AUSTRALIAN EGG CORPORATION LIMITED

# Virulence of layer farm or egg associated *Salmonella* isolates

## Final Project Report

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# Foreword

This study was conducted to evaluate the virulence of *Salmonella* serovars commonly isolated from egg layer farms. The virulence typing was performed by PCR, *in vitro* cell invasion assay. High, medium and low invasive *Salmonella* serovars were also tested by an *in vivo* mouse infection model. *Salmonella* free pullets were hatched and raised from day old to study the *in vitro* invasive ability of different *Salmonella* serovars in the oviduct. The whole genome sequences of selected *Salmonella* serovars were also analysed. *Salmonella* Typhimurium infection in laying hens under experimental conditions was also investigated.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

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# Abbreviations

| | |
|---|---|
| ATCC | American Type Culture Collection |
| DMEM | Dulbecco's Modified Eagle's Media |
| DT | Definitive Type |
| LB | Luria-Bertani |
| MLVA | Multilocus Variable Tandem repeat analysis |
| MOI | Multiplicity of Infection |
| NS | Normal Saline |
| NTS | Non-typhoidal *Salmonella* |
| OD | Optical Density |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PT | Phage Type |
| qPCR | Quantitative Polymerase Chain Reaction |
| SA | South Australia |
| SPI | *Salmonella* Pathogenicity Island |
| ST | *Salmonella* Typhimurium |
| XLD | Xylose Lysine Deoxycholate |

# Executive Summary

*Salmonella* infection is one of the most common food borne infections worldwide. In Australia eggs have been implicated in several *Salmonella* food borne outbreaks. The central hypothesis of this study was that not all *Salmonella* isolates/serovars isolated from layer farm environments or eggshell wash possess the same level of virulence for humans.

Twenty-three virulence genes known to be involved in adhesion, invasion, intracellular replication and survival were selected to generate a virulence gene profile of the 29 *Salmonella* serovars isolated from egg farm environments. The majority of *Salmonella* serovars were positive for the virulence genes selected for this profile. Data from *in vitro* cell culture experiments revealed that the invasion rate of enriched cultures of *Salmonella* serovars was between 4.5 and 355.5 fold higher than non-enriched cultures. *Salmonella* Typhimurium definitive types exhibited the greatest overall invasion in non-polarised cells and polarised human gut cells cultured *in vitro*. The *in vivo* virulence of *Salmonella* serovars exhibiting low, moderate, and high invasion *in vitro* was tested using a mouse infection model. Clinical signs of infection and mortalities were observed in mice inoculated with both low and high doses of *S*. Typhimurium DT9, DT44, DT135, DT170=108, DT193 and *S.* Typhimurium ATCC 14028. No evidence of morbidity or mortality occurred in medium or low invasive *Salmonella* serovars.

All serovars selected for this study had the ability to attach or colonise sections of the oviduct organ cultures prepared from *Salmonella* free hens. Interestingly, the *S.* Typhimurium definitive types were not found to possess greater invasive capacity than other serotypes. Interpretation of the results of this study is limited, largely because only the invasion ability of the selected serovars was assessed *in vitro*. Single gene sequence analyses of the five pathogenicity islands of twelve *Salmonella* serovars indicated that the five specific pathogenicity islands were highly conserved amongst the twelve *Salmonella* serovars. Sufficient amino acid substitution and deletions were observed across all the pathogenicity that could affect the *in vitro* invasion and *in vivo* pathogenicity of NTS serovars. *Salmonella* was not detected in egg internal contents of *Salmonella* Typhimurium DT9 infected hens. S. Typhimurium DT9 can persist in the gut of laying hens over a prolonged period of time. Hens can then shed bacteria during stressful events.

# Overall Conclusions

- Although PCR was a rapid method for *Salmonella* spp. detection, this tool was not conclusive for discriminating virulent or non-virulent *Salmonella* serovars. Bacteria may require a combination or cluster of virulent genes to colonise in the chicken gut and cause a disease in humans. The presence or absence of virulent genes could only provide limited information in terms of the virulence potential of bacteria.
- The *in vitro* results of virulence typing of *Salmonella* serovars indicated that the *Salmonella* Typhimurium definitive types DT44, DT135, DT170=108, DT193, as well as *S.* Virchow (unknown phage type), all remained highly invasive. However, this study was conducted using a single isolate of a selected serovar. The potential within-serotype variation in virulence is important in *Salmonella* pathobiology. Future studies should be directed to study the serotype variation
- The findings of intestinal epithelial invasive assays suggest that some strains of *Salmonella* require prior enrichment to stimulate virulence. Some *Salmonella* Typhimurium isolates used in this study were highly invasive upon enrichment. If there is any point during food preparation or storage that encourages the enrichment of *Salmonella* within the food item or supply chain, the risk of potential food poisoning increases. Future studies could be directed to investigate the conditions under which *Salmonella* is amplified in a food preparation or storage environment.
- The mice infection experiment suggested that *Salmonella* Typhimurium isolates (in both high and low doses) were pathogenic and likely represent the greatest public health risk. However, it is also important to note that this risk could vary according to the health status of humans. Future studies could be conducted to study the virulence potential of enriched vs. non-enriched *Salmonella* Typhimurium isolates using this infection model.
- It is possible to raise *Salmonella* free birds, provided that good hygiene, sanitation and biosecurity conditions are maintained.
- All *Salmonella* serovars used in this experiment showed variable invasive potential in the oviduct organ cultures. These oviduct organ cultures were prepared from *Salmonella*-free laying hens. This method is an excellent way to check the attachment or colonisation of different *Salmonella* serovars within a short time, and with relatively little labour. However, further studies are essential to test their virulence in commercially hens in full lay. It would be difficult to predict the interaction between *Salmonella* and the fully functional oviduct, as many other factors such as the bird's age and environmental conditions can influence the pathogenicity of *Salmonella*. Long-term infection trials using *Salmonella*-free birds are essential to study the vertical transmission, shedding, and colonisation of several definitive types of *Salmonella* Typhimurium.
- If *Salmonella*-free or naïve birds (that have not yet been exposed to *Salmonella*) are orally infected with *S.* Typhimurium, they can shed bacteria up to 16 weeks p.i.
- The findings of the current study suggest that there was intermittent and prolonged shedding of *Salmonella* in the faeces of laying hens, along with highest eggshell contamination at the onset of lay.
- The increased faecal shedding at week 5 p.i. in both infected groups could be due to the stress related to the onset of sexual maturity.
- Throughout the experiment, *Salmonella* was not detected in egg internal contents in either group.

# 1 Introduction

In Australia, annual egg consumption on a per capita basis is increasing, with an average of 213 eggs consumed per person recorded in 2013. However, this consumption rate is low compared to some European countries and the United States. In Australia, the main egg market is shell egg sales, which account for approximately 80-85% of all eggs consumed. Of these eggs, approximately 53% are sourced from intensive cage systems in Australia [1].

The contents of eggs are an ideal growth media for micro-organisms potentially hazardous to humans. Previous reports, however, suggest that the use of cracked eggs in uncooked food is a major risk factor contributing to outbreaks of food borne illness. Cultural diversity within Australia has contributed to a far wider selection of food, incorporating a greater range of raw egg based food into our diet. In 1997, the Foodborne Disease Working Party on examining the status of foodborne illness in Australia made 12 recommendations to reduce the pathogens in food including, under Recommendation 10, relating to the wider research and regulatory context, that research should be conducted into 'methods for reducing microbial contamination of agricultural products that enter the food supply chain.'

Major studies investigating the pathogenicity of *Salmonella* serovars for human epithelial cells have shown variation in virulence amongst different serovars. These studies however have for the most part been focused on *Salmonella* Enteritidis with limited investigation on *Salmonella* Typhimurium.

In Australia, since the 1950s, there has been a gradual increase in *Salmonella* notifications [2]. However, after the 1980s, cases of reported *Salmonella* infection have increased significantly [3]. The serovar *S.* Typhimurium (28-38%) was the most predominant *Salmonella* serovar reported to The National Enteric Pathogens Surveillance Scheme (NEPSS) during the period 1990-1995 [2]. From 2006 to 2010, eggs were implicated in 92 *Salmonella* food poisoning outbreaks that resulted in 1740 cases and the rate of hospitalisation was 23% (400 / 1740). New South Wales (37 cases) and Victoria (22 cases) recorded the highest number of outbreaks in this period. Outbreaks were more frequent in warmer months of the year (October to March). Out of 92 outbreaks, most (91%) were due to the various phage types of *S.* Typhimurium. *S.* Typhimurium definitive type (DT) 170 (31.5%) and *S.* Typhimurium DT 193 (19.6%) were the most frequently recorded DTs in egg-implicated outbreaks, followed by *S.* Typhimurium DT 9 (14%) and DT 135a (8.7%). Other *Salmonella* serovars such as Singapore (3.2%), Anatum (1.08%), Virchow (1.08%) and Montevideo (1.08%) were rarely reported in egg-related outbreaks [4-8]. During this period, *S*. Infantis was not directly observed responsible for egg related food poisoning outbreaks. However, in 2010, there was a 2.2 times increase in human cases of this serovar compared with 2009 [4].

There are several *Salmonella* intervention strategies that could be adopted on farm. Reducing the environmental load of *Salmonella* in the layer shed, by adopting good management practices (such as regular cleaning of sheds), could also reduce the incidence of egg contamination. On farm *Salmonella* control strategies include:

- genetic selection for resistance to *Salmonella*
- flock management involving sanitation, flock testing, and biosecurity
- vaccination
- use of natural antimicrobial products such as prebiotics, probiotics, and organic acid.

Post-harvest methods involve eggshell decontamination using:

- chemical methods, e.g. egg washing by sanitisers, ozone, electrolysed water
- physical methods, e.g. irradiation, U.V. light, gas plasma technology
- biological methods, e.g. plant extracts [9].

However, the control of *Salmonella* shedding on farm still remains a challenge. *Salmonella* control on farm requires a multi-pronged approach targeting all the possible sources of *Salmonella* contamination [10].

This study was designed to test the hypothesis that not all *Salmonella* serovars recovered from layer farm environments or eggshell wash have the same level of virulence for humans. Virulence of representative strains of different *Salmonella* serovars for humans was tested using *in vitro* and *in vivo* model systems. Thus the main objective of the proposed study was to identify *Salmonella* serovars isolated from egg wash and poultry environmental samples with high or low invasiveness for human epithelial cells and animal models, and also to study their ability to colonise or invade cells from chicken oviduct organ cultures.

# 2 General materials and methods

## 2.1 *Salmonella* isolates

All *Salmonella* serovars were obtained from the *Salmonella* Reference Laboratory, Institute of Medical and Veterinary Science (IMVS), Adelaide. The cultures were collected on Xylose Lysine Deoxycholate (XLD agar, Oxoid Australia) and incubated overnight at 37°C. Long-term stocks were generated by preparing a bacterial suspension in brain heart infusion broth containing 20% glycerol and storing at -80°C. The list of cultures is provided in Table 2-1.

## 2.2 DNA extraction and PCR

Overnight cultures of the *Salmonella* serovars selected for this study were grown at 37°C in 4 mL Luria Bertani (LB) broth DNA was purified from $10^9$/ mL of bacteria using the Promega Genomic DNA kit (Promega, USA). Purified DNA was quantified using a NanoDrop™ spectrophotometer (Thermo Scientific, Australia). Working DNA solutions were prepared by diluting stock solution to 5 ng/µl. Primers for each gene were designed using the primer design feature in GenBank or obtained from Hughes et al 2008. Primers were obtained from GeneWorks (Adelaide, South Australia) (Table 2-2). PCR mix was comprised of 1X Taq Polymerase Buffer (Fisher Biotec, Australia), 1.0 mM $MgCl_2$, 0.5 µm forward and reverse primers, 0.2 µM dNTPs, 0.3 units Taq Polymerase (Fisher Biotec, Australia) and 10 ng *Salmonella* DNA.

Product sizes for each of the genes were relatively similar therefore PCR reaction conditions were nearly similar. The primer annealing temperatures were the only variation during PCR reaction and are listed in Table 2-2. The general reaction protocol was Step 1: 95°C 5 minutes, Step 2: (30 cycle repeats): 95°C 30 seconds (melt), (See Table 2-2) °C 45 seconds (annealing), 72°C 1 minute (extension), Step 3: 72°C 4 minutes, Step 4: 8°C hold.

Twenty-nine genes known to be involved in *Salmonella* virulence or survival were selected. Primers were either designed using the genome of the reference strain LT2 and utilising the primer design feature in BLAST, or they were obtained from Hughes et al 2008 [11].

**Table 2-1 List of *Salmonella* serovars used in the current study**

| Serovar | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|
| **Typhimurium DT9** | 17 | 69 | 17 | 50 |
| **Typhimurium DT44** | 43 | 35 | 12 | 1 |
| **Typhimurium DT135** | 7 | 16 | 2 | 13 |
| **Typhimurium DT 170 (=108)** | 48 | 79 | 17 | 9 |
| **Typhimurium DT193** | 2 | 14 | 0 | 7 |
| **Adelaide** | 0 | 2 | 0 | 7 |
| **Agona** | 18 | 85 | 25 | 4 |
| **Anatum** | 16 | 46 | 0 | 13 |
| **Bredeney** | 0 | 1 | 0 | 0 |
| **Cerro** | 2 | 0 | 0 | 0 |
| **Chester** | 3 | 1 | 0 | 3 |
| **Havana** | 0 | 26 | 0 | 0 |
| **Infantis** | 27 | 122 | 9 | 7 |
| **Johannesburg** | 0 | 3 | 0 | 0 |
| **Kiambu** | 7 | 19 | 0 | 0 |
| **Lille** | 3 | 4 | 0 | 0 |
| **Livingstone** | 2 | 0 | 0 | 0 |
| **Mbandaka** | 11 | 10 | 1 | 129 |
| **Montevideo** | 12 | 49 | 1 | 0 |
| **Ohio** | 17 | 25 | 6 | 0 |
| **Orion** | 1 | 3 | 1 | 0 |
| **Orion var + 15, +34** | 10 | 28 | 0 | 0 |
| **Oranienburg** | 0 | 0 | 0 | 73 |
| **Senftenberg** | 3 | 5 | 0 | 0 |
| **Singapore** | 12 | 42 | 3 | 1 |
| **Subspecies I ser 4,12:d** | 6 | 8 | 1 | 4 |
| **Virchow** | 12 | 12 | 1 | 1 |
| **Worthington** | 0 | 46 | 12 | 125 |
| **Zanzibar** | 0 | 5 | 0 | 0 |

This list was obtained from *Salmonella* Reference Centre, IMVS, Adelaide, SA.
Numbers reported reflect the frequency of isolations from eggshell wash or egg farm environment.

## Table 2-2 PCR primers for amplification of virulence genes

| Virulence Gene | Forward primer (5' – 3') | Reverse Primer (5' - 3') | Product size (base pairs) | Annealing Temperature (°C) | Reference |
|---|---|---|---|---|---|
| *prgH* | GCCCGAGCAGCCTG AGAAGTTAGAAA | TGAAATGAGCGCCCC TTGAGCCAGTC | 755 | 55 | [11] |
| *InvA* | CTGGCGGTGGGTTTT GTTGTCTTCTCTATT | AGTTTCTCCCCCTCT TCATGCGTTACCC | 1062 | 60 | [11] |
| *sitC* | CAGTATATGCTCAAC GCGATGTGGGTCTCC | CGGGGCGAAAATAAA GGCTGTGATGAAC | 740 | 64 | [11] |
| *spiC* | CCTGGATAATGACTA TTGAT | AGTTTATGGTGATTG CGTAT | 300 | 56 | [11] |
| *sifA* | TTTGCCGAACGCGCC CCCACACG | GTTGCCTTTTCTTGC GCTTTCCACCCATCT | 448 | 62 | [11] |
| *misL* | GTCGGCGAATGCCG CGAATA | GCGCTGTTAACGCTA ATAGT | 540 | 58 | [11] |
| *orfL* | GGAGTATCGATAAAG ATGTT | GCGCGTAACGTCAGA ATCAA | 331 | 56 | [11] |
| *pipD* | CGGCGATTCATGACT TTGAT | CGTTATCATTCGGAT CGTAA | 398 | 58 | [11] |
| *Iron* | ACTGGCACGGCTCG CTGTCGCTCTAT | CGCTTTACCGCCGTT CTGCCACTGC | 1204 | 60 | [11] |
| *PefA* | GCGCCGCTCAGCCG AACCAG | CAGCAGAAGCCCAG GAAACAGTG | 154 | 58 | [11] |
| *spvC* | TCTCTGCATTTCGCC ACCAT | TGCACAACCAAATGC GGAAG | 563 | 58 | [12] |
| *sipA* | TACCCCTGCTGCTAC GTAAT | CTCCAGGGCTTTACG TATCA | 916 | 60 | [12] |
| *sipB* | TGGCAGGCGATGATT GAGTC | CCCATAATGCGGTTC GTTTC | 641 | 58 | [12] |
| *sipD* | TGCGTCAGCGTCTGT AATGT | GGCCTTATTTAGCGC TTCGC | 588 | 58 | This study |
| *fliC* | TACGCTGAATGTGCA ACAAA | TACCGTCATCTGCAG TGTAT | 553 | 58 | [12] |
| *sopA* | GCCCACGGTTTCTGA AGGTA | AAAGAGTCCGCTGTG AGTGG | 982 | 60 | This study |
| *sopB* | GAAGACTACCAGGC GCACTT | TTGTGGATGTCCACG GTGAG | 804 | 55 | This study |
| *AvrA* | ATACTGCTTCCCGCC GC | ACACCGAAGCATTGA CCTGT | 667 | 58 | This study |
| *SptP* | TTCACCCTATCCGCC AGGTA | GTGTAGCCCGGTTCT CACAA | 658 | 58 | This study |
| *hila* | CACCAACCCGCTTCT CTCTT | ATTGTGGTCCAGCTC TGTCG | 345 | 58 | [13] |
| *Xth-a* | CGAAAAACACCAGCC CGATG | CCGGCAGGAAGGAG CATTTA | 479 | 55 | [14] |
| *YafD* | CGGATCCGTATCCTC GTGTG | ATCGTCAGTGAAACG CACCT | 531 | 55 | [14] |
| *Stn* | CTTTGGTCGTAAAAT AAGGCG | TGCCCAAAGCAGAGA GATTC | 260 | 55 | [14] |

Primers used were designed using CLC Sequence Viewer to the strain, *Salmonella* Typhimurium LT2 (GenBank) or obtained from the literature.

