# The Epidemiology of *Salmonella* Transmission in Chicken Meat

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## Abstract

A longitudinal study was conducted between January 2013 and September 2014 in a vertically integrated chicken meat enterprise under commercial farming conditions. Using methods routinely used for *Salmonella* surveillance in poultry production systems, environmental sampling was conducted in two generations (parent and broiler) at multiple locations within the production system. Data was collected on all product movements during the study period and social network analysis was used to describe product movements and identify locations for the potential introduction and dissemination of *Salmonella* and targets for enhanced surveillance and intervention. Of the 4,219 samples collected thirty six percent were positive for *Salmonella*. Sixty-five percent of the isolates were identified as *S.* Typhimurium and of these isolates, 8 phage types (PT), 41 multi-locus variable-number tandem-repeats analysis (MLVA) profiles and 62 PT/MLVA combinations were detected. There was no difference between locations in the variant assemblage of these isolates and all were detected at the parent generation prior to detection in subsequent locations or generations indicating the parent site was the most likely point of introduction and dissemination to the rest of the enterprise. Phenotyping (serotyping and phage typing) and genotyping using MLVA profiling indicated that the introduction of at least 13 different *S.* Typhimurium isolates may have occurred during the study but the genetic relatedness of the variants remained unknown. Whole genome sequencing identified two major lineages of *S.* Typhimurium that were clonally disseminated between each generation and location within the study under purifying selection. Both lineages entered the enterprise at the same time and were present for the duration of the study. Single isolates of three unique *S.* Typhimurium genomes were also identified. Phenotyping failed to differentiate these isolates from the two major lineages; rather than 13 introductions, 5 introductions had occurred during the study.

Of critical importance is understanding if the *Salmonella* transmitted between generations within the poultry operation contributed to human infection for the concurrent period. De-identified human and non-human *S.* Typhimurium case submitted to the National Enteric Pathogen Surveillance Scheme (NEPSS) database for the period of the longitudinal study were obtained for comparative analysis. It was identified that, phage type and MLVA profile were poorly correlated particularly for the major phage types, which comprised greater than 90% of all isolates. The isolates from this study did not significantly cluster with the human cases, and the large number of isolates used for comparison strongly support these findings. Bovine and non-study poultry isolates were more tightly clustered with the human cases, but the remaining non-human data was so sparse that further resolution for source attribution was lost due to small sample size and the necessary aggregation of isolates from multiple sources. These results highlight the extreme limitations of using passive surveillance data to make conclusions on source attribution in the absence of strong epidemiological evidence.

In conclusion, this study identified that the structure of a vertically integrated enterprise enhanced the transmission of *Salmonella* between poultry generations and locations, even at very low prevalence, and that the hatchery is a critical point of amplification. The use of phenotyping (phage typing) and genotyping (MLVA) tools are not sufficient in the absence of good sampling (methodology or intensity) or epidemiological evidence to determine the source of introduction or dissemination within a complex environment. Whole genome sequencing allowed the genetic relatedness of the *S.* Typhimurium isolates to be elucidated and confirmed that transmission was occurring between generations within the enterprise with little to no change.

Finally, diversity and cluster analysis findings suggest that these salmonellae were not a significant source of infection to the human population during the study period. Further analysis of human and non-human isolates via whole genome sequencing is warranted to confirm these findings.


*Next: Declaration*
*It is all mine, I promise*

*Next: Preface*
*Information on publications and papers in progress. Conference attendences and talks about the content of this thesis.*



## Declaration

This is to certify that:

i. This thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. Due acknowledgement has been made in the text to all other material used.

iii. This thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signature: Helen K Crabb

# Preface

PUBLICATIONS (PUBLISHED/SUBMITTED/IN PROGRESS)

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Crabb, H. K., Allen, J. L., Devlin, J. M., Firestone, S. M., Stevenson, M. A., Wilks, C. R, Gilkerson, J. R. A comparison of *Salmonella* Typhimurium isolates using diversity analysis: human and non-human isolates. In preparation for submission to Emerging Infectious Diseases: Chapter 8

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Crabb, H. K., Allen, J. L., Devlin, J. M., Gilkerson, J. R. *The more we do the less we know: Intensive sampling fails to identify clonal dissemination of antibiotic resistance genes in poultry.* Antimicrobials 2018. 22-24 February 2018, Brisbane, Australia

Crabb, H. K. *Understanding Salmonella transmission in poultry using whole genome sequencing.* Australian Veterinary Association Conference. 13-18 May 2018, Brisbane, Australia.

Crabb H. K., Allen, J. L., Devlin, J. M., Firestone, S., Holt, K, Gilkerson. J. R. Comparing phenotyping and whole genome sequencing in und*erstanding the epidemiology of Salmonella spp. transmission in a vertically integrated broiler operation.* International Symposium on Salmonella and Salmonellosis. 06 - 08 June 2016. St Malo, France.

Crabb, H. K., Allen, J. L., Devlin, J. M., Firestone, S. M., Stevenson, M. A., Gilkerson. J. R. *The use of social network analysis to examine the transmission of Salmonella spp. within a vertically integrated broiler production system.* International Symposium Salmonella and Salmonellosis. 06 - 08 June 2016. St Malo, France.

Crabb, H. K., Firestone, S. M., Allen, J. L., Devlin, J. M., Valcanis, M., Holt, K., Gilkerson. J. R. *A comparison of serotyping, phage typing, MLVA and whole genome sequencing for describing relationships between Salmonella isolates of human and poultry origin in the food chain.* Australasian Veterinary Poultry Association Meeting, 11 – 14 October 2015, Queenstown, New Zealand.

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*What is contained herein.*



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*Next: List of Figures*
*Where to find all the Figures*



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*Next: Introduction*
*The beginning of the start of this project. The start of the journey and why we went to where we went to...*



## List of Abbreviations

| *Abbreviation* | *Description* |
|---|---|
| AGRF | Australian Genome Research Facility |
| BGA | Brilliant Green Agar |
| BPW | Buffered Peptone Water |
| $CD_{50}$ | Colonisation Dose 50% |
| CFU | Colony Forming Unit |
| CLED | Cystine Lactose Electrolyte Deficient Agar |
| CI | Confidence Interval |
| CrI | Credible Interval |
| $LD_{50}$ | Lethal Dose 50% |
| LIA | Lysine Iron Agar |
| HACCP | Hazard Analysis Critical Control Points |
| MDU | Microbiological Diagnostic Unit |
| MLVA | Multi-locus Variable-number Tandem-repeats Analysis |
| MPN | Most Probable Number |
| NEPSS | National Enteric Pathogen Surveillance Scheme |
| NNDSS | National Notifiable Diseases Surveillance System |
| ONPG | O–nitrophenyl–β–D–galacto–pyranoside |
| PT | Phage Type |
| ST | Serotype |
| SNA | Social Network Analysis |
| TSI | Triple Sugar Iron |
| XLD | Xylose Lysine Desoxycholate |

# General Introduction

This project was conceived to understand the complexities of *Salmonella* transmission within a vertically integrated poultry operation. It is not a risk factor, nor a prevalence study. This study was designed within the construct of a population surveillance strategy to maximise the detection of *Salmonella* within a poultry operation to answer a series of questions relating to the movement of salmonella within the population, indirectly addressing questions about possible points of entry and pathways of transmission.

To understand the potential sources and distribution of salmonella within an operation, the focus of these studies was to identify *Salmonella* at each point in the vertically integrated operation and compare the isolates for similarity using traditional typing tools such as phage typing and MLVA profiling. At the onset of this study whole genome sequencing was not considered feasible due to the high cost, but the rapid reduction in cost and acceptance of this tool for outbreak investigations allowed its use to be incorporated into the study. Different sampling and testing strategies would have been employed at the onset of the study if this has been known in advance.

Within a vertically integrated poultry production system multiple points of entry exist whereby *Salmonella* could enter and be disseminated within the enterprise. Very little has been published within the Australian broiler sector addressing these questions longitudinally through a structured surveillance strategy. A traceback or outbreak investigation strategy was employed with the aim of tracing the movement of *Salmonella* through the enterprise systematically via known pathways that are well described within the poultry sector.

Previously published investigations have tended to focus on parts of the system, but not the entire system at the same the same time. Many of these pathways are vertical, (true or pseudo), but the importance of these paths is rarely described or quantified. As vertical transmission of *Salmonella* such as *S.* Typhimurium is fiercely debated its relative importance as a transmission pathway is poorly understood. Most other international studies are focused on *S.* Pullorum or *S.* Gallinarum and *S.* Enteritidis and thus have more of a focus on either poultry disease and eradication, or human disease and its reduction. In Australia, *S.* Typhimurium is the most frequent cause of human foodborne salmonellosis, and poultry meat and eggs are considered the most important source of salmonellae. This presents a unique opportunity to study the transmission of *S.* Typhimurium in poultry meat without the complexities of other important vertically transmitted *Salmonella* serovars, as is the case overseas.

In Australia, poultry feed is not typically considered to be an important source of *S.* Typhimurium as it is infrequently (rarely) detected in raw or mixed feed ingredients submitted for testing. This is contrary to early reports from the mid 1970's that identified feed as an important source for the transmission of *Salmonella* including *S.* Typhimurium, but failed to describe or calculate the size of this risk at that time. Sampling of feed was specifically excluded from this study due to the complexity of the feed chain, as it requires a much greater study focus than was possible for the size of this study.

There are many points at which controls can be implemented to control the spread of disease, such as *Salmonella*, within a poultry operation and these have been well described in the literature. It is also well understood that *Salmonella* typically cause little, if any, disease in poultry and that prevalence varies over time. What is missing is an understanding of the complexity of the dynamics of poultry and poultry movements within the context of an organization at a larger scale, and the importance of detection at each point in the potential pathways so that attention can be focused on both surveillance and control options of practical importance developed within a commercial operation. As with all things, it is impossible to get the balance right and investigate all aspects of transmission in one study and answer all questions that arise.

These studies were conducted in a commercial setting, under commercial production conditions with all the limitations that arise because of this. Commercial decisions are made that impact the best laid plans and study designs must therefore be sufficiently flexible to accommodate the commercial imperative of these decisions.

Hopefully, this study enhances conversations about surveillance strategies and deepens our understanding of the limitations of our current microbiological tools for understanding this complex and fascinating microorganism.

*Next: Literature Review*
*This is where all the information you need to understand this project rests... there is a lot of it. Enjoy.*

# 1

CHAPTER ONE

# The Epidemiology of *Salmonella* spp. Transmission in Poultry and Consequences for Man: A Review of the Literature

*Introduction*
*And so the journey begins..*



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# Introduction

*Salmonella* are ubiquitous and found in all locations where people have looked for them. The presence of multiple species, subspecies and serovars, each with unique characteristics enable *Salmonella* to become host adapted or grow preferentially in specific niches, enabling the emergence of one dominant serovar to a specific host or location and enhances their discovery in more niches yet to be explored. This phenotypic or genomic plasticity and serovar redundancy means that eradication of one serovar without replacement by another to fill the niche left behind is an unlikely goal. A large proportion of the *Salmonella* genome may be involved in adaptation to a particular growth phase or stress related stimuli [1]. This has been evident with the eradication of *Salmonella enterica* subspecies *enterica* serovar Pullorum or *Salmonella enterica* subspecies *enterica* serovar Gallinarum from the poultry industry, for them to be replaced with *Salmonella enterica* subspecies *enterica* serovar Enteritidis.

The incidence of human salmonellosis associated with poultry products is considered to be a significant public health burden and the risks to human health from *Salmonella* in poultry and poultry products are well described [2-4]. As a consequence, *Salmonella* in poultry is the focus of intensive surveillance and regulatory intervention, particularly in Europe [5].

The Australian commercial poultry industry is free of both host adapted salmonellae (*S.* Pullorum and *S.* Gallinarum) and non-host adapted paratyphoid serovars such as *S.* Enteritidis and *Salmonella enterica* subspecies *enterica* serovar Heidelberg, offering a unique environment within which to study the dynamics of *Salmonella enterica* subspecies *enterica* serovar Typhimurium infection within the poultry industry. Globally until the mid 1980's *S.* Typhimurium was the most prevalent cause of human salmonellosis. Between 1988 and 2008 *S.* Enteritidis had taken over as the primary causative serovar [6] with the key exceptions of Australia and New Zealand where *S.* Typhimurium remained the predominant cause of human salmonellosis. Poultry meat and eggs are considered the most important source of foodborne outbreaks of human salmonellosis in Australia [7-9].

Studies on *Salmonella* in Australian poultry are limited and recent peer reviewed studies have not focused on the epidemiology or transmission of salmonellae in chicken meat flocks; rather these studies have focused on the pathogen in poultry litter [10-12] or in eggs or egg layers [13-17]. Studies in chicken meat have been limited to prevalence surveys at processing, farm or retail and a handful of descriptive epidemiological studies reported in the 1970's [18-21].

## 1. Biology of Salmonella

### 1.1 Introduction

Prokaryote members of the Enterobacteriaceae family, genus *Salmonella* are motile Gram-negative facultative, non-spore forming, rod shaped, anaerobes. Salmonellae are found in many environmental niches including the gastrointestinal tract of both warm and cold-blooded animals, insects, soil, plants, aquatic environments, and on a variety of surfaces [22]; thus they have been described as ubiquitous [23].

The genus *Salmonella* consists of two genetically distinct species: *Salmonella enterica* and *Salmonella bongori* [24-26]. *Salmonella enterica* is subdivided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* [27]. The subspecies are classified into serogroups based on the somatic (O) antigen and then further divided into serovars based on the flagellar (H) and the virulence (Vi) capsular K antigens [28, 29]. There are more than 2,579 *Salmonella* serovars defined, with 1,531 identified in the *Salmonella enterica* subspecies *enterica* group [30, 31], while a much smaller number of serovars (~80) are commonly associated with human disease internationally, and within each country a much smaller number again (~30 serovars) may account for over 90% of all serovars identified [30, 32].

### 1.2 Nomenclature

The taxonomy and nomenclature of the genus *Salmonella* is complex and controversial. The nomenclature is used in accordance with the

most current knowledge of the taxonomy and genetic relatedness of *Salmonella* species [24, 30, 33], and used here in accordance with the Journal of Bacteriology nomenclature.

### 1.3 Salmonella Growth and Survival

Salmonellae can replicate both in the gut of an animal, warm or cold blooded, intracellularly, bypass host defense mechanisms and not only survive but replicate in multiple environments such as water, soil, plants, internal egg contents and the surface of animate and inanimate objects (4). In general, salmonellae have been demonstrated to survive and be infective outside a host for many months (4.3, Table 1–9, Table 1–10). The resistance of salmonellae within biofilms to chemical disinfection is greater when they are older, and depends on temperature and pH [34, 35]. *Salmonella* spp. can survive high heat treatments in low water activity or high lipid content foods such as chocolate, peanut butter, nuts or animal feed [36-40]. Heat tolerance and reduction effects are dependent on the initial dose, with dose response relationships or biphasic inactivation kinetics occurring under the same conditions [41, 42].

*Salmonella* have been demonstrated to not only be absorbed passively into plants, but to infect plant material and for plants to actively mount an immune response to this invasion [43, 44].

The survival strategies of bacteria including salmonellae within different environments including the intracellular strategies for survival have been reviewed [45-47]. Survival strategies include, but are not limited to, different colony morphology, a viable non-culturable state, biofilm formation, thermal resistance and a sophisticated stress response.

In response to changes in the external environment, change of temperature, water, oxygen or other microbes, stress response regulatory systems are activated. In response to stress, particularly chronic stress, *Salmonella* can undergo morphological changes that result in improved survival in what would typically be identified as poor environmental conditions such as low pH or water activity. These conditions inhibit septation and cell division but do not stop bacterial growth or chromosomal replication. Growth continues with an increase in biomass and formation of long (>200 μm) multinucleate filaments. When conditions return to those suitable for septation, the formation of multiple daughter cells occurs rapidly, within a couple of hours [48, 49].

Stress responses appear to be regulated by key bacterial regulatory genes involved in RNA polymerase transcription and regulation such as *RpoS*, *PhoPQ*, *Fur* and *OmpR/EnvZ*. Mutation in these regulators modify virulence [50]. *RpoS* regulates the expression of at least 70 proteins including those produced in response to starvation, pH, temperature, regulation of virulence plasmid gene expression and DNA repair functions [1, 51-54]. In salmonellae, the composition of the RpoS regulon appears to differ significantly from *Escherichia coli*, and there is suggestion that a large proportion of the genome may be involved in adaptation to a particular growth phase or stress related stimuli [1].

The thermal resistance of *Salmonella* was reviewed by Doyle (2000). Heat resistance of salmonellae depends on the serovar and strain tested, experimental method, pre-treatment, culture conditions, recovery conditions and the composition of foods, media or surface attachment and the microbial complexity [36, 42].

The formation of complex biofilms on both biotic and abiotic surfaces may represent a long-term persistence strategy for environmental survival. Salmonellae within biofilms have been shown to survive chemical treatments and appear to have enhanced survival in harsh environments. Significant inter-serovar differences in their ability to form biofilms exist, but in general they form better on plastic materials than metal surfaces in processing environments and cut surfaces of plant material rather than intact surfaces [55-57].

## 2. Identification and Differentiation of *Salmonella* Isolates

Classical surveillance techniques for *Salmonella* rely on phenotypic methods such as serotyping, phage typing and antimicrobial susceptibility testing. Newer molecular techniques, such as pulse field gel electrophoresis (PFGE), the current gold standard, multi-locus variable number tandem repeat typing (MLVA), multi-locus sequence typing (MLST) and next generation sequencing have been more routinely utilized to further subtype isolates and thus inform the detection of related clusters. These newer tools have become more popular as the cost of these methods has declined. The use of these tools and their advantages and disadvantages have been reviewed [58, 59]. Key features of the major methods used for diagnostic and surveillance or outbreak investigation in Australia are highlighted below.

### 2.1 Phenotyping

Phenotyping tools include serotyping and phage typing. Serotyping differentiates *Salmonella* isolates into one of the five subspecies and to serovars within each the groups. There are 46 O antigens, and 85 H antigens resulting in ~2500 serovars within the *Salmonella* genus as described in the antigenic formulae [30, 31, 60]. In Australia, the Colindale scheme (aka Anderson) created by Felix and Callow then extended by Anderson is used for typing *S.* Typhimurium [61-63](M. Valcanis, pers comm, 2013).

Phage typing schemes were developed for the further differentiation of a limited number of *Salmonella enterica* serovars such as *Salmonella enterica* subsp. *enterica* serovar Typhi, *S.* Typhimurium and *S.* Enteritidis [63, 64].

The practice of phenotyping salmonellae for subspecies and serovar differentiation has been strongly established for many decades. However, key limitations to serotyping and phage typing exist. Both serotyping and phage typing require an extensive set of monoclonal antibodies and phages respectively to enable identification of all possible serovars. Drawbacks of phage typing are mainly associated with its practical disadvantages. Phage typing is limited to specialist laboratories and although useful in detecting outbreaks of rare or unique phage types the method is less discriminatory within phage types [65]. In addition to the presence of non-typeable isolates, phage conversion may occur (change from one phage type to another) [66]. The use of phage typing alone for outbreak investigations of *Salmonella* infections can be ineffective when a small number of phage types predominate in the geographic area [67, 68]. Consequently, typing is expensive and typically limited to a few specialised reference laboratories. In Australia, these services are provided by two public health reference laboratories; Microbiological Diagnostic Unit, Victoria (MDU) and the Institute of Medical and Veterinary Services, South Australia (IMVS). The global cessation of the supply of phage stocks has resulted in the cessation of phage typing services in Australia and the emergence of molecular typing methods such as whole genome sequencing will supersede traditional phenotyping.

### 2.2 Molecular Typing

Molecular typing has emerged as the new standard method of typing of salmonellae. Molecular typing tools have traditionally been employed to further discriminate between closely related isolates during surveillance and outbreak investigations (pulsed field electrophoresis (PFGE) and multi-loci variable-number tandem-repeats analysis (MLVA)). However, the introduction of multi-locus sequence typing (MLST) and whole genome sequencing (WGS) means that wholly molecular methods are available to both identify and discriminate between *Salmonella* isolates.

Pulse field gel electrophoresis remains the gold standard but is quickly being replaced by alternative methods such as whole genome sequencing. Multi-loci variable-number tandem-repeats analysis has been used to differentiate a limited number of serovars, *S.* Typhimurium and *S.* Enteritidis, as this can be done rapidly and does not require specific reference laboratory expertise. In practice it is difficult to ensure that all laboratories use the same naming conventions and apply the same interpretations at each of the 5 alleles so that results can be shared between states and countries [65, 69-71]. There has been urging by the Australian laboratory sector [72] for the poultry industry to embrace MLVA for serovar differentiation, but its usefulness as a typing method beyond linking isolates in an outbreak situation has yet to be established by long term epidemiological studies.

Multi-locus variable-number tandem-repeat analysis (MLVA) targets regions of high variability or hyper-variable tandem repeat regions (VNTR) within the inter-genomic regions of the *Salmonella* genome. Hyper-variable regions (highly polymorphic or exhibiting high

mutation rates) are considered ideal for discrimination between closely related isolates [69, 571]. However, homoplasy where alleles of the same size may arise by different mutational pathways (convergent evolution), may result in isolates being identified as the same or from the same source when they are not [70]. Multi-locus variable-number tandem repeat analysis (MLVA) has been rapidly introduced as an alternative method to discriminate between *Salmonella* strains and a number of schemes for *Salmonella* Typhimurium have been developed involving 5-, 7- and 8-VNTR loci [69, 571]. It is not an alternative method for phage typing.

MLVA has the advantage that it is amenable for high throughput, is highly discriminatory and repeatable is easily standardized between laboratories and particularly useful for discrimination between clones in outbreak situations [69]. It has been shown to have high discriminatory power between highly monomorphic *Salmonella* strains such as *Salmonella* Typhimurium DT104 [571]. The limitations of MLVA are that it is currently only able to be used for *Salmonella* Typhimurium isolates, it does not correspond well to current phage typing systems, there is reported instability of MLVA patterns and isolates may be incorrectly grouped. Additionally results may not be comparable between laboratories unless methodology and equipment, and reporting arrangements are standardised [65, 69, 70, 71].

Multi-locus sequence typing uses seven highly conserved housekeeping genes to identify Salmonella isolates. The seven loci are identified and sequenced from PCR products and the resulting sequences are designated into identified sequence types (ST) that cluster similarly related sequences into ebursts or genetically related groups. It can be automated and is aimed primarily at identification and grouping of isolates similarly to serotyping and not for fine resolution investigation of outbreak tracing [613]. MLST can be computed from next generation sequencing results and is typically incorporated into NGS pipelines as part of Salmonella identification.

Next generation sequencing of the whole *Salmonella* genome is rapidly being established as the future of *Salmonella* surveillance. Public health laboratories in Europe, such as Public Health England, and the Communicable Disease Centre and Food and Drug Agency (CDC/FDA) in the USA, have stopped phenotyping and are now using whole genome sequencing as the first line of typing for surveillance activity [73, 74]. Isolates are compared by genetic similarity according to either single nucleotide polymorphism (SNP) differences or core gene analysis, and the formation of phylogenetic trees to determine their genetic relatedness [75-79]. Further analysis by evaluating the pan-genome and comparison of the accessory genome is useful for establishing more detailed relationships between isolates. The biggest practical issue arising from the use of whole genome sequencing isolates relates to naming conventions. Differentiation or identification of individual isolates and the difficulties in reporting whether they belong to the same groups or sources, particularly in surveillance reports is a big issue. It is impossible to compare isolates with general surveillance data unless access to all genomic data is freely available [74]. Next generation sequencing, while quickly gaining acceptance as a standard surveillance tool, requires sophisticated analysis. This situation is changing as fast as the sequencing tools are being developed. However, they are currently beyond that of routine diagnostic or quality assurance laboratories particularly those in industry.

## 3. Epidemiology of *Salmonella* in poultry

It was recognised in the early 1940's that poultry are susceptible to many non host-adapted salmonellae from all serogroups [80, 81], commonly referred to as paratyphoid infections in poultry veterinary medicine [82]. Non-host specific salmonellae such as *S.* Typhimurium or *S.* Enteritidis are common causes of infection in poultry and may cause clinical or sub-clinical disease in susceptible poultry, and are considered important pathogens of poultry from a public health perspective [9]. The commercial Australian poultry industry is considered to be free of *S.* Enteritidis [83].

### 3.1 Pathogenesis of Salmonellosis in Chickens

The infectious dose, consequence of infection [84], pathogenicity [85, 86], and host specificity of salmonellae infection in chickens varies with serovar [86, 87], between strains or phage types of the same serovar [87-90], between routes of infection [88] and age at challenge [87, 91]. Different isolates of the same or similar serovars may behave differently, both in-vitro and in-vivo, with regard to tissue invasion [92], environmental survival, tolerance to heat and pH [92, 93].

Table 1–1. Infectious Dose of *Salmonella* for Chickens via Different Routes of Infection

| *Serovar [Ref]* | *Chicken Type* | *Age* | *Route of Infection* | *Infectious Dose (CFU)** |
|---|---|---|---|---|
| *S.* Typhimurium (23 serovars tested) [88] | Layer | 1 day | Oral | 4 - 5 |
| | Broiler | | | 9 (all serovars) |
| | | 7 weeks | | $10^8$ |
| *S.* Typhimurium [94] | Layer | 1 day | Intra cloacal | $LD_{50}$ 2.5 x $10^1$ |
| | | | Oral | $LD_{50}$ 1.2 x$10^5$ |
| *S.* Typhimurium (9 other serovars) [100] | Layer | 1-2 days | Aerosol | ~20 |
| *S.* Enteritidis [101] | Layer | 17 weeks | Conjunctiva | $10^2$ |
| *S.* Enteritidis PT4 [102] | Layer | 1 day | Airborne | 1 $\log_{10}/m^3$ |
| *S.* Typhimurium [96] | Broiler | 1 day | Oral | $10^2$ $CD_{50}$ $10^{2.4}$ |
| | | | Intra cloacal | 2 $CD_{50}$ $10^{0.6}$ |
| | | 3 days | Oral | $10^2$ $CD_{50}$ $10^{4.5}$ |
| | | | Intra cloacal | $10^1$ $CD_{50}$ $10^{2.6}$ |
| *S.* Typhimurium [103] | Broiler | 1 day | Oral | $10^2$ - $10^3$ |
| | | | Trachea | $10^2$ |
| | | | Eye | $10^2$ |
| | | | Cloaca | $10^2$ |
| | | | Navel | $10^3$ - $10^4$ |
| | | | Aerosol | $10^2$ - $10^3$ |
| *S.* Typhimurium [84] | Layer | 2 day | Oral | 100 |
| | | 1 week | | $10^2$ - $10^4$ |
| | | 2 week | | $10^2$ - $10^6$ |
| | | 4 week | | $10^6$ - $10^8$ |
| | | 8 week | | $10^8$ - $10^{10}$ |
| *S.* Typhimurium [104] | Broiler | 7 days | Intra tracheal | 1.5 x $10^4$ |
| *S.* Typhimurium [105] | Layer | 17 weeks | Aerosol | 2.8 x$10^2$ |
| | | | Oral | 5 x $10^6$ |

*CFU colony forming units, $CD_{50}$ Colonisation dose 50%, $LD_{50}$ Lethal dose 50%

#### 3.1.1 Infection and Route of Entry

As with all gut borne pathogens the oral-faecal route is an important pathway of transmission, but experimental evidence shows that poultry are also susceptible to infection with *Salmonella* via the eye (airborne), the respiratory tract and percloacally. A single organism may be infectious to a day-old chick by any of these routes (Table 1–1). The infectious dose appears to be unaffected by poultry type (layer or meat breed) when studied together [84, 88]. A strong relationship between age and infectious dose exists in poultry; as poultry age, they quickly become less susceptible to infection and require an increased dose of salmonellae to become infected [84, 88, 91, 94-96]. Poultry may also become infected via vertical

or pseudo-vertical transmission, via the egg, from breeder to progeny. Systemic infection of laying hens may result in invasion of reproductive tissues and colonization of different regions of the reproductive tract [97]. Additionally it is thought that "cloacal drinking" may enable the ascension of salmonellae into the reproductive tract via the cloaca enabling reproductive tract colonisation and subsequent contamination of the egg prior to lay [98, 99].

### 3.1.2 Colonization, Localization and Systemic Infection

There are observable differences in the site of phagocytosis [106], localization and colonization of tissues by different serovars and between strains of the same serovar [107].

#### 3.1.2.1 Colonisation and Infection

Within a day of infection, *S.* Typhimurium are visible on the mucosal epithelial surface, and the lamina propria of the caecum with marked heterophilic and mononuclear cell invasion into both the lamina propria and intestinal lumen. This is serovar dependent. Rapid phagocytosis of *S.* Typhimurium is reported and organisms were observed both within vacuoles and macrophages within the epithelium having migrated through the intestinal epithelial mucosa within a day [106]. In-vitro studies confirmed that *S.* Typhimurium was more adhesive and invasive to avian epithelial, ovary and kidney cells than *S.* Pullorum. Macrophage cells phagocytosed salmonellae within an hour of exposure and replication occurred within the phagosome. *S.* Typhimurium was able to survive longer after phagocytosis in both heterophils and macrophages [106].

Polymorphonuclear cells and monocytes appear quickly within the lumen of the intestine, caeca and bursa and appear to be important for clearing of salmonellae [106].

In *S.* Typhimurium, type 3 secretion systems (T3SS) present on *Salmonella* pathogenicity islands (SPI) 1 and 2, assist the colonization and tissue invasion of chicken epithelial cells. T3SS on SPI1 contributed to colonization of the caecum and spleen while SPI2 was only important in colonization of the spleen. Without SPI1, the presence of only SPI2 inhibited caecal colonization indicating SPI2 may be an important determinant for host specificity [108]. The role of T3SS in cell invasion has been reviewed in more detail [109].

#### 3.1.2.2 Systemic Dissemination

Differences between serovars in their ability to invade beyond the intestine have also been demonstrated [87, 94, 110, 111]. *Salmonella enterica* subsp. *enterica* serovar Infantis may penetrate the epithelial layer to the sub-epithelial region, while *S.* Typhimurium spread to the lamina propria basement membrane of the caecal mucosa [110].

After epithelial tissue invasion, salmonellae infected macrophages [110] are quickly detectable within the intestinal mucosa, bursa and liver, suggesting their importance in tissue dissemination [106]. The dissemination to multiple organs occurs rapidly within the first 24 hours to 2 days [94, 112-114] post infection and appears to vary both within strains of the same serovar and between serovar types [112], however dissemination does not appear to be dose nor route of administration dependent [87, 100, 115]. For example when infected per-cloacally proliferation in the bursa, caecal tonsils and liver had occurred as quickly as 1 [112] to 12 hours [94] post infection. It is speculated that rapid dissemination may be assisted in part by bursal lymphocytes [112]. Bacteraemia due to *S.* Typhimurium was not detectable till 2 to 3 days post oral infection, with little to no bacteraemia detectable by week 3 [87, 88, 100].

#### 3.1.2.3 Tissue Localization

After entry into the host, salmonellae colonize the distal ileum and caecum [95], but not until ~24 to 48 hours after inoculation [116]. However primary sites for localization after initial infection appear to be serovar specific. *S.* Pullorum rapidly located to and remained in the Bursa of Fabricius, while *S.* Typhimurium preferentially remained in the intestinal tissue [106].

A primary role of the caecum in the maintenance of intestinal infection with *S.* Typhimurium has not been established. Birds with caeca removed, shed *Salmonella* at higher rates and for as long a period as birds with intact caeca, however the caecal tonsil may be important [116]. Preferential localization of *S.*



Table 1–2. Concentration of Salmonellae in Faeces, Caeca and Crop Content of Infected Chickens

| *Serovar [Ref]* | *Sample Type* | *Concentration* |
|---|---|---|
| *S.* Typhimurium [128] | Caeca | 2 $\log_{10}$ /ml |
| | Faeces | 1 $\log_{10}$ - 2 $\log_{10}$/g |
| *S.* Typhimurium [129] | Faeces | 5 x$10^6$ /g |
| *S.* Typhimurium [130] | Caeca | 2.66 +/- 2.0 $\text{Log}_{10}$/g - 5.75 +/- 1.1 $\text{Log}_{10}$/g |
| *S.* Typhimurium [107] | Faeces | 5.4 $\log_{10}$ /g (5wk)<br>1.8 $\log_{10}$/g (8wk)<br>2.6 $\log_{10}$ CFU/g (10wk) |
| *S.* Infantis [131] | Caeca | $10^6$- $10^8$ /g |
| | Crop | $10^3$ - $10^6$ /g |
| | Small Intestine | $10^2$ - $10^6$ /g |

Typhimurium in the caeca were not dependent on flagellar (H) or somatic (O) antigens, haemagglutinins nor the presence of the virulence plasmid and appears to be due to non-specific or host factors [117].

*S.* Typhimurium is capable of significantly colonizing the liver [87, 110, 115] spleen and lungs [87, 100, 103], thymus tissue [112] and the bursa of Fabricius [95], while *S.* Infantis demonstrated the same capabilities but to a lesser degree [110, 112]. Persistence and subsequent recovery from internal organs appears to vary between serovars, with many tissue infections apparently cleared within a short time ~3 weeks post infection [87, 88, 112], while others persist for a longer period, up to many months [116].

#### 3.1.2.4 Clinical Signs and Pathogenicity

Infection and clinical presentation is affected by the age of bird, dose, route of infection, serovar and serovar variant and minor differences or disagreements between studies can typically be demonstrated to be due to differences in study design, variation between serovars, strains of the same serovar and phage type [86, 91, 107]. Clinical signs may consist of transient diarrhoea with or without mortality. Mortality varies from no or little mortality to significant mortality (up to 100%) with rates varying between batches of birds infected with the same organism by different routes [84, 87, 88, 95, 100, 114, 118-120]. Clinical signs associated with *S.* Typhimurium infection are frequently reported as only being associated with young chicks (< 2 weeks of age) with little to no clinical signs in older birds [84, 88, 91].

#### 3.1.2.5 Excretion

*Salmonella*e are readily detected in cloacal swabs within 1.5 to 24 hours of oral inoculation [84, 87, 88, 116]. The concentration of excreted salmonellae (Table 1–2) is initially high immediately post infection and decreases slowly, with older challenged chickens excreting lower concentrations of organism than younger birds [84, 95]. The higher the initial challenge dose the higher the rate of excretion or percentage of chickens positive [121]. In general the rate of excretion, both the number of birds shedding and the amount of bacteria shed, declines as birds get older, regardless of the initial infectious dose [117]. The duration of excretion varies depending on the serovar, strain and phage type, the initial infectious dose or challenge, the individual bird and poultry species (chicken or turkey), with persistence for many weeks or months [84, 88, 89, 91, 122-125].

A specific characteristic of infection is that poultry and other species may shed salmonellae intermittently. The duration of shedding appears to be dose and age dependent [126]. After periods of stress such as moult, feed or water deprivation, asymptomatic recovered birds that were *Salmonella* negative at the time of stress may commence shedding post the stress event. A stress event may not be needed for this phenomenon to occur and may sim-

Table 1–3. Salmonellae Contamination of Tissues after Primary and Secondary Infection ($log_{10}$ CFU/g) [123]

| *S. Typhimurium F98* | *2d* | *6d* | *10d* |
|---|---|---|---|
| Caecal content (1° infection) | 5.82 (+/- 0.29) | 7.45 (+/- 0.49) | 4.61 (+/- 0.70) |
| Caecal content (2° Infection) | 4.82 (+/-0.61) | 3.89 (+/- 1.01) | 0.49 (+/- 0.049) |
| Spleen (2° Infection) | 3.03 (+/- 0.05) | 3.23 (+/- 0.60) | <1 |
| Liver (2° Infection) | 3.32 (+/- 0.09) | 2.16 (+/- 0.62) | <1 |

ply be a feature of the infection [127].

### 3.2 Immunity

The immunobiology of systemic salmonellosis in poultry has been reviewed [132], and while concentrating on *S.* Pullorum and *S.* Gallinarum, compares the immune response to systemic infection with *S.* Typhimurium and *S.* Enteritidis. Immunity to salmonellae in poultry involves complex innate, humoral and cell mediated responses that are affected by the age at which the bird is first infected [113]. The serological response appears to vary between birds, with intestinal carriers or shedders frequently not detectable by serology [133]. Importantly while immunity is effective in preventing extensive systemic infection, and reducing the quantity of salmonellae found in the caecal content or tissues, the duration of shedding or tissue invasion, it is not fully protective, and it is possible for birds to be re-infected despite apparently protective immunity, and these birds then shed a significant quantity of salmonellae [95, 123].

The chicken immune system does not fully mature until some weeks after hatch Initial maturity of the Peyer's patches, caecal tonsil, expansion of the lamina propria and changes in gut associated immune cell composition (B, T cells) occur in the first 7-10 days post hatch [134, 135], with full maturity and immunocompetence occurring later, 6 to 9 weeks post hatch [123, 135-138]. Heterophils are present in the blood at day old but increase in phagocytic ability as the birds age, doubling in ability after day 7 [139]. A delay in feeding post hatch (24-72 hours) delayed the colonisation by T and B lymphocytes of gut associated lymphoid tissue in the caeca and bursa for two-weeks post hatch [140].

#### 3.2.1 Innate Immunity

The innate immune system of chicks at hatch is capable of recruiting leukocytes to the gut [141]. Pattern recognition receptors are central to the initiation of host immune response by recognition of the invading pathogen. In avian species, a number of toll like receptors (TLR) have been identified including, TLR-4 and TLR-5 that respond to the invasion of salmonellae. The TLR-5 receptor responds to flagellin and up-regulates Interleukin (IL-1B) responses limiting systemic infection by flagellated *Salmonella* serovars [142], while TLR-4 recognises the LPS in a complex multi-protein process. In addition the T3SS-1 and T3SS-2 structural proteins are also recognised by TLR-4 and TLR-2 [143]. Receptors and their roles in the initiation of the innate immune response to *Salmonella* infection have recently been reviewed [43].

In day old chicks infected with *S.* Typhimurium chemokine and cytokine levels, interleukin (IL-6, IL-8, IL-1α), chemokine (K60), and macrophage inflammatory protein 1B (MIP-1B), are up-regulated in both intestinal tissue and liver, but not in the spleen[113]. Expression of these chemokines and cytokines was correlated with the degree of inflammatory response with a subsequent invasion of heterophils to the intestine and caeca and associated inflammation and pathology [106, 144]. Responses are less rapid in the liver and spleen, with K60, IL-8 and MIP-1B first detectable in the first 12 hours, while bacteria are detected within 24 hours and may drive early hepatosplenomegaly [106]. These responses are similar to those found in mammals [145]. In 1-week-old birds, levels of proinflammatory cytokine and chemokine expression are not increased dramatically like in 1-day-old chicks [145]. Only inflammation and an influx of heterophils were detectable clinically and this may have been inhibited by the expression of TGF-B4 in the intestines. IL-6 is expressed later in the ileum and caecal tonsils and may be associated with the maturity of lymphocytes or macrophages rather than a primary inflammatory response [113].

#### 3.2.2 Passive Immunity

Maternal antibody may be transferred to chicks via the yolk. High levels of antibodies (IgY) are detectable in the yolk, and subsequently in hatched chicks, which is detectable for a period of ~2 weeks post hatch [146]. Maternal immunity is high in intestinal and caecal content at day 2 post hatch but declines over time and is substantially reduced by 9 days of age [147].

#### 3.2.3 Adaptive Immunity

##### 3.2.3.1 Humoral response

The humoral response to infection with *S.* Typhimurium depends on the age of the bird infected. The lack of immediate antibody response in young chicks is due to immaturity of T lymphocytes, as their maturity does not occur until ~2 weeks of age [134]. The humoral response is more rapid in older birds than in younger chicks [95], likely reflecting maturity of immunological system [113].

Following primary infection, immunoglobulin M (IgM), IgG (a.k.a IgY in poultry) and IgA antibodies are produced. High transient IgM is detectable from 6 days, with all birds seroconverting within 20 days post challenge. A subsequent rise of IgG and IgA peaks at 4 and 5 weeks post infection. IgG was detectable and persistent after clearance of bacteria from the GIT. Between 5 and 9 weeks post infection titres dropped with IgG titres persisting longest. In young birds IgA levels were not observed until 26 days post infection but were reported within 20 days in the older birds and was persistent post resolution of infection [95, 113, 123].

After re-infection a low, transient response was detectable to IgM, and IgG, with a rise in IgA. The associated IgA response is consistent with a mucosal associated response to clearance of *Salmonella* from the GIT [113]. The degree of immunity following secondary challenge was significant but not complete and varied between chickens [95]. The gastrointestinal tract was able to be recolonized, but shedding occurred for a shorter duration of time, < 10 days, but birds were not systemically infected [113]. The quantity of salmonellae detected in various tissues after secondary challenge is tabulated in Table 1–3 [123]. The importance is to demonstrate that while immunity is effective in preventing extensive systemic infection, and reducing the duration of both the quantity of salmonellae in the caecal content and tissues, it is not fully protective. Thus, indicating that it is possible for birds to be re-infected, and excrete a significant quantity of salmonellae despite apparent protective immunity.

Serological response to *Salmonella* infection varied by infectious dose with high levels of between and within group variation in response, regardless of infectious dos. The serological response was not correlated with terminal isolation of salmonellae [84, 114].

Serological response appears to vary between birds, with intestinal carriers or shedders frequently not detectable by serology (serum agglutination with *S.* Typhimurium somatic antigen) in one study, with only 13.7%, of carriers positive by serology (11 /80)[133].

It is important to note that the type of serological test (direct agglutination, indirect agglutination or ELISA) may be responsible for the apparently conflicting results reported in the literature and serological testing may be more suited to testing of flocks rather than indi-

Table 1–4. Feed Contamination (concentration of organisms per g) with *Salmonellae*

| *Location [Ref]* | *Salmonella Concentration* | *Number of Salmonella Serovars* | *Feed Type* |
|---|---|---|---|
| USA (1969) [156]. | 2.3 - 4.3 MPN[1]/ g | NS[2] | NS |
| Queensland (1980/1981) [157, 158] | < 1 - 147 MPN / 100 g | 37 | Finished feed |

[1]MPN: Most probable number, [2] NS: not specified or reported

vidual birds [114]. Cross reactivity to serovar antigens may occur and as a result specificity may be reduced [148]. Some treatments (e.g. furazolidone) may inhibit serological response to infection [114].

#### 3.2.3.2 Cell Mediated Immune Response

The invasiveness of salmonellae to the deeper regions of the lamina propria determines the character and degree of the cell-mediated immune response. The caecal immune response is characterised by a cellular influx of granulocytes and T cells. The size, day to peak and plateau of the response varied by serovar invasiveness with *S.* Enteritidis > *S.* Typhimurium > *S.* Infantis. Despite the differences in invasive capability, colonization within epithelial cells was associated with increases in Interleukin-2 (IL-2) and IL-2Rα and CD4-T cell influx [110].

Clearance of a primary infection involves an interferon (IFN-γ) mediated Th1 response. Increased expression of IFN-γ in the liver 7 to 14 days post infection is associated with increased T cells and lesions in the liver. CD8+, and CD4+ T cells are involved in lesion formation, including mononuclear cells, and are likely attracted by high MIP-1β expression from day 3 post infection. T cells decline in the spleen and are trafficked to the intestines [113, 123], where there are still high numbers of bacteria. Following re-challenge, there is little inflammation or chemokine or cytokine response, other than IL-6 increase in the intestinal tissue and MIP in the caecal tonsil and ileum. In older birds (10 weeks) there was little inflammatory or cytokine and chemokine response to infection, supporting the role of mature T cells in regulating pathogen induced inflammation [113].

## 4. Horizontal Sources of Salmonellae to Poultry.

Horizontal sources of salmonellae introduction to poultry include feed, vermin, equipment, litter, and environmental contamination between flocks.

### 4.1 Feed

Feed has been recognised as an important source of salmonellae to poultry since at least the 1940s. A small number of field studies have established the temporal correlation between feed as a source of poultry infection and subsequent vertical transmission between generations of birds [18-20, 149-151]. The contamination of feed is difficult to assess because rates of contamination are typically low (number of organisms per gram of feed) and contamination tends to be heterogeneously distributed within large quantities of feed (Table 1–4). *Salmonella*e may persist for extremely long periods of time (years) in seemingly adverse conditions in feed [36-40, 152-155].

#### 4.1.1 Sources of Feed Contamination

The sources of feed contamination have been extensively reviewed and include wild birds or rodent contamination of raw product, cross contamination during harvesting with shared equipment and contaminated by-products [159, 160]. Contaminated ingredients include those derived from animal proteins, such as meat and bone meal, fishmeal, and vegetable protein sources including cereal grains [149, 151, 153-155, 161-167]. Points of contamination of raw feed ingredients include pre-harvest, post-harvest during storage and/or transport, during processing or milling and post-processing during storage and/or transport. The internalization of salmonellae into plants has been reviewed and its role in foodborne illness examined but not with regard to contamination of feed for animal consumption [168-170]. In compound feeds, mixing of different sources of raw feed ingredients pose risk to final products depending on the type of raw feed ingredients, and cross contamination during milling, handling, storage and transport of the finished product [171]. Some feed ingredients may be handled multiple times prior to final shipment to the end user [150]. In Australia, shipping of cereal grain from farm may involve at least two or three handling steps on and off farm prior to delivery to the feed mill; Harvester to truck or temporary paddock storage, on-farm storage, delivery to bulk storage or delivery to feed mill [172]. Of particular note, is the use of ground storage in bunkers that is a unique feature of grain storage developed in Australia (J. Owens pers. comm.) This method of storage has never been reviewed or investigated as a potential

Table 1–5. Infectious Dose of Salmonellae in Artificially Contaminated Feed Sufficient to Colonise Poultry

| *Salmonella serovar [Ref]* | *Age* | *Feed Contamination Rate (organisms/g feed)* | *Number of Animals or Units Infected* |
|---|---|---|---|
| *S.* Kedougou & *S.* Livingston [125] | Day old | 0.1 | 25% groups<br>3-6% individuals |
| | | 100 | 100% groups<br>35-97% individuals |
| *S.* Montevideo [174] | Day old | 0.8 | 10.5% individuals cloacal swab<br>100% caecal tonsil |
| | | 0.04 | 20% individuals caecal tonsil |
| | | 5.0 | 70% individuals cloacal swab<br>86% caecal tonsil |
| *S.* Menston [173] | 1-3 weeks | 0.5 - 1 | 1.9 - 4.8% carriers |
| | 9 months | 1 | 6.7% eggs contaminated |

Table 1–6. Feed Sample Prevalence of *Salmonella* spp., Australia

| *State [Ref]* | *Year* | *Feed Type* | *Percentage of samples positive (%)* | |
|---|---|---|---|---|
| | | | *Salmonella spp.* | *S. Typhimurium* |
| Victoria [182] | 1960 | Bonemeal | 91 | Not reported |
| Queensland [19] | 1974 | Wheat | 35 | 35 |
| | | Sorghum | 23 | 7 |
| | | Maize | 30 | <1 |
| | | Finished Feed | 23 | 21 |
| Queensland [158] | 1980-1981 | Mash | 58 | 2 |
| | | Pellets/Crumble | 11 | |
| Queensland [183] | 2002 | Raw Feed Ingredients | 78 | Not reported |
| New South Wales [178] | 2010-2011 | Finished Feed[1] | 17 | 1 |
| | | Bulk Feed | 11 | 0 |

[1]At point of consumption

Table 1–7. Vermin and Vermin Faeces Contamination with *Salmonella* spp.

| *Reservoir [Ref]* | *Infectious Agent* | *Concentration* | *Infectious Period* |
|---|---|---|---|
| Gulls [200] | Faeces | 0.18 - 191 MPN / g | – |
| Cockroaches [187, 188] | Insect | $7.2 \times 10^5$ / insect | 20 - 44 d<br>60 d post death |
| | Faeces | $1.6 \times 10^7$ | 4 - 7d |
| Soil Nematode [185, 186] | Nematode | 10,000 / worm | – |
| Mealworm [201] | Larvae | $50 - 4 \times 10^5$ | 28 d |
| *Carcinops pumilio* [202] | Faeces | – | 14 d |
| Mice [203] | Faeces | $2.3 \times 10^5$ CFU / pellet | – |

– Not reported

source of *Salmonella* contamination of cereal feed grains in Australia.

### 4.1.2 Infectivity of Contaminated Feed to Poultry

Birds are susceptible to very low infectious doses of *Salmonella* spp. (Table 1–5) found in feed and even at a single organism per gram of feed, intermittently fed over a long period of time birds became infected [173]. Even at 1 organism per gram of feed the quantity of feed consumed by a hen (16-20 weeks) at the onset of egg production may be as much as 100g of feed per day and, as such, it would not take long for them to consume a sufficiently large infectious dose, while they are still young.

### 4.1.3 Feed Contamination in Australia

The importance of poultry feed as a source of *Salmonella* spp. to poultry has also been recognized for many decades in Australia [18, 20, 21]. Jackson et al (1971) described the transmission of *S.* Typhimurium through a vertically integrated poultry organization via feed contaminated with by-product (offal meal) produced from a known *Salmonella* infected flock. Offal- meal contaminated with *Salmonella* was the likely source of the introduction. McKenzie and Bains (1976) described the initial detection of *Salmonella* serovars in raw feed ingredients and the subsequent detection of the same serovars through a broiler production pyramid correlating feed sources with those detected in subsequent production steps (breeders, broilers) over several production cycles [19]. It was reported that 14 of the 17 *Salmonella* serovars detected were identified in the grain constituents of breeder feed: wheat, sorghum and maize [18]. *S.* Typhimurium was detected more frequently in wheat than maize, and was associated with clinical disease in broilers and detected in broiler carcass wash.

In Australia, key poultry feed ingredients are wheat/barley (cereal grains), animal and vegetable proteins [175]. Due to the ruminant feed ban implemented to control the spread of bovine spongiform encephalopathy (BSE), only monogastric species (pigs, poultry, cats and dogs) are permitted to eat feed containing ruminant animal protein products (restricted animal materials or RAM) such as meat and bone meal or meat-meal [175, 176]. Structured national surveillance of feed is not conducted in Australia and only passive laboratory surveillance or *ad-hoc* survey reports are available (Table 1–6). As with earlier studies, multiple different serovars continue to be detected in feed and feed ingredients and the prevalence of *S.* Typhimurium appears to have declined [157, 158, 177, 178]. In the first half

Table 1–8. Vermin Contamination with *Salmonella*

| *Reservoir [Ref]* | *Salmonella positive (%)* | | | *Salmonella serovar* |
|---|---|---|---|---|
| | *Internal* | *External* | *All* | |
| Lesser Meal Worm (*Alphitobius spp*) [197] | 5 | 43 | 14 | *S.* Othmarchen<br>*S.* Orangienberg |
| American Cockroach (*Perplaneta Americana)* [197] | 0 | 57 | 29 | *S.* Thompson<br>*S.* Heidelberg<br>*S.* Infantis |
| German Cockroach *(Blatella germanica)* [197] | 0 | 25 | 13 | |
| Mice [197] | 5 | – | – | *S.* Typhimurium |
| House flies [195] | – | 2[1] | – | *S.* Typhimurium |
| Snake [195] | 40 | – | – | |
| Cats [195] | 3 | – | – | |
| Wild bird [196] | 7 | – | – | ND[2] |
| Mice [196] | 4 | 8 | – | |
| Fly strips [196] | – | 19 | – | |
| Insects [196] | – | 3 | – | |
| "Animal" Faeces [196] | 3 | – | – | |
| Mice [203] | 32 | – | 32 | *S.* Typhimurium |
| Cat [189] | 50 | – | – | *S.* Enteritidis |
| Fox [189] | 25 | – | – | |
| Mouse [189] | 28 | – | – | |
| Beetles [189] | – | 38 | – | |
| Larvae [189] | – | 100 | – | |
| Darkling Beetle [204] | – | 14 | 14 | ND |
| Seagulls [200] | 13 | – | – | ND |

[1] Pooled prevalence calculated from paper data 1.7, (95% CI: [0.42, 4.5]), [2]ND Not described, – Not reported

Table 1–9. Environmental Survival Times for *Salmonellae*

| *Medium [Ref]* | *Survival Time* |
|---|---|
| Oxidation ditch [207] | 27 – 47 days |
| Manure pit –bottom [207] | 4 days |
| Manure pit – top [207] | 48 hours |
| Manure – slow dried [207] | 120 days |
| Wet manure [207] | 66 days |
| Manure-soil mix [207] | 29 days |
| Cattle manure [207] | 2 months |
| Soil [207] | 7 – 168 days |
| Poultry Litter [211] | 13 days – 18 months |
| Soil [209] | 65 days |
| Poultry Litter –stored [210] | 4 days |
| Poultry litter – spread [210] | 4 – 32 days |
| Manure - raw (*S.* Typhimurium) [212] | 204 days |
| Manure –composted [212] | 7 –14 days |

Table 1–10. Persistence of *Salmonella* spp. in Contaminated Poultry Environments

| *Study [Ref]* | *Material* | *Persistence* |
|---|---|---|
| UK 2003 [189] | Floor | 8 – 21 months |
| | Dust | 3 – 13 months |
| | Dried faeces | 21 – 26 months |
| | Litter | 21 – 26 months |
| | Water troughs | 2 months |
| | Feeder | 8 – 26 month |
| | Incubators | 3 months |
| | Soil | 8 months |
| UK 1995 [213] | Walls | 52 weeks |
| | Floor sweepings | 38 week |
| | Feed hopper | 53 weeks |
| | Litter | 53 weeks |
| | Feed | 26 months |

Table 1–11. Concentration of *Salmonella* spp. Detected in Litter Samples

| *Study Location [Ref]* | *Salmonella Serovar* | *Infectious dose* | *Litter Contamination* |
|---|---|---|---|
| USA [130] | *S.* Typhimurium | $10^2$ | 112 - 1,071 CFU/g |
| | | $10^4$ | 245 - 35,481 CFU/g |
| | | $10^6$ | 3,715 - 26,302 CFU/g |
| Australia [10] | *Salmonella* spp. | Used litter | 4.0 - 1.1 x$10^5$ MPN/g |
| USA [218] | *Salmonella* spp. | Used litter | < 1.0 - 3.6 MPN/g |

of 2014 (Jan – Jun), 36 different serovars were identified in raw feed ingredients including; canola meal, fish meal, meat and bone meal, meat-meal and chicken feed. Only one isolate (1/185, 0.5%), identified as *S.* Typhimurium Phage Type 9, was reported from an unknown feed ingredient. Testing results from cereal grains are rarely reported [179-181].

## 4.2 Vermin Reservoirs

Many investigators have reported *Salmonella* detection in vermin and insects found on contaminated poultry farms or conducted in-vivo infection experiments. The reported concentration of salmonellae in or on vermin or vermin faeces (Table 1–7, Table 1–8) may be quite high [184-188]. It is worth noting that the external contamination rates of vermin (Table 1–8) appear to be higher than internal contamination rates suggesting indirect surface contamination rather than simple infection.

Mice and rats are frequently associated with salmonellae detection on poultry farms and have been identified as positive for the same *Salmonella* serovars found in cereal grains on cereal grain farms [189]. However, with the exception of a few notable experimental studies which infected chicks with contaminated insects and demonstrated them to be a viable source of chick infection [190-194], most studies fail to demonstrate temporality of infection, and typically only demonstrate an association with detection rather than causation [189, 192, 195-198]. Frequently, only a few animals are sampled, typically only when infected flocks are present and rarely if ever between batches of birds and frequently only a few individuals are detected positive [199]. Seagulls were found to be contaminated with 27 *Salmonella* serovars (including *S.* Typhimurium and *S.* Infantis) that reflected those in the human population and implicated sewerage as an important source of infection as the prevalence was higher in samples collected near dumps and sewage outlets [200]. In all instances, the most plentiful food source in a poultry environment for all vermin is poultry feed. The constant availability of poultry feed to poultry and thus vermin, is what attracts vermin to poultry sheds.

## 4.3 Environment

Salmonellae have been shown to contaminate animal waste materials in high quantities, and can be detected in many different environments, including soils (Table 1–9, Table 1–10). The fate of pathogens in soils receiving animal wastes and environmental survival strategies employed by bacteria, including salmonellae, has been reviewed [45, 205, 206]. Factors affecting survival times in soil include physical structure, texture and pore space, porosity and chemical adsorption [207, 208]. Salmonellae may survive in soil in a viable but non-culturable state making detection difficult [209] and have been shown to be detectable in litter applied to soil for up to a month after spreading [210]. *Salmonella* survival and stress mechanisms may enable survival and replication in these environments.

Table 1–12. Airborne Concentration of *Salmonella* spp.

| *Salmonella [Ref]* | *Location* | *Age* | *Contamination* |
|---|---|---|---|
| *S.* Enteritidis [102] | Experimental rooms | 1 day | 10 CFU/$m^3$ |
| *Salmonella* spp. [225] | Hatcher exhaust[1] | 21 days | 23 CFU/plate |
| *S.* Enteritidis [226] | Layer Shed[2] | 67-76 weeks | 5 CFU/plate |
| *S.* Typhimurium [227] | Turkey Shed | 17 weeks | 2.6 x105 CFU/g |
| *Salmonella* spp. [12] | Broiler Shed | 0-55 days | 0.65 - 4.4 MPN/$m^3$ |
| *Salmonella* spp. [228] | Broiler Shed | 0-55 days | 2.5 - 3x106 CFU/$m^3$ |
| *Salmonella* spp. [229] | Processing Plant | Turkey | 2 - 598 CFU/$m^3$ |
| *Salmonella* spp. [230] | Processing Plant | Broilers | 1.5 x 104 CFU/$m^3$ |

[1]2 minute collection period, [2]24 hour collection period



Table 1–13. Internal and External Egg Contamination Rates with *Salmonella* spp. (%)

| *Salmonella Serovar [Ref]* | *Internal Contamination (95% CI)* | *External Contamination (95% CI)* | *Within Flock Prevalence* |
|---|---|---|---|
| *Salmonella* spp. [235] | 0.24 (0.13 - 0.37) | 0.53 (0.16 - 1.0) | 1%[1] |
| *S.* Typhimurium [235] | 0.23 (0.08 - 0.55) | 0.94 (0.23 - 1.60) | |
| All serovars [236] | – | 0.08 (0.05 - 0.31)[2] | – |
| *S.* Enteritidis [236] | – | 0.03 (0.0 - 0.63)[3] | – |
| *S.* Enteritidis[4] [237] | 0.49 (3.71 - 4.92)<br>Yolk 0.49 (0.31 - 0.57) | – | – |
| *S.* Menston [173] | 1.5 - 37 | 1.5 - 25 | 37 - 91 |
| | 0.44 | 0.57 - 0.87 | 21 - 59 |
| *S.* Heidelberg [133] | 0.47 | 6.6 | – |
| *Salmonella* spp. [238] | – | 3.3 (2.5 - 4.3) | – |
| *S.* Enteritidis [239] | – | 0.38 | – |
| *S.* Enteritidis [240] | 0.04 - 0.4 | 0.18 - 1.8 | – |

[1]4000 eggs tested, [2] 300 eggs tested, [3]1,000 eggs tested [4] Experimental study, – Not reported

## 4.4 Poultry Litter

Poultry litter contaminated with salmonellae has been demonstrated to be an important reservoir for the infection of poultry in the production chain [214], and contributes to the horizontal spread of infection between birds in houses [130] and reinfection of birds housed on contaminated litter [215]. The concentration in litter (Table 1–11) was related to initial infectious dose in chicks placed on the litter, with generally higher infectious doses resulting in higher litter contamination rates [130]. Litter amelioration by aeration did not decrease litter concentration and salmonellae were found to survive between batches of birds [216, 217]. Detection rates vary widely between studies and may reflect different study design and sampling methodology with litter core sampling versus, boot or drag swabbing and variable sample sizes and pooling methods. Observed differences regarding the effects of pH, ammonia and moisture content between new and old litter are confounded by poor descriptions of the "age" of litter; new versus aged or re-used litter [214, 217-224]. These conflicting results may simply reflect both the type of litter and how long litter is in use (one or two batches of birds versus years). Field studies typically report *Salmonella* survival, when reporting detection, and detection rates per sample taken, rather than population concentration (CFU or MPN) on litter with concurrently *Salmonella* positive flocks.

Litter in Australia may be re-used or new for each batch of birds. In laying hens, litter is typically new for a new batch of floor-reared birds. In the only studies published regarding the use of litter in Australian poultry, *Salmonella* was identified in a high proportion of either new or re-used litter (68% – 83%) and the concentration of salmonellae in new litter was lower (39 MPN/g vs 59 MPN/g). While this was not part of the reported study, it is important to note that day old chicks will consume litter and the concentration in a single gram of either new or used litter observed in this study is sufficient to infect a day-old chick. Additionally, it was noted that new litter was observed to have a higher number of *Salmonella* serovars detected than older litter.

The importance of this observation was not fully explained.

### 4.5 Airborne Contamination with Salmonellae

A small number of poultry studies have demonstrated the presence of salmonellae in air samples in contaminated sheds, hatchery rooms and processing plants (Table 1–12). There is considerable variation between the method of sampling and the number of organisms detected between locations, but there is sufficient contamination to potentially be infectious to day old chicks [12, 102, 225-230].

## 5. Vertical Transmission of Salmonellae in poultry

The pathways of egg contamination with *Salmonella* spp. have been extensively reviewed [231-233]. Systemic infection of laying hens may result in invasion of reproductive tissues and colonization of different regions of the reproductive tract. Non-host adapted *Salmonella* serovars including *S.* Typhimurium are able to colonize ovarian and peritoneal tissues, and may be isolated infrequently from these tissues, resulting in their potential to cause internal contamination of egg contents during egg development [97]. Infection in different parts of the reproductive tract results in deposition of salmonellae in different regions of the developing egg, yolk, vitelline membrane, albumen, external membranes or the shell surface. Vertical transmission may occur via the internal contamination of the egg, while pseudo-vertical transmission occurs via external contamination of the egg surface. Transmission from generation to generation via the egg is an important route of dissemination and most truly vertical infections are eliminated when there is elimination of infected breeders. This will not eliminate pseudo-vertical transmission. (The subtle difference between vertical and pseudo-vertical transmission of organisms via eggs in poultry are complex, and the differentiation between the two is unnecessary as for all intents and purposes both pathways result in vertical transmission).

