Molecular Epidemiology of *Salmonella* in the Broiler Industry of Sri Lanka

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Abstract

The increasing occurrence of non-typhoidal *Salmonella* in poultry is an emerging threat for public health in Sri Lanka, and salmonellosis has incurred massive economic loss for the poultry industry in the country. Thus, the thesis presented encompasses a comprehensive study to understand prevalence and possible risk factors for *Salmonella* carriage in broiler farms as well as whole-genome sequence-based population structure, phylogenetic relationships and antimicrobial resistance in *Salmonella* in Sri Lankan poultry.

The studies described in this thesis include a cross-sectional survey (i.e., sampling and questionnaire-based study) conducted from July to December 2017 in broiler farms (115) from poultry-dense areas and associated hatcheries (15) as well as an outbreak study (from 2010 to 2018), based on isolates and metadata from poultry salmonellosis outbreaks. After initial identification and PCR confirmation of a total of 164 *Salmonella* isolates, whole-genome sequencing was performed and antimicrobial resistance profiles of the isolates were determined.

Results revealed a *Salmonella* prevalence of 32.2%, CI 95% [23.6-40.7] in broiler farms and 66.7%, CI 95% [42.8-90.5] in the associated hatcheries. Litter management, rest period between flocks, feed storage, district and farmers' knowledge of sick birds were identified as risk factors for *Salmonella* carriage in the broiler farms, through multivariate logistic regression modelling. Eighteen different multi-locus sequence types of *Salmonella* were identified, including nine which were reported for the first time in Sri Lankan poultry. The most common serovars were *S*. Kentucky ST314 (26.8%, CI 95% [20.0-33.6]) and *S*. Enteritidis ST11 (19.5%, CI 95% [13.4-25.6]). A high percentage of quinolone resistance manifesting as resistance to nalidixic acid (41.5%, CI 95% [33.9-49.1]) and intermediate resistance to ciprofloxacin (45.1%, CI 95% [37.5-52.7]) and enrofloxacin (35.4%, CI 95% [28.0-42.7]) was found. The findings of this thesis, especially in the absence of previous comprehensive studies, will enable the design of control strategies to strengthen the national *Salmonella* control programme in Sri Lanka.

Presentations at Conferences

Molecular Epidemiology of *Salmonella* in the Broiler Industry in Sri Lanka: Preliminary Results: Poster presentation at the annual scientific sessions of Sri Lanka Veterinary Association (SLVA), held on 14 June 2018 in Kandy, Sri Lanka.

Motile *Salmonella* spp in Sri Lankan poultry: An impending public health threat? Poster presentation at the One Health Aotearoa Symposium, Wellington, New Zealand held on 12-13 December 2018

***Salmonella* in Poultry in Sri Lanka:** Oral Presentation at the 'Three Minute Thesis' Competition at the Annual Symposium of New Zealand Food Safety Science and Research Centre (NZFSSRC), held on 1 July 2019 in Christchurch, New Zealand.

Diversity of *Salmonella* isolates sourced from poultry in Sri Lanka: Oral Presentation at the Annual Symposium of the New Zealand Microbiology Society (NZMS) held on 25-28 November in Palmerston North, New Zealand.

Genotypic diversity of *Salmonella* in poultry in Sri Lanka: Poster presentation at the Lorne Genome 2020 Conference held on 16-18 February 2020 in Lorne, Australia.

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Abbreviations

AIC: Akaike Information Criterion

AMP: Ampicillin

AMR: Antimicrobial resistance

ASEAN: Association of Southeast Asian Nations

AST: Antibiotic Susceptibility Tests

BGA: Brilliant Green Agar

bp: base-pairs

C: Chloramphenicol

cgMLST: core-genome multi-locus sequence typing

cgSNP: core-genome single nucleotide polymorphisms

CI: Confidence Interval

CIP: Ciprofloxacin

CLSI: Clinical and Laboratory Standards Institute

CN: Gentamicin

CTX: Cefotaxime

DAPH: Department of Animal Production and Health

DNA: Deoxyribonucleic acid

DOC: day-old chicks

ECDC: European Centre for Disease Prevention and Control

EFSA: European Food Safety Authority

ENR: Enrofloxacin

EU: European Union

FAO: Food and Agriculture Organization

FT: Fowl typhoid

GDP: Gross Domestic Production

GOF: goodness of fit

GP: Grand Parent

GVR: Government Veterinary Range

iTOL: Interactive Tree Of Life

LRT: Likelihood-ratio tests

MDR: Multidrug resistant/ multidrug resistance

MEGA: Molecular Evolutionary Genetic Analysis

MEM: Meropenem

MLST: Multi-locus sequence typing

MLVA: multi-locus variable-number tandem repeat analysis

MPI: Ministry of Primary Industries

NA: Nalidixic Acid

NGS: next-generation sequencing

NTS: Non-typhoidal *Salmonella*

OIE: Office International des Epizooties (World Organization for Animal Health)

OR: Odds ratios

PC3: Physical Containment level 3

PCR: Polymerase chain reaction

PD: Pullorum disease

PFGE: pulsed-field gel electrophoresis

QC: Quality Control

rMLST: Ribosomal multi-locus sequence type

ROC: Receiver Operating Characteristic

RVS: Rappaport-Vassiliadis soy peptone

SE: Standard Error

Sistr: *Salmonella in silico* Typing Resource

SNP: Single Nucleotide Polymorphism

ST: Sequence type

SXT: Trimethoprim-Sulphamethoxazole

TE: Tetracycline

UNESCO: United Nations Educational, Scientific and Cultural Organization

VDCA: Veterinary Drug Control Authority

VIC: Veterinary Investigation Center

VRI: Veterinary Research Institute

WBAT: Whole Blood Agglutination Test

wgMLST: whole-genome multi-locus sequence typing

WGS: Whole genome sequencing

wgSNP: whole-genome single nucleotide polymorphisms

WHO: World Health Organization

XLD: Xylose Lysine Deoxycholate

Glossary of Terms Used in Poultry Industry in Sri Lanka

Buy-back: Unique feature in the broiler industry, where small-scale farmers are engaged in rearing for companies. Many leading broiler companies adopt this system, where they enter into a contract with small-scale farmers for rearing chickens.

Capacity of poultry farms: According to the department of Census and statistics in Sri Lanka, commercial poultry farms are broadly categorised into farms with greater than or equal to 1000 chicken (also called farms over 1000 chicken) and farms with fewer than 1000 chicken (also called farms below 1000 chicken). Farms over 1000 chicken are again categorised as small-scale (up to 5,000 birds), medium-scale (5,001-25,000 birds), large-scale (>25,000 birds).

Closed-house: environmental controlled (optimal temperature, humidity etc) poultry house in large-scale farms, often company-owned. The flock capacity can be 10,000 birds upwards with automated facilities and managed by trained staff.

DAPH Biosecurity Grading: All hatcheries are monitored annually and scaled from A to C (A-satisfactory, C- poor), based on their prevalent biosecurity levels, with the objective being the provision of technical expertise to improve to next level of biosecurity or to maintain the level if it is within the level of satisfactory.

Dead-in-shell eggs: Unhatched eggs with dead and/or live (weak) chicks or dead embryos, after completion of the usual incubation period (~21 days) in a hatchery.

Egg batch: eggs in a hatchery, which is the offspring of same batch of parent birds.

Eggshells: eggshells remain after hatched chicks were collected in the hatcher of a hatchery
Farm stage: single-stage/ one flock (i.e., all in all out) or multi-stage (more than one flock simultaneously)

Flock: birds of same age in a farm. Often a flock is housed together, however it can differ depending on the number of birds and space availability.

