lines indicate different production sites.

Animal health

The herd operated at high health with low average mortality rates (<3% per annum) among pre-weaned piglets. The herd did not have *mycoplasma*, dysentery or PCV2 associated disease problems during the period of the study. The herd had undergone a depopulation-repopulation to address embedded health problems, primarily *Mycoplasma*, in 2007, which had proved effective in resolving this issue. However, the herd experienced ongoing acute enteric disease problems, primarily scouring among young pigs, that culminated in a severe outbreak of enteritis among weaners in mid-2012, in which a large number of weaner pigs died. *Salmonella* had been isolated prior to the outbreak and was identified in samples taken from pigs affected during the outbreak. In response to the outbreak, and longer-term issues relating to gut health, the management introduced organic acids to weaner and grower diets at the recommendation of the consulting veterinarian. Weaner-grower water was supplemented with organic acids (Selko®-pH, to pH=4) aged three to seven weeks. From 8-12 weeks of age feed was supplemented with benzoic acid (5kg/t).

This change occurred approximately three months prior to the study. The addition of dietary organic acids appeared to have been highly effective in controlling the clinical disease problems. Over the course of the study very little scouring was observed in any production stage within the herd. During the period of study the management and veterinarian reported ileitis (laboratory confirmed *Lawsonia intracellularis*), coccidiosis, as well as sow lameness and occasional meningitis, among their main health concerns.

The herd had mixed levels of hygiene. The gestating sow sheds, farrowing shed and weaner rooms were in good condition; clean and protected from weather and birds. However, there was evidence of rodents. Weaner Shed 2 had very low hygiene standards. The shed had limited protection from the environment, poor quality surfaces and high levels of organic matter, furthermore, it was severely infested with rodents throughout the study period—an ongoing problem the management had tried but failed to address effectively. The grower-finisher shed was, relatively, more hygienic than the grower shed, however, the buildings were old with variable surface quality and rodents were also present in high numbers. The management had tried various rodent baiting schemes without success.

The operation effectively controlled traffic onto the sites, with ample signage. The herd employed a minimum 48 hours off-farm policy for entry. Boot and clothing changes were mandatory for all visitors. The production site was relatively isolated from other pig herds, the nearest commercial herd located more than 50km away. Some small-scale/hobby production operated nearer (<10km) but there were no direct links to these operations. The farm supported several academic institutions and accepted a steady flow of veterinary and agricultural/animal science students, as well as engaging in a number of research projects.

Herd 5

Management

The enterprise operated a single site farrow-to-finish production system producing finished hogs for market. The sow herd numbered in the range 400-600 head. The management introduced 20-25 gilts every two months, 120-150/year, procured from a single supplier. Incoming gilts were quarantined at an isolated site for five to six weeks prior to introduction. Semen was supplied from a single commercial supplier, unrelated to the gilt supplier.

All feed was provided in pelleted complete rations supplied from a single mill. Drinking water was sourced from a reservoir. The herd employed on-site effluent ponds and deep composting of culls.

Dry sows were housed in groups of 5-20 head in conventional sheds on concrete and slatted floors prior to service. Teaser boars were kept in the service shed. The herd employed batched mating, once confirmed gestating sows were moved to a straw-based deep-bedded shed. Sows were moved to conventional crates for farrowing and lactation in a single farrowing room adjoining the service pens and porker shed. The herd achieved average born alive of approximately 9.5 head and 17 pigs/sow/year. The herd weaned weekly in batches of approximately 100 head. The average weaning age was increased from approximately 21 days to 28 days between sampling batches B and C to increase average weights at weaning targeting >5kg per piglet, upon recommendation from the consulting veterinarian. Piglets were weaned into one of four weaner shelters on deep straw bedding. Cohorts were maintained to 12 weeks. At age 12 weeks growers were moved to a conventional porker shed with concrete and slatted floors housing pens of 20-30 head. Porkers had snout-to-snout contact with neighbouring pens through barred partitions. At 16 weeks growers were moved to a conventional concrete and slatted floor finishing and loading shed housing pens of approximately ten head each. Finished pigs were transported to a slaughtering facility approximately 150km from the site for slaughter at approximately 22 weeks and 100kgLW using the business' own transport. The slaughtering facility supplied both domestic and export markets.

The flows of pigs within the farm are depicted as a flow diagram (Figure 0-5).