*S.* Typhimurium and other *Salmonella* serovars may contaminate internal egg contents, albeit at varying rates (Table 1–13). Sexual maturity, feed withdrawal and moult affect the rate of contamination with rates increased after these associated stressors [234]. In all cases, internal contamination rates are low when compared with external contamination. The primary discussion associated with internal egg contamination appears to be associated with the risk of human infection rather than vertical transmission of salmonellae to progeny. Much of this discussion is meaningless in the context of dissemination and control of *Salmonella* in poultry systems or enterprises. *Salmonella* contamination of eggs via vertical transmission is a real risk. Whether the path is vertical or pseudo-vertical via contamination of the external surface and migration through the shell, the outcome to offspring at the hatchery is the same. It is impossible to sterilise the internal content of the fertile egg and hatch a live chick. Thus the life cycle of *Salmonella* in these populations is perpetuated.

## 6. Salmonellosis in Poultry Flocks

In Australia, chicken meat (broiler) production systems are fully integrated comprising two generations of poultry production (parent stock and broiler progeny). Parent stock are purchased as day old birds from the primary breeding sector and reared for egg production. Each integrator owns parent stock production (pullet rearing and egg production also known as breeders), hatcheries, broiler production sites and chicken meat processing operations. All components of the integration are owned and operated by a single entity [241, 242]. In the table egg production sector, full integration is limited to a small number of large producers, with most producers single site operations. Only a single generation of birds is reared for commercial table egg production, with commercial birds purchased from the primary breeding sector either as day old birds for rearing and production or as point of lay hens for egg production. Depending on the scale of the operation producers may grade eggs on site or send eggs to a commercial grading floor [243].

### 6.1 Integrated Chicken Meat Production Systems

A limited number of studies have investigated or described in detail the identification, dis-



Table 1–14. Egg Laying Hen *Salmonella* Prevalence Studies

| *Location [Ref]* | *Year* | *Farm Type* | *Prevalence* | *Serovar* |
|---|---|---|---|---|
| France [261] | 2004-2005 | Cage | 0.31 | All |
| | | Cage | 0.12 | SE[1], ST[2] |
| | | Barn | 0.12 | All |
| | | Barn | 0.04 | SE, ST |
| | | Free Range | 0.06 | All |
| | | Free Range | 0.05 | SE, ST |
| France [262] | 2004-2005 | All | 0.18 | All |
| | | Cage | 0.31 | All |
| | | Floor | 0.08 | All |
| Australia [260] | 2013 | Cage Farm[3] | 0.57 | All |
| | | Cage Farm | 0.13 | ST |
| | | Cage Flock[4] | 0.44 | All |
| Australia [178] | 2013 | Cage Farm | 0.45 | All |
| | | Cage Farm | 0.20 | ST |
| | | Cage Flock | 0.49 | All |
| UK [263] | 2004-2005 follow-up | Cage | 0.48 | All |
| | | Cage | 0.66 | ST |
| | | Free Range and Barn | 0.64 | All |
| | | Free Range and Barn | 0.40 | ST |
| UK [264] | 2004-2005 | Cage | 0.26 | All |
| | | Barn | 0.08 | All |
| | | Free Range Organic | 0.05 | All |
| | | Free Range | 0.07 | All |
| USA [265] | 1989-1990 | NS[5] | 0.76 | All |
| USA [266] | 1989-1990 | Cage Flock | 0.92 | All |
| Belgium [267] | 2005 | Cage | 0.30 | All |
| | | Free Range/Barn | 0.01 | All |
| USA [268] | 1999 | Cage | 0.07 | SE |
| Canada [269] | 1989 | Cage | 0.53 | All |
| | | Cage | 0.03 | SE, ST |

[1]SE: *Salmonella* Enteritidis, [2] ST: *Salmonella* Typhimurium, [3]Farm Prevalence, [4]Flock Prevalence, [5] Not Stated

semination and prevalence of *Salmonella* serovars across all major parts of an integrated broiler production system from the feed mill through to processing [20, 151, 197, 244]. One study identified feed and feed mills as a major source of multiple *Salmonella* serovars, but only identified 2 serovars present in all parts of the production system, concluding that transmission through the poultry production system of a limited number of serovars may be an important but rarely described or acknowledged indirect route of transmission [197], particularly with reference to *S.* Typhimurium. A single Australian study, described the introduction of *S.* Typhimurium via contaminated feed and subsequent dissemination from breeders through to processing [20]. Most studies are limited to parts of the production system independently of each other, either breeders, broilers [245, 246] hatchery [247], processing [248] or breeder to hatchery, hatchery to broilers [196, 249-251] or broilers to processing [21, 252].

## 6.2 Parent Breeder Flocks (Fertile Egg Production)

There is a paucity of information about *Salmonella* prevalence or transmission in hens producing fertile eggs and most information reviewed regarding egg laying flocks was derived from the commercial layer sector [253, 254]. Parent flocks produce fertile eggs for hatching. Parent flocks may be infected with more than one serovar of *Salmonella* and may contribute to more than one flock at grow out. As such they may provide a source of infection (via hatching eggs) that may contaminate a hatchery and infect multiple progeny grower flocks [217]. The prevalence of *Salmonella* in eggs varies by study, serovar and whether internal or external egg contamination rates are considered [235], but a strong correlation between environmental contamination and egg contamination was reported [236, 255].

A number of studies implicate indirect transmission of *Salmonella* from feed via breeding flocks to progeny, via contaminated eggs, as a primary source of contamination through integrated poultry operations [151, 173, 197, 256, 257]. The risk of broiler infection was increased if parent flocks were infected with *Salmonella* and the associated hatcheries were also affected [258]. Elimination of feed as a source of contamination for the breeder flocks, substantially reduced vertical transmission to broilers [151].

## 6.3 Egg Laying Flocks (Table Eggs for Human Consumption)

### 6.3.1 Prevalence Studies

A brief summary of a few *Salmonella* prevalence studies in egg laying flocks ( Table 1–14) reveals that flock prevalence is highly variable, varies by serovar and study, even studies in the same location and year demonstrated different *Salmonella* prevalence, and a high level of variation between farm and flock prevalence (even on the same sites) and housing types is evident [259]. Similar findings have been made in Australia. Two separate studies reported farm prevalence varying from 45-57% with 13-20% of flocks on farm infected [178, 260].

### 6.3.2 Risk Factors Associated with Flock or Farm Infection

None of the risk factors identified with regard to transmission or the detection of *Salmonella* were unique to farms producing eggs for human consumption. The key risk factors for infection identified in all studies were feed, manure management, flock or farm size, farm house type, farm hygiene and biosecurity and the use of *Salmonella* vaccination [240, 262, 264, 267, 268, 270-272].

Key differences between studies are the level of detail focused on some aspects of production such as biosecurity indicators including the use of foot baths, visitor and vehicle access and parking, and sharing of common equipment between houses. Studies typically focus on hygiene (cleaning, rodent and fly activity), contamination of egg processing environments, contamination of houses prior to flock placement, while a smaller number of studies focused on previous flock infection or pullet infection [240, 263, 273]

Rearing pullets on the floor was associated with increased risk of infection [268], but no studies reported purchasing of infected pullet flocks as a risk factor for farm infection. Presumably the status or infection of the production flock is most likely due to risk factors on the production farm rather than introduction via the importation of live birds. The source of pullet flocks, their *Salmonella* testing history, or their location of rearing (on-site or off-site) are rarely discussed. This is an important area for greater discussion and study.

The vaccination of flocks for *Salmonella* and the housing type are the two most substantial areas of disagreement in the studies. The vaccination status of a flock as a risk factor varied by study and it is known that exposure to high dose challenge will overwhelm the efficacy of vaccination [263, 267]. Additionally it is also known that vaccination is not fully protective, not cross protective against multiple serovars and does not prevent, nor eliminate egg contamination [274, 275]. The consequence of this is that blanket vaccination programs will have some effect, but in the absence of understanding within or between site epidemiology and understanding of transmission, blanket vaccination is unlikely to be effective on its own.

The influence of housing system on *Salmonella* infection in hens has been reviewed [276] and differences in the frequency of isolation of *Salmonella* between cage, conventional cage and enriched cage, and cage-free systems have been reported, with cage systems frequently reported to have higher prevalence than floor or free-range systems (Table 1–14) [262, 264, 267, 271, 277, 278]. In cage sheds, equipment is frequently shared between houses, conveyors, egg packing houses and walkways link houses and the efficacy of cleaning and disinfection is complex [279]. Factors such as farm and flock size may be more important than house type. Caged farming systems tend to have larger flock sizes, have multiple flocks on a single site and the larger the farm size the more likely it is to have multiple flock ages on the same site [262, 280]. Flocks may be managed either in multi-aged houses or as single aged flocks. National surveys are often stratified by flock size and free range or barn flocks tend to be smaller and less frequently sampled [267, 271, 273, 281].

Reduced risk was associated with operating farm sites as all-in all-out, vaccination, cleaning and disinfection [268] and biosecurity [264] and, in non-cage systems, the presence of cats and dogs. It is known that domestic animals can excrete *Salmonella* so their presence may be contradictory and fly in the face of good biosecurity practices [240]. Factors associated with reported *Salmonella* prevalence in egg laying flocks is complicated by within-flock variation over time [259, 283, 284].

### 6.3.3 Within Farm Flock Prevalence

Farm level flock prevalence increases with increasing flock size [262, 264, 268, 280], flock age [236], moulting [268, 284], facility age [273] and multi-age stocking [262, 264]. High stocking density and poor sanitation is associated with increased flock and egg prevalence [235, 236, 264, 271]. The key risk factors associated with a reduction in within farm flock prevalence or a decreased risk in transmission include operating farms as all-in all-out (single age), cleaning and disinfection. However, again there was no discussion on the purchase or rearing of *Salmonella* free pullets.

### 6.3.4 Dissemination Within a House

Dissemination of infection within a house occurs quickly (1-3 weeks) and this has been demonstrated with multiple *Salmonella* serovars including *S.* Typhimurium regardless of housing type [221, 223, 285, 286]. However, the speed of spread may vary between serovars [287]. Spread to adjacent flocks within the same shed was slow [133, 221]. Airborne transmission by dust or dry particles may explain dissemination within cage houses better than mechanical or fomite transmission and this is supported by some experimental studies [102, 226] and observations made in other risk factor studies [281]. Environmental contamination with salmonellae was greater in the lower levels of houses [213] or multi-story frames and decreased with flock age but the *Salmonella* concentration per sample increased as the flock aged [16]. It is likely that different housing systems will enhance or reduce the speed of dissemination within a house [280] and that the interaction of house design variables and management may influence spread. However, better information on exactly what design features or management practices lead to greater concentrations of *Salmonella* in certain areas of sheds or in flocks is required.

## 6.4 Horizontal Transmission Between Flocks

Transmission between flocks, houses or production sites may occur horizontally via con-

tact between infected flocks and susceptible flocks via the common use of shared equipment, such as mobile plant or common egg conveyors, people or vermin, or the carryover of infection between flocks by the introduction of susceptible birds into contaminated environments (shed carryover). Studies rarely focus on risk factors for transmission between flocks and reports are contradictory. Two studies reported that spread to adjacent sheds did not occur despite common stockpersons or shared equipment between sheds [133, 221].

#### 6.4.1 Carry-over Infection between Flocks

Many studies have focused on the importance of cleaning and disinfection as a means of preventing the carryover of infection between flocks via contaminated sheds, which appears to be considered to be one of the most important pathways of infection to newly placed flocks [213, 240, 259, 268, 270, 279, 288]. A single study reported the identification of identical clones found in flock houses, production facilities and egg washing over a longitudinal period which suggests that ongoing introductions of infection from outside the flock are more important than continuance of an original infection [284]. This study failed to identify temporally the source of the contamination as carryover infection.

The introduction of pullets into a contaminated environment did not always result in infection in the birds [189, 213] and in most studies it appears to be presumed that infection in flocks is only due to shed or environmental contamination with no consideration given to status of flocks prior to shed entry, bringing into question the issue of detection versus infection. As already mentioned, most if not all studies of this type do not investigate or report the presence or absence of *Salmonella* in point-of-lay flocks prior to placement so the temporality of houses as a source of infection is unknown. *Salmonella* survival in the environment is known to occur for long periods of time (Table 1–9, Table 1–10) but sampling is typically not differentiated by direct contact exposure surfaces so the route of exposure in carryover infections is largely speculative. Environmental contamination may not reflect flock contamination or excretion by the birds in that environment [278, 289]. Additionally, the detection of a *Salmonella* serovar is not sufficient evidence to confirm that the source of infection is the shed unless there is further typing of the isolates or confirmation that both the shed contamination and the infection in the flock are the same *Salmonella* isolates.

### 6.5 Hatchery

Hatcheries have been identified as an important source of salmonellae to progeny stock, whether commercial broiler or pedigree operations [290-292]. Chicks at day old can be infected vertically from infected parent flocks, or horizontally during hatching, loading and transport to the farm. Conflicting reports describe either the same or different serovars at the hatchery as those found at broilers or processing [217, 249, 250, 293-295]. Many of these discrepancies are likely to be due to sampling methodology and study design. Typically, sampling strategies describe surveillance sampling (the detection of *Salmonella*) rather than an attempt to ascribe contamination to specific hatchers/sets of eggs or donor flocks at either the hatchery or broiler flock. Only a single study to date has attempted to describe these relationships and only from the hatchery to broilers [217].

Multiple *Salmonella* serovars are detected during hatchery sampling and very high rates of contamination of both sample type and contamination dose have been reported, despite intensive cleaning and disinfection programs [217, 291, 292, 296, 297].

Up to 9% of chicks leaving the hatchery have been reported as *Salmonella* positive [197]. It can be quickly observed (Table 1–15) that the number of potentially infected chicks at day of hatch varies considerably depending on the study and the sample taken. Variation in *Salmonella* prevalence occurred between flock placements (0 - 87% chicks), hatchery (12.5 - 50% flocks infected) and company (2.9 - 10.7% chicks infected) [298]. The correlation between hatchery and broiler house *Salmonella* serovars was high ($R^2 = 0.885$, $P < 0.01$), indicating that the hatchery and potentially breeder flocks are a significant source of broiler farm contamination [217].

Risk factors for the role of the hatchery in broiler contamination include the following; young birds at high risk of infection with a low infectious dose, high risk of dissemination from infected to susceptible chicks, cross contamination at multiple points in the hatchery, multiple sources of donor flocks, and hatchery hygiene. One study found that variability in chick prevalence was not attributable to hatcheries either between or within a company, indicating factors other than hatchery management *per se* were important [298].

#### 6.5.1 Donor Flocks and hatching eggs

Breeder flocks (aka donor or parent flocks (depending on the generation in question)) via vertical or pseudo-vertical transmission have been implicated as an important source of salmonellae but direct investigations at breeder generation are rarely reported [299-301]. Studies beyond the parent generation (primary breeding stock in grandparent generations) are absent from the literature. Eggs typically have a very low contamination rate either internally or externally, therefore only a small proportion of hatching eggs are likely to be infected on entering the incubator at the hatchery (Table 1–13). Washing and fumigation of any egg is imperfect and a small proportion of eggs are likely to remain contaminated after treatment [302, 303]. In particular, hatching egg handling is critical for maximising hatchability, so treatments are modified to account for this. Floor eggs (dirty eggs) have a higher risk of residual bacterial contamination prior to incubation [304].

Multiple sources of eggs (donor parent flocks) may supply a hatchery, and eggs from different donor flocks may be mixed in the hatchery, resulting in multiple donor flocks contributing to day old flock placements. A single contaminated donor flock has the potential to contaminate multiple hatchers, chicks and thus chick placements, potentially with more than one *Salmonella* serovar [217, 298]. Sampling of tray liners (chick papers) identified 12.41% of samples positive, but when sampling was considered by source of the chicks (breeder flock) 42% of the progeny of a donor flock were positive for *Salmonella*, implying that infection was associated with the source of the eggs rather than simple environmental contamination and transmission within the hatchery. Flock prevalence in day old chicks was associated with number of parent flocks contributing to the broiler flock on delivery to the farm. The more donor flocks the greater the probability of infection [298]. Additionally, multiple *Salmonella* serovars were identified simultaneously from the same breeder lots implying that breeders may be an important source for hatchery contamination and subsequent broiler infection [217, 293, 294]

Sample size and sampling methodology for hatchery sampling is critical for *Salmonella* detection within a hatchery. As illustrated above, the way samples are both collected and analysed, taking into consideration the complex movements and egg management processes that occur in a hatchery is important for determining the role the hatchery plays in the transmission of salmonellae from parent to progeny flocks. For example in one study 9.4% of chick yolk sacs sampled were positive for *Salmonella*, yet despite a high number of samples collected only a single positive hatcher sample was detected [197].

#### 6.5.2 Age of Birds

Young birds are at greater risk than older birds of becoming infected at the hatchery if contamination is present due to the relatively low infectious dose required for infection. The presence of airborne *Salmonella* in hatchery dust or fluff and relatively long exposure periods (24-48 hours) prior to transport in highly contaminated environments such as hatcher cabinets or holding rooms increases this risk [196]. Eggs infected with *Salmonella* hatch normally and can contaminate non-infected chicks prior to removal from the hatcher, demonstrable by intestinal contamination [290, 305]. These chicks are infectious and act as "seeder" chicks to in-contact non-infected chicks [305]. Large numbers of contaminated chicks are reported infected on the day of hatch, but varies widely depending on the study from 9-50% [173, 197, 306].

#### 6.5.3 Cross Contamination and Dissemination

It is estimated that there is at least a 50-fold to 100-fold increase in salmonellae incidence from eggs to chicks in the hatchery: 1 contaminated egg per 50 to 100 chicks [290, 297]. One infected chick in a modern commercial

Table 1–15. Hatchery Samples and Chick Detection of ***Salmonella*** spp.

| *Location Date [Ref]* | *Time of Sampling* | *Sample Type* | *Salmonella Positive %* |
|---|---|---|---|
| Japan 1955-1958 [296] | Day of hatch | Hatchery Fluff | 53 |
| USA 1982 [291] | Day of hatch | Hatch Debris | 71 |
| | | Chick Belt | 81 |
| | | Chick Paper | 74 |
| USA 2003-2006 [315] | Day of hatch | Chick Papers | 13 - 50 |
| USA 2005 [316] | Day of hatch | Chick Papers | 95 |
| USA 1998 [196] | Day of hatch | Chick Papers | 51 |
| USA 1992/93 [311] | Prior to pip | Chicks | 0 |
| | | Yolk sac | 2 |
| | Post pip | Chicks | 15 |
| | | Yolk sac | 8 |
| USA 1999 [217] | Day of hatch | Yolk sac | 9 |
| USA 2003-2006 [298] | Day of hatch | Chick Paper | 39 |
| | | GI Tract | 1 - 87 |
| UK 1965 [173] | Day of hatch | Cloacal swab | 50 |
| Japan 1955-1958 [317-320] | Day of hatch | Yolk Sac | 7 - 48 |
| | | Yolk Sac | 2 - 7 |
| | | Yolk Sac | 1 - 12 |

broiler hatcher may potentially expose more than 100,000 eggs or chicks to infection in the same hatcher. Peak microbial air contamination and egg cross-contamination occurs at pipping and removal of chicks from hatching cabinets [304, 307, 308]. The quantity of air contamination is directly proportional to the level of surface contamination and hatchery hygiene [309, 310].

Following experimental contamination of the external surface of eggs prior to incubation, 100% of shells and membranes were *Salmonella* positive after inoculation, but only 38% remained positive after 17-20 days of incubation. No chicks were positive before pipping, but 15% of chicks were positive post pip. Additionally, only 2% of yolks were positive prior to pip, while 8% were positive post pip [311].

Chicks in trays above and below infected chicks were contaminated by both shell debris and aerosol contamination [305]. Oral contamination (oral swabs) was detected in over half the chicks indicating this was also an important path of dissemination during hatch [290], The wet peri-cloacum in newly hatched chicks is also a potential route of contamination in contaminated chick trays [291] while the respiratory tract has also been identified as an important route for infection [104].

High levels of salmonellae contamination (100 to 1000 organisms per sample) were detected in some hatchery samples [291, 292]. *Salmonellae* were detectable in hatcher fluff kept for 4 years at room temperature at high concentrations 104 -106 organisms/g fluff. The same *Salmonella* serotypes detected in hatcher fluff were also detected in dead embryos examined at the same time [296]. Fan assisted airborne transmission through the hatcher from contaminated environment has been shown to be an important source for dissemination, assisted by susceptible wet chicks enabling high contamination rates at day of age [297]. Horizontal spread may also occur during chick processing or chick holding from the excreta of contaminated chicks or contaminated fluff or egg shell [291, 298].

### 6.5.4 Hatchery Management

Several key factors in hatchery management are not described nor widely discussed in *Salmonella* transmission studies that involve the hatchery. These management factors include the number of parent donor flocks supplying the hatchery (and under what arrangement e.g. contract egg purchase or company owned and managed), the age of those flocks, the day of set, the number of donor flocks contributing to the set in the same incubator or hatched together, the use of fumigation or egg washing during incubation or hatch, coolstore storage times and temperature, vaccination (in-ovo or spray), chick handling and lairage time, and chick delivery management. The *Salmonella* status of the parent flocks and age are rarely reported. As parent flocks age, egg hatchability declines and therefore more eggs may either be held longer if set as single flocks or more donor flocks contribute to a single set when incubated. Egg contamination may increase in older flocks and it is known egg shell characteristics change as flocks age [312]. Parent flocks are managed to ensure there are no large peaks or troughs in egg supply and thus hatchability, to ensure continuity of chicks for broiler flock placements.

Hatcheries may not hatch chicks every day of the week, the smaller the number of days hatching, means more eggs are stored, as the week progresses eggs are taken from store for setting, with composite progeny flocks more likely as eggs become shorter in supply. Good egg management to maintain hatchability recommends that lower storage temps are used when eggs are stored for longer, so high egg storage temps (> 18°C) are likely associated with short storage and have been shown to increase *Salmonella* survival [313, 314]. Single stage or multistage incubation affects hatchery hygiene and the risk of cross contamination during the incubation phase; no studies have described the incubation type.

Fluff and faeces in chick trays may not reflect hatchery hygiene *per se*, but may reflect longer chick standing times in the hatchery, transportation times prior to delivery which were positively associated with *Salmonella* gut contamination [298]. Multiple batch deliveries (multiple flocks to different farms) indicate that deliveries wait until the vehicle is loaded before transportation thereby increasing chick holding times at the hatchery, multiple sources of donor flock exposure during both lairage and transportation and chicks processed late in the day have likely spent longer in the hatcher, possibly increasing exposure times to *Salmonella*. No studies have sufficiently demonstrated that the source of *Salmonella* during loading or transport of day old chicks is anything other than the hatchery.

## 6.6 Chicken Meat Prevalence

As with non-fertile egg laying flocks, the reported *Salmonella* prevalence in broilers (by farm/flock) varies significantly by study, time period, country and serovar reported (Table 1–16). Farm prevalence ranges tend to be high but vary considerable depending on method of detection, notably however the prevalence doesn't appear to have changed markedly over time [320]. There are differences between farm and shed/flock prevalence with some sheds positive on positive farms frequently reported [214, 298, 321]. In general, mul-

Table 1–16. Prevalence of *Salmonella* in Broilers

| *Study Location [Ref]* | *Year* | *Broiler Flock/Farm Prevalence* | *Sample Type* |
|---|---|---|---|
| Netherlands [258] | 1989 | 94% | Pooled faeces |
| | | *S.* Typhimurium 25% | |
| Netherlands [323] | 1999 - 2002 | 11.2 - 22.8% | Pooled faeces |
| USA[1] [214, 299] | 2003 - 2006 | 38% | Drag Swab |
| | | 29% | Litter sample |
| Canada [324] | 2007 | 50%, 95% CI: [37%, 64%] | Caecal content |
| Denmark [302] | 1993 | 16.8% | Caecal tonsil |
| Australia [321] | 2010 | 46.8% Flocks | Pooled faeces |
| | | 84.6% Farms | |
| Australia [325] | ~1984 | 60% | Caeca |

[1]Empty Shed

tiple *Salmonella* spp. are detected on broiler farms [196, 197, 217, 218, 306, 315, 322], suggesting multiple sources of infection or a source that potentially contains multiple serovars. The frequency of detection appears to vary significantly between batches of birds, with the same serovars detected from batch to batch in the same houses but not consistently [219, 322]. It has been estimated that *Salmonella* positive broiler farms were less profitable than *Salmonella* negative farms by 0.37cents/lb liveweight (~0.17c/kg) [219].

### 6.6.1 Salmonella Prevalence Surveys in Australian Broilers

Epidemiological information on the incidence or prevalence of *Salmonella* contamination in the chicken meat industry in Australia is relatively sparse. Other than infrequently published studies or those conducted by specific states, there are no formal national surveys of chicken meat conducted in Australia [321, 325-328]. The last national survey conducted occurred as part of the baseline study prior to the implementation of the primary production and processing standard (PPPS) and not all states participated in the study [321]. Some details from human outbreak investigations in the various States may be reported [9, 329, 330]

Microbiological surveys have failed to show any real significant reduction in poultry meat contamination between 2005 and 2014 regardless of the study or study design. Carcase prevalence ranged from 12.5 – 77.8% while chicken piece prevalence ranged from 5 – 64.3% [321, 325-328]. Multiple *Salmonella* serovars may be detected and more than one serovar may be detected in a single sample [325, 327]. Only South Australia (SA) routinely conducts surveys of poultry meat, and these studies are frequently small. They do however provide a continuous source of information about chicken meat sold into the SA market and these studies would indicate that despite assertions to the contrary (20 year old study [331]) there has likely been no real difference in the overall prevalence of *Salmonella* observed in poultry meat. As with all surveys of retail meat reported the prevalence varies between studies by study design and methodology and location (State).

*Salmonella enterica* subsp. *salamae* serovar Sofia detection in chicken meat may be very high and frequently confounds surveys of poultry meat prevalence in Australia [332] when reporting is not serovar specific [331-333]. *S.* Sofia isolates in Australia are rarely identified as causing disease in humans [334], therefore careful interpretation of carcass *Salmonella* prevalence results are required.

### 6.6.2 Risk Factors

Sources of infection for broilers can be defined as those entering the poultry house; infection arriving with day old chicks, people and biosecurity, or those present before chicks arrive such as carryover infection, vermin and litter [306]. In most cases the same risk factors described for egg laying flocks are identified [196, 197, 213, 214, 258, 306, 322, 335-337] with the key exception of the following: vehicles for the transport and pick up of broilers such as trucks, pallets and crates, catching equipment (rarely the people) [196, 197, 337-339], litter [218, 250, 306, 336], breeder (parent) flocks [197, 217, 258], and the hatchery [258, 322]. The status of day old chicks is frequently described as the most important risk factor for *Salmonella* infection during the rearing period [130, 196, 197, 298, 306, 339-342].

Rapid spread of *Salmonella* throughout the house via drinkers, feeders and environmental contamination may occur at a time when birds are most susceptible to infection with a low infectious dose [252]. An infectious dose sufficient to infect a single bird will enable transmission to pen mates [128]. As few as 5% of broilers infected at day old infected more than half the contact group in grow out conditions. Those chicks infected with a larger dose ($10^6$) were able to infect more in-contact birds [121, 130], but reproductive ratios have not been described for salmonellosis. In natural infections shedding declines after the first 14 days, and reduces until slaughter [121, 252, 306, 343], this is consistent with increased resistance as birds age [84, 88].

Risk areas within houses include areas around drinkers, which may promote environmental multiplication of *Salmonella* spp. [220]. Aeration of litter increases air contamination with dust and may promote survival of bacteria within a dry matrix [344]. High stocking density, incorrect ventilation, and poor litter quality are reported to increase *Salmonella* shedding and favour multiplication and spread, particularly at the end of rearing when shed density increases as bird weight gain increases and litter quality declines [258, 345]. However the complexity of this shedding and transmission, such as continued ongoing transmission to susceptible in contact pen mates, young birds and their short lives are rarely described. The use of mash feed, more than 4 feed changes, consumption of a starter feed and the use of salinomycin (coccidiostat) in the feed were also associated with increased risk of flock infection [346].

Factors associated with a decrease in flock or shed prevalence included increasing downtime between batches of birds [217, 298] and increased biosecurity status [298, 323]. Vaccinations, particularly coccidial vaccination decreased the odds of infection on farm [315], consistent with reports demonstrating increased prevalence of infection in chicks with concurrent coccidiosis [347, 348]. Rarely are longitudinal studies conducted in these groups and reports are typically limited to presence absence or serovar reporting and so temporal analysis and transmission is limited by the study design and reporting.

## 6.7 Processing

Poultry processing is a continuous operation consisting of two stages; primary and secondary processing. Primary processing includes the slaughter and production of a chilled dressed whole carcass, while secondary processing converts the dressed whole carcasses into smaller portions for sale. Secondary processing may occur on the same site as primary processing but may be done externally. It is important to note differences between processing studies depend on the sampling methodology, location of sampling (position in processing line) and the sample testing method (Table 1–17). It is well established that the primary source of *Salmonella* into the processing plant is derived from the birds arriving at the plant, however factors associated with bird supply influence both the prevalence of carcass contamination and the level of contamination [149, 196, 299, 338]. Poor transport crate cleaning and truck or catching equipment hygiene is associated with the transfer of *Salmonella* serovars from processing to broiler farms. Transmission is not unidirectional (both to and from processing), and contamination of uninfected broilers can occur during transport and at processing [149, 196, 337,339, 349]. The *Salmonella* status of broiler carcasses at processing is influenced by the day of placement, *Salmonella* status (hatchery derived infection), and differences between grow-out farms were more important than company or complex [324, 350, 351].

Time in lairage affects both internal and external contamination [338, 352, 353]. Feed withdrawal prior to slaughter showed no effect on *Salmonella* contamination of carcasses post-chill [354]. Contamination of poultry products (carcass or pieces) at the end of pro-

**Table 1–17. Proportion of Carcases Contaminated with *Salmonella* at different Locations in the Processing Chain***

| *Location [Ref]* | *Sampling location* | *Positive Carcasses (%)* |
|---|---|---|
| USA [359] | Post-pluck | 23 |
| | Pre-chill | 20 |
| | Post chill | 19 |
| Vietnam [370] | Pre-chill | 43 |
| USA [196] | Post-chill | 2 - 24 |
| Australia [321] | Post-chill | 48 (NSW)[1] |
| | | 13 (WA)[2] |
| | | 52 (SA)[3] |
| | | 44 (Qld)[4] |

[1]New South Wales, [2]Western Australia, [3]South Australia, [4]Queensland, * All tested using carcase rinse sampling method

Table 1–18. Environmental Sample Types for *Salmonella* spp. Surveillance or Surveys

| *Study (Location) [Ref]* | *Sample Type* | *Volume/Area* | *Method* |
|---|---|---|---|
| Cage Layer Flocks | Dust/litter | 10-15g | Sweeping |
| (UK) [372] | Surface Swabs | 0.5-1.0m$^2$ | 6 gauze surgical swabs |
| Hatchery (USA) | Surface Swabs | 0.9m$^2$ | 58 cm$^2$ sterile cheesecloth |
| [291] | Hatchery chick belts | | |
| | Egg Fragments | 10 g | Hatching trays |
| | Chick papers | 1 | Chick trays |
| Broiler Processing | Surface Swabs | 300cm$^2$ | 4 x 4" gauze swabs |
| (USA) [196] | Transport crates | | |
| Broiler Farm (USA) | Dust, litter, feed hoppers dirt | 50 to 100g dry material | "aseptically collected" |
| [196] | Surface Swabs | 100cm$^2$ | 4 x 4" gauze swabs |
| | Wall, boot, fan | | |
| | Water line, water cups | End of drinker line, cups or nipples | 4-5 cotton tip swabs |
| | Drag Swab | House Length 3 x | Commercial drag swab assemblies |
| | Chick papers | 25 | Chick trays |
| | Caecal droppings | 5 | 2-3 cotton tipped swab per sample |
| | Faeces | 25 | 50ml pooled pot |
| Layer Farms | Faeces | 5 x 200-300g | Pools |
| (EU) [374] | Dust | 2 x 250ml | "Evenly distributed from within shed" |
| NCP (EU) [374] | Faeces | 2 x 150g | |
| | Dust | 1x 250ml | |
| VLA (UK) [374] | Dust | 10 x 15g | |
| | Faeces | 10 x 25g | |
| Broiler [388] | Faeces | 300 faeces | 60 pools |
| | Boot Swab | 1800m$^2$ | 1 – 5 pairs |

cessing can occur at two points in the processing environment either via the introduction of contaminated birds into the poultry plant or cross-contamination within the processing plant [197, 248, 306, 351, 355, 356].

There is poor to moderate agreement between sampling methods for the detection of carcass *Salmonella* prevalence and this affects sample size requirements for assessing efficacy of interventions or prevalence surveys and the results between studies [357, 358]. Poultry carcass *Salmonella* prevalence is underestimated by sampling either the external carcass or the caeca alone [128, 197]. Non-specific aerobic bacterial counts, Enterobacteriaceae, nor coliform or *Escherichia coli* counts are correlated with either the quantity of *Salmonella* present nor the number of *Salmonella* positive carcasses [359, 360].

Cross contamination of poultry carcasses at processing due to the high volume and speed of poultry processing is high [361-363]. Sources of contamination during processing are dirty feathers, gut content and crop content [128, 350, 351, 353, 361, 363-367]. Crop content may contain very high numbers of salmonellae and there is a positive relationship identified between positive crops and proportion of carcasses found to be contaminated when sampled post chill [350, 368]. Carcass to carcass variability accounted for 73.2% of the total variability in bacterial load, with between lot within-supplier and between-supplier accounting for the remaining 14.2% and 12.6% of the variability [352, 353, 369 ].

Relationships between infected broiler flocks and carcass contamination post chilling are lost (particularly during wet chilling), thereby eliminating the effect of any pre-processing *Salmonella* controls [345, 350, 355]. Australian studies demonstrate that regardless of the source of broiler contamination to the processing site that carcass contamination rates can be significantly reduced from 100% to <1% of carcasses contaminated post chill providing adequate processing controls, such as the use of inside outside washes or chlorination of chiller water, are implemented during processing [251, 321, 361, 364-367].

## 7. Environmental Sampling

Sampling strategies in poultry are primarily aimed at determining flock status, positive or negative, rather than the individual status of the bird. The knowledge of the within-flock prevalence of infection has no real practical value, as control or regulatory responses to infection is based on flock status rather than prevalence, unless measuring the effect of an intervention. The difficulty of sampling poultry flocks for the detection of *Salmonella* spp. and subsequent control of salmonellosis in the human food chain has been the subject of a significant number of studies. Key issues identified include adequate sample size, methodology of sample collection, sample processing and quantitative versus qualitative testing [371]. Environmental sampling is more sensitive and cost effective in detecting or monitoring flock infection in breeder flocks, egg layers and broilers than individual bird sampling and the different techniques have been critically reviewed [257, 283, 336, 372-375]. A large number of different sampling methods, composite sample types and strategies have been proposed for different environments [196, 213, 291, 376], a few of which are presented in Table 1–18. Surface swabs and boot or litter swabs collect a small sample of the two major surface contaminants, either faeces or dust and possibly those organisms present in biofilms on the respective surface [196]. The sensitivity of environmental samples varies between sample types (dust or faeces). It is easier to recover *Salmonella* from dust than faeces [375] but *Salmonella* counts in dust were lower (by $10^5$ CFU) than in faeces despite more dust samples being positive [266, 288]. There appears to be good agreement between the level of environmental contamination, flock prevalence and egg contamination in layer operations [235, 289, 374, 377]. Therefore, composite sampling (multiple sample types within a shed) is employed as the best sampling strategy. The power of composite samples to detect infected flocks is strongly influenced by the within-flock prevalence. This is particularly true when within flock prevalence is low, especially less than 10% [235, 255, 261, 263, 372, 374, 376-379]. National sampling strategies involving composite environmental sampling methods have been developed and implemented in Europe, the USA and Australia [380-382].

### 7.1 Limitations of Pooling Environmental Samples

The sensitivity of pooling environmental sam-

Table 1–19. *Per capita* (100,000) Notifications of Human Salmonellosis All Sources*

| *Country [Ref]* | *Per capita Notification Rate /100,000 (% S. Typhimurium)* | | | |
|---|---|---|---|---|
| | *1990's* | *2000* | *2010* | *2013-14* |
| USA [389] | 14.46[1] | 14.08 | 17.55 | 15.45 |
| Australia [390] | 31.9 | 32.2 | 54.0 | 69.7 |
| New Zealand [391] | - | 48.1 (51.3) | 26.2 (51.9) | 21.2 (41) |
| Denmark [392] | 82.3[2](31.8) | 43.3(18.8) | 28.7(32) | 19.9(38) |
| EU [393, 394] | - | 34.6[3] (12.9) | 21.5 (22.4) | 20.4 (20.2) |

[1]1996, [2]1994, [3]2006, *Cross country and time comparisons of raw data is limited as methodologies for calculating incidence, sampling and reporting vary between countries and years so rates are indicative only.

pling depends on the dilution effect of mixing negative samples with positive samples. Dilution factors may include; lower concentration of *Salmonella* in the final pool tested, greater impact of competing micro-organisms in the pool, heterogeneous distribution of organism in the pool, and an increasing proportion of inhibitory factors to the portion of positive sample [378, 383]. Pooling of samples reduces the total cost of sampling, but also reduces the sensitivity of testing, and the number of serovars detected [257].

Sampling strategies or models for determining sample sizes assume that strains growing on a plate are representative of the initial concentration of each strain in the original sample. However, this has been demonstrated to not be the case [384, 385]. Dilution enrichment is considered important to reduce the inhibitory effects of selective media and loss of low numbers of salmonellae through the overgrowth of competitor organisms [386, 387].

## 8. Epidemiology of Human Non-Typhoidal Salmonellosis

The epidemiology of non-typhoidal salmonellosis in humans is complex. Many *Salmonella* serovars are pathogenic to humans and infectious pathways include person-to-person transmission, zoonotic, waterborne or foodborne transmission.

The annual global burden of non-typhoidal salmonellosis circa 2006 was estimated to be 93.8 million cases of which 80.3 million cases (86%) were foodborne [395]. In a recent review, 32% to 95% of human salmonellosis was attributed to the ingestion of contaminated food [396, 397]. Regardless of the study methodology or location the source of *Salmonella* most frequently identified was the consumption of eggs (3-63%), chicken meat (0.09-48%) or pork (0.08-60%), in either sporadic and outbreak associated human salmonellosis [392-399]. Infection rates (notified cases) of salmonellosis vary between countries ( Table 1–19). In Australia, *per capita* rates of salmonellosis appear to be increasing annually [405], while in New Zealand and Europe per capita rates have been declining [391, 393], and in the USA rates have been stable since the mid 1990's with no major trends up or down in overall prevalence, despite changes in survey methodology [391, 406]. The key changes are in serovar prevalence, with a decline in *S.* Typhimurium, and a large increase in *S.* Infantis [389]. Globally, *S.* Typhimurium was the predominant cause of salmonellosis by the end of the 1970's, however, there was an increase in *S.* Enteritidis as the dominant serovar in humans during the 1980's primarily associated with eggs and poultry meat. This has not occurred in Australia, which has remained free of *S.* Enteritidis in the commercial poultry industry and most human cases are attributed to overseas travel [405, 407].

Table 1–20. *Salmonella* Contamination (concentration) in Foodstuffs Associated with Outbreaks of Human Disease

| *Salmonella Serovar [Ref]* | *Food Stuff* | *Level of Contamination* |
|---|---|---|
| *S.* Heidelberg [414] | Cheese | 0.36 - 1.8 MPN/100g |
| *S.* Typhimurium [415] | | 0.015 - 0.091 MPN/g |
| *S.* Typhimurium [54] | Chocolate | 10 CFU |
| *S.* Napoli [416] | | 2 - 23 MPN/g |
| *S.* Eastbourne [417] | | 2.5 MPN/g |
| *S.* Agona [418] | Maize Snack | 2 - 45 MPN/25g |
| *S.* Saint Paul, *S.* Javiana, | Potato Chips | 0.04 - 0.45 MPN/g |
| *S.* Rubislaw [419] | Paprika, spice mix | |
| *S.* Mbandaka [420] | Peanut Butter | < 3 - 4 MPN/g |
| *S.* Newport [421] | Beef (ground) | 6 - 23 MPN/100g |
| *S.* Typhimurium [422] | Roast Pork | $4.3 \times 10^4$ - $2.6 \times 10^5$ MPN/g |
| *S.* Infantis [423] | Ham | $1 \times 10^6$ - $2 \times 10^6$ organisms/g |
| *S.* Typhimurium PT2a, PT2b [424] | Ice Cream | 113 - 11,300 MPN/75g |
| *S.* Typhimurium PT10 [425, 426] | Water | 17 - 1000 MPN/L |
| *Salmonella* Spp. [427] | Sausages | < 30 – 120 CFU/g |
| *S.* Ealing [428] | Dried milk (Infant) | 1.6 MPN/450g |



Infection with non-typhoidal salmonellae may cause clinical disease such as gastroenteritis, bacteraemia, focal or asymptomatic infection [408, 409]. Typically, infection is mild and self-limiting [410]. The clinical presentation, and outcome of disease may vary by serovar, and some patients become long term chronic carriers [411, 412]. The infectious dose required to cause human infection in a healthy individual is thought to be in the order of $10^6$-$10^8$ colony forming units [54] and was reviewed by Blaser (1982), however epidemiological evidence suggests the infectious dose can be substantially lower. Published levels of source contamination are difficult to find and a few are presented for illustration in Table 1–20. The infectious dose is influenced by a number of factors including the serovar, host resistance, age (young or old), immunocompetence, meal content, including the food type or food matrix (high fat) that is ingested and the physiological state of the bacterial cells; pre-exposure to heat or acid may increase survival in the gut of the host [39, 408, 409, 412, 413]. Two key risk factors for human exposure to *Salmonella* in eggs and poultry meat are under cooking and cross-contamination of which the former is the more important for eggs and the latter for poultry meat [430]. The role of carriers and food handlers as a source of infection has also been reviewed and the role of passive transfer during active infection is considered to be of greater importance in a food-handling environment than the role of carriers [412, 431]. *Salmonella* was demonstrated to survive on fingertips for up to 3-hours post contamination even after hand washing. The smaller the initial dose, the less time it was recoverable [432].

### 8.1 Epidemiology of Human Non-Typhoidal Salmonellosis in Australia

Information on human salmonellosis in Australia may be obtained from a number of disparate sources. The National Notifiable Diseases Surveillance System (NNDSS) reports monthly counts of salmonellosis notifications and rates per capita without serovar differentiation, while the National Enteric Pathogen Surveillance System (NEPSS) publishes annual summary reports for human and non-human salmonellosis, environmental and food testing and the Australian *Salmonella* reference centre (IMVS) reports quarterly and annual *Salmonella* laboratory testing results [390, 432, 433]. The NEPSS reports contain some of the same data reported by IMVS. Quarterly and annual foodborne disease reports are published by OzFoodNet Australia. All of the annual reports are now published either with a significant lag or not published at all, with the last OzFoodNet annual report, 2011, published in 2015 [405, 434, 435]. Each state health authority may publish *Salmonella* case or outbreak information but this information is limited in nature and more complete data must be sought from the health departments. The last Victorian annual *Salmonella* surveillance report was published for 2010 however monthly summaries of human salmonellosis counts by *Salmonella* serovar are published [436, 437].

In Australia in 2014, there were 16,354 cases of salmonellosis reported by the NNDSS. Between 1991 and end 2014 the mean per capita notification rate increased from 31.9 to 69.7 cases per 100,000 people [390] (Table 1–21). Significant endemic foci for a number of *Salmonella enterica* serovars are present in Australia: Northern Territory (*S.* Ball) Tasmania (*S.* Mississippi), Western Australia (*S.* Choleraesuis) and NSW (*S.* Wangata), NSW and Queensland (*S.* Birkenhead) [438-440].

The cost of foodborne illness to Australia was estimated to be in the order of 1.25 billion dollars per annum, with largest proportion of cost attributable to loss of productivity and lifestyle (62% of cost) with each notified *Salmonella* infection estimated to cost $1,387 per infection [396]. There is a strong seasonal

Table 1–21. Australian Salmonellosis Notification Rates, 2008-2017

| | *2008* | *2009* | *2010* | *2011* | *2012* | *2013* | *2014* | *2015* | *2016*[1] | *2017*[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| Rate per 100,000[2] | 38.3 | 43.5 | 53.6 | 54.6 | 49.1 | 55.0 | 69.2 | 71.1 | 74.1 | 67.9 |
| All salmonellosis[2] | 8,288 | 9,501 | 11,912 | 12,274 | 11,245 | 12,788 | 16,354 | 16,957 | 18,071 | 16,431 |
| *S.* Typhimurium | 3,481[2] | 3,895[2] | 5,241[2] | 5,940[2] | 4,812[3] | 5,469[3] | 7,680[3] | 7,180[3] | 6,026[3] | –[4] |
| *S.* Typhimurium (%) | 42 | 41 | 44 | 48 | 43 | 43 | 47 | 43 | 33 | – |
| Number of outbreaks[5] | 35 | 59 | 58 | 61 | – | – | – | – | – | – |
| Outbreak associated cases[5] | 486 | 765 | 793 | 920 | – | – | – | – | – | – |

[1]Data incomplete due to timing of annual update (June), [2] Report summary NNDSS, [3]Public Dataset NNDSS,
[4]– Annualised figures not reported to date, [5]OzFoodNet annual reports [405, 434, 435]

pattern with more notifications in the summer and autumn and per capita rates tend to be higher in Northern states [438]. Cases in young children (< 5) are notified more frequently than all other age groups [405]. However, it is noted that ascertainment bias may be responsible for more cases being reported in young children, the elderly or immunocompromised and are rarely representative of the true population burden. Additionally, there are differences between states with regard to investigation and attribution of cases, and testing regimes between laboratories [390, 441].

Using expert elicitation for the year circa 2000 it was estimated that between 70%, 95% Credible Interval (CrI) [38, 88%] and 87%, 95% CrI [80, 93] of all non-typhoidal *Salmonella* notifications were transmitted by food [442-444]. Other transmission pathways were environment 15% (5 - 25%), water 5% (1 - 5%), person 5% (1 - 5%) and zoonotic 4% (1 - 9%) [444]. Expert elicitation is frequently employed to estimate the source of *Salmonella* in the absence of epidemiological studies. The issues with regards to source attribution based on expert elicitation experts are subject to overconfidence and fail to identify the true estimate of known values, at least half the time [445, 446].

## 8.2 Source of Infection

In Australia, *Salmonella* Typhimurium is the most frequently identified cause of foodborne salmonellosis and has been for a number of years [405]. The proportion of cases attributable to this serovar had appeared to be slowly increasing, but this appears to have stabilised and may be declining (Table 1–21). However, there is considerable variation in top 5 *Salmonella* serovars and phage types reported both nationally and locally (Table 1–22).

In Victoria, *S.* Typhimurium phage types are frequently in the top 5 whereas in states such as Tasmania, *Salmonella enterica* subsp. *enterica* serovar Mississippi is frequently the top cause of human illness [405], suggesting different epidemiology in each state.

The last published annual report for Australia was for 2011. That year 12,271 cases of human salmonellosis were notified (54.3 cases per 100,000) [405]. Of those notified cases, 7.5% (917/12,271) were attributed to outbreaks (n = 61). *S.* Typhimurium was identified as the cause of illness in 92% (56/61) of outbreaks [405]. Of the outbreaks tied to a single food, eggs (29/33) and poultry meat (4/33) were the attributed vehicle. However, the remaining 92.5% (11,354/12,271) cluster or so called "sporadic" *Salmonella* associated cases were not attributed to a source, either foodborne or other.

Interpretation of foodborne illness from annual reporting information requires great care as not all sources of *Salmonella* are considered and reported and differentiation of outbreaks associated with transmission pathways other than food are not discussed.

## 8.3 Source Attribution Studies

Three Australian studies have been published attempting to correlate the source of *Salmonella* spp. with human cases. A source attribution study conducted using the NEPSS human and non-human surveillance data concluded that 35% of cases were attributable to poultry and eggs, other meats (31%), plant foods (10%) and 24% to other sources. It was also noted that pets may also be an important source of infection. Limitations of this study were that it was not based on random sampling, rather used surveillance data, therefore it was not representative of available foods, biased toward those foods sampled during outbreaks and failed to take into account that not all cases are foodborne [448]

A similar serovar matching study investigating the prevalence of *Salmonella* serovars on poultry meat and the incidence in humans before and after the introduction of hazards analysis and critical control points (HACCP) based processing standards concluded that there had been no substantial impact on human health as a result of the introduction of HACCP standards and that poultry *S.* Typhimurium serovars were similar to those attributed to human salmonellosis at the same period [331]. Significantly, the prevalence of *Salmonella* serovars in poultry did not match the top human cases, indicating that the relationship between poultry meat contamination and human disease is not simply associated. A case-control study was conducted in Adelaide comparing human *Salmonella* infections with a retail survey of poultry meat and egg *Salmonella* contamination over the same period of time. Despite 38.8% of poultry meat and 3.5% of eggs sampled being positive and 49% of the *Salmonella* serovars identified in humans were also identified in eggs and poultry meat, the exposure variables (including egg or poultry meat consumption) were not significantly different between cases and controls in this study. This is not surprising as the consumption of either chicken meat or eggs was reported by 62.2% of participants in the week prior to the onset of illness [327].

A Bayesian source attribution study evaluated the source of human salmonellosis in South Australia from food animals using the modified-Hald approach excluding cases acquired overseas, and including passive surveillance data [394]. Unsurprisingly, chicken meat and eggs were attributed as the key source of both sporadic and outbreak associated cases of salmonellosis in South Australia. It was estimated 35% (95% CrI [20%, 49%]) and 37%,(95% CrI [23%, 53%]) of sporadic cases were attributable to poultry meat or eggs respectively, with eggs attributed to 59% (95% CrI [29% ,75%]) of outbreak cases [449]. One model was discarded as not likely, where chicken meat did not conform to expected transmission values [449]. Does this represent confirmation bias? Confirmation bias is an important factor in causal attribution models. Experts are prone to flawed or biased interpretation of causality and applicable evidence [450] and the removal of identified transmission paths because they do not conform to perceived assumptions is a form of confirmation bias and is not discussed. The passive surveillance data presented in the supplementary data includes all South Australian sourced *Salmonella* results. It is unlikely that the epidemiology of human salmonellosis in South Australia is representative of all of Australia, for the reasons described above. Additionally, the number of chicken isolates reported were considerably higher in the ten year study period i.e. ~180 *Salmonella* isolates per year versus 11, 22 or 50 isolates per year for pigs, sheep and cattle respectively [451]. In 2012 – 2013 there were 1,626 sheep, 1,702 beef and dairy, 90 poultry and 93 pig single species farms in South Australia [452, 453]. Given the respective size of the agricultural industries in South Australia, in farm numbers alone, there was a larger number of *Salmonella* samples from the chicken industry (equivalent to ~2 samples/

Table 1–22. Top 5 *Salmonella* Serovars reported in Humans, Australia (2009-2011)

| *Salmonella serovar* | *% Salmonella cases by serovar [Ref]* | | |
|---|---|---|---|
| | *2009 [447]* | *2010 [9]* | *2011 [405]* |
| *S.* Typhimurium | 41 | 44 | 48 |
| *S.* Enteritidis | 9 | 7 | 7 |
| *S.* Virchow | NR[1] | 5 | 5 |
| *S.* Saintpaul | 3 | 4 | 3 |
| *S.* Infantis | NR | 3 | NR |
| *S.* Paratyphi B biovar Java | NR | NR | 2 |

[1]NR Not reported in that calendar year

year/farm) than the other industries. The key question is do these numbers accurately reflect the relative risk from the source populations or simply sampling bias? Sparse or small numbers will result in low precision, and bias in estimates using the modified-Hald approach [399].

## 9. Key Research Gaps Identified in the Literature

During this review, several critical gaps were identified in the literature that are relevant to the understanding of the epidemiology and transmission of *Salmonella* within poultry populations and particularly within the Australian context.

### 9.1 Source Attribution in Humans

Source attribution from outbreaks is frequently linked to the consumption of poultry and eggs, but cases non-attributed to a source are typically excluded from analysis, but comprise 95% of Australian cases. Where do they come from, is serotyping sufficient for source attribution studies where a limited number of phage types predominate?

Why are human cases in Australia not declining nor is there a reduction in the seasonal distribution of cases? Cannot be a simple case of chickens or eggs have more *Salmonella*. There appears to be little temporal or annual change in *Salmonella* prevalence in intensively sampled flocks? Is there an age effect or a seasonal effect in *Salmonella* infection in poultry flocks in Australia that may contribute to this effect?

### 9.2 Litter

Does the use of litter amelioration increase the prevalence of *Salmonella* within flocks housed on the floor? What about the timing of litter turning, does this affect flock prevalence? Limited studies report structured sampling to determine the heterogeneity of *Salmonella* distribution within floor reared house types.

### 9.3 Hatchery Design

What is the effect of different fertile egg treatments on the prevalence of *Salmonella* in progeny: Effect of "dirty" eggs from infected parent flocks vs "clean" eggs. Few if any studies specifically track breeder eggs within the hatchery and no discussion is made of hatchery type (multi-stage versus single stage incubation) or design or management factors on flock infection.

### 9.4 Egg Laying Flocks (Fertile or Table Egg)

Are the risk factors in parent breeding flocks (fertile eggs) the same as those in the laying (table eggs) industry? Are there effects of breed or egg production type (fertile, table) on egg contamination?

Is there a difference in flock or farm prevalence due to housing type and flock sizes or are these results confounded by shed and farm size? Temporal and molecular confirmation that the source of infection was the shed environment and not a new introduction from another source is required in future studies.

Intensive longitudinal sampling of flocks and or sheds of known *Salmonella* status by following product (eggs, chicks, broilers) from these flocks to confirm source of infection.

There are key gaps in sampling methodology regarding homogeneous or heterogeneous population distribution in sheds over time. Is shed contamination and dissemination a flock or shed factor influenced by the environment only or by flock shedding. Sampling is currently conducted assuming homogeneous distribution of either *Salmonella* within sheds or within flocks and appropriate sampling methodology.

Shed description of housing types does not describe the manure removal systems. Do birds in cages have access to the manure belt above them?

Information on the status of pullets prior to placement in a new production environment. No studies report sampling in pullet rearing environments, or the status of pullets prior to placement. These results confound assumptions of carryover infection being the primary source of infection particularly when birds are reared in one location and moved to another.

The efficacy of wet and dry cleaning in the reduction of *Salmonella* contamination in a shed and the effect on subsequent flock contamination is reported, but not for Australia, how many *Salmonella* are present in sheds and are they the same isolates for reinfection?

Temporality of infection derived from vermin into flocks and the contamination of flock from vermin by molecular typing has not yet been confirmed. Do vermin represent environmental contamination (indicators) by eating the same source of infection as chicks or birds in addition to being a potential source of infection?

### 9.5 Feed

There is no annual surveillance nationally for feed ingredients (including cereal grains) other than passive surveillance conducted by feed mills. Storage of cereal grains on the ground is a primary method of bulk storage during harvest and poses some risk for contamination of cereal grains particularly. No published studies have described the consequence of using poultry manure as a potential source of Salmonella into cereal grains in Australia. Why is *S.* Typhimurium not reported in feed very frequently now compared to previously? Is this a real change?

## 10. Research Aims

This project attempts to further our understanding of the transmission of salmonellae and *S.* Typhimurium, within a vertically integrated chicken meat organization. The first part of the study describes the transmission paths within the integrator using social network analysis (Chapter 3). The second part of the study describes the sampling results at each location using phenotyping (serotyping and phage typing) and MLVA profiling (Chapter 4) and the results of intensive sampling within the parent flocks and environmental factors that may affect these findings (Chapter 5). A comparison is made of the different phenotyping and genotyping methods used to describe the *Salmonella* isolates detected at each of the sampling locations using diversity analysis (Chapter 6) and then the use of whole genome sequencing to further characterize the *S.* Typhimurium isolates (Chapter 7). The final chapter makes a comparison of the *S.* Typhimurium isolates detected in this study with those found in human and non-human populations reported to the NEPSS database for the coincident period (Chapter 8).

2

CHAPTER TWO

# Methodology: Longitudinal Study Design and Sample Processing

*(next spread)*
*How the study was designed and the samples collected and processed.*



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# Introduction

A small number of surveys have been conducted in Australia focused on identifying *Salmonella* in eggs at retail, egg production systems or poultry meat contamination at processing or retail. There have been no studies published describing transmission in Australian broilers or integrated chicken meat companies since the mid 1970's [18-20]. These studies were limited to descriptive analysis at the *Salmonella enterica* serovar level and focused on the attribution of *Salmonella* to feed sources. A small number of international studies describe the transmission of *Salmonella* through integrated broiler operations from the breeders to processing [197, 244], with hatcheries frequently identified as the most significant source for dissemination to broilers [196, 217], but again limiting *Salmonella enterica* subspecies *enterica* identification to the serovar level [196, 197, 295].

Chicken meat is grown in all Australian states with production concentrated within a small number of vertically integrated companies. Nearly one billion meat birds are processed annually by seven companies, with two of these enterprises growing and supplying 70% of Australian chicken meat. The remaining companies supply the rest of the market [242]. The majority of chicken meat is sold as fresh poultry (60%) and is distributed daily via major supermarkets (~70%), or direct to butchers (~18%) from processors [454]. Approximately 3% of poultry meat is exported and no fresh poultry meat is imported into Australia [242]. Australian chicken meat consumption has been increasing 2.0% per annum since the mid 1960's and in 2006, per capita chicken meat consumption surpassed beef, with current annual consumption estimates of ~49 kg per person [242].

The Australian chicken meat industry is vertically integrated [333]. Depending on the size of the integrator they may own primary breeding operations, breeder farms, hatcheries, broiler farms, processing plants and feed mills. Modern poultry production and processing has essentially remained the same for over 40 years with a number of sequential stages occurring [455]. Parent broiler breeders are purchased from primary breeding companies, reared to produce eggs, which are transferred to a hatchery for incubation. After 21-days incubation, day old chicks are transported to broiler houses where they are reared on the floor and fed ad lib until they reach market weight ~1.6 kg (dressed) at 5-6 weeks of age. Processing companies produce day old broilers for grow-out, organize transportation of the birds for processing and process chickens for human consumption. Broiler grow-out in Australia is typically done under contract by independent broiler growers. Contract broiler farmers own their own properties, sheds and equipment [242].

*Salmonella* contamination of broilers or chicken meat at processing or retail is well documented and multiple pathways for the entry of *Salmonella* into an integrated broiler operation exist, both vertical and horizontal. In addition, multiple points within a poultry operation exist where amplification and dissemination of *Salmonella* may occur. Sources of *Salmonella* can be defined as those entering the poultry house; infection with the chicks (vertical), contaminated feed (horizontal) during a batch and those residing within the poultry house present before chicks arrive such as carryover infection or contaminated reservoir rodent or insect populations [196, 306]. Chicks at day old can be infected vertically from infected parent flocks or horizontally during hatching, loading and transport to the farm [217]. Rapid spread of *Salmonella* throughout the house post placement may occur at a time when birds are most susceptible to infection with a low infectious dose [123, 130, 252]. Following natural infection shedding in broilers peaks around 14 days and declines until slaughter [252], but may persist to 10-12 weeks of age [123], well beyond the age for processing a commercial broiler (32 – 35 days of age at 1.6kg carcase weight). High stocking density, incorrect ventilation, and poor litter quality may increase *Salmonella* shedding and favour multiplication and spread, particularly at the end of rearing when shed density increases as a result of bird weight gain and declining litter quality [258].



When considering only the potential pathways for the vertical dissemination of *Salmonella* through a poultry organization, even within a single operation where all aspects of the production process are managed within the same organization, the relationships are complex.

It is unknown if *Salmonella enterica* serovars, particularly *Salmonella* Typhimurium, detected in Australian integrated operations are clonally transmitted through the operation or are introduced from multiple different sources.

## 1. Aims/Purpose

The purpose of this study was to investigate the relationships between *Salmonella* spp. isolated within a vertically integrated poultry production system. This chapter describes the integrator and study methodology developed to investigate the transmission of *Salmonella* within such an operation. The sampling method was designed to identify *Salmonella* positive flocks at pullet rearing and follow these flocks and their progeny longitudinally for the productive life of the breeder flocks. Eggs and broilers produced from these flocks were also sampled longitudinally with the aim of identifying points of introduction and/or dissemination within the integrator system by phenotyping and genotyping of the *Salmonella* isolates detected.

## 2. Units of Study

To maintain integrator confidentiality commercially sensitive details regarding the operation have been omitted.

### 2.1 Integrator Description

A schematic of the organization of the vertical integrator is presented in Fig. 2–1. The integrator is the second largest chicken meat supplier in Victoria and supplies approximately ~6% of the Australian chicken meat market. The integration is typical of all vertically integrated chicken meat operations in Australia , which are similar in arrangement to the USA integrator models. The company comprises (Fig2-1 C.) a single hatchery and processing plant, multiple parent (pullet rearing and egg production) and broiler grow-out farms.