Fluff: debris remain after hatching (e.g., down feathers) in the hatcher of a hatchery

Government Veterinary Range: refers to a geographical area under the purview of one senior government veterinarian (who may have one or two veterinarians reporting to him/her). Government veterinary ranges roughly correspond to the administrative

divisions of the country. In a government veterinary range, the senior veterinarian-in-charge is responsible for providing the required services (health, breeding, extension etc.) for government sector and small-scale private sector ventures as well as for households, though not for large-scale private enterprises that employ their own veterinarians.

Hatch-date: Estimated hatched out of approximately 90% eggs in an egg batch. Usually pull out in a hatchery is carried out after completion of incubation: between 504 to 512 hours (~21 days).

Hatcher: A cabinet used for egg incubation in the hatchery

High deaths: refers population wise mortality of 5% or more

Open-house: poultry pen with half walls of approximately 0.5 m height, with a wire mesh to complete the walls up to roof.

Poultry belt: Western and North-Western provinces are best known for poultry production in Sri Lanka. This region, consisting of Colombo, Gampaha and Kalutara districts of Western province and Kurunegala and Puttalam districts of the North-Western province, is known as the 'poultry belt'.

Rest period: lapse of time of having an empty poultry house after cleaning and disinfection, between two successive flocks on the same holding

Routine medication: refers to administration of probiotics, multivitamins, prophylactic antibiotics etc.

Routine vaccination: refers vaccines against viral diseases such as Infectious Bursal Disease and Newcastle Disease

Turnaround time: Total time (including cleaning, disinfection and rest period) between two successive flocks on the same holding

Chapter 1: Introduction

1.1. Background

Salmonellosis is a major food-borne public health concern worldwide (Antunes *et al.*, 2016; Hohmann, 2001; Yachison *et al*., 2017) with an estimated 95.1 million (95% CI 41.6-184.8) cases of enterocolitis and 50,771 (95% CI 2,820–130,000) deaths in 2017 (Stanaway *et al*. 2019). In Sri Lanka, although the magnitude is not well understood due to lack of publicly available statistics, *Salmonella* is considered one of the main causes of foodborne illnesses (Ministry of Health, 2013).

The main reservoirs of food-borne salmonellosis are often the intestinal tracts of food-producing animals (Barrow *et al*., 2012; Crump *et al*., 2015). In Sri Lanka, chicken is the most popular meat contributing 70% of the total meat industry (De Silva *et al*., 2010); while eggs are the most affordable source of animal protein (DAPH Livestock Statistics, 2015). Consequently, poultry is important as a potential major reservoir and source of food-borne salmonellosis in the country.

In Sri Lanka, the poultry industry is the most established among all livestock industries (DAPH Livestock Statistics, 2015). Over the past few decades, it has shown a high growth, and at present Sri Lanka is self-sufficient in poultry meat production with a minute surplus being exported as well (DAPH Poultry forecast, 2017). A major drawback for the expansion of Sri Lankan export trade is the failure of local chicken products to conform to the international hygienic standards, for which a principal cause is the prevalence of *Salmonella* in the poultry supply. Further, salmonellosis with high morbidity and mortality remains a significant cause of economic loss in the poultry industry in Sri Lanka (DAPH Annual report, 2018; 2020). This comprises of the loss of birds (i.e., production loss) as well as the cost of veterinary services, medicines for treatment and disposal of dead birds.

1.2. Motivation

The Sri Lankan Veterinary Research Institute, the main national laboratory for livestock research in the country, is responsible for providing technical expertise for the national *Salmonella* control programme. As a veterinarian in this institution, my diagnostic and research efforts have delved mainly into this topic. From my point of view, scarcity of reliable and up to date research-based information has resulted in an impasse in broadening the knowledge on salmonellosis in Sri Lanka, thus hindering the achievement of expected results through the national control programme. It is in this context that this comprehensive study into the *Salmonella* in poultry industry was designed, to understand its prevalence, possible risk factors, whole-genome sequence (WGS) based population structure and antimicrobial resistance (AMR). Thus, the ultimate aim of the study is to inform prevention and control strategies and strengthen the national *Salmonella* control programme.

1.3. Research Questions

The research questions along with corresponding hypotheses, that are discussed in each analytical chapters (chapters 3, 4, 5 and 6 respectively) of the thesis are outlined below:

1. What is the prevalence of *Salmonella* carriage in broiler farms in Sri Lanka?

The primary method to assess prevalence is to carry out a survey on broiler farms. In Sri Lanka, there aren't any such previous studies done at the farm level. However, two previous studies of *Salmonella* prevalence in broiler carcasses by Weerasooriya *et al* (2008) and Kottawatta *et al* (2014) revealed 16.7% (24/144) and 9% (18/200) respectively.

2. What are the factors that determine *Salmonella* carriage in farms in Sri Lanka?

Poor hygienic conditions in the farms and housing with minimal infrastructure in Sri Lanka could be the most possible reasons regarding the Sri Lankan context. Rifky *et al* (2016) reported out of the 100 small-scale broiler farms in their study, 56% had clay floors and 52% had cadjan (mats woven from coconut palm leaves) as a roofing material.

3. What are the common practices about antibiotic use in broiler farms in Sri Lanka?

The use of antibiotics for prophylaxis is considered common in commercial broiler operations in Sri Lanka. Two recent studies by Herath *et al* (2015) and Lowe *et al* (2019) stated that all the participating farms (i.e., 11 farms and 39 farms respectively) had been using prophylactic antibiotics from the age of day one of their broiler chicks (Herath *et al.*, 2015; Lowe *et al*., 2019).

4. What is the genetic diversity and population structure of *Salmonella* in poultry in Sri Lanka?

With lack of comprehensive analysis using WGS data to study *Salmonella* in Sri Lankan poultry, not much is known about the diversity or the population structure. However, as per the previous findings based on confirmed and/ or suspected salmonellosis outbreaks, poultry specific, non-motile *S*. Gallinarum and/ or *S*. Pullorum are predominant in the Sri Lankan poultry (Gunawardena *et al*., 2006; Priyantha *et al*.,2007; Liyanagunawardena *et al*.,2012).

1.4. Research design

The research was designed as a collaboration between the Veterinary Research Institute, Sri Lanka and the School of Veterinary Science, Massey University, New Zealand. This study was comprised of two stages, of which the first part was carried out in Sri Lanka and the second in New Zealand. The first stage in Sri Lanka involved field visits for sampling and data collection. This encompassed a pilot study (in the highest poultry dense district) to provide baseline data which then was followed by cross-sectional study (in five poultry dense districts) in broiler farms and associated hatcheries[1], reviving known poultry outbreak isolates with available metadata, and initial laboratory identification. Subsequently, material was transferred to a PC3 facility (Massey University) in New Zealand. The second stage

[1]Associated hatcheries: In the case of *Salmonella* positive farms, the broiler hatcheries from which the chicks

consisted of further identification and characterisation of *Salmonella* through WGS-based analysis, AMR, use of bioinformatics and statistical analysis.

1.5. Thesis outline and aims

This thesis is formed of an introductory chapter (chapter 1), a literature review (chapter 2), four analytical chapters (chapters 3 to 6), and a general discussion (chapter 7). The current chapter (chapter 1) introduces the topic, significance, study design and the structure of the thesis. The literature review surveys the contemporary knowledge on poultry salmonellosis, food borne salmonellosis and molecular epidemiological tools in the global context; followed by an overview of the Sri Lankan context - the country's poultry industry with a focus on the broiler industry and poultry salmonellosis.