## 2.3 Tissue culture

A human colonic carcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC). The human intestinal Caco-2 cell line has been widely used as a model of the intestinal barrier. The cell line was originally obtained from a human colon adenocarcinoma. Caco-2 cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) (HyClone, Australia) supplemented with 10% foetal bovine serum (HyClone, Australia) containing 100 U/mL penicillin and 100 µg/mL streptomycin (HyClone, Australia). Caco-2 cells were expanded and maintained as frozen stocks. Cell line stocks tested negative for mycoplasma. Cells were used between passage 4 and 9 for gentamicin-protection invasion assays [19].

## 2.4 Gentamycin protection *in vitro* invasion assay

The gentamycin protection assay was performed using Caco-2 cells and used to characterise the invasive capacity of the selected *Salmonella* serotypes into epithelial tissue. Polarised monolayers of Caco-2 cells were obtained by seeding cells into each well of a 48 well tray. The progression to polarisation was monitored by measuring alkaline phosphatase production. When alkaline phosphatase levels plateaued (after 12-13 days), cells were considered to be polarised and were used within 24 hours. Cell culture medium was changed every other day during polarisation. All invasion assays were repeated five times.

For the invasion assay experiments, bacteria were plated fresh 24 hours prior to experiment. Suspensions were prepared in 0.9% NaCl by selecting individual colonies from agar plates. The optical density (OD) 600 of the suspensions for each serovar was measured and adjusted to between 0.150-0.200 corresponding to $10^8$ cfu/mL. An inoculum check was performed by doing a dilution series of the stock on nutrient agar plates.

Invasion assays were also performed using bacteria prepared in LB suspensions. These were prepared by selecting a single colony from a pure culture and placing it into 3 mL LB broth. Tubes were incubated at 37°C for 6 hours with shaking at 50 rpm. 10 µl of this suspension was then added to 4 mL LB and incubated overnight at 37°C with shaking at 50 rpm. This method yielded bacterial cultures that were in a stationary phase of growth [19].

On the day of the invasion assay, Caco-2 cells were lifted from a single well from each of two 48 well trays and counted using a haemocytometer. An average number of cells was obtained and used to calculate the multiplicity of infection (MOI). An MOI of 100 was selected to statistically distinguish between low, mid, and high invasive *Salmonella* serovars.

Prior to the addition of bacteria, Caco-2 cells were washed three times with DMEM containing no supplements to remove any residual culture media antibiotics or foetal bovine serum (FBS). The final DMEM wash was replaced with 500 µl fresh DMEM. Bacteria were added to individual wells. Experiments were performed in replicate. Following the addition of *Salmonella*, Caco-2 cells were incubated with bacteria for 2 hours at 37°C + 5% $CO_2$. Cells were then washed three times with DMEM with no supplements. To kill any adherent or extracellular bacteria, 400 µg/mL gentamycin diluted in DMEM was added to the Caco-2 cells. Cells were incubated in gentamycin for 15 minutes at 37°C + 5% $CO_2$. Cells were then washed three times with calcium and magnesium-free Phosphate Buffered Saline (PBS). Finally, 1 ml of sterile 1% Triton X 100 was added to each well to lyse the cells and collect the bacteria. A serial 10-fold dilution series were prepared from the 1 mL of bacteria/cell lysate. 100 µl of each dilution was plated on to nutrient agar plates. Plates were incubated at 37°C overnight and colonies were counted for each dilution of each serovar.

## 2.5 Mouse infection trial with selected isolates

The BALB/c mouse strain was selected for these studies. Mouse challenge studies were performed according to animal ethics protocol approved by the University of Adelaide Animal Ethics Committee (UA Approval No. S2013-157). Six to eight week old, specific pathogen free, female mice weighing between 10-14 g were obtained from Laboratory Animal Services (University of Adelaide). Mice were raised in isolator cages and fed on a *Salmonella*–free commercial diet. Sterilised food and water were supplied *ad libitum* until the end of the experiment.

*Salmonella* serovars with different cell-invasion potential (as determined from data obtained from *in vitro* experiments) were used for *in vivo* experiments (Table 2-3). Bacterial isolates were grown overnight in Luria-Bertani (LB) broth and serially diluted in PBS to obtain the required cell counts. Mice from each treatment group were inoculated by oral gavage with either $10^3$ or $10^5$ CFU/mL µL of LB containing *Salmonella* culture, which was within a range that has been detected on the surface of eggs contaminated with *Salmonella* [33]. Control mice were inoculated with sterile LB. Each treatment group contained seven animals. To exclude observer bias in the interpretation of results, all clinical and bacteriological assessments were conducted by personnel blinded to the identity of the challenge isolates.

Faecal pellets were collected from each mouse at days 3, 6, 9, 12, 15 and 18 post infection (p.i.) and processed for enumeration of challenge isolates. Challenged mice were observed at least twice daily for mortality and clinical parameters of disease including ruffled fur, hunching behaviour and lethargy for up to 21 days p.i.. Mice showing all of the above symptoms were considered moribund and euthanised by carbon dioxide asphyxiation. An experiment was terminated at day 21 p.i. when all the surviving mice were euthanised. The ATCC *Salmonella* Typhimurium strain 14028 was included as a positive control. This ATCC strain of S. Typhimurium was selected as a positive control because of its known virulence. Negative control was LB broth.

**Table 2-3 Treatment groups for *Salmonella* infection trial in mice**

| ***Salmonella* Serovar/phage type** | **Invasive ability based on *in vitro* assay** |
|---|---|
| *Salmonella* Typhimurium DT 9 | High |
| *Salmonella* Typhimurium DT 44 | High |
| *Salmonella* Infantis | Low |
| *Salmonella* Senftenberg | Moderate |
| *Salmonella* Orion | Low |
| *Salmonella* Adelaide | Low |
| *Salmonella* Typhimurium DT 170 | High |
| *Salmonella* Typhimurium DT 193 | High |
| *Salmonella* Typhimurium DT 135 | High |
| *Salmonella* Bredeney | Moderate |
| *Salmonella* Lille | Low |
| *Salmonella* Cerro | Low |
| *Salmonella* Oranienburg | Moderate |
| *Salmonella* Virchow | Moderate |
| *Salmonella* Montevideo | Moderate |
| *Salmonella* ATCC strain (control positive) | High |
| Sham control (negative control) | |

Seven animals were used per treatment group including negative control.

## 2.6 DNA extraction from faecal samples for qPCR analysis

DNA from faeces was extracted using a QIAamp DNA stool mini kit (Qiagen, Australia). Faecal samples (0.2 g) collected from 0 to 18 days post infection (p.i.) from each treatment group were weighed and dispensed into microcentrifuge tubes containing 2 mL of Aamp stool lysis buffer. The samples were vortexed and heated in a 70°C water bath for 5 min. The samples were centrifuged at 4,800 × *g* for 10 min and 120 µL of the supernatant was transferred to another clean microcentrifuge tube containing an InhibitEx tablet (Qiagen, Australia). The samples were vortexed and stored at room temperature for 1 min. The samples were then centrifuged at 4,800 × *g* for 10 min and 200 µL of the resulting supernatant was treated with 15 µL of proteinase K and 200 µL of Aamp lysis buffer. The mixture was reheated at 70°C and transferred to a spin column. Washing and elution was performed according to the manufacturer's instructions. The elution volume was 100 µL. Extracted DNA was quantified using a spectrophotometer (NanoDrop™ ND 1000) and stored at −70°C until used for real-time PCR (RT-PCR). Five nanograms of faecal DNA were used for the real-time PCR reaction.

## 2.7 qPCR (real-time PCR)

*Salmonella* shedding in faecal material was quantified using real-time PCR (RT-PCR). RT-PCR was performed using a Rotor Gene 3000 real time PCR machine (Qiagen, Australia) and a TaqMan® *Salmonella enterica* Detection Kit (Applied Biosystems, Australia). Each reaction contained 9 µl of qPCR supermix and 6 µl of DNA template (12 ng) in a total reaction volume of 15 µl. The cycling parameters were 95°C for 10 min, then 40 cycles at 95°C for 15 s followed by 60°C for 60 s. All real time PCR runs included a negative and positive control. The data were analysed by two-way ANOVA.

A standard curve was generated by preparing a serial dilution of a *Salmonella* strain. Bacteria were resuscitated on XLD agar overnight at 37°C. The individual isolated colonies were then suspended in 2 mL of PBS and matched with a 0.5 McFarland standard (bioMerieux Australia). Serial dilutions were performed to achieve $10^8$ CFU/mL. The CFUs were confirmed by spreading serial dilutions on XLD agar plates. In order to determine the limit of detection of qPCR, faecal samples were spiked with various concentrations ($10^8$ CFU/mL to $10^0$ CFU/mL) of *Salmonella.* qPCR was performed on serial dilutions ($10^8$ to $10^0$) of genomic DNA and a proportionality relationship was produced by plotting the Ct value against the logarithm CFU number. *Salmonella* copies were calculated using a standard curve prepared by serial 10-fold dilution of a cultured *Salmonella* spp.

## 2.8 Raising *Salmonella* negative pullets for oviduct invasion assay

Fertile eggs were obtained from commercial brown layer parent flocks. Eggs were fumigated with formaldehyde gas (immediately up on incubation with vents closed for 20 mins) and incubated in the laboratory over the period of 21 days at 100.4 $^0$F with relative humidity of 45-55 % up to day 18 and then 55-65% up to hatching. The chicks (n=26) were hatched at day 21 and housed in positive pressure rooms at Roseworthy campus, the University of Adelaide. These rooms had been previously decontaminated with F10 SC veterinary disinfectant and then fumigated with formaldehyde. All animal pens, cages, trays, feeders, equipment, floor and walls of the rooms were cleaned and disinfected with FoamClean S (Chemetall, Australia) followed by a wash in Sanigard (Chemetall, Australia). Waterlines were internally cleaned using acid solution (Circacid, Chemetall, Australia). All equipment was then moved into each of two rooms and then rooms were fumigated with Sanigard (Chemetall, Australia).

Over the 26 week period, workers wore sterilised overalls, head-covers, shoe covers, masks, and gloves while working with the chickens. Feed was sterilised by either gamma irradiation by Steritech or by fumigation with formaldehyde. Water was treated by the addition of chlorine tablets. *Salmonella* positive or negative status of the birds was monitored by traditional *Salmonella* isolation methods as well as PCR testing [33]. Sterilised or fumigated feed and water were tested for presence of *Salmonella* spp. at regular intervals. Faecal sample, and water and feed samples were tested at regular intervals. The animal trial was terminated at week 30 of flock age. All housing procedures and experiments were performed according to animal ethics protocol approved by the University of Adelaide (UA) Animal Ethics Committee (UA Approval No. S-2012-170).

## 2.9 *In vitro* oviduct invasion assay

The invasion potential of Non-typhoidal *Salmonella* (NTS) serovars into sections of the oviduct was performed *in vitro* using methods similar to the Caco-2 invasion assay protocol. Birds were humanly euthanised by a high dose of intravenous Lethabarb injection and the entire oviduct from infundibulum to vagina was removed from each bird. The oviduct was divided into sections containing only infundibulum, isthmus, magnum, shell gland or vagina. Using a 5 mm biopsy punch, sections of the infundibulum, isthmus and vagina were collected. For collecting sections of magnum and shell gland, a 10 mm biopsy punch was used. Punches were placed into the wells of a 48 well tissue culture tray (Nunc, ThermoScientific Australia) containing DMEM.

Bacteria cultures were started 48 hours prior to oviduct invasion experiments. Forty-eight (48) hours prior to the experiments bacteria were resuscitated from frozen culture and streaked on to nutrient agar plates. The day prior to the experiments, a single colony from each culture was placed in to 5 ml of LB broth and was cultured at 37°C with

shaking at 50 rpm for 6 hours. 10 µl of this suspension was then added to 4 mL LB and incubated overnight at 37°C with shaking at 50 rpm. This method yielded bacterial cultures that were in a stationary phase of growth. The optical density (OD) 600 of the suspensions for each serovar was measured and adjusted to between 0.150-0.200 corresponding to$10^8$ cfu/mL. An inoculum check was performed by doing a dilution series of the stock on nutrient agar plates.

$10^6$ CFU of each serovar were added to each well containing an oviduct punch. Experiments were performed in replicate. Following the addition of *Salmonella*, oviduct punches were incubated with bacteria for 2 hours at 37°C + 5% $CO_2$. Oviduct punches were then washed three times with DMEM containing no supplements. To kill any adherent or extracellular bacteria, 400 µg/mL gentamycin diluted in DMEM was added to the tissue punches and incubated for 15 minutes at 37°C + 5% $CO_2$. Tissue punches were then washed three times with DMEM. Punches were collected and homogenised at 10,000 rpm for 10 minutes in a Bullet Blender® (BioTools, Australia) using sterile stainless steel beads (0.5-2.0 mm in diameter). Serial 10-fold dilution series were prepared from the 1 mL of bacteria/tissue homogenate. 100 µl of each dilution was plated on to XLD plates. Plates were incubated at 37°C overnight and colonies were counted for each dilution of each serovar.

## 2.10 Whole genome sequencing

Whole genome sequencing was performed on the nine (9) *Salmonella* serovars selected for this study. *Salmonella* were cultured on nutrient agar. A single colony was selected and grown overnight at 37°C with shaking (100 rpm) in Brain Heart Infusion Broth. Bacterial DNA was purified from the overnight culture using the Promega Wizard Genomic DNA Purification Kit (Promega, Wisconsin, USA). The quality of DNA was assessed by NanoDrop™ and agarose gel. Sequencing was performed by the Australian Genomic Research Facility (AGRF, Melbourne, Victoria Australia) using the Illumina MiSeq platform.

## 2.11 Sequence analysis

Sequences were analysed using CLC Genomics Workbench (Version 7.0.4). Sequences were trimmed and de novo assembly was performed to obtain large contigs of no less than 1000 base pairs. Single gene analysis of five *Salmonella* specific pathogenicity islands (SPI) 1, 2, 3, 4 and 5 was performed using *Salmonella* LT2 (NC_003197) as a reference strain.

## 2.12 Statistical analysis

The data were normally distributed. One-way Analysis of Variance (ANOVA) was used with Tukey's correction for multiple comparisons. Kaplan-Meier survival curves were generated for the *in vivo* mouse infection experiment. A log-rank Mantel-Cox test was used to compare survival curves. All tests were run using either SPSS Version 21 or GraphPad Prism Version 6.0.

# 3 Virulence profiling of *Salmonella* serovars

## 3.1 Introduction

The *Salmonella* genome encodes numerous virulence factors that enable the bacterium to gain access to intestinal epithelial cells but also replicate within those cells as well as evade the host immune response. The majority of these genes are located on the chromosome within highly conserved specific pathogenicity islands or in a virulence plasmid. Genomic variability amongst bacterial strains arises primarily as a consequence of horizontal gene transfer [15]. This inherent variability is likely the source of variable virulence amongst non-typhoidal *Salmonella* strains (*Salmonella* strains other than *Salmonella* Typhi). Characterisation of virulence gene repertoire by has been used by several groups to profile virulence of *Salmonella* [11, 16]. The aim of this study was to identify specific genes responsible for controlling virulence capacity. This information would ultimately be used to develop a PCR pathotyping method that would enable the rapid characterisation of virulent verses non-virulent foodborne outbreak related *Salmonella* serovars.

## 3.2 Results

Twenty-three virulence genes known to be involved in adhesion, invasion, intracellular replication and survival were selected to generate a virulence gene profile of the 29 *Salmonella* serovars selected for this study. PCR detection of these genes has been widely used as a predictive measure for *Salmonella* virulence [16, 17].

Results obtained are presented in Table 3-1. Interestingly, no PCR product was obtained for *avrA* in *S.* Adelaide, *S.* Kiambu, *S.* Lille, *S.* Livingston, *S.* Montevideo, *S.* Ohio, *S.* Oranienburg or *S.* Orion. *S.* Lille was negative for *sopB*. *S.* Adelaide, *S.* Bredeney, *S.* Chester, *S.* Havana, *S.* Infantis, *S.* Johannesburg, *S.* Kiambu, *S.* Lille, *S.* Mbandaka, *S.* Montevideo, *S.* Oranienburg, *S.* Senftenberg and *S.* Zanzibar were negative for *sptP*. The pLST virulence plasmid genes *pefA* and *spvC* as well as the flagellar gene, *fliC,* were only detected in the *S.* Typhimurium DT9, DT44, DT135, DT193 and *S.* Worthington. Of note *S.* Typhimurium DT170=108 (DT170 is also well known as DT108) was negative for these three genes.

**Table 3-1 Virulence gene profile of 29 *Salmonella* serovars**

| Virulence Gene | Adelaide | Agona | Anatum | Bredeney | Cerro | Chester | Havana | Infantis | Johannesburg | Kiambu | Lille | Livingston | Mbandaka | Montevideo | Ohio | Oranienburg | Orion | Orion var +15, +34 | Singapore** | Senftenberg | Subspecies 14,5 12:d | Typhimurium DT9 | Typhimurium DT44 | Typhimurium DT135 | Typhimurium DT170=108 | Typhimurium DT193 | Virchow PT34 | Worthington | Zanzibar |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *avrA* | - | + | + | + | + | + | + | + | + | - | - | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + |
| *hilA* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *invA* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *prgH* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *sipA* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *sipB* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *sipD* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *sopA* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *sopB* | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *sptP* | - | + | + | - | + | - | - | - | - | - | - | + | - | - | + | - | + | + | + | - | + | + | + | + | + | + | + | + | - |
| *sitC* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *spiC* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *sifA* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *misL* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *orfL* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *pipD* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Iron* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *pefA* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | - | + | - | + | - |
| *spvC* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | - | + | - | + | - |
| *fliC* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | - | + | + | + | - |
| *xthA* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *yafD* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *Stn* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

## 3.3 Discussion

The *Salmonella* genome possesses pathogenicity islands (PI), which are genetic elements within the bacterial genome that encode for genes associated with virulence. The presence or absence of evidence of genetic polymorphisms can be used as an index of virulence for different *Salmonella* serovars. Therefore, many groups have utilised PCR amplification of virulence genes from different pathogenicity islands to characterise potential virulence of different *Salmonella* serovars. Our results showed that the majority of the *Salmonella* serovars tested were positive for the virulence genes.