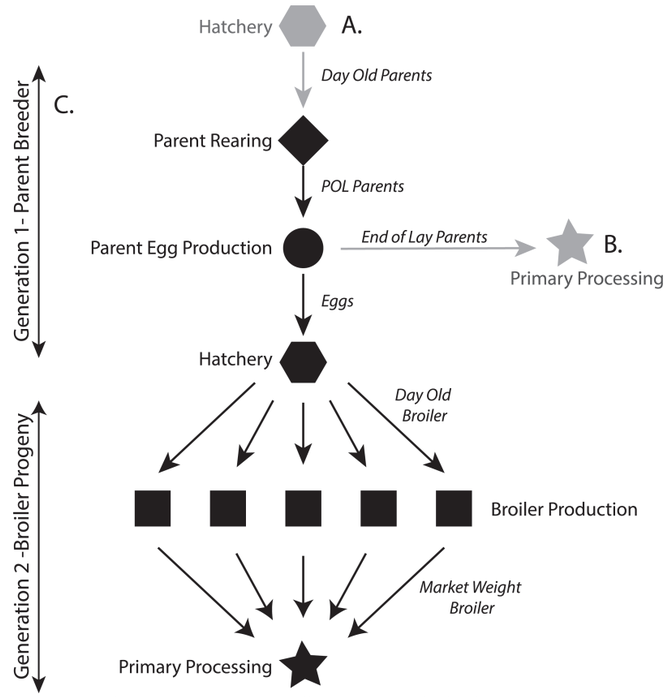


**Fig. 2–1. Schematic of a Vertically Integrated Chicken Meat Enterprise**

**A.** Primary breeder supplier; **B.** External Poultry Processor; **C.** Vertical integrator comprises parent farms (pullet rearing and egg production), hatchery, broiler farms and a processing plant. Major movement paths of key products (eggs and birds) are drawn. POL Parents: point of lay parent breeders

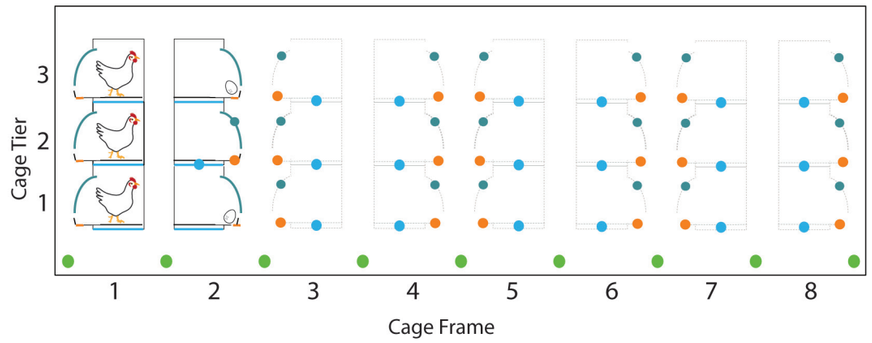


**Fig. 2–2. Sampling Locations within Colony Cage House by Spatial Reference to each Colony Cage Frame and Tier**

Each cage frame is identified along the horizontal axis (1-8), and each tier of cages per frame is identified on the vertical axis (1-3). The frame outline and position of birds within each frame relative to the sampling location are indicated on the left. Potential sampling locations are indicated by a point with the colour representing the sample type. Colours indicate the sample type; Boot Swab = Green, Manure Belt = Blue, Egg Belt = Orange and Dust = Dark Blue.

Broiler production comprised both contract grower and company owned farms. Day old breeding birds were supplied by a single primary breeding company (Fig 2–1 A.) and end of lay breeders were processed by an external processing company (Fig 2–1 B.). Feed was supplied by a single feed supplier from multiple feed mills. A full description of the system is described in Chapter 3. Birds were housed in compliance with the Australian model code of practice for poultry [456] and state legislation [457]. All sheds were tunnel ventilated with cool pads for heat management. Sampling was conducted at breeder (pullet rearing and production) farms, the hatchery, broiler farms and the poultry processing plant.

## 2.2 Parent Farms (Broiler Breeder)

### 2.2.1 Rearing Farms

For each placement cohort, a single primary breeding company supplied the day-old chicks. Each placement cohort comprised four flocks; 3 hen flocks and 1 rooster flock (one flock per shed) reared on a single site (farm). Birds were reared on the floor with bedding material of rice hulls or wood shavings. Birds were housed in a single age all-in all-out system with pan feeders and nipple drinkers. Each cohort was treated as a single management unit of the same biosecurity status. At the end of rearing pullets were transferred to the parent production farms for the onset of lay.

### 2.2.2 Parent Production Farms

During egg production parent birds were either housed in multi-tiered frames in enriched colony cages (400 birds per colony) one cage width per frame, Fig. 2–2 or in barns with a colony nest box system.

Parent production flocks were transferred from pullet rearing to production facilities at the point of lay (POL) between 19-21 weeks of age. Each breeder flock (females plus 10% males) was managed as a single age group, with all-in all-out management. Routine sampling of all breeder production flocks occurred during the study period and more than one flock may have been sampled per shed. All production flocks transferred during the study period were sampled at least once. On each breeder production farm eggs were packed and identified by breeder flock and date of lay.

## 2.3 Hatchery

A single hatchery received eggs from breeder farms owned by the integrator only. Eggs were set and hatched in a single stage incubation system. Eggs were managed by donor flock throughout the incubation process. On delivery to the hatchery, egg traceability was managed throughout the incubation process from storage to chick hatch and broiler placement using the farm identification. Egg transport and chick placement was managed by the hatchery and planned delivery schedules were implemented in company owned vehicles.



## 2.4 Broiler Production Farms

Broilers were floor reared in single age tunnel ventilated sheds on new litter. Litter comprised either rice hulls or wood shavings supplied by external providers. Broiler production farms were either contract growers or company owned farms. All sheds were managed as all-in, all-out production system with flocks ranging in size from 7,000 to 60,000 birds. Birds were processed at market weight starting at 1.6 kg dressed weight at approximately 32 days of age. Multiple pick-ups from each flock may occur until the flock is completely depopulated.

## 2.5 Primary Processing

A single processing plant was supplied by all broiler production sites. Broiler processing, pick up schedules and processing was organized by the integrator. Collection of broilers was contracted but conducted using integrator supplied vehicles, crates and equipment. Primary processing, slaughter and production of a dressed carcass and secondary processing, production of secondary processed product, packaging, chilling and product distribution occurred from a single site. Processing schedules were managed to meet daily and weekly fresh meat market demand.

Depopulated parent flocks (at the end of egg production) were processed by an external processor. Samples were not collected from this location.

## 2.6 Biosecurity, Cleaning and Disinfection

### 2.6.1 Biosecurity

Strict biosecurity processes were in operation during the time of this study. All visiting persons external to the integrated operation must have had at least 72 hours down-time from visiting other poultry or pig operations, and were only permitted to visit with management authorisation. No staff or contractors are permitted to have contact with poultry or birds at home. Staff movement between locations is strictly managed according to risk. No staff move between generations of birds (Parent rearing or egg production and broiler production) without stand down periods of at least 24 hours between locations and only when absolutely necessary. Vehicle movements only occur between locations of the same biosecurity status and dedicated vehicles are utilised for each specific task. At the highest biosecurity sites automated truck washes are used prior to all vehicle entry to site. On high biosecurity sites (pullet rearing) dedicated site vehicles are provided for movement within a site. These vehicles do not leave the site location.

### 2.6.2 Cleaning and Disinfection

At the end of each batch of birds flocks are depopulated for processing. Manure or bedding material is emptied from the shed prior to cleaning. All non-fixed plant and equipment is removed from the shed prior to dry clean-

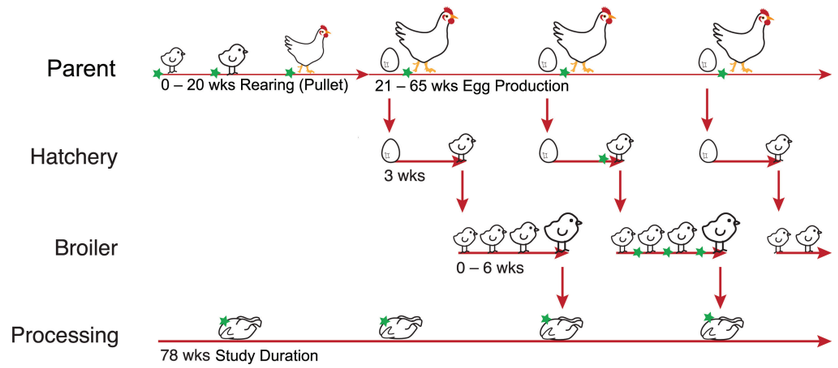


**Fig. 2–3. Study Sampling Design Schematic Illustrating the Longitudinal Sampling Interval from Breeder Flocks through to Processing for the Duration of the Study**

The time of sampling at each location is indicated by a green star. Sampling was repeated three weekly during the parent life, at day of hatch in the hatchery, on four occasions prior to collection for processing during broiler production. Samples from processing were provided every month for the study duration.

ing. Dry cleaning entails the removal of all gross organic material from the environment by sweeping and compressed air. On completion wet cleaning is initiated. Detergent is applied to all surfaces by foam application in accordance with the manufacturers instructions. At the time of the study Foamclean S™ (Chemetall Pty Ltd, sulphamic acid organic salt) was used as the primary detergent. The detergent is rinsed off all surfaces prior to drying by agitation (high pressure water). After surfaces were dry, disinfectant was applied and left to dry. Cleaned surfaces were disinfected with acidified glutaraldehyde (Chemetall Pty Ltd, Glutachem™) applied at 10ppm until the surface was saturated and drying occurred over no less then 15minutes contact time. At the end of the cleaning process all sheds were inspected for compliance with a cleaning score card. Any failures resulted in either re-cleaning or reapplication of final disinfectant.

## 3. Study Design

A longitudinal study was conducted over an eighteen-month period between January 2013 and July 2014. The study was designed to follow longitudinally *Salmonella* positive parent flocks, identified at rearing, through production to the end of life. Where possible, samples were collected from the equivalent sampling date at the hatchery and several batches of chicks produced from the sampled breeder flocks were followed to the broiler farm. A schematic of the sampling design is illustrated in Fig. 2–3.

### 3.1 Parent Rearing and Production

For the duration of the study, environmental sampling was conducted at 3 weekly intervals at all parent rearing sites. All parent production flocks were sampled at least twice during production.

#### 3.1.1 Flock Recruitment into the Longitudinal Study

On detection of a *Salmonella* positive parent flock at rearing, the flock was purposefully recruited for inclusion into the longitudinal study. Two cohorts comprising three parent flocks identified as *Salmonella* Typhimurium positive during rear were recruited into the study. These two cohorts were transferred into four production sheds (A-D). The four flocks were followed longitudinally and were sampled every three weeks for the duration of the study until the end of production.

### 3.2 Hatchery

Between August 2013 and July 2014, chick papers or hatch debris from flocks A – D were collected from eggs produced the same week as on farm sampling occurred, when possible. For a 12-week period, between February and May 2014, chick papers were collected from all production flocks hatched each week.

### 3.3 Broiler Production Farms

For a 16-week period, February to May 2014, 46 broiler flocks were sampled longitudinally from day old to end of rearing. For each flock, sheds were sampled three times: at 3 weeks of age, at the end of the batch prior to shed clean-out and post cleaning. Four flocks were sampled weekly until first pick-up.

### 3.4 Primary Processing

A random selection of suspect *Salmonella* spp. positive carcass or portion rinse samples were obtained from primary processing between July 2013 and July 2014. Carcass rinse, caecal and crop samples were obtained from a primary processing process control survey conducted in May 2013 from a single production line. *Salmonella* isolates and results for this enterprise from a retail survey conducted in South Australia in October 2013 were obtained from the principal investigator [326].

### 3.5 Production Records

During the study period, daily production records were obtained for all production sites that were sampled. Major movements of products such as feed, eggs and birds between each of the sites and within sites were obtained where possible. Full details of the records collected are described in Chapter 3. Historical *Salmonella* testing results were obtained for a 5-year period prior to the onset of the study and during the study period routine testing results were supplied by the integrator.

### 3.6 Weather Data

Weather data for the breeder production location was obtained from records kept by the Australian Bureau of Meteorology (www.bom.gov.au) for the weather station closest to the farm (~6 km). Daily records were downloaded for the sampling period and summarized for rainfall (mm), temperature (°C) and solar radiation (MJ/m$^2$). Internal shed temperature and humidity observations were collected but were incomplete so were not utilized.

## 4. Ethics Statement

No birds were directly sampled or handled during this study. In accordance with the University of Melbourne Animal Ethics requirements this study did not require animal ethics approval as all sampling was conducted as part of routine veterinary care and agricultural practice.

## 5. Sampling Methodology

### 5.1 Parent (Broiler Breeder) Sites

#### 5.1.1 Rearing Farms

Samples were collected from pullet flocks placed during the study period at day old, via chick papers (paper that lines the inside of a chick transport box), and sampled four times during rear using drag swabs. All samples were tested to detect *Salmonella* spp. Ten chick papers, were randomly collected from each placement into a sterile plastic bag. Drag swabs comprise 2 to 4, 10 x 10 cm gauze swabs tied to a 1-2m length of string that are dragged systematically across the floor of a shed. Four drag swabs were collected from each shed at each sampling event with one swab covering an area of approximately a quarter of the shed. The four swabs were pooled into a sterile plastic bag, identified by shed, age and flock ID, refrigerated and processed on the same day.

#### 5.1.2 Egg Production Farms

All longitudinally studied parent flocks were housed in a colony cage system as depicted in Fig. 2–2. To ensure sampling repeatability and to meet occupational health and safety requirements, all samples of the cage system were collected within easy reach and no high platform equipment was utilized. The four longitudinally sampled production sheds (A-D) were sampled prior to placement, immediately post cleaning, and every three weeks until the start of flock depopulation (58 - 62 weeks). The last sampling date occurred on the day of pick-up prior to the arrival of catching crews, so that contamination with *Salmonella* spp. from other sheds via catching crews was unable to confound results.

Samples were systematically collected between 7 am and 10 am, from each cage frame, with the same locations sampled at each sampling event. Four 10 x 10 cm cotton gauze swabs (pre-moistened with buffered peptone water) were used to collect each egg belt, manure belt and dust sample by wiping each respective surface. Egg belt samples were collected from the bottom of the egg belt surface (height: 1.00 - 1.20 m). Dust samples were collected from the uppermost surface of the nest box (height: 1.20 - 1.40 m) and manure belt samples were collected from all exposed manure belt surfaces within reach at the end of each frame (height: 0.50 - 1.80 m). Dust and egg belt samples were collected from each row of cages along the horizontal length of each frame while manure belts were sampled at the end of each frame of cages.

While sampling each shed, two pairs of boot swabs were worn. Boot swabs were worn over clean dry boots, and new plastic boot covers. A pair of boot swabs was exchanged after half the shed was walked for a new pair of boot covers and boot swabs. Each boot swab was processed as a single sample (4 boot swab samples per shed per sampling event).

Each sample-type was pooled separately by frame and cage row into a Whirlpak™ bag and identified by shed, flock, sample type and row ID. Samples were immediately refrigerated and transported to the laboratory for processing on the same day. Fresh whole eggs were collected when available. At the end of production culled birds from sheds A and B were necropsied and fresh samples collected on farm. Samples of liver, spleen, ovarian tissue and caecal and cloacal swabs were collected aseptically. Each sample was identified by bird, flock and sample type, refrigerated and transported for same day processing.

### 5.2 Hatchery

One pool of hatch debris or chick papers, were collected for each breeder flock of interest on hatch-day. Chicks were placed into baskets lined with newsprint paper for transport. After ~1 hour, the paper was retrieved from 10 baskets (per donor flock) and placed into a sterile plastic bag.

Hatch debris (egg shell and adhering material) was collected from chick transfer trays at the point of chick sorting, until a minimum equivalent of 100 eggs was collected into a sterile plastic bag. Hatch debris was collected from at least 10 trays and multiple trolleys from the same donor flock. All hatchery samples were identified with date and breeder flock details and transported unrefrigerated to the lab. Source breeder flock identification was maintained throughout chick processing and delivery to farm.

### 5.3 Broiler Production Farms

Chick papers were collected at the hatchery from the donor flock supplying the flocks placed during the study period.

All sheds were sampled in the morning before the arrival of farm staff, where possible. Three environmental sample types were collected: boot swabs, wall and fan dust. Boot swabs were worn over dry, cleaned and disinfected, boots and new plastic boot covers. Boots were exchanged between sheds. Two pairs of boot swabs were worn per shed, each pair pooled into a single Whirlpak™ bag. Boot swabs were worn while collecting other samples and were exchanged at the end of the shed, with each pair covering an area of approximately half the shed.

Wall dust was collected by wiping twelve 10 x 10 cm gauze swabs along the wall the length of each side of the shed. Fan dust was collected by wiping twelve 10 x 10 cm gauze swabs over the surface of all shed fan guards. For each sample type, pools of six swabs were placed into a single Whirlpak™ bag. Each sample was identified by shed, date, flock ID, refrigerated immediately and processed the same day.

### 5.4 Primary Processing

Carcass rinse and portion rinse samples are collected daily from each production shift as part of routine primary processing quality assurance testing in accordance with the Australian Standard AS4465 [458]. Positive samples were provided monthly during the study period.

On a single occasion, from a single production line, 10 carcasses were randomly selected from four points in the processing line: post plucking, post evisceration, prior to chilling, and post chilling. A carcass rinse sample was taken from each bird. From the ten carcasses obtained post plucking, the entire caeca and crop were collected aseptically into a sterile Whirlpak™ bag. Each sample was identified by date, and carcass ID, refrigerated immediately and processed the same day.

Table 2–23. Primary Sample Unit Description and Pool size or volume by Location

| *Location* | *Sample Type* | *Sample Material* | *Pool Size/ (No. or volume)* |
|---|---|---|---|
| Parent Egg Production Farm | Dust | Gauze Swab | 2 |
| | Manure Belt | Gauze Swab | 2 |
| | Egg Belt | Gauze Swab | 2 |
| | Boot Swab | Boot Swab | 1 |
| | Egg | Whole Egg | 1 to 10 |
| | Cloacal Swab | Cotton Tip Swab | 1 |
| | Caecal Swab | Cotton Tip Swab | 1 |
| | Fresh tissue | Ovary, Liver, spleen | 1 |
| Hatchery | Chick Paper | Basket Liner | 10 |
| | Hatch Debris | Hatch remnants | 100 g |
| Broiler Farm | Boot Swab | Boot Swab (ea) | 2 |
| | Dust - Wall | Gauze Swab | 6 |
| | Dust - Fan | Gauze Swab | 6 |
| Processing | Carcass Rinse | Rinsate | 10 mL |
| | Portion Rinse | Rinsate | 10 mL |
| | Crop | Crop content | 10 mL |
| | Ceaca | Caecal content | 1 g |



## 6. Sample Processing

### 6.1 Primary Samples

The primary sample types for each location, material used for sample collection and the sample pool sizes for microbiological testing are summarized in Table 2–23. Details of the treatment of primary samples for microbiological analysis and incubation conditions at each stage of processing are described below.

#### 6.1.1 Chick Papers

To each sample of ten chick papers 900 mL of buffered peptone water (BPW) was added and the sample left at room temperature to incubate for approximately 30 minutes. The sample was then macerated manually by massaging the paper within the bag and incubated.

#### 6.1.2 Hatch Debris

Each sample of hatch debris was crushed manually and mixed thoroughly. Three sub-samples (100 g) were taken randomly from each primary sample and BPW (500 mL) was added to each and incubated.

#### 6.1.3 Boot Swabs

Each pair of boot swabs collected from breeder farms were processed as individual swabs, while boot swabs collected from broiler farms were processed as a pair of boot swabs. BPW (200 mL) was added per boot swab with minimal mixing and incubated.

#### 6.1.4 Eggs

Individual whole eggs (not cracked or broken as determined by candling) were placed in a sterile Whirlpak™ bag containing BPW (10 mL). The surface of the egg was gently massaged for 1-2 minutes to remove as much surface debris, as possible. The rinsate was collected aseptically from the Whirlpak™ bag into a sterile McCartney bottle, while the egg was transferred to a new sterile Whirlpak™ bag containing BPW (50 mL). Each egg was manually macerated and the contents gently mixed for 1 – 2 minutes.

Table 2–24. *Salmonella* spp. Growth Characteristics on Culture Media

| *Test Media* | *Colony Morphology* | *Agar Colour Reaction* | *Other growth characteristics* |
|---|---|---|---|
| XLD (Oxoid CM0469) | Red with black centre | Red | Non $H_2S$ fermenters red colonies |
| CLED (Oxoid CM0301) | Flat blue colonies | No change | – |
| BGA (Oxoid CM0263) | Red-Pink-white opaque colonies | Red | Lactose fermenters pink colonies |

Table 2–25. Biochemical Reaction for *Salmonella* spp. Confirmation

| *Test Media* | *Slant* | *Butt* | *Gas* | *$H_2S$ Production* |
|---|---|---|---|---|
| TSI | Red or no change | Yellow | Yes | Black |
| LIA | Purple | Purple | No | Black |

Table 2–26. Biochemical Reaction for *Salmonella* Sofia Differentiation

| *Test media* | *Start Color* | *Salmonella Sofias* | *Salmonella spp.* |
|---|---|---|---|
| ONPG | Colourless | Yellow | No change |
| Mannitol | Blue-Green | Yellow | No change |

### 6.1.5 Egg Pools

Pools of 4 - 10 eggs were placed in a sterile Whirlpak™ bag containing BPW (200 - 400 mL). Eggs were macerated manually and the contents gently mixed for 1 - 2 minutes. Egg pools were incubated at 37°C for 48 hours with sub-samples collected for testing at 24 and 48 hours.

### 6.1.6 Carcass or Portion Rinse

Carcass rinse samples were collected in accordance with the Australian Standard AS4465 [459]. A fresh chicken carcass was collected from the processing line or chiller into a sterile plastic bag. BPW (500 mL) was added to a single bagged chicken, and massaged gently for 2 - 3 minutes. A sub-sample (10 mL) of the rinsate was collected as the primary sample.

Portion rinse samples were collected by adding a fresh chicken portion into a sterile bag containing BPW (200 mL) and massaged gently for 2 - 3 minutes. A sub-sample (10 mL) of the rinsate was collected as the primary sample.

## 7. Microbiological Testing

Microbiological testing was conducted as per the Australian Standard : 5013.10-2009: Food Microbiological Method 10: Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002, MOD) [459]. To each primary sample BPW was added with little mixing and each suspension was statically incubated at 37°C for 18-24 hours. After incubation, three aliquots (33 µL: total 0.1 mL) were taken from each primary sample and inoculated onto Modified Semi-solid Rappaport Vassiliadis (MSRV) plates.

MSRV plates were incubated aerobically at 41.5°C and visually examined at 12, 24 and 48 hours post inoculation. Plates with evidence of swarming growth have a grey-white turbid zone extending from the inoculation point with a clearly defined edge. Positive plates were sub-cultured by streaking in duplicate onto Xylose–Lysine–Desoxycholate agar (XLD), and either Brilliant Green Agar (BGA) or Cystine Lactose Electrolyte Deficient agar (CLED). XLD, BGA and/or CLED plates were incubated for 24 hours at 37°C. When swarming growth was observed after the first 24 hours on XLD or BGA/CLED, no further plating from MSRV was conducted.

Suspect *Salmonella* spp. positive colonies on XLD, BGA or CLED (Table 2–24) were confirmed biochemically in triplicate using Triple Sugar Iron agar (TSI) and Lysine Iron agar (LIA), incubated for 24 hours at 37°C (Table 2–25). Biochemical differentiation of *Salmonella* Sofia isolates was conducted in duplicate with suspect positive isolates that tested positive for O-nitrophenyl-β-D-galacto-pyranoside (ONPG) and able to ferment mannitol presumed to be *Salmonella* Sofia (Table 2–26). *Salmonella* positive samples were selected for typing. If more than one morphologically distinct colony type was observed in the primary culture then additional colonies were selected. All positive samples were sub-cultured into Tryptone Soya broth (Oxoid CM0129) containing 30% glycerol, and stroed at -80°C, or *Salmonella* maintenance media for long-term storage.

### 7.1 Serotyping, Phage Typing and MLVA

Serotyping, phage typing and multi-locus variable tandem repeat analysis (MLVA) were conducted at the Victorian Salmonella Reference Laboratory (Microbiological Diagnostic Unit). All samples were initially screened using the H antigen (flagella). Tentative *S.* Typhimurium isolates (H = i) were fully serotyped, phage typed and multi-locus variable tandem-repeat analysis (MLVA) sequence typed. For each sample that was not H=i, at least one sample per shed per submission was fully characterized. Isolates were serotyped using the Kauffman–White–Le Minor scheme [30] and phage typed using the Anderson phage typing scheme [63]. MLVA analysis was conducted in accordance with the European MLVA protocols [69] on all submitted *S.* Typhimurium strains.

Table 2–27. Multiplex PCR primers for Screening *Salmonella spp.*, *S.* Typhimurium, *S.* Infantis

| *Gene* | *Primer* | *Primer sequence (5'- 3')* | *Product Size (base pairs)* | *Reference* |
|---|---|---|---|---|
| *STM* | STM4497F | GGAATCAATGCCCGCCAATG | 523 | [460] |
| | STM4497R | CGTGCTTGAATACCGCCTGTC | | |
| *Fli*B | 878F | TTGCTTCAGCAGATGCTAAG | 413 | [461] |
| | 1275R | CCACCTGCGCCAACGCT | | |
| *Inv*A | 139-141F | ACAGTGCTCGTTTACGACCTGAAT | 244 | [462] |
| | 139-141R | AGACGATGGTACTGATCGATAAT | | |



Table 2–28. Antibiotic Disc Potency and Interpretative Zones of Inhibition and MIC (mg/L)

| *Antibiotic Disc* | *Disc Concentration (µg)* | *Minimum inhibitory concentration (mg/L)* | *Susceptibility interpretation zone (mm)* |
|---|---|---|---|
| Ampicillin | 25 | ≤ 8 | > 6 |
| Apramycin | 15 | ≤ 8 | > 4 |
| Cefotaxime | 5 | ≤ 1 | > 6 |
| Chloramphenicol | 30 | ≤ 8 | > 6 |
| Ciprofloxacin | 2.5 | ≤ 1 | > 6 |
| Naladixic Acid | 30 | ≤ 4 | > 6 |
| Neomycin | 30 | ≤ 8 | > 4 |
| Streptomycin | 25 | ≤ 16 | > 6 |
| Sulphafurazole | 300 | ≤ 64 | > 6 |
| Tetracycline | 10 | ≤ 4 | > 4 |
| Trimethoprim | 5 | ≤ 4 | > 6 |

### 7.2 Serotyping using PCR

Isolates, where it was not possible to determine a serotype using conventional serotyping methods, were used to develop a multiplex PCR to screen for *Salmonella* spp., *S.* Typhimurium, *S.* Infantis and *S.* Sofia. Published primers and methods were used as described (Table 2–27). Each suspect colony was suspended in sterile water (200 µL), and incubated at 100°C for 2 minutes and then centrifuged, once cooled, at 16000x g for 5 minutes and the supernatant was used as template for PCR. The PCR was performed in a final volume of 20 µL containing 1 µL template, dNTPs (1.25 mM), oligonucleotide primers (10 mM), MgCl2 (25 mM), 5 x GoTaq® buffer, water and 1 x GoTaq® enzyme (Promega). The reaction cycling conditions were as follows; initial denaturation step 95°C for 5 minutes, then 35 cycles of denaturation at 95°C for 1 minute, primer annealing at 62°C for 30 seconds and extension at 72°C for 1 minute, followed by the final extension step at 72°C for 10 minutes. PCR products were electrophoresed on a 1.5% agarose gel, prepared in 0.4x TBE with SyberSafe® DNA gel stain (Invitrogen) at 80 V/cm for 20-30 minutes. Hyperladder-IV (Bioline) was used as a DNA molecular weight marker.

### 7.3 Antimicrobial Susceptibility Testing

*Salmonella* Typhimurium positive samples were tested for antimicrobial susceptibility using disc diffusion in accordance with the Calibrated Dichotomous Susceptibility (CDS) method [463]. Selected isolates were incubated aerobically overnight on nutrient agar plates (Oxoid CM0003) at 35 - 37°C. Antimicrobial susceptibility testing was conducted on sensitest agar (Oxoid CM0409) plates [464] using a panel of eleven antibiotic discs, with *Escherichia coli* NTCC 10418 [465] used as a control. Sensitest agar plates were inoculated with test medium, antimicrobial disc

and grown in air overnight at 35 - 37°C, with the zones read the next day. The isolates were interpreted as susceptible or resistant in accordance with the method. The standard interpretation zone for susceptibility, > 6mm, was used unless otherwise indicated. The antibiotic discs used, the disc concentration and the minimum inhibitory concentration (MIC) for each, as outlined by the CDS method are listed in Table 2-28.

## 8. Next Generation Sequencing

### 8.1 DNA Extraction

*Salmonella* Typhimurium isolates selected for sequencing were recovered from storage and grown overnight at 37°C in Tryptone Soya Broth (Oxoid, CM0129) (10 mL). Genomic DNA was extracted, using the Roche High Pure PCR Template Preparation Kit (Roche). The resulting genomic DNA was purified and concentrated using a DNA clean and concentrator kit™ (Zymo) with a final elution volume of 30 µL genomic DNA. All kits were used in accordance with the manufacturers' instructions, unless otherwise stated.

Genomic DNA quality was assessed by gel electrophoresis using a 1% agarose gel made with SYBR® Safe DNA gel stain (Invitrogen) in 0.5 x TBE buffer (45 mM Tris, 45 mM borate, 1.0 mM EDTA, pH 8.3). A 2 µL DNA sample was loaded into each well and gels were electrophoresed at 80 mV for 20 - 30 minutes. The Hyperladder-1 (Bioline) DNA molecular weight marker was used to determine DNA fragment size and visually assess DNA integrity. The agarose gels were visualized using Imagelab (v3.0) in a ChemiDoc XRS+ Molecular imager (Bio Rad Laboratories). Extracted DNA was quantified using the Qubit® 3.0 Fluorometer (Life Technologies) with the dsDNA BR kit, and the RNA/DNA ratio checked using the Nanodrop (v3.8.1) spectrophotometer (Thermo Fisher Scientific). Extracted genomic DNA (30 µL) was stored in 96 well plates at -20°C until sequencing.

### 8.2 Whole Genome Sequencing

Next generation sequencing was outsourced to either the Australian Genome Research Facility (AGRF) or the Microbiological Diagnostic Unit (MDU). At the completion of sequencing all next generation sequences were supplied as raw reads in fastq files for analysis.

#### 8.2.1 AGRF

Extracted DNA from each isolate was supplied to AGRF for Illumina short read sequencing Chapter 7 ( Table 7-57, Run 3, 4, 5, 6, 9). Genomic DNA quality assurance testing and library preparation using the Nextera XT DNA library kit v3.0 was conducted in accordance with the manufacturer's instructions. Multiplex sequencing (100bp, paired end) was conducted on the Illumina HiSeq 2500 platform using the HiSeq reagent kit v3.0, with 96 isolates per lane. Image analysis was performed using HiSeq control software (HCS) v2.2.38 and Real Time Analysis (RTA) v1.1.61.

Eight isolates failing the AGRF HiSeq quality assurance process were re-sequenced by Illumina MiSeq in a single run (Chapter 7, Table 7-57, Run 7). Image analysis was performed by MiSeq Control Software (MCS) v2.4.1.3 and base calling was implemented by Real Time Analysis (RTA) v.18.54. For both HiSeq and MiSeq runs sequence data was generated using the Illumina bcl2fastq 1.8.4 pipeline and the CASAVA pipeline version 1.8.2. Illumina HiSeq 125bp Paired End sequencing was conducted using HiSeq control software (HCS) v2.2.68 and real time analysis v1.18.66.3. The Illumina bcl2fastq 2.18.0.12 pipeline was used to generated the sequence data.

#### 8.2.2 MDU

**Ilumina short read sequencing:** *Salmonella* genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen) on a QIAcube robot (Qiagen). Genomic DNA quality assurance and library preparation using the Nextera XT DNA library kit v2.0 was conducted in accordance with the manufacturers instructions. Multiplex sequencing was conducted on the Illumina MiSeq platform using the MiSeq reagent kit v2.0, with 20 isolates per lane. (Chapter 7, Table 7-57, Run 1)

**Pacbio sequencing:** Two *Salmonella* Typhimurium isolates were supplied to MDU for Pacbio sequencing. Genomic DNA extraction and sequencing was conducted on a Pacbio RSII sequencer in accordance with the manufacturer's instructions. A single SMRT cell was used per isolate.

# 3

CHAPTER **THREE**

# The Use of Social Network Analysis to Examine the Transmission of *Salmonella* spp. within a Vertically Integrated Broiler Enterprise

Crabb, H.K., Allen, J. L., Devlin, J. M., Firestone, S. M., Stevenson, M. A., Gilkerson, J. R., (2018). *The use of social network analysis to examine the transmission of Salmonella spp. within a vertically integrated broiler enterprise.* Food Microbiology, 71(5), 73-81.

*(next spread)*
*Only two locations (breeder or feed nodes) were identified where the transmission of a single Salmonella clone could theoretically percolate through the network to the broiler or processing nodes.*



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## Abstract

To better understand factors influencing infectious agent dispersal within a livestock population information is needed on the nature and frequency of contacts between farm enterprises. This study uses social network analysis to describe the contact network within a vertically integrated broiler poultry enterprise to identify the potential horizontal and vertical transmission pathways for *Salmonella*. Nodes (farms, sheds, production facilities) were identified and the daily movement of commodities (eggs, birds, feed, litter) and people between nodes were extracted from routinely kept farm records. Three time periods were examined in detail, 1- and 8- and 17- weeks of the production cycle and contact networks were described for all movements, and by commodity and production type. All nodes were linked by at least one movement during the study period but network density was low indicating that all potential pathways between nodes did not exist. *Salmonella* transmission via vertical or horizontal pathways can only occur along directed pathways when those pathways are present. Only two locations (breeder or feed nodes) were identified where the transmission of a single *Salmonella* clone could theoretically percolate through the network to the broiler or processing nodes. Only the feed transmission pathway directly connected all parts of the network.



*Keywords:*
*Salmonella* , Poultry, Social network analysis, Broiler, Chicken


## 1. Introduction

Information on the nature and frequency of contacts between farm enterprises is essential to accurately understand factors influencing infectious agent dispersal within and between livestock populations. Social network analysis (SNA) provides an analytical framework for these data, allowing observed patterns of contact to be described and quantified. Social network analysis is a method of investigating the relationships between entities that make up a system. In a poultry enterprise, the entities are the locations (farms or production sites–hatchery or processing facilities) that make up the system and the relationships between them including the movement of commodities, equipment or people. The relationships that may link common entities create paths where endpoints may be indirectly linked to the origin. Indirect links are a powerful mechanism by which disparate parts of a system may affect or be affected by other network entities [466]. Previous use of social network analysis techniques in poultry populations has largely been limited to identifying patterns of live bird movement that might influence the spread of avian influenza [467-469]. In these studies spatial and temporal resolution is focused to regional [468, 470, 471] or national cross sectional studies [472-474] rather than targeted at daily movement patterns. A small number of notable exceptions have also included the movement of other poultry commodities [469, 475]. Identifying entity relationships and understanding the maximum potential geospatial range of livestock dispersal is important when investigating highly transmissible or emergency animal diseases where spread is rapid, the consequence of spread is high and disease control activities must be timely to prevent further dissemination. The topology, density and dynamic nature of network relationships are important in understanding how diseases may enter the system, how they might spread within it, and where to target surveillance and control activities. Through identifying possible transmission pathways, SNA allows surveillance and control strategies to be developed based on evidence rather than hypothesis [476, 477]. The use of social network analysis to investigate other disease transmission dynamics within poultry populations has not yet been reported.

In Australia, salmonellosis in humans is the second leading cause of foodborne illness and chicken meat is the second most frequently attributed source of illness. Chicken meat is the most frequently consumed meat in Australia with an average of ~49kg of meat consumed per capita per annum. The chicken meat industry is vertically integrated with a small number of companies producing all chicken meat. No fresh chicken meat or live birds are imported due to strict biosecurity requirements. The epidemiology of *Salmonella* spp. transmission within a poultry enterprise is complex, with vertical and horizontal pathways, multiple sources of the agent (including other animals, insects or feed) and multiple locations where both introduction and cross-contamination may occur [478]. Salmonellosis in poultry typically presents as an asymptomatic or sub-clinical disease. Control of salmonellosis requires multiple control strategies to be applied at different levels of the enterprise. Strategies for the eradication of host specific strains such as *Salmonella enterica* serovar Pullorum have been successfully implemented [380] but have yet to be fully successful for non host-specific *Salmonella* serovars such as *Salmonella enterica* serovar Typhimurium [479, 480].

The aim of this study was to define the characteristics of the dynamic contact network within a vertically integrated chicken meat enterprise at a fine temporal resolution and to estimate the spread of a *Salmonella* spp. clone within the network in real-time. This increased understanding will enable potential patterns of *Salmonella* spp. transmission to be elucidated and thereby inform appropriate surveillance and control.

## 2. Materials and Methods

### 2.1 Study Population

The study population comprised the components of a single vertically integrated chicken meat enterprise in Australia, including pullet rearing and breeder production farms, hatchery, meat processing plants, contract broiler farms, contractors and feed mills. All sites where the movement of poultry or poultry products and external contractors or suppliers occurred within the system were identified.

### 2.2 Data Collection, Validity and Verification

Between January 2013 and July 2014, daily movements were collated from routinely kept paper and electronic farm records. These records included daily production records, delivery dockets, and pick-up schedules. All data were collated into a standardized electronic format using Excel™ [481] and FileMaker Pro Advanced v13.0 [482]. All data were verified at the time of entry by visual examination of records. After data entry, summaries were made using Tableau™ [483], Excel™ or R [484] to identify outliers, missing data, spelling errors or unusual data fields. Where an outlier or unusual event was detected, event details were clarified or corrected where necessary. Missing data that could be entered based on other available sources were included. Further requests for missing data were made if they were considered to comprise important observations.

### 2.3 Social Network Analysis

#### 2.3.1 Network construction

From the daily production records locations associated with the movement of people, live birds, hatching eggs, feed, and litter delivery and removal were identified. In all networks, nodes represented geolocations where a movement of a poultry commodity or people was identified as occurring to or from, during the 18– month data collection period. Multiple sites within a single geographical location may be represented by a separate node for example truck washes, shower facilities or fumigation rooms. Each room or location (hatcher, setter or chick holding room) within the hatchery was modeled as a separate location where birds or eggs were held for significant periods of time or commodities followed different paths. These locations were selected as representing sites where the introduction of *Salmonella* spp. or mixing of poultry commodities or people could occur or control activities to limit the spread of disease may occur. Nodes were created to represent teams of people that moved as a unit such as vaccination or cleaning crews.

#### 2.3.2 The Contact Network

A 17– week (121 days) contact network was constructed using Gephi v0.9.1 [485] where the edges were formed from the daily movement between locations of the following:

1. Live birds — day old pullet chicks, point of lay pullet transfer, end of lay breeder depopulation, day old and market weight

broilers

2. Hatching eggs — packing, fumigation, storage and transport to the hatchery, hatchery storage, setting, transfer and chick processing
3. Feed delivery — broiler, breeder production and pullet rearing
4. People — external contractors for cleaning, vaccination and pick-up crew movements. Movement of company employees between locations were not included
5. Manure removal and litter delivery

This study period was purposely selected to encompass the same period of time broiler field work was conducted and contained the most complete farm movement data for all commodity movement types. This period was equivalent to 2 broiler production cycles, one pullet rearing placement or ¼ of the annual production cycle. The date of movement was included to maintain temporal relationships between movements and locations.

### 2.3.3 Network Analysis

From the 17–week time period a series of 17, 1– week, two 8– week and the entire 17–week time periods were examined to evaluate the temporal features of the network. In Australia, broilers are processed and sold fresh daily, with commodity sales occurring daily and production movements weekly. In this enterprise, a broiler production cycle – all broiler sites placed and subsequently processed, occurred every 8–10 weeks. In addition, from the complete 17–week network eight subnets were evaluated, one for each production type —breeder, hatchery and broiler, and movement type — feed, manure, people, chickens and hatching eggs.

For each contact subnet, descriptive metrics for network- and node-level parameters were calculated. All locations identified during the 18-month data collection period were used to calculate the number of nodes, number of connected nodes, number of directed movements, diameter, degree, mean path length, eccentricity, centrality, betweenness, clustering coefficient and density. Network modularity, community detection, weak and strong network components were also calculated. Communities are collections of highly inter-connected nodes. These interconnected communities may be joined to other communities by strong (more than one movement between communities) or weak ties (single movements or bridges connecting communities). Weakly connected communities are vulnerable to attack at the ties (movements) that join these communities together [486]. For the production type networks these metrics were calculated for the locations identified to each production type only. Network- and node-level parameters were aggregated for each of the 1– and 8–week time series. A brief description of these metrics are presented in Table 3–29. For a more comprehensive description of these measures and their use the reader is referred to a couple of recent reviews [487, 488].

Contact network graphs were visualised in Gephi v0.9.1 using the Fruchterman Reingold force–based algorithm [489]. High degree and betweenness locations within the topological structure were visualised for each of the graphs.

### 2.3.4 Network Topology

Network topology, "small-world-ness" and power law assumptions were investigated.

Disease spread on small world networks is characteristed by a small number of long range interactions in a locally structured population, resulting in clustered spread of disease within a susceptible population and long range transmission to new susceptible populations [490]. In contrast networks with power law properties are heterogeneous (high degree range) and single nodes may have a great influence on the spread of disease within the network. Disease spread will occur within the network even with low reproductive ratio, but these networks are vulnerable to attack at these important nodes [491]. Power law assumptions were tested using the Kolmogorov Smirnoff test of goodness of fit using a bootstrapping procedure with 5000 simulations using the poweRlaw v1.4.9 [492] package in R [484]. A network was determined to have degree parameters suggestive of a power law if the goodness of fit p–value was greater than 0.1, in accordance with previously accepted methods [493]. "Small-world-ness" was assessed by comparing each subnet to a Erdös-Rényai random network with the same node number and edge density and the "small-world-ness" value calculated. A "small-world-ness" value ($S^{\Delta}$) greater than one indicated the presence of a "small-world-ness" network topology [494].

### 2.3.5 Network Heterogeneity and Reproductive Ratio

Network degree heterogeneity may affect disease transmission as the presence of a small subset of nodes with very high degree values in a network may amplify the spread of a disease through the network. Nodes with high in-degree are more likely to gain infection from incoming movements while nodes with high out-degree can infect a large number of other nodes. "Super-spreader" nodes with positively correlated in– and out–degree values are at higher risk of both acquiring and disseminating disease [495]. Network heterogeneity was assessed by calculating the coefficient of variation (CV) for each node degree value including in– and out–degree for each subnet examined. CV is highly influenced by high degree nodes in the network. The coefficient of variation for each degree value is calculated as the ratio of the standard deviation and mean degree values estimated for each contact subnet. The impact of the degree heterogeneity on potential disease spread was evaluated for each subnet by calculating the reproductive ratio ($R_0$) multiplier using the following formula [496]:

$$R_0 \propto 1 + CV_{in} CV_{out}\, r$$

$CV_{in}$ and $CV_{out}$ are the coefficient of variation of in– and out– degree respectively for the subnet while r is the correlation between the in- and out–degree values for all nodes in

Table 3–29. Social Network Analysis Network- and Node-level Parameters and Brief Description

| Parameter | Description |
|---|---|
| **Network size** | |
| Number of nodes | Each node represents a location in the poultry enterprise associated with the movement (to or from) of a commodity or people. |
| Connected nodes | Number of locations connected by the movement of a commodity or people in the network. |
| Directed movements | Total number of movements (unidirectional) between locations. |
| Network diameter | The largest geodesic distance between all reachable pairs of nodes in the network |
| Mean path length | The mean number of shortest geodesic distances between pairs of locations in the network. It is measured only among reachable pairs in the network. |
| Eccentricity | Geodesic distance from a given starting location to the furthest location from it in the network. |
| **Network centrality** | |
| Betweenness | How frequently a location appears in the shortest path between pairs of reachable locations in a network. |
| Degree | The mean number of all movements in and out of a location in the network (median reported if highly skewed) |
| In-degree | The mean number of all movements into a location in the network (median reported if highly skewed) |
| Out-degree | The mean number of all movements out of a location in the network (median reported if highly skewed) |
| Closeness centrality | Mean distance from a given starting location to all the other reachable locations in the network. |
| **Network cohesion** | |
| Density | The proportion of all movements that are present in the network, of all those that could be present. A complete network where all movements and locations are present has a density of 1. |
| Clustering coefficient | Measure of the density of movements between locations in the network. Estimated for directed networks as the mean proportion of possible movements actually present amongst the neighbourhood of locations that each location outwardly connects to. The clustering coefficient in a complete network, where all locations are connected to the other, will be 1. |
| Communities | Groups of highly inter-connected locations (nodes) within the network |
| **Network topology** | |
| "Small-world-ness" | A network topology characterized by high local clustering and a short average path length. Multiple highly internally connected 'small worlds' connected by infrequent inter-world movements. |
| Scale free | A network topology characterized by a power law relationship in the number of connections (degree) of locations in the network. In such networks, no threshold exists below which an epidemic won't occur, eradication of disease is only possible if transmission is reduced to zero. |

the subnet. If there is a positive correlation between the in- and out-degree value then $R_0$ will increase and conversely if there is a negative correlation the $R_0$ will decline.

### 2.3.6 Transmission Times for Salmonella spp. Spread

To examine the influence of different pathways of transmission within the network it is necessary to have an understanding of the time required for the spread of *Salmonella* spp. clone within a contact network. The distribution of possible times for a *Salmonella* spp. clone to spread within the network was evaluated. Vertical (transovarian or pseudo vertical) and horizontal transmission (direct and indirect) paths were considered. A path is a sequence of movements between locations from source to end, with no repeated movements or locations [497]. Vertical paths with a source at pullet or breeding locations and horizontal paths with a source at feed, litter, or people locations ending at the poultry processing location were considered. The time for a *Salmonella* spp. clone to spread via the identified path was calculated by taking into account the potential duration of exposure at each location (length of production) and the number (and duration) of movements in each path from the putative entry point (source location) to poultry processing. The mean duration that each poultry class (live bird or egg) remained at each production site was calculated from the production data based on the placement and depopulation dates obtained during the 18–month study period. Other potential modes of local spatial spread that may result in spatial clustering, such as windborne transmission, were not considered in this analysis, as there was no evidence to indicate other modes of transmission occurred.

Table 3–30. Social Network Analysis of Daily Movements in a Vertically Integrated Poultry Enterprise. Summary network-level and node-level metrics for time (1–, 8– and 17– week), production type (breeder, hatchery and broiler), people and commodity (feed, live birds, eggs, manure and litter) contact sub-networks

| Network metrics | Production Type | | | People | Commodity type | | | |
|---|---|---|---|---|---|---|---|---|
| | Breeder | Hatchery | Broiler | | Eggs | Feed | Manure/ Litter | Live Birds |
| **Network Size** | | | | | | | | |
| Number of nodes | 63 | 64 | 191 | 310 | 310 | 310 | 310 | 310 |
| Connected nodes | 63 | 64 | 191 | 193 | 92 | 189 | 189 | 196 |
| Directed movements | 883 | 1457 | 5599 | 1661 | 916 | 2244 | 702 | 2355 |
| Diameter | 6.00 | 4.00 | 8.00 | 8.00 | 6.00 | 1.00 | 2.00 | 3.00 |
| Mean path length | 2.06 | 1.62 | 3.66 | 3.68 | 3.52 | 1.00 | 1.05 | 1.88 |
| Eccentricity (range) | 2.04 (0-6.00) | 2.79 (0-4.00) | 5.89 (0-8.00) | 3.29 (0-8.00) | 0.53 (0-6.00) | 0.01 (0-1.00) | 0.91 (0-2.00) | 0.74 (0-3.00) |
| **Network Centrality** | | | | | | | | |
| Median degree (range) | 19.00 (1-451) | 22.50 (1-565) | 44.00 (2-1111) | 16.00 (2-158) | 17.00 (1-329) | 12.00 (0-1073) | 4.00 (1-126) | 12.00 (0-1201) |
| Median In–degree (range) | 17.00 (1-120) | 9.00 (1-565) | 27.00 (3-1111) | 9.00 (2-79) | 8.00 (1-50) | 12.00 (7-19) | 2.00 (1-120) | 3.00 (1-1111) |
| In-degree CV* | 1.02 | 2.96 | 2.53 | 0.94 | 1.09 | 0.25 | 3.17 | 6.96 |
| Median Out–degree (range) | 2.00 (1-451) | 14.50 (1-324) | 18.00 (2-972) | 8.00 (1-104) | 9.00 (1-324) | 1050.00 (121-1073) | 2.00 (1-126) | 8.00 (1-636) |
| Out-degree CV | 3.63 | 1.74 | 2.88 | 1.26 | 3.02 | 0.73 | 3.19 | 3.65 |
| Reproductive ratio multiplier | 5.36 | 4.12 | 5.15 | 1.84 | 1.22 | 0.98 | 0.64 | 11.97 |
| Closeness centrality | 0.57 | 0.69 | 0.19 | 0.17 | 0.17 | 0.00 | 0.89 | 0.57 |
| Betweenness (range) | 3.84 (0-30) | 46.57 (0-1682) | 539.30 (0-4836) | 224.47 (0-10036) | 13.13 (0-1653) | 0.00 – | 0.09 (0-0.67) | 8.11 (0-2400) |
| **Network cohesion** | | | | | | | | |
| Density (directed) | 0.23 | 0.36 | 0.15 | 0.02 | 0.01 | 0.02 | 0.01 | 0.03 |
| Clustering coefficient | 0.02 | 0.00 | 0.19 | 0.16 | 0.00 | 0.00 | 0.00 | 0.00 |
| Modularity (range) | 0.41 | 0.14 | 0.31 | 0.55 | 0.16 | 0.55 | 0.68 | 0.25 |
| Number of communities | 4 | 3 | 4 | 124 | 226 | 124 | 126 | 118 |
| Weakly connected components | 1 | 1 | 1 | 120 | 219 | 124 | 125 | 117 |
| Strongly connected components | 63 | 64 | 41 | 160 | 310 | 310 | 310 | 310 |
| **Network Topology** | | | | | | | | |
| "Small-world-ness" | No | No | No | Yes | No | No | No | No |
| Scale Free | Yes | No | No | No | Yes | No | Yes | No |



## 3. Results

Three hundred and ten nodes were identified during the 18– month study period. For the 17– week network a total of 7,939 movements occurred between 308 of the 310 identified network nodes. Network and node–level descriptive metrics for each production, commodity and people movement subnet are summarized in Table 3–30. Breeder, hatchery and broiler farms comprised 82% of the nodes identified with 71% of the directed movements going to or from the broiler production nodes. All movements between locations were directed and acyclic (unidirectional) and all networks had a low density (range 0.005 – 0.260) indicating that only a few of all the possible pathways for the movement of people or commodities between locations occurred during the study period.

### 3.1 Contact Networks by Time

The number of movements on the network varied by day (range: 4 –115) and week (range: 353 – 593) respectively: Supplementary Fig. 3–8 and Supplementary Fig. 3–9. Most of the movements between nodes occurred on weekdays except for hatchery associated movements, egg collection and egg transfer movements which occurred all days of the week. Aggregated network- and node-level metrics for each time period are summarized in Supplementary Table 3–32. The dynamic nature of these movement events is presented in the animation Supplementary Fig. 3–11.

The number of movements between locations increased as the time period examined increased. From 1– to 8–weeks the movements increased 6.20– to 10.50–fold but the number of connected nodes only increased 1.19 - 2.00 times. Between 8– and 17– weeks the number of movements between nodes doubled while the number of additional connected nodes increased only slightly (303 to 308 connected locations, respectively).

The diameter of the network decreased from 15 to 11 steps when the 1– or 17– week networks were examined but there was little difference in the mean path length (5.35 to 4.13 mean path length, respectively). A large proportion of the nodes (74%, range: 49 -82%) had movements occur between them in any one week. When examining the contact network by 1– week intervals the network

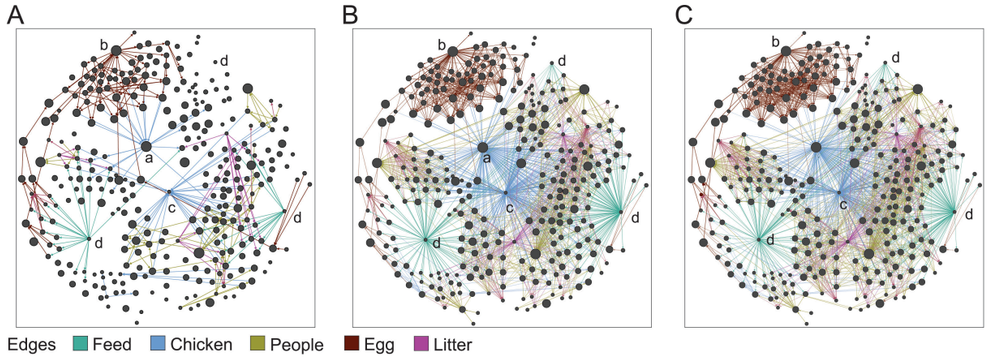


Fig. 3–4. Social Network Analysis of Daily Movements in a Vertically Integrated Poultry Enterprise

A single network from each time period is presented for illustrative purposes only. **A** 1–week contact network for daily movements of commodities and people. Contact network graphs are constructed using the force–based Fruchterman Reingold layout. Locations (nodes) are sized according to their betweenness centrality, indicating the potential importance of each location in flow within the 17-week network. Four node types are identified: **a** hatchery chick–holding **b** hatchery coolstore **c** processing **d** feed mill. All movements are directed, and edges are coloured by movement type. **B** 8– week contact network. **C** 17– week contact network.

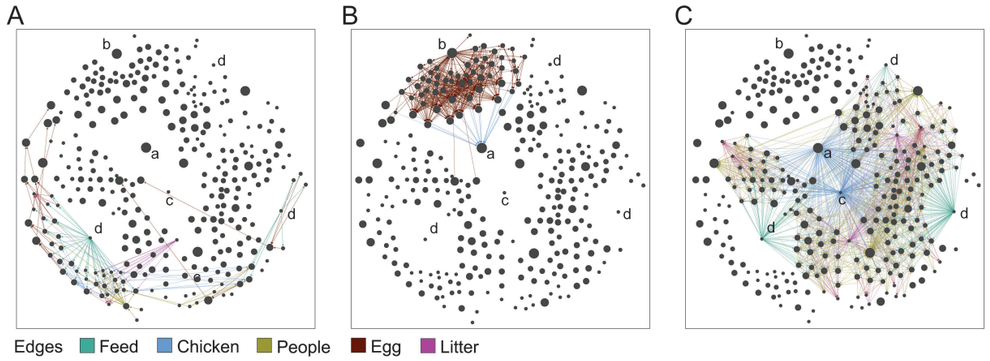


**Fig. 3–5. Social Network Analysis of Daily Movements in a Vertically Integrated Poultry Enterprise by Production Type**

**A** Breeder production contact network for all movements (16– weeks). Contact network graphs are constructed using the force–based Fruchterman Reingold layout. Locations (nodes) are sized according to their betweenness centrality, indicating the potential importance of each location in the flow within the network. Four node types are identified: **a** hatchery chick–holding **b** hatchery coolstore **c** processing **d** feed mill. All movements are directed, and edges are coloured by movement type. **B** Hatchery network and **C** broiler production network for all movement types: commodities and people.

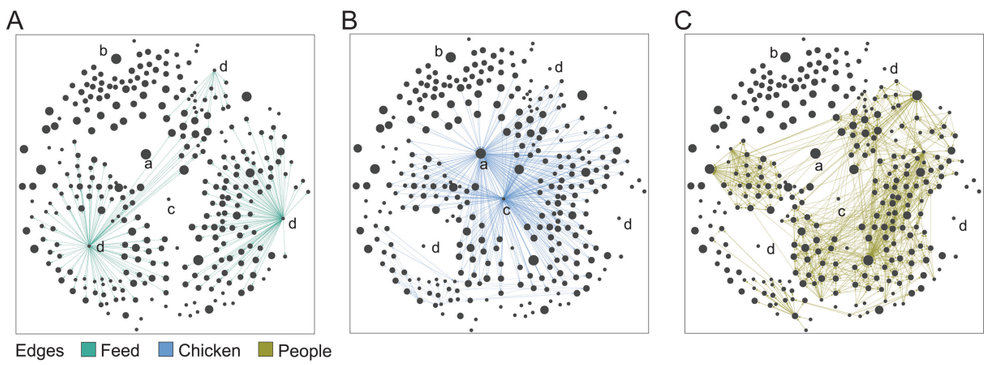


**Fig. 3–6. Social Network Analysis of Daily Movements in a Vertically Integrated Poultry Enterprise by Commodity Type**

**A** Feed contact network for all feed movements (16–weeks). Contact network graphs are constructed using the force–based Fruchterman Reingold layout. Locations (nodes) are sized according to their betweenness centrality, indicating the potential importance of each location in flow within the 17-week network. Four node types are identified: **a** hatchery chick–holding **b** hatchery coolstore **c** processing **d** feed mill. All movements are directed and edges are coloured by movement type. **B** Live bird contact network and **C** People contact network (contractor movements only).

was incomplete and at no point were pullet nodes connected to the rest of the network. Only one 8– week and the 17–week contact network linked all parts of the pullet-broiler production system, with 86% of the locations descending from pullet nodes. Only 14.5% of the locations descended from the most connected node (highest degree) in the network, chick–holding. Contact networks for each time period of interest (1–week, 8–weeks and 17–weeks) are presented in Fig. 3–4.

## 3.2 Network Topology

The people movement subnet was the only contact network to have a "small-world-ness" topology; high local clustering and short mean path length. Suggesting that people movements within the broiler network are potentially important for both local and long distance spread between communities within the network.

A single 1-week subnet demonstrated scale-free degree (degree, in– and out– degree) properties but 4 different, 1-week subnets had scale free in-degree or out-degree topology (2– weeks respectively). In the 8-week and the 17– week networks only the out-degree values



of each subnet showed scale free properties. The egg and litter commodity networks and the breeder production network all demonstrated scale free degree properties, while the hatchery and broiler production networks had out-degree scale free properties.

As the time examined increased both the network modularity and the number of communities identified within the network decreased. As the length of time examined increased from 1– to 17- weeks the number of weakly connected components declined from 83 to 3. All networks had significantly skewed degree, in–degree and out–degree network values with a heavy right tail. Coefficient of variation varied considerably for each subnet with the chicken movement (CV: 6.96) and the feed network (CV: 0.25) having the highest and lowest in-degree heterogeneity respectively. Reproductive ratio multipliers were highest in the breeder, hatchery and broiler production networks (Range: 4.12-5.36). The manure commodity network had the lowest $R_0$ (0.64) while the chicken movement network had the highest $R_0$ multiplier value (11.97).

## 3.3 Contact Networks by Production Type

The 17–week contact subnetworks for production–type, breeder, hatchery and broiler, are presented in Fig. 3–5. The breeder, hatchery and broiler contact networks comprised 18.6%, 29.3%, 63.6% of the locations identified, respectively. The density of movements in the breeder, hatchery and broiler networks were higher (4.00– 36.00 times greater) than the other 17-week networks investigated, despite each network comprising only a proportion of the locations present in the whole 17–week contact network. The broiler network had the highest clustering of all networks and a single location (broiler farm) had the highest betweenness value each week examined. This reflected the number of pick-ups that was occurring during the production week. Some commodity origins or sources were shared between the production networks; feed, litter source and manure removals, but people, hatching eggs and live birds were not. The only commodities to move between production types were hatching eggs which move from the breeder to the hatchery network and day old chicks which move from the hatchery to the broiler network. The hatchery is an important bridge between the broiler and the breeder production networks.

The diameter and mean path length of the network was greatest in the broiler network, whilst the network with the highest closeness (shortest distance between locations from any location) was the hatchery.

## 3.4 Contact Networks by Commodity Type

The contact networks for feed, live birds and people are presented in Fig. 3–6. The egg contact network had both the longest diameter and mean path length. There was no clustering present and the network density was very low (0.008 – 0.026) in all commodity contact networks, as the movements of most commodities move in a single step from origin to destination at a single point in time. The exception to this is the movement of eggs.

### 3.4.1 Live Bird Contact Network

The live bird contact networks were closed. The input of live birds occurred at two points in the complete network; pullet rearing as day old chicks and day old chicks produced at the hatchery. Live bird movements were directed and movement flowed in a single direction through the production system. No bird movements were reciprocated between pairs of locations and the movement of live birds was limited to within the breeder or the broiler contact networks, with no overlap (i.e. no live birds moved between the production-type 17-week contact networks).

### 3.4.2 Egg Contact Network

The egg contact network, Supplementary Fig. 3–7, which had a scale-free topology, was also closed and eggs were only sourced from the breeder production network. Eggs were the only commodity that was moved every day; from each breeder flock node to farm coolstore and then to the hatchery. Eggs had the longest mean path length (3.52) and diameter (6.00) of all commodities moved. Eggs were the only commodity that moved between the breeder network and the hatchery network. The hatchery coolstore had both the highest number of degrees and the highest betweenness value in the 17-week egg contact network

and like all other commodity movement networks had no clustering.

### 3.4.3 Litter and Manure Contact Network

Litter and manure movements, Supplementary Fig. 3–7, only occurred in the broiler and breeder contact networks. Litter and manure movements had a low median degree (4.00) and the smallest range of all commodity movements, typically 1 movement on and off each location. The same litter source was utilized by the majority of locations (66%). The manure and litter movements were not clustered and had both a short diameter and mean path length (2.00 and 1.05, respectively). Litter supply was common to both the breeder and broiler contact networks, but did not occur at the same time or by the same supplier in both networks. However, a single common manure movement was identified that was shared between the breeder and broiler contact networks. As with the litter supply this movement did not occur at the same time in each network.

### 3.4.4 Feed Contact Network

The feed contact network had the shortest diameter and mean path length. Feed was delivered direct to location, there was no clustering in the contact network. The number of movements to each location only occurred when birds were present on the farm and the median number of feed deliveries onto each node was 12.00 (range 7.00 – 19.00) deliveries in the 17–week network. Common feed locations supplied both the breeder and broiler contact network, however feed was delivered using different vehicles and on different days of the week so while the feed supply network overlapped spatially it did not overlap temporally.

## 3.5 People Contact Network

Movement of contractors associated with cleaning, bird depopulation (catching crews) and vaccination were identified. Movements of company personnel between locations was not recorded. The people network density was low (0.02) but clustered (0.16) and the diameter of the network was the highest of all the movement types. Both the diameter and the mean path length were higher than those of the other commodities, as unlike the feed, litter and live bird movements more than one node may have been visited in a single day. People movements did not occur between the broiler and breeder contact networks.

In the people contact network, contractor movements associated with live bird catching crews (broiler only) and broiler locations, were identified as having the highest betweenness values. The nodes associated with the largest number of movements in the 17–week people movement network were broiler sites contacted with catching and cleaning crews. The location with the highest number of people movements changed from week to week depending on the pick-up schedule.