Analytical chapters of this thesis are presented as complete articles in preparation for publication, i.e., with an introduction, methodology, results and discussion for each chapter, due to which some repetition and overlapping across chapters is unavoidable. The four analytical chapters utilised microbiology, genomics, molecular epidemiology and statistics to reach a better holistic understanding of salmonellosis in Sri Lankan poultry. The research presented in this thesis was centred on the cross-sectional study in broiler farms and associated hatcheries, thus the results compiled in chapters 3, 4 and 5 are based on different aspects such as *Salmonella* prevalence, risk factors for *Salmonella* carriage and antibiotic usage at the farm level. Chapter 6 focuses on WGS-based population structure of *Salmonella,* phylogenetic relationships and AMR of *Salmonella*, which includes all the *Salmonella* isolates (from both the cross-sectional study and from outbreaks) sourced in the present study.

Chapter 7 is the general discussion that combines the outcomes of the four analytical chapters, how they inform existing knowledge of *Salmonella* in Sri Lankan poultry and potential avenues for future research.

Chapter 2: Literature Review

This literature review is twofold: the first section (i.e., 2.1 to 2.3) provides an overview of the global context on poultry salmonellosis, non-typhoidal salmonellosis (NTS) in humans and the use of molecular epidemiology in diagnosis, outbreak investigations and surveillance of *Salmonella*, while the second section (i.e., 2.4 to 2.8) reviews the Sri Lankan context focusing on the broiler industry, poultry salmonellosis, human NTS and control strategies to date.

2.1 Overview: Poultry salmonellosis

2.1.1 Introduction

Salmonella is a Gram-negative rod belonging to the family Enterobacteriaceae. There are two species *Salmonella bongori* and *Salmonella enterica* with over 2500 serotypes or serovars. The present classification of *Salmonella*, White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007), is based on conventional serotyping or antigenic classification of *Salmonella* upon antibody reaction of surface antigens: somatic (O) and flagellar (H) antigens with specific antisera. Other microbial typing with more discriminative ability have been developed such as phage typing (Hood, 1953), and subsequently DNA-based methods such as multilocus sequence typing (MLST) (Maiden *et al.*, 1998) with more discriminative ability have also been developed. This is discussed below (under 2.3) in detail. Moreover, all serotypes that are of most interest to veterinary and human health belong to *Salmonella enterica* (Barrow *et al.*, 2012). These serotypes can be divided into two broad groups, NTS and typhoidal salmonellosis, based on their pathogenesis (Wigley *et al.*, 2001).

2.1.2 Non-typhoidal salmonellosis in poultry

NTS consists of many serotypes, including *Salmonella enterica* serovar Enteritidis (*S*. Enteritidis) and *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) that can cause gastrointestinal disease in a range of hosts including poultry and humans. NTS rarely causes human systemic disease except in very young, old or immunocompromised individuals while

in poultry it occurs with concurrent or subsequent to viral infections (Barrow, 2007). NTS infection in poultry often appears as largely asymptomatic (Van Immerseel *et al.*, 2005; Barrow and Methner, 2013), but the course of infection and/or clinical signs may vary according to the serotype involved (Barrow and Methner, 2013). Nevertheless, both symptomatic and asymptomatic infected birds can transmit organism within and between flocks[2] (Barrow and Methner, 2013). Further, NTS serotypes can colonise the alimentary tract of infected birds, subsequently causing widespread contamination at slaughter/ processing plants (Barrow, 2007). This has major implications for public health and is discussed in detail under 2.2.

2.1.3 Typhoidal salmonellosis in poultry

Typhoidal salmonellosis or systemic "typhoid-like" infections can occur in a restricted range of host species, with the presence of a few serotypes such as *Salmonella enterica* serovar Gallinarum (*S*. Gallinarum) in poultry, *Salmonella enterica* serovar Typhi in humans and *Salmonella* enterica serovar Dublin in cattle (Wigley *et al.*, 2001). The pathogenesis caused by serovar Gallinarum in poultry results in the involvement of the monocyte-macrophage series and rarely intestinal colonization (Van Immerseel *et al.*, 2005).

Fowl typhoid (FT) and Pullorum disease (PD) are septicaemic diseases specific for avian species, caused by poultry-adapted *S*. Gallinarum and *S*. Pullorum respectively (Shivaprasad, 2000). *S*. Gallinarum and *S*. Pullorum are non-motile, while almost all *Salmonella* are motile via peritrichous flagella. Through serotyping *S*. Gallinarum and *S*. Pullorum are indistinguishable, hence they are known as biotypes of serovar Gallinarum (Christensen *et al.*, 2007). FT usually affects older birds while PD is more common in young birds. Acute FT is manifested by generalised septicaemia while its chronic form comprises anaemia,

[2]Flock: birds of same age in a farm. Often a flock is housed together, however it can differ depending on the number of birds and space availability.

depression, laboured breathing and diarrhoea causing adherence of faeces to the vent. PD causes high mortality in newly hatched chicks and birds of up to two-three weeks old. Peritonitis with generalised congestion of tissues and an inflamed unabsorbed yolk sac are common post-mortem signs of newly hatched chicks affected with PD (OIE, 2018a). In older birds PD may be inapparent except for reduced egg production and hatchability (Wigley *et al.*, 2005). Both diseases cause variable to high mortality, associated with endemic sporadic mortality or outbreaks of high mortality up to 100% (Barrow and Freitas Neto, 2011).

Birds that survive FT and PD often become lifelong carriers (Shivaprasad, 2000), with the localization of pathogens in the reproductive tract, liver, spleen and occasionally in the caecal tonsils (OIE, 2018a). Such carrier birds produce persistent infections with the intermittent shedding of the pathogen; thus, it is an important mode of disease transmission in the epidemiology of FT and PD. Trans-ovarian infection among survivors can cause vertical transmission between generations (i.e., from the infected hen to its eggs and subsequent chicks) (Haider *et al*., 2014) in addition to horizontal transmission among birds of the same flock.

2.1.4 *Salmonella* control

In the early 20th century, both FT and PD caused a significant economic impact on the poultry industry worldwide. Since then, control programmes have been implemented, with the main focus of eliminating carriers of FT and PD (Shivaprasad, 2000). Additionally, control attempts through vaccination with SG 9R live *Salmonella* vaccine[3] (Silva *et al.*, 1981) has been also used in places where complete elimination is not possible, such as South Korea (Barrow *et al.*, 2012). By mid-20th century, countries with developed poultry industries such

[3]SG 9R vaccine: Rough strain *S*. Gallinarum 9R (Smith, 1956), which was developed initially for immunization against *S*. Gallinarum. Also used for active immunization against *S*. Pullorum and for active cross protection against infections caused by other salmonellae of group D such as *S*. Enteritidis.

as countries in Europe and North America achieved success in controlling these two diseases through 'test-and-cull' policies. However, still there can be occasional outbreaks such as an FT outbreak which occurred in the UK in 2005 (Cobb *et al*., 2005). In other parts of the world, FT and PD remain endemic causing a negative economic impact on their poultry industries (Shivaprasad, 2000; Barrow and Freitas Neto, 2011; Wigley, 2017).