Because of the conserved nature of specific pathogenicity islands, it was not surprising that for the majority of virulence genes tested, PCR products were detected in all serovars. Multiple serovars were, however, found to be PCR negative for *avrA*, *fliC*, *pefA*, *sopB*, *sptP*, *spvC*, and *fliC*. No PCR amplification was, however, observed in any samples for *sipC*, *sopD*, *sopE*, or *ssph1* (data not shown). It is known, however, that there is significant genetic variability of virulence gene sequences between *Salmonella* serovars [18]. As such, it is likely that PCR results do not indicate that the *Salmonella* serovars tested were negative for these genes but more likely possess sufficient genetic variability preventing primer annealing and subsequent amplification. Further full genome investigation of *Salmonella* serovars is essential to confirm this.

# 4 *In vitro* invasion potential of non-typhoidal *Salmonella* serovars

## 4.1 Introduction

During infection, *Salmonella* gains access to the host by invading the cells of the intestinal epithelia. Many virulence genes encoded within the bacteria enable it to adhere to intestinal epithelial cells and invade. Deletions or mutations in one or more of these genes can limit the ability of *Salmonella* to invade host tissues, thereby reducing their overall pathogenicity. Thus, characterising the invasiveness of different serovars can serve as a parameter of bacterial virulence.

The studies regarding the invasive potential of *Salmonella enterica* serovars have been primarily limited to *S.* Enteritidis [19], *S.* Typhimurium [20] and *S.* Virchow [21]. There are, however, over 1500 members of subspecies I yet there has been only limited characterisation of their overall virulence [22, 30]. To date, only limited characterisation of the pathogenicity of other non-typhoidal *Salmonella* (NTS) serovars has been performed. While NTS serovars have been shown to have variable *in vitro* invasive capacity, the serovars selected were isolated directly from human clinical case samples [22].

For the experiments described in this chapter, 29 NTS serovars commonly isolated from Australian egg layer farms were selected. These bacteria were isolated directly from various point sources (e.g., dust, faeces, egg belts, eggshells) in the layer hen environment. Several of these serovars including the *S.* Typhimurium definitive types (DT) as well as S. Infantis, S. Mbandaka, and S. Virchow (unknown phage type) are frequently associated with disease in humans, however, many of the others are generally considered "environmental" *Salmonella* serovars. The invasive disease potential of NTS was studied during this experiment for a greater understanding of their general biology, which will ultimately contribute to their control.

## 4.2 Results

### 4.2.1 *Salmonella* invasion of polarised human intestinal epithelial cells

Human exposure to *Salmonella* can occur as a consequence of acquiring a strain or strains from the eggshell surface or egg internal contents where the bacterium would be in a quiescent state. The invasive potential of *Salmonella* serovars grown on nutrient agar and then suspended in 0.9% saline was examined. The invasive capacity of the 29 selected *Salmonella* serovars was determined using the human intestinal epithelial cell line, Caco-2. A multiplicity of infection of 100 was selected to distinguish between low, mid, and high invasive *Salmonella* serovars. Suspensions of *Salmonella* strains were prepared and co-incubated with the Caco-2 cells for two hours. Data obtained are represented as percent recovery, determined by the ratio of the amount of bacteria recovered to the initial MOI. Invasive assays were repeated five times.

Overall cell invasion was low for all serovars, with mean percent recoveries ranging from 0.03 to 0.75%. There were significant differences detected amongst *Salmonella* serovars tested. *Salmonella* Typhimurium DT170=108 exhibited the greatest invasive capacity (mean percent recovery, 0.75 ± 0.16) and was found to be significantly more

invasive than all other serovars ($p > 0.05$) (Figure 4-1). DT44, DT135, DT193, and Virchow exhibited moderate invasion while most other serovars had very low invasive capacity. Percent invasion for *S.* Adelaide and *S.* Cerro was not statistically significantly different from the non-invasive *Escherichia coli* control strains EC006 and ETEC (Figure 4-1).

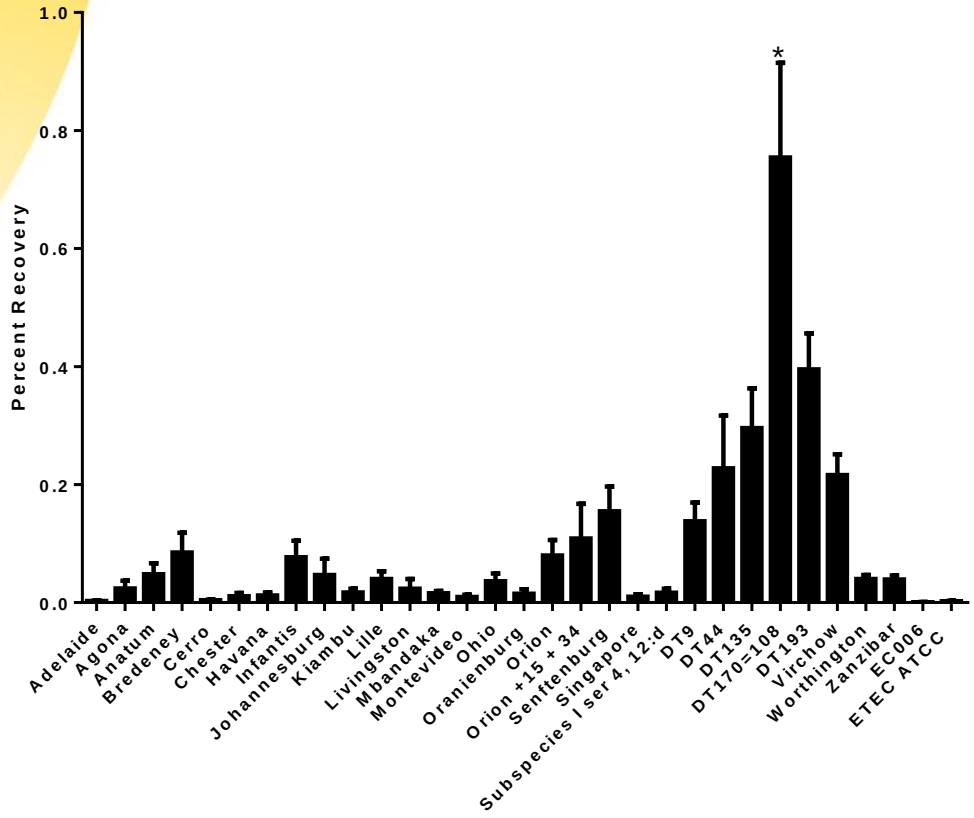


**Figure 4-1 *Salmonella* invasion of Caco-2 cells**

Bacteria were suspended on 0.9% saline and added to an MOI of 100.
Bacterial invasion of the Caco-2 cells was for two hours followed by treatment with gentamycin to kill any adhered bacteria.
Cells were lysed and serial dilutions of lysate were prepared.
Data are represented as percent recovery.
Significantly higher invasive capacity was observed for *Salmonella* Typhimurium DT170=108 ($p < 0.05$).

*Salmonella* infection can occur following the consumption of contaminated food that likely has been prepared or stored in a manner that enables bacteria to survive and/or multiply. Furthermore, it is likely that certain egg-based food products provide a nutritive environment that contributes to the expression of genes required for invasion. It has been widely reported that *in vitro* growth media substantially affects the expression of genes required for *Salmonella* invasion in cultured epithelial cells [20, 23]. The gentamycin invasion protection experiment was therefore repeated with bacteria cultured to stationary phage in LB broth.

**Table 4-1 *Salmonella* invasion matrix**

| Serovar | Before Enrichment | After enrichment |
|---|---|---|
| Adelaide | 0.002895 | 0.2246 |
| Agona | 0.02377 | 2.207 |
| Anatum | 0.04786 | 2.036 |
| Bredeney | 0.08475 | 0.5571 |
| Cerro | 0.003972 | 1.412 |
| Chester | 0.01079 | 1.407 |
| Havana | 0.01195 | 1.71 |
| Infantis | 0.07699 | 0.9906 |
| Johannesburg | 0.04692 | 1.497 |
| Kiambu | 0.01714 | 1.411 |
| Lille | 0.04002 | 0.1818 |
| Livingston | 0.02333 | 0.6838 |
| Mbandaka | 0.01607 | 1.825 |
| Montevideo | 0.009497 | 3.018 |
| Ohio | 0.03618 | 1.907 |
| Oranienburg | 0.01517 | 2.032 |
| Orion | 0.07966 | 1.187 |
| Orion +15 + 34 | 0.1088 | 1.49 |
| Senftenberg' | 0.1547 | 1.382 |
| Singapore | 0.01002 | 2.006 |
| Subspecies 45 12:d | 0.0168 | 2.669 |
| DT9 | 0.138 | 6.521 |
| DT44 | 0.2274 | 3.938 |
| DT135 | 0.2958 | 5.684 |
| DT170=108 | 0.7544 | 4.65 |
| DT193 | 0.3951 | 4.021 |
| Virchow | 0.2163 | 4.885 |
| Worthington | 0.03998 | 0.5874 |
| Zanzibar | 0.03915 | 3.357 |
| EC006 | 0.0008302 | 0.03263 |
| ETEC ATCC | 0.002195 | 0.1186 |

Data presented are mean percent recovery from bacteria suspended in saline or grown to stationary phase in LB.
The icon colour indicates the invasive ability based on the risk matrix before and after the enrichment of bacteria.
Red icon indicates highly invasive (High risk up on enrichment in LB), Yellow icon indicates medium invasive (moderate risk up on enrichment in LB) and Green icon are low invasive (Low risk up on enrichment in LB).

Consistent with previous reports, a substantial increase in invasive capacity was observed for all serovars grown in LB broth as compared with suspensions in physiological saline. Increases in invasive capacity ranged from 6.6 to 355.5 fold, with *S.* Cerro and *S.* Montevideo exhibiting the greatest change of 355.5 and 317.8 fold respectively (Table 4-1). Greater than 90-fold increase in invasion for *S.* Agona, *S.* Chester, *S.* Havana, *S.* Mbandaka, *S.* Oranienburg, *S.* Singapore and *S.* Subspecies 45 12:d was also observed.

As with results obtained using physiological saline, the *S.* Typhimurium definitive types DT9, DT44, DT135, DT170=108, DT193 as well as *S.* Virchow exhibited the greatest invasive potential. DT9 and DT135 had the highest mean percent recoveries, 6.6 ± 1.5 and 5.9 ± 1.8 respectively, and were significantly more invasive than *S.* Adelaide, *S.* Bredeney, *S.* Lille, *S.* Mbandaka or *S.* Worthington ($p < 0.05$) (Figure 4-2). Interestingly, despite growth in enriched media *S.* Adelaide, *S.* Bredeney, *S.* Lille and *S.* Worthington retained very low invasion capacities (Figure 4-2).

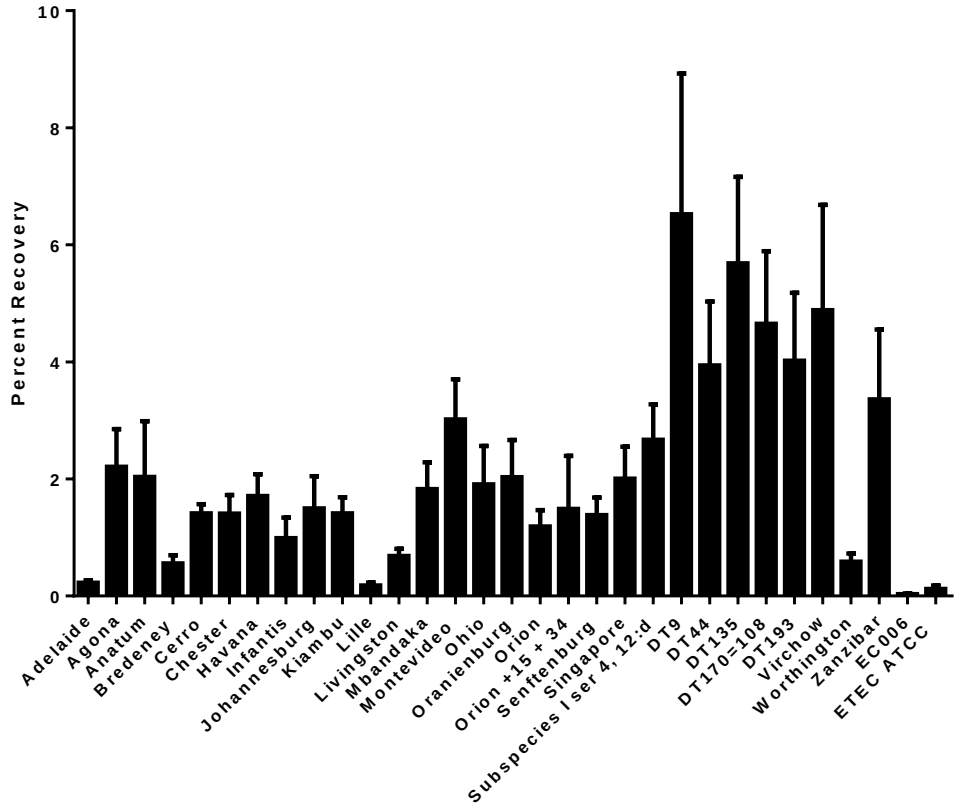


**Figure 4-2 Effects of growth enrichment on *Salmonella* invasion**

*Salmonella* serovars were grown to stationary phase in LB broth and then added to Caco-2 cell monolayer to an MOI of 100.
Data are mean percent recovery of bacteria.
The *Salmonella* serovars selected for this study exhibited significantly different invasive capacities ($p < 0.01$) in the *in vitro* intestinal epithelial cell model.
*S.* Typhimurium DT9, DT135, DT170=108 and *S.* Virchow exhibited the highest mean percent recoveries.

### 4.2.2 Invasion potential of *Salmonella* in to non-polarised epithelial cells

The invasion potential of NTS serovars in to non-polarised epithelial cells was also investigated. As above, bacteria were suspended in 0.9% saline and added to an MOI of 100 to non-polarised Caco-2 cells that were plated 48 hours prior to the start of the experiment.

Overall invasion of the 29 NTS serovars was low (Figure 4-3). A significant effect of serovar was observed ($p < 0.001$). The *S.* Typhimurium definitive types 44, 135, 108=170, 193, *S.* Senftenberg and *S.* Virchow exhibited the greatest invasion into non-polarised Caco-2 cells. No significant difference in invasion potential was observed for bacteria suspended in 0.9% saline between polarised versus non-polarised cells.

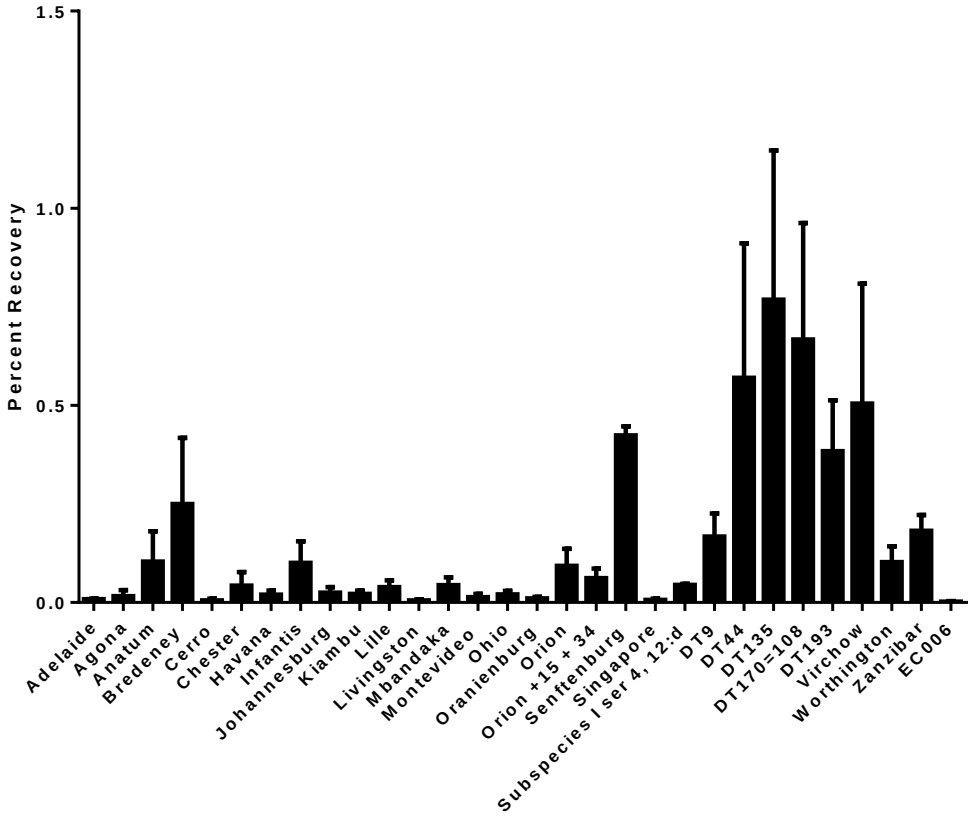


**Figure 4-3 Invasion of NTS serovars into non-polarised intestinal epithelial cells**

*Salmonella* serovars were suspended in 0.9% saline and then added to Caco-2 cell monolayer to an MOI of 100.
Data are mean percent recovery of bacteria.
The *Salmonella* serovars selected for this study exhibited significantly different invasive capacities ($p < 0.01$) in the *in vitro* intestinal epithelial cell model.
*S.* Typhimurium DT44, DT135, DT170=108, DT193, *S.* Senftenberg and *S.* Virchow exhibited the highest mean percent invasion.

## 4.3 Discussion

The *Salmonella* species *S. enterica* is comprised of over 2500 different serovars. It is representative serovars from this group that are most commonly isolated during outbreaks of foodborne salmonellosis including *S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, and *S.* Infantis. Virulence potential of many other *Salmonella* serotypes commonly isolated from eggs, egg-related products and layer farms remains largely unknown. In the present study, we have examined *in vitro* cell invasion as a pathogenic parameter of 29 *Salmonella* serovars recently isolated from egg farm environment.