## 3.6 Contact Network and Production Cycle Length

The mean time that each production class resided at each production location within the enterprise is presented in Table 3–31. Where a movement occurred between locations (two nodes) within a single day on a path the minimum duration for that component of the path was calculated as 1 day.

### 3.6.1 Vertical Pathways

Two vertical transmission pathways were considered; pullet to processing and breeder to processing. As hatching eggs did not enter the network from any location other than the breeder contact network nodes only those vertical pathways were considered. The longest possible path that could exist between the pullet and processing node was 11 steps; this complete path was not present in the 17–week network. If a *Salmonella* spp. clone was to enter the network at a pullet node and pass vertically through the contact network the mean time taken until it might be detected at the poultry processing location was 247 days (range 235 - 276 days). If a *Salmonella* spp. clone was to enter the network at a breeder location via the pullet flock, then the mean time taken until that clone could be detected at the processing location would be 100 days (range 95 – 115 days). If a *Salmonella* spp. clone entered the network horizontally at a breeder location and spread vertically the mean time taken until that clone could be detected at processing was estimated to be 65 days (range 53 – 94 days). If a *Salmonella* spp. clone was shed continuously (or intermittently) via a vertical pathway for the life of a breeder flock, then the mean duration that clone may be detected at the processing location was 169 days (range 130 - 227 days).

### 3.6.2 Horizontal Pathways

A number of horizontal pathways were identified within the network; feed to processing, people to processing, broiler to processing, and hatchery to processing. Complete horizontal paths were present in all of the contact networks except the 1–week network. All horizontal pathways were indirect from the source to the processing location except the people contact network associated with catching crews. The shortest possible time for a *Salmonella* spp. clone to be detected at processing was 1 day if the source was people, despite a mean path length of 3.68 steps. The shortest indirect horizontal path identified in the network occurred between the broiler and processing locations (2.88 steps) via the feed contact network. The length of the horizontal path is dictated by the duration of the broiler production cycle; the mean time for a clone to be detected at processing was 42 days (range 32–56 days). The length of time a clone may be detected at processing from a single broiler source was short (24 days).

# 4. Discussion

The contact networks identified in this vertically integrated poultry enterprise were dynamic and the movement of commodities and people between locations were acyclic. A cross-sectional study at a single point in time would fail to identify all nodes and movements between nodes within the contact network, and severely underestimate the complexity of the contact processes. Even though all nodes were identified from the 18-month study period, not all nodes were connected during the 17-week temporal period.

Table 3–31. Production length.

| *Production Site (poultry class)* | *Mean duration (days)* | *Range (days)* |
|---|---|---|
| Pullet Rearing (birds) | 147 | 140-161 |
| Breeder Production (birds) | 286 | 259-315 |
| Hatchery (eggs) | 23 | 21-38 |
| Broiler Production (birds) | 42 | 32-56 |

The duration (days) that birds or eggs spend at each production location within the contact network

Nearly all locations were linked by at least one movement type in the 8– and 17– week contact networks but the overall network density was low, indicating that all the potential pathways between locations do not exist and were not present at all times. Both the network modularity and the number of communities declined as longer time periods were considered for analysis from 1-week to 8-weeks. From 8-weeks to 17-weeks there was very little change in either the number of communities detected or the modularity (13.00 to 8.00 and 0.48 to 0.47 and respectively).

The density of the network was highest within production types (breeder, hatchery or broiler), indicating that disease transmission dynamics may be different within in each component of the network compared to across it. This is supported by the presence of different network topologies when the 17– week contact network was considered by production and commodity type, with the breeder and egg networks having scale free properties and the contacts by people having small-world properties.

Nearly all nodes (98.6%), but not all movements between nodes present in the 17– week network, were identified within the 8– week movement network. The degree distribution of the movements were highly skewed with three specific locations having a greater number of movements associated with them: hatchery, processing and feed mills. Movements occurred frequently between locations and were of short duration (1 day).

Disease transmission via vertical or horizontal pathways can only occur along potential pathways when those pathways are present. Not all paths were present at all times and even when a pathway was present, this does not necessarily mean that transmission will occur along it. The contact network was used to identify the number of steps required for the horizontal and vertical transmission of a *Salmonella* spp. clone within the contact network. The use of the contact network alone without an understanding of both the network dynamics and the length of the production cycle could lead to erroneous assumptions being made about the transmission of disease and so these factors were also considered in our analyses.

Transmission along the longest potential path in the network (pullet to processing) could not occur when only 17– weeks of the contact network were considered. Only the feed contact network directly connected all production types in the network, however clearly not all locations are reachable. Despite a common source of feed to each production type, the feed contact network was separated by both vehicle and delivery scheduling so the contact networks did not actually overlap. In addition, numerous standard biosecurity interventions (movement restrictions, vehicle cleaning) are in place along each recognised transmission path to limit transmission along those paths.

Topology is important for the dissemination and maintenance of infection within a network. In scale–free networks the degree distribution of a network is skewed and may approach a power law distribution, meaning that a few nodes have many contacts or connections, while most nodes have few or a limited number of contacts. Networks with scale-free topology included the breeder production network, the egg network and the out-degree topology of the hatchery broiler and litter networks. High degree nodes or hub locations may act as "super-spreaders:" [498]. Hub locations with high betweenness values representing important locations enabling flow within the network were identified at the hatchery. In human disease network studies, hierarchical networks containing hubs are important in the transmission of disease as the existence of hubs reduce the epidemic threshold to zero so that even weakly infectious diseases will spread and prevail in a contact network with this structure. Hubs are important in bridging many small communities into an integrated network [499]. A number of real-world biological networks have been described as having small world characteristics; tightly clustered nodes and a short mean path length, similar to a matched random graph [494]. Only the people contact network showed evidence of having small world characteristics in this study. The reproductive ratio multipliers in all networks were >1 except for the litter and feed contact networks indicating that even without the presence of hub and high degree nodes even poorly infectious diseases could spread readily within this network. The presence of scale free topology within both the litter and the feed contact networks mean that this remains a viable pathway for the entry of an organism such as *Salmonella* spp. within the network as long as the infection probability is sufficiently high.

Using the number of steps identified in each transmission path from the network and the production length it was possible to estimate the time it might take for a *Salmonella* spp. clone to spread through the network. These time frames can then be used to investigate the potential source of a clone. A reasonable prediction for the source of a *Salmonella* spp. based on the analysis presented above is that horizontally acquired *Salmonella* spp. clones at broiler production might only be present in the network for short durations when sampling is focused at poultry processing as broilers are removed from the system quickly. In contrast, vertically transmitted clones may remain detectable for considerably longer periods of time at processing locations.

The importance of this finding for surveillance and control is that if people are involved in the transmission of *Salmonella* spp. movements occur to disparate locations within the network quickly and spread will occur before surveillance activities are implemented.

## 5. Conclusions

The contact network structures identified in this study confirm that the movement of commodities within a vertically integrated poultry enterprise are both hierarchical and complex. There was a surprisingly large number of movements with a very complex and dynamic nature which is only fully explained by considering multiple periods of time. Examination of the network at a single point in time would fail to identify both the complexity and the dynamic and changing nature of these relationships. As a number of the contact networks demonstrate scale–free characteristics this may explain how after introduction into such a system a new *Salmonella* spp. can rapidly become endemic and be maintained within a poultry enterprise, despite all the best measures for control being implemented, as very low reproductive ratios are required to maintain infection within networks with scale–free topology.



Ongoing investigations are focused on field sampling across this contact network to validate the hypotheses generated in this analysis about transmission pathways. The contact network described in this study provides a detailed picture of within-enterprise dynamics that will be highly informative for models of infectious disease spread across the industry or regions.

## Funding Sources

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## Acknowledgements

We gratefully acknowledge the support of the poultry company and its staff for allowing us access to their farming operation during the course of the study. Without their support this project and associated research could not be conducted.

## Appendix A. Supplementary Figures

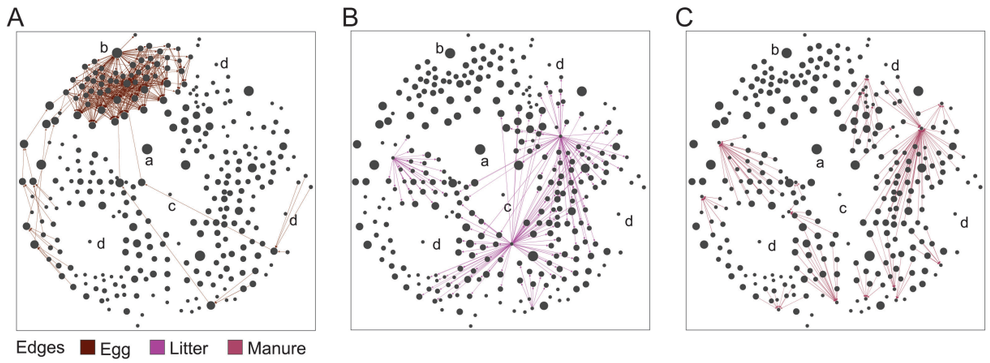
Supplementary data related to this article can be found at http:// dx.doi.org/10.1016/j.fm.2017.03.008.

Supplementary Table 3–32. Social Network Analysis of Daily Movements in a Vertically Integrated Poultry Enterprise
Summary network-level and node-level metrics for the full network aggregated by 1-, 8- and 17-week time periods

| *Network Metrics per time period* | *Time Period* | | |
|---|---|---|---|
| | *1 Week*[1] | *8 Weeks*[1] | *17 Weeks* |
| **Number of networks (n)** | 17 | 2 | 1 |
| **Network Size** | | | |
| Number of nodes | 310 | 310 | 310 |
| Connected nodes (range)[2] | 230 (152-254) | 303 (301-304) | 308 |
| Directed movements (range)[2] | 497 (353-593) | 3751 (3703-3799) | 7939 |
| Diameter | 15.24 ± 3.07 | 12.00 ± 0.00 | 11.00 |
| Mean path length | 5.35 ± 0.91 | 4.47 ± 0.15 | 4.13 |
| Eccentricity (range) | 3.41 ± 0.94 | 5.75 ± 0.70 | 5.66 ± 2.39 |
| **Network Centrality** | | | |
| Mean degree | 4.40 ± 0.49 | 24.79 ± 0.27 | 38.00 (IQR:28.25) [2] |
| Degree range | 824.00-1186.00 | 7407.00-7598.00 | 2.00-1201.00 |
| Mean In-degree | 2.43 ± 0.30 | 13.59 ± 0.11 | 23.00 (IQR:16)[2] |
| In-degree range | 412.00-593.00 | 3703.00-3799.00 | 1.00-1111.00 |
| In-degree CV | 2.12 ± 0.17 | 2.57 ± 0.24 | 2.61 |
| Mean Out-degree | 4.08 ± 0.31 | 13.77 ± 0.42 | 16 .00 (IQR:9.75)[2] |
| Out-degree range | 412.00-593.00 | 3705.00-3799.00 | 1.00-1073.00 |
| Out-degree CV | 2.15 ± 0.31 | 3.42 ± 0.50 | 3.48[2] |
| Reproductive ratio multiplier | 1.71 ± 0.30 | 2.44 ± 0.53 | 2.22 |
| Closeness centrality | 0.12 ± 0.02 | 0.25 ± 0.02 | 0.27[2] |
| Mean Betweenness | 74.80 ± 33.16 | 466.59 ± 98.96 | 164.33[2] |
| Betweenness range | 0.00-4360.00 | 0.00-16425.00 | 0.00-17810.00 |
| **Network cohesion** | | | |
| Density (directed) | 0.005 ± 0.001 | 0.04 ± 0.001 | 0.09 |
| Clustering coefficient | 0.01 ± 0.07 | 0.06 ± 0.01 | 0.15[2] |
| Modularity | 0.63 ± 0.03 | 0.48 ± 0.005 | 0.47 |
| Number of communities (range) | 89.00 (62-168) | 13.00 (12-14) | 8.00 |
| Number of weakly connected components (range) | 83.00 (57-161) | 8.00 (7-10) | 3.00 |
| Number of strongly connected components (range) | 266.00 (258-276) | 165.00 (163-166) | 160.00 |
| **Network Topology** | | | |
| "Small-world-ness" | No | No | No |
| Scale Free | Yes | Yes | Yes |

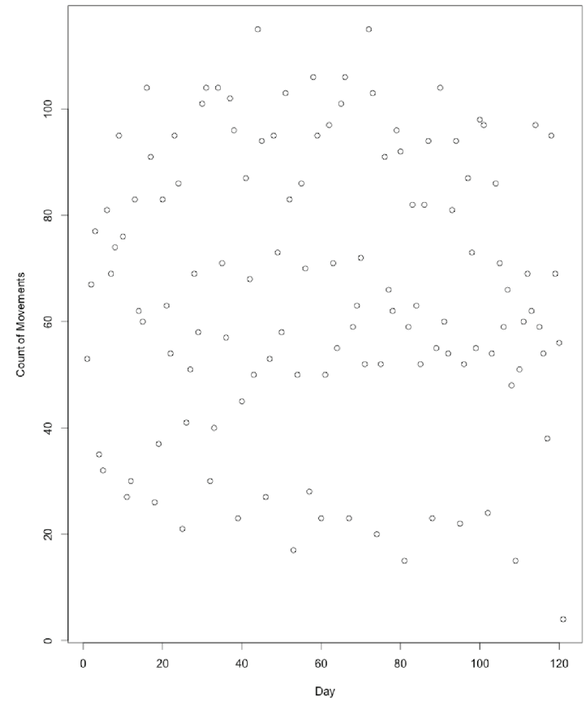
[1]For each time period the mean value and standard deviation is reported for the interval measured [2] Median value and interquartile range reported for skewed variables



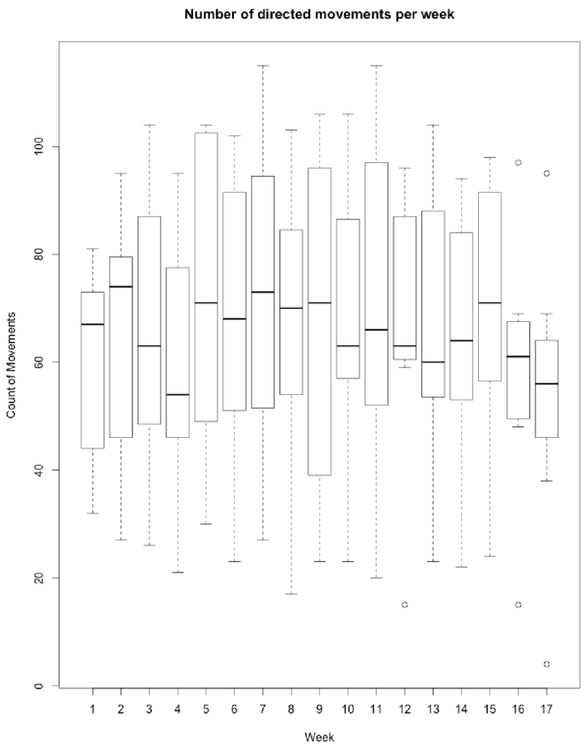



Supplementary Fig. 3–7. Social Network Analysis of Daily Movements in a Vertically Integrated Poultry Enterprise by Commodity Type

**A** Egg contact network for all egg movements (17-weeks). Contact network graphs are constructed using the force–based Fruchterman Reingold layout. Locations (nodes) are sized according to their betweenness centrality, indicating the potential importance of each location in flow within the 17-week network. Four node types are identified: **a** hatchery chick–holding **b** hatchery coolstore **c** processing **d** feed mill. All movements are directed and edges are coloured by movement type. **B** Litter contact network and **C** Manure contact network.

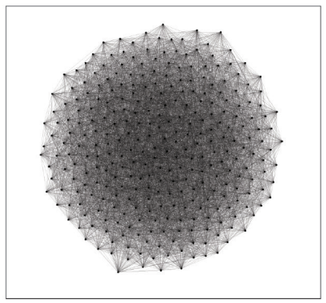



Supplementary Fig. 3–8. Number of Directed Movements per Day
The number of commodity and people movements per day for the complete 17-week network.




**Supplementary Fig. 3–9. Number of directed movements per week**

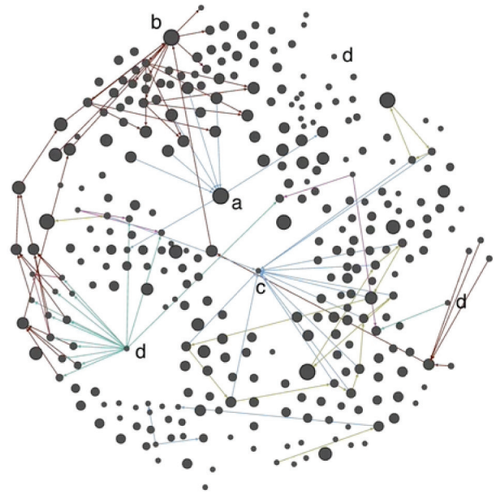
The number of daily commodity and people movements aggregated per week for the complete 17-week contact network.



**Supplementary Fig. 3–10. Erdös-Rényi Random Graph**

An Erdös-Rényi random graph with 310 nodes and 7939 edges, equivalent in both the number of nodes and edges to the complete 17-week network.






**Supplementary Fig. 3–11. Animation of all daily movements in a vertically integrated poultry enterprise**

The contact network graph is constructed using the force–based Fruchterman Reingold layout. Locations (nodes) are sized according to their betweenness centrality, indicating the potential importance of each location in flow within the 17-week network. Four node types are identified: **a** hatchery chick–holding **b** hatchery coolstore **c** processing **d** feed mill. All movements are directed and edges are coloured by movement type.

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# 4

C H A P T E R **F O U R**

# *Salmonella* spp. Transmission in a Vertically Integrated Poultry Enterprise: Clustering and Diversity Analysis using Phenotyping (Phage Typing ) and Genotyping (MLVA)

Crabb, H.K., Allen, J. L., Devlin, J. M., Firestone, S. M., Wilks, C. R., Gilkerson, J. R., (XXXX) *Salmonella spp. transmission in a vertically integrated poultry operation: clustering and diversity analysis using phenotyping (Phage Typing) and genotyping (MLVA).* Submitted to PlosONE, 22 Jan 2018

*(next spread)*
*Despite the high level of apparent diversity, cluster analysis revealed that the S. Typhimurium detected at all generations of the integrated enterprise likely arose from the same source.*



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## Abstract

The transmission of *Salmonella* spp. within a vertically integrated poultry operation was investigated longitudinally over an 18-month period (2013-2014). Thirty six percent of all samples collected (1,503 of 4,219) were positive for *Salmonella* and seven *Salmonella enterica* subsp. *enterica* serovars were detected. *Salmonella enterica* subsp. *enterica* Typhimurium was the most frequently detected serovar (63% of serotyped samples) with 8 phage types (PT) and 41 multi-locus variable-number tandem repeats analysis (MLVA) profiles identified. A total of 62 PT/MLVA combinations were observed. Despite the high level of apparent diversity, cluster analysis revealed that the *S.* Typhimurium detected at all generations of the integrated enterprise likely arose from the same source. The breeder site was identified as the most likely point of introduction of *S.* Typhimurium (either via introduction of day old chicks or in the feed) into the production system with subsequent dissemination to the broiler flocks via the hatchery. The use of phage typing and MLVA profiling, on their own or in combination, were inadequate to understand the complexity of the epidemiological relationships between locations within this production system. This complexity is unable to be resolved in the absence of intensive sampling programs at all generations of the production system.


## 1. Introduction

The epidemiology of *Salmonella* transmission within a vertically integrated chicken meat operation is poorly described. Risk factors associated with *Salmonella* infection or contamination on broiler farms, during carcase processing and putative pathways of transmission within integrated broiler systems have been well characterized but studies are typically limited by consideration of individual parts of the integrated system in isolation, and frequently limited to description at the *Salmonella enterica* serovar level [258, 295, 299, 340, 342, 350]. Three Australian publications from the 1970s describe the dissemination of *Salmonella* within integrated broiler operations [18-20]. These studies were a descriptive analysis of the occurrence of *Salmonella enterica* serovars at each component of the production system and implicated feed as the common source of introduction. There are a small number of studies that characterize *Salmonella enterica* strains beyond serovar identification across entire poultry meat production systems but none have been conducted in Australia [244, 500-502]. Australia is unique in that *Salmonella enterica* subsp. *enterica* Enteritidis is not endemic within the commercial poultry industry, which offers a novel opportunity to understand *Salmonella* Typhimurium transmission in its absence [83].

Understanding the relationships between the various *Salmonella* strains that are transmitted between generations of birds within a vertically integrated poultry production system, beyond serovar identification, is important to quantify the effectiveness of specific control strategies at critical points in the production system. This is especially important when there are a limited number of dominant *Salmonella enterica* serovars, as differentiation between isolates of the same serovar is critical for understanding the transmission epidemiology within complex production systems. When strict biosecure separation exists between generations (single source of day old chicks and eggs supplied for broiler production from this parent generation) the only route by which *Salmonella* can be introduced into the system via the importation of live birds is at the parent generation. Entry points for *Salmonella* via feed exist at both the parent and/or broiler flocks [493]. Further characterization of isolates is required to better understand points of introduction, subsequent dissemination and the effectiveness of controls within a poultry organization.

The key aim of this study was to identify the introduction of *Salmonella* Typhimurium into the operation and to follow putative transmission paths within the enterprise by intensive repeated sampling of identified infected flocks or locations for 18 months.

## 2. Materials and Methods

### 2.1 Study Population

This study was conducted within a single vertically integrated poultry operation. A full description of the integration and the complexity of poultry and commodity movements within the system is published elsewhere [503], but in brief the system operates with separate sites for parent bird rearing and egg production, the hatchery, broiler production and meat processing. All sites are operated under strict biosecurity with no sharing or movements of equipment or people between production types within the enterprise. The only commodities entering the system during the study period were day old parent chicks and feed [503]. This study was conducted under normal farming operating conditions to evaluate routinely conducted sampling procedures for surveillance purposes. All environmental sampling was conducted as part of routine production standard operating procedures in accordance with standard industry procedures and guidelines [381, 456, 458, 504]. As all samples were collected as part of routine veterinary care and agricultural practice this study did not require ethics approval [505]

### 2.2 Study Design

A prospective cohort study was conducted longitudinally over an eighteen-month period between January 2013 and July 2014. A stratified sampling approach was taken, to ensure samples were collected from each generation of the enterprise and from all identified transmission or bottle neck locations such as the hatchery or processing. Environmental samples were collected systematically from parent sites (rearing and egg production), the hatchery and broiler farms. All parent flocks were routinely sampled at least six times during the study period: four were intensively sampled 3 weekly for their productive life (20-63 weeks). Samples were collected from the hatchery and 68 broiler chick placements were followed to the broiler farms (Fig. 4–12). Samples were supplied monthly from the processing plant for the duration of the study.

### 2.3 Sampling Methodology

**Parent Bird Rearing.** Samples were collected from 44 deliveries of day-old chicks and the flocks established with these chicks. Ten chick box liners, referred to as chick papers, were obtained from the transport boxes on the day of each chick delivery. Four drag swabs, each sampling ¼ shed, were collected per flock on each sampling date. In total, there were 220 sampling events in 44 flocks, comprising 44 pooled chick paper samples and 352 drag swab samples.

**Parent Egg Production.** Twenty-eight samples were systematically obtained from each shed at each sampling event. Manure belt, egg belts, and nest box surfaces were systematically sampled by swabbing each surface the length of the shed from multiple locations to ensure the full length and width of the shed was sampled. Boot swabs were worn each sampling occasion. The same sites were sampled on each sampling occasion. In total 324 boot swabs, 533 dust swabs, 500 egg belt and

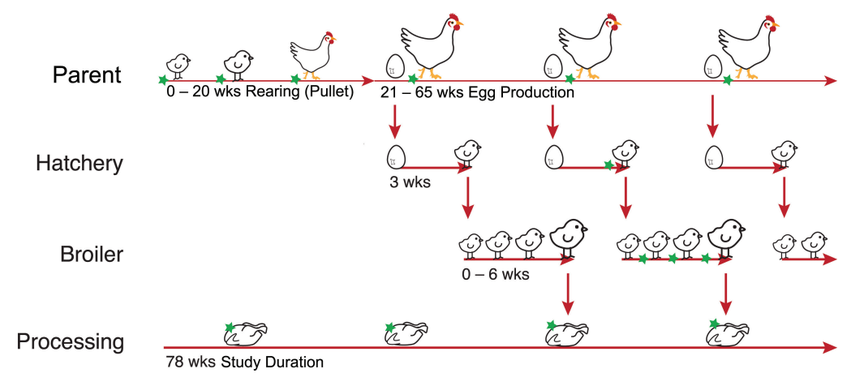


**Fig. 4–12. Study Design Schematic**
Sampling occurred longitudinally (for the life of each parent flock) within each of the generations of the integrated system. Positive samples at one generation were followed to the next via the vertical paths as indicated. Timing of sampling is indicated by a green star.

500 manure belt samples were collected from 22 flocks.

**Broilers.** At the hatchery, chick papers were collected indirectly from day old broiler chicks on the day of hatch, by the collection of chick papers randomly selected from 10 hatching trays of processed chicks. Samples were collected by donor flock (parent flock supplying the chicks) for each broiler flock subsequently established with these chicks. In total 708 chick paper samples from 16 parent flocks were collected.

Broiler flocks were sampled on farm at least three times during production prior to processing. Dust samples were collected by swabbing walls the length of the shed and all internal fan guards. Two pairs of boot swabs were collected per shed. In total, 263 boot swabs, 136 dust, and 138 fan swabs were collected from 68 broiler flocks.

**Processing.** Suspect positive *Salmonella* spp. isolates (183 samples) from carcass or portion rinse samples collected from daily production lines at the primary processing plant were provided monthly for the duration of the study. These samples were provided from those samples collected for routine quality assurance testing in accordance with Australian Standard AS4465 [458]

### 2.4 Salmonella Isolation

All samples were processed on the day of collection in accordance with the Australian Standard 5013.10-2009 Horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002, MOD) [459]. Buffered peptone water was added to each primary sample with little mixing and each suspension was statically incubated at 37°C for 18-24 hours. After incubation, three 33 µL aliquots were taken from each primary sample and inoculated onto Modified Semi–solid Rappaport Vassiliadis (MSRV) plates. Inoculated MSRV plates were incubated under aerobic conditions at 41.5 °C and visually examined after 12, 24 and 48 hours. Plates with swarming growth, indicative of *Salmonella* spp., were sub-cultured onto Xylose–Lysine–Desoxycholate agar (XLD), and either Brilliant Green Agar (BGA) or Cystine Lactose Electrolyte Deficient agar (CLED) in duplicate. All plates were incubated for 24 hours at 37°C. Suspect *Salmonella* spp. colonies were confirmed biochemically in triplicate using Triple Sugar Iron agar (TSI) and Lysine Iron agar (LIA), incubated for 24 hours at 37°C. Further biochemical testing was conducted in triplicate to differentiate *Salmonella* Sofia isolates. Isolates that tested positive for O–nitrophenyl–β–D–galacto–pyranoside (ONPG) and able to ferment mannitol were presumed to be *Salmonella* Sofia and eliminated from further phenotypic or genotypic analysis [507].

### 2.5 Salmonella Typing

*Salmonella* isolates were confirmed positive by real time PCR in accordance with previously published methods [460]. A random selection of isolates was submitted to the Victorian Salmonella Reference Laboratory for serotyping, phage typing and MLVA profiling. Isolates were serotyped using the Kauffman–White–Le Minor scheme [30] and phage typed using the Anderson phage typing scheme [63]. MLVA analysis was conducted in accordance with the European MLVA protocols [69] on all *S.* Typhimurium positive isolates. MLVA results are presented in accordance with the Australian naming convention [508].

### 2.6 Statistical Analysis

All statistical analyses were conducted in the R statistical package unless otherwise stated [484]. Ecological diversity and cluster analysis was conducted using "vegan" [509], "BiodiversityR" [510], "FactoMineR" [511], "factoextra"[512] and "pvcluster" [513]. The distribution of phenotypes/genotypes between study locations was visualized using venn diagrams created in the "VennDiagram" package [514].

#### 2.6.1 Multi-locus Variable-number Tandem-repeats Analysis (MLVA)

MLVA analysis was conducted using the goeBURST algorithm in "Phyloviz 2.0" [515-517]. The *S.* Typhimurium MLVA relationships were visualized using minimum spanning trees (MST) of single and double locus variants. Manual MLVA profile curation was conducted to group related MLVA profiles. MLVA profiles with a single locus or double locus variant of no more than two tandem repeat differences, in the three shortest loci (STTR-5, STTR-6 and STTR-10) were combined [72].

#### 2.6.2 Differences in Species Composition between Locations

The diversity of the species composition at each location was measured using the Shannon-Weiner index of diversity (H´). Shannon's index (H´) is a non-parametric measure of heterogeneity combining both evenness and richness in a single measure, but is sensitive to sample size. This index accounts for both abundance and heterogeneity of the species present. The null hypothesis under investigation was that there was no difference in *S.* Typhimurium typing profiles (PT, MLVA or PT/MLVA combination) between parent, broiler and processing locations. All *S.* Typhimurium results were aggregated by location for all sampling events to maximize sample size and analytical power. It was determined, based on the variance in MLVA diversity (H´) between and within location, that a minimum of 50 samples per location was required to provide 80% power assuming more within-location ($s^2$ = 21.1) than between-location ($s^2$ = 2.0 -3.0) variance.

#### 2.6.3 Dissimilarity and cluster analysis

Analysis of species composition was evaluated by measuring the ecological distance between locations. The ecological distance (dissimilarity) between sites that share the most *S.* Typhimurium typing variants is small, whereas the ecological distance between sites sharing only a few typing variants in common is large. Location dissimilarity was calculated using the Chao index [518]. The Chao dissimilarity index attempts to take into account the number of unseen species pairs that may not be detected due to the limitations of sampling where complete inventories of all "species" are potentially impossible to collect [519]. Agglomerative clustering was conducted using three methods on each distance matrix: unweighted pair group average (UPGMA), single and complete linkage. The cophenetic correlation was used to evaluate which of the three clustering methods best represented the original matrix ecological distance. Statistical significance of the correlation was estimated using bootstrapping to calculate the approximately unbiased (AU) P-value and 95% confidence intervals. If the AU P-value was greater than 95% then the clustering was considered to be supported by the data [513].

#### 2.6.4 Principal Component Analysis (PCA)

The *S.* Typhimurium typing profile matrix for each site (either "location"; parent, broiler or processing, or "species"; PT, MLVA or PT/MLVA combination) was transformed to a "species profile" proportion of typing profiles (PT, MLVA or PT/MLVA combination per site) to remove the effect of extremely high or low abundance typing profiles while maintaining the original composition of the location matrices [520]. The adequacy of the normalisation of the species transformation was assessed using the Shapiro test and visualization of the quantile- quantile plot (qqplot )[521]. A qqplot is used to determine if the two datasets come from the same population by plotting the quantiles of each on the same plot. Euclidean distances were calculated on the transformed species profile matrices. The ecological distance between sites was evaluated using principal component analysis. The number of significant principal components was assessed using the broken stick test. Principal components were included where the cumulative eigenvalue was greater than the equivalent broken stick value, sufficient to include no less than 75-80% of the total variance and visualized using a screeplot [521]. Typing profiles significantly contributing to the ordination were identified using the circle of equilibrium [522] and their square cosine values [523].

## 3. Results

### 3.1 Sample Testing Summary

Thirty six percent (1,503) of the 4,219 samples collected were positive for *Salmonella* spp. (Table 4–33). The proportion of positive samples varied according to location of sample collection, with a higher proportion of *Salmonella* positive samples collected at the processing plant compared to other sites.

### 3.2 Salmonella Serotyping Results

Ninety six percent of the *Salmonella* isolates were serotyped and seven *Salmonella enterica* subsp. *enterica* serovars were identified: *S.* Typhimurium (62.8%), *S.* Infantis (23.5%), *S.*

Agona, *S.* Mbandaka, *S.* Senftenberg, *S.* Tennessee and *S.* Worthington. *Salmonella enterica* subsp. *salamae* serovar Sofia (11.6%) was also identified. Five percent of samples contained more than one serovar. *S.* Typhimurium and *S.* Infantis were the only serovars detected at all locations, while *S.* Sofia was only detected at either the broiler farms or at the processing plant. *S.* Sofia was never detected in day old broiler chicks at the hatchery. *Salmonella* serovar diversity (H′) was greater at the broiler location than all others (Broiler > Processing > Parent) (Table 4–33).

## 3.3 Salmonella Typhimurium Typing

### 3.3.1 Phage Typing

Forty five percent (421/944) of the *S.* Typhimurium samples were phage typed. Eight phage types (PT) were identified with PT135a found most frequently in 60% of the samples tested. The three most frequently identified phage types (PT135a (60%), DT135 (19.5%), DT193 (11%) were detected at all locations. Two phage types (DT9 (2.1%) and DT141 (2.4%)) were only detected at the onset of the study and one (PT30) was identified at processing on a single occasion. Phage types PT12 (1.2%) and Untypeable (3.5%) were also identified. *S.* Typhimurium phage type diversity (H′) was greatest at the broiler locations (Broiler > Processing > Parent) (Table 4–33).

### 3.3.2 Multi-locus Variable-number Tandem-repeats Analysis (MLVA)

All *S.* Typhimurium phage typed isolates were MLVA profiled (n = 421). A total of 41 MLVA profiles were identified with two MLVA profiles (03-14-10-09-525 and 03-15-11-11-525) comprising 60% of all tested isolates, detected at all locations (Fig. 4–13A).

MLVA profile analysis identified a single minimum spanning tree comprising all MLVA profiles with twenty-nine of the MLVA profiles single or double locus variants of the two dominant MLVA profiles. A single MLVA profile (03-09-04-14-525) was identified as intermediary between the two dominant MLVA profiles. MLVA profile diversity (H′) was highest at the processing location (Processing > Broiler > Parent) (Table 4–33).

### 3.3.3 Phage Type/MLVA Combinations

MLVA profiles were not found exclusively in one phage type. More than 83% of the isolates identified as DT12, DT135, PT135a and DT193 or Untypeable shared at least one MLVA profile with an isolate from another phage type. *S.* Typhimurium phage types DT9 (MLVA = 4) and DT30 (MLVA = 1) isolates also shared a MLVA profile. DT141 isolates were the only isolates to have unique (not shared with other PT) MLVA profiles (MLVA = 5). In total, there were 62 PT/MLVA combinations identified from the 421 isolates that were phenotype and genotyped. The relationship between phage type and MLVA profile is illustrated using the MLVA minimum spanning tree (Fig. 4–13B). Each tie within the MST represents a single locus difference between isolates. Manual curation of the MLVA profiles reduced the number of unique MLVA profiles from 41 to either 7 MLVA profiles or 17 PT/MLVA combinations containing possibly genetically related isolates. Three dominant PT/MLVA combinations were identified using principal component analysis (see below). Manual curation or PCA did not change the overlap between phage types. All MLVA profiles were detected at the breeder production sites.

Table 4–33. Summary of all Study Samples Tested for *Salmonella* by Location

| | *Number of Samples collected at each sampling location (%)* | | | |
|---|---|---|---|---|
| | *Parent* | *Broiler* | *Processing* | *Total* |
| No. Samples | 2,769 | 1267 | 183 | 4,219 |
| No Sites sampled | 66 | 69 | 1 | |
| No. positive samples (%) | 447 (16.1) | 916 (72.3) | 140 (76.5) | 1,503 (35.6) |
| *Salmonella enterica* Serovars | 3 | 7 | 4 | 8 |
| Shannon's Index (H′)[1] | 0.71 | 0.91 | 0.78 | 0.95 |
| *Salmonella* Typhimurium (%) | 209 (46.7) | 638 (69.7) | 97 (69.2) | 944 (62.8) |
| Phage Types[2] | 7 | 6 | 6 | 8 |
| Shannon's Index (H′) | 0.74 | 1.43 | 1.29 | 1.22 |
| MLVA types[1] | 22 | 16 | 15 | 40 |
| Shannon's Index (H′) | 1.52 | 1.96 | 2.39 | 2.26 |
| PT/MLVA combinations[1] | 27 | 30 | 19 | 61 |
| Shannon's Index (H′) | 1.85 | 2.67 | 2.61 | 2.75 |

[1]Shannon's Index (H′) Measure of species diversity accounting for both sample heterogeneity and abundance. [2]Number of *Salmonella* Typhimurium isolates both Phage Typed and MLVA profiled (n=421)



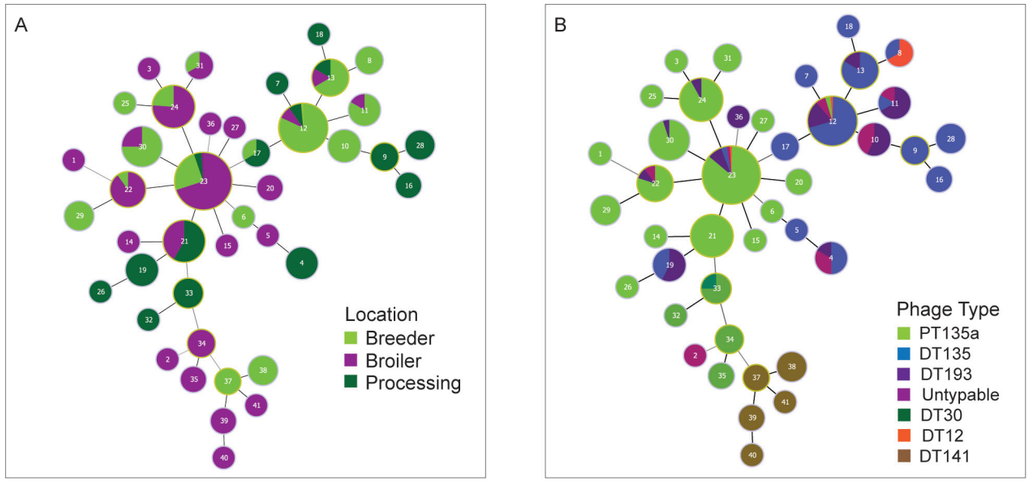


**Fig. 4–13. Salmonella Typhimurium Multi-locus Variable-number Tandem-repeats Analysis (MLVA) Minimum Spanning Tree (imputed using goeBURST)**

Each MLVA profile is represented as a single pie chart. The size of the pie chart is proportional to the number of isolates represented by that MLVA profile and the pie segments represent either the number of locations or number of phage types associated with each MLVA profile. **A** MLVA profiles coloured by location from where the sample was collected. **B** MLVA profiles coloured by Salmonella Typhimurium Phage Type associated with that profile. DT9 is not identified.

## 3.4 Clustering by Location

The number of *S.* Typhimurium phage types and MLVA profiles identified at each location (parent, broiler or processing) are illustrated in Fig. 4–14. Seven of the eight PTs were identified at the breeder location and one unique PT was identified at processing. No PT were unique to the hatchery only. MLVA profiling indicated only seven of the 41 MLVA profiles were shared between locations and 30/41 MLVA profiles were unique to any one location. Using agglomerative and hierarchical

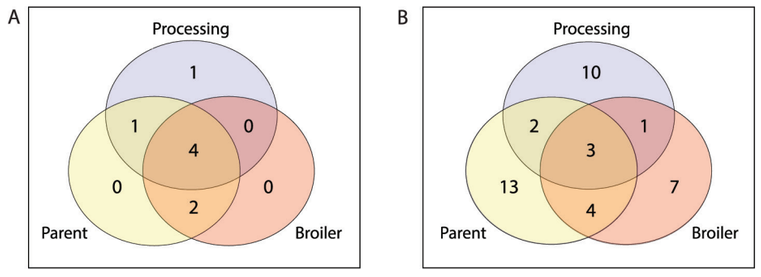


**Fig. 4–14. The number of unique *Salmonella* Typhimurium isolates identified by typing method detected at each location**

**A** Phage Type. **B** MLVA profile.

Table 4–34. Complete Linkage Agglomerative Clustering Results for each *Salmonella* Typhimurium Typing Methods

| | *Agglomerative Coefficient* | *Height* | *Cophenetic Correlation* | *P value* | *Dissimilarity (Chao) Mean (min-max)* | *Median* |
|---|---|---|---|---|---|---|
| PT | 0.547 | 0.024 - 0.136 | 0.607 | 0.333 | 0.067 (0.024 - 0.136) | 0.040 |
| MLVA | 0.483 | 0.203 - 0.741 | 0.964 | 0.333 | 0.513 (0.203 - 0.741) | 0.594 |
| PTMLVA | 0.319 | 0.405 - 0.770 | 0.905 | 0.333 | 0.599 (0.404 - 0.777) | 0.618 |

clustering there was a clear difference between locations in the composition of *Salmonella enterica* serovars (AU P = 0.997, 95% CI [0.995, 0.999] but not *S.* Typhimurium phage types (AU P = 0.949, 95% CI [0.933, 0.965]). The *S.* Typhimurium composition did not differ between locations in when either MLVA (AU P = 0.889, 95% CI [0.857, 0.921]) or PT/MLVA profiling combinations (AU P = 0.893, 95% CI [0.878, 0.908]) were considered (Table 4–34, Fig. 4–15A). The *Salmonella enterica* serovar and *S.* Typhimurium phage type diversity (measured using Shannon H´) was highest at the broiler location and lowest at the parent location (Broiler > Processing > Parent) (Table 4–33).

### 3.5 Principal Component Analysis for Salmonella Typhimurium differentiation

After removal of the outlying *S.* Typhimurium phage types (PT9, PT141, PT30, n = 20 isolates) the complexity of the PT/MLVA combination relationships between locations and the correlation between PT and MLVA profiles (DT12, DT135, PT135a, DT193 and Untypeable) was examined. Eighty-eight percent of the variation between the two typing methods could be explained in 2 dimensions (Table 4–35).

Three MLVA profiles were significantly correlated with the two dimensions; 03-15-11-11-525 ($\rho_{component1}$ = -0.99, P = 0.001), 03-14-10-09-525 ($\rho_{component1}$ = 0.978, P = 0.003) and 03-13-10-09-526 ($\rho_{component2}$ = 0.95, P = 0.012) and these MLVA profiles were positively correlated (in the same quadrant) with PT135Aa, DT135, and DT12 respectively (Fig. 4–15B). The remaining MLVA patterns did not contribute significantly to the analysis. The relationship between phage type and MLVA profile in the PCA demonstrates there is substantial overlap between *S.* Typhimurium isolates depending on the typing method used. This indicates substantial uncertainty in their true relationships when these phenotypic or genotypic typing methods are considered alone or in combination (Fig. 4–16).

## 4. Discussion

In this intensive longitudinal study of a sin-

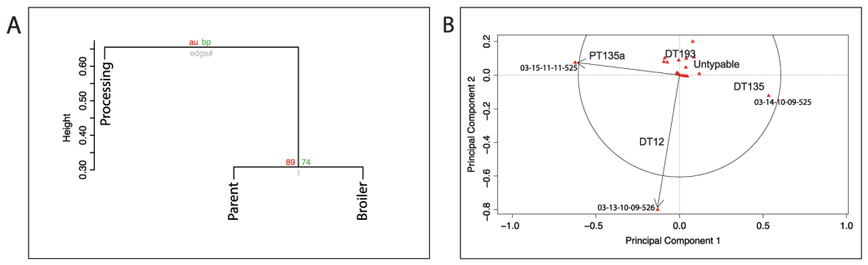


Fig. 4–15. Cluster and Principal Component Analysis of *Salmonella* Typhimurium Isolates by Location and Typing Method

**A** Agglomerative hierarchical clustering by location (Parent, Hatchery and Processing). Approximately unbiased (AU=red) and bootstrap probability (BP = green) values are indicated. A p-value less than 0.95 indicates clustering is not statistically significant. **B** PCA factor plot with MLVA profiles plotted as points (red triangles) and position of phage type identified by phage type designation. Significant MLVA profiles lie outside or close to the circle of equilibrium (black arrows). Distance between isolates is representative of the Euclidean distance between phage types and MLVA profiles on the two significant principal component axes.



Table 4–35. Phage Type and MLVA Principal Component Analysis Versus Broken Stick Results

| | *Principal Component 1* | *2* | *3* | *4* |
|---|---|---|---|---|
| Eigenvalue | 0.085 | 0.034 | 0.013 | 0.0002 |
| % of Variance | 63.08 | 25.20 | 9.82 | 1.89 |
| Cumulative % of Variance | 63.08* | 88.29* | 98.11* | 100* |
| Broken Stick % of Variance | 52.08 | 27.08 | 14.58 | 6.25 |
| Broken Stick cumulative % | 52.08 | 79.16 | 93.75 | 100 |

*Component variance is greater than the broken stick variance indicating statistically significant component

gle vertically integrated poultry operation the breeder site was the most likely point of introduction of *Salmonella* Typhimurium isolates into the integrated production system and the breeder transmission pathway (parents -> eggs -> hatchery -> broiler -> processing) was the most likely path of dissemination to all other locations receiving commodities via this path (eggs or day old chicks). The highly comparable degree of clustering between sites, the dominance of two *S.* Typhimurium PT/MLVA combinations identified at all locations and most of the unique PT and MLVA types (as indicated by PCA) identified at the parent sites supports the observation that parent sites were the most likely point of *S.* Typhimurium introduction into the enterprise. The identification of two dominant *Salmonella enterica* serovars (*S.* Typhimurium and *S.* Infantis) and *S.* Typhimurium phage types (DT135, PT135a) simultaneously across all sites was an unexpected finding, and highlights the importance of intensive sampling within a complex epidemiological environment.

MLVA typing on its own indicated the presence of a much higher number of unique *S.* Typhimurium introductions than phage typing. MLVA profiling indicated the potential introduction of at least 13 unique *S.* Typhimurium variants when considered with phage typing, 17 when using phage typing and MLVA profile combinations, or 8 introductions if only phage typing was considered. The apparently high level of diversity based on the MLVA profiles was unexpected. The variation in MLVA profiles within six of the eight

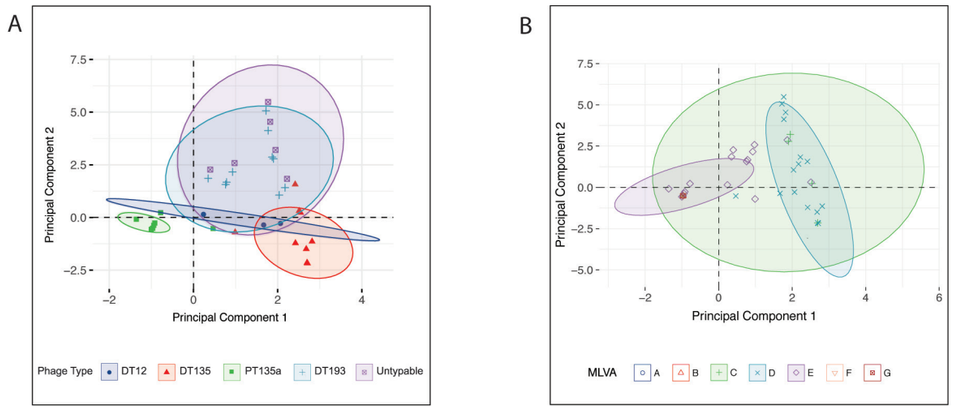


Fig. 4–16. Principal Component Analysis of *Salmonella* Typhimurium Isolates by Typing Method, Phage type and MLVA profile

Distance between each isolate is representative of the Euclidean distance between isolates on the two principal component axes. Concentration ellipses illustrate isolate membership of each typing profile. Isolates are plotted at the same coordinates in both charts. **A** Ellipses identifying isolates grouped by phage type. **B** Ellipses identifying isolates grouped by MLVA profile. Only three MLVA profile groups contained sufficient members to enable ellipses to be generated (MLVA profile groups A, C, E).

phage types made it difficult to resolve the epidemiological relationships between isolates. MLVA profiling alone was not a useful tool for epidemiological investigation even when all complexities were known.

In this study multiple samples were collected and processed at each location to ensure sufficient confidence to detect *Salmonella*, assuming a low prevalence, and to ensure sampling was as representative of the microbiological population diversity as possible. To avoid selection bias due to sample testing methodology, multiple samples from the same environmental site, rather than multiple isolates from the same sample were typed [384-386, 524]. Our results highlight that, while the MLVA profiles were indicative of potential dissemination within the enterprise, they were too variable to be used without considerable curation and interpretation of their relatedness. This has been reported as a potential problem in other Australian studies [525].

Only two potential sources for the introduction of *Salmonella* into this integrated enterprise exist at the parent generation, either live birds (day old chicks) or feed [503]. These findings are comparable to other studies that demonstrate, via genotyping of *Salmonella* Typhimurium isolates, that the breeder sites may be an important source for dissemination throughout an operation [244, 500 501]. In contrast, studies limited to only *Salmonella enterica* serovar identification, frequently implicate the hatchery and its subsequent persistent contamination as a primary source of contamination to the broiler chain but not the parent flocks as the source of contamination to the hatchery [290, 295, 350]. Differences in the supply of parent eggs to the hatchery (such as contract versus company owned supply) may explain the subtle differences in the importance of the hatchery as a source of contamination to the broiler chain.

The limited usefulness of MLVA in the absence of other phenotyping or genotyping methods for epidemiologically complex investigations is unquestionable. Beyond confirmation of similarity in outbreak situations, under strict case definitions, the amount of variation, particularly that observed at the shortest MLVA loci (STTR-5, STTR-6, STTR-10), may be misleading over longer time periods. Substantial variation at these loci has been observed and whether this is mutation, evolution or variation due to the method of analysis (measurement error) remains to be fully explained [71, 72, 526, 527]. Further genetic characterization of isolates by whole genome sequencing is required to understand if the relationships identified by phage typing and MLVA profiling or PT/MLVA combination are real.

As with the findings reported elsewhere, Australia (Chapter 7) has specific dominant *Salmonella* Typhimurium phage types that are also observed overseas. The *S.* Typhimurium strains detected in this study are the same as the dominant strains detected in both the human and animal populations [72]. Additional tools for strain differentiation are essential for epidemiological investigation and outbreak or source identification. Given the close apparent relationships between strains and the over differentiation observed in this study when MLVA is applied it would appear that the rapid introduction of other methods is not just important but essential.

## 5. Conclusion

The relationships between the isolates detected in this study were revealed by intensive repeated longitudinal sampling within the same environment and the combined application and interpretation of multiple typing methods. Considered on its own, each novel MLVA profile identified could potentially misidentify the most likely transmission paths between parts of the integrated system. Without relatively sophisticated computational analysis a substantial level of manual curation is required to utilize MLVA profiling for understanding the putative relationships between isolates. This level of curation is beyond the simple field surveillance routinely employed in most poultry production systems. Extreme caution is advised when each typing method is used alone or in combination without significant sampling and epidemiological insight. To fully understand the relationships between the different isolates of the different phage types and MLVA profiles, given the substantial overlap between isolates detected in this study, further molecular characterization such as whole genome sequencing of the isolates is required.

## Funding Sources

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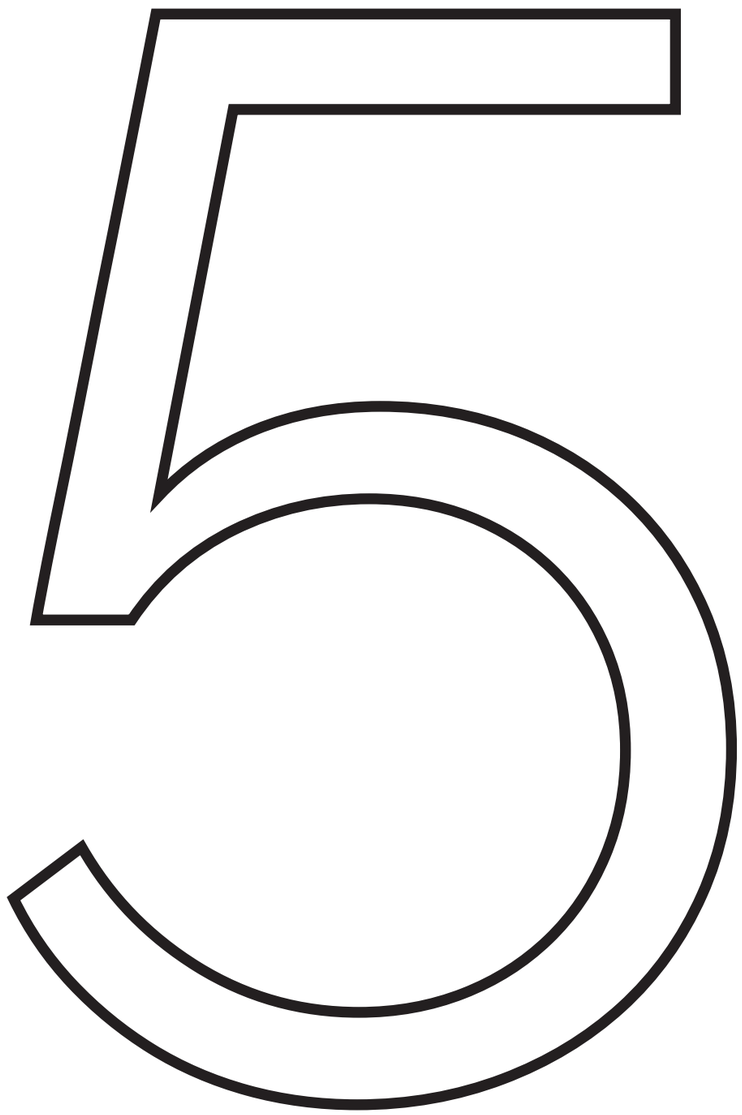
## Acknowledgements

We gratefully acknowledge the support of the poultry company and its staff for allowing us access to their farming operation during the course of the study. Without their support this project and associated research could not be conducted.



C H A P T E R **F I V E**

# The Effectiveness of Sampling in Colony Cage Environments; Environmental Factors Affecting *Salmonella* spp. Detection

Crabb, H.K., Allen, J. L., Devlin, J. M., Wilks, C. R., Gilkerson, J. R., (XXXX) *The effectiveness of sampling in colony cage environments; environmental factors affecting Salmonella spp. detection.* In progress for submission to Applied Environmental Microbiology

*(next spread)*
*The spatial distribution of salmonellae is not homogeneous within a shed. S. Typhimurium was more evenly distributed across the shed space than S. Infantis.*

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## Abstract

Intensive longitudinal sampling within colony caged sheds revealed that both the number of samples collected, and where the samples are collected within this environment was important for ensuring the best chance of detecting *Salmonella*. Multiple *Salmonella enterica* serovars were detected in each shed, and 6% of all samples contained more than one serovar. Samples collected on the north side of the shed (OR =1.77, 95% CI [1.17, 2.68]), on the sheltered side of the shed (OR = 1.90, 95% CI [1.26, 2.89]) and those collected during winter (OR = 48.41, 95%CI [23.56, 104.19]) were more likely to be positive for *Salmonella*. The distribution of salmonellae within a shed was not homogeneous and differences in the within-shed distribution of *Salmonella enterica* subspecies *enterica* serovar Typhimurium ($\chi^2$ (27, 1,538) = 54.4, P = 0.001), and *Salmonella enterica* subspecies *enterica* serovar Infantis ($\chi^2$ (27, 1,538) = 79.8, P = 0.0001), were identified. The difference in *Salmonella enterica* serovar prevalence by spatial location within a shed indicates that there are important micro-environmental or biological factors that influence their survival or distribution. These factors should be taken into consideration when undertaking environmental surveillance for *Salmonella* in flocks housed in caged sheds.


## 1. Introduction

The method of choosing environmental sites for sampling within a cage shed environment is a non-trivial part of surveillance design. Important information about the distribution of *Salmonella* and other pathogens within the environment and how this may affect surveillance strategies beyond simple presence or absence of detection is largely unknown or undescribed in the literature.

In spite of this lack of information, routine surveillance for salmonellae in poultry typically involves environmental sampling as it has long been established as both the most cost effective and sensitive method for detection [376] when compared to individual bird sampling [375, 378, 528]. Environmental sampling is indicative of flock infection with *Salmonella* and the level of environmental (both semi-quantitative and sample prevalence) contamination and egg prevalence may be correlated with the prevalence of infection in the flock [235, 374]. As flock prevalence declines the number of environmental samples required to detect infection must increase to maintain adequate test sensitivity [263, 377]. Importantly animal welfare and production effects associated with live bird handling are minimised allowing environmental sampling to be conducted at frequent intervals with little to no flock interference.

A variety of environmental sampling methods and strategies have been developed for the detection of *Salmonella* in poultry environments [278, 372, 376] and the principal features have been incorporated into national surveillance and control programmes [379-382, 479]. While extensive knowledge has been gained regarding environmental sampling for the identification of infected flocks for control purposes, only a small number of studies have investigated the longitudinal distribution of *Salmonella* within infected flocks or environments [259, 529 - 531]. It is known that *Salmonella* will survive for extensive periods of time in contaminated environments under optimal conditions and an increase in sample prevalence has been observed in winter periods [530], although it is not clear whether this is due to increased shedding from infected birds or multiplication of *Salmonella* in the environment. In addition, multiple *enterica* serovars may be detected in flocks at a single environmental sampling event [531].

Despite extensive discussion regarding sample type and sample pooling, the method of sample site selection and sample collection within a cage shed environment is infrequently described nor discussed [278, 532]. Additionally, no studies have repeatedly sampled flocks within the same environment at a high frequency (3 weekly) for the lifespan of the flocks contained therein. Thus, it is not known what, if any, environmental or flock factors may influence the repeated detection of *Salmonella* during the life of the flock, or what the influence of post-cleaning decontamination on subsequent flock infection or detection of environmental salmonellae.

In veterinary science, prevalence calculations typically use herd or flock size to calculate the appropriate sample size to detect infection at a given prevalence [533] so that the sampling strategy chosen is sufficiently powerful to provide sufficient confidence of detecting infection at this determined prevalence [371]. When extrapolating this information to environmental sampling for the detection of a specific pathogen, thus indirectly sampling the flock, several unknowns are encountered. Does repeated detection reflect flock shedding of *Salmonella* organisms, or rather the repeated detection of the same salmonellae that are persisting and possibly multiplying in the contaminated environment? Is any failure to detect *Salmonella* due to inadequate sample site selection, insufficient sample size, or differences in *Salmonella* distribution (possibly reflecting flock shedding) within a caged shed environment. How does one ensure that sufficient samples are collected that adequately represent both the environment and the distribution of infection within a flock assuming infection may not be homogeneously distributed? None of these issues have been addressed in flocks housed in colony style cages, so this study was undertaken to investigate some of the variables that may affect the sensitivity of environmental sampling to indirectly detect infection in flocks in colony cage sheds in Australian conditions.

## 2. Materials and Methods

### 2.1 Study Population

Four sheds were intensively sampled within a single farm located in south eastern Australia. Each shed comprised a single flock of birds housed within a colony cage system [625]. Each flock was managed in an all-in all-out fashion and placed in the shed after the cleaning and disinfection of the shed and prior to the onset of lay at 19 - 21 weeks of age. Each shed housed ~24,000 birds in eight frames, 3-4 tiers high, in 48 colonies each containing ~500 birds. All sheds were identical in design and construction, only varying in their positioning next to another shed and whether the long length of the shed was exposed or sheltered by another shed, Fig. 5–17. All sheds were placed with the same orientation to the sun, with the length of the shed east-west (Southern Hemisphere). Sheds were ~130m x 30m in dimension with concrete floors. Walls had short concrete walls from the floor ~ 600 mm topped by an aluminium bonded insulated panel to the ceiling with no exposed joists or framing. Ceilings were constructed of the same material as the walls and greater than 6 m in height and unable to be reached without equipment. Sheds were climate controlled with evaporative cooling (cool pads and fans) and a heat exchange unit for warming. Eggs were automatically collected from nest boxes and travelled to a central egg packing room.

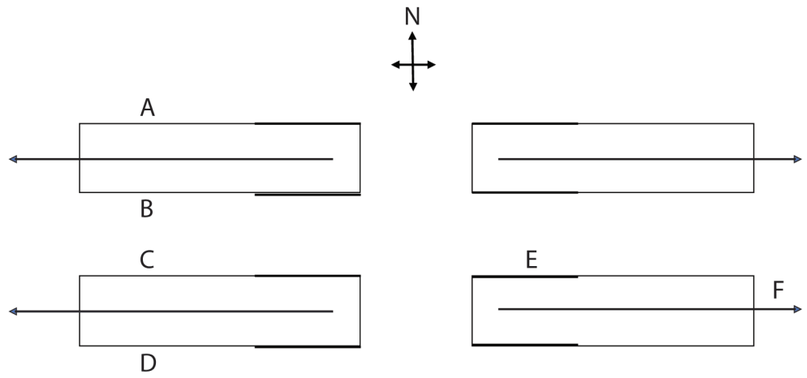


Fig. 5–17. Shed Arrangement and Orientation

**A.** Northern aspect, **B.** Southern aspect between shed, **C**. Northern aspect between shed, **D**. Southern aspect, **E**. Cool pad location, **F.** Tunnel ventilation direction of airflow.

Manure was collected on belts under the cages and removed at least weekly from one end of the shed.

### 2.2 Environmental Sampling Sites

Sheds were sampled post cleaning, prior to flock placement, and subsequently at 3-week intervals until the end of flock production at ~65 weeks of age. A total of 168 potential sampling sites were identified within each shed. These sampling sites included manure belts, frame surfaces, feed lines or feeders, nest box surfaces, fan covers, floor and wall surfaces, egg belts. Sites were identified as either bird contact areas immediately within the bird surrounds such as manure belt, egg belt, nest box surfaces or the tier framing, or non-bird contact areas indirectly contaminated by dust, dander or feed residue such as walls, floors, fan covers, feed lines. It was hypothesized that sites closest to the birds may be more likely to reflect flock infection status rather than more distant sites which may only reflect the shed contamination status. All possible bird contact sampling locations are indicated in Fig. 5–18.

### 2.3 Sample Size Calculations

Sample size was estimated for three design prevalence estimates of 1, 5 or 10 *Salmonella* positive units per 100 units at risk (1. Birds, 2. Cages or 3. Environmental sites) with 95% confidence that the estimated prevalence was within 5% of the true prevalence in the unit tested, assuming an imperfect test. Two sample size calculations were used. The sample size to estimate freedom from infection using an imperfect test [534, 535] was used to calculate the number of birds to sample and for the number of cage or environmental sites required the sample size to estimate a true prevalence assuming an imperfect test was used [533, 536].