Since the elimination of FT and PD in the developed world, control attempts have frequently targeted *Salmonella* of public health importance that cause foodborne zoonotic infection (Wigley, 2017). Control strategies comprise biosecurity and hygienic measures on the farm and in the slaughterhouse to restrict entry of pathogens into the human food chain as well as vaccination to reduce *Salmonella* infection among breeder and layer flocks (Van Immerseel *et al.*, 2005). Further, with the *S*. Enteritidis epidemic in 1980s, which initially occurred as egg associated outbreaks in the UK, legislation was made at the European Union level (Directives 92/117, 2160/ 2003 and 1168/2006) to implement control plans in each member state to monitor *S*. Enteritidis and *S*. Typhimurium in poultry. As a consequence, *S*. Enteritidis declined drastically from 1992 to 2010 in the UK: from hundreds of infected flocks to a few flocks and from over 20,000 human cases to fewer than 5,000 (Barrow *et al.*, 2012). As such, Barrow *et al* (2012) state that the rise and fall of *S*. Enteritidis has been the greatest epidemiological change within *Salmonella* in the last few decades.

2.1.5 Antimicrobial resistant *Salmonella*

Antimicrobial therapy is in use for PD and FT in some countries; however, as birds remain infected after cessation of chemotherapy, infection is not eliminated from the flock (Barrow and Freitas Neto, 2011). Extensive use of antibiotics for disease control in poultry globally may have led to the development and persistence of antimicrobial resistance in *Salmonella* species (Tollefson and Miller, 2000). Further, multi drug resistance (MDR), frequently defined as resistance to at least three classes of antimicrobials (Magiorakos *et al.*, 2011) has

also been detected in several serotypes of *Salmonella* such as *S*. Typhimurium (Barrow, 2007).

Pan *et al* (2009) has evaluated the changes in antimicrobial resistance of *S.* Pullorum in China between 1962 and 2007 using 450 isolates. Over half of the isolates (56.2%) exhibited MDR (i.e., resistant to four or more antimicrobials) and there was an increasing trend in MDR between 2000 and 2007 (Pan *et al.*, 2009). A substantial trend of MDR development has been observed mainly in the serovar Typhimurium since as early as 1980s, specifically associated with S. Typhimurium definitive type 104 (DT104) (Threlfall, 2002). this was considered important due to its high resistance to a wide range of frequently used antimicrobials: ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Mather *et al*., 2013). Another important resistance trend in NTS has been the development of resistance to quinolones/ fluoroquinolone (such as nalidixic acid and ciprofloxacin). In Southeast Asia, there is documented evidence of resistant *Salmonella* strains (Lee *et al*., 2009) while it is much lower in high-income countries (Crump *et al*., 2011). Development of quinolone resistance is of critical importance as it is used for treatment of NTS in humans. Further, resistance to extended-spectrum cephalosporins (such as ceftriaxone) (Miriagou *et al*., 2004; Lee *et al*., 2009), which could be used as a drug of choice for treating invasive *Salmonella* infections, points to a public health concern.

2.2 Non-typhoidal salmonellosis in humans

NTS are important foodborne zoonotic pathogens, causing gastroenteritis worldwide (Hohmann, 2001; Antunes *et al.*, 2016; Yachison *et al.*, 2017) and rarely bacteraemia (Feasey *et al.*, 2012; Crump *et al*., 2021). The mode of NTS transmission in high-income countries is primarily by poultry/ animal faecal contamination of food products through the production chain, while it is less well understood in low and middle-income countries due to lack of systematic surveillance and fragmentary data (Crump *et al*., 2021).

NTS infections often cause self-limiting diarrhoea in individuals with rare fatality. NTS were responsible for an estimated 95.1 million (95% confidence interval (CI) 41.6-184.8) cases of enterocolitis and 50,771 (95% CI 2,820–130,000) deaths globally in 2017 (Stanaway *et al*., 2019). NTS can also invade other sites of the body, resulting in bacteraemia or bloodstream infections manifested by non-specific febrile illnesses with a higher case fatality rate, known as invasive NTS disease. Bacteraemia occurs in approximately 6% of paediatric and/or geriatric patients and particularly in immunocompromised patients with diarrheal enterocolitis (Crump *et al.*, 2015). In sub-Saharan Africa, predisposing factors such as malaria, malnutrition and HIV-infection are associated with NTS bacteraemia among infants, children and young adults (Feasey *et al.*, 2012). The estimate is that 535,000 (95% CI 409,000–705,000) cases of NTS invasive disease with 77,500 (95% CI 46,400–123,000) deaths occurred worldwide in 2017 (Stanaway *et al*., 2019). Usually, antibiotic therapy is not needed for NTS gastroenteritis in healthy individuals, while it is recommended for bacteraemia as well as for populations at risk of bacteremia or invasive NTS disease (Crump *et al.*, 2015).

The egg associated *S*. Enteritidis outbreak in the 1980s in the UK was reported as one of the foremost issues of foodborne NTS. During the late 1980s to early 1990s, reported cases worldwide peaked, and phage typing demonstrated transmission of several different lineages (Barrow *et al.*, 2012). In the late 1990s, *S*. Enteritidis and *S*. Typhimurium were the most frequently isolated serotypes accounting for ∼50% of isolates from patients in the United States, though by 1999 there was a decrease in the *S*. Enteritidis infections possibly due to increased public awareness (Hohmann, 2001). In the United States between 2004 and 2012, most NTS serotypes isolated from humans comprised Enteritidis (19%), Typhimurium (18%), Newport (11%), Heidelberg (4%), and other serotypes (48%) (Medalla *et al*., 2017). In 2006, in the European Union, *S*. Enteritidis and *S*. Typhimurium were identified in

approximately 60% and 14% (respectively) of the human cases, while other serotypes found were, Infantis, Virchow, Newport, Hadar, Stanley, Derby, Agona and Kentucky. Moreover, while the *S*. Enteritidis infection in humans is primarily egg associated, certain serotypes such as S. Infantis, Hadar and Virchow are mostly associated with broiler meat (EFSA, 2007). Globally, *S.* Enteritidis remains the most important serovar associated with foodborne salmonellosis, despite being successfully controlled in the UK and some other countries (Barrow *et al.*, 2012). Serotypes Typhimurium and Enteritidis are most commonly found to be associated with invasive NTS across Africa (Gordon *et al*., 2001; Gordon *et al*., 2008). One of the most common causes of invasive NTS in sub Saharan Africa is *S*. Typhimurium ST313, which has a human-to-human transmission route (Mather *et al.*, 2015).

2.3 Molecular Epidemiology

2.3.1 Introduction

Amongst the subspecialties of epidemiology, molecular epidemiology is the application or use of techniques of molecular biology to the study of infectious disease epidemiology (Tompkins, 1994). Molecular techniques may be applied to study diseases that cannot be approached or would be more expensive, and/or time consuming to address by conventional techniques (Foxman and Riley, 2001). In addition, molecular epidemiology has great potential to provide better understanding of disease in relation to disease surveillance, outbreak investigations and identifying transmission patterns and sources (Bloomfield *et al.*, 2017; Mather *et al.*, 2015; Saravanan *et al.*, 2015).

2.3.2 Molecular tools

Molecular tools are used to identify the genome of an organism, parts of a genome or proteome, and also to distinguish isolates based on differences in their genome or proteome. Some of the molecular tests that are in use today are polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), multi-locus variable-number tandem repeat

analysis (MLVA), MLST and whole genome sequencing (WGS). Microbial typing to distinguish isolates is increasingly used in diagnostics and epidemiological investigations. MLST (Maiden *et al.*, 1998) is widely used and usually examines nucleotide variation in sequences of internal fragments of seven housekeeping genes, although the number of genes may differ among species. MLVA on the other hand, uses polymorphic repeated sequences instead of housekeeping genes, and can provide higher resolution than MLST (Pérez-Losada *et al.*, 2018).