The invasive potential of *Salmonella* spp. and the underlying mechanisms controlling bacterial invasion have been widely studied using the intestinal epithelial cell line, Caco-2 [20, 24]. The previous work, however, was limited to those serovars most commonly associated with human disease. Polarised monolayers of the human intestinal epithelial cell line, Caco-2, display morphologic features strikingly similar to the single layer of epithelial cells that line the small intestine including the formation of distinct apical and basolateral membranes, expression and construction of functional tight junctions and the production of microvilli, which are finger-like projections supported by a highly organised actin network (reviewed in [25]). Polarised tissue culture models are relevant to human disease as they more closely approximate the initial site where *Salmonella* interacts.

In this study, the invasive capacity of 29 non-typhoidal *Salmonella* serovars commonly isolated from the Australian egg industry was characterised under varying conditions. Data from our experiments revealed that the invasion rate of enriched cultures of *Salmonella* serovars was between 4.5 and 355.5 fold higher than non-enriched cultures. It has been shown that culture conditions affected invasion efficiency. *Salmonella* spp. aerobically grown at late exponential phase were more invasive [23]. Similar findings were recorded in this study.

Patterson et al. (2012) reported up-regulation of all genes in the *Salmonella* pathogenicity island 1 of *S*. Typhimurium up on enrichment [26]. Based on findings of the current work, it could be hypothesised that similar pattern could be observed in other *Salmonella* serovars. However, further investigations are necessary. These results indicate that although egg products are major sources of infection for humans, not all isolates of *Salmonella* recovered from poultry may be equally invasive for humans even after enrichment.

It has also been widely characterised *in vitro* that *Salmonella* invades polarised epithelial cells more efficiently than non-polarised cells [24, 27]. The non-polarised cell invasion of the serovars selected for this study did not differ significantly for bacteria suspended in 0.9% saline. Future studies to characterise the invasion capacity of enriched *Salmonella* serovars into non-polarised cells are required. Human gut is colonised by several gut bacteria. Age and immune status of an individual may contribute to polarity of intestinal epithelial cells [28] and as a consequence may alter susceptibility to infection or invasion by pathogenic gut bacteria. It is important to note that the *S*. Typhimurium definitive types exhibited the greatest overall invasion in non-polarised cells.

The findings from these intestinal epithelial invasive assays could have significant implications for egg handling in the kitchen during food preparation as they suggest that some strains of *Salmonella* require prior enrichment to stimulate virulence. Moreover, these results also indicate that many *Salmonella* serovars, in particular the *S*. Typhimurium definitive types, may have constitutively active virulence gene(s) that enable invasion under enriched or non-enriched conditions. Future studies are intended

to be aimed at determining the molecular and cellular mechanisms responsible for these differences.

# 5 Pathogenicity of non-typhoidal *Salmonella* serovars in mice

## 5.1 Introduction

*Salmonella enterica* is subdivided into six subspecies: *enterica* (subspecies I), *salame* (II), *arizonae* (IIIa), *diarizonae* (IIIB), *houtenae* (IV) and *indica* (VI). Serovars within subspecies I are largely responsible for causing the majority of human salmonellosis cases [29]. Furthermore, many subspecies I serovars have established ecological niches within multiple agricultural, livestock and food based systems. It is important to characterise the disease potential that these serovars represent so that their risk to public health can be established.

Currently, there are several widely used animal models of human salmonellosis that enable the study of *in vivo* virulence of *Salmonella* [31]. Inbred, specific pathogen free mouse strains, in particular, are used to investigate the underlying disease processes of human salmonellosis. There is a large range of inbred mouse strains with varied susceptibilities to *Salmonella*, which have facilitated the characterisation of both host and bacterial determinants of disease [32]. In this study, the BALB/c mouse strain was selected to investigate the comparative virulence of 15 *Salmonella* serovars commonly isolated from layer hen environments. Fifteen *Salmonella* strains selected for the mice infection trial were representative strains from low, medium and highly invasive categories (categories were based on the cell culture experiment). BALB/c mouse strain is highly susceptible to *Salmonella* infection.

## 5.2 Results

### 5.2.1 Morbidity and mortality of mice infected with different *Salmonella* serovars

To test whether the differences in the *in vitro* measure of virulence are a measure of *in vivo* virulence, the susceptible mouse strain BALB/c was selected. Groups of mice were inoculated with either $10^3$ or $10^5$ CFU of individual serovars. These doses were selected because they represented the load of bacteria commonly found on the eggshell surface of eggs laid by *Salmonella* infected hens [33]. Mice were inoculated by oral gavage and were monitored for signs of clinical disease. Mice exhibiting early removal criteria, such as hunching, ruffled coat, lack of movement, dehydration, or not eating, were humanely euthanised.

Morbidity of mice was characterised using a clinical scoring system. Data obtained for the morbidity scores are presented as mean clinical scores ranging from zero to five taken for an entire experimental group. Results are summarised in Figure 5-1. Animals inoculated with either $10^3$ or $10^6$ CFU of *S.* Adelaide, *S.* Bredeney, *S.* Cerro, *S.* Infantis, *S.* Lille, *S.* Montevideo, *S.* Oranienburg, *S.* Orion, *S.* Senftenberg and *S.* Virchow did not exhibit any clinical signs of infection over the course of the entire 21 day experiment. As such, their clinical scores were zero and not included in Figure 5-1.

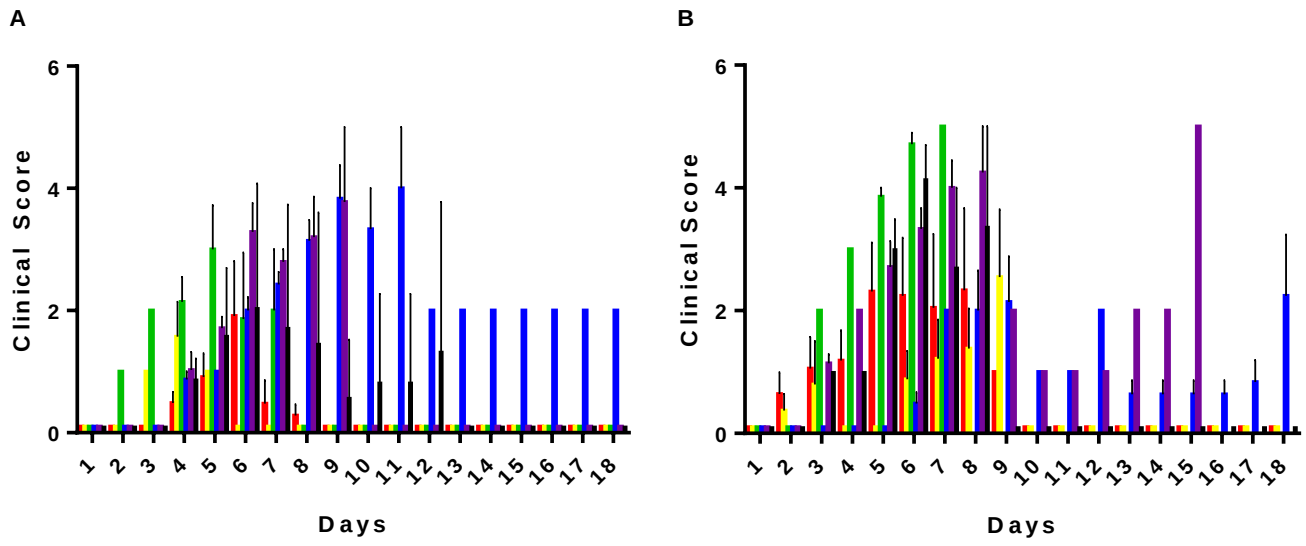


**Figure 5-1 Morbidity in mice infected with *Salmonella* Typhimurium definitive types**

Clinical signs of infection appeared from day 2 with $10^3$ CFU of individual serovars (A).
Peak morbidity in low dose animals ranged between day 6 and 10 post infection.
Morbidity was observed in animals inoculated with $10^5$ CFU starting on day 1 p.i. and peaked between days 5 and 9 p.i. (B).
DT9 (red), DT44 (yellow), DT135 (green), DT170=108 (blue), DT193 (purple), ATCC 14028 (black).

Mice inoculated with either $10^3$ (Figure 5-1 A) or $10^5$ CFU (Figure 5-1 B) of S. Typhimurium DT9, DT44, DT135, DT170=108, DT193 and ATCC 14028 exhibited the greatest amount of morbidity. Mice inoculated with the low dose of *Salmonella* exhibited a range of clinical symptoms over the course of the experiment. DT44 cause the least morbidity amongst the Typhimurium definitive types selected for this study. DT44 caused very mild clinical symptoms in mice from Day 3 to 5 but by Day 6 p.i. all animals had recovered. Amongst mice receiving the low dose inoculum, the greatest morbidity was observed in DT135, DT170=108 and DT193 (Figure 5-1 A). Clinical evidence of infection was observed from Day 2 post-inoculum (p.i.) for animals infected with $10^3$ CFU of DT135. Morbidity for this group increased rapidly from Day 3 to Day 6.

Three mice infected with DT135 recovered after seven days and survived to the end of the experiment. Animals infected with either DT170=108 or DT193 exhibited morbidity from day 4 which increased till day 10 by day 11 all but one animal in each group had been euthanised. Animals infected with DT9 and ATCC 14028 exhibited clinical symptoms from day 4 p.i. Morbidity in these groups peaked between days 7 and 9. Five animals infected with DT9 and three with ATCC 14028 recovered from infection and did not show further clinical signs of infection.

Morbidity in mice inoculated with $10^5$ CFU of *S.* Typhimurium definitive types was observed from either Day 2 or 3 depending on strain (Figure 5-1 B). The peak of morbidity was observed earlier and clinical scoring was higher than in mice inoculated with $10^3$ CFU. Mice inoculated with the high dose of DT9 and DT44 exhibited similar patterns of morbidity. Clinical signs of infection for these two groups were observed from day 2 p.i. and peaked between days 5-9. Two mice in the $10^5$ CFU DT9 group and three in DT44 recovered from infection and remained till the end of the experiment (Figure 5-1 B). DT193 and ATCC 14028 caused significantly greater morbidity than

either DT9 or DT44 ($p < 0.01$). For both serovars, clinical symptoms were observed from day 3 and morbidity scores peaked between Day 4-8 p.i. DT135 exhibited the greatest morbidity and was significantly different from all other definitive types ($p < 0.01$). The majority of animals in the DT170=108 group exhibited moderate morbidity over the course of the experiment. Two animals exhibited a clinical score of 5 between Day 7-9 p.i. and were euthanised. All five other animals in the DT170=108 group remained over the course of the experiment.

Survival curves were generated to compare survivability of the infections. All Typhimurium definitive types were significantly different in their survival curves (Mantel-Cox log rank test, $p<0.001$) than the other serovars at both doses (Figure 5-2 A and B). There were significant differences in the capacity of the different Typhimuriums to limit survival. At the low dose infection with DT9, DT135, DT170=108, DT193 and ATCC 14028 all resulted in significantly greater mortality than DT44 ($p < 0.0001$) (Figure 5-2 A). At the high dose, DT135 was found to be more virulent than the other S. Typhimurium strains selected for this study ($p < 0.01$) (Figure 5-2 B).

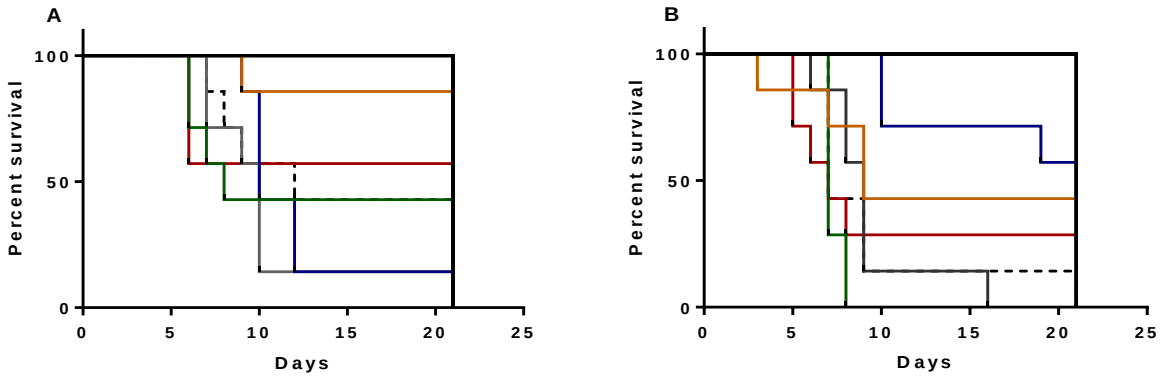


**Figure 5-2 Survival curves for mice inoculated with either $10^3$ or $10^5$ CFU of *S.* Typhimurium definitive types**

*S.* Typhimurium definitive types DT9 (red), DT44 (orange), DT135 (green), DT170=08 (blue), DT193 (grey) and ATCC 14028 (black hashed) exhibited significantly greater mortality at both the $10^3$ (A) and $10^5$ (B) dose, than all other serovars (black line) tested in this study.
Mice inoculated with 103 CFU of DT9, DT135, DT170=108, DT193, or ATCC 14028 all had significantly greater mortality than DT44.
The greatest mortality was observed for mice inoculated with $10^5$ CFU of DT135.

## 5.2.2 Detection of faecal shedding of *Salmonella* serovars

*Salmonella* persistence is an important parameter for investigation as continued bacterial shedding perpetuates the oral-faecal route of transmission. As such, the shedding of each *Salmonella* serovar in faecal material was monitored both by culture isolation and quantitative qPCR methods. *Salmonella* was consistently detected in faeces of all groups by one or both detection methods, over all time points and at both low (Table 5-1) and high doses (Table 5-2).

**Table 5-1 qPCR and culture detection of *Salmonella* from mice inoculated with $10^3$ CFU**

| Low Dose | Day 0 | | Day 3 | | Day 6 | | Day 9 | | Day 12 | | Day 15 | | Day 18 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Serovar** | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture |
| Adelaide | LD | - | 3730 ± 20 | + | LD | + | 53795 ± 295 | + | LD | - | LD | - | 540.5 ± 2.5 | - |
| Bredeney | LD | - | 1070 ± 160 | + | 540 ± 540 | + | LD | + | 1670 ± 580 | + | LD | + | LD | + |
| Cerro | LD | - | 3630 ± 370 | + | 1875 ± 435 | + | 1205 ± 45 | + | 4050 ± 1180 | + | 3550 ± 160 | + | 2285 ± 285 | + |
| Infantis | LD | - | 1055 ± 65 | + | 4.5E4 ± 2505 | - | 1470 ± 240 | + | LD | - | LD | - | LD | - |
| Lille | LD | - | 1245 ± 145 | + | 500 ± 500 | + | LD | + | LD | - | 805 ± 15 | - | LD | - |
| Montevideo | LD | - | 4115 ± 565 | + | 870 ± 30 | + | 825 ± 405 | + | 560 ± 40 | - | 645 ± 65 | + | 1005 ± 15 | + |
| Oranienburg | LD | - | 1555 ± 1005 | + | LD | - | LD | + | LD | - | LD | + | LD | - |
| Orion | LD | - | 1480 ± 940 | + | LD | + | 730 ± 160 | + | LD | - | LD | + | LD | + |
| Senftenburg | LD | - | LD | - | 1.3E8 ± 1.3E8 | + | 940 ± 100 | - | 2620 ± 2020 | + | 3.5E5 ± 2.0E5 | - | 1035 ± 95 | - |
| Virchow | LD | - | 8165 ± 265 | + | LD | + | LD | + | LD | + | LD | + | LD | + |
| DT9 | LD | - | 8210 ± 2500 | - | 4805 ± 1035 | + | LD | - | 1475 ± 595 | - | 2025 ± 1735 | + | 1235 ± 365 | - |
| DT44 | LD | - | 1535 ± 495 | - | 605 ± 15 | + | 5940 ± 1020 | + | 3015 ± 3015 | - | 2035 ± 1525 | - | 4.7E5 ± 4.7E5 | - |
| DT135 | LD | - | 3150 ± 130 | + | LD | + | LD | + | LD | + | 575 ± 75 | + | 4175 ± 495 | + |
| DT170=108 | LD | - | 4.4E5 ± 4.7E4 | + | 1355 ± 395 | + | 3.3E4 ± 4185 | + | LD | + | 2.4E4 ± 7575 | + | 1.5E4 ± 8290 | + |
| DT193 | LD | - | 2.7E4 ± 1605 | + | 870 ± 870 | + | 635 ± 165 | + | 300 ± 300 | + | 1245 ± 1005 | + | LD | + |
| 14028 | LD | - | 2520 ± 460 | + | 3480 ± 1430 | + | 1.E4 ± 8730 | + | 5.7E4 ± 5.5E4 | + | 1.1E4 ± 5845 | + | 3450 ± 3210 | + |

LD = limit of detection for qPCR method.
qPCR data are represented as mean CFU/g ± standard error of the mean.

Persistent shedding of *Salmonella* in faeces was observed over all time points in mice inoculated with $10^5$ CFU (5-2). Fewer groups were culture positive for bacteria over the course of the experiment for mice infected with $10^3$ CFU (Table 5-1). No significant difference in bacterial load (CFU/g, qPCR) was detected between the low and high doses. The highest bacterial load was of observed at Day 6 p.i. in mice inoculated with $10^3$ CFU of *S.* Senftenberg with a mean of $1.3 \times 10^8 \pm 1.3 \times 10^8$ CFU/g faecal material. Other high shedding time points were observed for DT44 at day 18 ($4.7 \times 10^5 \pm 4.7 \times 10^4$ CFU/g faeces) and DT170=108 low dose day 3 ($4.4 \times 10^5 \pm 4.8 \times 10^4$ CFU/g faeces).