### 2.4 Environmental Sampling Site Selection

The sampling sites were systematically selected from the bird contact sites identified on tier two of each frame within the shed. These sites were purposefully selected because they were within easy reach. Birds at this height are exposed to more interaction with people as they were in easy view and may experience more stress, resulting in more shedding of *Salmonella*. Specific sampling sites were chosen to ensure the location and type of sampling was repeatable on all sampling occasions by all samplers.

Multiple different sites, and thus sample types, were selected from each frame to maximize the opportunity to detect *Salmonella*. Manure belt samples were obtained at the end of each frame. A single egg belt from tier 2 per frame was selected. Dust samples were collected from the same tier. Floor samples were collected from each half of the shed (Frames 1-4 and 5-8). All sampling sites were selected away from the main working thoroughfare of the shed in the nest box rows. It was hypothesized that these sites were less likely to be cross contaminated by people movements in and out of the shed.

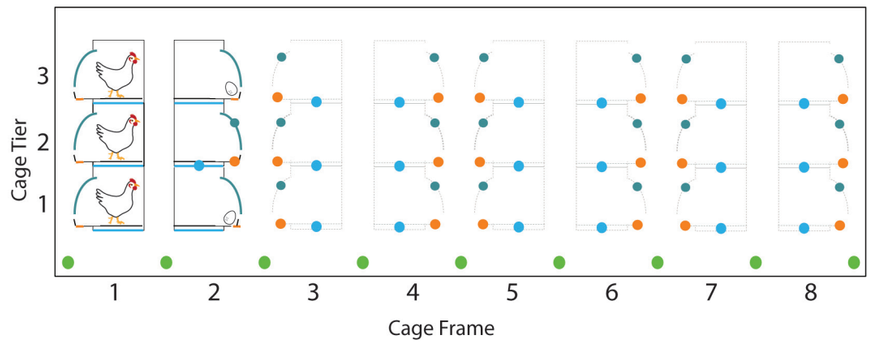


**Fig. 5–18. Sampling Locations within Colony Cage House by Spatial Reference to each Cage Frame and Tier**

Each cage frame is identified horizontally (1 - 8), and each tier of cages per frame is identified vertically (1-3). Potential sampling locations within each cage frame and tier are indicated by a coloured point representing the sample type. The frame outline and location of birds within each frame are indicated on the left. Colours indicate the sample type; Boot Swab = Green, Manure Belt = Blue, Egg Belt = Orange and Dust = Dark Blue.



### 2.5 Environmental Sampling Methodology

Within each cage shed a total of 28 samples, 8 egg belt, 8 dust, 8 manure belt and 4 boot swabs were collected on each sampling occasion. Four 10 x 10 cm cotton gauze swabs (pre-moistened with buffered peptone water) were used to collect each surface sample.

Manure belt samples were collected by wiping the exposed edge of all belts at one end of the shed, avoiding excessive shed "dust" and debris, concentrating on exposed faecal material. A single egg belt in each frame was sampled by wiping the length of the "returning" surface of the egg belt from a single tier. Dust samples were collected by wiping the surface of the nest box the length of the frame. Clean dry boots, with new plastic boot covers were worn on entry to the shed. Two pairs of boot swabs, were worn while walking within the shed during sample collection. The second pair was exchanged at the shed sampling midpoint (Frame 4 – 5). Each sample-type was pooled separately by frame and cage row into a Whirlpak™ bag and identified by shed, flock, sample type and sample location. Samples were refrigerated immediately after collection.

### 2.6 Sample Processing

All samples were collected and returned to the laboratory for processing the same day. Each sample was processed separately, no samples were pooled. All sample processing and laboratory methods are described in the Supplementary Material.

### 2.7 Weather Records

Daily weather records for study period were obtained from the closest weather observation station to the farm location (~ 10 km) [537]. The following daily observations were obtained: total rainfall (mm), mean, maximum and minimum temperature (°C), and global solar exposure (MJ/m2). Humidity and wet bulb temperature readings were not available. Daily data was aggregated by week of sampling and 3 weeks prior to sampling for each of the variables.

The following months were aggregated for each southern hemisphere season: Summer, December – February; Autumn, March – May; Winter, June – August; and Spring, September - November.

### 2.8 Statistical Analysis

All analyses were conducted in the R statistical package unless otherwise stated [484]. Spatial analysis was conducted using the following packages: "spsurvey" [538], "rgeos" [539] , "sparr" [540] and "spatstat" [541, 542]. Measures of association, odds ratios and positive predictive values were estimated using "epiR" [543]. The R packages "nlme", "lme4" and "aod" were used for hierarchical mixed effects model building [544-546].

#### 2.8.1 Sample Prevalence Calculations

Prevalence estimates based on the results of testing (apparent prevalence) were distinguished from prevalence estimates corrected on the basis of imperfect diagnostic test performance (true prevalence) [547, 548]. For the calculation of true and apparent prevalence the test sensitivity was assumed to be 0.88 and specificity 0.995. For sample size calculations, diagnostic test sensitivity and specificity for *Salmonella* by culture was assumed to vary between 0.88 - 0.98 and 0.99 - 1.00 respectively. [549-551].

#### 2.8.2 Sample Site Homogeneity

The spatial distribution of *Salmonella enterica* serovars within each shed was evaluated by aggregating all sampling events for each shed. Spatial heterogeneity was evaluated by spatial point pattern analysis and calculating the spatial density (kernel) of each of the sampled sites using "spatstat" [541, 542]. The distribution of *Salmonella enterica* within the shed was visualized using heat maps which plotted the calculated density at each sampling location within a two-dimensional representation of the shed environment. Spatial autocorrelation was evaluated using Moran's I from the "ape" package [552] and the resulting variogram visualised using "geoR" [553].

### 2.8.3 Univariate Analysis

There were two outcomes of interest: 1. Binary, the presence (1) or absence (0) of *Salmonella*; and 2. The true sample prevalence at a given sampling event. Only explanatory variables associated with the sampling methodology and the housing environment were considered in this analysis (Supplementary Table 5–40). Measures of association between the binary outcome of interest and the explanatory variables were computed using the odds ratio. For categorical or continuous variables, simple linear or logistic regression was used where appropriate. If an explanatory variable was statistically associated with the binary outcome of interest in the univariate analysis ($P < 0.1$) it was considered for inclusion in the multivariable analysis.

### 2.8.4 Hierarchical mixed effects multivariable logistic regression

A fixed-effects multivariable logistic regression model was built where the probability of a sample being positive for *Salmonella* at a sampling event was parameterised as a function of the significant explanatory variables ($P < 0.1$) identified in the univariate analysis. The first 13 sampling events of all sheds were included in this analysis. The model was built in a stepwise manner with non-significant explanatory variables removed sequentially from the model, until all variables retained in the model were significant at alpha < 0.05. Variables removed from the model were retested in the final model and retained if their inclusion changed the regression coefficients significantly, using the Wald test. Where no significant difference was observed between two models then the most parsimonious model was selected. Biologically plausible interactions were considered. Autocorrelated measures were identified and tested individually in the model, only one was kept in the model if it remained significant.

Due to the hierarchical and longitudinal nature of the data (Supplementary Table 5–41), samples clustered within sampling events and sampling events clustered within sheds, the model was extended to include sample event– and shed – level random effect terms. Factors influencing the detection of *Salmonella* were categorized into three classes: those operating at the sample level, the sample event level and the shed level. Sample level effects considered included the sample type, sampling location, whether the sample was collected from the northern aspect of a shed or whether the sampling location within a shed was between two sheds (Fig. 5–17). Sampling event level effects included the month of sampling, the season of sampling event and the weather (including rainfall, temperature and solar radiation at the time of sampling or the three weeks prior to sampling). For this analysis, the shed level effects considered included whether *Salmonella* were detected in the shed prior to the onset of the study and the flock. The *Salmonella* status of sheds were the same, i.e. *Salmonella enterica* serovars were present in all sheds prior to the onset of the study, but not detected at the post cleaning sampling event. The shed is a proxy variable for the flock as this is the only different variable at the shed level. The assumptions of normality and homogeneity of variance of the final model were checked using frequency histograms of the residuals, and plots of the residuals versus predicted values.

## 3. Results

### 3.1 Sample Size Calculations

The number of samples required to be 95% confident that the estimate of prevalence was within 5% of the true population prevalence for each unit of interest (1. Birds, 2. Cages or 3. Environment) are summarised in Supplementary Table 5–42. At the lowest design prevalence, at either diagnostic test sensitivity, the number of birds to be sampled was greater than the precision of the sample calculation and unable to be calculated. At least 28 samples (cage or environmental) were required to estimate the *Salmonella* prevalence with sufficient confidence at the lowest design prevalence. The number of cages to be sampled at a design prevalence of 5% or 10% is higher than the number of cages present in the shed. Twenty-eight cages were estimated as the equivalent of sampling ~14,000 birds in a shed of this size and design.

Table 5–36. Area ($m^2$) of Shed Sampled per Sample Type on each Sample Event

| *Sample Type* | *Area sampled per sampling unit ($m^2$)* | *Total Area per shed ($m^2$)* |
|---|---|---|
| Dust/Egg Belt | 5 | 80.00 |
| Manure Belt | 2 | 16 |
| Boot Swab | 29 | 116.00 |
| Total Area | 40.60 | 209 |

### 3.1.1 Surface Area Sampled

Four sheds were sampled. The total surface area of each shed sampled on each sample event by sample type is presented in Table 5–36. A total of 209 $m^2$ of shed surface area was sampled per shed on each sampling occasion, this was estimated as equivalent to 24 cages or half the flock.

### 3.2 Univariate Analysis

### 3.2.1 Sample Event

Each shed was sampled on 13 -15 occasions during the flock production period with 1,538 environmental samples collected (Table 5–37); of these 24% were positive for *Salmonella*. All sheds were negative for *Salmonella* prior to the onset of flock placement. Five percent of the samples contained more than one *Salmonella enterica* serovar. *Salmonella enterica* were detected in all sheds on most sampling events (69 – 100%). However, the number of sampling events that were positive for each sample type varied for any one shed (Supplementary Table 5–43). The percentage of *Salmonella* positive samples also varied by shed (8 – 56%), the difference in sample prevalence between sheds was statistically significant ($\chi^2$ (3, 1,538) = 246.8, $P < 0.0001$) and varied by *Salmonella* serotype. Both *Salmonella* Typhimurium and *Salmonella* Infantis were detected in all sheds, but in 3 of the 4 sheds *Salmonella* Typhimurium was isolated more frequently (Table 5–37).

Those sheds with fewer positive sampling events had fewer *Salmonella* positive samples. In sheds A and C (high environmental prevalence sheds) any combination of boot or dust sample would have detected that these were sheds positive for *Salmonella* on any sampling event. Whereas in sheds B or D (low environmental prevalence sheds) fewer than half the samples were positive on any sample event regardless of the sample type. Only by combining the results of each sample type on each sampling event did the probability of detecting *Salmonella* increased, 9– to 12– fold in sheds B or D respectively.

### 3.2.2 Sample Type and Predictive Values

There was a significant difference between sample types in the detection of *Salmonella* ($\chi^2$ (3, 1,538) = 76.0, $P < 0.0001$). All sample types were superior to egg belt swabs for the detection of *Salmonella*. Boot swabs (OR = 5.90, 95% CI [3.86, 9.17]) were better than both dust and manure belt swabs for the detection of *Salmonella*, but there was no significant difference between dust and manure belt sample types (Table 5–38).

The positive and negative predictive values of the sampling methodology for detecting *Salmonella* was high (PPV = 0.98, 95%CI [0.96, 0.99], NPV = 0.99, 95%CI [0.98, 1.00]).

Table 5–37. Summary of Environmental Sampling Results for each Shed

| | *Shed* | | | |
|---|---|---|---|---|
| | *A* | *B* | *C* | *D* |
| Sampling Period[1] | 36 | 36 | 38 | 41 |
| Mean Sampling Interval[2] (Range) | 2.8 (2.0 - 3.0) | 3.0 (0) | 2.8 (2.0 - 3.0) | 2.8 (2.0 - 3.0) |
| Total Sampling Events | 13 | 13 | 14 | 15 |
| Positive Sampling Events[3] (%) | 13 (100) | 9 (69) | 14 (100) | 1 (73) |
| Total Number of Samples | 364 | 362 | 392 | 420 |
| Total Samples Positive | 203 | 35 | 103 | 33 |
| True Prevalence: *Salmonella* (95% CI) | 0.63 (0.57 - 0.69) | 0.10 (0.07 - 0.14) | 0.29 (0.25 - 0.35) | 0.08 (0.06 - 0.12) |
| True Prevalence: *S.* Typhimurium (95% CI) | 0.39 (0.34 - 0.46) | 0.09 (0.06 - 0.12) | 0.006 (0.00 - 0.02) | 0.07 (0.05 - 0.11) |
| True Prevalence: *S.* Infantis (95% CI) | 0.19 (0.15 - 0.24) | 0.01 (0.003 - 0.04) | 0.28 (0.23 - 0.34) | 0.002 (0.00 – 0.02) |

[1] Total number of weeks over which sampling was conducted. [2] The interval between sampling events in weeks. [3] The number of sampling events where at least one of 28 samples were positive.

However, it should be noted that the positive predictive value of the sampling method differed between *Salmonella enterica* serovars, with the probability of detecting *S.* Typhimurium (PPV=0.91, 95%CI [0.86, 0.96]) lower than *S.* Infantis (PPV = 0.94, 95%CI [0.90, 0.98]).

When accounting for both serovar and sample type there were differences between the sample types in the detection of each *Salmonella enterica* serovar ($\chi^2$ (3, 374) = 25.2, P<0.0001). Boot (OR = 5.48, 95% CI [2.4, 13.12]) and dust swabs (OR = 2.14, 95%CI [1.00, 4.83]) were more likely to be positive for *Salmonella* Infantis. Manure swabs were not significantly different for either serovar (OR = 1.41, 95%CI [0.66, 3.21]).

### 3.2.3 Salmonella Distribution within a Shed

There was a significant difference between locations within a shed for the detection of *Salmonella* ($\chi^2$ (27, 1,538) = 96.6, P < 0.0001). Samples taken on the north side of the shed (OR = 1.95, 95% CI [1.54-2.49]), and those collected from locations on the between side of a shed were more likely (OR = 1.92, 95% CI [1.52 - 2.45]) to be positive for *Salmonella*. Thirteen of the 28 sampling locations were more likely to be positive for *Salmonella enterica* regardless of the serovar (Supplementary Table 5–45). *S.* Typhimurium was more likely to be found in seven of the twenty-eight locations ($\chi^2$ (27, 1,538) = 54.4, P < 0.001), and *S.* Infantis was more likely to be identified in 7 different locations ($\chi^2$ (27, 1,538) = 79.8, P < 0.0001). The detection of a *Salmonella* positive sample at one location was not influenced by the detection of another positive sample (spatial autocorrelation) in the same or similar spatial location (Moran I = -0.026, P = 0.343). The frequency that each serovar was identified at a location is illustrated in Fig. 5–19A. The sample prevalence for each serovar, illustrated in a 2D space using a heatmap, is presented in Fig. 5–19B and Fig. 5–19C. The difference in sample prevalence by location is readily visualised with higher prevalence locations having a warmer colour. The height of the 3D perspective plot indicates the prevalence at the corresponding spatial location. The spatial distribution of *Salmonella* is not homogeneous within a shed, with *S.* Typhimurium more evenly distributed across the entire space than *S.* Infantis, which was only detected in one half of the shed.

Table 5–38. Univariate Results for all Statistically Significant Shed and Location Specific Variables

| *Variable* | *Code* | *OR* | *95%CI* | $\chi^2$ | *DF* | *P value* |
|---|---|---|---|---|---|---|
| Shed | A | 3.54 | 2.61 – 4.82 | 246.8 | 3 | < 0.0001 |
| | B | 0.30 | 0.19 – 0.45 | | | |
| | C | 1.00[1] | – | | | |
| | D | 0.23 | 0.16 – 0.36 | | | |
| Location | 17-77[2] | – | – | 96.6 | 27 | < 0.0001 |
| Aspect | North | 1.95 | 1.54 – 2.49 | 30.4 | 1 | < 0.0001 |
| | South | 1.00[1] | – | | | |
| Betweenness | Yes | 1.92 | 1.52 – 2.45 | 29.1 | 1 | < 0.0001 |
| | No | 1.00[1] | – | | | |
| Sampling Event | 1-13[3] | – | – | 62.5 | 12 | < 0.0001 |
| Sample Type | Boot Swab | 5.90 | 3.86 – 9.17 | 76.0 | 3 | < 0.0001 |
| | Dust | 4.20 | 2.87 – 6.27 | | | |
| | Manure Belt | 4.04 | 2.76 – 6.05 | | | |
| | Egg Belt | 1.00[1] | – | | | |
| Season | Autumn | 1.00[1] | – | 54.3 | 3 | < 0.0001 |
| | Spring | 2.07 | 1.48 – 2.93 | | | |
| | Summer | 2.16 | 1.54 – 3.05 | | | |
| | Winter | 4.30 | 2.92 – 6.38 | | | |
| Rain | | 1.26 | 1.14 – 1.39 | 20.1 | 1 | < 0.0001 |
| Prev3MaxT | | 0.97 | 0.95 – 0.99 | 12.6 | 1 | 0.004 |
| Prev3MinT | | 0.94 | 0.91 – 0.97 | 15.2 | 1 | < 0.0001 |

[1]Reference variable, [2] Full results Supplementary Table 5–45, [3] Full results Supplementary Table 5–46



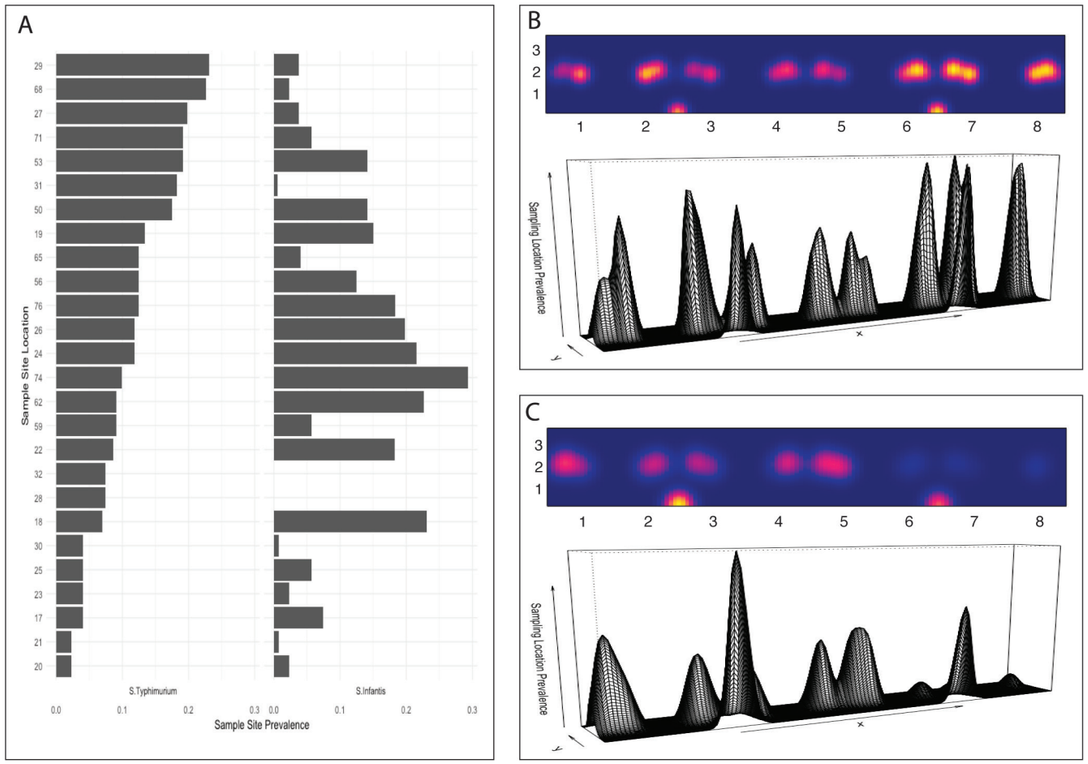


**Fig. 5–19.** Heterogeneity of *S. Typhimurium and S.* Infantis Distribution within a Colony Cage Environment

The environmental prevalence for each serovar is calculated from all samples collected from that sampling location. **A.** Salmonella serovar prevalence at the sampling location within the shed environment for S. Typhimurium and S. Infantis separately. Length of the bar indicates serovar prevalence at that sampling location. Sampling location is ordered by the site with the highest S. Typhimurium prevalence. **B**. Heat map indicating S. Typhimurium prevalence in a two-dimensional space at each sampling location. The warmer the colour the greater the prevalence at that location. Corresponding perspective plot of S. Typhimurium prevalence, height is proportional to the prevalence at the sampling location. **C.** Heat map indicating S. Infantis prevalence in a two-dimensional space at each sampling location. The warmer the colour the greater the prevalence at that location. Corresponding perspective plot of S. Infantis prevalence, height is proportional to the prevalence at the sampling location.

### 3.2.4 Weather observations

All weather observations are summarised in Supplementary Table 5–44. The mean rainfall during the study period was low (0.60 – 2.00 mm) and rainfall was significantly different on each sampling event (Adj-$R^2$ =0.41, F (14,1523) = 79.82, P < 0.001). Rainfall varied by season (Adj-$R^2$ = 0.042, F (3,1534) = 23.77, P < 0.0001), with a significantly higher rainfall in winter (Fig. 5–20). Both the mean minimum (Adj-$R^2$ = 0.50, F (14,1523) =112.7, P < 0.0001) and maximum temperatures (Adj-$R^2$ = 0.70, F (14,1523) =262.9, P < 0.0001) were significantly different on each sampling event. The mean maximum temperature was highest in the summer (mean

= 27.56 °C) and lowest in winter (mean = 5.90 °C). As with all the weather variables the amount of solar irradiation also varied significantly by season (Adj-$R^2$ = 0.47, F (3, 1534) =458.2, P < 0.0001), being highest in summer, and differed significantly on each sampling event (Adj-$R^2$ =0.75, F (14, 1523) = 339.1, P < 0.001).

Only three weather variables were significantly associated with the detection of *Salmonella* in samples; Rainfall on the week of sampling and the minimum and maximum temperature in the three weeks prior to sampling (Supplementary Table 5–47, Supplementary Table 5–48). As the rainfall increased more samples were positive for *Salmonella* and a higher temperature in the three weeks prior to sampling reduced the number of positive samples. However, there was a difference between *Salmonella enterica* serovars with *S.* Typhimurium more likely to be detected when rainfall was higher (OR =1.36, 95% CI [1.20, 1.54]), while *S.* Infantis was more likely to be detected when there was more solar irradiation (OR = 1.05, 95% CI [1.06 – 1.09]) and higher temperatures, particularly high minimum temperatures (OR = 1.08, 95% CI [1.04-1.13]). There was a statistically significant difference between seasons in the number of *Salmonella* positive samples detected ($\chi^2$ (3, 1538) = 54.3, P < 0.001) with more positive samples likely to be detected in winter (OR = 4.30, 95% CI [ 2.92, 3.67]) than any other season.

### 3.3 Multivariable Modelling

The univariate results for all statistically significant explanatory variables considered for multivariable modelling are summarized in Table 5–38. The final multivariable model considered the detection of a *Salmonella* positive sample irrespective of serovar. Estimated regression coefficients for the final model are provided in Table 5–39.

A two-level hierarchical model was considered to account for both sample event– and shed– level effects. The most parsimonious model was selected, which only accounted for the difference in the shed-level effects, as there was no significant difference between the two models. The residual variation due to unknown shed effects (variation partition coefficient) was estimated as 1.858/(1.858 + 3.29) = 0.39. A standard logistic regression model was built without taking into account the random effects associated with the shed–level effects. There was no statistically significant difference between the two models (Log-Lik= -98.2, DF=10, P=1 ) with or without the random effects terms for shed. The results from the most parsimonious model with shed as a fixed term are presented. The Hosmer-Lemeshow goodness of fit test was significant at small values of g=5 to 10 ($\chi^2$ d.f. (3) = 2.18, P = 0.54, d.f. (8) =13.85, P = 0.086) indicating a relatively good fit to the data with this model. The area under the ROC curve (Figure 5-21) or diagnostic accuracy was estimated as 0.884 95%CI: [0.864 -0.904], indicating a good prediction of the outcome by the model. The sensitivity and specificity of the model was estimated as 0.875 and 0.771 respectively. The post fit model diagnostic plots are presented in Figure Samples that were collected from a location on the north side of a shed or within the shed on a side between two sheds were 1.79 (95% CI [1.16 -2.68]) and 1.90 (95% CI [1.25, 2.89]) times more likely to be positive for *Salmonella*. Both the actual location of sample collection and sample type were important but only sample type was included in the final model. The odds of

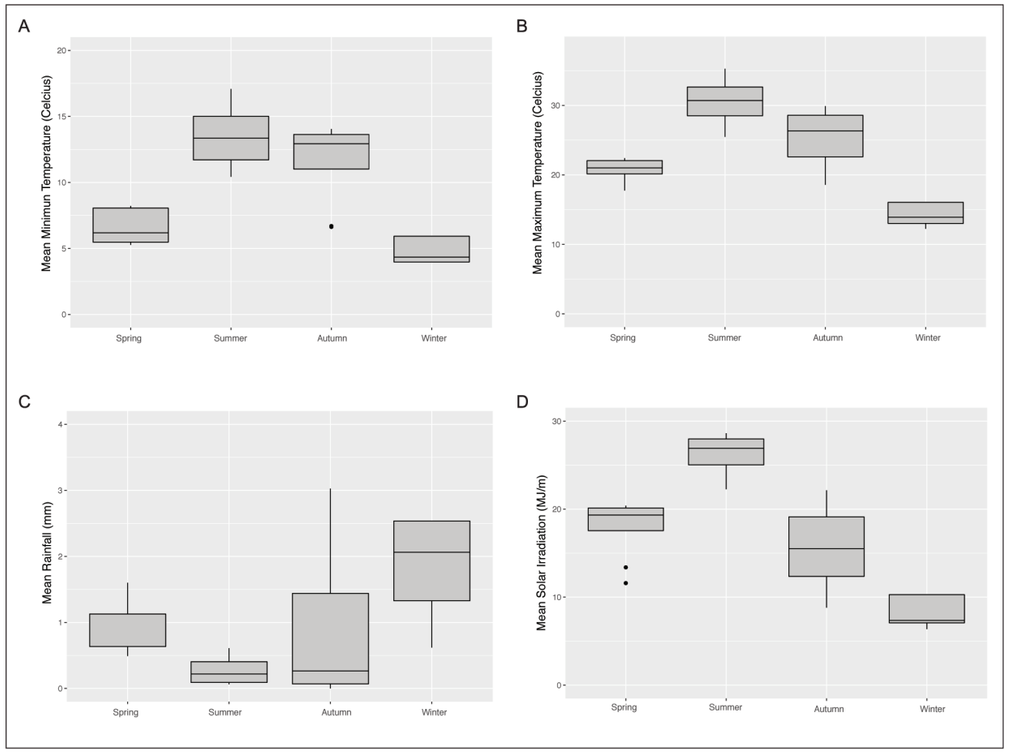


Fig. 5–20. Mean Seasonal Weather Observations for the Study Period (2013-2014)
**A.** Mean minimum daily temperature (°C); **B.** Mean maximum daily temperature (°C); **C.** Mean daily rainfall (mm); D. Mean daily solar irradiation (MJ/$m^2$)

Table 5–39. Mixed-effects Logistic Regression Results for Environmental Risk Factors (2013-2014)

| Variable | Positive | Total | Coefficient (SE) | Z | P-value | OR (95% CI) |
|---|---|---|---|---|---|---|
| Intercept | 367 | 1,458 | -4.14 (0.32) | – 12.96 | <0.001 | |
| Aspect | | | | | | |
| North | 229 | 728 | 0.58 (0.21) | 2.71 | 0.007 | 1.78 (1.16 – 2.68) |
| South | 138 | 726 | Reference | | | 1.00 |
| Betweenness | | | | | | |
| Yes | 230 | 728 | 0.65 (0.21) | 3.06 | 0.002 | 1.90 (1.25 – 2.89) |
| No | 137 | 726 | Reference | | | 1.00 |
| Sample Type | | | | | | |
| Egg Belt | 39 | 415 | Reference | | | 1.00 |
| Dust | 126 | 415 | 2.14 (0.25) | 8.47 | <0.001 | 8.53 (5.26 – 14.22) |
| Manure Belt | 123 | 416 | 2.11 (0.25) | 8.31 | <0.001 | 8.24 (5.08 – 13.75) |
| Boot Swab | 79 | 208 | 2.68 (0.28) | 9.39 | <0.001 | 14.52 (8.41 – 25.73) |
| Season | | | | | | |
| Autumn | 63 | 448 | Reference | | | 1.00 |
| Winter | 74 | 112 | 3.92 (0.38) | 10.25 | <0.001 | 50.22 (24.29 – 108.92) |
| Spring | 113 | 446 | 0.92 (0.21) | 4.47 | <0.001 | 2.52 (1.81 – 3.79) |
| Summer | 117 | 448 | 0.99(0.20) | 4.82 | <0.001 | 2.70 (1.81 – 4.07) |
| Shed | | | | | | |
| 1 | 98 | 364 | Reference | | | |
| 2 | 203 | 364 | 1.13 (0.19) | 6.02 | <0.001 | 3.09 (2.15-4.49) |
| 3 | 31 | 364 | -1.45 (0.23) | -6.18 | <0.001 | 0.23 (0.14 -0.36) |
| 4 | 35 | 362 | -2.48 (0.29) | -8.26 | <0.001 | 0.08 (0.04 -0.15) |

a sample being positive was greater for boot swabs (OR= 14.52, 95%CI [8.41 – 25.73]) than any other sample type. As each sample type was collected by location they could be interpreted as a proxy variable for location. Individual weather variables were not significant but the effect of season was very important as samples collected in the winter months were OR = 50.22, 95% CI [24.29, 108.92] times more likely to be positive than those collected in the other seasons. There was a significant effect of shed on the results with samples from Shed 2, 3 times more likely to be positive than in any other shed (OR=3.09, 95%CI [2.15, 4.49]).

## 4. Discussion

This study investigated the performance of environmental sampling for the detection of *Salmonella* in a colony-caged environment under Australian environmental conditions. Sample size estimations demonstrated that at least 28 samples were required to detect *Salmonella* at a design prevalence of 1% with an imperfect test. Because individual bird sampling for *Salmonella* is known to be less sensitive than environmental sampling [518], the number of birds to sample was estimated using the strictest criteria, proof of freedom of disease. This comparison (bird vs environment) was made to demonstrate the relative efficiency of environmental sampling versus individual bird sampling as a screening tool. Both the logistics and practicality of selecting and testing hundreds of birds from each colony at regular intervals repeatedly throughout production precludes this from being a real option for routine surveillance purposes. It also highlighted that the number of samples (birds) required at very low flock prevalence is greater than the design criteria when using an imperfect test. This has important implications for confirmatory testing of the *Salmonella* status in flocks after a positive environmental sampling result especially when the flock prevalence is low.

Analysis of the field results where these considerations were applied demonstrated that, not only is the number of samples taken critical for detecting *Salmonella* within a caged environment, but where the samples are collected is also important. Despite the small number of sheds sampled, many samples were collected frequently from the same locations within the sheds for over 12 months. The analysis clearly demonstrated that *Salmonella enterica* serovars were heterogeneously distributed within the shed and that the distribution differed by serovar. The identification of specific locations that were more likely to be positive for *Salmonella* such as those on the northern side of the shed or on sampling locations aspects located on a sheltered sides of the shed (between sheds), suggested that there may be

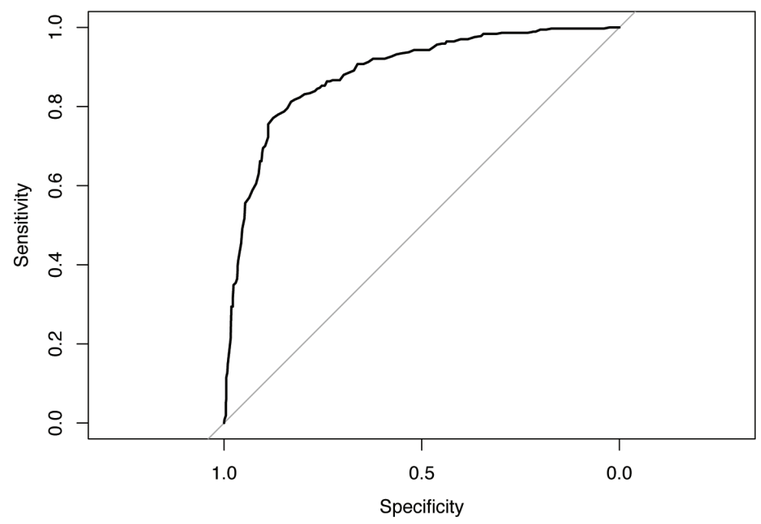


Fig. 5–21. ROC for the Final Logistic Regression Model.



specific micro-environmental conditions that favour the detection or survival of *Salmonella* in these environments and that these may favour survival or growth of one serovar over another.

The importance of the specific location within the shed is a new observation. Consequently, the specific site where sampling is to be conducted within the shed is critical for detection, as the presence or survival of salmonellae within the shed is not a random event. The effectiveness of detecting *Salmonella* varied by shed and this variation was related to the overall prevalence of infection in the flock and therefore level of contamination in a particular shed.

Samples were more likely to be positive in the winter and univariate analysis indicated that decreasing temperature and increasing moisture also increased the odds of detection. Critically, the winter in this location was dry and cool (2.00 mm, 7.95 -21.95°C) but not wet or hot. These results are consistent with other studies that have demonstrated a seasonal effect on the detection of *Salmonella* [278].

The use of a multi-sample type sampling strategy is not novel and this approach was used to allow comparison with international studies using similar methods . However, the strategy for collecting samples was novel. A critical gap identified in the literature was where to collect samples within the shed (other than randomly) and there is robust discussion about the value of taking a small sample more frequently from multiple locations rather than a large pooled sample of, for example, dust [374, 376].

Samples were deliberately collected systematically from within the shed at person height to ensure that all frames of birds were sampled within easy reach assuming dust samples are heavy and that any dust present at one level is from the tier sampled and the tiers above. It is important to note that while obvious, birds housed in caged environments cannot roam freely within the whole shed space. Additionally, birds are housed both vertically and horizontally. This means that if infection within the flock is not homogeneously distributed then failing to sample the entire shed space may bias the sampling results, particularly if the prevalence is low. Other studies have reported that *Salmonella* prevalence may be lower in birds housed in higher tiers of the frames [16].

The positive predictive value of the overall sampling strategy was high (98%), even when accounting for an imperfect test. Each of the sample types performed well, with the key exception of egg belt swabs in this study, where the likelihood of detection was lower. Egg belts in this environment were constructed of a polyethylene plastic material and this may have hindered detection. These egg belts have a smaller surface area as they are perforated and not absorbent like fabric egg belts; this may mean a greater degree of contamination is required for detection of *Salmonella*. Also, it is likely that insufficient egg belt surfaces were swabbed. The egg belt is the only surface in the shed that is not as readily cross contaminated with dust or environmental material as the other exposed surfaces, as it is protected from above.

A novel approach in this study was to include the use of boot swabs on concrete floors. Boot swabs are typically used on litter or slatted floors in free range layer sheds or broiler production sheds but this study has demonstrated that they are a very sensitive method of sampling in the cage environments. Boot swabs were more likely to test positive for *S.* Infantis than for *S.* Typhimurium. Finally, manure samples were collected by directly sampling the ends of the manure belts, where they are more easily accessed, rather than collecting a pooled sample of faeces. Depending on the shed design, access to fresh manure under cages can be very difficult, additionally the timing of sampling is critical as manure belts may have been cleaned prior to sampling which will limit access to an adequate, representative manure sample. These results indicate that manure belt samples were as effective as dust samples for the detection of *Salmonella* and there was no difference in detection of the two *Salmonella enterica* serovars. Surface swabs of the manure belt were preferred to dealing with large quantities of manure when considering sample collection, handling, laboratory testing and disposal. Additionally, there was no difference in serovar detection with manure belt swabs, unlike dust swabs where *S.* Infantis was more likely to be detected.

A number of other potential factors not included in this study, but may affect the presence of *Salmonella* within the shed as a consequence, or reflection of, bird stress. Areas where birds are more stressed, such as the hotter more exposed sides of the shed (northern aspect) or those with poorer air flow or recirculation (sheltered side of the shed between other sheds), may shed more *Salmonella*, which may be reflected in a higher prevalence of positive samples collected from these sites within the shed or it may indicate the preferential survival of *Salmonella* under those environmental conditions. Unfortunately, internal shed environmental records were only available for the whole shed, rather than for specific areas of the shed, so these effects could not be investigated further.

## 5. Conclusion

This study confirmed that the number of samples chosen is important for *Salmonella* detection, but of more importance is how samples are collected and from where within a caged flock shed environment. A multi-sample type approach has high positive predictive value even when the diagnostic test sensitivity is low. *Salmonella* distribution within a shed is not homogeneous and the location of *Salmonella* within a shed does not appear to be random. Factors influencing detection include the season, the weather (rainfall and moderate temperatures) and the shed aspect (north or south side) or sheltering by another building. *Salmonella enterica* serovars were detected in different spatial locations within the shed indicating that specific micro-environments may enhance survival, and possibly multiplication, and hence detection. All these factors should be accounted for when designing a surveillance strategy for the detection of *Salmonell*a.


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## Acknowledgements

We gratefully acknowledge the support of the poultry company and its staff for allowing us access to their farming operation during the course of the study. Without their support this project and associated research could not be conducted.

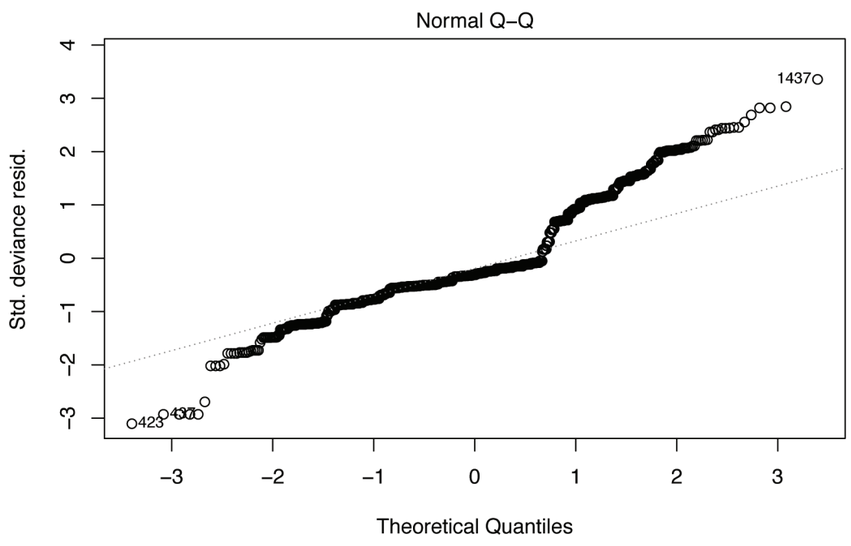




Fig. 5–22. Final Post-Fit Logistic Regression Model QQ-Plot

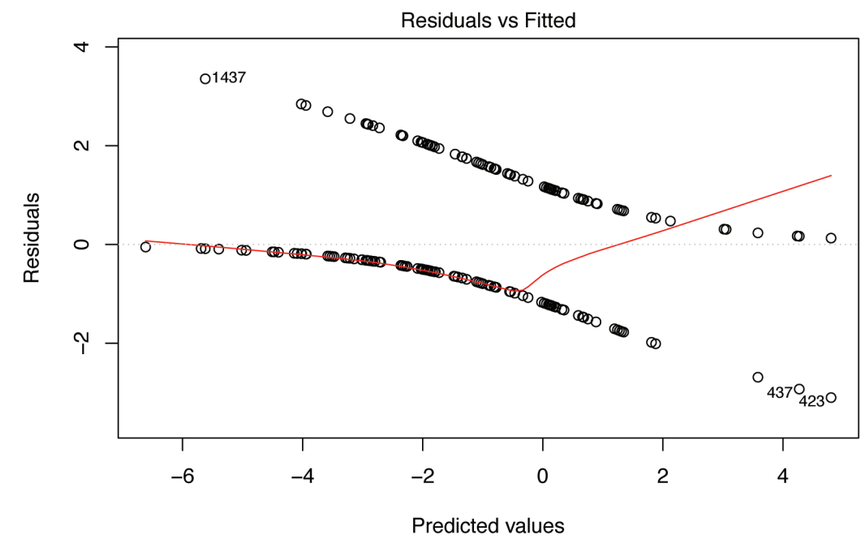



Fig. 5–23. Final Post-Fit Logistic Regression Model Residuals versus Fitted Plot

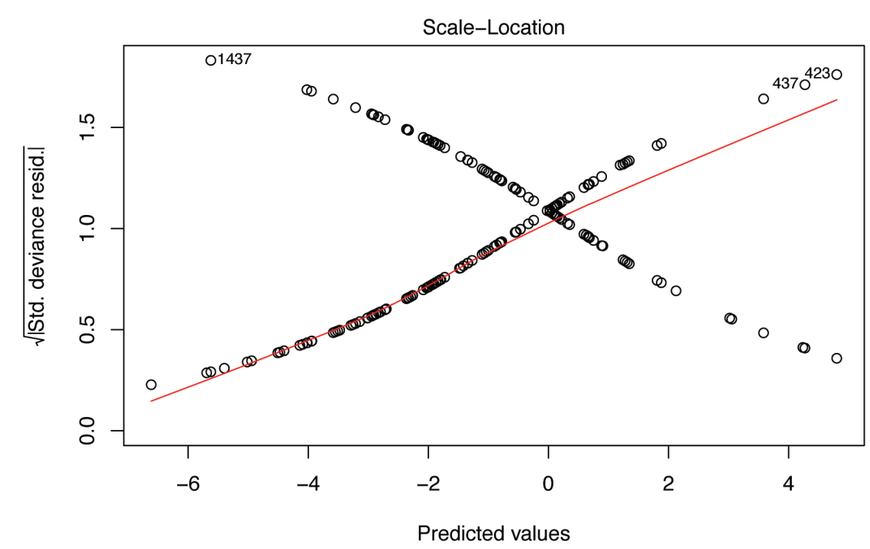



Fig. 5–24. Final Post-Fit Logistic Regression Model Scale-Location Plot

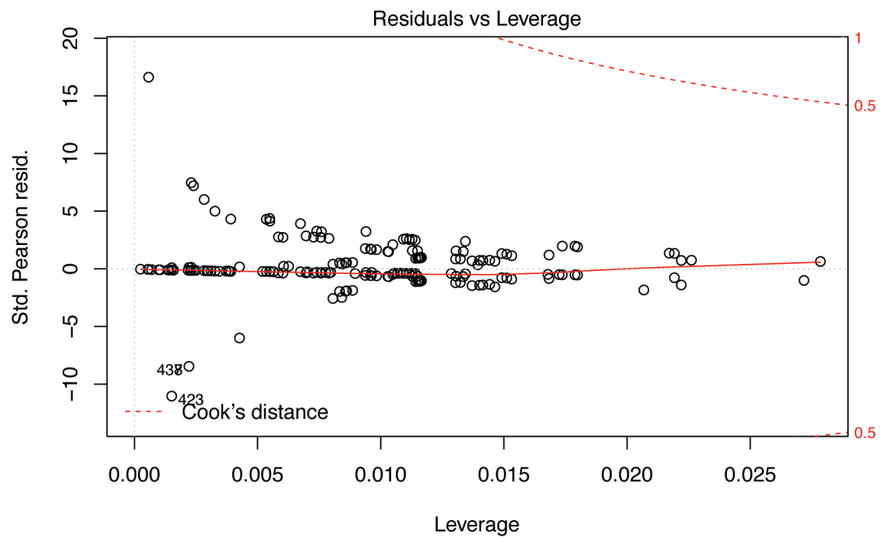



Fig. 5–25. Final Post-Fit Logistic Regression Model Residuals versus Leverage Plot

## Supplementary Material

### 1. *Salmonella* Isolation

All samples were processed on the day of collection in accordance with the Australian Standard 5013.10-2009 Horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002, MOD) [459]. Buffered peptone water was added to each primary sample with little mixing and each suspension was statically incubated at 37°C for 18-24 hours. After incubation, three 33 µL aliquots were taken from each primary sample and inoculated onto Modified Semi–solid Rappaport Vassiliadis (MSRV) plates. Inoculated MSRV plates were incubated under aerobic conditions at 41.5 °C and visually examined after 12, 24 and 48 hours. Plates with swarming growth, indicative of *Salmonella* spp., were sub-cultured onto Xylose–Lysine–Desoxycholate agar (XLD), and either Brilliant Green Agar (BGA) or Cystine Lactose Electrolyte Deficient agar (CLED) in duplicate. All plates were incubated for 24 hours at 37°C. Suspect *Salmonella* colonies were confirmed biochemically in triplicate using Triple Sugar Iron agar (TSI) and Lysine Iron agar (LIA), incubated for 24 hours at 37°C.

### 2. *Salmonella* Isolate Confirmation and Serotyping

*Salmonella* isolates were confirmed positive and differentiated as *Salmonella* Typhimurium, *Salmonella* Infantis or other *Salmonella enterica* subspecies by real time PCR in accordance with previously published methods [460 - 462].



Supplementary Table 5–41. Explanatory Variable Descriptions

| *Variable* | *Variable Description (number or levels)* |
|---|---|
| Shed | Unique shed identifier (n=4) |
| Sample Type | Sample type used for sampling (1 = Boot Swab; 2 = Dust; 3 = Manure Belt; 4 = Egg Belt) |
| Location | Unique identifier for each sampling location (n = 28) |
| Aspect | Sample site on the northern or southern aspect of the shed (1 = North; 0 = South) |
| Betweenness | Sample site location is on the long aspect of the shed between two sheds (1 = Yes; 0 = No) |
| Month | Month of sampling (1–12; 1 = Jan 12 = Dec) |
| Event | Sampling event number (1–15) |
| Shed Status | Shed *Salmonella* status prior to flock placement post cleaning (1 = Positive; 0 = Negative) |
| Rain | Mean rainfall (mm) the week of sampling |
| Prev3Rain | Mean rainfall (mm) in the 3 weeks prior to sampling |
| 3wkTotRain | Total rainfall (mm) in the 3 weeks prior to sampling |
| MaxTemp | Mean maximum temperature (°C) in the week of sampling |
| Prev3MaxTemp | Mean maximum temperature (°C) in the 3 weeks prior to sampling |
| MinTemp | Mean minimum temperature (°C) in the week of sampling |
| Prev3MinTemp | Mean minimum temperature (°C) in the 3 weeks prior to sampling |
| SolarRad | Mean solar exposure (MJ/m$^2$) the week of sampling |
| Prev3SolRad | Mean solar exposure (MJ/m$^2$) the 3 weeks prior to sampling |
| 3wkTSolRad | Total solar exposure (MJ/m$^2$) in the 3 weeks prior to sampling |

Supplementary Table 5–40. Hierarchical Structure of the Study Data

| Level | Number | Number at next highest level | |
|---|---|---|---|
| | | *Mean* | *Range* |
| Shed (highest level) | 4 | - | - |
| Sampling Event | 52 | 13 | - |
| Samples | 1, 458 | 27.5 | 26 - 28 |

Supplementary Table 5–42. Number of Samples Required for each Sampling Unit at the given Design Prevalence and Test Sensitivity (Fixed Test Specificity 0.995).

| *Test Sensitivity* | *0.88* | | | *0.98* | | |
|---|---|---|---|---|---|---|
| *Design Prevalence* | *0.01* | *0.05* | *0.10* | *0.01* | *0.05* | *0.10* |
| Birds | –[1] | 169 | 63 | _ | 131 | 57 |
| Cage /Environment | 28 | 94 | 169 | 24 | 83 | 149 |

[1]Sample size is unable to be calculated because the false positive rate (1 - sensitivity) is higher than the design prevalence x sensitivity

Supplementary Table 5–43. Positive Sampling Events for Each Shed by Sample Type

| *Sample Type* | *Salmonella spp. positive sampling events (%)* | | | |
|---|---|---|---|---|
| | *A* | *B* | *C* | *D* |
| Boot Swab | 13/13 (100) | 5/13 (39) | 14/14 (100) | 7/15 (47) |
| Dust | 13/13 (100) | 3/13 (23) | 14/14 (100) | 6/15 (40) |
| Manure Belt | 13/13 (100) | 4/13 (31) | 11/14 (79) | 7/15 (47) |
| Egg Belt | 9/13 (69) | 1/13 (8) | 6/14 (43) | 1/15 (7) |
| Sample Positive Events[1] | 13/13 (100) | 9/13 (69) | 14/14 (100) | 11/15 (73) |

[1]Number of sampling events where at least one sample type was positive for *Salmonella* spp. and the flock status would be regarded as positive

Supplementary Table 5–44. Mean Seasonal Rain and Temperature for each Weather Variable (Standard Deviation)

| *Season* | *Rain (mm)* | *Rain Prev3 (mm)* | *Min Temp (°C)* | *Min Temp Prev3 (°C)* | *Max Temp (°C)* | *Max Temp Prev3 (°C)* | *Solar Rad (MJ/m²)* | *Sol Rad Prev3 (MJ/m²)* |
|---|---|---|---|---|---|---|---|---|
| Spring | 0.60[a] (1.04) | 0.95[a] (0.34) | 5.90[a] (3.30) | 6.62[a] (1.14) | 21.77[a] (4.42) | 20.42[a] (1.58) | 23.61[a] (6.33) | 17.47[a] (3.05) |
| Summer | 0.50[a, b] (0.87) | 0.28[b] (0.22) | 9.95[b] (4.37) | 13.45[b] (2.39) | 27.56[b] (4.60) | 30.49[b] (3.52) | 28.94[b] (8.97) | 26.11[b] (2.35) |
| Autumn | 0.40[b] (0.76) | 0.95[a, c] (1.23) | 10.30[a, c] (3.66) | 11.65[c] (2.94) | 21.84[a, c] (4.35) | 25.22[c] (4.33) | 16.50[c] (5.89) | 15.79[c] (4.70) |
| Winter | 2.00[c] (2.01) | 1.78[d] (0.66) | 7.95[d] (1.36) | 4.80[d] (0.80) | 21.95[d] (2.67) | 14.03[d] (1.38) | 10.40[d] (2.38) | 7.97[d] (1.49) |

Variables with different superscripts are significantly different from each other ($P < 0.05$)

Supplementary Table 5–45. Odds Ratio (95% Confidence Interval) for each Sampling Location by *Salmonella* Serovar

| *Location* | *Sample Type* | *Odds Ratio (95% Confidence Interval)* | | |
|---|---|---|---|---|
| | | *Salmonella spp.*[A] | *S. Typhimurium* | *S. Infantis* |
| 17 | Egg Belt | 1 | 1 | 1 |
| 18 | Dust | 3.26 (1.28 – 9.17)[2] | 1.33 (0.28 – 7.05) | 3.86 (1.26 – 14.55)[2] |
| 19 | Dust | 3.00 (1.17 – 8.46)[2] | 2.89 (0.78 – 13.80) | 2.13 (0.62 – 8.40) |
| 20 | Egg Belt | 0.38 (0.08 – 1.45) | 0.63 (0.08 – 3.95) | 0.23 (0.01 – 1.59) |
| 21 | Egg Belt | 0.39 (0.08 – 1.48) | 0.64 (0.08 – 4.02) | 0.00 |
| 22 | Dust | 3.00 (1.16 – 8.46)[2] | 1.70 (0.39 – 8.64) | 2.77 (0.86 – 10.69) |
| 23 | Egg Belt | 0.67 (0.19 – 2.25) | 0.98 (0.17 – 5.51) | 0.23 (0.01 – 1.63) |
| 24 | Dust | 3.84 (1.52 – 10.71)[3] | 2.48 (0.65 – 12.01) | 3.49 (1.22 – 13.02)[2] |
| 25 | Egg Belt | 0.98 (0.31 – 3.07) | 0.98 (0.17 – 5.51) | 0.98 (0.22 – 4.35) |
| 26 | Dust | 3.27 (1.28 – 9.17) | 2.08 (0.52 – 0.29) | 3.49 (1.12 –13.20)[2] |
| 27 | Dust | 2.07 (0.78 – 5.99) | 4.25 (1.24 – 19.67)[2] | 0.47 (0.06 – 2.53) |
| 28 | Egg Belt | 0.54 (0.13 – 1.89) | 1.36 (0.29 – 7.19) | 0.00 |
| 29 | Dust | 2.29 (0.87 – 6.56) | 5.26 (1.57 – 24.06)[2] | 0.23 (0.01 –1.63) |
| 30 | Egg Belt | 0.53 (0.13 – 1.86) | 0.98 (0.17 – 5.51) | 0.23 (0.01 – 1.63) |
| 31 | Dust | 1.53 (0.54 – 4.53) | 3.86 (1.10 – 18.04)[1] | 0.00 |
| 32 | Egg Belt | 0.82 (0.25 – 2.65) | 2.08 (0.52 – 10.29) | 0.00 |
| 50 | Manure Belt | 3.27 (1.28 – 9.17)[2] | 3.78 (1.08 – 17.63)[1] | 2.13 (0.62 – 8.40) |
| 53 | Manure Belt | 3.54 (1.39 – 9.92)[2] | 3.78 (1.08 – 17.63)[1] | 2.44 (0.74 – 9.52) |
| 56 | Manure Belt | 2.29 (0.87 – 6.56) | 2.89 (0.78 – 13.80) | 1.53 (0.41 – 6.29) |
| 59 | Manure Belt | 1.31 (0.45 – 3.96) | 2.08 (0.52 – 10.29) | 0.72 (0.13 – 3.43) |
| 62 | Manure Belt | 3.84 (1.52 – 10.71)[3] | 2.08 (0.52 – 10.29) | 3.87 (1.26 – 14.55)[2] |
| 65 | Manure Belt | 1.68 (0.61 – 4.93) | 2.89 (0.78 – 13.80) | 0.47 (0.06 – 2.52) |
| 68 | Manure Belt | 4.15 (1.65 – 11.55)[3] | 8.27 (2.57 – 37.17)[3] | 0.47 (0.06 – 2.52) |
| 71 | Manure Belt | 2.29 (0.87 – 6.56) | 4.25 (1.23 – 19.67)[2] | 0.47 (0.06 – 2.52) |
| 73 | Boot Swab | 4.82 (1.93 – 13.41)[3] | 1.33 (0.28 – 7.05) | 6.08 (2.07 – 22.40)[3] |
| 74 | Boot Swab | 4.82 (1.93 – 13.41)[3] | 2.08 (0.52 – 10.29) | 5.59 (1.89 – 20.66)[3] |
| 76 | Boot Swab | 3.84 1.63 – 10.71)[3] | 2.89 (0.78 – 13.80) | 3.12 (0.98 – 11.19)[1] |
| 77 | Boot Swab | 2.75 (1.06 – 7.79)[2] | 2.08 (0.52 – 10.29) | 2.78 (0.86 – 10.70) |

[A] Full results for Table 5–37, [1] $P < 0.05$, [2] $P < 0.01$, [3] $P < 0.001$

Supplementary Table 5–46. Sampling Event Odds Ratio and 95% Confidence Intervals (Full results for Table 5–38)

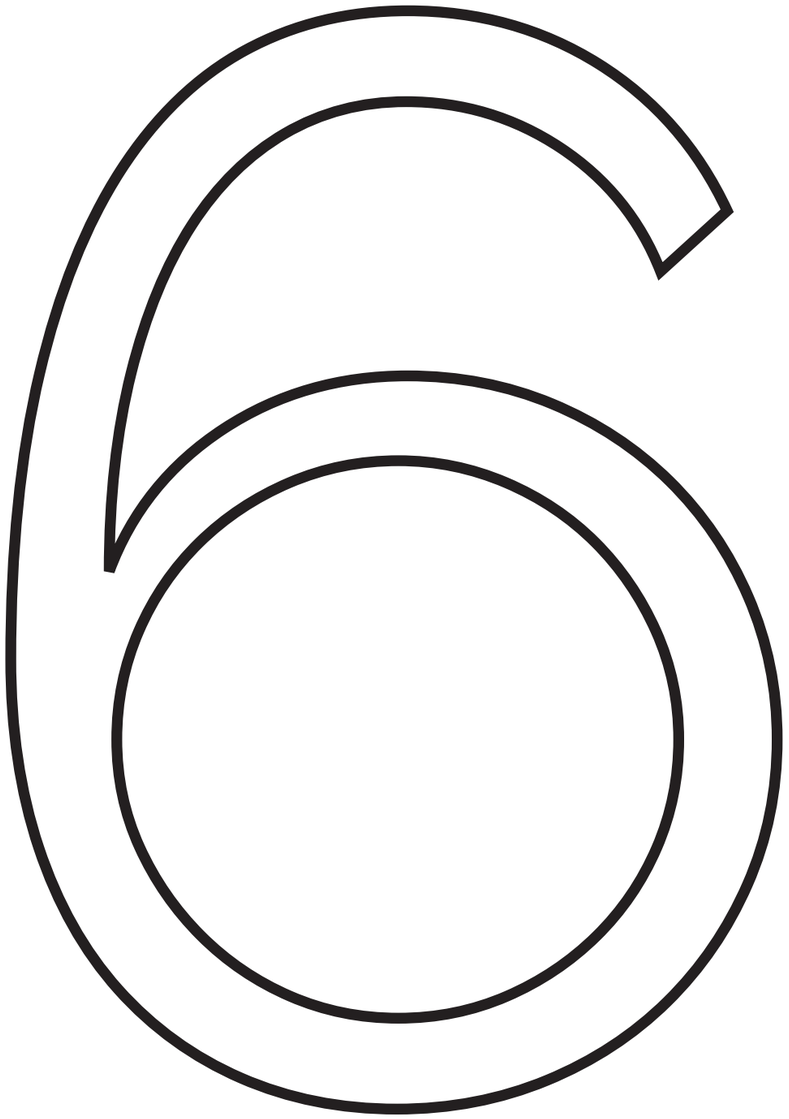
| *Sample Event Number* | *OR* | *95%CI* | $\chi^2$ | *DF* | *P value* |
|---|---|---|---|---|---|
| 1 | 1 | | 62.5[1] | 12 | $8.0 \times 10^{-9}$ |
| 2 | 0.77 | 0.51 – 1.09 | | | 0.339 |
| 3 | 0.65 | 0.45 – 1.31 | | | 0.120 |
| 4 | 0.38 | 0.37 – 0.39 | | | 0.001 |
| 5 | 0.58 | 0.21 – 0.95 | | | 0.053 |
| 6 | 0.71 | 0.33 – 1.09 | | | 0.217 |
| 7 | 0.46 | 0.41 – 0.51 | | | 0.008 |
| 8 | 0.40 | 0.26 – 0.81 | | | 0.002 |
| 9 | 0.36 | 0.22 – 0.71 | | | 0.001 |
| 10 | 0.24 | 0.19 – 0.65 | | | < 0.001 |
| 11 | 0.17 | 0.12 – 0.44 | | | < 0.001 |
| 12 | 0.24 | 0.12 – 0.44 | | | < 0.001 |
| 13 | 0.25 | 0.13 – 0.47 | | | < 0.001 |

Supplementary Table 5–47. Odds Ratio of a Sample being Positive for each Weather Variable by *Salmonella* serovar

| *Variable* | *S. Typhimurium* | | *S. Infantis* | |
|---|---|---|---|---|
| | *OR* | $\chi^2$ *(P)* | *OR* | $\chi^2$ *(P)* |
| Rain | 1.36 (1.20 – 1.54) | 8.5 (0.004) | 0.87 (0.73 – 1.02) | 2.8 (0.093) |
| Prev3Rain | 1.32 (1.13 – 1.55) | 12.0 (0.005) | 0.71 (0.56 – 0.87) | 10.2 (0.001) |
| MinTemp | 0.99 (0.96 – 1.03) | 0.04 (0.83) | 1.01 (0.97 – 1.05) | 0.31 (0.58) |
| Prev3MinTemp | 0.85 (0.81 – 0.89) | 52.3 (0.0001) | 1.08 (1.04 – 1.13) | 14.3 (0.0001) |
| MaxTemp | 0.98 (0.96 – 1.02) | 0.59 (0.44) | 1.04 (1.01 – 1.07) | 8.5 (0.004) |
| Prev3MaxTemp | 0.90 (0.88 – 0.93) | 56.5 (0.0001) | 1.06 (1.04 – 1.09) | 22.2 (0.0001) |
| SolRad | 0.97 (0.96 – 0.99) | 10.7 (0.001) | 1.05 (1.03 – 1.07) | 22.1 (0.0001) |
| Prev3SolRad | 0.94 (0.92 – 0.96) | 27.4 (0.0001) | 1.07 (1.04 – 1.10) | 27.2 (0.0001) |

Supplementary Table 5–48. Non-significant Weather Variables for *Salmonella* Detection

| *Variable* | *OR* | *95%CI* | $\chi^2$ | *DF* | *P value* |
|---|---|---|---|---|---|
| Prev3Rain | 1.04 | 0.91-1.18 | 0.40 | 1 | 0.53 |
| SolarRad | 1.00 | 0.99 -1.01 | 0.04 | 1 | 0.84 |
| Prev3SolarRad | 0.99 | 0.97-1.00 | 1.5 | 1 | 0.21 |
| MaxTemp | 1.02 | 0.99-1.04 | 2.6 | 1 | 0.11 |
| MinTemp | 1.00 | 0.97-1.03 | 0.1 | 1 | 0.75 |



CHAPTER **SIX**

# Diversity Analysis of *Salmonella* spp. Isolates by Location

*(next spread)*
*The method used to differentiate Salmonella serovars (phenotype or genotype) is critical for describing the population diversity and potentially ascribing a source or pathway of transmission.*



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## Abstract

Ecological measures of microbial population diversity confirmed that the *Salmonella enterica* diversity and intra-serovar diversity of *Salmonella* Typhimurium assemblage varied by typing method. *Salmonella enterica* diversity was highest at the broiler location. The *Salmonella* Typhimurium diversity varied according to the typing method (phage type, MLVA profile or PT/MLVA combination) and while no significant difference in the intra-serovar composition could be detected between locations, the relationships between locations differed depending on the typing method used. These results indicate that the current methods used for intra-serovar differentiation (phenotype or genotype), were not sufficient for ascribing a source or pathway of transmission in a well sampled population (n = 1,468) in the absence of epidemiological information. The high level of intra-serovar differentiation observed may be a consequence of naturally occurring random variation or phenotypic plasticity in the sampled population. The use of more specific tools for genotyping the *Salmonella* Typhimurium isolates (such as whole genome sequencing) is indicated.