WGS allows a more comprehensive, high-resolution analysis by providing the highest possible discrimination of the genome. Further, it yields all the available genetic data of the organism (Pérez-Losada *et al.*, 2018). Next-generation sequencing technologies (i.e., Illumina sequencing, Ion Torrent sequencing, Oxford Nanopore technologies and Pacific Biosciences sequencing), have become cost-effective and popular platforms in the field of genomic epidemiology. WGS-based typing approaches (e.g., single nucleotide polymorphisms-based and allele-based methods) as well as different analytical tools and software have become increasingly available for analysing WGS data. This is discussed in detail in chapter 6 (under 6.1).

2.3.3 Use of molecular tools in *Salmonella* research

Molecular tests can differentiate between strains of microorganisms that are usually indistinguishable by conventional methods. This has made it easier to distinguish *Salmonella* strains causing an outbreak or to do surveillance of a certain serovar from closely related strains. WGS provides sequence data of the entire genome, not only to distinguish between different isolates, but also to provide other information such as the presence or absence of gene coding for antimicrobial resistance or virulence factors. National health organisations around the world such as Public Health England (Ashton *et al.*, 2016), National Surveillance in Canada (Yachison *et al.*, 2017) and US Food and Drug Administration Center *Salmonella*

(Pettengill *et al.*, 2014) have established WGS as a routine typing tool for their public health surveillance of *Salmonella*. Further, a WGS approach towards investigations of outbreaks and disease transmission of salmonellosis show promising results (Bloomfield *et al.*, 2017; Mather *et al.*, 2015; Saravanan *et al.*, 2015).

2.4. The poultry industry in Sri Lanka

2.4.1 Introduction

The poultry industry is the most developed livestock industry in Sri Lanka (DAPH Livestock Statistics, 2015). Over the past few decades, it has grown from a backyard industry into commercial status, thus providing livelihood for many. In addition, the country is self-sufficient in the production of meat and eggs, with a small surplus being exported (DAPH Poultry forecast, 2017). Further, chicken is the most consumed, as well as the cheapest of all meats, while eggs are the most affordable source of animal protein in Sri Lanka (DAPH Livestock Statistics, 2015).

2.4.2 Distribution

Although the poultry industry is well established throughout the country, it is the Western and North-Western provinces that are best known for poultry production (Abeyratne, 2007). This region, consisting of Colombo, Gampaha and Kalutara districts of Western province and Kurunegala and Puttalam districts of the North-Western province, is known as the 'poultry belt'. The majority of breeder and commercial farms, as well as associated industries (such as animal feed, pharmaceutical, meat-processing plants), are found in these provinces. Several breeder and numerous commercial level farms are located in the Central province (Kandy and Matale districts) as well. With a new trend of development in Northern, North-Central and Eastern provinces, commercial farms are currently in operation throughout the country. The distribution of commercial poultry farms (with over 1000 chickens) and breeder farms in Sri Lanka is presented in figures 2.1 and 2.2 respectively.

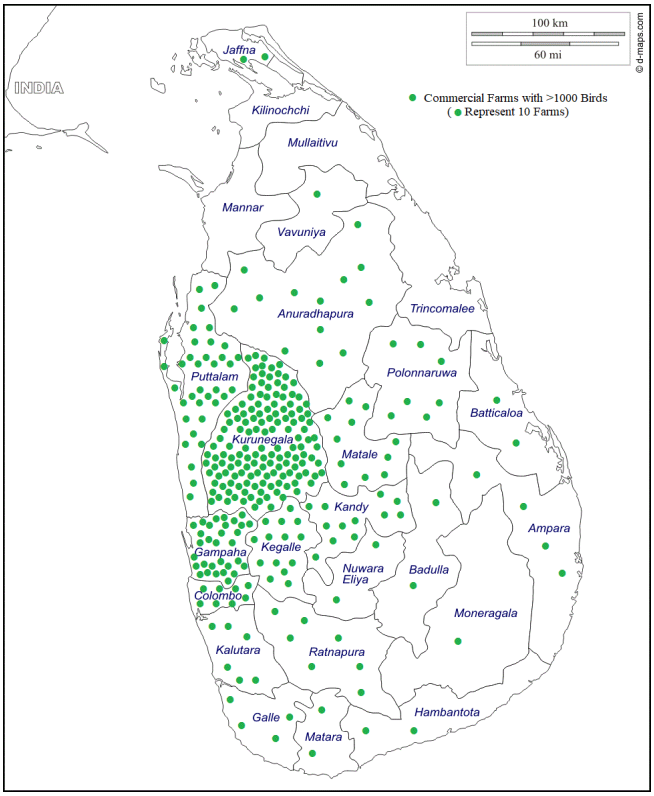


Figure 2.1: Map of Sri Lanka showing locations of commercial poultry farms with over 1000 chickens (Dept of Census and Statistics, 2015).

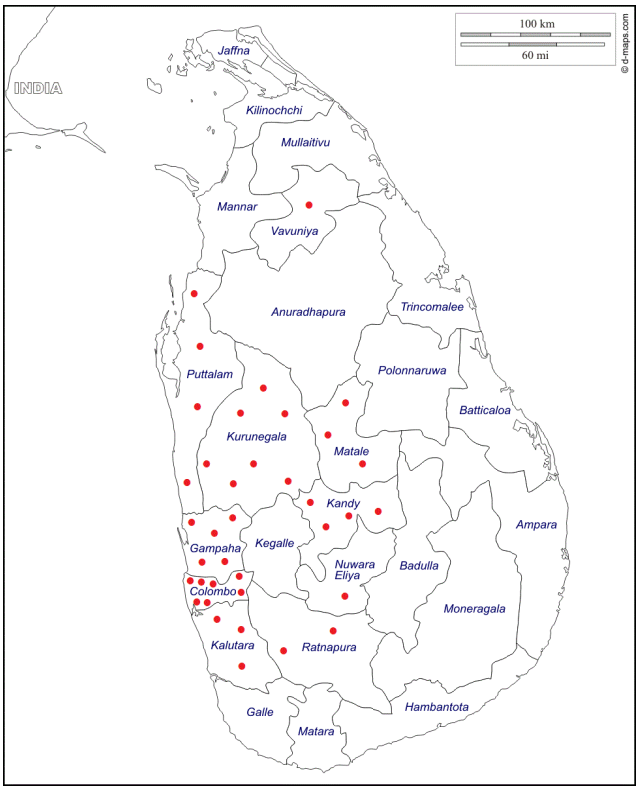


Figure 2.2: Map of Sri Lanka showing locations of Poultry Breeder Farms (DAPH Annual report, 2016).

2.4.3 Primary structure

In the poultry industry, roles of the private and government sectors are clearly demarcated. More than 90% of the poultry industry comes under the private sector, while the rest is operated by governmental or semi-governmental authorities. As reported by Abeyratne (2007), private sector engagement occurs throughout the industry from farm to product supply chain, with 75% of the industry being owned by fewer than ten large scale companies. My observation is that little has changed since then with regard to the percentage of ownership within the private sector. At present, the government functions as the regulatory body through the Department of Animal Production and Health (DAPH), providing technical leadership, expertise and implementing a range of statutes pertaining to the livestock sector as a whole. In this regard, technical expertise in the form of diagnosis and technology transfer is provided mainly by the Veterinary Research Institute (VRI), which is the main laboratory of DAPH and Veterinary Investigation Centers (VIC) which are the peripheral laboratories of DAPH (DAPH Annual report, 2015).