**Table 5-2 qPCR and culture detection of *Salmonella* from mice inoculated with $10^5$ CFU**

| High Dose | Day 0 | | Day 3 | | Day 6 | | Day 9 | | Day 12 | | Day 15 | | Day 18 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Serovar** | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture | CFU/g | Culture |
| Adelaide | LD | - | LD | + | 539 ± 1.0 | + | 6465 ± 835 | + | LD | - | 6.6E4 ± 3.0E4 | - | LD | - |
| Bredeney | LD | - | 2560 ± 100 | + | 2945 ± 45 | + | 2.0E4 ± 2130 | + | 695 ± 45 | + | 1630 ± 40 | + | 1210 ± 490 | + |
| Cerro | LD | - | 4915 ± 4275 | + | 9305 ± 115 | + | 690 ± 250 | + | 3805 ± 2615 | + | 2280 ± 780 | + | 6625 ± 585 | + |
| Infantis | LD | - | LD | + | LD | + | 1160 ± 340 | + | LD | + | 615 ± 65 | + | LD | + |
| Lille | LD | - | 1575 ± 265 | + | 970 ± 240 | + | 1165 ± 35 | - | LD | - | LD | + | LD | + |
| Montevideo | LD | - | 1940 ± 480 | + | 3880 ± 580 | + | 2895 ± 25 | + | 4885 ± 3155 | + | 4755 ± 1455 | + | 1.4E4 ± 915 | + |
| Oranienburg | LD | - | 5590 ± 590 | + | 1645 ± 605 | + | 1235 ± 415 | - | 745 ± 125 | + | 1925 ± 45 | + | LD | + |
| Orion | LD | - | 1010 ± 50 | + | 5240 ± 4210 | + | 2290 ± 160 | + | 985 ± 15 | + | LD | + | 1745 ± 655 | - |
| Senftenburg | LD | - | 1155 ± 395 | + | LD | + | LD | - | 985 ± 25 | + | 8359 ± 7335 | + | 6555 ± 2095 | + |
| Virchow | LD | - | 5655 ± 975 | + | 630 ± 260 | + | 750 ± 140 | + | LD | + | LD | + | 2250 ± 610 | + |
| DT9 | LD | - | 5990 ± 150 | - | 650 ± 30 | + | 1.1E4 ± 505 | + | 265 ± 155 | - | LD | - | 4740 ± 970 | - |
| DT44 | LD | - | 720 ± 50 | + | 9515 ± 1015 | + | 2.1E4 ± 180 | + | LD | + | LD | - | LD | - |
| DT135 | LD | - | 3230 ± 460 | + | 2420 ± 660 | + | | | | | | | | |
| DT170=108 | LD | - | 3.0E4 ± 2255 | + | 1655 ± 1345 | + | 2605 ± 2265 | + | 9325 ± 4485 | + | 3240 ± 1620 | + | 1920 ± 480 | + |
| DT193 | LD | - | 4945 ± 525 | + | 475 ± 475 | + | 1.8E4 ± 1.1E4 | + | LD | + | 9.0E4 ± 2500 | + | | |
| 14028 | LD | - | 1.6E4 ± 3130 | + | 1775 ± 1135 | + | 830 ± 830 | + | 1435 ± 1125 | + | 1855 ± 955 | + | 5575 ± 785 | + |

LD = limit of detection for qPCR method.
qPCR data are represented as mean CFU/g ± standard error of the mean.
Cells filled with black hashed lines indicate no animals remained in that group.

## 5.3 Discussion

Based on the results obtained in the *in vitro* invasion experiments (Chapter 4), the *in vivo* virulence of *Salmonella* serovars exhibiting low, moderate, and high invasion was tested using a mouse infection model. BALB/c mice were inoculated with either $10^3$ or $10^5$ CFU of bacteria, a range that has been detected on the surface of eggs contaminated with *Salmonella* [33]. Clinical signs of infection and mortalities were observed in mice inoculated with both low and high doses of *S.* Typhimurium DT9, DT44, DT135, DT170=108, DT193 and *S.* Typhimurium ATCC 14028. No evidence of morbidity or mortality occurred in any other serovar. Interestingly, *S.* Virchow exhibited high invasion capacity in Caco-2 cells but lacked virulence in BALB/c mice. This result was somewhat unexpected as S. Virchow has been commonly isolated during human outbreaks of salmonellosis and is globally, among the top ten serovars responsible for causing disease [34].

The lack of virulence in mice observed for *S.* Montevideo is consistent with previous studies [30]. This study is, however, the first to demonstrate that *S.* Adelaide, *S.* Bredeney, *S.* Cerro, *S.* Infantis, *S.* Lille, *S.* Oranienburg, *S.* Orion and *S.* Senftenburg isolated from Australian egg layer farms have limited virulence in mice. The reason for their lack of virulence is currently unknown. In humans, age and immunocompetence are important factors that can predispose an individual to succumb to *Salmonella* infection [28, 34]. Further investigation with mice of variable age as well as immunosuppressed knockout mice would lead to a better understanding of the public health risk that each serovar represents.

Host to host transmission is an important element in the lifecycle of a pathogen. For *Salmonella*, the mechanisms that enable it to establish a persistent infection are largely

unknown [35]. It, however, involves a complex process that requires *Salmonella* to both outcompete the resident gut microbiota and invade into host epithelial cells to establish a long term infection [35]. An important outcome from this study was the detection of persistent faecal shedding of all serovars over the course of the 21 day experiment in mice. These results demonstrate that while many NTS serovars may have low disease potentials, they retain the ability to persist within the host. Further exploration of the mechanisms of *Salmonella* persistence is important to developing control strategies and improved therapies.

# 6 *Salmonella* invasion of sections of layer hen oviducts

## 6.1 Introduction

Worldwide, *Salmonella enterica* infection is among the leading causes of foodborne gastrointestinal disease and represents an important public health issue [36, 37]. An egg may become contaminated through two major mechanisms – through environmental surface contamination or during development in the oviduct. Surface contamination occurs during lay when an egg contacts *Salmonella* positive faecal material present in the environment [38-40]. Internal contamination of the egg can occur if the bacterium penetrates through the eggshell under poor storage or handling conditions [41-43].

Layer hens shed *Salmonella* bacteria in faecal material intermittently, a pattern which has been linked to physiological and environmental stressors [44-46]. Stress caused by onset of lay, in particular, has been widely characterised for layer hens [47, 48]. During this period, point-of-lay leads to suppression of the bird's cell mediated immune response [49, 50]. In particular, dramatic reduction in lymphocyte populations in the reproductive mucosal surfaces occurs during this time, which may contribute to the colonisation of the layer hen oviduct by bacteria [49]. Furthermore, longitudinal epidemiological investigation of *Salmonella* in layer flocks has correlated point-of-lay with peak bacterial loads in faeces [33].

It has been widely studied that ovary and oviduct tissues can become colonised by some *Salmonella* serovars such as *S.* Enteritidis, which could potentially lead to contamination of the yolk, albumin, eggshell membranes or eggshell prior to oviposition [41, 51]. It is however important to note that *S.* Enteritidis is not endemic in commercial poultry industry in Australia. Infection of the layer hen reproductive tract can arise through one of two mechanisms. The first mechanism involves migration of bacteria through the vagina and further up the oviduct where they may invade the local tissue [41]. This has been documented. Studies using *S.* Enteritidis, *S.* Heidelberg and *S.* Hadar have shown that oviduct tissue may become colonised as a result of systemic infection [51-53].

It is important, then, to understand the range of invasive abilities of different *Salmonella* serovars into different regions of the layer hen oviduct and determine whether they can establish colonisation of the reproductive tract. The aim of this study was to characterise the ability of multiple *Salmonella* serovars to invade the reproductive tract of two commercial brown egg laying hens, commonly used in the Australian egg industry. Tissue punches of the infundibulum, isthmus, shell gland, magnum and vagina were collected and used to compare the *in vitro* invasion of 18 *Salmonella* serovars commonly isolated from egg layer farms. Understanding the ability of *Salmonella* to contaminate eggs and the underlying mechanisms that are involved in this process will aid in the development of improved control measures.

## 6.2 Results

### 6.2.1 Maintaining *Salmonella*-free layer hens

Commercial-Brown layer hens were hatched, raised and maintained free from *Salmonella* for 30 weeks. Water, feed and faecal samples were tested weekly using the culture method for *Salmonella* isolation. DNA was extracted from peptone water and a specific PCR was used to determine the presence of *Salmonella*. All samples were negative for *Salmonella* over the course of the experiment.

### 6.2.2 Oviduct invasion potential differs amongst *Salmonella* serovars

The invasive ability of eighteen *Salmonella* isolates of representative serovars into explants of five oviduct segments was studied. An *in vitro* approach was selected over *in vivo* experiments because it permitted the testing of a greater number of *Salmonella* isolates with fewer birds raised under *Salmonella* free conditions. Moreover, an *in vitro* whole organ approach has advantages over cell culture in that the mucoid secretions were still present. Data were normalised to total punch area and are presented as colony forming units (CFU)/mm$^2$. No effect of breed was detected. Data obtained from both layer hen breeds were subsequently pooled for further analyses. A significant effect of serovar ($p < 0.0001$) was observed for all oviduct tissues included in this experiment (Figure 6-1). Mean *Salmonella* invasion of the infundibulum ranged between 39.60 ± 12.81 and 353 ± 77.96 CFU/mm$^2$. *S.* Bredeney exhibited the least invasion while *S.* Infantis, *S.* Montevideo and *S.* Senftenberg exhibited the highest (Figure 6-1). Mean invasion of *S.* Montevideo was significantly greater than *S.* Adelaide, *S.* Bredeney, *S.* Cerro and *S.* Worthington ($p < 0.01$).

Invasion of the magnum ranged between 38.15 ± 7.33 and 219.4 ± 26.33 CFU/mm$^2$ (Figure 6-1). As with the infundibulum, *S.* Infantis and *S.* Montevideo were found to be the most invasive. *S.* Bredeney and *S.* Typhimurium DT170=108 exhibited the lowest invasive capacity, and were significantly different from *S.* Infantis ($p < 0.05$). Mean *Salmonella* invasion of the isthmus ranged between 85.27 ± 44.22 and 411.9 ± 182.0 CFU/mm$^2$. *S.* Bredeney was the least invasive while *S.* Infantis and *S.* Typhimurium DT193 were the most invasive (Figure 6-1). No significant difference in invasion of the isthmus was detected between isolates. The overall invasion potential of *Salmonella* into the shell gland was low but exhibited a 10-fold variation amongst isolates. Mean invasion into the shell gland ranged between 14.15 ± 3.66 and 149.2 ± 106.5 CFU/mm$^2$ (Figure 6-1). *S.* Adelaide exhibited the lowest overall invasion capacity and was significantly different from *S.* Lille, *S.* Orion, and *S.* Senftenberg ($p < 0.05$). *S.* Virchow was the most invasive isolate in the shell gland. The highest invasion capacities were observed in the vagina. *S.* Montevideo was found to have the greatest invasion potential (461.0 ± 129.2 CFU/mm$^2$) while *S.* Bredeney exhibited the lowest (53.74 ± 15.02 CFU/mm$^2$); the difference between these two strains was significant ($p < 0.05$) (Figure 6-1).

*S.* Infantis and *S.* Montevideo consistently exhibited the highest invasion overall into sections of the oviduct while *S.* Adelaide, *S.* Bredeney, *S.* Cerro and *S.* Worthington all had the lowest. It is interesting to note that, in general, the *S.* Typhimurium definitive types included in this study did not possess substantially greater invasive abilities compared with other strains.

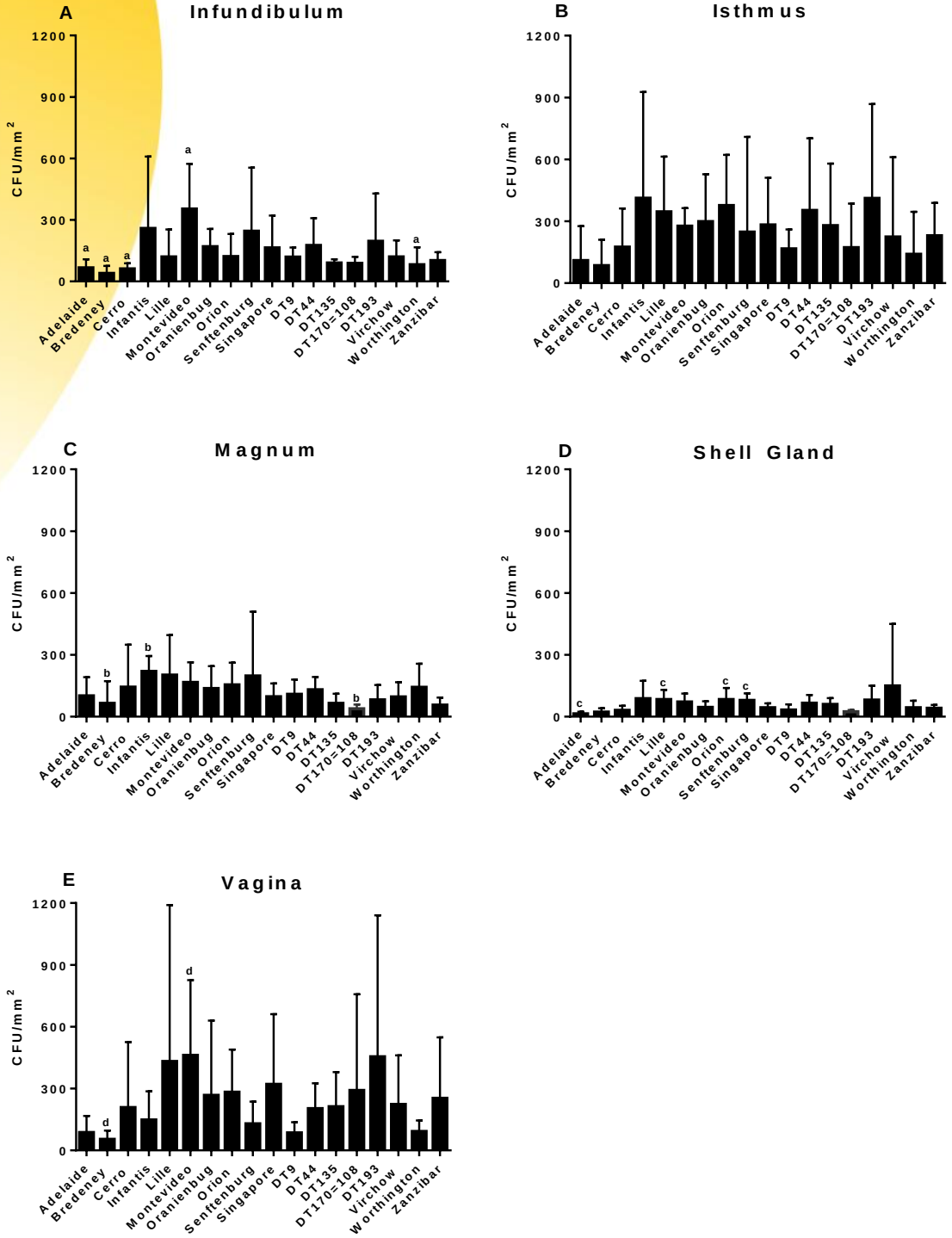


**Figure 6-1 Invasion of *Salmonella* serotypes into sections of the layer hen oviduct**

$10^5$ CFU of *Salmonella* were added to punches of the layer hen oviduct. Data are presented as mean CFU/mm$^2$ of tissue. All serovars included in this study exhibited similar invasion potential. In the infundibulum (A), *S.* Montevideo exhibited significantly higher invasion than *S.* Adelaide, *S.* Bredeney, *S.* Cerro and *S.* Worthington (a; $p < 0.01$). No significant differences were detected amongst strains in the isthmus (B). Invasion of the magnum (C) was greatest for *S.* Infantis, which was significantly different from *S.* Bredeney (b; $p < 0.05$) *S.* Adelaide exhibited the lowest invasion in the shell gland (D) and was significantly different from *S.* Lille, *S.* Orion and *S.* Senftenberg (c; $p < 0.05$). The vagina (E) exhibited the highest mean invasion across all strains. *S.* Montevideo had the highest invasion and was significantly different from S. Bredeney (d; $p < 0.05$).

### 6.2.3 Oviduct *Salmonella* invasion into layer hen oviduct tissue

To characterise the overall tropism for the *Salmonella* strains included in this study, mean invasion for each oviduct section was pooled across all strains and compared. The highest bacterial invasion was observed in the isthmus and vagina, while the lowest was observed in the shell gland. Across all strains a significant effect on invasion was observed for oviduct segment ($p < 0.0001$) (Figure 6-2). Mean overall *Salmonella* invasion was 143.0 ± 18.93 CFU/mm$^2$ in the infundibulum, 254.7 ± 23.66 in the isthmus, 122.3 ± 12.44 in the magnum, 58.84 ± 7.724 in the shell gland, and 233.5 ± 29.72 in the vagina. *Salmonella* invasion of the isthmus was significantly higher than that observed for both the magnum ($p < 0.0001$) and shell gland ($p < 0.001$) (Figure 6-2). Mean invasion of the vagina and infundibulum was significantly greater than the shell gland ($p < 0.001$). No difference in bacterial invasion was observed between the magnum and shell gland.

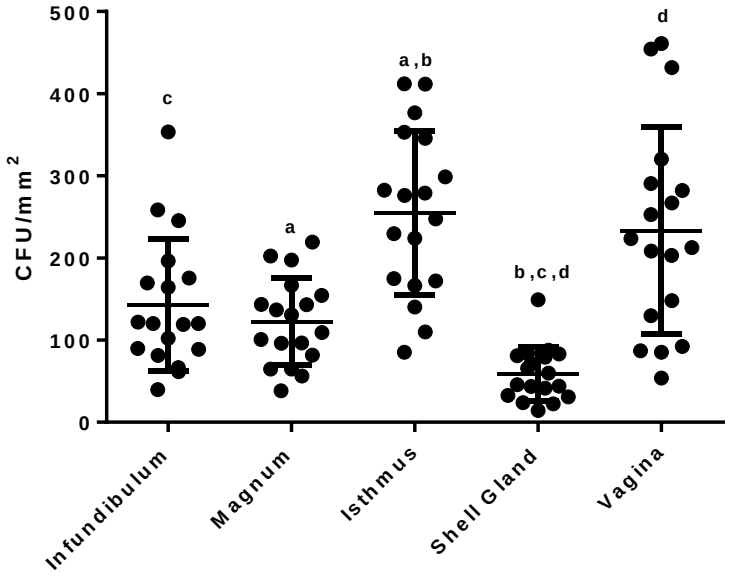


**Figure 6-2 Invasion of *Salmonella* spp. into sections of the layer hen oviduct**

Data were pooled across all strains to determine which oviduct segment exhibited the highest overall bacterial invasion.
Bacterial invasion into the isthmus and vagina were highest.
A significant effect on invasion was detected by Kruskal-Wallis ANOVA for oviduct segment ($p < 0.0001$).
Bacterial invasion of the isthmus was significantly higher than either the magnum (a, $p < 0.0001$) or shell gland (b, $p < 0.001$).
Mean invasion of the infundibulum and vagina were significantly greater than the shell gland (c, d, $p < 0.001$).