## 1. Introduction

This chapter explores the use of traditional ecological measures of microbial population diversity for the *Salmonella enterica* and *Salmonella* Typhimurium variants identified during this study. This study uses terms borrowed from the field of ecology (Table 6–49) and focuses on *Salmonella* intra-species diversity or within serovar *S.* Typhimurium assemblage within the sampling locations as defined by traditional phenotyping (serotype, phage type, antibiogram) and newer genotyping methods (MLVA). The phenotypic and genotypic diversity (MLVA) of the *Salmonella* Typhimurium isolates are explored in Chapter 4. and the genomic diversity identified by whole genome sequencing is explored in Chapter 7. A key aim was to identify differences in the *Salmonella* Typhimurium assemblage structure between sampling locations within the production system.

As indicated in previous chapters spatial, temporal and phenotypic heterogeneity of *Salmonella* was identified both within and between sampling locations. This was not surprising, as it would be reasonable to expect that any taxa, animal, plant or otherwise, is not randomly or homogeneously distributed within a natural environment [554]. *Salmonella* are diverse, may grow both in and out of an animal host, and possess many genotypic and phenotypic traits enabling their differential survival and persistence in both animal and environmental niches [39, 54]. Ecological measures of diversity are used to describe population variation and distribution within or between environments [555]. Population characteristics may be described at many spatial and taxonomic scales and typically involves the count of species: counts of individual species or the number of individuals within species. The application of ecological theory to microbiological populations is still evolving and the use of ecological theory was cited as a potential area where epidemiology could be further developed [556]. Microbial diversity refers to one of three levels: within species diversity (phenotypic or genetic), species diversity (number of) and community (ecological) diversity. This diversity may be measured in terms of richness; the number of species or taxonomic units present, or evenness; the distribution of individuals within the species or taxonomic unit [557].

The application of traditional ecological diversity measures to microbial populations have been reviewed and their limitations described [554, 556-559]. Some unique features of microbial populations should be noted; the ability of microorganisms to remain inactive or dormant for prolonged periods of time, the presence of significant phenotypic plasticity in some species and their rapid population expansion and evolution [554]. Ecological measures of population or community diversity may be validly applied below species criteria under specific conditions [560] and in some cases bacterial communities may behave similarly to animal and plant communities [561].



Table 6–49. Ecological Terms Used in this Chapter: Definitions

| *Term* | *Definition* | *Reference(s)* |
|---|---|---|
| Community | Group of *Salmonella enterica* that co-occur in space and time (synonym for location) | [555] |
| Assemblage | Defined group (Phylogenetic, phenotypic or genotypic) of *Salmonella enterica* serovars or strain variants that co-occur in space and time | [562] |
| Species | Synonym for higher level taxonomic unit measures of *Salmonella enterica* subspecies, serovar or intra-serovar. Use of the term species has been maintained for consistency with the literature but is use is synonymous with the above. | [557, 560, 561] |
| Richness | Number of taxonomic units in a community. Measures include Richness or Chao diversity | [562] |
| Rarefaction | Number of taxonomic units in a community standardised to the number of individuals sampled | [562] |
| Evenness | A measure of the similarity of the taxonomic unit abundance in a community. Measures include Shannon Evenness, Simpson Evenness, Hill Ratio | [555] |
| Diversity | Function of richness and evenness. Less diverse communities are typically less even than richness would indicate. Measures include Shannon Diversity, Simpsons Diversity and Hills Diversity numbers | [555] |
| Structure | Number of taxonomic units in. a community and their relative abundance | [554] |
| Alpha Diversity | Diversity observed within a location | [555] |
| Beta Diversity | Change in diversity between locations | [555, 563] |

## 2. Aim/Purpose

1. Use the *Salmonella enterica* and *Salmonella* Typhimurium phenotyping (serotype, phage type, antibiogram) and genotyping (MLVA) results to describe the microbiological diversity, intra-species richness and structure, of *Salmonella enterica* community and *S.* Typhimurium assemblage within each sampling location (α–diversity).
2. Examine the ß–diversity between the sampling locations (breeder, broiler, hatchery, processing) and identify differences in the *Salmonella enterica* assemblage structure between sampling locations within the production system.

The null hypotheses investigated was that there were no differences in the composition of *Salmonella* Typhimurium assemblages at each location (breeder, hatchery, processing)

## 3. Materials and Methods

### 3.1 Bacterial isolates

A total of 1468 fully typed *Salmonella* enterica isolates by phenotyping (serotype, phage type, antibiogram) and genotyping (MLVA) from four different population locations (Parent, Hatchery, Broiler and Processing) were used for diversity comparison. The methods used for phenotyping and genotyping are as described in Chapter 2.

### 3.2 Taxonomic Units of Interest

Data were aggregated for all sampling events for each sampling location (Parent, Hatchery, Broiler and Processing). For this analysis, it was assumed that the taxonomic distance between or within *Salmonella* Typhimurium phage types and MLVA profiles, have a similar taxonomic distance from each other. As specific variants were most abundant, this higher level taxon identification (phage type and MLVA profile) was utilized for assessing community variation, assuming the variation between and within groups was similar [72, 555-557]. It has been demonstrated that higher taxon surrogates (subspecies, serovar or intra-serovar) may perform best in communities with a few common "species" [551].

Diversity measures were calculated for the seven typing methods commonly used to differentiate *Salmonella* isolates;

**Group A.** All *Salmonella enterica* serovars (taxonomic unit: subspecies and serovar) serotyping only (ST), serotyping plus phage typing (STPT) and;

**Group B.** *Salmonella* Typhimurium (taxonomic unit: intra-serovar), phage typing (PT), MLVA profile (MLVA), antibiogram (Ab), phage typing plus MLVA profile (PTMLVA), phage typing and antibiogram (PTAb). PTMLVA was used to categorise phage type specific MLVA profiles as a proxy of distinct genotypes within a phage

These typing methods were compared as each is used by industry and public health laboratories as a method for the differentiation of *Salmonella enterica* strains or isolates during epidemiological studies. Regarding *Salmonella* Typhimurium specifically these typing methods are utilized to further differentiate between isolates to assist epidemiological investigation as this differentiation is considered to aid identification of sources of *Salmonella*. However, it is unknown how effective these methods truly are within the bacterial population under study.

### 3.3 Assemblage Population Distribution

To assess the validity of using intra-species (in this case serovar and intra-serovar) typing methods as the taxonomic unit for microbial population diversity analysis the population distribution for each of the typing methods was assessed. The distribution was estimated using the abundance curve calculator [567] based on calculations described by Magurran and McGill (2011).

### 3.4 Measures of Diversity

#### 3.4.1 Salmonella Richness and Diversity

Alpha diversity (α-diversity) indices of species richness (S) were estimated for each location and then rarefied to account for the effect of sample size. Species richness is dependent on sample size, thus comparisons may not be valid if sample sizes are different within each community. The rarefied value is the expected number of variants that might be detected from a smaller number of samples drawn at random from the larger sample [555]. Confidence intervals (95%) were calculated for each value. Rarefaction curves can be utilised to evaluate sampling adequacy for detecting population variation. When a rarefaction curve reaches a horizontal asymptote, sufficient samples have been collected from a site to detect all the possible variants within the population. Abundance measures were calculated using Simpsons index ($D_S$) and the Shannon-Wiener index of diversity (H′), Shannon's index of diversity (H′) is a non-parametric measure of heterogeneity combining both evenness and richness in a single measure, but is sensitive to sample size. Simpsons index ($D_S$) is a measure of evenness within a sample or community, and measures the probability that any two individuals drawn at random from an infinitely large community belong to the same group [555].

#### 3.4.2 Community Similarity

Beta diversity (β–diversity), indices of community similarity, was assessed using dissimilarity analysis to explore the variability in community composition. The Rényi similarity index was calculated for each assemblage and the Rényi diversity profile plotted for each. The community diversity and the distance between communities was estimated using the binomial index, Raup-Crick (RC) dissimilarity index and Chao index [555]. The binomial index is based on likelihood theory, and tests whether communities differ in their composition and relative abundance of species [568]. The Raup-Crick dissimilarity index is less sensitive to differences in species richness among sites than other dissimilarity metrics as it uses the total species pool of each dataset (γ-diversity) to calculate diversity. RC indices range from -1 to +1, ranges from -1 to < 0 or > 0 to +1, indicate locations are more dissimilar than expected by chance [563]. A value of 0, indicates that there is no difference in the observed dissimilarity from the null value. The Chao index is a probabilistic index based on presence absence data taking into account the effect of unseen shared species [519]. All diversity analyses were conducted in the R statistical package [484] using the "vegan" package [509] and "Biodiversity.R" [510].

## 4. Statistical Methods

### 4.1 Cluster Analysis

To evaluate the null hypothesis, that there was no difference in the composition of *S.* Typhimurium assemblages between locations, cluster analysis was conducted for each assemblage. *S.* Typhimurium typing matrices for each site were transformed using the Hellinger distance and species profile to maintain meaningful measures of ecological distance between sites [520]. Transformation was conducted to minimize the effect of abundance of the presence of one species variant over another due to differences in sample size at each sampling location, while ensuring that the effect of either plentiful or rare variants was not over represented in the resulting analysis, but rare variants were not ignored in the analysis.

Clustering was performed using the Agnes method for agglomerative nesting [569]. The Agnes clustering coefficient was used to assess the significance of the clustering result, the higher the clustering coefficient the stronger the clustering present in the dataset. The output of the hierarchical clustering was viewed as a dendrogram. Results were considered robust if both the species profile distance and Hellinger distance resulted in the same hierarchical clustering. The cophenetic distance (the distance between each cluster) for each dendrogram was measured. The cophenetic distance measures the distance between two observations that have been clustered and measures the intergroup dissimilarity. The correlation between the original site Hellinger distance and the cophenetic distance was assessed using the Mantel test of significance with 1000 permutations. The Mantel test is used to measure whether the distance calculated between clusters is the same as the original distance measures. If the results are significantly highly correlated then the agglomerative clustering is a good representation of the distance between sites in the original data set [570].

### 4.2 Principal Component Analysis

Unconstrained principal component analysis (PCA) was used to further compare site species composition. Hellinger distances computed for the clustering analysis was used for PCA. Goodness of fit measures were cal-

Table 6–50. *Salmonella* Assemblage Population Distribution Pattern for each Location

| Group | Assemblage | Breeder | Hatchery | Broiler | Processing |
|---|---|---|---|---|---|
| A | ST | Log | Log Normal | Geometric | Log Normal |
| | | | | Log Normal | |
| | STPT | Geometric | Log Normal | –[1] | Log Normal |
| | | Log Normal | | | |
| B | PT | Log Normal | Log Normal | – | Log Normal |
| | MLVA | Log Normal | Log Normal | – | Log Normal |
| | Ab | Log Normal | Log Normal | Log Normal | Log Normal |
| | PTMLVA | Geometric | Geometric | – | Log Normal |
| | | Log Normal | Log Normal | | |
| | PTAb | Geometric | Log Normal | Log Normal | Log Normal |
| | | Log Normal | | | |

[1]– No samples typed using this method from this location

culated for each site; the percentage of total variance explained for each site in the ordination graph. The significance of each principal component (PC) for each variant analysis was compared using the broken stick distribution. Significant components have larger variation than the corresponding broken stick variation for that PC. Species significantly contributing to the ordination were evaluated using the equilibrium circle plotted over the plotted ordination graph. Values lying outside the equilibrium circle significantly contribute to ordination [520].

### 4.3 Sample Size Estimation for Between Location Comparisons

To ensure the sample size at each location was sufficient to detect a difference between two locations with 95% confidence and 80% power the *S.* Typhimurium MLVA profile (assemblage variant with the highest variation) variation between and within sites was estimated. This variation was used to estimate the sample size required at each location to detect a true difference between locations using an anova power calculation for multiple groups [521].

## 5. Results

### 5.1 Assemblage Population Distribution

All typing assemblages conform to a log normal or geometric population distribution pattern as indicated (Table 6–50). These population distributions are typical for species population distributions so the application of species diversity measures for investigating population or community diversity within each assemblage and location is appropriate.

### 5.2 Alpha–Diversity

#### 5.2.1 Salmonella Species Richness

*Salmonella enterica* richness and the rarefied values for each assemblage at each sampling site are presented in Table 6–51. When using the different phenotypic and genetic typing methods for comparing the site richness, species richness varied by typing method or combination of typing methods. For example, *Salmonella enterica* richness by serotyping was highest at the broiler sites, whereas when serotype and phage type were considered the breeder site had the highest species richness. When considering the *S.* Typhimurium assemblages PT had the least amount of richness between sites, versus PTAb demonstrating the highest richness. Rarefaction of each assemblage improved the consistency of the species richness between the sites, with the *S.* Typhimurium assemblages all having a higher richness at the hatchery. The *Salmonella enterica* assemblages (Group A) richness values did not vary significantly with the non-rarefied values as all confidence intervals generated included the original richness value. In contrast the species richness values for Group B (*S.* Typhimurium assemblages) were substantially smaller than the raw site richness for MLVA, Ab, PTMLVA and PTAb, indicating that sample size is likely to have substantial influence on the variant richness detected at these sites.

Table 6–51. Group Richness for Each Assemblage by Location (95% Confidence Intervals)

| *Group* | *Assemblage* | | *Breeder* | *Hatchery* | *Broiler* |
|---|---|---|---|---|---|
| Richness (S) | | N | | | |
| A | ST | 1468 | 3 | 4 | 6 |
| | STPT | 1468 | 11 | 7 | 10 |
| B | PT | 421 | 7 | 6 | – |
| | MLVA | 419 | 22 | 16 | – |
| | PTMLVA | 419 | 28 | 30 | – |
| | Ab | 375 | 13 | 13 | 7 |
| | PTAb | 375 | 26 | 42 | 8 |
| *Rarefied (S)* | | $N_{min}$ | | | |
| A | ST | 137 | 2.32 (1.41 - 3.27) | 3.37 (2.18 - 4.54) | 5.22 (4.14 - 6.30) |
| | STPT | 137 | 8.05 (5.79 - 10.31) | 5.49 (4.03 - 6.95) | 8.62 (6.79 - 10.45) |
| B | PT | 35 | 5.61 (3.93 - 7.28) | 5.81 (5.00 - 6.61) | – |
| | MLVA | 61 | 11.83 (8.19 - 15.47) | 11.94 (9.30 - 14.59) | – |
| | PTMLVA | 61 | 14.8 (10.8 - 18.9) | 20.0 (16.2 - 23.8) | – |
| | Ab | 35 | 7.5 (5.3 - 9.7) | 8.9 (6.6 - 11.4) | 6.9 (6.5 - 7.4) |
| | PTAb | 35 | 11.16 (7.67 - 14.65) | 20.39 (16.34 - 24.44) | 7.89 (7.27 - 8.50) |



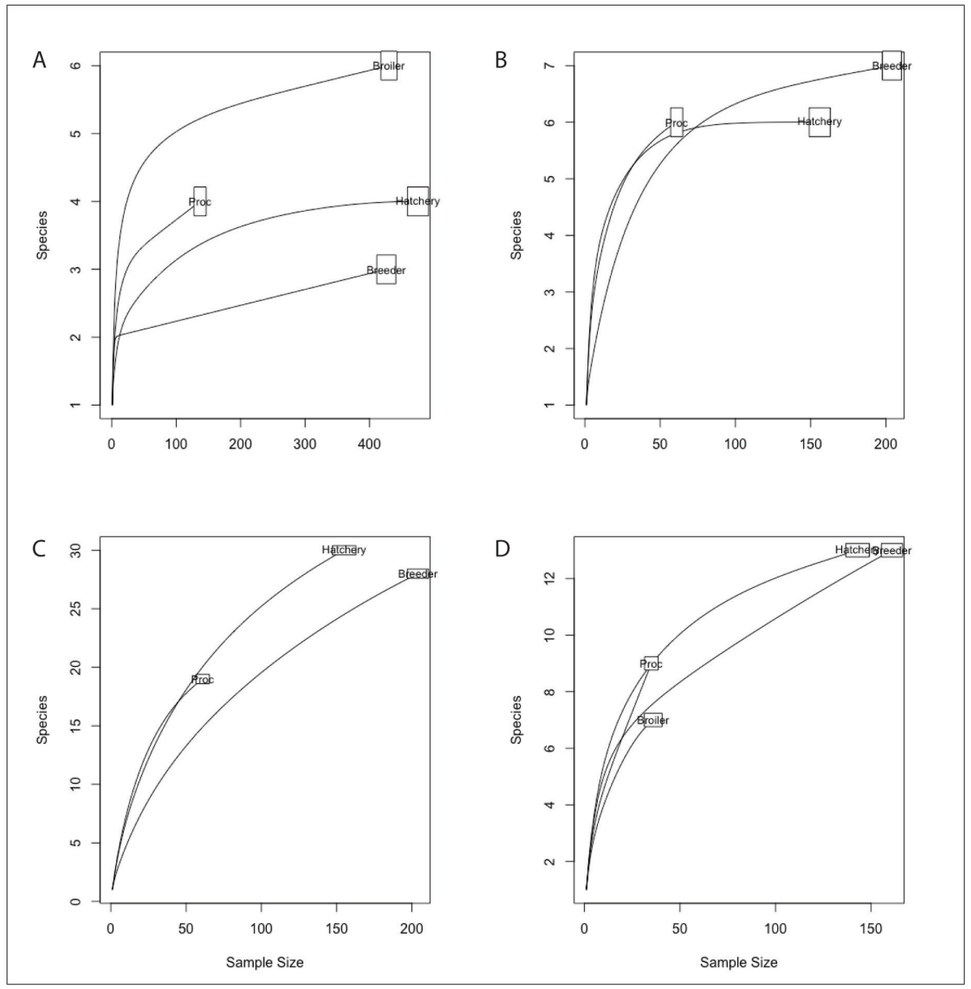



Fig. 6–26. Rarefaction Curves for Each Group Assemblage at Parent, Hatchery and Broiler and Processing Sites

**A.** *Salmonella* spp. serovar **B.** *S.* Typhimurium PT **C.** *S.* Typhimurium PTMLVA **D.** *S.* Typhimurium Antibiogram

#### 5.2.2 Rarefaction Curves

Rarefaction curves were produced for each of the assemblages. Four of these curves are illustrated in Fig. 6–21. A horizontal asymptote was reached for *Salmonella enterica* serotyping Fig. 6–21A (at ~200 samples) and *S.* Typhimurium phage typing Fig. 6–21B (at ~90 samples) at the hatchery site only. Despite more than 400 isolates identified at either the breeder and broiler locations, rarefaction indicated that this was insufficient samples to detect all the possible *Salmonella enterica* serotypes present at these sites, whereas at the hatchery site this was sufficient. For the remaining sites and typing methods (Ab, MLVA, PTMLVA or PTAb) the sample size was not sufficiently large for a horizontal asymptote to be reached, indicating again that all the population variation had not been detected at these locations using these methods

of differentiation.

### 5.2.3 Species Abundance and Homogeneity

Both the Shannon-Weiner and Simpson's indices were calculated for each assemblage and location. Both indices were chosen because their diversity measures either account for, or ignore the importance of rare variants in the community (Table 6–52). As with species richness the measure of diversity varied between locations depending on the typing method.

Group A assemblages varied in species abundance and homogeneity from location to location and the order of relationship between the locations varied between typing methods. Serotype diversity ($D_S$) decreased from Hatchery > Processing > Breeder > Broiler, whereas STPT diversity decreased from Breeder > Hatchery > Broiler > Processing. Group B assemblage diversity ($D_S$) also varied by typing method for each location. The heterogeneity (H') of Group A and Group B assemblages also varied by typing method and location. Homogeneity varied across locations by the assemblage. Serotyping and phage typing were the most homogeneous across all locations (lowest values) whereas heterogeneity increased when other typing methods were applied. For Group B, the order of increasing heterogeneity was PT < MLVA < Ab < PTMLVA < PTAb. The results indicate that the method used to describe the isolates (phenotype or genotype) is critical for describing the population diversity and potentially ascribing a source or pathway of transmission.

### 5.2.4 Rényi Diversity Profiles

A comparison of the diversity between locations was visualized using Rényi diversity profiles for each assemblage to rank the locations from highest to lowest diversity. The advantage of this method is that a comparison of the relative diversity between locations for each assemblage is possible. The diversity of one location can only be considered greater than the other locations when the Rényi diversity values are higher at all estimates in the series for that location and an example of this is illustrated in Fig. 6–22. Evenness in assemblage composition is indicated by a flat horizontal line in the Rényi diversity accumulation plot. As with the individual measures of diversity at each location the Rényi diversity series varied by assemblage and interpretation of site *Salmonella enterica* or *S.* Typhimurium diversity was again dependent on the typing method. No assemblage had an even distribution across the Rényi profile indicating uneven species composition at all sites regardless of the assemblage. Ab was the only typing method where ranking of diversity between each of the sampling locations was demonstrable (Hatchery > Breeder > Processing > Broiler). All other assemblages were unable to be ranked.

Table 6–52. *Salmonella* and *S.* Typhimurium Variant Assemblage, Abundance and Homogeneity for each Location

| *Group* | *Assemblage* | *Breeder* | *Hatchery* | *Broiler* | *Processing* |
|---|---|---|---|---|---|
| Simpsons Index ($D_S$) | | | | | |
| A | ST | 0.50 | 0.26 | 0.62 | 0.44 |
| | STPT | 0.58 | 0.62 | 0.66 | 0.81 |
| B | PT | 0.31 | 0.72 | – | 0.66 |
| | MLVA | 0.56 | 0.79 | – | 0.89 |
| | PTMLVA | 0.65 | 0.88 | – | 0.89 |
| | Ab | 0.81 | 0.82 | 0.65 | 0.73 |
| | PTAb | 0.85 | 0.95 | 0.65 | 0.78 |
| *Shannon-Wiener (H')* | | | | | |
| A | ST | 0.71 | 0.48 | 1.17 | 0.78 |
| | STPT | 1.11 | 1.47 | 1.18 | 1.83 |
| B | PT | 0.74 | 1.43 | – | 1.29 |
| | MLVA | 1.52 | 1.96 | – | 2.39 |
| | PTMLVA | 1.87 | 2.67 | – | 2.61 |
| | Ab | 1.86 | 2.00 | 1.36 | 1.61 |
| | PTAb | 2.31 | 3.32 | 1.40 | 1.98 |



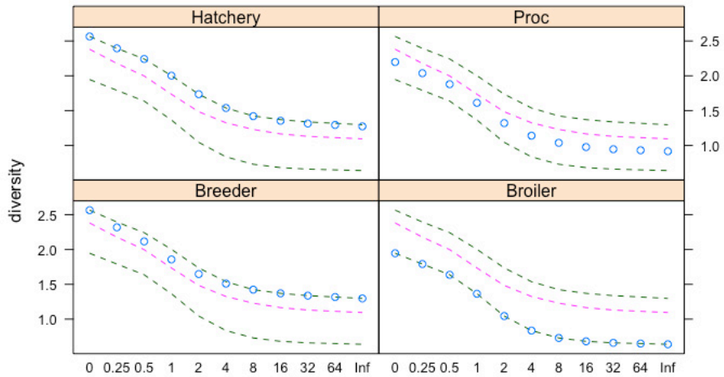



Fig. 6–27. Rényi Diversity Profile Plots for *S.* Typhimurium Assemblage Ab

For all panels the dotted lines indicate the median (pink), lower and upper bounds (blue) of the diversity profile for all locations in the comparison. The Rényi values in the series are presented as open circles within the location panel. A location is considered more diverse if all the Rényi values in the series for that location are greater than those at another site. In this example, the Ab diversity was greater at all series values Hatchery > Breeder> Processing > Broiler.

## 5.3 Beta-diversity

ß–diversity between sampling locations was only conducted for the Group B, *S.* Typhimurium assemblages. Investigations focused on clustering and principal component analysis.

### 5.3.1 Cluster Analysis

Analysis was conducted on both raw species profiles and "Hellinger" transformed species profiles. Hierarchical clustering was based on the distance matrix: the distance between two locations in species composition.

Zero distance between locations indicates all the same species are shared, and if this distance is shared then they vary by the same species and abundance from the other location. The Agnes Coefficient measures the strength of the clustering structure obtained by the group average linkage; the smaller the co-efficient the weaker the clustering. The only assemblage that demonstrated moderately significant clustering between sampling locations was Ab (Mantel r 0.862, P = 0.083). No other assemblages were significantly clustered (Table 6–53). At best, the remaining agglomerative clustering results describe the output but not the true species variation between sites. The distance matrix and dendrogram between locations for the Ab assemblage are illustrated (Table 6–54, Fig. 6–23). The distance matrix and associated dendrogram are interpreted as the breeder and hatchery sites are equidistant from each other, contain the same variant composition and abundance, but are equidistant from the processing plant which is distant from the broiler site. These distances are small, yet significant.

Table 6–53. Agnes Coefficient and Mantel r for each *S.* Typhimurium Assemblage

| *Assemblage* | *Agnes Coefficient* | *Mantel r* | *P value* |
|---|---|---|---|
| PT | 0.114 | 0.955 | 0.333 |
| MLVA | 0.268 | 0.972 | 0.333 |
| PTMLVA | 0.667 | 0.500 | 0.667 |
| Ab | 0.268 | 0.862 | 0.083 |
| PTAb | 1 | 0.489 | 0.333 |

Table 6–54. Cophenetic Distance Matrix for *S.* Typhimurium for the Antibiogram (Ab) Assemblage

| *Location* | *Breeder* | *Broiler* | *Hatchery* |
|---|---|---|---|
| Broiler | 0.587 | | |
| Hatchery | 0.335 | 0.587 | |
| Processing | 0.460 | 0.587 | 0.460 |

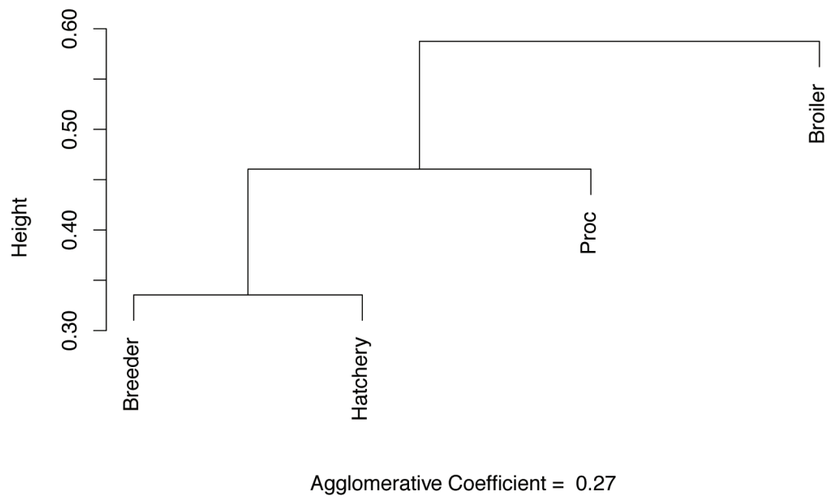



Fig. 6–28. *Salmonella* Typhimurium Antimicrobial (Ab) Assemblage Dendrogram (Hierarchical Clustering)

### 5.3.2 Principal Component Analysis

Unconstrained principal component analysis results for each of the *S.* Typhimurium assemblages also indicated the total variation between sites was affected by the *S.* Typhimurium typing method (Table 6–55) (total variance: PTAb > PTMLVA > MLVA > Ab > PT). PT and Ab had the least amount of variation explained in species composition between sites (PT < Ab).

Only the Ab assemblage had a significant principal component (PC1), where 62% of the variation at each of the locations was explained by the Ab typing method. No other assemblages had a good fit to the data. Even for the assemblage with the most significant clustering (Ab), the proportion of total variation explained at the hatchery and the breeder sites, was very small. Ordination graphs for *S.* Typhimurium Ab assemblage by location are illustrated in Fig. 6–25 and Fig. 6–24. The distance between all sites and all species is small (< 0.5). When a location and antibiogram profile (Fig. 6–24 are in the same quadrant this indicates that this variant is contributing more to the variation at that location. The influence of a single Ab variant (ApSSu) is illustrated in Fig. 6–25. A line is drawn to the Ab variant and then the relationship of each location with that variant is indicated by a perpendicular line.

## 5.4 Sample Size Analysis

The variation in *Salmonella* Typhimurium MLVA profiles within the dataset indicated

Table 6–55. Summary of Principal Component Analysis between Locations, Total Variance explained by the Variant Measure and the Eigenvalue of each Identified Principal Component

| *Variant* | *Total Variance* | *Eigenvalue (Proportion variation explained)* | | |
|---|---|---|---|---|
| | | *PC1** | *PC2* | *PC3* |
| PT[1] | 0.126 | 0.078 (0.627) | 0.047 (0.372) | –[2] |
| MLVA[1] | 0.129 | 0.089 (0.683) | 0.040 (0.316) | – |
| Ab | 0.132 | 0.082 (0.621) | 0.032 (0.242) | 0.018 (0.136) |
| PTMLVA[1] | 0.526 | 0.343 (0.653) | 0.182 (0.347) | – |
| PTAb[1] | 0.585 | 0.346 (0.592) | 0.137 (0.235) | 0.101 (0.172) |

*PC1, PC2, PC3 Principal component 1, 2 or 3. [1]Not significant. [2]"–" Component not identified.



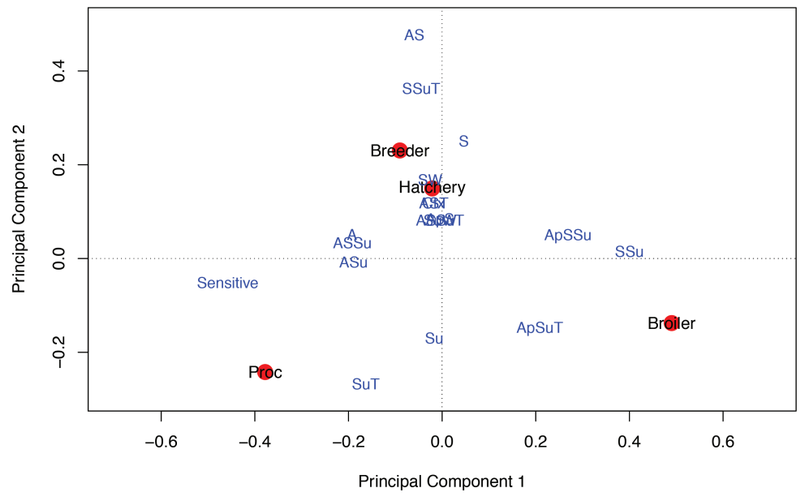



Fig. 6–29. Ordination Graph for *S.* Typhimurium Ab Assemblage Variation between Locations

The closer the locations (e.g. breeder and hatchery) are to each other the smaller their Euclidean distance, and more similar their species composition. The broiler and hatchery sites are best represented in this analysis (comprising respectively 20.3% and 70.8% of the total variation). Variant positions (Ab phenotype) indicate the relative distance of each of the Ab variants from origin. If variants are in the same quadrant as the locations, they have larger than average expected values for that variant at that location.

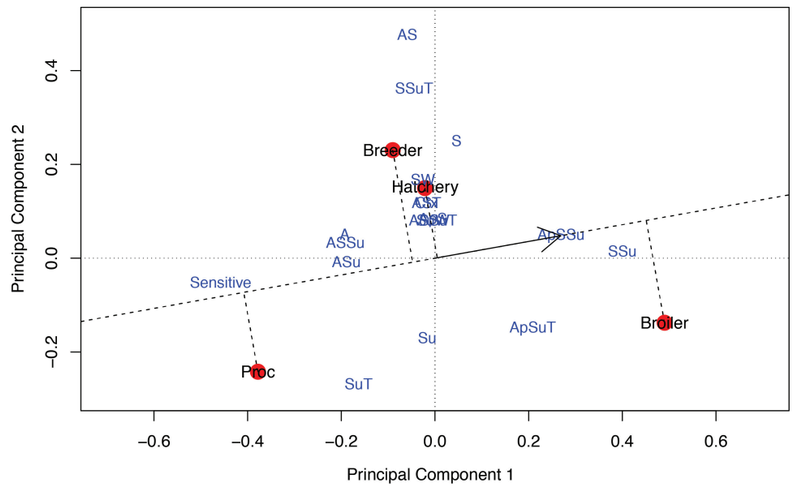



Fig. 6–30. Ordination Graph for *S.* Typhimurium Ab Assemblage between Locations with Lines Indicating Relationship to ApSSu Variant

A vector is drawn for the ApSSu variant and shows the direction from the origin for locations that have a greater abundance for this variant. The projections to the locations indicate the ranking of the sites from low (processing) to high (broiler) abundance for the variant.

there was greater within-location ($s^2$ = 21.1) than between-location variance ($s^2$ = 2.0 -3.0). To provide sufficient analytical power to detect a difference between locations using these estimates of variance, a minimum of 50 samples per typing method per location was required.

## 6. Discussion

The distribution of *Salmonella enterica* and *S.* Typhimurium variants were shown to be consistent with species abundance distribution patterns (log-normal or geometric series) and therefore appropriate [555] for measuring the abundance or diversity of *Salmonella* in this study. Ecological diversity measures were applied to answer a key question regarding whether the high population diversity observed both within the *S.* Typhimurium isolates and between each location is due to true biological diversity, such as the introduction via multiple sources, or if it is a function of the typing method. If the population diversity is a function of the typing method, it is possible that only a single source of contamination exists and that the observed diversity is artefactual due to the typing method.

These results confirm that the diversity of *Salmonella enterica* subspecies serovars (ST) between sites varies, with greater richness at the broiler location (5.22, 95%CI [4.14, 6.30]). The results for the *S.* Typhimurium isolate variant assemblages are less conclusive. The population diversity measures varied according the assemblage. Assemblages were used to group "like isolates" based on the assumed phenotypic or genetic relationships inferred by the typing methods. It is quickly apparent however, that the richness and the apparent diversity within locations differs depending on the method used for typing (assemblage). This is problematic as the relationships between locations differ depending on the typing method used for analysis, when it is known that these isolates only vary by the typing method. If only one of the phenotyping methods was used for inference the relationships between locations would be resolved. The uncertainty generated by the typing methods indicates that the true genetic relationships between the isolate variants is unknown using these phenotyping tools and that the real relationships between locations can only be resolved with high intensity sampling and isolate testing. More sophisticated analytical tools, agglomerative clustering and principal component analysis, utilized to measure the ß-diversity, demonstrated that there was no difference between locations in the *S.* Typhimurium population composition; only antibiogram phenotyping demonstrated a small degree of clustering by location.

Rarefaction analysis, suggested that not all variants had been identified at each location. Implying that insufficient samples were examined at each location to identify all possible assemblage variants with confidence; despite more than 400 isolates tested at either the breeder or broiler locations. Only at the hatchery site were sufficient samples tested (ST and PT only) for a horizontal asymptote to be reached. Sample size was not sufficiently large at any of the locations, for any of the *S.* Typhimurium typing methods (Ab, MLVA, PTMLVA or PTAb), to reach a horizontal asymptote indicating that if more samples were tested more variants were likely to be detected using any of these methods. Sample size analysis indicated that at least 50 isolates were required from each location to differentiate with sufficient confidence a true difference between locations if it was present. Sample size in this study was more than sufficient to meet this objective.

It is known that incidence based indices are biased by sample size, particularly when the assemblage/community has high species richness and a large proportion of rare species [519]. Measures that avoid these assumptions (presence-absence data) may be more robust [519, 568]. The Raup-Crick metric is "appropriate when comparisons are made among communities that can reasonably be considered to be a part of the same regional species pool" [563]. In this study, multiple locations were sampled and the results aggregated by management type within the production system. The use of the phenotyping or genotyping information alone, where a single dominant species, in this case *S.* Typhimurium, comprised a large portion of the samples failed to demonstrate there was a difference in species composition between sites. This suggests that either there is a single source of *S.* Typhimurium distributed between all locations over the within the study duration or that the sample size at each location had insufficient power to differentiate between the locations. It is also possible that both of these scenarios exist.

Consideration might be given to another possible source of variation within the data that could create the impression that insufficient samples were collected for this analysis. The assumption of any typing tool is that the phenotype and genotype (MLVA) of the isolates is fixed and as a consequence dissemination between locations can be tracked using these methods of differentiation. It must be recalled however that *Salmonella* may remain inactive or dormant for long periods of time and show significant phenotypic plasticity, more than 2500 *Salmonella* serovars and 300 *Salmonella* Typhimurium phage types have been identified [30, 62]. The failure of phage typing to reliably differentiate *Salmonella* Typhimurium is one reason that the adoption of other typing methods has occurred [67]. All these features must be considered when taking into account the reliability and interpretation of the results presented [554]. MLVA profiles are derived from short tandem repeat regions within the intergenic regions in the *Salmonella* genome and associated plasmids [571]. The variation in MLVA profile is due to random addition or deletion of these hypervariable tandem repeat regions during transcription [572]. It has been reported that in clonal outbreaks there is very little change in the MLVA profile and this feature is useful for tracing, but MLVA profiles may not be sufficiently stable for more complex investigations of longer duration [71]. The same may apply to the appearance of multiple phage types. The identity of a phage type depends on the carried phages and their lysogenicity [62]. The acquisition or loss of phages, and consequential change of phage type, or phage type conversion is a well recognised feature of some *Salmonella* serovars including *S.* Typhimurium [67, 68, 573, 574]. Again, the acquisition of these new phage types may reflect the random gain or loss of phages or other changes in the isolate rather than true genetic diversity in the *S.* Typhimurium population.

While it was technically possible to do more sample testing for phenotyping and genotyping at all locations other than the breeder locations (all samples were tested), it is unclear whether this would make the results of this analysis more conclusive. The amount of variation observed and the failure to reach an asymptote in the rarefaction analysis may be a consequence of the plasticity of the *S.* Typhimurium genome rarely observed in smaller studies that limit analysis to only a few samples and one of the powerful aspects of this study. A key question remains regarding the reproducibility of these results in other settings in a similar environment under the same conditions. No studies have addressed the issue of reproducibility with regards to the use of PT, MLVA or PT/MLVA typing within an epidemiological context. The large sample size and results described here demonstrate that large sample sizes are required to interpret with sufficient confidence results that would at first glance appear to be contradictory or counter-intuitive. The rarefaction analysis supports the conclusion that more samples would only lead to further differentiation of the isolates beyond that which would be considered economical or practical in a real-world investigation. Additionally these results support the assertion that these methods are not adequate for complex epidemiological investigations for source attribution over long time frames.

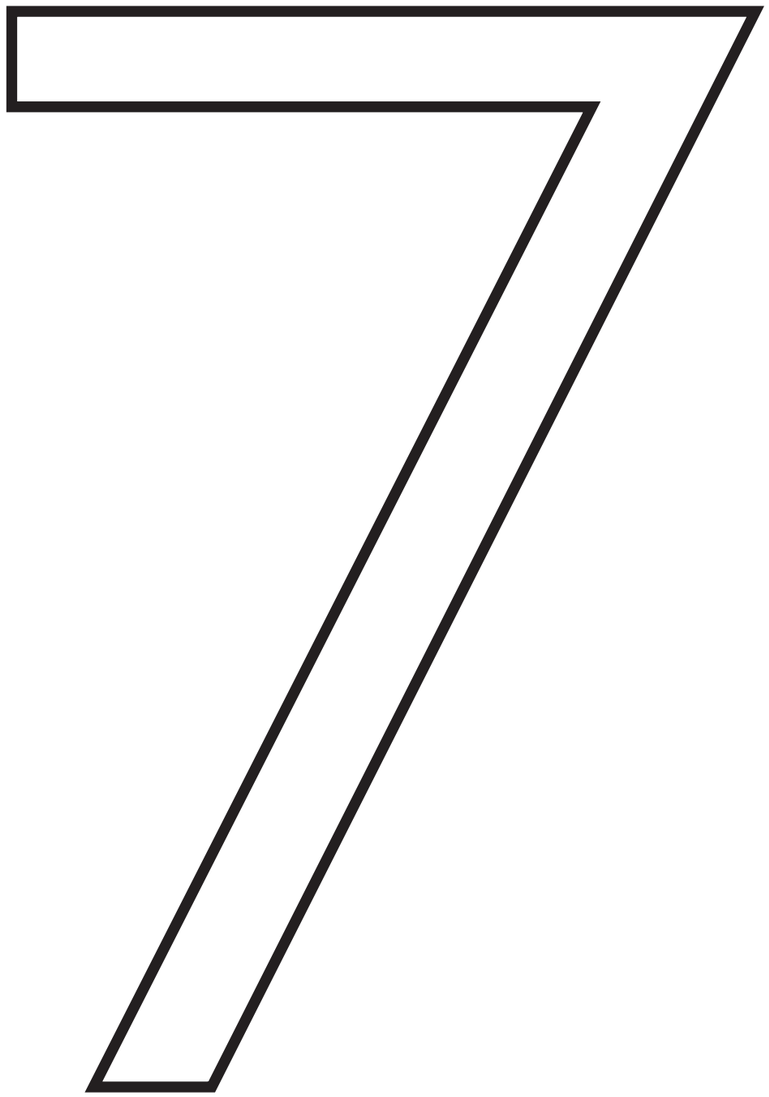
## 7. Conclusion

This analysis assessed the diversity of the *Salmonella enterica* and *S.* Typhimurium isolates within and between locations. *S.* Typhimurium diversity varied depending on the typing method used for analysis. There was no difference in species composition between communities, but the sample size assessment using rarefaction implied that insufficient samples were collected at each site, to detect all variants present. Two biologically plausible possibilities for these findings exist, either there really is no difference in species composition between sites and that all variation observed is due to random events (genomic plasticity) or that insufficient samples were collected to describe the variation present.

This current study involved evaluating 1468 *Salmonella* isolates from single vertically integrated broiler production enterprise. Flocks were sampled repeated at different generations

yet diversity varied depending on the typing method used. If the results of this analysis suggest that the study design had insufficient sample size to detect all possible variants then studies with small source populations or source attribution studies from carefully curated collections are highly unlikely to yield an accurate assessment of the biological relatedness or diversity within the populations.

With these findings in mind, further analysis of the *S.* Typhimurium isolates by different analysis methods such as whole genome sequencing is required to determine if the phenotyping and genotyping conclusions made above truly represent the population and transmission epidemiology.



CHAPTER **SEVEN**

# Whole Genome Sequencing of *Salmonella* Typhimurium Isolates

*(next spread)*
*The method used to describe the isolates (phenotype or genotype) is critical for describing the population diversity and potentially ascribing a source or pathway of transmission.*



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## Abstract

Whole genome sequencing of 411 *Salmonella* Typhimurium isolates from a longitudinal study of a vertically integrated poultry operation over an 18-month period identified an ancestral phylogeny of 204 unique isolates with two major clonal lineages encompassing 99% of the isolates. Isolates within the two lineages differed between 2 – 11 SNPs across the genome. The two lineages were evolving at different rates under purifying selection (Lineage I: 0.433 and II: 0.389), with a gain of 0.8 – 2.45 SNPs per annum. No recombination events were identified in any of the sequences and the virulence plasmid pSLT (NC_017720) was transmitted intact with the genome with little variation. The first identification of both clones initially at the parent generation supports the pullet rearing site as the most likely point of introduction into the production system. The finding of little to no temporospatial change in the genome during the study supports the rapid dissemination, by vertical transmission via the hatchery, of these clones to subsequent generations of birds.


## 1. Introduction

The tracing of *Salmonella* strains within poultry operations or in foodborne disease outbreak investigations has traditionally been based on phenotyping, serotyping and phage typing of isolates, and more recently a large array of genotyping tools. A very small number of these genotyping tools have become routine laboratory tools for the identification of isolates with similar genomic characteristics such as pulsed field gel electrophoresis (PFGE), or multi-locus variable-number tandem-repeats analysis (MLVA). They are either very time consuming and limited to comparison of a small number of isolates at a time (PFGE) or the genetic relatedness of isolates is unknown unless epidemiologically linked (MLVA) such as in outbreak situations. The advantages and disadvantages of these methods for epidemiological investigation has been well documented [58, 571, 575, 576].

A small number of studies that have investigated or described the transmission of *Salmonella enterica* within integrated poultry operations are frequently limited to one generation of the system, but few have described transmission beyond characterizing *Salmonella enterica* to the serovar level [197, 250, 299, 577]. Those studies that have further differentiated *Salmonella* by molecular typing tend to focus on *Salmonella* Enteritidis or important foodborne serovars relevant to the country of study [244, 253, 500, 501, 578, 579]. *Salmonella* Enteritidis is not considered endemic within the commercial chicken industry in Australia and cases acquired overseas are the predominant source of infection to humans in Australia. *Salmonella* Typhimurium is the predominant cause of salmonellosis in people (48%) and outbreaks are frequently linked to the consumption of chicken eggs or meat [405].

In Australia, three studies reported transmission of *Salmonella* Typhimurium between generations of poultry within an integrated organization, but did not report results beyond the serovar level [18-20]. The origin of *Salmonella* in these studies was identified as feed but the pathway of transmission between generations was implied but not detailed.

The advent of whole genome sequencing and the recent reduction in cost has enabled the rapid production of large quantities of genome sequence data. This change has occurred very rapidly, whereby countries are now moving from traditional phenotyping methods to whole genome sequencing as the standard method of investigation for outbreaks of foodborne illness [73, 74]. This rapid accumulation of publicly available genome sequences means it is now possible to gain powerful insight into transmission paths not previously available.

Whole genome sequencing of *Salmonella* has been used to document the global transmission of antimicrobial resistant lineages of *Salmonella* and resistance gene containing plasmids and to investigate and document *Salmonella* outbreak transmission paths and the source of outbreaks both internationally and in Australia [75, 77, 580]. The advantage of whole genome sequencing in transmission and epidemiological studies is the opportunity to identify relationships between isolates beyond simple type matching offered by phenotyping or genotyping tools (PFGE or MLVA) which enable discrimination between isolates on different evolutionary time scales, but which are limited in discriminatory power between highly similar isolates [581]. Whole genome sequencing enables evaluation of the rate of evolutionary change across the complete genome and estimation of the divergence of closely related isolates if temporal sampling has occurred. A more precise investigation of the time of introduction or putative entry point or source of *Salmonella* in carefully designed studies is possible by whole genome sequencing and opportunities abound for understanding bacterial pathogenesis, host adaptation and gene to phenotype associations using this technology [582, 583].

In Chapter 3 the potential transmission paths for *Salmonella enterica* within this enterprise have been described, in Chapter 4 the relationships between the phenotype and genotypes of the *Salmonella* Typhimurium isolates have been investigated and in Chapter 6 the similarity of *Salmonella* Typhimurium isolates between locations has been assessed using phage type, MLVA and antimicrobial sensitivity profiling. This work has demonstrated the parent site as an important point of introduction of *Salmonella* Typhimurium into the operation, with at least 13 introductions detected based on these analyses. However, due to the large amount of variation, and the apparent close relatedness of the isolates, further discrimination of these isolates is warranted. The aim of this study is to describe the core genomic relationships between *Salmonella* Typhimurium isolates detected within the studied poultry population and evaluate the usefulness of whole genome sequencing in context with the findings of the previous chapters.

## 2. Aim/Purpose

Describe and compare the relationships between poultry isolates identified at each location within the longitudinal study population using the core genome to identify the putative source or point of introduction and pathways of transmission within the production enterprise. Exploration of the accessory genome and causes of phenotypic variation will be conducted elsewhere.

## 3. Materials and Methods

### 3.1 DNA Extraction and Sequencing

*Salmonella* Typhimurium isolates (437) from the longitudinal study were purposefully selected for whole genome sequencing (Table 7–56). A selection of fully typed sequences were selected from each sampled site based on source, phage type and MLVA profile. Sample selection criteria included: A single representative isolate from each sample where duplicate isolates were obtained (more than one was selected if phenotype or genotype was different), donor flock, phage type, MLVA type and location. Broiler samples (not phenotyped) were stratified by site and randomly selected from those that grew after resuscitation from storage. DNA extraction and next generation sequencing methodology for each run is described in Chapter 2, Section 8.

### 3.2 Quality Assurance and Run Performance

Each short sequence read file (fastq) was assessed for data quality using FastQC V-0.11.6 [575]. A summary of all sequencing runs and output is provided in Table 7–57. Comparison of the sequence quality scores (Phred), to-

Table 7–56. Isolates Selected for Whole Genome Sequencing from Longitudinal Study

| *Location* | *DT135* | *PT135a* | *DT9* | *DT193* | *DT12* | *Unknown* | *No Isolates* | *Proportion* |
|---|---|---|---|---|---|---|---|---|
| Parent | 13 | 140 | 4 | 7 | - | 8 | 172/208 | 82.7% |
| Hatchery | 41 | 50 | _ | 29 | 4 | 60 | 184/411 | 44.8% |
| Broiler | 1 | - | - | - | - | 46 | 47/227 | 21.6% |
| Processing | 10 | 6 | - | - | - | 18 | 34/98 | 34.7% |
| Total | 65/65 | 196/196 | 4/4 | 36/37 | 4/4 | 85/122 | 437/944 | 46.3% |

tal sequence reads, insert mean and nucleotide composition (A, T, C, G, N), reference genome coverage, reference genome depth and SNP calling for each run was conducted in R using analysis of variance where normality assumptions were not violated and the Kruskal-Wallis rank sum test if they were. Multiple comparisons between runs were evaluated using Tukeys multiple comparison test in "multcomp" [484, 544, 546, 585].

Sequences excluded from analysis (Supplementatry Table 1) were identified as follows: Isolates that failed the RedDog run criteria, identified as another *Salmonella enterica* serovar(not *S.* Typhimurium) or contaminant via MLST, had poor quality assemblies or poor quality assessment during core genome assembly or failed sequencing (no/insufficient sequence generated) (as above).

### 3.3 Core Phylogeny Analysis

A *Salmonella* Typhimurium core phylogeny was identified. Single nucleotide polymorphisms (SNPs) were identified using the RedDog pipeline v2b103 [586]. The *Salmonella* Typhimurium SL1344 genome NCBI reference sequence NC 016810.1 and associated plasmid sequences NC_017719.1, NC_017718.1 and NC_017720.1 were used as the reference genome [587]. This genome has previously been assessed as the closest match to Australian origin *Salmonella* Typhimurium PT135a [77]. Each short read sequence was mapped to the reference strain using Bowtie2 [588], and single nucleotide polymorphisms (SNPs) called using SAMtools and BCFtools [589, 590] in accordance with the pipeline methodology. Criteria used for sequences to pass the initial pipeline were set at default values as follows: the minimum read depth for calling a SNP equals 5, more than 50% of the sequence reads map to the reference genome, the average read depth across the whole reference genome must be greater than 10 and more than 50% of all sequence reads must map to the reference genome. A sequence was identified as an "outgroup" if the number of SNPs called was greater than 2 times the standard deviation of the mean count of SNP calls. Heterozygous SNP calls were removed and the conservation of alleles was set to 95%, whereby 95% of the SNPs called with a missing allele are removed.

Repeat sequences, insertion sequences and phage locations in the reference genome (SL1344) were identified using the nucmer tool in MUMmer v3.23 [591] and PHAST [592] respectively. The final core alignment was filtered, by removing SNPs identified in the repeat regions, insertion sequences and phage locations, and cleaned by removing those SNPs within 3 base pairs of each other and 3 or more SNPs (trio's) called within a 10-base pair window (possible recombination events).

The filtered core alignments were used to identify highly recombinant areas in each sequence using "Gubbins" v2.0.0 [593]. The default value of 3 base substitutions was used to identify a recombination, and the maximum number of iterations was set to 100 with iterations halted when convergence met the recombination criteria.

The final core phylogenetic tree was inferred from the concatenated alignment of SNP alleles by maximum likelihood using RAxML v 8.2.11 [594, 595]. The GTRGAMMA substitution model was specified with 100 rapid bootstrap replications. Tree output was visualized and annotated in the R statistical package using "ggtree" [484, 596].

### 3.4 Adaptive Evolution

Selection with the phylogeny was assessed by calculating the ratio of non-synonymous to synonymous mutations (dN/dS) within the genome. Analysis was conducted using CodeML, within PAML v4.9e [597]. A null model (Model 0) was inferred first to estimate the average dN/dS ratio for each branch. The null model was compared to Model 1 which inferred the dN/dS ratio for each branch. The significance of the dN/dS estimates were evaluated using the likelihood ratio test. A whole genome alignment of 204 unique isolates was used for analysis.

### 3.5 Time to most recent common ancestor (TMCRA)

The temporal structure of the phylogeny was evaluated for the whole phylogeny (204 unique isolates) and each of the two lineages (I: 97 and II: 106 isolates) using a dated concatenated SNP alignment for each analysis in TempEst v1.5.1 [582]. Each isolate was dated with the date of sample collection or where duplicate identical isolates (5.4.4) were collected the date the first isolate of that group was isolated was used. Dates were measured in days from 1900. The substitutions per variable site per day was scaled to genome-wide units of substitutions per site per annum according to the following calculation: number of substitutions per variable site per day by constant k: k = n/N x 365 where n = the number of SNPs and N= length of the genome for SNP calling.

### 3.6 Assembly

*De novo* assembly of each sequence was conducted using SPAdes v with the resulting contigs scaffolded using SSPace v3.0 [598, 599]. GapFiller vv1.10 was used to close gaps within scaffolds [600]. Scaffolds were organized against the reference genome using ABACAS v1.13 and Prokka v1.11 was used for genome annotation [601, 602].

### 3.7 Short Read Sequence Screening

All sequences were screened for multilocus sequence type (MLST), plasmid and antimicrobial resistance gene content using SRST2 v0.2.0 in accordance with the published method [603]. MLST profiles [604] were identified for each isolate using the seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, *thrA*). Sequences were screened for antimicrobial resistance genes using the ARG-Annot database [605]. Small plasmids were identified using the plasmid database (PlasmidFinder [606]) included within SRST2.

## 4. Results

### 4.1 Sequencing Quality Analysis

A total of 437/944 (46.3%) *S.* Typhimurium identified poultry isolates from the longitudinal study were sequenced in 7 sequencing runs using both Illumina MiSeq (n=2) and HiSeq (n=5) platforms. Details of the output from each of the sequencing runs are summarized in Table 7–58. A total of 1.625 x$10^9$ paired end reads were generated in all runs (mean 2.3 x $10^8$, range: 2.6 x $10^7$ - 4.6 x $10^8$). There was a statistically significant difference in the number of reads generated between sequence runs (Adj-$R^2$ = 0.124, F (6, 404) = 10.67, P < 0.0001). The mean Phred score (base calling quality) for all isolates was 35.02 (S.D. +/- 0.374) and there was a small but statistically significant difference between the runs in sequence quality scores with Run 5 and 9 significantly poorer and Run 7 significantly better than Run 1 (Adj-$R^2$ = 0.344, F (6, 404) = 36.93, P < 0.0001). Insert mean length was 239.95 (S.D. +/- 67.201) bases. There was a statistically significant difference between all runs in the insert mean length (Adj-$R^2$ = 0.887, F (6, 404) = 539.7, P < 0.0001).

The mean GC content for all sequences was 51.57% (S.D. +/- 0.45%). For each nucleotide call (A, T, G, C) there was also a small but statistically significant difference between each run (A: Adj-$R^2$ = 0.369, F (6, 404) = 41.01, P < 0.0001; T: Adj-$R^2$ =0.359, F (6, 404) = 39.41, P<0.0001; C: Adj-$R^2$ =0.366, F (6, 404) = 38.97, P<0.0001, G: Adj-$R^2$

Table 7–57. Next Generation Sequencing (NGS) details for each Sequencing Run

| *Run ID* | *Sequencer* | *NGS Platform* | *Isolates Final Analysis* | | | *Total Number of Isolates Sequenced in Run* |
|---|---|---|---|---|---|---|
| | | | *Number* | *Mean Paired End reads* | *Standard Deviation* | |
| 1 | MDU[1] | Illumina MiSeq | 10 | 3,069,826 | 287,984.4 | 12 |
| 3 | AGRF[2] | Illumina HiSeq | 92 | 5,032,142 | 834,717.4 | 96 |
| 4 | AGRF | Illumina Hiseq | 92 | 4,662,979 | 644,250.0 | 96 |
| 5 | AGRF | Illumina HiSeq | 88 | 5,238,724 | 1,134,834.8 | 96 |
| 6 | AGRF | Illumina HiSeq | 78 | 5,611,285 | 2,299,073.6 | 96 |
| 7* | AGRF | Illumina MiSeq | 7 | 3,805,249 | 1,617,418.8 | 8 |
| 9 | AGRF | Illumina HiSeq | 44 | 4,403,554 | 691,412.8 | 96 |

[1]MDU Microbiological Diagnostic Unit, [2]Australian Genome Research Facility, *Isolates failing Sequencer quality assurance in Run 6 were resequenced in Run 7

=0.374, F (6, 404) = 41.92, P < 0.0001). The mean number of N calls (mean 0.003, S.D. +/- 0.002) also differed significantly between runs (Adj-$R^2$ =0.989, F (6, 404) = 6681, P < 0.0001). There were no N calls made in Run 7.

### 4.2 Failed Sequences

Twenty-six sequences (5.8%) were removed from further analysis; 17 of these isolates were identified by MLST as *Salmonella* Infantis. A further 7 isolates were mixed containing both *Salmonella* Typhimurium and *Salmonella* Infantis (Supplementary Table 7–60). Despite serotyping (O-antigen) prior to sequencing 5.5% of the isolates were misidentified (3.9%) or contained mixed *Salmonella* serovars (1.6%).

### 4.3 Multi locus sequence typing (MLST)

All *Salmonella* Typhimurium isolates (411) had the same MLST type ST19, 10-7-12-9-5-9-2, with 100% sequence identity and no variations. All *Salmonella* Infantis isolates were MLST type ST32, 17-18-22-17-5-21-19.

### 4.4 Core Phylogeny Analysis

A total of 411 *Salmonella* Typhimurium isolates were included in the ancestral phylogeny. The number of isolates included from each generation of the production system were: Parent = 164, Hatchery = 168, Broiler = 46, Processing = 33. Core phylogeny run summary statistics are summarized in Table 7-59. Coverage of the core genome (Fig. 7–26) was high (mean = 98.93, S.D. +/- 0.07) as was the depth of reads across the genome (mean = 68.61, S.D. +/- 21.25) ensuring that SNP calls were likely to be called with a high level of accuracy across the genome.

There was no statistically significant difference between runs in the reference genome coverage (Adj-$R^2$ = -0.010, F (6, 404) = 0.304, P = 0.934). There was a small but significant difference in mean depth (Range: 51.82 – 99.75) across the reference genome between runs (Adj-$R^2$ = 0.290, F (6, 404) = 28.94, P < 0.001), and a significant difference between runs in the average number of reads mapped to the reference genome (Adj-R2 = 0.169, F (6, 404) = 14.9, P < 0.001). Runs 5 and 6

Table 7–58. Sequencing quality statistics per sequence for each next generation sequencing run

| Run | *Mean Quality (Standard Deviation)* | | | *Max Length (bp)* | *Mean nucleotide (%) composition of sequence* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *No Reads* | *Insert Size* | *Base Quality* | | *A* | *T* | *G* | *C* | *N* |
| 1 | 2,982,290.50 (331,717.08) | 428.71 (50.39) | 34.88 (0.52) | 251 | 23.57 (0.11) | 23.59 (0.11) | 26.48 (0.12) | 26.35 (0.11) | 0.0001 (0.0001) |
| 3 | 5,032,142.5 (834,717.40) | 172.193 (16.46) | 35.04 (0.29) | 100 | 24.33 (0.12) | 24.35 (0.12) | 25.68 (0.12) | 25.62 (0.12) | 0.0062 (0.0001) |
| 4 | 4,662,979.3 (644,250.00) | 253.38 (20.55) | 35.21 (0.28) | 100 | 24.66 (0.18) | 24.29 (0.18) | 25.75 (0.18) | 25.69 (0.17) | 0.0017 (0.0002) |
| 5 | 5,239,723.6 (1,134,834.84) | 221.66 (22.87) | 34.86 (0.30) | 100 | 24.24 (0.13) | 24.25 (0.13) | 25.79 (0.13) | 25.71 (0.13) | 0.0021 (0.0002) |
| 6 | 5,576,247.5 (2,243,053.38) | 223.73 (14.34) | 35.19 (0.25) | 100 | 24.13 (0.13) | 24.15 (0.13) | 25.88 (0.14) | 25.82 (0.13) | 0.0018 (0.0001) |
| 7 | 4,007,057.0 (1,602,539.68) | 233.76 (9.48) | 35.52 (0.38) | 100 | 24.00 (0.05) | 24.02 (0.05) | 26.02 (0.06) | 25.95 (0.04) | 0.000 (0.000) |
| 9 | 4,415,081.7 (3,986,496.71) | 370.06 (37.94) | 34.59 (0.38) | 125 | 23.97 (0.32) | 24.01 (0.32) | 26.05 (0.33) | 25.95 (0.31) | 0.0015 (0.0001) |



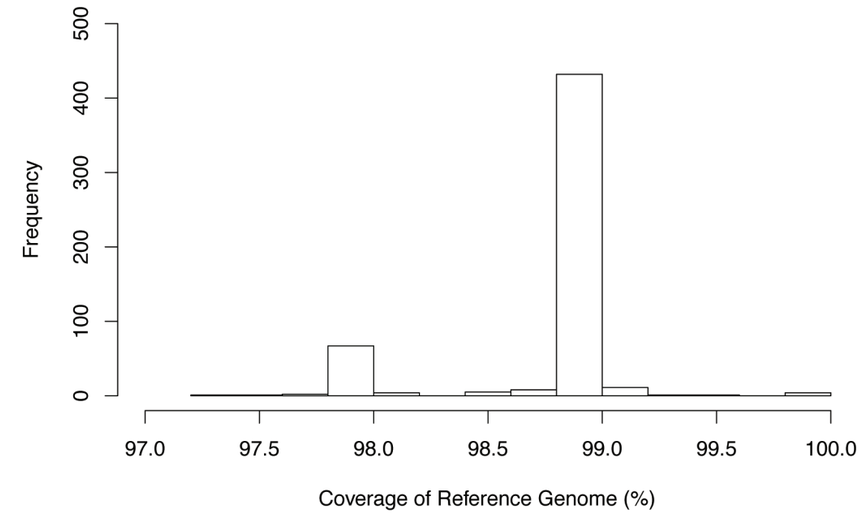



Fig. 7–31. Coverage of the Reference Genome for all Sequences in the Core Phylogeny RedDog Run

had significantly fewer reads mapped to the reference, and this is likely due to the higher number of mixed *Salmonella* isolates contained within these runs.