Similar to elsewhere in the South Asian region, the Sri Lankan poultry industry consists of broiler, layer and backyard or village chicken farming sectors, which are organised as separate operations. In 2015, there were 195,462 poultry farms including 10,408 broiler farms, 55,410 layer farms and 139,344 homesteads engaged in backyard chicken farming (Dept of Census and Statistics, 2015). Broiler and layer sectors, in particular, are subdivided into breeder or parent farming, commercial farming and poultry processing. Backyard chickens, on the other hand, are reared for both eggs and meat (called curry chickens) and often kept as breeder stock as well.

The poultry industry possibly accounts for the largest work force engaged in livestock in Sri Lanka. As reported by Priyantha (2009), poultry production has provided livelihood for more than 500,000 families directly and indirectly. In 2017, commercial poultry farms alone

account for 43% (195,462/453,886) of the total livestock enterprises in Sri Lanka (Dept of Census and Statistics, 2015). Although accurate data is not available, the actual employment status should be higher than this when including poultry breeder farms and other associated commodities.

2.4.4 Production status

In 2016, the poultry industry supplied more than 50% of the total contribution of Sri Lankan livestock sector to the GDP (DAPH Poultry forecast, 2017). Total poultry population has increased from 13.77 million in 2007 to 36.72 million in 2016 (DAPH Key Statistics, 2016). As a result, there is a rise in the annual chicken meat production from 100,000 MT in 2007 to 214,200 MT in 2018, while annual egg production has also increased from 1,800 million in 2007 to 2,853 million in 2018 (DAPH Key Statistics, 2016; DAPH Livestock Outlook, 2018).

There is a parallel increase in the annual per capita consumption of poultry products from 2007 to 2018: from 4.98kg to 9.73kg of poultry meat, and from 89.8 to 131.39 with regard to eggs. Similarly, the export of poultry meat and eggs displays a massive growth. From 2007 to 2018, poultry meat exports have risen from 55 MT to 3810 MT, while export of poultry eggs has increased from 0.76 million to over three million (DAPH Key Statistics, 2016; DAPH Livestock Outlook, 2018).

2.5. The Broiler sector in Sri Lanka

2.5.1 Introduction

The substantial growth in the broiler sector over the past decade is due to a number of reasons. The cessation of civil war in the country in 2009 resulted in subsequent development of the poultry industry in new areas, more investment in large-scale enterprises and an increase in tourism. It is suggested that the government declaration of chicken meat as an essential food item, with retail price revisions subjected to prior approval in 2007 (DAPH Livestock Statistics, 2010), has enhanced further development of the sector.

Consumption of chicken, unlike consumption of pork and beef is not closely associated with the popular ethno-religious beliefs of the multi-ethnic population (Alahakoon *et al*., 2016; De Silva *et al*., 2010) As such, chicken meat accounts for 70% of the total meat production in the country (De Silva *et al*., 2010).

The production potential of the broiler industry is currently projected to increase annually from 3-5% (DAPH Poultry forecast, 2017), and several recent initiatives have focused on expanding export markets. Chicken is the only meat type being exported from Sri Lanka. Most of the meat and processed products are currently being sent to Maldives, India and several Middle Eastern countries (DAPH Annual report, 2015).

2.5.2 Broiler breeder stocks

Broiler breeder stocks are supplied by Sri Lankan Grand Parent (GP) and parent farms, as well as some imported parent bird stocks. In 2016, local GP farms were able to provide 76% of the parent bird requirement while the rest was imported as day-old chicks (DOC) (DAPH Poultry forecast, 2017). In 2016, there were 32 parent farms in operation (DAPH Annual report, 2016). These breeder stocks operate under a regular comprehensive disease monitoring and surveillance system, with a special emphasis on poultry specific salmonellosis. From there, the DOC are distributed as broiler birds to commercial level farms.

2.5.3 Commercial broiler farms

There are 10,708 commercial broiler farms throughout Sri Lanka with the majority being situated in the 'poultry belt'. Kurunegala district has the highest number of broiler farms at 1488/10,708 (13.9%). Western and North-Western provinces account for 3732/10,708 (34.9%) farms. Additionally, Kegalle, Anuradhapura and Kandy districts also have high numbers of broiler farms (Dept of Census and Statistics, 2015). Commercial farms have been categorised into two: farms with greater than or equal to 1000 chicken (also known as

farms with over 1000 chicken) and farms with fewer than 1000 chicken (also known as farms with below 1000 chicken). At present, there are 1847/10,708 (17.2%) farms with over 1000 chickens, and 8861/10,708 (82.8%) with below 1000 chickens (Dept of Census and Statistics, 2015). Farms over 1000 chickens can be further categorised as small (1001-5000), medium (5001-25,000) and large scale (>25,000). Large scale farms may consist of a maximum flock size of 50,000 with several flocks per farm. Such farms mostly function as environmental controlled houses with automated facilities and cutting-edge technology that are seen in with the large global broiler-producers.

The buy-back system in the broiler industry is a unique feature where small-scale farmers are engaged in rearing for companies. Many leading broiler companies adopt this system, where they enter into a contract with small-scale farmers for rearing chickens. Accordingly, most inputs such as DOC, feed, veterinary and extension services are supplied by the contractor, who buys the chickens back after 38 to 42 days based on live weight. In return, farmers are to provide the housing facilities, electricity, litter material and the labour required for rearing the broilers to market age. In Kurunegala district, where most of the broiler farms are located, 60% are buy-back farms (Rifky, 2016).

2.5.4. Processing of broiler chicken and meat products

Similar to that of many low and middle-income countries, the majority of chicken is sold in chilled or frozen form in the domestic market with little further processing. This is largely due to the local consumer preference of consuming meat as a spicy curry rather than processed products (Alahakoon *et al*., 2016). Most broilers are processed mechanically in semi-automated plants where standard methods such as pre-processing, slaughter, evisceration, secondary processing, packing and shipping are followed. Usually, the batch capacity at a semi-automated facility is over 1000 birds (DAPH Annual report, 2015). On the other hand, small-scale processors also known as ‘wet market’ in most of the South East Asia

can be seen confined to sub-urban and rural areas in Sri Lanka. These small-scale processors supply limited amounts of chicken depending on the daily requirement due to lack of logistics in refrigeration. However, there is no appropriate regulatory system for the poultry processing component at present similar to many other low and middle-income countries. This was identified as one of the priorities in recent amendments to the control programme (DAPH *Salmonella* policy, 2017).

2.5.5 Limitations in the broiler industry

Amongst the concerns that affect the profitability and potential growth of broiler farming in Sri Lanka, cost of feed is a pivotal factor. This accounts for nearly 70% of the cost of production of chicken and is further aggravated by the high import dependency of feed raw materials. In response, domestic maize production has been identified as a potential solution that requires government facilitation (DAPH Livestock Statistics, 2010).

Diseases and health-related issues are another major constraint. According to Bandara and Dassanayake (2006) and Rifky (2016), mortality, cost of veterinary services and medicine significantly impact broiler farming. These include preventive and/or treatment medication for significant diseases affecting poultry such as salmonellosis (mentioned in 2.6). If these are to be minimised, proper vaccination and biosecurity measures need to be implemented. Poor hygienic conditions in broiler plants remains a foremost drawback in providing consumer-safe end products. This is particularly associated with small-scale plants, where processing and retail outlet areas are in close contact (Alwis *et al.*, 2014). Therefore, it is important that small-scale processors prioritise hygienic production to secure consumer safety.