## 6.3 Discussion

*Salmonella* contamination of an egg can occur through both horizontal and vertical mechanisms. Horizontal contamination occurs when an egg comes in contact with a contaminated environment or faecal material [38, 41]. In contrast, vertical transmission of *Salmonella* requires bacterial colonisation of the oviduct and the ability of the bacterium to survive within this tissue as well as the egg itself [40, 41].

Colonisation of the oviduct arises as a consequence of an ascending infection from the cloaca or following systemic infection [40, 41]. Investigation of the mechanisms driving oviduct colonisation and subsequent egg contamination has in large part been focused

on the serovar *S.* Enteritidis. Indeed, *S.* Enteritidis possesses many unique abilities that enable it to colonise oviduct tissue and survive within the egg environment. *S.* Enteritidis, however, is not prevalent within the Australian layer industry. However, many other *Salmonella* serovars including *S.* Typhimurium, *S.* Infantis and *S.* Virchow which are frequently associated with food poisoning outbreaks have been isolated from layer farms and causally associated with table related food safety incidents. The potential for these Australian *Salmonella* serovars and others to colonise the layer hen oviduct was unknown. In this study, the invasion potential of 18 Australian *Salmonella* serovars for the oviduct was assessed *in vitro*.

All serovars selected for this study had the ability to invade sections of the oviduct. Overall, the highest levels of invasion were observed in the isthmus and the magnum. The least invasion was observed for the shell gland. At this time, it is unclear what mechanisms may be responsible for this result. Interestingly, the *S.* Typhimurium definitive types were not found to possess greater invasive capacity than other serotypes. *S.* Infantis exhibited the greatest invasion potential in the infundibulum, isthmus and shell gland.

Interpretation of the results of this study is limited, largely because only the invasion ability of the selected serovars was assessed *in vitro*. As such, these *in vitro* experiments are strictly a measure of the ability of the bacteria to invade oviduct tissue. To colonise the oviduct and ultimately contaminate an egg, bacteria first need to overcome host physical and immunological barriers to gain access to the epithelial cells. They then must invade, replicate and ultimately survive within the environments of the oviduct and egg [41]. The results obtained from this study establish that these serovars have the ability to invade layer hen oviduct tissues but have not determined whether these serovars can establish colonisation through either the cloaca or systemically. It should be noted however, that despite colonisation of the oviduct, rates of egg internal contamination with *S.* Enteritidis remain low [54].

The mechanisms that enable *Salmonella* spp. to colonise the oviduct are currently unknown. A recent report investigated five virulence loci that are found only in a limited number of poultry specific *S.* Enteritidis strains [55]. While they found that the virulence loci mutants had attenuated ability to colonise the liver and spleen, no affect was observed in tissues of the oviduct [55]. It is likely that it is a multifactorial process that requires a unique combination of host and bacterial elements. Future investigation of this process will enable the identification of oviduct colonisation factors and their significance in the overall contribution to food poisoning episodes causally associated with eggs, It is important that risk mitigation procedures are focused on the epidemiological areas that will have the greatest impact in reducing egg contamination with *Salmonella,* which could potentially result in food safety outbreaks.

# 7 Comparison of specific pathogenicity islands 1, 2, 3, 4, and 5 from twelve non-typhoidal *Salmonella* serovars

## 7.1 Introduction

Enteritidis comparative genomic analyses of *Salmonella* spp. have revealed that there is considerable variation in virulence elements across the species as a whole [18]. *S.* Typhimurium, for example, lacks multiple virulence genes that are found in *S.* Enteritidis [56]. Many of these genes are responsible for conferring increased virulence to *S.* Enteritidis [56]. Analysis of sequence variation can identify sources of genomic variation that are responsible for phenotypic variation and overall pathogenicity. PCR testing conducted on *Salmonella* strains included in this study (as described in chapter 2) suggested the presence of several virulent genes irrespective of their high, medium or low invasive status. In order to study the gene sequence in more detail, whole genome sequencing of 12 serovars (representative of low, medium and high invasive serovars identified based on cell culture experiment) was performed and the sequences of five specific pathogenicity islands were analysed and compared with *Salmonella* Typhimurium LT2.

## 7.2 Results

In order to identify any genomic variability responsible for invasive verses non-invasive *Salmonella*, whole genome sequencing was performed on each of the 12 isolates. A comparative single gene analysis of the specific pathogenicity islands (SPI) 1, 2, 3, 4 and 5 was performed and compared with the reference strain, *Salmonella* Typhimurium LT2 (NC_003197)(further referred to as LT2).

Specific pathogenicity islands are known to be highly conserved across *Salmonella enterica*. The specific pathogenicity island 1 (SPI-1) contains genes that are involved in the formation of the Type III transmembrane secretion system (T3SS). Of the 39 genes in SPI-1, 79.5% of the genes of 12 *Salmonella* serovars had greater than 98% amino acid homology with LT2. The greatest sequence variation in SPI-1 was observed in *avrA*, *srpB*, *orgC*, *prgI, sptP, and sipA* (Figure 7-1).

Variability in the amino acid sequence was observed for *orgC*, *sipA* and *prgI* amongst several serovars. Minor sequence variation was observed for *orgC*. All four *S.* Typhimurium definitive types exhibited 100% homology to the LT2 orgC sequence. Amino acid substitutions were observed for all other serovars. *S.* Adelaide and *S.* Bredeney exhibited the greatest variability in the *orgC* sequence and were 97.35% homologous with LT2. The percent homology with LT2 for *sipA* ranged between 97.96-100% with the greatest variability observed in the *S.* Bredeney and *S.* Senftenberg sequences. As with *orgC* and *sipA*, only a few amino acid substitutions were observed for *prgI* and *srpB*. The greatest variability in prgI was observed for the *S.* Orion sequence, which had five amino acid substitutions. *S.* Cerro and *S.* Orion exhibited the highest number of amino acid substitutions in *srpB* and were found to be 97.62% homologous with LT2.

Major sequence variations were observed in the SPI-1 genes, *avrA*, *orgB*, *sptP* and *SipD* in *S.* Adelaide, *S.* Bredeney, *S.* Cerro, *S.* Orion and *S.* Senftenberg. *avrA* encodes a protein that is 303 amino acids in length and is important for survival of the bacterium in

host epithelial cells and macrophages. The SPI-1 coding sequence for *S.* Adelaide and *S.* Orion lacked the *avrA* gene. *S.* Bredeney had a deletion of a single base pair that shifts the open reading frame, which results in a premature stop codon at 292. Base pair substation in the *avrA* sequence of *S.* Virchow also results in a premature stop at amino acid 251. Interestingly, S. Typhimurium DT44 had a triple base pair deletion in *avrA* that truncates the protein by one amino acid but did not have an effect on the open reading frame.

*orgB* encodes a protein that is 227 amino acids in length in LT2. An insertion of 10 base pairs at the 3' end of the *orgB* sequence was observed in S. Adelaide, S. Bredeney, S. Cerro, S. Orion and S. Senftenberg. This leads to a premature stop at amino acid 224 and disrupts the remainder of the open reading frame in this protein. These five serovars are lacking the 3' terminal VIR amino acid sequence that is observed in the *S.* Typhimurium definitive types and LT2.

Minor amino acid variability was observed for the sptP sequence of *S.* Adelaide, *S.* Bredeney and *S.* Senftenberg. A single base pair substitution was detected in the *sptP* sequence of *S.* Virchow, which creates a premature stop at position 128 of a 544 amino acid protein. For *sipD*, the *S.* Bredeney protein sequence was found to be highly variable and had 88.37% amino acid homology with LT2. In addition, a nine base pair deletion was detected in the *sipD* sequence for *S.* Bredeney. While this results in a 3 amino acid deletion in the protein coding sequence, it does not disrupt the open reading frame.

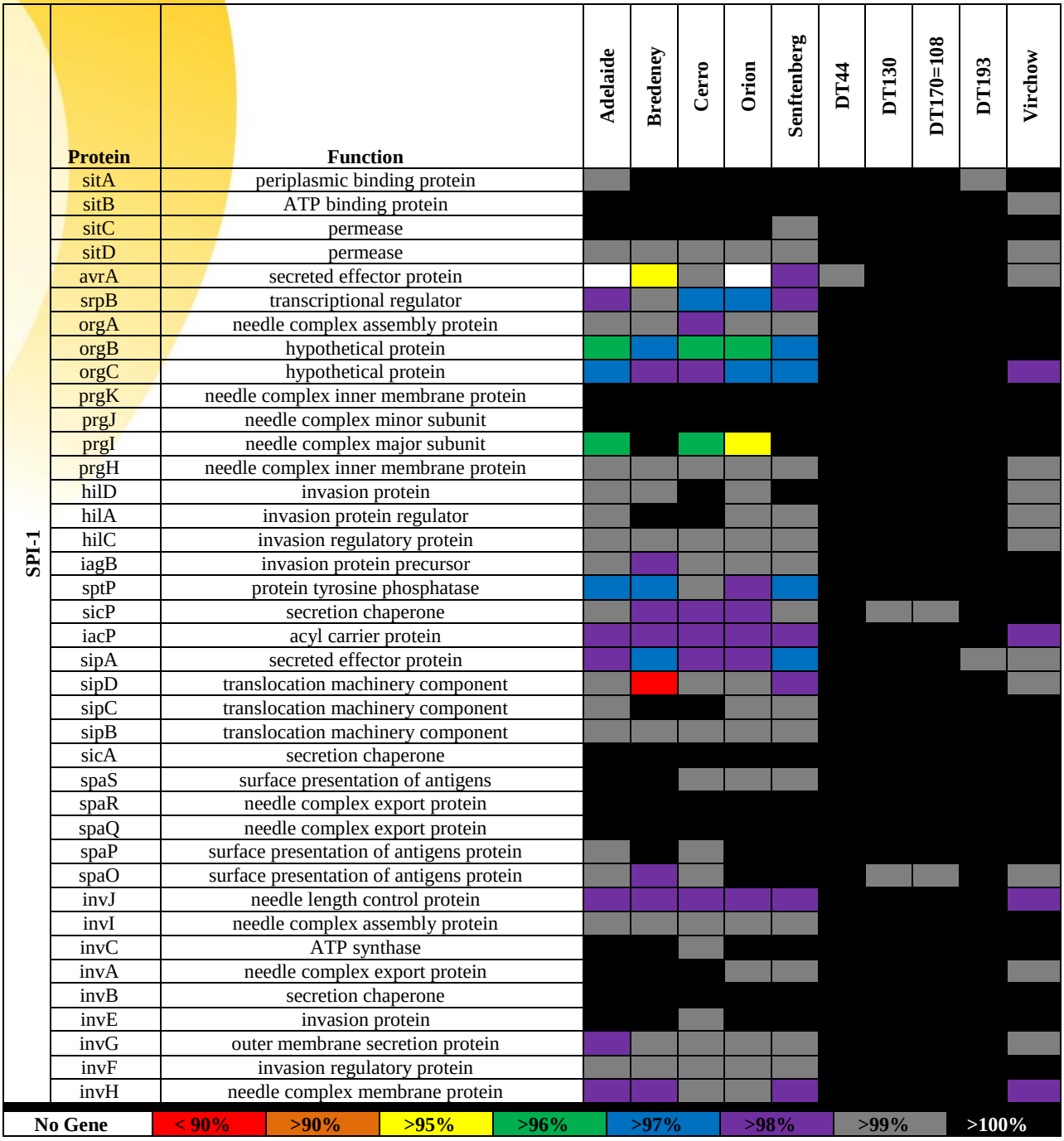


**Figure 7-1 Comparative analysis of specific pathogenicity island one from twelve non-typhoidal *Salmonella* serovars**

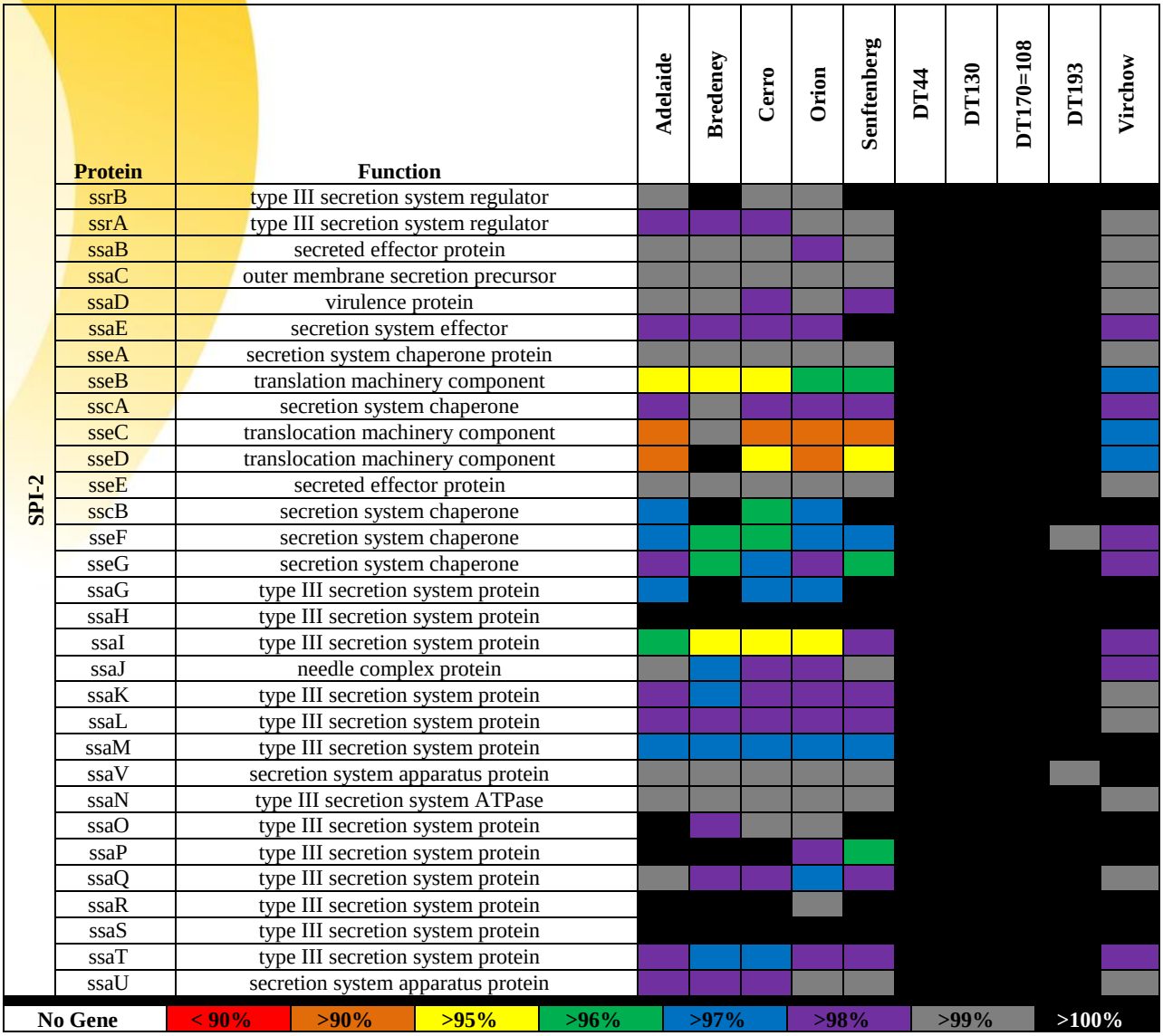


**Figure 7-2 Sequence variation of SPI-2 from twelve non-typhoidal *Salmonella* serovars**

A second T3SS is encoded by SPI-2 and is found in all members of *Salmonella enterica* (reviewed in [57]). Of the 31 SPI-2 genes analysed, 54.8% of the genes were found to have greater than 98% homology. Sequence variability observed in genes of SPI-2 was largely amino acid substitutions. No deletions or insertions were observed in any SPI-2 genes. The highest amount of amino acid variability was observed in the *sse* and *ssa* operons, primarily in *sseB*, *sseC*, *sseD*, *sseE*, *sseF*, *sseG* as well as *ssaG* and *ssaI*. This variability was observed most predominantly in *S.* Adelaide, *S.* Bredeney, *S.* Cerro, *S.* Orion and *S.* Senftenberg and to a lesser extent in *S.* Virchow (Figure 7-2).