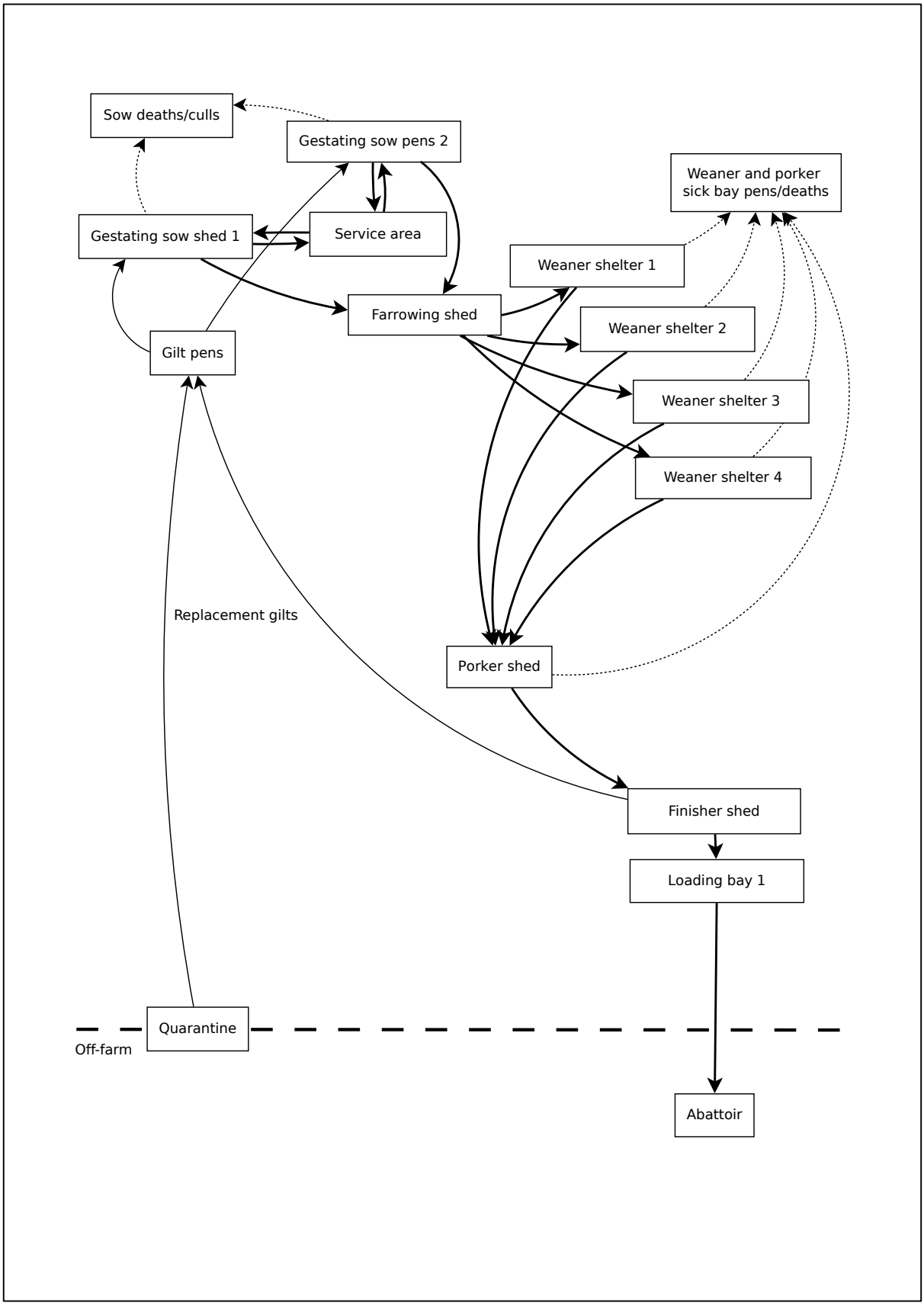

Figure 0-5. Flow diagram of animal movements within Herd 5. Thick links indicate the majority of the batch, finer links indicate gilts for replacement or sale, dashed links indicate movement of sick pigs, deaths or sow culls, as indicated. Larger dashed lines indicate different production sites.

Animal health

The herd operated at relatively high health status with low average mortality rates (<3% per annum) among pre-weaned piglets. The herd did not have *Mycoplasma*, but had experienced dysentery and PCV2 associated disease. *Salmonella* had been isolated on several occasions prior to the study. Occasional scouring was observed in farrowing sheds and among weaners during sampling. Colitis and Glasser's disease was reported from post-mortem examination of grower pigs during the study. The consulting veterinarian reported previous ileitis concerns, which had been resolved. No disease outbreaks were observed or reported during the study period. The herd vaccinated against PCV2 (CircoFLEX®) and *Lawsonia intracellularis* (Enterisol®) to control ileitis.

Herd 5 controlled traffic onto the site with signage. The pig shelters were accessible to the road, as no gate was present. However, the herd was located in a quiet area with very little passing traffic. The herd employed a minimum 24 hours off-farm policy for visitor entry. Signing in and boot and clothing changes were mandatory upon entry to the site. There were no other pig herds within 50km of the operation. The herd had mixed hygiene levels. The deep bedding and conventional dry sow sheds, service pens and grow-out shelters were relatively clean and well managed, as were the weaner-grower ecoshelters. It was clear that straw was effectively removed, surfaces cleaned (high pressure hose) and disinfected (Virkon S®) and straw replaced between batches. However, the conventional buildings were old with variable quality surfaces. Conventional sheds effectively protected animals from weather conditions and birds. Rodent baiting was employed and rodents appeared to be well controlled throughout the study period.