Moreover, there is a public health global concern of salmonellosis and campylobacteriosis especially with regard to the international food trade. Barrow *et al* (2012) state that

Salmonella remains a problem in many poultry meat producing countries and the current globalisation of trade will increase pressure on those countries in reducing levels of *Salmonella* infection to comply with the expectations of the consuming countries. Therefore, the occurrence of *Salmonella* in local breeder as well as commercial broiler setups (mentioned in 2.6) would be a major hindrance for the expansion of Sri Lankan export trade.

2.6 Poultry salmonellosis in Sri Lanka

2.6.1 Significance to Sri Lankan poultry

Salmonellosis at present is one of the most significant conditions affecting poultry, third only to Infectious Bursal Disease and Newcastle Disease (DAPH Annual report, 2016; 2018; 2020). It being the only bacterial disease reported, reflects the present status of poultry salmonellosis as the most harmful bacterial pathogen for the industry. According to the DAPH annual reports, salmonellosis occurs mainly due to *S.* Gallinarum and *S.* Pullorum, causative agents of FT and PD respectively (DAPH Annual report, 2016; 2018; 2020).

Economic losses due to FT and PD can be very high (Shivaprasad, 2000), although the figures are not available for Sri Lanka. This consists of the loss of birds, as well as the cost of veterinary services, medicines, feed and disposal of dead birds. In terms of public health, salmonellosis in humans is typically a food-borne zoonotic disease. With chicken meat and eggs being the commonest source of animal protein popularly accessed by Sri Lankans, salmonellosis could develop into a potential threat for human health (Tay *et al.*, 2019).

2.6.2 Occurrence of poultry disease

Actual disease occurrence in the field is not well documented in Sri Lanka. The number of cases reported in commercial poultry in 2018 and 2020 were 78,928 and 50,642 respectively while mortality due to disease were 738 and 900 respectively (DAPH Annual report, 2018; 2020). The cases reported could involve individual birds or several birds from a flock. It is noteworthy that these are not confirmed cases: they are largely based on clinical signs and

post-mortem lesions rather than on laboratory confirmation. Further, the mortality owing to disease is arguably higher than these figures indicate, as the number of deaths is recorded as per the information received at case presentation. In general, there is no proper follow-up on field outbreaks, thus there is information inconsistency within the reported cases.

Case confirmation is performed mainly by laboratory findings at VRI and VICs. Between 1998 and 2003, a total of 100 *Salmonella*-confirmed cases were detected from an unreported number of chicken carcasses submitted to VRI (Gunawardena *et al.*, 2006). Liyanagunawardena *et al* (2012) confirmed 56 *Salmonella* outbreaks in commercial poultry through laboratory isolation during a 2010-2012 study. According to a detailed description on chicken carcasses presented to VIC- Pannala in 2010, 13% (45/342) were diagnosed as salmonellosis on post-mortem. Subsequently, out of 847 samples taken for bacteriology, 390 (46%) could be confirmed by conventional culture, including 97 (25%) identified as *Salmonella* (Wijemanna, 2011). This demonstrates that the actual organism isolation is less than 40% of post-mortem salmonellosis diagnoses. It is s highly recommended that fresh chicken carcasses should be sampled to improve *Salmonella* isolation rates (OIE, 2018a), yet it is not practical to do this in Sri Lanka. It usually takes several hours to reach the closest facility available, and if they arrive after hours the carcasses have to be kept in freezers. Further, antibiotic administration up to a few days prior to death could also interfere with the isolation procedure.

2.6.3 *Salmonella* in breeder flocks

Salmonella-carrier status in birds recovered following FT and PD is an important mode of disease spread for both vertical and horizontal transmission (Shivaprasad, 2000). Hence, through the direct involvement of DAPH, the breeder flocks are closely monitored for carrier status for *S*. Gallinarum and *S*. Pullorum using Whole Blood Agglutination Test (WBAT), while isolation of *Salmonella* is performed on samples taken from hatchery premises. Certain

parent farms have shown evidence of carrier status from 2008 to 2016, while *Salmonella* organisms were isolated from some parent hatcheries from 2011 to 2014 (DAPH Annual report, 2008; 2011; 2014; 2016). However, it is not clear whether there is any association between these *Salmonella*-positive hatcheries and *Salmonella*-carrier parent flocks. On the other hand, evidence of salmonellosis in certain parent farms and hatcheries from 2011 to 2014 and outbreaks of disease at commercial farms from 2013 to 2016 could possibly be related to both vertical and horizontal transmission of the infection. After 2015, there was no carrier status identified or *Salmonella* organisms detected from any breeder stock through surveillance (DAPH Annual report, 2015; 2016; 2018).

With regard to GP flocks, there has been no detectable *Salmonella*-carrier status between 2008 and 2016 in any of the farms through routine testing (DAPH Annual report, 2008; 2011; 2014; 2016). Similar findings were reported by Liyanagunawardena *et al* (2016a) investigating *Salmonella* carrier status of GP flocks at the age of 20 weeks. A retest at 35-40 weeks after the birds reach the peak egg production was recommended in consideration of the physiological stress during egg production which could significantly increase the susceptibility to *Salmonella* infection (Liyanagunawardena *et al*., 2016a).

2.6.4 *Salmonella* serotypes

Salmonella in commercial and breeder flocks

Case or outbreak studies in commercial poultry have been carried out to broaden understanding of common *Salmonella* serotypes among poultry in Sri Lanka (Gunawardena *et al*., 2006, Priyantha *et al*., 2007, Liyanagunawardena *et al*., 2012). However, identification and characterisation of these were based on conventional methods. Out of 100 *Salmonella* outbreak isolates obtained during 1998-2003, Gunawardena *et al*. (2006) reported 78 as *S.* Gallinarum and the remainder as *S.* Enteritidis. Another study on identification of *Salmonella* isolates from poultry post-mortem samples revealed 87% (95/109) and 13% (14/109) as *S.*

Gallinarum and *S.* Enteritidis respectively (Priyantha *et al*., 2007). In both these studies, the authors have considered biovars *S.* Gallinarum and *S.* Pullorum together under serovar *S.* Gallinarum. Liyanagunawardena *et al*. (2012), confirmed 28/56, 23/56 and 5/56 of the *Salmonella* outbreaks in commercial poultry of the country were due to *S.* Gallinarum, *S.* Pullorum and *S.* Enteritidis respectively, thus pointing to the recurrence of previously reported serotypes.

Wijemanna *et al* (2008) reported *S.* Enteritidis in three out of four breeder farms previously positive for *Salmonella*-carrier status and then in two out of four hatcheries consisting of their progeny. From 2011 to 2012, *Salmonella* isolation in breeder farms had resulted in 3/7, 3/7 and 1/7 isolates as *S.* Gallinarum, *S.* Enteritidis and *S.* Typhimurium respectively. In the same study it was 4/6, 1/6 and 1/6 as *S.* Enteritidis, *S.* Gallinarum and *S.* Pullorum respectively at hatchery level (Liyanagunawardena *et al.,* 2013).

Salmonella in poultry carcasses, meat and eggs

There is limited information on *Salmonella* contaminated poultry meat and eggs in Sri Lanka. Weerasooriya *et al* (2008) investigated possible contamination of broiler carcasses with *Salmonella* in a modern processing plant with ISO accreditation. *Salmonella* was isolated from 24 of 144 (16.7%) ceacal samples, comprising *S* Typhimurium (18/24), *S* Pullorum (4/24) and other motile *Salmonella* (2/24) which could not be serotyped (Weerasooriya *et al.*, 2008). A similar study with a greater number of pooled caecal samples (200) from 11 districts by Kottawatta *et al* (2014) revealed 9% (18/200) positive for *Salmonella* and these isolates represented somatic sero-groups A, B, C and E; *S*. Nitra, *S*. Typhimurium, *S*. Chester, *S*. Paratyphi, *S*. Saintpaul, *S*. Kentucky and *S*. Muenster.