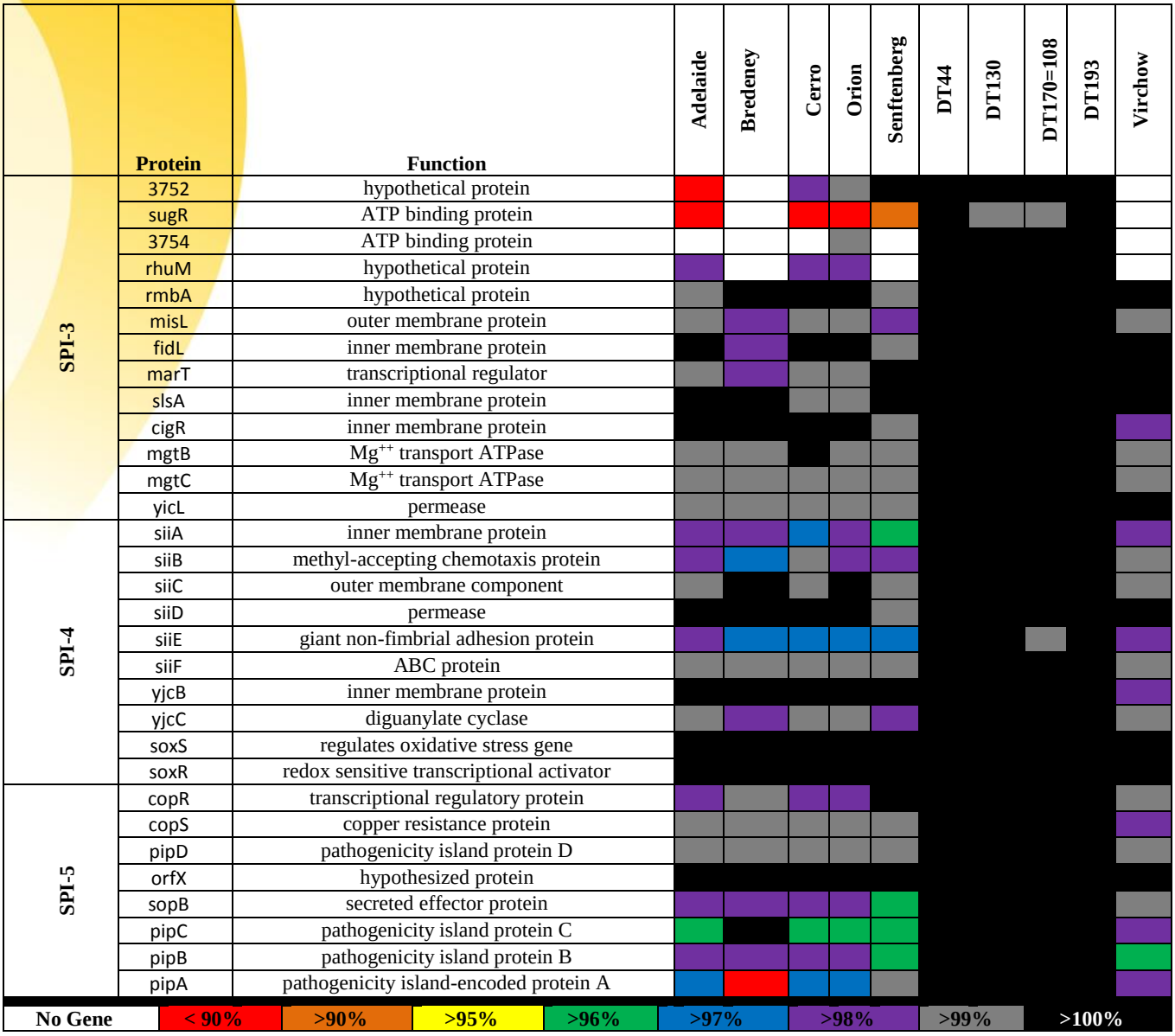


**Figure 7-3 Sequence variation of SPI-3, SPI-4, and SPI-5 from twelve non-typhoidal *Salmonella* serovars**

The genes encoded within SPI-3 are not functionally related with each other and are in general less well studied than those of the SPI-1 or SPI-2. Of the 13 SPI-3 genes, 69.2% of them had greater than 98% amino acid homology with LT2. The region of SPI-3 from STM3752 and *rhuM* was found to be highly variable for *S.* Adelaide, *S.* Cerro, *S.* Orion and *S.* Senftenberg. *S.* Adelaide, *S.* Cerro and *S.* Senftenberg were found to lack multiple genes within the STM372-*rhuM* region. STM3754 was absent in *S.* Adelaide, *S.* Cerro and *S.* Senftenberg (Figure 7-3). *S.* Senftenberg lacks the gene *sugR*. Neither *S.* Virchow nor *S.* Bredeney possessed the entire region (Figure 7-3). *S.* Singapore was found to have a deletion of two amino acids in *misL*.

SPI-4 has six open reading frames that are within a single operon *siiABCDEF,* which has recently been found to play a role in early interaction with the intestinal epithelium. In general, the 12 NTS serovars sequenced in this study were highly conserved for SPI-4; 70% of the genes were found to have greater than 98% homology with LT2. *siiA*,

*B*, and *E* exhibited the greatest variability in amino acid sequence for *S.* Bredeney, *S.* Cerro, *S.* Orion and *S.* Senftenberg (Figure 7-3).

Genes within SPI-5 code for effector proteins that are translocated by the T3SSs of both SPI-1 and SPI-2 [58]. The greatest amount of variability is seen in the *pipABC* operon. *S.* Bredeney possesses a 232 base pair deletion at the 5' end of *pipA* which completely disrupts the open reading frame. Minor amino acid variability was also observed for *pipA*, *pipB* and *pipC* have amino acid variability for *S.* Adelaide, *S.* Cerro, *S.* Orion and *S.* Senftenberg. Additionally, amino acid substitutions were observed for *sopB* in *S.* Senftenberg (Figure 7-3).

## 7.3 Discussion

The *Salmonella* genome possesses five major regions within the chromosome that encode virulence determinants. These regions, known as pathogenicity islands, are collections of genes that enable *Salmonella* to invade host cells, replicate and evade the immune response (Review article [59]). Genomic variability within these pathogenicity islands may contribute to the wide range of virulence observed for members of *S. enterica*. Twelve *Salmonella* serovars were selected based on their *in vitro* and *in vivo* virulence potentials, and their genomes were sequenced. Single gene sequence analyses of the five pathogenicity islands were performed and compared with the ATCC reference strain LT2.

The genes within SPI-1 are involved in invasion of host cells and the secretion of effectors that are involved in the modulation of the immune response. This is accomplished by the expression of a type III translocation secretion system (T3SS) that is encoded by SPI-1. The *prg, org, inv* and *spa* operons encode the needle complex of the T3SS while the *sic* and *sip* operons encode effector proteins [59]. The highest amino acid variability was observed for *orgB*, *orgC* and *prgI* in all serovars except the Typhimurium definitive types. *orgB*, along with *orgA*, is part of the effector sorting platform of the SPI-1 T3SS [60]. It is not clear whether the premature stop observed in *S*. Adelaide, *S*. Bredeney, *S*. Cerro, *S*. Orion and S. Senftenberg would have any effect. The needle complex is formed by 120 copies of *prgI,* which interacts with the tip protein *sipD* to form the pore structure of the T3SS that embeds in host cell membranes [61]. The amino acid sequence for *prgI* in S. Adelaide, *S*. Cerro and *S*. Orion exhibited considerable amount of variability, which may affect how the needle complex in these serovars is formed. The formation of the pore structure may also be affected if *sipD* is not able to interact normally. Interestingly, *S.* Bredeney had a nine base pair deletion in *sipD*, which leads to a three amino acid truncation of the protein. This deletion may also lead to a defect in pore formation by the bacterium and may contribute to the low invasion capacity observed for *S.* Bredeney.

A second T3SS is encoded by SPI-2 that is involved in the translocation of effectors across the *Salmonella*-containing vacuole in infected macrophages and epithelial cells [57]. The highest variability was observed in the *sse* and *ssa* operons. The greatest variability was observed in amino acid sequences of *sseB*, *sseC*, *sseD*, *sseE*, *sseF*, *sseG* as well as *ssaG* and *ssaI* for *S.* Adelaide, *S.* Bredeney, *S.* Cerro, *S.* Orion and *S.* Senftenberg, *S.* Worthington. Fewer amino acid substitutions were observed in *S.* Singapore and *S.* Virchow. The *ssa* operon that encodes the T3SS mutations in these genes may result in functional defects in its formation.

Future functional studies would need to be performed to identify whether the mutations observed here have any phenotypic effects. Proteins encoded by *sseB*, *C* and *D* are secreted on to the surface of the bacterium and are required for the translocation of effectors [59, 62]. Serovars that have mutations in these genes are severely attenuated

and are unable to secrete effectors through the T3SS (reviewed in [62]). It is interesting to note that *sseB* is a highly antigenic protein and in humans elicits a strong CD4 T cell response post inoculation with *S.* Typhimurium [63] and is currently being developed as a potential vaccine [64]. The degree of variability observed for *sseB* amongst the *Salmonella* selected for this study indicates that such a vaccine would likely be serovar-specific.

SPI-3 has ten open reading frames that encode virulence determinants with highly diverse functions. The *mgtBC* operon is involved in bacterial survival within macrophages as well as virulence in mice [65]. However, *misL* is an autotransporter protein that is involved in *Salmonella* adhesion, and acts as an intestinal colonisation factor in both chicks and mice [66]. Future studies need to focus on the most probable candidates in chickens for the colonisation attributes of *Salmonella*. All serovars included in this study exhibited high homology for the majority of SPI-3 genes. Substantial sequence variation was, however, observed in the region between STM3752 and *rhuM* in *S.* Adelaide, *S.* Bredeney, *S.* Cerro, *S.* Orion, *S.* Senftenberg, *S.* Singapore, and *S.* Virchow. *S.* Bredeney, *S.* Singapore and *S.* Virchow completely lack this portion of SPI-3. Sequence diversity has been demonstrated for this region across *S. enterica* by several groups (reviewed in [59]). It is unclear whether mutation or deletion within this region would have any effect on virulence as gene functions are unknown. Interestingly, *S.* Worthington, which exhibited low invasion capacity in Caco-2 cells, exhibited 100% homology with LT2.

SPI-4 is a 27 KB region within the *Salmonella* genome [67] that is comprised of six genes that are within a single operon *siiABCDE* [68]. During infection, SPI-4 acts in consort with SPI-1 to initiate invasion into host epithelial cells [69]. Deletion of SPI-4 attenuates the virulence of both *S.* Typhimurium and *S.* Enteritidis in mice [70]. The gene *siiE* encodes a giant, non-fimbrial adhesion protein that enables the bacterium to adhere to the apical surface of a host cell a process that is required for SPI-1 T3SS mediated invasion [69, 71, 72]. The *siiE* amino acid sequence found in *S.* Bredeney, *S.* Cerro, *S.* Orion and *S.* Senftenberg had 97% homology with LT2 but it is unknown whether this variability would have any functional effect on the adhesion. Similar levels of amino acid variability were observed in the *siiA* sequence for *S.* Cerro, *S.* Senftenberg and *S.* Worthington as well as the *siiB* sequence of *S.* Bredeney and *S.* Singapore. It has recently been shown that *siiA* and *siiB* form a proton channel within the inner membrane of the bacteria and that they are regulatory proteins for *siiE* [73]. The amino acid variability observed in the *siiA* and *siiB* sequences of the *Salmonella* included in this study may have an effect on the regulation of *siiE* in these serovars but this requires further functional experiments to determine.

The pathogenicity island SPI-5 encodes six genes that play a role in the enteropathogenesis of *Salmonella* [74]. They are induced by distinct regulatory cues that occur at different stages phases of infection [58]. Amino acid sequence variability in SPI-5 was observed primarily for *pipA*, *pipB*, *pipC* and *sopB*. The largest variation was observed for *pipA* in *S.* Bredeney. *S.* Bredeney has a large deletion in *pipA* that alters the open reading frame. *pipA* is an effector protein that is translocated across the SVC by the SPI-2 T3SS and is important for the development of systemic disease in mice [58]. Considerable amino acid variation was also observed in the *pipA* sequence of *S.* Adelaide, *S.* Cerro and *S.* Orion but it is not known whether this amino acid variability could have any effect on mice. Additional variability was observed in *pipC* for *S*. Adelaide, *S*. Cerro, *S*. Orion and *S*, Senftenberg. The *pipC* protein functions either as a chaperone or is involved in the stabilisation of *sopB* [75].

Overall, the sequences of the five specific pathogenicity islands were found to be highly conserved amongst the 12 *Salmonella* serovars selected for this study. Sufficient amino acid substitution and deletions were observed across all the pathogenicity that could affect

the *in vitro* invasion and *in vivo* pathogenicity of NTS serovars. Further investigations are, however, required to identify the outcomes of the sequence variability and its effects on virulence.

# 8 Dynamics of *Salmonella* Typhimurium definitive type 9 shedding from early to peak lay in laying hens

## 8.1 Introduction

Globally, eggs and raw egg products are frequently linked to outbreaks of human salmonellosis. In Australia, *Salmonella* Typhimurium (*S*. Typhimurium) is the most frequently reported serovar in egg related food poisoning [4]. *Salmonella* enters the gastrointestinal tract by ingestion and establish persistent infection in the caeca of chickens [78]. The persistence and extent of flock colonisation is dependent on several factors such as the bacterial strain, dose of initial inoculum, age, genetic line and immune status of the birds [51, 52]. At oviposition, 90% of eggs are free of bacteria [79]; however, eggshell surface contamination can occur during contact with any surface. Previous studies reported that an increase *Salmonella* shedding in faeces could increase the likelihood of eggshell contamination [33]. Studies conducted under field conditions showed that 39.3% of *Salmonella* positive flocks had at least one positive eggshell with a total of 1.05% of eggshells testing positive for *Salmonella* [80].

Stress has a profound impact on the host immune response. Laying stress, in particular, can affect the avian immune response, making them more susceptible to *Salmonella* infection and contributing to increased *Salmonella* shedding [50]. Gole *et al* [33] conducted a study on a single age layer flock for detection of *Salmonella* shedding at the onset of lay and found the highest prevalence of *Salmonella* in faeces (82.14%) was at onset of lay (18 weeks). However, there is a paucity of literature investigating dynamics of *Salmonella* Typhimurium shedding from the early to peak lay stage in experimentally infected birds.

Our epidemiological investigation on caged layer farms indicated that *S*. Typhimurium definitive type 9 (DT9) was consistently isolated from the egg farms in South Australia. It was also observed that the *Multiple Loci VNTR Analysis* (MLVA) pattern of *S* Typhimurium DT9 changed in the same flock sampled over period of ten months. The MLVA patterns of DT9 isolated from egg layer farms and human outbreaks reported in South Australia were similar. Moreover, there are very few reports in Australia that have examined the contamination of egg internal contents of DT9 in infected laying hens. Our hypothesis was that MLVA of *S* Typhimurium DT9 changes over the period of time due to selection pressure of the gut microflora.

The present experiment was conducted to study the vertical transmission of *S.* Typhimurium DT9 as well as the shedding pattern of *S*. Typhimurium DT9 in eggs and faeces of laying hens. In addition the effects of co-infection with *S*. Mbandaka, on the MLVA stability of DT9 loci were also studied.

## 8.2 Materials and methods

### 8.2.1 Experimental chickens

Fertile eggs were obtained from commercial Hy-Line brown layer parent flocks and were hatched in animal facilities at Roseworthy campus, the University of Adelaide. A total of 32 pullets were raised in pens up to week 10 and then divided in to three treatment groups consisting of control (C), *Salmonella* Typhimurium (T group) and *S*. Typhimurium + *S*. Mbandaka combination (TM group), and housed in individual cages in three separate positive pressure rooms for the duration of the experiment. Feed was gamma irradiated, and water was treated with chlorine and provided *ad libitum*. Strict biosecurity measures were maintained to ensure the birds remained free of *Salmonella* until inoculation and to avoid cross-contamination throughout the experiment. Prior to infection, feed, water and faecal samples were screened for *Salmonella* spp. fortnightly.

All experimental birds were negative for *Salmonella* spp. before infection. All the experiments were performed according to the Australian code for the care and use of animals for scientific purposes and approved by the Animal Ethics Committee of the University of Adelaide (UA-S-2014-008A).

### 8.2.2 Bacterial inoculations

At 14 weeks of age, control birds received only sterile Luria-Bertani (LB) broth, other hens were orally inoculated with $10^9$ colony forming units (CFU) of either *S*. Typhimurium definitive type DT9, MLVA type 03 24 11 12 523 (T group) or a combination of *S*. Mbandaka and DT9 (TM group) suspended in LB broth (Oxoid Australia). *Salmonella* isolates used in this study were originally isolated from Australian layer farms Gole *et al*. (2014 b) and typed at the Institute for Medical and Veterinary Science (IMVS), Adelaide, Australia.

### 8.2.3 Faecal sampling for bacteriology and most probable number

Faecal samples were collected aseptically from individual hens in Whirl-Pak™ plastic bags (ThermoFisher Scientific, Australia) on day 0 followed by day 1, 3, 6 and then at weekly intervals from 1 to 16 weeks post-infection (p.i.). All faecal samples were cultured for *Salmonella* isolation using the previously described method [33], and processed for enumeration of *Salmonella* by the most probable number (MPN) method as described earlier [81, 82].

### 8.2.4 Bacteriological analysis of eggshell wash and internal contents

All birds started to lay at 5 weeks p.i.. Eggs were collected from an individual hen during 5 to 16 weeks p.i.. Each egg was collected in a separate sterile Whirl-Pak™ plastic bag to avoid cross-contamination. Eggshell wash and internal egg contents were processed separately for *Salmonella* isolation as described previously [33].

### 8.2.5 Collection of bacterial colonies for MLVA typing

Upon enumeration, suspected cultures were streaked on *Xylose lysine deoxycholate agar* (*XLD*, Oxoid, Australia) plates. Ten colonies from each infected bird, both from T and TM group were collected. DNA extraction from all *Salmonella* isolates was

performed using 6% Chelex® (Bio-Rad, Sydney, NSW, Australia) and prepared in TE. Multiplex PCR was performed to differentiate between *S.* Typhimurium and *S.* Mbandaka [33]. The confirmed *S.* Typhimurium colonies from the mixed infected group (TM) from weeks 1, 3, 5, 9, 14, 15 and 16 were sent for MLVA typing. MLVA typing was performed at the *Salmonella* Reference laboratory, Institute of Medical and Veterinary Sciences (IMVS). Eleven (11) *S*. Typhimurium cultures were tested for MLVA.

### 8.2.6 Statistical analysis

All MPN values were expressed as per gram of wet faeces and data were analysed statistically using two way ANOVA, using SAS© 6.0 software. P values < 0.05 were considered statistically significant.

## 8.3 Results and discussion

T and TM group birds did not show clinical signs of infection except for mucoid or blood tinged faeces for 48 hours p.i. There were no mortalities. Clinical signs were not recorded in the control group.

### 8.3.1 *Salmonella* populations and count in the faecal samples

The dynamics of *Salmonella* shedding from one (1) day to 16 week p.i. hens are presented in Figure 8-1. A total of 608 faecal samples were processed for the isolation and enumeration of *Salmonella*. None of the faecal samples from control hens was *Salmonella* positive. For both the *S.* Typhimurium (T group) and *S*. Typhimurium + *S*. Mbandaka combination (TM group) overall, no significant difference ($P > 0.05$) in *Salmonella* spp. was detected between the two treatment groups (Mean ± SE of 22.85 ± 4.09 and 28.93 ± 4.05 MPN/g for T and TM group, respectively), and no significant difference ($P > 0.05$) was found between weeks p.i. The overall *Salmonella* counts in faeces ranged from 1.53 to 48.53 MPN/g of faeces for birds infected in T group and 0.30 to 73.60 MPN/g in the birds infected in TM group. *Salmonella* shedding in birds was variable. At one week p.i. hens showed the highest MPN count, which could be due to the early stage of the infection and a large number of bacteria in the intestine. The degree to which intestinal, hepatic, splenic, or reproductive tissues are colonised by ST or SE isolates following oral inoculation does appear to vary substantially [83].