#### 4.4.1 Single Nucleotide Polymorphisms

The mean number of raw SNPs called per sequence was 355 (Median 350, Range: 49-1286) with a moderate amount of variation between isolates (CV% = 24.19), but no statistically significant difference between runs in the number of SNPs called (Adj-R2 = 0.014, F (6, 404) = 1.97, P < 0.069). Run 5 had a higher number of SNPs called compared to all the other runs (Mean +48.80 SNPs, P = 0.088), but was only statistically higher than Run 3 (Mean +38.79, T-value = 3.046, P = 0.033).

#### 4.4.2 Recombination

There were no recombination blocks identified in the input sequences (411 isolates). The final 204 sequence output tree contained a mean of 0.93 SNPs (Range 0 – 11 SNP) per clonal branch, and no SNPs were identified within a recombination block of 3 or more bases. The recombination to mutation (r/m) ratio equaled zero and the rho/theta ratio also equaled zero indicating all variations between isolates were due to point mutation events rather than recombination.

#### 4.4.3 Ancestral Phylogeny

The final sequence alignment comprised 204 unique sequences; approximately 50% (207/411) of the sequences were identical (4.4.4). The number of isolates from each generation of the production system represented in the final phylogeny were: Parent = 70, Hatchery = 83, Broiler = 26, Processing = 25. Isolate 3214 was identified as an outgroup for tree building and rooting. The final maximum likelihood tree had an optimized likelihood of -3261.623 with tree length of 1.137 nucleotide substitutions per site from root to tip. Estimated substitution rates were A <-> C: 0.898, A <-> G: 2.459, A <-> T: 0.503, C <-> G: 0.126, C <-> T: 2.482, G <-> T: 1.00. The frequency of each nucleotide was pi(A): 0.199, pi(C): 0.291, pi(G): 0.309, pi(T): 0.200.

The ancestral phylogeny comprised a single clade comprising isolate III and clonal lineage II, (105 isolates), and subgroup comprising isolate IV and clonal lineage I. Branch support for the two major clonal lineages was 100%, and 90% for the subgroup containing isolate IV and Lineage I and 61% for the Outgroup (Fig. 7–27).

#### 4.4.4 Identical sequences

Thirty-six isolates within the ancestral phylogeny had identical sequences (207) removed from the ancestral analysis. The number of clones per sequence group ranged from 2 to 53 isolates per group (mean = 8.87, median = 4 isolates per group). Further analyses of these sequences identified eight 8 sequence groups (17 identical isolates) were from samples collected from the same location or sample type on the same sampling date. Of these groups

only 3 (6 isolates, 2 of each sequence) had the same phenotype. The remaining 11 isolates had different phenotypes; their reason for inclusion in the initial analysis. Twenty of the sequence groups were identified at different sampling times; 4 sequence groups were identified in different sample types or locations on the same date. The length of time these clones were identified in the sample population ranged from 4 to 334 days (mean =90, median = 62 days). Ten sequence groups were identified in a single location (parent = 9, hatchery = 1 sequence groups) for a duration of up to 327 days (median= 62, range = 8 to 327 days) from the first detection at that location. Fourteen of the sequence groups (155 isolates) were identified at more than one location and all were detected at a preceding generation prior to detection at the next generation, and were detected for up to 334 days (median = 62 days, range 4 - 334 days) from first detection. There was no statistically significant difference between the length of time a clone was detected and the number of locations where a clone was detected ($R^2$ = -0.038, F (1, 22) = 0.143, P=0.705). Sequence groups containing more clones were detected for a longer duration ($R^2$ = 0.175, F (1, 22) = 5.896, P = 0.024).

#### 4.4.5 Plasmid Content

Core genome SNP analysis was conducted on the three plasmids identified within SL1344, NC_017719.1, NC_017718.1 and virulence plasmid pSLT (NC_017720.1) (Table 7–59). All sequences contained plasmid pSLT with very little variation between sequences (11.2 +/- 1.25 SNPs). Plasmid NC_017719 was not identified in any of the sequences, while Plasmid NC_0177818 was detected intact in 63 sequences with substantial SNP variation (1508 +/- 104 SNPs). The phylogeny of the pSLT plasmid had the same branching topology as the phylogeny of the core genome with division into the same core lineages.

#### 4.4.6 Antimicrobial Resistance Gene Content

Seven ß-lactamase antimicrobial resistance gene variants were detected in 26 of the 411 isolates. All were TEM-1 variants identified as TEM-30, TEM-70, TEM-76, TEM141, TEM-143, TEM-191 and TEM-198. The TEM class A ß-lactamase genes identified in *Salmonella* have been demonstrated to have enzymatic activity against ampicillin [598]. Eighteen of the twenty-six isolates had been phenotyped using the CDS method (Chapter 2, Section 7.3), with 16 phenotypically resistant to ampicillin. No isolates were resistant to cephalosporin. Further analysis of these genes and transmission pathways will be conducted elsewhere.

### 4.5 Adaptive Evolution

The dN/dS ratio (w) for the entire phylogeny was estimated as 0.396, and varied significantly across all branches but was < 1 indicating no positive selection within the sampled population or individual linages during the study period ($\chi^2$ = 450.81, df = 403, P < 2.2 x $10^{-16}$). The dN/dS ratio for lineage I: 0.433 and II: 0.389 indicates both branches were under purifying selection with Lineage II purification occurring at a slower rate than Lineage I which is consistent with the divergence estimates.

### 4.6 Time to most recent common ancestor (TMRCA)

The complete phylogeny of 204 unique isolates did not identify a clear temporal signal, with a

Table 7–59. Core phylogeny RedDog Run Summary Statistics

| Core Phylogeny | Sequences | Mean (+/- Standard Deviation) | | | |
|---|---|---|---|---|---|
| | | Coverage (%)[1] | Depth (bases) | Reads Mapped (%)[2] | SNPs (bases) |
| Core Genome NC 016810 | 411 | 98.93 (0.07) | 68.61 (21.25) | 90.31 (8.92) | 355.59 (86.01) |
| Plasmid NC 017720 | 411 | 99.98 (0.20) | 69.11 (40.82) | 1.74 (0.71) | 11.13 (1.39) |
| Plasmid NC 017718 | 407 | 14.69 (31.18) | 14.98 (41.83) | 0.273 (0.79) | NA |
| Plasmid NC 017719 | 3 | 3.01 (1.34) | 1 (0) | NA | NA |

[1]Percentage of replicon covered by mapping, [2] Percentage of all reads mapped to the replicon



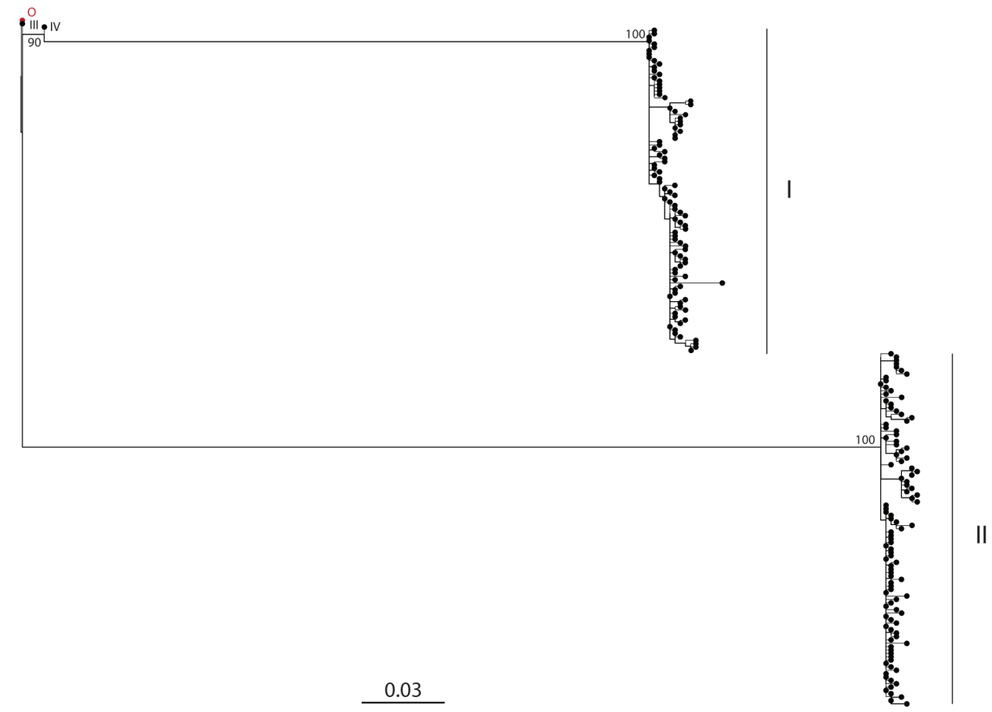



Fig. 7–32. Ancestral Phylogeny of Poultry Isolates

The two major clonal lineages (I, II) are identified plus the outgroup isolate (O) and singleton isolates III and IV. The distance (SNPs) between isolates within each clone ranged from 1-11 SNPs. Branch support is indicated for the three major branches.

reduction in the number of substitutions per site per year of -0.0001 ($R^2$ = 0.20, RMS = 3.28 x $10^{-4}$) and a TMCRA after the onset of sampling, estimated as 2028 (Supplementary Fig. 7–28). Consequently, each lineage was assessed separately. Lineage I was detected for a total of 336 days. The correlation between time of collection and temporality was poor (R = 0.154, $R^2$ = 2.37 x$10^{-2}$, RMS = 1.56 x$10^{-5}$), indicating there was no to little temporal signal within this lineage (Supplementary Fig. 7–29). The TMCRA was estimated to be 6/7/1930 (95%CI: 17/5/1930 - 24/8/1930), and the rate of substitutions per site per year estimated at 8.82 x $10^{-6}$. Lineage II was detected for a total of 483 days. The correlation between time of collection and temporality was moderate but greater than lineage I, (R = 0.31, $R^2$ = 9.89, RMS = 4.91 x $10^{-6}$) indicating that there was also little to no temporal signal within the lineage (Supplementary Fig. 7–30). The TMCRA was estimated to be 21/12/1893 (95%CI: 06/03/1876 - 27/08/1904), and the rate of substitutions per site per year estimated at 6.32 x $10^{-6}$. Due to the homochronous temporal signal within these lineages, further analysis by more sophisticated analysis methods was not deemed to be fruitful. The k value for Lineage I k=747/4487272 x 365 = 0.061 and Lineage II k=375/4487272 x 365 = 0.031 gives an estimate of 5.36 x $10^{-7}$ substitutions per site per year or 2.4 SNPs per genome in Lineage I and a rate of 1.92 x $10^{-7}$ substitutions per site per year or 0.86 SNPs per genome in Lineage II.

## 5. Discussion

Two major clonal lineages were identified within the sampled population despite considerable observed phenotypic variation as described in previous chapters (Chapter 4, Chapter 6). These results clearly demonstrate that phenotyping was insufficient for this epidemiological analysis. At the time this study was initiated, phenotyping was the standard method for *Salmonella* epidemiological stud-

ies. Next generation sequencing was not considered to be cost effective nor likely to be used as a standard method for epidemiological studies of this nature dues to the prohibitive costs at the time. This very rapidly changed and these findings reflect the understanding that phenotyping tools are now both insufficient and largely inappropriate for complex epidemiological investigations. However, until the issues around sequencing are resolved it will not become standard practice in Australia despite rapid adoption elsewhere for a while yet.

The presence of two dominant clonal lineages with so little variation between isolates was an unexpected finding. Previous analysis using traditional typing tools and diversity analysis indicated that there may have been at least 13 introductions of unique *Salmonella* Typhimurium isolates into the study population. This analysis indicates that there were at least five introductions of *Salmonella* Typhimurium into the production system, four of which were detected at the parent site, based on core genome analysis. Only two introductions (Lineage 1 and II) were detected for the duration of the study, despite sampling all parent flocks for the duration of the study period. No other *S.* Typhimurium introductions were detected in this parent population during the study period. Three introductions occurred, one prior to the onset of the study (Isolate III) and two were detected at end of the study (Isolate IV and Outgroup). Isolate III was detected in a previous parent flock at depopulation, and never detected subsequently. The other two introductions (Isolate IV, and the Outgroup) were detected at the end of the study one at the parent generation in a new pullet flock and the other at processing after tracing had ceased. The two major Lineages I and II were present for the entire study duration with the first samples collected at the parent sites. The samples collected from the pullet rearing location were identical to both clonal lineages indicating that the most likely point of introduction for these lineages into the poultry population occurred here.

Both Lineage I and II were under purifying selection indicating that they had likely been present in the host population for an extended time-period as there was no selection pressure being exerted on this population. It is possible that this may represent host adaptation of these *Salmonella* Typhimurium strains to the host population. The estimates of time to MRCA were long (Lineage I: 1930 and Lineage II: 1893) due to the low temporal signal within the samples, but the substitution estimates were consistent in magnitude with those estimated from outbreak studies in Australia with the same or similar *S.* Typhimurium phage types (DT135 and PT135a) [77], albeit at the lower end of their estimated SNP accumulation rates. These rates are consistent however, with those reported in recrudescent *S.* Typhimurium infection in HIV infected humans [608] and those in *Salmonella* Agona [609].

Reasons for the difference in magnitude (nearly half the rate reported by Hawkey et al, 2013) may be due to the intense sampling within the same host population over time, or indicating the lineages are well adapted to the host population. Or similarly to that proposed by Okoro et al, 2012 in an immunosuppressed population, may reflect recrudescence (or ongoing intermittent shedding in the poultry population) of infection from the parent population and subsequent vertical transmission, whereby no or little change over time would be expected to occur. This hypothesis is supported by the fact that half of the isolates were exact replicates of the studied population, and the lack of temporospatial variation.

The faster rates identified by Hawkey et al, 2013 may reflect introduction into multiple new host populations, both farm and human in an outbreak situation, even though purifying, not positive selection pressure was supported in the analysis.

Limitations of this analysis.

This analysis is restricted to the core genome of the *S.* Typhimurium isolates only. Further detailed analysis on the accessory genome will be presented elsewhere. As most of the accessory genome is generally composed of transmissible components of the genome such as plasmids, phage and prophage detailed presentation of this information and consideration of its importance in transmission paths and location separation is worthy of separate analysis.

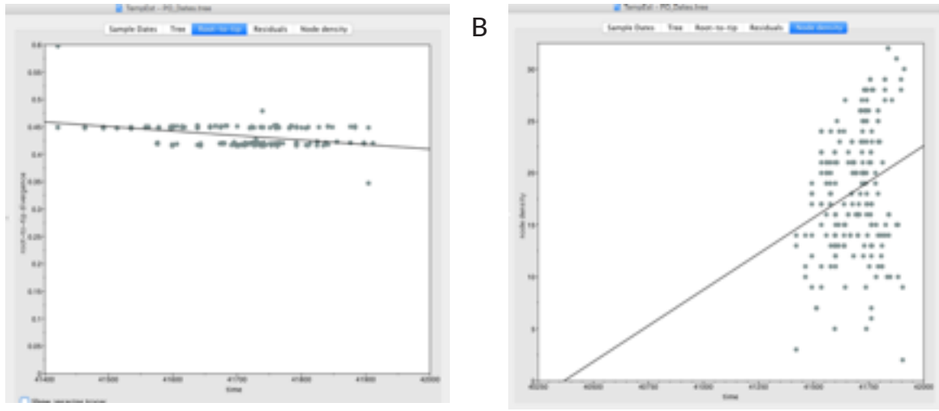
Further analysis will be conducted with additional *S.* Typhimurium isolates from more animal species, the environment and over a greater temporospatial period to contextualize these findings with regards to this pathogen in other animal and human hosts. The highly clonal nature of the phylogeny was unexpected given the large number of samples taken, the length of time considered and the disparate locations samples were collected. The hatchery, as indicated in the previous chapters (Chapter 3), is a critical hub for the dissemination of a successful pathogen between generations and this is evident by the successful transmission of identical *Salmonella* Typhimurium lineages between generations as identified in this analysis.

## 6. Conclusion

This analysis revealed a much closer relationship between *S.* Typhimurium isolates within a well-defined study population than was identified using traditional phenotyping and genotyping analyses in the previous Chapters. The parent sites were confirmed as the most likely site of introduction and subsequent dissemination, both temporally and genetically, most likely via vertical transmission, through the integrated broiler operation. This is supported by identifying ongoing purifying selection occurring in the two lineages identified, the slow rate of SNP accumulation in both lineages and the detection of new *S.* Typhimurium lineages at the parent generation at opposite ends of the study on four of five occasions.

It is apparent from this analysis that there is still much to learn about the dynamics of transmission of *Salmonella* within the chicken host and the complexity of transmission between hosts.

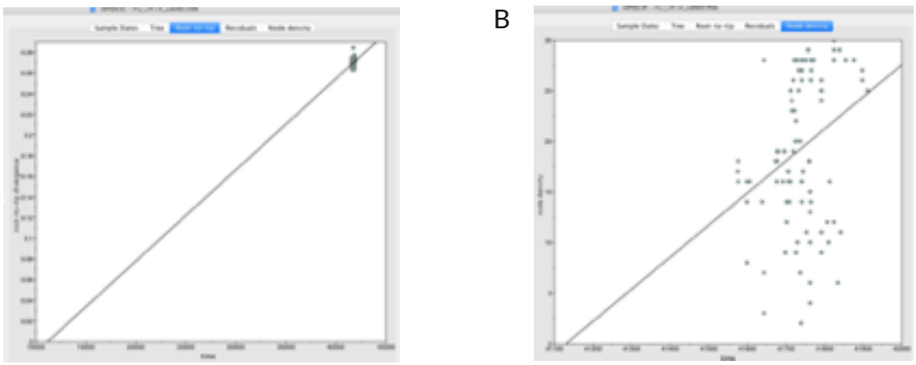
A B



**Supplementary Fig. 7–33. Tempest Screen shot of Root-to-tip Divergence for all Poultry Isolates**

**A**. Root to tip divergence (RtT) of all lineages. Three outlying sequences are evident at time 41400, RtT 0.6, time 41700, RtT 0.35, and time 41700, RtT 0.47. These isolates represent the outgroup isolate, Isolate IV and III respectively. The two lineages I and II are present across the entire sampling period with little divergence evident in Lineage II (RtT 0.45, top line) and Lineage I (RtT 0.4, bottom line). **B**. Node density of all isolates within the dated phylogeny demonstrating the lack of temporal signal within the phylogeny.

A B

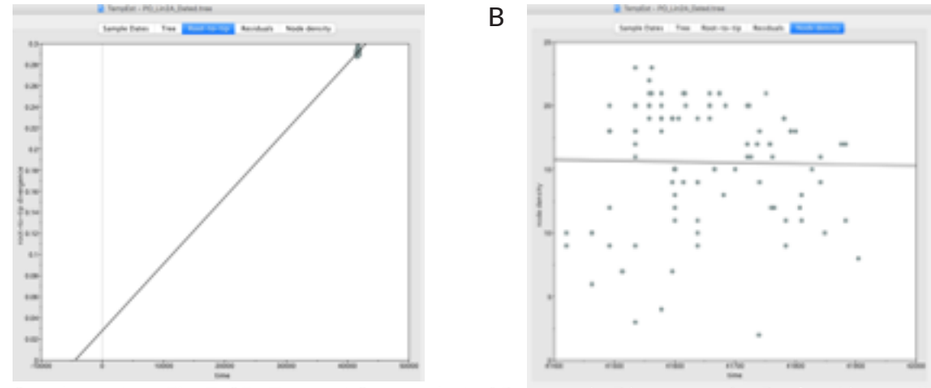


**Supplementary Fig. 7–34. Tempest Screen shot of Root-to-tip Divergence for Poultry Lineage I**

**A.** Root to tip divergence (RtT) of Lineage I, correlation R = 0.15. **B.** Node density of all Lineage I isolates within the dated phylogeny demonstrating the lack of temporal signal within the phylogeny.



A B

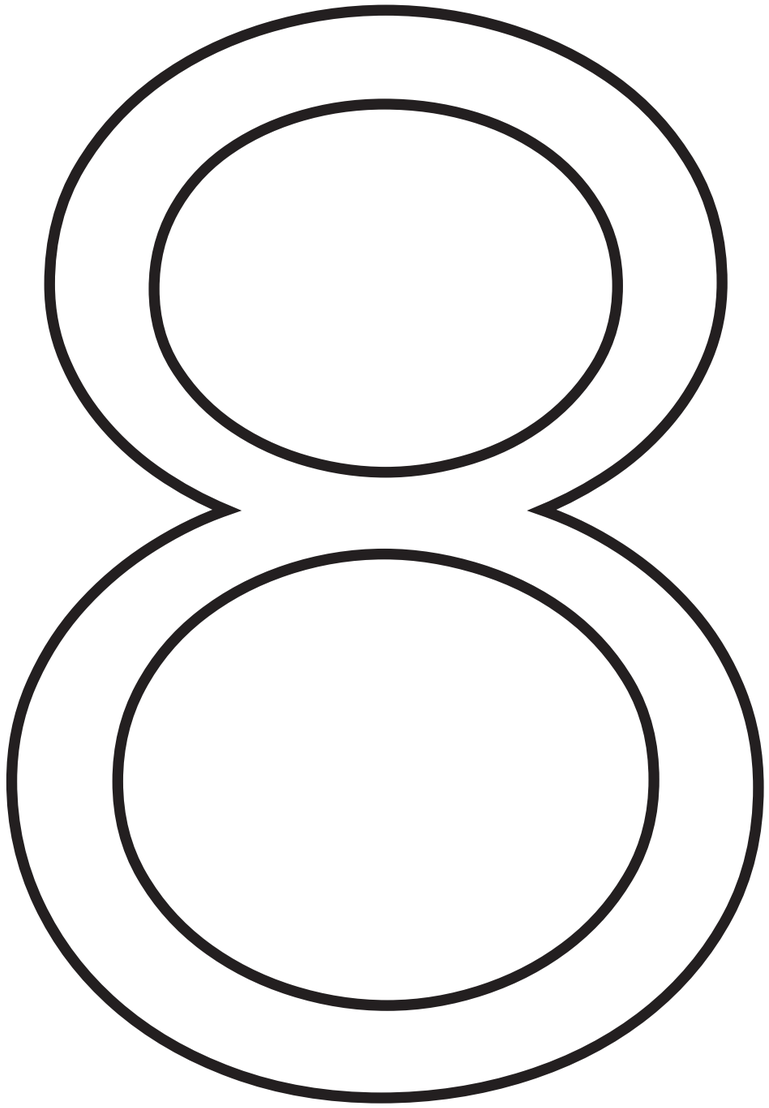


**Supplementary Fig. 7–35. Tempest Screen shot of Root-to-tip Divergence for Poultry Lineage II**

**A**. Root to tip divergence (RtT) of Lineage II, correlation R = 0.31. **B.** Node density of all Lineage II isolates within the dated phylogeny demonstrating the lack of temporal signal within the phylogeny.

**Supplementary Table 7–60. Poultry Study Isolates Removed from Subsequent Analysis**

| *Isolate ID* | *Run* | *Coverage* | *Depth* | *SNPs* | *Hets* | *Removal Reason* |
|---|---|---|---|---|---|---|
| 34 | 5 | 92.07 | 71.15 | 41974 | 341 | *S.* Infantis |
| 36 | 5 | 92.13 | 88.50 | 41946 | 361 | *S.* Infantis |
| 44 | 5 | 92.10 | 59.94 | 124869 | 516 | *S.* Infantis |
| 83 | 5 | 92.25 | 106.68 | 41833 | 372 | *S.* Infantis |
| 111 | 5 | 92.10 | 73.39 | 41275 | 400 | *S.* Infantis |
| 119 | 5 | 92.24 | 76.49 | 41575 | 425 | *S.* Infantis |
| 350 | 5 | 92.04 | 52.44 | 41430 | 417 | *S.* Infantis |
| 351 | 5 | 91.99 | 53.60 | 41654 | 361 | *S.* Infantis |
| 551 | 6 | 91.90 | 67.68 | 41371 | 383 | *S.* Infantis |
| 648 | 6 | 98.93 | 54.29 | 207 | 6298 | Mixed Serotype |
| 660 | 6 | 98.93 | 59.55 | 236 | 219 | Mixed Serotype |
| 664 | 6 | 98.93 | 60.06 | 213 | 284 | Mixed Serotype |
| 665 | 6 | 98.93 | 60.71 | 253 | 138 | Mixed Serotype |
| 669 | 6 | 91.35 | 14.68 | 104870 | 6 | Not *Salmonella* |
| 672 | 6 | 98.98 | 61.57 | 402 | 41638 | *S.* Infantis |
| 686 | 6 | 92.00 | 77.88 | 41867 | 332 | *S.* Infantis |
| 695 | 6 | 98.97 | 83.46 | 359 | 17944 | Mixed Serotype |
| 874 | 7 | 98.96 | 43.01 | 348 | 46922 | Mixed Serotype |
| 997 | 3 | 92.15 | 59.09 | 41385 | 189 | Mixed Serotype |
| 1059 | 3 | 95.59 | 60.12 | 40099 | 623 | *S.* Infantis |
| 1061 | 3 | 94.47 | 60.84 | 39976 | 938 | *S.* Infantis |
| 1301 | 6 | 91.93 | 52.82 | 40836 | 409 | *S.* Infantis |
| 1318 | 3 | 92.25 | 71.08 | 41476 | 249 | *S.* Infantis |
| 2529 | 4 | 92.19 | 75.28 | 41706 | 322 | *S.* Infantis |
| 3016 | 4 | 84.81 | 59.94 | 124869 | 516 | *S.* Infantis |
| 3099 | 9 | 12.23 | 1.2 | 0 | 0 | Poor Sequencing |



CHAPTER **EIGHT**

# A Comparison of *Salmonella* Isolates using Traditional Typing Methods: Human and Non-Human Isolates

*(next spread)*
*The method used to describe the isolates (phenotype or genotype) is critical for describing the population diversity and potentially ascribing a source or pathway of transmission.*



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## Abstract

The 2010 global burden of foodborne non-typoidal *Salmonella enterica* was estimated to affect 78 million people. Between 1991 and the end of 2014 the per capita notification rate of salmonellosis in Australia increased from 31.9 to 69.7 cases per 100,000 people with *Salmonella* Typhimurium the most frequently identified cause of human salmonellosis. An ecological study was conducted to compare all *Salmonella* Typhimurium isolates (421) that had been identified using phage typing (PT) and MLVA profiling from a previous large-scale poultry study (chicken meat) were compared using cluster analysis with those reported to the National Enteric Pathogen Surveillance Scheme (NEPSS) from the coincident human (3,950 isolates) and non-human populations (558 isolates). This study was conducted to determine if the study-origin poultry isolates could be identified as the source of human cases of salmonellosis, and to assess the usefulness of the current typing methods for source attribution. There was limited phage type diversity within all populations with cluster analysis revealing a significant lack in specificity between PT and MLVA profile combinations. The study-origin poultry isolates were not significantly clustered with the human cases and at least 7 other sources of *S.* Typhimurium with similar PT/MLVA profiles could also be implicated as a source of human cases during the same period. MLVA profiling and phage typing alone or in combination were not sufficient to resolve the source of human cases. Our results indicate that more intensive surveillance across all food production systems is required to better understand the epidemiology and source attribution of *Salmonella* Typhimurium in Australia and that the currently available *Salmonella* Typhimurium typing tools are insufficient to resolve anything, but well defined outbreaks.


## 1. Introduction

The 2010, global human health impact of all foodborne associated non-typhoidal *Salmonella enterica* infections was estimated to be the equivalent of 6.43 million (95% UI 3.08–13.2 million) disability adjusted life years (DALYs) [610]. Between 1991 and end 2014 the per capita notification rate of salmonellosis in Australia increased from 31.9 to 69.7 cases per 100,000 people [390] with *Salmonella* Typhimurium the most frequently identified cause of foodborne salmonellosis. The proportion of cases attributable to this serovar is slowly increasing from 42% in 2008 to 47% in 2014 [405, 447]. In contrast, the rate of foodborne salmonellosis has either declined or remained static in most other developed countries (Table 8–61) [389-392, 394, 611, 612]. *Salmonella* Enteritidis is not endemic in the Australian commercial poultry industry [83].

In 2011, only 7.5% of *Salmonella* diagnosed cases (clustered cases) could be attributed to a source and where that source was identified, eggs and poultry meat were frequently implicated. The remaining 92.5% of *Salmonella* associated cases could not be attributed to a single food source [405, 434, 435].

The characterization of *Salmonella enterica* serovars by phenotyping and genotyping methods such as phage typing (PT), pulse-field gel electrophoresis (PFGE), multi-locus variable-number tandem-repeats analysis (MLVA), multi-locus sequence typing (MLST) and whole genome sequencing (WGS) have become standard techniques for investigating the epidemiology of *Salmonella* and associated outbreaks of human salmonellosis in Australia and elsewhere [75, 329, 527, 613]. The advantages and disadvantages of each technique have been reviewed and it is generally accepted that MLVA profiling is the most discriminatory method for outbreak investigations but its value in complex epidemiological investigations or general surveillance is not clear [59, 67, 614, 615].

Chicken meat in Australia is produced locally in each state and distributed nationally by 7 vertically integrated chicken meat enterprises, 2 of which operate in all states and produce 70% of all chicken meat. Five enterprises produce the remaining 30% of chicken meat in Australia and fresh chicken meat is not imported [241-242].

Results of a previous study into the transmission of *Salmonella* within a poultry population identified the parent population as a primary site of introduction, with subsequent inter-generational transmission via the hatchery a key pathway for the spread of *Salmonella* Typhimurium within the integrated poultry enterprise [503, 616]. Phage typing and MLVA profiling were unable to resolve the source of *Salmonella* Typhimurium within this intensively sampled population without additional epidemiological information [616]. This study was instigated to compare the *Salmonella* Typhimurium findings from the poultry study with those reported to the National Enteric Pathogen Surveillance Scheme (NEPSS) for the same period to determine if the study-origin poultry isolates could be identified as a source of human cases of salmonellosis, and to assess the usefulness of current typing methods for source attribution purposes.

## 2. Materials and Methods

### 2.1 Study-Origin Poultry Isolates

A prospective cohort study investigating the transmission of *Salmonella enterica* within a single vertically integrated poultry enterprise was conducted between January 2013 and September 2014. Poultry supplied from this enterprise comprise ~6% of Australian chicken meat production. Parent and broiler generation, hatchery and processing sites (60% of all locations) within the enterprise were longitudinally sampled for the duration of the study. Thirty-six percent of the samples collected were positive for *Salmonella*, of which 63% were identified as *Salmonella* Typhimurium [616]. The study-origin poultry isolates selected for comparison comprised all *S.* Typhimurium isolates (n = 421) fully identified using the same laboratory methodologies as the isolates reported to the NEPSS.

Table 8–61. Comparison of *per capita* (100,000) Notifications of Human Salmonellosis (1990-2013)[1]

| *Country* | *Number of notifications per 100,000 head of population (% Salmonella Typhimurium)* | | | |
|---|---|---|---|---|
| | *1990's* | *2000* | *2010* | *2013-14* |
| Australia [390] | 32 | 32 | 54(44) | 70 (43-47) |
| USA [389] | 14[2] | 14 | 18 | 15 |
| New Zealand [391] | NA | 48 (51) | 26 (52) | 21(41) |
| Denmark [392] | 82[3] (32) | 43 (19) | 29 (32) | 20 (38) |
| EU [393,394] | NA | 35[4] (13) | 22 (22) | 20 (20) |

[1]Rates are sourced from national surveillance data but between country comparisons are indicative only. Cross country and time comparisons of raw data is limited as methodologies for calculating incidence, sampling and reporting vary between countries and years. [2]1994, [3]1996, [4]2006

Table 8–62. Comparison of *S.* Typhimurium Phage Types (%) from the NEPSS Datasets with Study-origin Isolates for the Coincident Study Period (2013-14)

| *Population* | *Percentage of Salmonella Typhimurium isolates by Phage Type detected in each population* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *DT9* | *DT170* | *DT135* | *PT135a* | *DT44* | *Other* | *Total Samples* |
| Human | 25 | 19 | 19 | 20 | 5 | 15 | 3950 |
| Bovine | 23 | 15 | 4 | 13 | 5 | 41 | 256 |
| Poultry | 27 | 24 | 21 | 11 | 7 | 11 | 200 |
| Study-origin | 2 | ND[1] | 20 | 60 | ND | 18 | 421 |
| Other | 18 | 4 | 18 | 23 | 3 | 35 | 102 |

[1]ND Not detected in this study

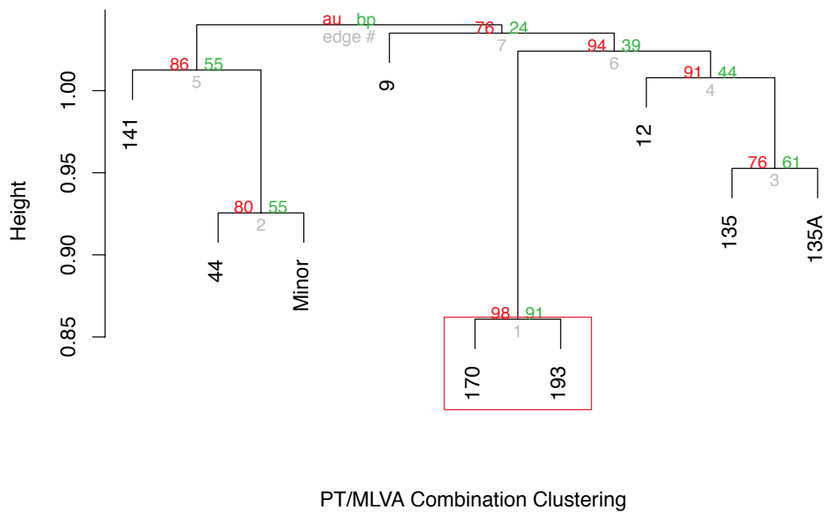



**Fig. 8–36. Hierarchical Cluster Dendrogram of PT/MLVA Combinations**

Clusters with approximately unbiased P-values (AU) greater than 95% are strongly supported. A single cluster containing DT170 and DT193 is statistically supported by the dataset (Highlighted in red). Branch support for each cluster is labelled with the AU value (red = au) and bootstrap support (bp = green).

A full description of the enterprise is published elsewhere, but in brief the integration operates with separate sites for parent production (rearing and egg production), the hatchery, broiler production and meat processing [503].

### 2.2 Human and Non-Human Surveillance Data

A de-identified extract of all *Salmonella* Typhimurium records submitted to the NEPSS for the period January 2013 to September 2014 was obtained for comparison [617]. *Salmonella* Typhimurium phage type, MLVA profile, isolation date and origin (human or non-human) metadata was provided. All records with complete *S.* Typhimurium typing information, phage typing and MLVA profiling were used for comparison. A total of 3,950 human and 558 non-human records were available for analysis. Non-human sources were divided into four groups: bovine, poultry, companion animal and other.

### 2.3 Statistical Analysis

All statistical analyses were conducted in the R statistical package unless otherwise stated [474]. Ecological diversity and cluster analysis was conducted using "vegan" [509], "BiodiversityR" [510], "FactoMineR" [511], "factoextra"[512] and "pvcluster" [513].

#### 2.3.1 Minimum Spanning Tree Analysis

The *Salmonella* Typhimurium MLVA relationships were analyzed and visualized using minimum spanning trees of single and double locus variants conducted using the goeBURST algorithm in "Phyloviz 2.0" [515-517].

#### 2.3.2 Sample Size Estimates

To ensure the sample size within each group was sufficient to detect a true difference between groups with 95% confidence and 80% power the *S.* Typhimurium MLVA profile variation between ($s^2$ = 2.0 – 3.0) and within ($s^2$ = 21.1) groups was estimated. Using an anova power calculation for multiple groups it was estimated that at leat 50 isolates per group was required to provide sufficient analytical power for between group comparisons [510]. Sources within the non-human NEPSS dataset were aggregated where less than 50 samples were present within the category. The source population diversity for each typing method (PT, MLVA, PT/MLVA) was estimated using the Shannon-Weiner Index of diversity (H´) [555]. The null hypothesis under investigation was that there was no difference in *Salmonella* Typhimurium typing profiles (PT or MLVA, PT/MLVA) between the study-origin poultry isolates and the human and non-human NEPSS dataset.



#### 2.3.3 Cluster analysis

The *Salmonella* Typhimurium typing matrices for each source (human, non-human or poultry study) was transformed to a "species profile" proportion of typing profiles (PT, MLVA or PT/MLVA combination) to remove the effect of extremely high or low abundance typing profiles while maintaining the original composition of the source matrices [520]. The adequacy of this normalization was tested using the Shapiro test and visualization of the qqplot [521]. Euclidean distances were calculated on the transformed matrices. The difference in *Salmonella* Typhimurium population diversity was evaluated by measuring the ecological distance between sources, where the ecological distance (correlation) between sources sharing many *Salmonella* Typhimurium variants is small and the distance between sources sharing only a few variants is large. Hierarchical clustering was conducted using complete linkage on each species transformed matrix. The significance of the clustering correlation was conducted using multiscale bootstrapping (n = 1000) to estimate the approximately unbiased (AU) P-value and 95% confidence intervals. If the AU P-value is greater than 95% then clustering is supported by the data [513].

#### 2.3.4 Principal Component Analysis (PCA)

The ecological distance between sites was further evaluated using principal component analysis. The number of significant components was assessed using the broken stick test [522]. Principal components were included where the cumulative eigenvalue was greater than the equivalent broken stick value, sufficient to include no less than 75-80% of the total variance and visualized using a screeplot [521]. Typing profiles significantly contributing to the ordination were identified using the circle of equilibrium [522] and their square cosine values [523].

## 3. Results

### 3.1 Study-origin Poultry Isolates

A total of 421 *Salmonella* Typhimurium isolates from the poultry study were used for this comparison. Briefly, phenotyping (PT) and genotyping (MLVA) of 421 *Salmonella* Typhimurium isolates identified 8 phage types with 41 MLVA profiles or 62 PT/MLVA combinations. Nearly 80% of these isolates were either DT135 (19.6%) or PT135a (59.95%). MLVA profiles were not specific to individual phage types with more than 83% of the MLVA profiles shared across 5 of the 8 phage types (Table 8–62).

### 3.2 Human Isolates (NEPSS dataset)

Between January 2013 and September 2014, 3950 human cases of *Salmonella* Typhimuri-

**Table 8–63. Summary of Human and Non-Human *S.* Typhimurium PT and MLVA Combinations from the NEPSS Dataset for 2013 and 2014[1]**

| *Phage type* | *No. isolates* | *No. MLVA profiles* | | *Most Frequent MLVA Profile* | | *Shannon H´* | *MLVA present in 2013 remaining in 2014 (%)* |
|---|---|---|---|---|---|---|---|
| | | *2013* | *2014[1]* | *2013* | *2014* | | |
| DT9 | 1,122 | 159 | 149 | 03-24-13-11-523[1] | 03-24-13-11-525 | 4.51 | 37 / 160 (23) |
| DT135 | 813 | 76 | 88 | 03-15-09-12-523 | 03-14-10-09-525 | 3.70 | 19 / 82 (23) |
| PT135a | 866 | 116 | 103 | 03-15-11-11-523 | 03-15-11-11-525 | 4.35 | 30 / 116 (26) |
| DT170 | 734 | 131 | 77 | 03-09-07-13-523 | 03-09-07-13-525 | 3.68 | 31 / 131 (24) |
| DT44 | 233 | 24 | 20 | 03-10-08-10-523 | 03-10-08-10-523 | 2.63 | 7 / 24 (29) |
| DT12 | 97 | 32 | 17 | 06-11-15-11-490 | 05-15-16-00-492 | 3.34 | 1 / 32 (3) |
| DT141 | 90 | 30 | 19 | 05-15-14-12-490 | 05-15-14-12-490 | 3.52 | 4 / 30 (13) |
| DT193 | 58 | 35 | 20 | 04-14-10-00-491 | 04-14-10-00-492 | 3.91 | 3 / 35 (9) |
| All | 4,508 | 737 | 626 | 03-09-07-13-523 | 03-24-13-12-525 | 5.89 | 163 / 737 (22) |

[1]2014 is only partial to the end of September. [2]MLVA presented in the standard Australian reporting method as reported in NEPSS

um were fully characterised in the NEPSS dataset. Sixty-two phage types were reported and 85% of the isolates were from the top five most frequently identified phage types DT9, DT135, PT135a, DT170 and DT44 (Table 8–62). Phage types DT12, DT3, DT141 and DT193 comprised the next most frequently reported (7.8%) phage types with the remaining 53 phage types (Other) comprising less than 1% of the cases each. A total of 918 MLVA profiles and 1076 PT/MLVA combinations were described in the human dataset.

### 3.3 Non-Human Isolates (NEPSS Dataset)

For the coincident period, there were 558 non-human cases of *Salmonella* Typhimurium from 25 different sources. Bovine and poultry sources comprised 81.7% of all isolates reported in the NEPSS dataset (Table 8–62). A total of 34 phage types with 313 MLVA profiles and 371 PT/MLVA combinations were described. As with the human dataset the top five most frequently identified phage types accounted for a large proportion of the isolates (72%). The top four phage types were the same as those in the human population DT9, DT135, PT135a, DT170, but the fifth most frequently detected phage type was DT141 rather than DT193. The remainder of the top ten most frequently reported phage types differed by source and were not reported with the same frequency as those detected in humans and included DT197, RDNC, DT29, DT8, DT4.

### 3.4 Phage Typing and MLVA Profiling

A comparison of the most frequently reported phage types in the NEPSS dataset identified a high amount of MLVA diversity within each phage type (H′ = 5.89) (Table 8–63). Phage types with the highest diversity were DT9 and PT135a while DT44 had the least diversity in MLVA profiles. The most frequently detected MLVA profiles in 2013, were also the most frequently detected profiles in 2014, but not all the MLVA profiles detected in 2013 were present in 2014. The proportion of profiles present in both years varied by phage type and ranged from 9 - 29%.

Hierarchical cluster analysis identified a single cluster containing DT193 and DT170 PT/MLVA combinations (AU > 0.991, 95%CI: 0.997 – 0.985) but did not strongly support

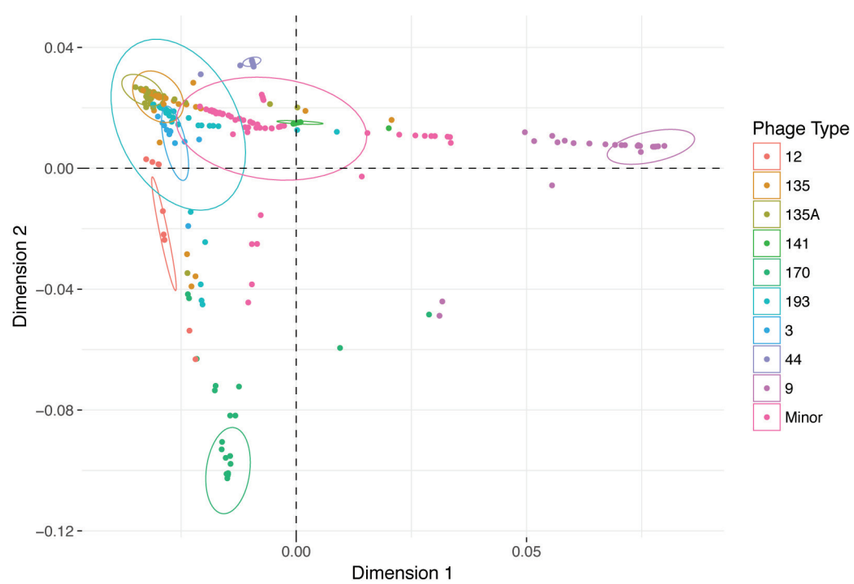



**Fig. 8–37. Principal Component Analysis of S. Typhimurium Isolates by PT and MLVA profile**

Individual locations represent the position of the MLVA profile and phage type in a two-dimensional space with 95% confidence ellipses around individual MLVA patterns with the same phage type. The variation explained within each dimension is low, this is highlighted by the large confidence ellipses around many of MLVA profiles and overlapping of phage type ellipses, indicating that MLVA profile is not unique to specific phage types.



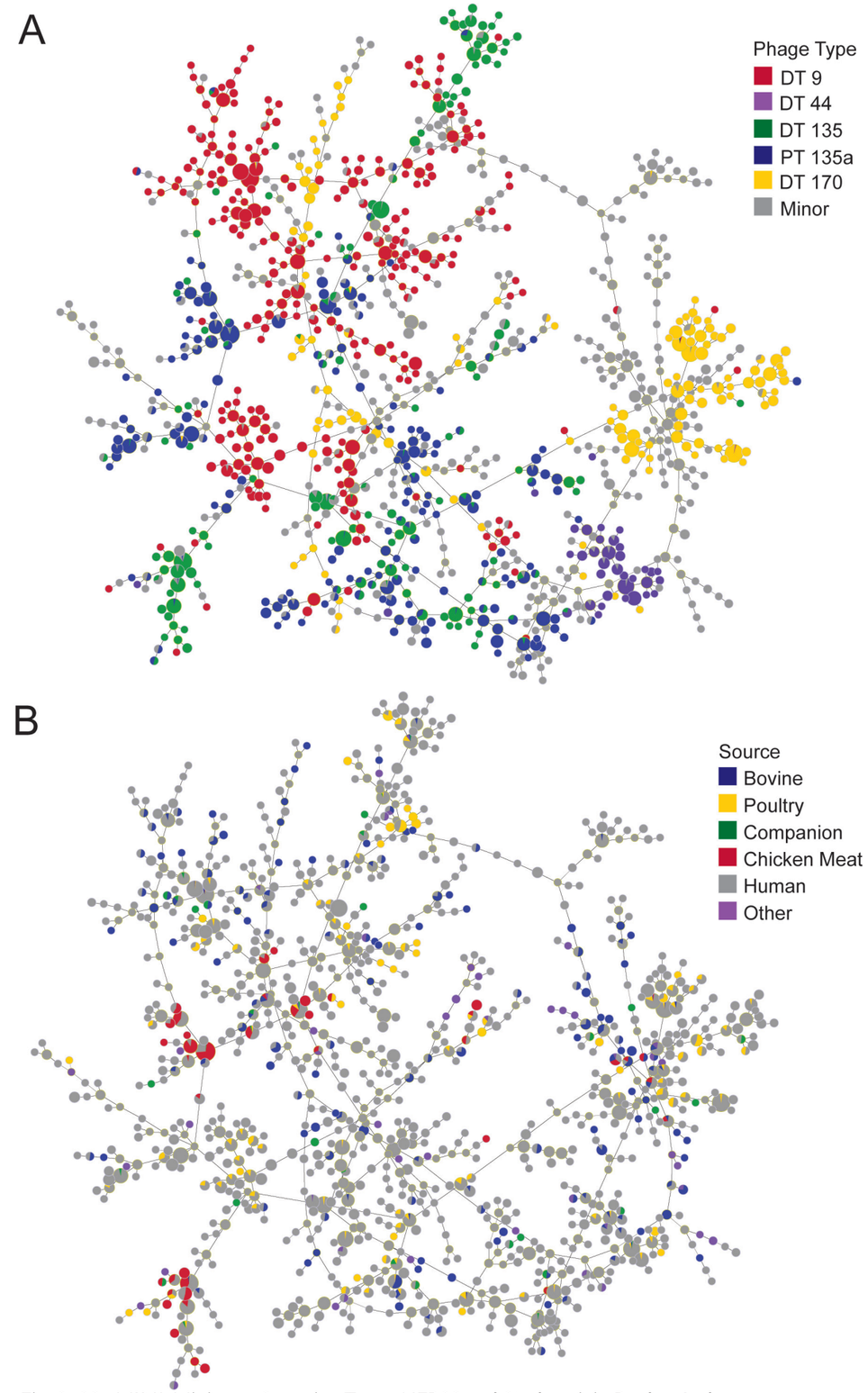



**Fig. 8–38. MLVA Minimum Spanning Trees; NEPSS and Study-origin Poultry Isolates**

All data combined into a single MST with 984 MLVA profiles joined by a single locus within the MLVA profile. A. MLVA profiles are coloured by phage type associated with that isolate. B. MLVA profiles coloured by source of isolate.

clustering of the other PT/MLVA combinations within the dataset (Fig. 8–31). The relationship between PT and MLVA profiles by PCA identified ten significant principal components that explained only 24.7% of the variance between phage type and MLVA profiles (Supplementary Table 8–64). There was significant overlap between phage types and MLVA profiles, with multiple MLVA profiles contained within each of the phage type confidence ellipses (Fig. 8–32). Only subsets of the PT/MLVA combinations (DT9, PT135a and DT170) were well defined within the PCA. These results indicate that the MLVA profile was not well correlated with phage type, so further analysis was not conducted.

### 3.5 Comparison of Salmonella Typhimurium Sources

Using PCA and minimum spanning trees (MST) the relationships between the sources of *Salmonella* Typhimurium were investigated. All MLVA patterns were linked into a single minimum spanning tree with 984 MLVA profiles linked by a single locus, 72 by two loci and 9 by three loci. The distribution of phage types within the MST is largely clustered by phage type but DT135 and PT135a were identifiable within multiple clusters within the MST (Fig. 8–33A). Non-human isolates are scattered within the human cases (Figure 4B), particularly the bovine origin isolates which appear across all branches of the MST. In contrast, the study-origin poultry isolates are tightly clustered within the tree. Hierarchical clustering and principal component analysis demonstrated that there was a difference between the typing methods (PT, MLVA or PT/MLVA combinations) in the clustering of *Salmonella* Typhimurium cases by source (Supplementary Fig. 8–34). PCA determined that the poultry study isolates and companion animal cases clustered separately from all other sources. The study-origin poultry isolates were the only source to significantly contribute to the PCA results (Supplementary Fig. 8–35).

### 3.6 Comparison of Study-origin Poultry Isolates with those Reported from NEPSS

Four of the five most frequently detected *Salmonella* Typhimurium phage types identified in the longitudinal study were also detected in the NEPSS dataset (PT9, DT135, PT135a and DT193) (Table 8–63). The study-origin poultry isolates could putatively be attributed to 511 (12.9%) of the human cases for the coincident period (Fig. 8–33B), however further cluster investigation identified seven additional sources for these isolates. Only 74 of the human cases (1.8%) were temporally associated, within a month of detection, with those isolates from the longitudinal study (Supplementary Table 3). Four other potential sources existed at the same time period.

## 4. Discussion

This study highlights several critical limitations with the epidemiological investigation of potential sources of *Salmonella* Typhimurium using the available passive surveillance data, particularly for the non attributable cases which comprise up to 93% of human cases of salmonellosis in Australia [405, 434, 435]. Firstly, the variation in phage types present in the human and non-human datasets is small. Four phage types (DT9, DT135 and PT135a, DT108/170) were the most frequently detected in both years in all data including the study-origin poultry isolates (except for DT108/170). Less than 11% of the isolates within the NEPSS dataset are from the 56 least frequently detected phage types (56/62). The dominant phage types are routinely identified in Australia from many sources as indicated in the results and other surveillance reports and studies [72, 432, 618]. This predominance of a few phage types means that further differentiation of isolates is required to cluster isolates to a common source.

Hierarchical clustering and principal component analysis demonstrated that there was a significant overlap between phage type and MLVA profile within the *Salmonella* Typhimurium cases reported to the NEPSS database, indicating that MLVA profile is not specific to a phage type, which is consistent with the biology of the tandem-repeats region. MLVA profiling of the isolates increased the diversity, but as the MLVA profiles are not unique to a phage type, does not improve attribution of the cases to a single source. This supports the phenotypic and genotypic findings of our previous poultry only study [616].



Therefore, when attempting to attribute the source of *Salmonella* Typhimurium from the longitudinal study with those isolates within the NEPSS dataset there was poor correlation between cases and sources as demonstrated by hierarchical clustering and PCA. These results demonstrate that the use of MLVA and phage typing on their own, or in combination, are inadequate, in the absence of strong epidemiological evidence, for source attribution.

Finally, this analysis is limited by the quantity of the source information obtained from the non-human NEPSS data. There were nearly as many cases from the study-origin poultry (n = 421) as from the NEPSS non-human dataset (n = 558), indicating the relative paucity of non-human data available for comparison for the coincident period. Most of the cases were from bovine (45.8%) and poultry (35.8%) sources. Grouping of source isolates (n > 50) was required to ensure that any of the clustering or PCA analysis was conducted with sufficient power to determine any associations between sources with sufficient confidence, which consequently caused loss in resolution within the dataset as most groups contained less cases than required for robust analysis. The data contained within the non-human database is comprised of opportunistically collected cases that may arise out of trace-back outbreak investigations, animal disease or environmental isolates submitted for *Salmonella* typing [617, 619]. There is no nationally coordinated structured surveillance for *Salmonella* in food animals in Australia [620]. Microbiological monitoring of the food supply occurs as part of routine HACCP based quality assurance schemes monitored by suppliers and food regulators. Thus, the non-human dataset it not a representative sample of all possible sources of *Salmonella* for human cases. Therefore, the attribution of human cases to only those sources contained within the non-human dataset should be made with extreme caution in the absence of epidemiological data.

The comparison of the study-origin poultry isolates with those identified within the NEPSS database confirmed that isolates detected in the longitudinal study may be entering the human population and contributing to human infection. However, the identification of multiple potential sources of the same or very similar *Salmonella* Typhimurium isolates supports the assertion that tools such as whole genome sequencing are necessary to differentiate their true relationships. Additionally, the disparity between the NEPSS poultry isolates and the study-origin isolates indicates that not all poultry *Salmonella* are the same and substantial source information is missing. These results are supported by similar research findings [326, 327], indicating that much remains unknown about sources of *Salmonella* Typhimurium in Australia and this would likely explain why human *Salmonella* cases in Australia mostly remain unattributed to a source.

## 5. Conclusion

The interpretation of phenotyping (phage typing) and genotyping (MLVA) results of *Salmonella* Typhimurium cases from national surveillance data for source attribution is limited by the homogeneous distribution of *Salmonella* phage types within the data. Opportunistic surveillance data from non-human sources is limited in usefulness due to the small number of cases available from many potential sources. Source attribution, in the absence of epidemiological information, using these data should be made with extreme caution. A single large source of isolates (study-origin poultry isolates) available for comparison demonstrated these isolates were not a significant source of human infection with high confidence. This study highlights the limitations of the current epidemiological tools available for the identification of salmonellosis cases non-attributable to a source and the necessity of the rapid introduction of alternate typing methods such as whole genome sequencing for the identification of all isolates.

# Supplementary Material

## 1. Hierarchical Cluster Analysis of Typing Methods

Depending on the *Salmonella* Typhimurium typing method employed different interpretations of the clustering of the sources may be made (Supplementary Figure 1). Clustering of companion animal and other source cases were strongly supported by phage typing (AU > 0.980, 95% CI: 0.970 - 0.990), clustering of companion animal and study cases was strongly supported by MLVA typing (AU > 0.972, 95% CI: 0.962 - 0.982) and PT/MLVA combinations (AU > 0.988, 95% CI: 0.980 - 0.996). PT/MLVA combination clustering also supported clustering of human and poultry isolates (AU > 0.862, 95% CI: 0.854 – 0.870), but clustering of bovine and other sourced isolates with these cases was poorly supported (AU > 0.816, 95% CI: 0.806 - 0.826).

## 2. Principal Component Analysis by Source

All the variance in the *Salmonella* Typhimurium typing (PT/MLVA combination) by source was explained in 5 principal components, with 2 components explaining 82% of the variation (Supplementary Table 8–65). The study isolates and the companion animal cases are clustered separately (different quadrants) from the bovine, human, poultry and other sources of *Salmonella* Typhimurium (Supplementary Fig. 8–35).

## 3. Cluster Analysis of Poultry Cases

The number of cases (511 (12.9%)) in the NEPSS dataset exactly matching those found in the poultry study population were identified in 7 other sources including humans. Twenty-seven (65%) of the MLVA profiles observed in the study population were detected in at least one human or non-human source population but only 24 of the 62 PT/MLVA combinations matched those in the NEPSS dataset exactly. Four large clusters containing poultry study cases were identified in the MLVA minimum spanning tree and assessed further (Supplementary Table 3). The four clusters included 465 human cases, from 4-6 sources. Between 14 and 23 PT/MLVA combinations were identified within each of the clusters, indicating that limiting cluster identification or putative source to a single matching PT or MLVA combination is flawed. Only 74 / 465 (15.9%) of the human cases could be temporally (occurring within a month of detection in the poultry study and within the human population) associated with cases from the poultry study population.

Supplementary Table 8–64. Principal Component Analysis for MLVA and Phage Type Comparison; First 10 Significant Components

| *Principal Component* | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* |
|---|---|---|---|---|---|---|---|---|---|---|
| Eigenvalue | 0.051 | 0.031 | 0.029 | 0.026 | 0.023 | 0.021 | 0.016 | 0.016 | 0.015 | 0.014 |
| % Variance | 5.100 | 3.109 | 2.97 | 2.65 | 2.27 | 20.9 | 1.88 | 1.61 | 1.56 | 1.46 |
| Cumulative % Variance | 5.100 | 8.21 | 11.18 | 13.83 | 16.10 | 18.19 | 20.8 | 21.69 | 23.25 | 24.71 |
| Broken stick % | 0.709 | 0.61 | 0.57 | 0.54 | 0.51 | 0.49 | 0.48 | 0.46 | 0.45 | 0.44 |
| Broken-stick cumulative % | 0.709 | 1.32 | 1.89 | 2.43 | 2.94 | 3.44 | 3.46 | 3.91 | 4.38 | 5.28 |

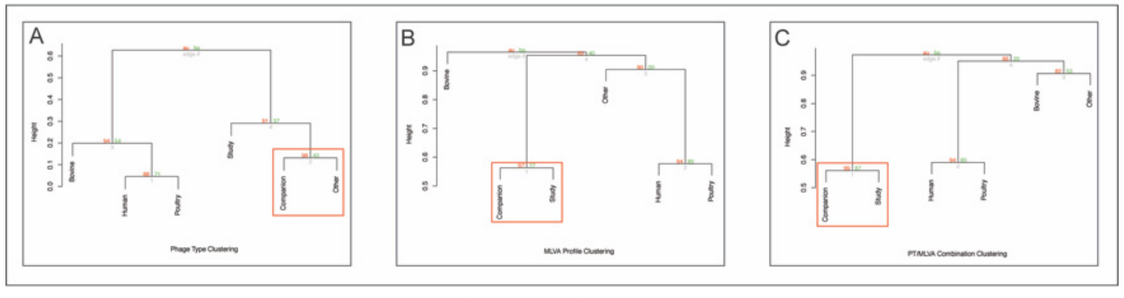


Supplementary Table 8–65. Principal Component Analysis for *Salmonella* Typhimurium Typing (PT/MLVA combinations) by Source of Isolate

| *Principal Component* | *1* | *2* | *3* | *4* | *5* |
|---|---|---|---|---|---|
| Eigenvalue | 0.026 | 0.004 | 0.003 | 0.002 | $9.47 \times 10^{-4}$ |
| % Variance | 70.35 | 12.10 | 9.11 | 5.89 | 2.54 |
| Cumulative % Variance | 70.35 | 82.45 | 91.57 | 97.46 | 100.00 |
| Broken stick % | 45.67 | 25.66 | 15.67 | 9.00 | 4.00 |
| Broken-stick cumulative % | 45.67 | 71.33 | 87.00 | 96.00 | 100.00 |

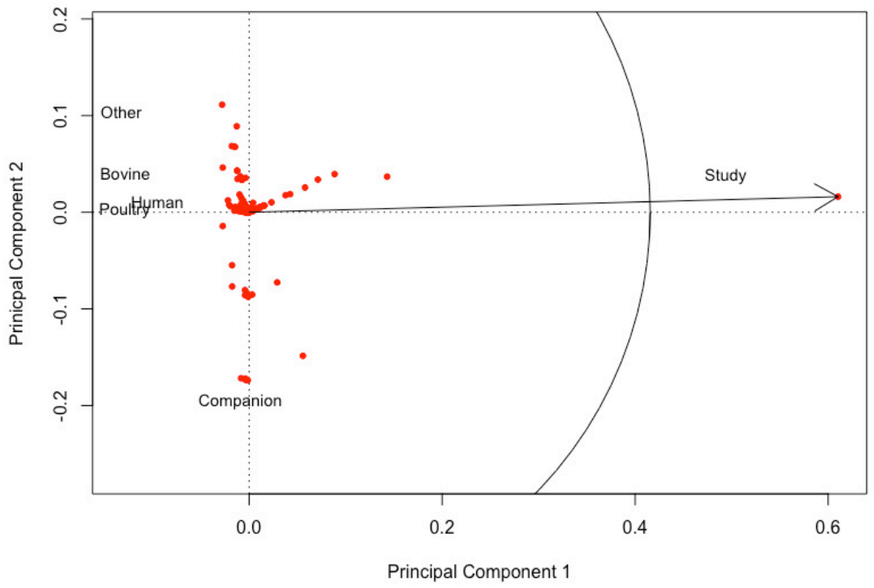
Supplementary Table 8–66. Cluster Identification and Temporal Comparison of Study-origin Poultry Isolates with MLVA profiles and Phage Types Matching Human Cases

| | | | *Human and Non-Human NEPSS Data* | | | | *Poultry Study* | |
|---|---|---|---|---|---|---|---|---|
| *Cluster* | *Year* | *Human Cases* | *Sources* | *MLVA* | *Phage Types* | *PT/MLVA Combinations* | *Within cluster attributable cases /total cases* | *Number of variants present /total variants* |
| 1 | 2013 | 80 / 2109 | 6 | 8 | 6 | 19 | 0 / 54 | 0 / 5 |
| 2 | 2013 | 45 / 2109 | 4 | 12 | 6 | 19 | 25 / 39 | 1 / 11 |
| | 2014 | 75 / 1841 | 4 | 14 | 7 | 21 | 49 / 63 | 2 / 8 |
| 3 | 2013 | 76 / 2109 | 5 | 28 | 8 | 33 | 0 / 76 | 1 / 1 |
| 4 | 2013 | 65 / 2109 | 5 | 8 | 6 | 14 | 0 / 25 | 0 / 8 |
| | 2014 | 124 / 1841 | 6 | 17 | 8 | 24 | 0 / 109 | 0 / 10 |
| All | 2013-2014 | 511 / 3950 | 7 | 1065 | 65 | 1286 | 111 / 171 | 11 / 51 |



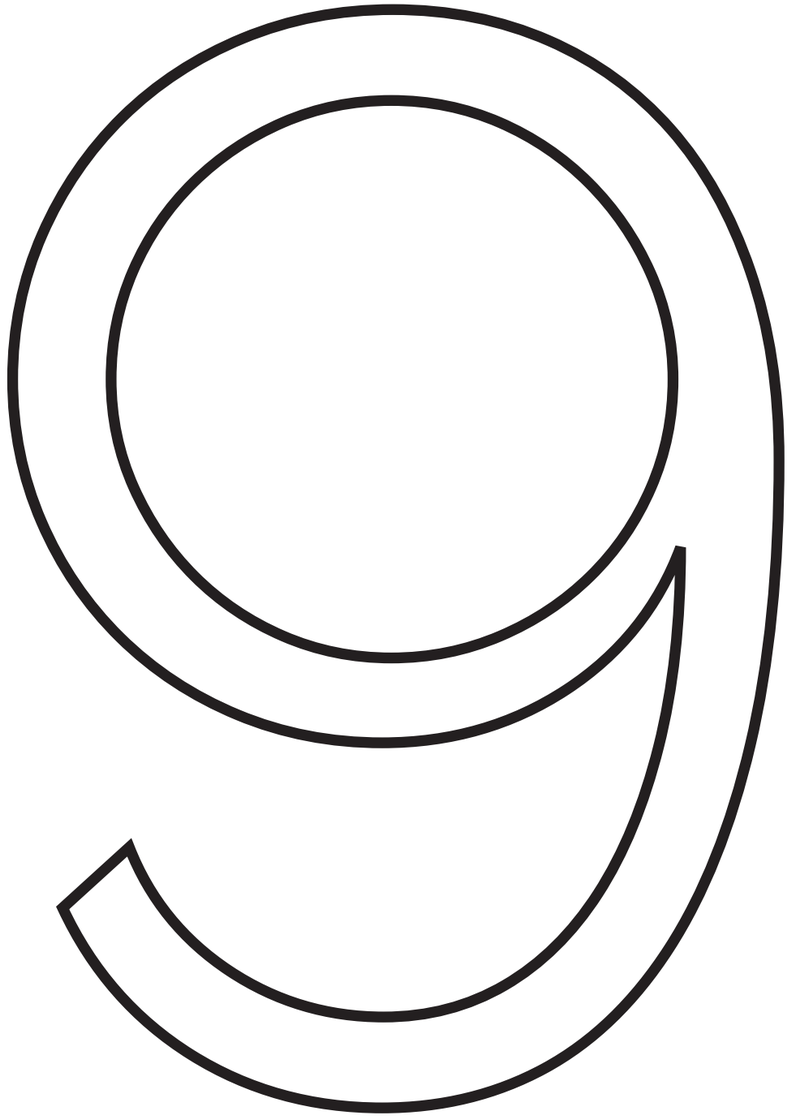

Supplementary Fig. 8–39. Hierarchical Cluster (correlation) Dendrogram of *Salmonella* Typhimurium cases by Source for each Typing Method

Significant clusters (AU < 0.05) supported by the data are highlighted in red. **A.** Clustering by Phage Type, supports clustering of the cases from companion animal and other sources (AU < 0.02). **B.** Clustering by MLVA profile, supports clustering of cases from the companion animal and study sources (AU < 0.03) and **C.** Clustering by PT/MLVA combination, also supports clustering cases from companion animal and study sources (AU < 0.01).