At six weeks p.i. there was an increase in the MPN count, which could be a result of stress and impaired immune response induced by the onset of lay, suggesting that laying stress had reactivated the bacteria. However, further investigation is necessary to conclude these findings. The MPN count was increased at the 12th, 14th and 16th week p.i. for the T group, which indicated that *Salmonella* can persist in the intestinal tract of birds for a prolonged period of time. Similar findings were recorded by several researchers [84, 85, 86, 87].

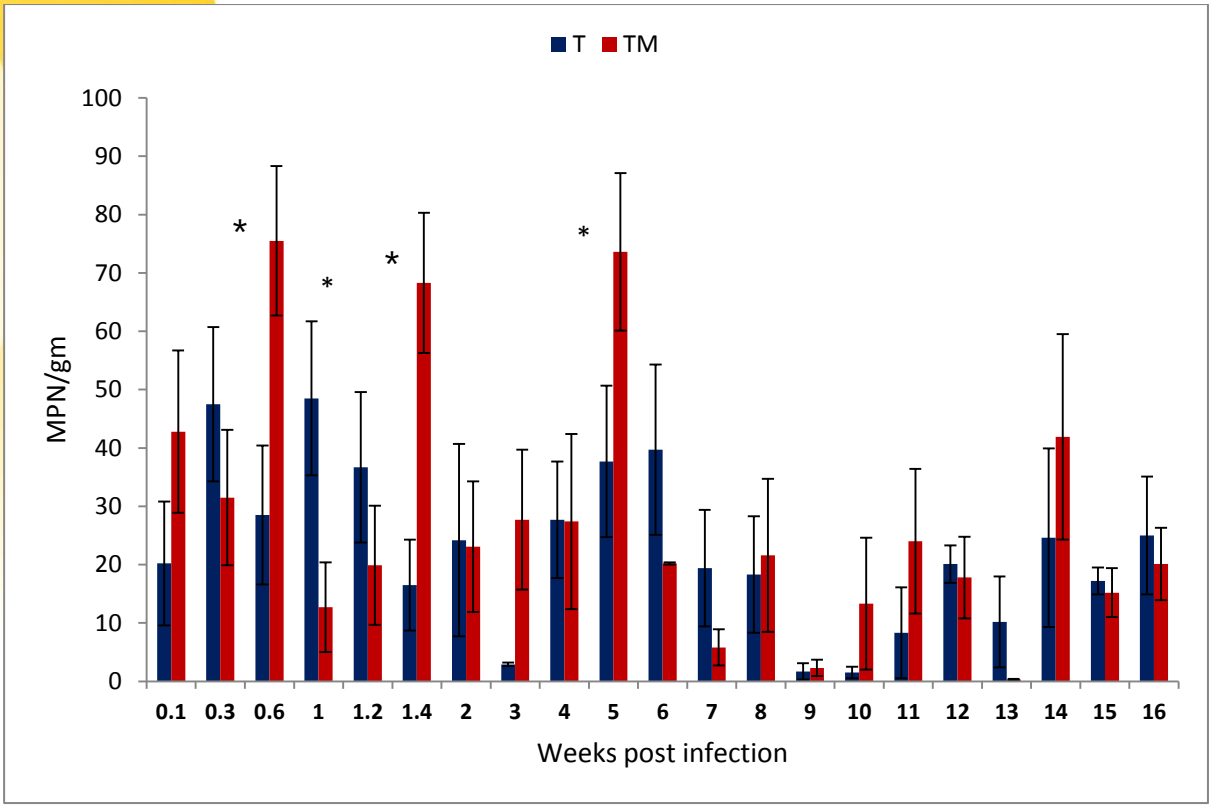


**Figure 8-1 Populations and Most Probable Number/g of faeces at different weeks post infection in *Salmonella* Typhimurium (T) and *S*. Typhimurium + *S*. Mbandaka (TM) in experimentally infected hens**

* indicates the significant differences between groups.

### 8.3.2 Detection of *Salmonella* from eggshell wash and internal contents

A total of 1931 egg samples was tested for *Salmonella*. None of the samples from control hens was *Salmonella* positive. The frequency of eggshell contamination ranged from 0 to 21.74% in the T group and 16.6 to 33.33% in the TM group (Figure 8-2). *Salmonella* was isolated from the start of laying (i.e. sixth week p.i. to fourteenth week p.i.) from the eggshell wash of experimentally infected hens of both T and TM groups. The percentage of shell contamination was highest in both groups at six weeks p.i. and lowest at eight week p.i.. Over the course of the experiment, *Salmonella* was not detected in egg internal contents of either group. It is important to note that at least one out of 14 hens (7.1%) laid an egg with a contaminated eggshell at every sampling (except for the T group at 7 weeks p.i.). This finding could be commercially very important if extrapolated to the larger number of birds in a commercial flock.

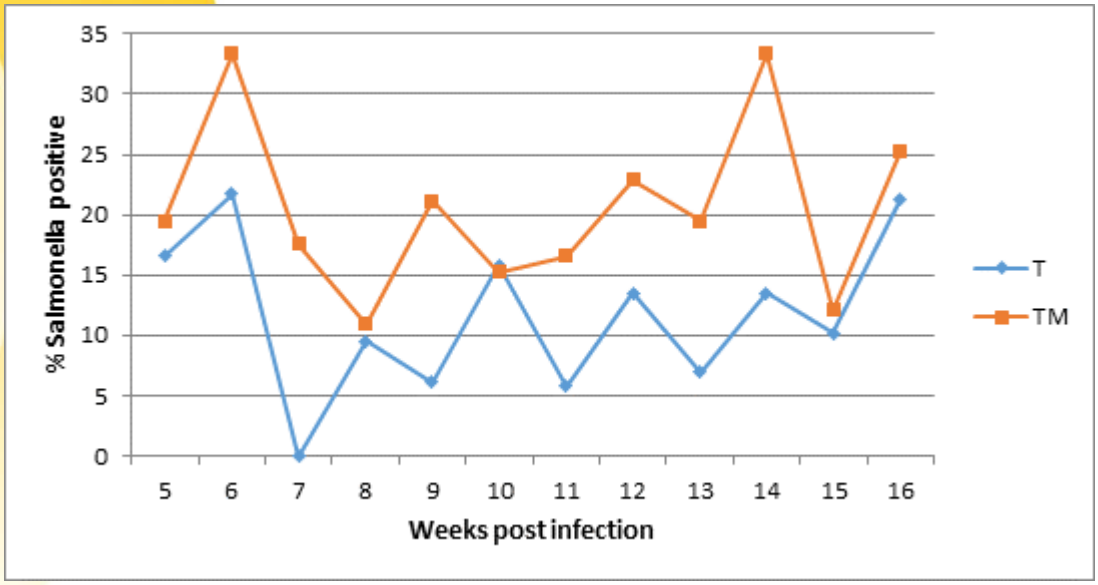


**Figure 8-2 Percent eggshell surface contamination at different weeks post infection by *Salmonella* Typhimurium (T) and *S.* Typhimurium + *S.* Mbandaka (TM) in experimentally infected hens**

### 8.3.3 Stability of MLVA

The MLVA pattern of 11 representative *S.* Typhimurium DT9 cultures did not show any variation. However, further investigation of the number of colonies is essential to rule out the stability or instability of MLVA loci.

# 9 General discussion

Many enteric bacterial pathogens are an inherent part of the food chain. They are present in many food production systems and are able to pass from domestic animals to humans with the potential for causing disease. *Salmonella* spp. have established niches within many food production systems and can contaminate food items consumed by humans. Once infected with *Salmonella*, humans may develop gastroenteritis and under particular circumstances septicaemic disease.

The genus *Salmonella* is highly diverse. It is comprised of two species: *S. bongori* and *S. enterica*. Members of both *S. bongori* and *S. enterica* have the ability to invade host epithelial cells, an ability that is conferred by the presence of specific pathogenicity island 1. However, it is the members of *Salmonella enterica*, in particular subspecies I, that are most commonly isolated from mammals and birds. Human salmonellosis can be caused by 90% of the *Salmonella* classified within subspecies I. Globally however, 70% of disease is caused by 12 of the most virulent serovars [29].

Some *Salmonella* spp. have established a niche within poultry environments. Many serovars from subspecies I are able to infect, colonise and establish persistent infection within layer hens. Periodic shedding of bacteria can occur if a bird is particularly stressed (physiologically or environmentally) which may lead to either the horizontal or vertical transmission of *Salmonella* to an egg [41, 46]. The experiments described in this report were designed to compare the virulence of NTS serovars commonly isolated from Australian layer hen farms with the aim of identifying which serovars posed the highest overall public health risk. Four separate aspects of virulence were investigated: virulence gene profile, *in vitro* invasion of human intestinal epithelial cells, *in vivo* virulence in a mouse model, and the comparative genomics of *Salmonella* specific pathogenicity islands. The *in vitro* invasive potential of *Salmonella* serovars in the different parts of the oviduct collected from *Salmonella* free hens was also studied.

Virulence gene profiles were generated for each of the 29 *Salmonella* serovars selected for this study. Genes involved in adhesion, invasion, intracellular replication and survival were amplified using standard PCR. An aim of this study was to generate a pathotyping profile that could be used to distinguish between virulent and avirulent *Salmonella* serovars. Comparison of these results with the invasion assay data (discussed in Chapter 4) did not, however, reveal any pattern associated with virulence. This outcome was unexpected as pathogenicity has previously demonstrated correlated with the presence or absence of virulence genes [76, 77]. These studies, however, have not included avirulent serovars nor have they included serovars known not to have previously circulated or passaged in the human population (or isolated from humans). The authors believe that a molecular based pathotyping method for *Salmonella* is achievable, but possibly with a different subset of genes.

The findings from the intestinal epithelial invasive assays described in Chapter 4 could have significant implications for egg handling in the kitchen during food preparation, as the study suggested that some strains of *Salmonella* require prior enrichment to stimulate virulence. The environment of many freshly prepared raw egg containing food products (e.g. poor storage conditions, presence of salt and sugar) comprises components that may enable certain serovars not only to survive and replicate but also to express virulence factors prior to human ingestion. Moreover, these results also indicate that many *Salmonella* serovars, in particular the Typhimurium definitive types, may have constitutively active virulence gene(s) that enable invasion under any conditions. This may provide a selective advantage for these strains. Future work should

be focused on determining the molecular and cellular mechanisms responsible for these differences.

The inbred mouse strain, BALB/c was selected for *in vivo* virulence studies of serovars possessing low, moderate or high invasive capacities in the Caco-2 model. Mice infected with the Typhimurium DT9, DT44, DT135, DT170=108 or DT193 exhibited the greatest morbidity and mortality in the mouse model. No clinical symptoms were observed for any other serovar included in the mouse trials. *S.* Adelaide, *S.* Bredeney, *S.* Cerro and *S.* Lille, however, also exhibited very low levels of *in vitro* invasion. However, these "low" or moderately virulent *Salmonella* serovars were able to invade the mouse intestinal epithelium, survive and replicate to sufficient levels that enabled shedding, albeit sometimes intermittently, over the course of the entire experiment. The results obtained in this study are consistent with those obtained for other non-typhoidal serovars isolated from human cases [30]. The results obtained from comparative *in vivo* investigation of *Salmonella* virulence indicate that the Typhimurium definitive types were more invasive for humans. It is important to note, however, that only one isolate of each serovar was used in these studies. It has been established that there can be considerable within serovar variation in virulence [21]. Also the mice were infected with enriched *Salmonella* serovars (enriched in LB). Future studies could be directed at the virulence potential of enriched vs. non-enriched *Salmonella* serovars in the mouse infection model.

The invasive potential of *Salmonella* isolates was investigated in the oviduct but not ovary. The birds were raised in *Salmonella*-free conditions. The *Salmonella* positive or negative status of hens used in reported studies was confirmed by either serological tests or testing faecal samples (drag swabs) at a single point prior to commencement of the experiment. It is important to note that there are 2500 different *Salmonella* serovars and commercial antibody testing kits available in the market could provide false negative results due to extensive antigenic variation amongst *Salmonella* serovars. The culture method is sensitive and ideal for detection/isolation of *Salmonella* serovars, however, a frequent (longitudinal) sampling is required before confirming the *Salmonella* positive or negative status of the flock. Our epdemiological work with Gore et al. [46] showed variation in *Salmonella* detection amongst a known *Salmonella* positive flock tested over a period of ten months. *In vitro* experiments suggested that there was a variation in the invasive ability of *Salmonella* serovars in the oviduct explants. *S.* Infantis was highly invasive compared to other *Salmonella* isolates. However, future *in vivo* studies are essential to confirm these *in vitro* findings.

Whole genome sequencing was performed on 12 of the serovars selected for this study. The sequences of five specific pathogenicity islands were compared with the ATCC reference strain Typhimurium LT2. All serovars possessed all five specific pathogenicity islands. Overall, high homology was observed across all sequences analysed. Amino acid variability and deletions were observed in several genes involved in the formation of both the SPI-1 and SPI-2 T3SS, as well as amongst their effector proteins. The sequence analyses performed thus far demonstrate that the Tyhpimurium definitive types are nearly 100% conserved for all genes but the "low" virulent genes are variable. This variability is, not, however consistent amongst the serovars. Therefore, it is the summative contribution of all of the genomic variation observed that contributes to "low" virulence. Current sequence analyses are focused on variation in flagellar, fimbrial and LPS genes to determine the contribution of these gene sets to virulence. Many genes within the *Salmonella* genome have redundant functions but are regulated via different pathways. We are also investigating whether "low" virulent serovars have defects in regulatory pathways.

*S*. Typhimurium DT9 infected birds did not show any clinical signs apart from the presence of mucoid faeces for 48 hrs. There was intermittent and prolonged shedding of

*Salmonella* in the faeces and eggs (eggshell and internal contents). *S*. Typhimurium DT9 can persist in the gut of laying hens over a prolonged period of time. Hens can then shed bacteria during stressful events. The MLVA pattern of 11 representative *S.* Typhimurium DT9 cultures did not show any variation. However, further investigation of the number of colonies is essential to rule out the stability or instability of MLVA loci. Change of MLVA loci by one or two base pairs was reported during field investigation [33], however, variation in the human virulence of *S*. Typhimurium strains with destabilised MLVA pattern is yet to be investigated.

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# 11 Plain English Summary

| **Project Title** | Virulence of layer farm or egg associated *Salmonella* isolates |
|---|---|
| AECL Project No. | 1UA121 |
| Researchers | Kapil Chousalkar and Andrea McWhorter |
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| **Objectives** | This work was conducted to study the pathogenesis of egg/layer farm-related *Salmonella* serovars in a well-differentiated human intestinal cell culture model (i.e. *in vitro*) followed by a mouse model (*in vivo*). The ability of *Salmonella* serovars to colonise the chicken oviduct organ cultures developed from *Salmonella* free laying hens was also investigated. The *Salmonella* serovars were classified as high, medium and low invasive serovars. The genome of selected high, medium and low *Salmonella* serovars was also studied. |
| **Background** | *Salmonella* infection is one of the most common foodborne infections worldwide including Australia. Despite the fact that eggs produced in Australia are of high quality and safety, eggs have been implicated in several *Salmonella* foodborne disease outbreaks. Major studies that investigated the pathogenicity of *Salmonella* serovars for human epithelial cells have been mainly focused on *Salmonella* Enteritidis or *Salmonella* Typhimurium to some extent. Our hypothesis was that not all *Salmonella* isolates/serovars recovered specifically from layer farm environments or eggshell wash have the same level of virulence. Thus the main objective of the proposed study was to identify the *Salmonella* serovars isolated from egg wash and poultry environmental samples with high or low invasiveness for human epithelial cells and animal models, and also to study their ability to colonise or invade cells from the chicken oviduct. |
| **Research** | The predominant *Salmonella* serovars were used in this study. The isolated *Salmonella* serovars were tested by PCR for virulence genes. The invasion potential of the *Salmonella* serovars was tested in a well-differentiated human intestinal cell culture model and also by cell invasion assay. The results were further confirmed by *in vivo* invasion assay in a *Salmonella* mouse model. The *Salmonella* serovars with high invasive ability were also tested for their ability to invade the oviduct organ culture. *Salmonella* Typhimurium infection in laying hens was also investigated. |
| **Outcomes** | PCR was a rapid method for *Salmonella* spp. detection. This tool was not conclusive for discriminating virulent or non-virulent *Salmonella* serovars. *In vitro* results of virulence typing of *Salmonella* serovars indicated that the *Salmonella* Typhimurium definitive types DT44, DT135, DT170=108, DT193, as well as *S.* Virchow, all remained highly invasive. The findings of intestinal epithelial invasive assays suggest that some strains of *Salmonella* require prior enrichment to stimulate virulence. It is possible to raise *Salmonella* free birds, provided that good hygiene, sanitation and biosecurity conditions are maintained. S. Typhimurium DT9 was able to persist in the gut of laying hens for up to |

| | |
|---|---|
| | 16 weeks post infection. All egg contents were negative for S. Typhimurium. |
| **Implications** | *Salmonella* Typhimurium isolates used in this study were highly invasive upon enrichment. If there is any point during food preparation or storage that encourages the growth or enrichment of *Salmonella* within the food item or supply chain, the risk of potential food poisoning increases. Future studies could be directed to investigate the conditions under which *Salmonella* is amplified in a food preparation or storage environment. |
| **Key Words** | eggs; hens; PCR; *Salmonella*; *Salmonella* Typhimurium; *Salmonella* invasion; virulence of *Salmonella* serovars |
| **Publications** | • Evaluation of virulence in *Salmonella* isolates from egg farms. Submitted to Applied and Environmental Microbiology.<br>• Virulence of Non Typhoidal *Salmonella* Serovars. Pending approval from AECL for submission to MBIO. |