**Supplementary Fig. 8–40. Ordination Plot of *Salmonella* Typhimurium Typing (PT/MLVA combinations) by Source of Isolate.**

*S.* Typhimurium cases are indicated by red circles and the source is identified by text. The circle of equilibrium identifies the study-origin poultry cases (Study) as significantly contributing to the analysis (arrow indicating direction) in these two components and different from the other sources. Human, bovine, poultry and other cases are in the same quadrant indicating they are clustered together, while companion animal cases (Companion) cluster separately.



CHAPTER **NINE**

# General Discussion

*(next spread)*
*"If you always do what you've always done, you will always get what you always gotten."*
*Jessie Potter*



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# Introduction

The aim of these studies was to investigate the transmission of *Salmonella* within a vertically integrated poultry meat enterprise and use phenotyping and genotyping tools to identify and compare the *Salmonella* isolates identified within the population. The initial intention was to evaluate the usefulness of phenotyping with MLVA profiling in identifying putative entry points and transmission pathways within the enterprise. As stated in the introduction, this was not intended to be a prevalence study and so sampling was not structured to detect how much *Salmonella* was present at each part of the poultry enterprise. Rather, it was designed to reveal what *Salmonella* was present over time in each of the levels of the enterprise, from parent flock to processing, and how isolates were related to one another phenotypically and genetically. Sufficient literature in the field tells us that *Salmonella* prevalence varies widely between farms, between hosts, within hosts, over time, by serovar, sample type and method. This study was intentionally designed using previously described methods to enable comparison with other published studies.

## 1. Why is this Important?

Salmonellosis of humans is the second most frequent cause of foodborne illness in Australia. *Salmonella* Typhimurium is attributed to more than half the cases of human foodborne illness and chicken eggs and meat are frequently implicated in those outbreaks (5% of cases) when a source is identified. However, around 95% of cases are not attributable to a specific source [405]; frequently described as "sporadic cases". In all other countries, with the notable exception of Australia, the incidence of salmonellosis per 100,000 people is either declining or has not changed for a number of years (Table 1–19). The incidence in Australia, however, has been increasing annually for more than 10 years and the proportion of cases attributed to *S.* Typhimurium has also increased (Table 1–21). While the seasonal differentiation of foodborne disease is declining in most industrialised countries [621], in Australia the swing for large seasonal outbreaks of disease, particularly in summer, appears to be increasing [622].

As chicken products are frequently implicated as a source of salmonellosis, controls in chicken production systems to prevent transmission and subsequent dissemination to humans needs to be effective. A good understanding of not only the risk factors for introduction into a poultry operation, but also the dynamics of *Salmonella* transmission within a poultry operation is required. It is not sufficient to just understand what *Salmonella enterica* serovars are present and in how many birds or flocks (prevalence studies). More typing information (phenotyping or genotyping) to differentiate isolates is required to understand the temporospatial distribution and transmission of *Salmonella* strains within a vertically integrated poultry operation.

## 2. What Did I Find?

Social network analysis (Chapter 3) was used to describe the movement of poultry and poultry products within the production enterprise. All movements between the four locations (parent, hatchery, broiler, processing) were directed and acyclic, with low density, indicating only a few of the possible pathways were used at any one point in time. The pullet location was connected to 86% of the network, but only when 8-week or 17-week time intervals were considered. The pattern of movement differed by commodity (feed, eggs, live birds and people) and most moved in a single step from origin to destination on a single day, except eggs which had the longest path (moved within the production farm in multiple steps then to the hatchery and within the hatchery on multiple occasions over a period of three weeks). The network density and topology of the network (small world or scale free) varied by production system (parent, hatchery, broiler) indicating disease dynamics and transmission may be different within each component of the production system. The reproductive ratio multipliers for all commodity networks were >1, except for the feed and litter, indicating that *Salmonella* could be readily disseminated by all commodity paths, even at very low prevalence.



Sampling of the parent (egg production) flocks housed in caged facilities (Chapter 4) identified important aspects of sample design not previously reported elsewhere. Parent egg production flocks were the most intensively sampled sites, with sampling occurring every three weeks for the duration of the life of 4 production flocks. In well cleaned sheds, it was possible to observe a change in the prevalence of environmental *Salmonella* contamination as the flocks aged. This change in prevalence varied by flock, but increased over the first couple of weeks in production then declined to the end of life. Multiple sample types were superior for detecting *Salmonella* particularly at very low environmental prevalence, and boot swabs worn on concrete floors were as effective as manure belt samples in detecting *Salmonella*. Additionally, *Salmonella* was heterogeneously distributed within the sampling environment. This means that the way a shed is sampled is critical for detecting *Salmonella*, again particularly when the environmental prevalence is low. Multiple *Salmonella enterica* serovars (*S.* Typhimurium and *S.* Infantis) were found in the cage sheds. The location (row and tier) where these serovars were detected within the same shed were different, indicating that micro-environmental factors within the shed may influence differential serovar survival and subsequent detection. This further emphasizes the importance of sampling design and sample number in the detection of *Salmonella* particularly within a cage environment.

Field sampling across the entire enterprise (Chapter 5) confirmed the presence of multiple *Salmonella enterica* serovars (8) within the poultry population. A total of 4,219 samples were collected and processed and 36% (1,503) were positive for *Salmonella*. *S.* Typhimurium, *S.* Infantis, and *S.* Sofia accounted for 99% of the isolates identified with *S.* Typhimurium the most frequently detected serovar (65.1%). Only two serovars, *S.* Typhimurium and *S.* Infantis, were detected at all locations during the study. Serovar diversity was higher at the broiler location. Differentiation of *S.* Typhimurium isolates via phage typing and MLVA profiling identified eight phage types and 41 MLVA profiles, a total of 62 PT/MLVA combinations. Diversity and cluster analysis did not reveal any statistically significant difference in *S.* Typhimurium PT/MLVA combinations between locations, however it did reveal the limitations of PT and MLVA profiling within the study context. The parent sites (pullet or breeder egg production) were the most likely point of introduction of *S.* Typhimurium into the enterprise. Based on PT/MLVA profiling a total of 13 separate introductions of *S.* Typhimurium were likely to have been made into this point in the system. Diversity and cluster analysis was conducted to identify putative sources of *Salmonella* based on the phenotyping (serovar, phage type, antimicrobial resistance testing) and genotyping (MLVA) analyses (Chapter 6). As indicated above, diversity and cluster analysis did not assist in the identification of a putative source of the *S.* Typhimurium isolates. Only serotyping revealed clustering by location, with the broiler site having more *Salmonella enterica* serovar diversity than any other location. The methods used to differentiate *S.* Typhimurium isolates (PT, MLVA, PT/MLVA, Ab) is critical for describing the population diversity and ascribing a potential source or path of transmission, or relationship of one location to another. There was no apparent differences between locations based on these phenotyping and genotyping methods, indicating that either that there was truly no difference in the population composition between locations or that not all variants had been identified at each of the sampling locations. Only antimicrobial sensitivity phenotyping (Ab) indicated a moderately significant difference in clustering between the locations. Despite the large sample sizes collected, species accumulation curves indicated that not all variants had been detected (depending on typing method) within the study, highlighting the importance of sample size in influencing the results.

*S.* Typhimurium was a significant pathogen detected within the production enterprise but differentiation of isolates using traditionally employed laboratory tools was insufficient to identify the putative pathways of transmission. There was a high level of apparent diversity within the *S.* Typhimurium isolates as indicated by phenotyping and genotyping techniques but this was insufficient to deter-

mine whether or not multiple different, but similar, *Salmonella* was entering the enterprise at several different points or was being disseminated from a single point within the system. Since none of the methods employed identify the true genetic relationships between the *S.* Typhimurium isolates, whole genome sequencing was utilised to identify the genetic relationship between the isolates (Chapter 7). Two clonal lineages of *S.* Typhimurium were identified. These lineages were detected at all locations throughout the enterprise for the duration of the study and were first detected at the parent generation. The spatiotemporal findings indicate the most likely source of introduction was the parent site (pullet rearing). Half the isolates, despite being detected at different times, in different sample types or different locations, were identical, and the remaining half differed by only 2 to 11 SNPs within each lineage, indicating that there was very little change in the *S.* Typhimurium isolates throughout the study period. The two most biologically plausible explanations for this lack of variation is either: 1. Rapid dissemination with little change over a short period of time, outbreak type spread, or 2. Continuous transmission of a highly host adapted or environmentally stable variant(s) from a single point source for the duration of the study. The amount of MLVA variation (41 variants) during the study period indicates a single transmission event was unlikely. The most likely pathway of transmission is via an intergenerational path (true or pseudo vertical) path from the parent generation right through to processing. As both lineages were under neutral or purifying selection this supports ongoing transmission from a single source via the hatchery into the subsequent generations and the processing plant during the study period. Five different lineages (two clonal and three singleton isolates) of *S.* Typhimurium were detected during the study. The detection of 4 of the 5 lineages at the parent generation prior to its detection in subsequent generations, provides additional support that the parent generation was the source of *S.* Typhimurium to the subsequent locations within the enterprise. The results suggest that it is possible to differentiate between introductions of very similar isolates that are not distinguishable by phage typing or MLVA profiling but are readily grouped by whole genome sequencing.

Finally, a comparison was made between the *S.* Typhimurium isolates found in the study population and human and non-human isolates reported to the NEPSS. The poultry study isolates clustered within the human isolates, but did not contribute significantly to human infection. Analysis of the potential sources of *Salmonella* to humans (and vice-versa) showed that the usefulness of the study and non-human NEPSS data was limited by both the lack of sample numbers and *S.* Typhimurium diversity within the populations. There was considerable overlap between phage types and MLVA profiles within the dataset particularly for two most frequently reported phage types (DT135 and PT135a). This lack of phage type specificity means that source attribution in the absence of strong epidemiological evidence will remain poor unless other typing tools are implemented and surveillance in all food sources is enhanced. Additionally, the size of the dataset available from the putative source population (non-human) was small. Sample size analysis indicated that a minimum of 50 samples per group was required to adequately identify if the populations are similar. In this case only three groups could be compared with sufficient confidence and the nuance of the putative source population variation was lost.

## 3. Study Limitations

One of the key limitations of this study was not estimating *S.* Typhimurium prevalence at the hatchery from a small number of samples collected during the sampling period (pooled prevalence estimate of chick contamination via the chick papers). If this had been done it would be possible to estimate the level of *Salmonella* transmission (R0) from parent to broiler flock and estimate if the risk changed over time in the same way that the environmental prevalence was observed to change in the parent egg production flocks. Quantification would enable an estimation of the size of the transmission risk from the respective parent flocks that could be used for parameterisation of future transmission models. This was not part of the original study design, as the detection of a change in environmental contamination at the parent sites was an unexpected, yet clearly important, finding.

Egg contamination with *S.* Typhimurium was low (1-10% of eggs tested, on a single occasion), and several attempts to identify *Salmonella* contaminated eggs were conducted, but unfortunately the sampling events were opportunistic due to the high value (egg shortage) of hatching eggs at the time. As with the chick contamination estimates this information would have been very informative to enable estimation of the size of the risk as the flocks aged.

This study only collected environmental samples from all production flocks (parent, broiler) and it might be considered inadequate to determine with sufficient confidence that the source of *S.* Typhimurium between generations was the parent flocks. Confirmation of *Salmonella* infection is conducted by flock sampling for control purposes in national control programs [469]. Environmental sampling has long been established as a more sensitive indicator of flock infection and the level of environmental contamination (sample prevalence) is reflective of the level of flock prevalence. Confirmation of flock prevalence was attempted at the end of life in two parent flocks after culling but infection was not detected (results not presented, 0/700 cloacal swabs), although egg contamination was (see above), supporting the decision to confine this study to environmental samples only. These results confirm the limitations of individual bird sampling and the value of environmental sampling as the first choice for surveillance.

## 4. Conclusions

In context with what was known prior to the onset of this study, this work supports international findings regarding the complexity of *Salmonella* transmission within a vertically integrated poultry production system. Additionally, it provides valuable new information gained from both the intensive sampling and the longitudinal approach taken. Intensive sampling of parent flocks in clean sheds demonstrated that not only was the sampling method important, but both the frequency and the number of samples collected are also important because *Salmonella* distribution within shed is not homogeneous. The importance of repeated sampling within 3 weeks of a previously negative test and not only sampling at the end of flock life is highlighted.

The phenotyping (phage type) and genotyping (MLVA) results were insufficient to reveal likely entry point and transmission of *Salmonella enterica* within this production system. Both intensive sampling and whole genome sequencing results were needed to support the finding that the parent site was the most likely point of introduction and source for transmission of *S.* Typhimurium to the other locations. The social network analysis of the putative transmission paths within this enterprise show that the spread of *Salmonella* within a vertically integrated operation can occur very quickly, even at a very low prevalence, and the hatchery is a critical point for multiplication and distribution within the system. It is the only point at which clones from one generation can be disseminated to another generation within the enterprise.

Whole genome sequencing demonstrated unequivocally that phage typing of *S.* Typhimurium was insufficient for source attribution as both clonal lineages shared multiple phage types and MLVA profiles. Only the collection of many isolates enabled the identification of the putative source location. The observed phenotype and genotype diversity was not substantiated when whole genome sequencing was applied. MLVA profiling is also insufficient to determine either phage type or the source of infection within a complex epidemiological environment.

The key gap identified in this study is the source of infection or mode of introduction into the production system. Biologically plausible hypotheses suggest that either feed or primary breeding (day old) stock are the most likely source of introduction at the parent generation. If ongoing introductions from multiple sources occur it is plausible there would be changes in the *S.* Typhimurium population detectable by whole genome sequencing. Four of five potential introductions (i.e. lineages) were detected at the parent generation indicating that detection of genetically distinct populations by sequencing is possible and that different sources could be further investigated using whole genome sequencing. What

is currently unknown is how much variation in *S.* Typhimurium isolates exists in Australia. Are poultry isolates able to be fingerprinted to different sources such as companies or farms (and as putative outbreak sources for humans) or are poultry simply a very efficient multiplier of multiple sources of *Salmonella*? The vertically integrated poultry system is a victim of its own success. The features of an integrated enterprise that make production efficient and chicken meat cheap for the consumer and in many ways has driven the successes of the chicken meat industry make them particularly vulnerable to the introduction of an efficient pathogen such a *Salmonella*.

## 5. Where to Next?

Critical questions identified from this study that remain unanswered and important for our understanding of *Salmonella* transmission and subsequent control in Australia that I hope to address in my future work include the following:

1. Is the transmission path between the parent flocks and the hatchery vertical or pseudo-vertical? In other words, is *S.* Typhimurium present in the internal content of hatching eggs prior to entry to the hatchery? Is fumigation of eggs therefore a sufficient control?
2. Given the high reproductive ratio determined from the poultry movements, are interventions for the control of *Salmonella*, such as vaccination, sufficient to reduce the transmission of *Salmonella* from parent flock to the hatchery?
3. How much variation is present in *S.* Typhimurium in Australia? Can we use whole genome sequencing to confirm the findings made regarding the amount of variation in *S.* Typhimurium observed in the NEPSS data?
4. Additionally, how much variation is present in the Australian poultry population? Are they just an amplifying host effectively transmitting salmonella between species via feed or are they an important disseminator of host adapted *S.* Typhimurium lineages?
5. Given that the highly clonal lineages detected within the poultry enterprise have characteristics similar to those reported for host adapted *S.* Typhimurium populations. Are these two key lineages host adapted?

To address some of these questions the following activities have been initiated and/or completed.

1. The Poultry CRC funded an intervention study "Field trials to evaluate the efficacy of a *S.* Typhimurium live vaccine in egg layers". Poultry CRC Sub-Project 3.2.7. I was the principal investigator for the Victorian component of this project which was conducted Dec 2017 to June 2018. Using the sampling design strategy developed from the work presented above, an intervention study was conducted in 10 caged flocks. Flocks were longitudinally sampled from 0 to 40 weeks of age and eggs were sampled four times during the production period. The correlation between environmental and egg contamination and the efficacy of vaccination was evaluated. *S.* Typhimurium was detected at a very low prevalence but was highest immediately post placement at the onset of lay, consistent with hypotheses made from the findings of this current work. Additionally, it was confirmed that *S.* Typhimurium may be detected in the internal content of eggs in Australia. The final report for this project has been submitted to the Poultry CRC [612] and two manuscripts are in preparation for publication. The results of this work have been presented as follows:

- Crabb, H. K., Browning, G. F. Field trials to evaluate the efficacy of a *Salmonella* Typhimurium live vaccine in egg layers. Poultry CRC Sub-Project 3.2.7. Poultry CRC Ideas Exchange. 27 – 29 October 2016. Gold Coast, Australia.
- Crabb, H. K., Browning, G. F. Field trials to evaluate the efficacy of a *Salmonella* Typhimurium live vaccine in egg layers. Poultry CRC Sub-Project 3.2.7. Australian Egg Corporation Industry Forum. 24 -25 May 2017 Sydney, Australia.
- Crabb, H. K., Browning, G. F. Field trials to evaluate the efficacy of a *Salmonella* Typhimurium live vaccine in egg layers. Poultry CRC Sub-Project 3.2.7. Eggs Australia Regulators Forum. 3 October 2017 Sydney, Australia.
- Crabb, H. K., Browning, G. F. *Salmonella* spp. contamination of table eggs: Does the age of the hen matter? 4 - 8 September 2017. 20th World Veterinary Poultry Association Congress. Edinburgh, Scotland.

2. A genome wide association study (GWAS) is underway using the *S.* Typhimurium sequencing results obtained from the PhD study. A case control study using sensitive and phenotypically resistant isolates from the sample collection will be used to determine the correlation between putative SNPs identified in the GWAS and minimum inhibitory concentration (MIC). This work will be conducted in conjunction with the project described below. Initial investigations have identified non-clonal dissemination of β-lactamase genes within this *Salmonella* Typhimurium population. The preliminary results of this work were presented: Crabb, H. K., Allen, J. L., Devlin, J. M., Gilkerson, J. R. *The more we do the less we know: Intensive sampling fails to identify clonal dissemination of antibiotic resistance genes in poultry.* Antimicrobials 2018. 22-24 February 2018, Brisbane, Australia
3. A new study has been initiated with the PhD collaborators to investigate the transmission of *Salmonella* resistance genes in the absence of antimicrobial use. Using the sampling design developed in the PhD work and isolates obtained during the project, *Salmonella* surveillance will be repeated within the enterprise. Phenotyping, whole genome sequencing and minimum inhibitory concentration (MIC) assays will be conducted on *S.* Typhimurium isolates for antimicrobial resistance genes. The effect of antimicrobial selection pressure on changes in the *Salmonella* population structure with regards to (presence of / absence of) transmissible antimicrobial resistance genes will be investigated. Initial sampling has been conducted and phenotyping has commenced.
4. A whole genome sequencing study has been initiated to investigate the *S.* Typhimurium population diversity in non-human Australia isolates. Isolates have been collected from the University of Melbourne historical collection and *Salmonella* projects underway within the APCAH microbiology group at the University of Melbourne. One hundred and seventy isolates have been collated and sequenced to date. These isolates will be compared to isolates obtained from this PhD study, the Poultry CRC study and published sequence data available for Australia. A comparison of sequence diversity will be made. It hoped that this collection will enable an assessment of both the diversity of the *S.* Typhimurium population in animals and poultry and whether host adaptation is present in the PhD study population.
5. To investigate hypotheses proposed regarding the putative sources of *Salmonella* introduction via the parent population a new study is currently being designed in conjunction with the PhD project collaborators and the feed supplier. Funding for this work is currently being sought.

A

A P P E N D I X **A**

# Conference Abstracts

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## ANTIMICROBIALS 2018

Crabb, H. K., Allen, J. L., Devlin, J. M., Gilkerson. J. R. *The more we do the less we know: Intensive sampling fails to identify clonal dissemination of antibiotic resistance genes in poultry.* Antimicrobials 2018. 22-24 February 2018, Brisbane, Australia

Objectives
This study evaluated the usefulness of antimicrobial sensitivity testing via a phenotypic method and whole genome sequencing for the epidemiological investigation of the dissemination of *Salmonella* Typhimurium within a well sampled poultry population.
Methods
A prospective longitudinal study (18 months) was conducted in an intensive poultry production organization. No antimicrobials were used in the studied population for 18 months prior to, or for the duration of the study period. Sixty-six parent and sixty-nine broiler sites were intensively sampled, 3 weekly, for the duration of the study. Three hundred and twenty-seven purposefully selected *Salmonella* Typhimurium isolates were screened for antimicrobial susceptibility using the calibrated dichotomous sensitivity test (CDS) method [453]. All isolates were whole genome sequenced using Illumina HiSeq and sequence reads were screened for carriage of known antibiotic resistance genes using SRST2 [594].
Results
The phenotypic test identified 16.5% of isolates susceptible to all antimicrobials and three resistant phenotypes; sulphafurazole (68.5%), streptomycin (56.5%), and ampicillin (10.1%). No fluoroquinolone, cephalosporin or ESBL producing phenotypes were identified. Genotyping identified four ß-lactamase resistance genes (TEM-30, TEM-70, TEM-143 and TEM-191) in 11 isolates (3.4%). Nine of the 11 isolates were resistant to ampicillin (MIC < 8mg/L). Temporal and spatial investigation identified these genes in two parent flocks on two separate occasions at the beginning and end of egg production but nowhere else in the production system.
Discussion
Clonal dissemination of known *Salmonella* antimicrobial resistance genes within the population was not detected, despite the transmission of a highly clonal population of *Salmonella* Typhimurium. The usefulness of antimicrobial susceptibility phenotyping and genotyping for tracing isolates within a well sampled population was poor. The presence of phenotypic resistance (streptomycin), in the absence of known resistance genes, at minimum inhibitory concentrations for antimicrobials both not permitted nor available for use in poultry for nearly 20 years [613] suggests factors other than direct antimicrobial use maintain phenotype presence in this *Salmonella* Typhimurium population.
This study highlights the complexity of transmission dynamics of epidemiologically related isolates in a well- sampled population with an absence of antimicrobial selection pressure. Further work is required to understand these relationships. The more we know the less we understand.

## AUSTRALIAN VETERINARY ASSOCIATION CONFERENCE

Crabb, H. K. *Understanding Salmonella transmission in poultry using whole genome sequencing.* Australian Veterinary Association Conference. 13-18 May 2018, Brisbane, Australia.

Current routine surveillance activities for *Salmonella* spp. in commercial poultry production rely on the use of phenotyping (serotyping and phage typing) tools to determine the entry of *Salmonella* into populations. The usefulness of MLVA typing (*Salmonella* Typhimurium) for surveillance beyond outbreak investigation and trace-back to farm have not been substantiated in field studies. The rapid introduction of whole genome sequencing means some of these tools are rapidly becoming unavailable. Despite many years of surveillance and research, we still know very little about the genetic relationships between *Salmonella* Typhimurium phage types and MLVA profiles and their usefulness in routine field surveillance in commercial production settings.
In this study, I compared the use phenotyping (phage typing) and genotyping (MLVA) with whole genome sequencing to understand points of introduction and transmission of *Salmonella* Typhimurium within two poultry enterprises, egg layer and chicken meat.

## 5TH INTERNATIONAL ONE HEALTH CONGRESS

Crabb, H. K., Allen, J. L., Devlin, J. M., Gilkerson. J. R. A *longitudinal evaluation of Salmonella Typhimurium AMR prevalence and transmission using whole genome sequencing and phenotyping in a poultry population with no antimicrobial selection pressure.* 5th International One Health Congress. 22-25 June 2018, Saskatoon, Canada

Background
Australia released its first National Antimicrobial Resistance Strategy in 2015 and the implementation plan for this strategy in 2016. The use of antimicrobials in food producing animals in Australia is strictly regulated and few antimicrobial classes are available for use in poultry. It is currently unknown what the long term impact of the reduction in use of antimicrobials will have on antimicrobial resistance in specific pathogens. An ongoing longitudinal study investigating the transmission of *Salmonella* Typhimurium within an "antibiotic free" vertically integrated chicken meat enterprise has been conducted. This study evaluated the impact of antimicrobial prescribing on the prevalence of antimicrobial resistance in *Salmonella* Typhimurium isolates in this poultry population.
Methods
No antimicrobials have been used in the studied population for a period of 5 years. Three hundred and twenty-seven *S.* Typhimurium isolates were screened for antimicrobial susceptibility using the calibrated dichotomous sensitivity test (CDS) method. Four hundred and eleven isolates were whole genome sequenced using Illumina HiSeq and sequence reads were screened for the carriage of known antibiotic resistance genes using SRST2.
Results
Two clonal lineages of *S.* Typhimurium were identified in this population. The phenotypic test identified 16.5% of isolates susceptible to all antimicrobials tested and three resistant phenotypes; sulphafurazole (68.5%), streptomycin (56.5%), and ampicillin (10.1%). No fluoroquinolone, cephalosporin or ESBL producing phenotypes were identified. Genotyping identified four TEM ß-lactamase resistance genes in 11 isolates (3.4%). Nine of the 11 isolates were resistant to ampicillin (MIC ≥ 8mg/L). These genes were identified on two occasions only. No transmissible genes conferring resistance to sulphonamides or streptomycin were identified in this population.
Conclusions
Two *S.* Typhimurium lineages were clonally disseminated through a vertically integrated poultry operation with their origin at the parent sites. Whole genome sequencing failed to identify the similar dissemination of antimicrobial resistance genes within this Salmonella population. The presence of phenotypic resistance (streptomycin MIC ≥ 16mg/L), in the absence of known resistance genes, at minimum inhibitory concentrations for antimicrobials not permitted nor available for use in poultry for nearly 20 years suggests factors other than direct antimicrobial use maintain phenotype presence in this *S.* Typhimurium population. These findings have important ramifications with regards to the current drive for the reduction in the use of antimicrobials in food producing animals and its' impact on the prevalence of antimicrobial resistance and subsequent transmission via the food chain.

## INTERNATIONAL SYMPOSIUM SALMONELLA AND SALMONELLOSIS

Crabb H. K., Allen, J. L., Devlin, J. M., Gilkerson. J. R. *Where, and How You Sample Really Does Matter.* International Symposium of *Salmonella* and Salmonellosis. 25-27 September, 2018. St Malo, France.

The method of choosing environmental sampling sites is a critical part of surveillance design. This study investigated which method of choosing sampling sites (simple random, stratified, or GRTS) ensured spatial homogeneity within a structured poultry housing environment (cage, barn or aviary). At least 28 samples were required to ensure spatial evenness when sampling in a poultry house. When sample size was small (n = 5), simple random sampling was the best method for site selection, but spatial evenness was unable to

be achieved. When the sample size was large (n = 28), all methods performed equally. Nineteen cage houses were sampled longitudinally, post cleaning and during production. A total of 2,879 samples were collected on 105 sampling events. All sheds were positive for *Salmonella* spp. on at least one sampling event and multiple *Salmonella* serovars were detected. The true sample prevalence differed significantly by *Salmonella* serovar, with 38.7% (95%CI [35.5, 41.8] of all samples positive for *Salmonella* spp., 9.0% (95%CI [7.6, 10.5]) positive for *S.* Typhimurium and 14.4% (95%CI [13.0,15.8]) positive for *S.* Infantis. The positive predictive value of the sampling method was high (PPV = 0.98), with boot swabs (OR=7.7, 95%CI [5, 11]) more likely to be positive for *Salmonella* spp. than all other sample types. *S.* Infantis was more likely to be detected from boot swabs (OR = 3.5, 95%CI[3.4,7.9]), while *S.* Typhimurium was more likely to be detected from manure belt swabs (OR=2.9, 95%CI[1.9,4.7]). In wet washed houses, the sample prevalence was lower immediately post cleaning, and houses were 1.4 times (95%CI [0.4, 4.8]) more likely to have *Salmonella* spp. negative sampling events during the subsequent flock production period than sheds that were dry cleaned. Both *S.* Infantis (OR = 8.5, 95%CI [3, 22]) and *S.* Typhimurium (OR = 3.2, 95%CI [1.0, 9.9]) were less likely to be detected in wet washed sheds. Field sampling demonstrated that *Salmonella* spp. were heterogeneously distributed within a house, and that the location of detection differed by *Salmonella* serovar. Samples collected on the north side (OR= 1.5, 95%CI [1.0, 1.8] or the side sheltered by another house [OR=1.4, 95%CI [1.2, 2.0] were more likely to be positive for *Salmonella*.

Crabb H. K., Allen, J. L., Devlin, J. M., Gilkerson. J. R. *The persistence of antimicrobial resistance in Salmonella Typhimurium in a poultry population with no antimicrobial selection pressure*. International Symposium of *Salmonella* and Salmonellosis. 25-27 September, 2018. St Malo, France.

With increased scrutiny on the appropriate use of antimicrobials in intensive livestock, a key question revolves around what effect will a reduction in antimicrobial use have on antimicrobial resistance in pathogens such as *Salmonella* spp. A prospective longitudinal study was conducted in an intensive poultry production organization, in 2013-2014. Sampling was repeated in 2017-2018 from the same sample populations within the organization. No antimicrobials, for treatment or growth promotion, were used in the studied population(s) for 18 months prior to, or during the period between sampling dates (5 years).

Four hundred and seventy five *Salmonella* Typhimurium isolates were phenotyped for antimicrobial susceptibility using the calibrated dichotomous sensitivity (CDS) test method. A sample (n = 327) of phenotyped isolates were whole genome sequenced and reads were screened for carriage of known transmissible antimicrobial resistance genes.

On the first sampling event the phenotypic test identified 16.5% of isolates susceptible to all antimicrobials and three resistant phenotypes; sulphafurazole (68.5%), streptomycin (56.5%), and ampicillin (10.1%). On the second sampling event no susceptible Salmonella isolates were identified and two resistant phenotypes were identified; sulphafurazole (100%) and streptomycin (50%). No fluoroquinolone, cephalosporin or ESBL producing phenotypes were identified. Two clonal *Salmonella* Typhimurium lineages undergoing purifying selection were identified on the first sampling event. Isolates phenotypically resistant to ampicillin were more likely (OR = 145, 95%CI [7.7, 2745]) to contain an IncI-1 plasmid (pcol1B9) and a TEM resistance gene. Only 26 isolates contained the plasmid and genotyping identified four ß-lactamase resistance genes (TEM-30, TEM-70, TEM-143 and TEM-191) in 11 isolates. There was no evidence of clonal dissemination of the ß-lactamase resistance genes within this population. No transmissible sulphonamide or streptomycin resistance genes were identified in the sequenced isolates.

Despite no selection pressure for antimicrobial resistance within the host population, there was an increase in the sulphonamide resistant phenotype in the most recently tested isolates. The causal relationship between decreased use and a reduction in resistant phenotypes in the same population(s) remains unknown. Work continues to identify what host or bacterial population characteristics support the maintenance of this resistant phenotype in the *Salmonella* Typhimurium population.

Crabb H. K., Allen, J. L., Devlin, J. M., Firestone, S., Holt[1], K, Gilkerson. J. R. *Comparing phenotyping and whole genome sequencing in understanding the epidemiology of Salmonella spp. transmission in a vertically integrated broiler operation.* International Symposium of *Salmonella* and Salmonellosis. 06 - 08 June, 2016. St Malo, France.

[1]The University of Melbourne, Centre for Systems Genomics and Department of Biochemistry and Molecular Biology, Melbourne, Australia. Collaborator.

Introduction

*Salmonella enterica enterica* serovars are an important cause of foodborne illness in Australia, accounting for 16,354 disease notifications in 2014. Seventy percent of human salmonellosis cases are attributed to food and 35% of these cases have been attributed to chicken meat and eggs. This study evaluated the use of serotyping, phage typing, multivariable tandem repeat analysis (MLVA) and whole genome sequencing to compare *Salmonella* isolates identified in a longitudinal study conducted in a vertically integrated chicken meat company from Jan 2013 to July 2014.

Materials and Methods

Environmental samples were collected from breeder rearing, production, hatchery, broiler and poultry processing sites and tested for *Salmonella* spp. All *Salmonella* positive isolates were serotyped and phage typed. *Salmonella* Typhimurium isolates were MLVA typed and 396 isolates were selected for whole genome sequencing.

Results

*Salmonella* Typhimurium and *Salmonella* Infantis were the most frequently identified serovars. Seven *Salmonella* Typhimurium phage types and 42 MLVA profiles were identified. MLVA profiles were not unique to phage type and more MLVA profiles were detected at the hatchery than any other site.

The phylogenetic tree inferred from whole genome sequencing showed the *Salmonella* Typhimurium isolates clustered into 4 distinct clonal groups. All four clonal groups were detected at breeder production, and two of the four clonal groups were detected in all locations sampled during the study.

Conclusion

MLVA profiling indicated that the biological diversity increased implying the introduction of new *Salmonella* Typhimurium organisms. Whole genome sequencing demonstrated however that there were only four clonal groups present during the study and we were able to distinguish between clones of the same and different phage types indicating it may be useful for determining source attribution in epidemiological studies where complex transmission pathways exist. Vertical transmission was a more likely explanation than horizontal transmission for two of the *Salmonella* Typhimurium clones as they were detected in all locations over the entire study period.

Crabb, H. K., Allen, J. L., Devlin, J. M., Firestone, S. M., Stevenson, M. A., Gilkerson. J. R. *The use of social network analysis to examine the transmission of Salmonella spp. within a vertically integrated broiler production system.* International Symposium of *Salmonella* and Salmonellosis. 06 - 08 June 2016. St Malo, France.

Introduction

To better understand factors influencing infectious agent dispersal within a livestock population information is needed on the nature and frequency of contacts between farm enterprises. Social network analysis provides an analytical framework for these data, allowing observed patterns of contact to be described and quantified. This study describes the contact network within a vertically integrated broiler company over an 18-month period to identify the potential horizontal and vertical transmission pathways for *Salmonella* spp.

Materials and Methods

Nodes (farms, sheds, production facilities) were identified and the daily movement of products (eggs, birds, feed), people and vehicles between nodes were extracted from routinely kept farm records. A 1-week and 8-week production cycle was examined in detail and a contact network was described and modelled

using R.
Results
Both 1-week and 8-week networks had low graph density and despite a similar number of connected nodes (199 versus 251) there was a substantially lower density (32x) in the 1-week network. Paths were directed, and only two locations (breeder or feed nodes) were identified where the transmission of a single *Salmonella* spp. clone could theoretically percolate through the network to the broiler or processing nodes. Transmission along the longest path could not occur when only one week of data was examined. Only the feed transmission pathway directly connected all parts of the network.
Conclusion
Transmission pathways identified in a vertically integrated poultry organisation network were dynamic and directed. All nodes were linked by at least one movement during the study period but network density was low indicating that all potential pathways between nodes did not exist. Disease transmission via vertical or horizontal pathways can only occur along directed pathways when those pathways are present. Not all paths were present at all times and the presence of a pathway does not mean that transmission will occur.

## 3RD INTERNATIONAL ONE HEALTH CONGRESS

Crabb, H. K., Firestone, S. M., Allen, J. L., Devlin, J. M., Valcanis, M[1]., Holt, K.[2], Gilkerson. J. R. *A comparison of serotyping, phage typing, MLVA and whole genome sequencing for describing relationships between Salmonella isolates of human and poultry origin in the food chain.* 3rd International One Health Congress, 15 - 18 March 2015. Amsterdam, Netherlands. Also, presented at Australasian Veterinary Poultry Association Meeting, 11 – 14 October 2015, Queenstown, New Zealand.
[1]Microbiological Diagnostic Unit, Department of Microbiology and Immunology, The University of Melbourne, Parkville, Victoria 3010, Australia.
[2]Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria 3010, Australia.

Background
*Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most frequent causes of foodborne illness in Australia, accounting for 44% of all *Salmonella* notifications in 2010. This study evaluated the use of serotyping, phage typing, multi-locus variable number tandem repeat analysis (MLVA) and whole genome sequencing (WGS) to describe *Salmonella* isolates identified in a longitudinal study conducted in a vertically integrated chicken meat company from Jan 2013 to end July 2014, with isolates of similar subtypes (phage type or MLVA profile) identified in the local human population.
Method
Human and non-human salmonellosis data (serotype, phage type and MLVA) were extracted from the National Enteric Pathogen Surveillance System (NEPSS) database and compared with the data collected from the poultry longitudinal study. Thirty-two, *S.* Typhimurium definitive type (DT) 135 and local variant phage type (PT) 135a isolates were selected for WGS; ten poultry from the longitudinal study, two animal and twenty human isolates. An additional 12 isolates from an earlier disease outbreak were also included in the analysis. *S.* Typhimurium SL1344 was used as a reference genome and single nucleotide polymorphisms (SNPs) were determined. A maximum likelihood phylogenetic tree was inferred using the identified SNPs.
Results
In the longitudinal study, *S.* Typhimurium DT135 and PT135a were the dominant phage types identified with 22 MLVA profiles defined. These MLVA profiles were not unique to these phage types only. During the study period, 40% of the 3,000 human *S.* Typhimurium cases notified in Victoria, Australia were identified as phage types DT135 or PT135a. Among the human isolates, 253 unique MLVA profiles were present with 20 of the 22 MLVA profiles identified in the longitudinal study detected in 283 human cases. The phylogenetic tree showed that the ten *S.* Typhimurium DT135 and 135a poultry isolates and one human case clustered in a tight clonal group with less than 8 SNP differences between pairs of isolates. The remaining 33 isolates clustered separately from this group.
Conclusion
Whole genome sequencing and SNP analysis of the *S.* Typhimurium DT135 and PT135a isolates was able to identify closely related isolates and potential epidemiological links, despite no known attribution. These relationships were unclear using serotyping, phage typing or MLVA alone. These findings are informing further research into the relationships between human, poultry and other animal isolates.
N.B. This same presentation was also delivered to the Australasian Veterinary Poultry Association Meeting, 11 – 14 October 2015, Queenstown, New Zealand.

## AUSTRALIAN ASSOCIATION OF VETERINARY LABORATORY DIAGNOSTICIANS

Crabb, H. K., Devlin, J. M., Firestone, S. M., Allen, J. L., Gilkerson. J. R. Pilot Study: *Comparison of different sampling methods for the detection of Salmonella spp. in poultry sheds.* Australian Association of Veterinary Laboratory Diagnosticians Meeting. 28 November 2013. Geelong, Australia.

A pilot study was conducted to evaluate different environmental sampling strategies for the detection of *Salmonella* spp. in poultry sheds. Two known *Salmonella* spp. positive sheds, one barn and one cage type, were identified for sampling. Seven sampling methods were evaluated: drag swabs, boot swabs, collecting dust, dust swabs, faeces, manure belt swabs and egg belt swabs.
Boot swabs were the most effective method in both shed types, with 6 of 8 boot swabs positive in the cage shed, and one swab positive in the barn shed. Sampling faeces and dust were unsuccessful in detecting *Salmonella* spp. in either shed.

# B

APPENDIX **B**

# Flock Housing Details

## PARENT SITES

### PARENT REARING

Each batch (placement) of pullets reared for egg production was supplied from the primary breeding company on the day of hatch. Each batch was placed as a single biosecurity unit comprising three pullet flocks (7-10,000 hens each) and one rooster flock (5-7000 males) housed in four separate sheds. Each shed was constructed with concrete floors, 1.2 m short concrete walls, topped with bonded aluminium sandwich panel walls. The roof was steel colour-bond with a clear span design. Each shed had fully automated climate control via tunnel ventilation with evaporative cooling and pan feeders. Birds were reared on wood shavings or rice hulls (litter).

### PARENT PRODUCTION FLOCK COMPOSITION

Two *S.* Typhimurium positive pullet flock placements were selected for the longitudinal study during pullet rearing. At the point of lay (19-21 weeks) birds were transferred from the pullet rearing facility to the production facility. Pullet flocks supplying the cage production facility were divided into two parent production flocks (~25,000 birds) of equal size, comprising one half of the hens (1.5 sheds), and one half of the roosters (1/2 shed).

### PARENT PRODUCTION FACILITY

The cage production facility comprised 8 sheds (each containing a single age production flock) of ~130 m x 30 m with a fully automated feeding chain feeding, egg collection and climate controlled environment with evaporative cooling and heat exchange unit for warming, with concrete floors. Birds were housed in a Vencomatic™ colony cage system [625] consisting of 8 frames 3 tiers high, containing 1000 birds per tier in colonies of ~500 birds. Eggs were automatically collected (continuously) from nest boxes and travelled to a central egg packing room. Manure was collected on belts under the cages and removed weekly from one end of the shed (opposite direction to egg collection). The shed configuration identifying cage rows, doors, fans and ventilation openings, people thoroughfares and direction of travel for manure and eggs is illustrated in Fig. B–36. Sampling sites used for sample collection are illustrated in Fig. B–37.

## BROILER PRODUCTION SITES

Each broiler production site (farm) comprised multiple sheds (2-10) each containing a single flock. Flock sizes per shed ranged from 7,000 to 80,000 birds. All flocks and sites were managed as-all in all-out.

Each broiler shed comprised fully automated tunnel ventilated sheds with evaporative cooling. All sheds were clear span (no centralised vertical supports holding the roof), with short concrete walls (1.2 metre) topped by refrigerated sandwich panel walls and a colour-bond troof with exposed crossbeams. Fan placement was either in tunnel formation with mini-vents along the sidewalls and fans at the end of the shed, or arranged in a cross ventilation orientation. All floors were a hard clay surface and covered with fresh bedding material of either dried wood shavings or rice hulls at the start of each batch of birds.



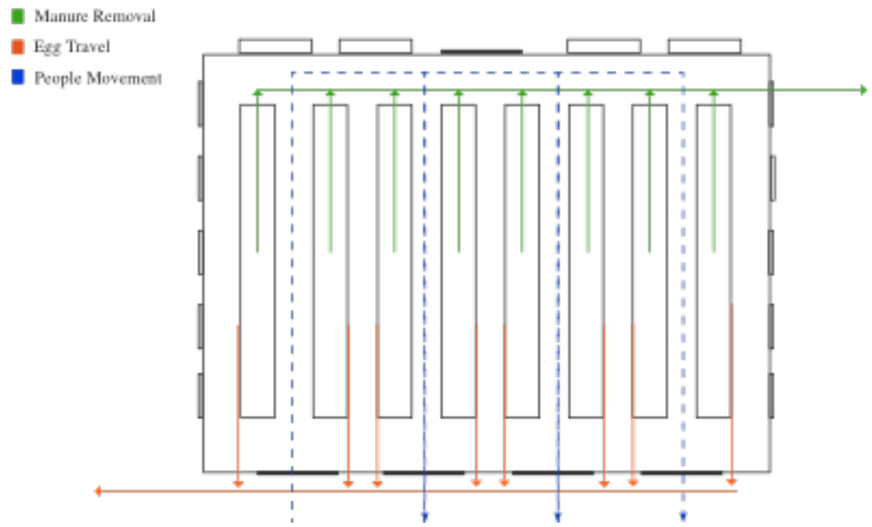



**Fig. B–41. Caged Parent Production Shed Configuration**
Direction of travel of people, eggs, and manure is indicated by the arrows on each coloured line.

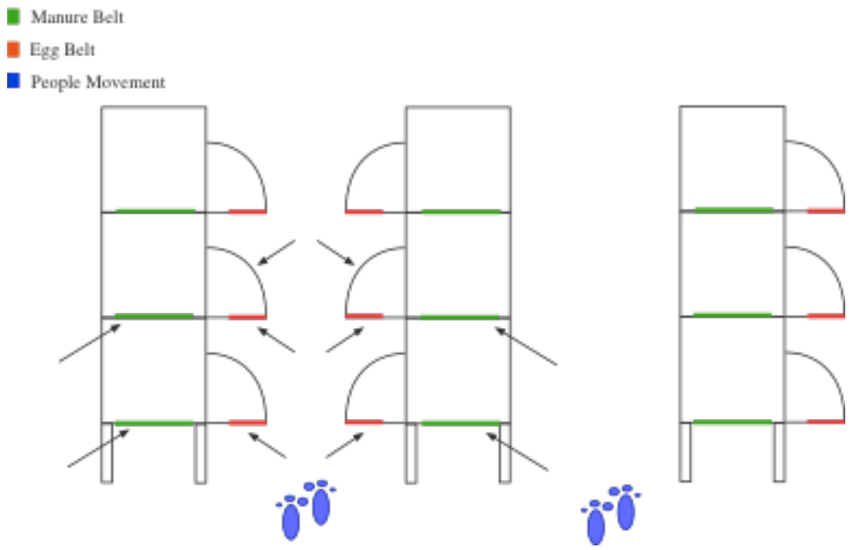
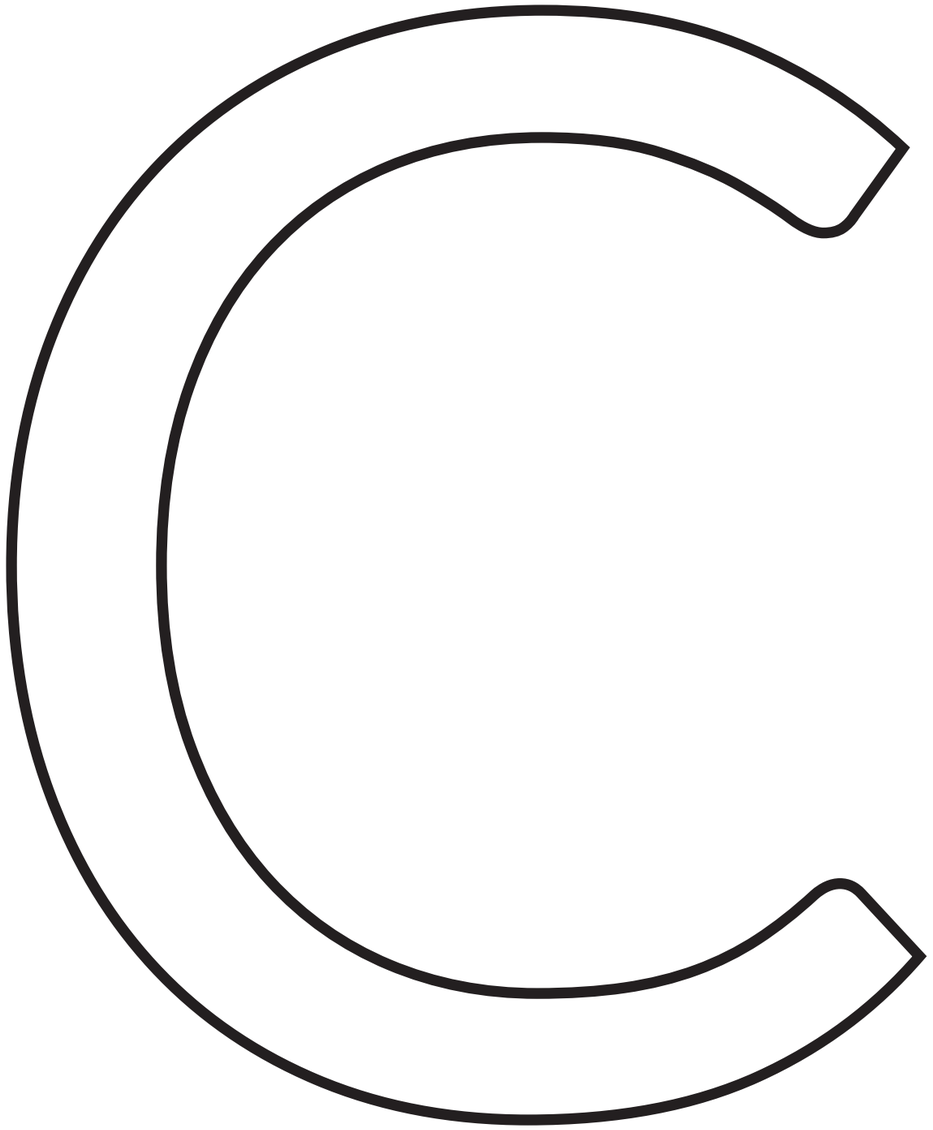



**Fig. B–42. Caged Parent Shed Sampling Locations**
Sampling locations for manure belt, egg belt and dust sampling sites are illustrated on each cage frame and tier. Walkways between cage frames are indicated.



A P P E N D I X **C**

# Media and Equipment Suppliers

Table C–67. Chemical Reagents Consumables

| | *Product Name* | *Quantity* | *Product Code* | *Batch/Lot* | *Expiry* | *Supplier* | *Address* |
|---|---|---|---|---|---|---|---|
| 1 | Novobiocin Supplement | 10mg/ml | SR0181E | 1441964 | 2016/01 | Thermo Fisher | Oxoid Ltd, Bassingstoke, Hampshire, England |
| 2 | XLD Medium | 500g | CM0469 | 1399007 | 2016/09 | | www.oxoid.co.uk |
| 3 | Modified semi-solid Rappaport Vassiliadis (MSRV) medium base | 500g | CM0910 | 1461131 | 2015/02 | | |
| 4 | Nutrient Agar | 500g | CM0003 | 1384650 | 2018/08 | | |
| 5 | CLED Medium | 500g | CM0301 | 1208548 | 2017/06 | | |
| 6 | Buffered Peptone Water | 2.5kg | CM0509 | 1390833 | 2018/09 | | |
| 7 | Tryptone Soya Broth | 500g | CM0129 | B270818 | 2007/05 | | |
| 8 | Sodium Hydroxide Pellets. Analytical Reagent. (Caustic Soda) | 500g | SA178 | 38-50 | 22/8/12 | Chemsupply | Bedford St, Allman South Australia, 5013 |
| 9 | N-Lauroyl-Sarcosine, Sodium salt | 250g | L9150 | 107H0265 | – | Sigma | |
| 10 | Sodium Chloride for Analysis. | 2 kg | 7647 14.5 | K43208004 210 | – | Merk | |
| 11 | Tris (Hydroxymethylamine) Analytical Reagent. (Tris Buffer) | 500g | TA034 9326410029358 | (10) 265552 | Rev 11/03 | | Gilman SA, 5013 |
| 12 | SeaPlaque® Agarose. Low melting temp agarose | 20g | 50101 | AG4653 | 05/08 | Cambrex BioScience | Rockland, ME USA. |
| 13 | EDTA Analytical Reagent. Ethylenediaminetet-ra-acetic acid disodium salt | 500g | K97130 | 0811430 | – | Ajax Finechem Pty Ltd | NSW, Australia |
| 14 | Lysosyme: Lysozyme egg white Ultra-Pure Grade | 10g | 0663-10G | 3116B007 | – | Amresco | Solon Ohio 44139 |
| 15 | Proteinase K Recombinant PCR grade | 25ml | 03 115 844 001 | 10130700 | Apr 2015 | Roche Diagnostics | Indianapolis, IN, USA 428 |
| 16 | Syber™ Safe DNA gel stain | 4L | S33101 | 1658547 | – | Invitrogen | |
| 17 | DNA Clean and Concentrator Kit™ -5 V1.2.1 | 50 Preps | D4003 | ZRC181086 | | Zymo Research | zymoresearch.com/m/D4003 |
| 18 | High Pure PCR Template Preparation Kit V. 20 | 100 | 11 796 828 001 | 10558200 | Oct 2015 | Roche Diagnostics | GmbH, Mannheim, Germany www.roche-applied-science.com |
| 19 | Qubit® dsDNA BR Assay kit | 500 Assays | Q32853 | 1618300 | – | Life Technologies | Eugene, Oregon, USA |
| 20 | Qubit® dsDNA BR Buffer | 500 Assays | Q32856 | 1618300 | – | | |
| 21 | HyperLadder™ 1kb | 100 lanes | BIO33025 | H1-1081 | – | Bioline | Bioline (Aust) Pty Ltd, Alexandria NSW 1435, Australia |
| 22 | DNA Loading Buffer (5x)(Blue) | 1ml | BIO37045 | HLBB108J | – | | www.bioline.com |
| 23 | Agarose Molecular grade | 500g | Bio-41825 | ES520 | – | | |

Table C–68. Consumables Supplier Details

| | *Product Name* | *Quantity* | *Code* | *Batch/Lot* | *Expiry* | *Supplier* | *Address* |
|---|---|---|---|---|---|---|---|
| 1 | Nasco Whirl-Pak® 532ml | 500 | B00736 | – | – | Thermo Fisher | Nasco-Modesto<br>Salida, California, USA<br>www.whirl-pak.com |
| 2 | Nasco Whirl-Pak® 710ml | 500 | B01297 | – | – | | |
| 3 | Carnivore Hair Nets<br>(polypropylene with double elastics) | 1000 | C933 | – | – | Start Food-Tech Australia | Leakes Rd, Laverton North Victoria AU<br>www.startfoodtech.com.au |
| 4 | Cotton Gauze Swab (Emh40<br>Non-sterile) 10 x10 cm x 8 ply | 200 | G5201 Emh40/0404 | 13201901 | – | Veterinary Companies of Australia P/L | Blacktown, NSW 2148, AU<br>emerham@vcaworld.com |
| 5 | Rayon tipped swab | 100 | 8155CIS | 015G51/DR0K01 | 2016-12 | Interpath Services P/L | Heidelberg West, Victoria 3081, AU |
| 6 | Mirella Research Sterile Bags<br>(400mm x 500mm) | 500 | AAF2 | SB 15R | – | Mirella Research Pty Ltd | Brunswick Victoria 3056, Australia<br>www.mirellaresearch.com |
| 7 | Kimiwipes (Kimtech Science Brand)<br>11 x 21 cm | 280/carton | 34120 | FL42282A | – | Kimberley Clark | Kimberley Clark Worldwide Inc USA |

Table C–69. Laboratory Equipment Model and Software

| | *Equipment* | *Model* | *Software Version* | *Manufacturer/Supplier* | *Address* |
|---|---|---|---|---|---|
| 1 | Qubit® 3.0 Fluorometer | 3.0 | APPv0.66, MCU v0.20, 2014 | Life Technologies | Thermo Fisher Scientific Eugene, Orgeon, USA |
| 2 | Nanodrop spectrophotometer | ND1000 | V3.8.1, 2010 | Thermo Fisher Scientific | Wilmington, Delaware 19810, USA |
| 3 | Electrophoresis Power supply | EPS 500/100 | N/A | Pharmaat | Fine Chemicals |
| 4 | ChemiDoc™ XRS+ Molecular Imager | AU500968 | ImageLab V3.0 build 11, 2010 | Bio-Rad | Life Science Bio-Rad Laboratories Pty., Ltd. Gladesville, NSW 2111, AU |

G L O S S A R Y

# Glossary

| *Term* | *Definition* |
|---|---|
| **Boot Swab** | Absorbent boot cover worn over boots to sample floor environments (litter, dirt or concrete). |
| **Breeder** | Hen laying fertile eggs for hatching. |
| **Breeder (Egg Production) Flock** | Flock of in-lay breeder hens and fertile males (egg production flock) that supply fertile eggs to a hatchery. Synonymous with donor flock or parent flock. |
| **Broiler** | Meat chicken from day old until ~50 days of age |
| **Chick Paper** | Absorbent material, newsprint or cardboard, used to line the inside of a chick transport basket. |
| **"Cloacal Drinking"** | Opening and closing of the cloacal opening, by which faecal material are collected ("drunk") back into the body via reverse peristalsis |
| **Day Old Chick** | Male (rooster) or female (hen) chicken on the day of hatch (~1 day old) |
| **Donor Flock** | Breeder production flock that is the source of eggs for setting (to the hatchery), or day old chicks for placement (broilers, replacement stock– pullets). See Breeder Production Flock. |
| **Drag Swab** | Cotton gauze swabs (2 - 4) tied to a long piece of string and dragged over the floor or litter surface to collect an environmental sample. |
| **Hatch Debris** | Remnants of the hatching process. Contains shell and membrane, meconium chick fluff. |
| **Para-Vertical Transmission** | See Pseudo-vertical transmission. Refers to the transmission of *Salmonella* via contamination of the internal or external surface of the egg during the hatching process. The source of chick infection is unable to be distinguished from internal or external contamination of the egg. In any other species this would be termed vertical transmission i.e. transmission from one generation to another via the dam. |
| **Parent Flock** | A term used to describe the breeding generation that produces fertile hatching eggs for the production of the commercial generation. In broiler operations parents produce hatching eggs for broiler production, in egg laying operations parent flocks produce hatching eggs to produce commercial egg laying birds. The generation preceding the parent flock is a grandparent flock. |
| **Placement** | A delivery of birds (batch or flock) to a farm site or shed. May comprise day old chicks, breeders or broilers, or transfer from one facility to another, pullets to production. A single placement may comprise birds from a single or multiple sources. |



| *Term* | *Definition* |
|---|---|
| **Placement Date** | Delivery date of a placement (delivery) of birds to a farm site or shed. For production purposes flocks are typically aged from the day of delivery to site on their placement date, with hatching day considered to be day 1. |
| **Point of Lay** | The age of an egg laying bird just prior to the onset of egg production. This is also the latest date (age) at which a transfer from one facility to another (rearing to production) is typically undertaken without compromising the onset of egg production. In broiler breeder flocks this is optimally no later than ~22 weeks of age. |
| **Production Period** | This is the time that a breeder flock (males and females) producing hatching eggs is housed on the production site from the point of lay to the end of egg production. For broiler breeder flocks this is approximately ~ 45 weeks. |
| **Pseudo-Vertical Transmission** | In poultry only, the internal contamination of an egg via penetration of the egg surface, not transovarial contamination. Unable to be distinguished from transovarial contamination other than by retrieving eggs from the oviduct of the hen at the point of lay. |
| **Pullet** | Young hen (female) from day old until the point of lay. General term used to describe both male and female chickens during the rearing period. |
| **Set** | A batch of eggs placed in a setter on a single day. Each group of eggs set in the same batch are identified by donor flock (Flock ID and date eggs laid) and setter combination. |
| **Transfer** | The movement of a flock from one location to another. Typically relates to the movement of pullet flocks from rearing facility to a production site. Transfer of pullets typically occurs at or on the point of lay. |
| **Sister Flock** | A broiler breeder production flock, the same age and source (cohort) as another breeder flock in production. Sister flocks contain birds reared on the same site or location. |
| **Vertical Transmission** | In poultry, this typically refers to the specific instance of transovarial contamination of the internal content of an egg during egg formation prior to lay |
| **Water Activity** | Water activity ($a_w$) is the partial vapour pressure of water in a substance divided by the standard state of partial vapor pressure of water. This measure is utlised to indicate the available water for growth of microbiological organisms in dry products such as grain or animal feed. |

R

R E F E R E N C E S

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