Alwis *et al* (2014) demonstrated an overall 21% (12/57) *Salmonella* prevalence only through conventional isolation methods (without further identification such as serotyping), in the

sampled retail chicken meat outlets in Kandy, Sri Lanka. Another study by Kalupahana *et al* (2017) based on one pooled sample of eggs each from 100 retail outlets in Kandy revealed an overall *Salmonella* prevalence of 15%. Subsequently, serotyping identified NTS of public health importance such as *S*. Mbandaka (46%), *S*. Braenderup (26%), *S*. Corvallis (20%) and *S*. Emek (7%). It is noteworthy that *S*. Mbandaka is known to be responsible for numerous reported human outbreaks (Scheil *et al*., 1988; Paine *et al*., 2014) while *S*. Braenderup could be linked to a multistate cluster of outbreaks in humans in the United States in 2012 (Nakao *et al.*, 2015). A more recent study using WGS reported occurrences of 33 NTS: Agona ST13 (16), Corvallis ST1541 (9), Kentucky ST314 (4) and Newport ST31 (4), in raw chicken meat sampled from Colombo (Tay *et al.*, 2019). While all NTS revealed through Tay *et al* (2019) are known human pathogens, the predominant S. Agona is one of the most commonly isolated NTS from humans in Europe and was responsible for the Pan- European outbreak in 2008 (McCusker *et al*., 2014).

2.6.5 Antibiotic use and antimicrobial resistance in *Salmonella*

The use of antibiotics is believed to be common in commercial poultry in Sri Lanka (FAO, 2013; Liyanage and Pathmalal, 2017), especially as a prophylactic measure (Herath *et al.*, 2015; Lowe *et al*., 2019). The use of antibiotics for prophylaxis in broiler operations allows them to continue their production until 38-42 days, although this has not been comprehensively studied. Owing to lack of a proper compensation system, culling is not performed in *Salmonella* positive cases in Sri Lankan commercial poultry.

As revealed by a questionnaire survey in medium-scale broiler farms (2,000 birds per flock) in Kandy district, all the eleven participants used antibiotics as a sub therapeutic measure via drinking water. Tetracycline, enrofloxacin and amoxicillin were the most common antibiotics used by these farmers during the first three weeks of the broilers (Herath *et al.*, 2015). Another recent study, based on 39 broiler farms owned by six leading broiler chicken

producers in Sri Lanka, reported the participants' administering amoxicillin, enrofloxacin or tylosin for prophylaxis during the first few days of the broiler life cycle (Lowe *et al*., 2019).

Few studies to date have looked at antimicrobial resistance in *Salmonella* in Sri Lanka. Gunawardena *et al* (2006) has tested 50 Sri Lankan *Salmonella* isolates derived from outbreaks; 38 Gallinarum and 12 Enteritidis, for susceptibility (as per National Committee for Clinical Laboratory Standards- NCCLS) to five antibiotics by the disk diffusion method. Susceptibility was found to be 100% to chloramphenicol, 98% to ciprofloxacin, 78% to amoxycillin, 68% to ampicillin, and 64% to tetracycline. Antibiotic susceptibility test profiles in a study by Kottawatta *et al* (2014) reported 33.3% (6/18) resistance of *Salmonella* against ampicillin. All the isolates of this study were susceptible to gentamicin and enrofloxacin (Kottawatta *et al.*, 2014). A more recent study investigated susceptibility of *Salmonella* isolates from eggs to 12 antimicrobials; ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, ceftazidime, tetracycline, tigecycline and trimethoprim. The relevant minimum inhibitory concentrations revealed one isolate to be resistant to nalidixic acid and another to a third-generation cephalosporin, while all other isolates (13/15) were sensitive to the tested antimicrobials (Kalupahana *et al*., 2017).

2.7 Human non-typhoidal salmonellosis in Sri Lanka

Salmonella is considered the main bacterial pathogen causing foodborne illnesses in Sri Lanka, followed by *Campylobacter jejuni, Shigella, Escherichia coli* etc (Ministry of Health, 2013). Nevertheless, the burden of foodborne salmonellosis in Sri Lanka is not well understood. Surveillance on food and water borne diseases in Sri Lanka are carried out together with a major emphasis on enteric fever, viral hepatitis and dysentery (Ministry of Health, 2018). In 2017, there were 439 cases of enteric fever and 2187 of dysentery while the reported incidence of food poisoning was 969 cases (Ministry of Health, 2018). Hence,

the number of foodborne illnesses that attributed to NTS in Sri Lanka remains unknown. Usually, the recorded figures on Epidemiological Reports comprise the patients being admitted or treated in the out-patient department in state hospitals, thus those being treated by private medical practitioners would not be included. Further, cases with mild symptoms or where medical intervention occurred early might not be recorded. In general, foodborne diseases in Sri Lanka are considered of little clinical significance given the high impact of other infectious diseases such as dengue (180,601) and leptospirosis (3632) which were the top two highest counts of cases in Sri Lanka in 2017 (Ministry of Health, 2018).

There is limited publications and/or publicly available statistics on NTS in humans in Sri Lanka. There had been a protracted infection associated with *S*. Bareilly over 27 months in a maternity hospital in Colombo as early as 1970s (Mendis *et al*., 1976). Further, there are a few case studies on bacteremia or invasive NTS infections among immunocompromised patients. They comprise three clinical cases of bacteremia caused by *S*. Typhimurium (Piyasiri *et al.*, 2017), four cases caused by *S*. Enteritidis and *S*. Corvallis (Mubarak and Chandrasiri, 2013), one case of sepsis each due to *S*. Enteritidis (Fernando *et al*., 2011) and *S.* Weltevreden (Gunasena and De Silva, 2021). Further, a more recent study identified 40 NTS through conventional serotyping and WGS, from human clinical specimens. The serotypes comprised Enteritidis (ST11), Corvallis (ST1514), Chester (ST2063) and Mbandaka (ST1602), providing insights into a potential public health threat (Tay *et al.*, 2019).

It is also likely that cooking habits of Sri Lankans happen to play an important role as a control measure against food-borne NTS. For there is hardly any consumption of raw meat or egg, and the use of spices and long cooking times could contribute to the low reported prevalence of NTS affecting humans. However, with known human pathogens being isolated from Sri Lankan poultry over the recent years (Alwis *et al.*, 2014; Kalupahana *et al.*, 2017; Tay *et al.*, 2019), there remain potential public health concerns. Consequently, with the

popularity of chicken meat and eggs among Sri Lankans as well as with the changes in lifestyles, and increased consumption of fast foods, microbiological quality assurance of poultry products is important to ensure consumer safety.

2.8 *Salmonella* control programme in Sri Lanka

PD and FT are notifiable diseases in Sri Lanka under the Animal Diseases Act No: 59 of 1992, the principal enactment that deals with the control and spread of important animal diseases in the country. Since poultry salmonellosis (i.e., PD and FT) re-emerged in 1998, a control programme was launched in 2001 in breeder farms to ensure DOC are free of *Salmonella* (Gunawardena *et al*., 2003). It was subsequently implemented as a national control programme.

The national control programme is carried out in all registered breeder farms and progress is recorded in the annual reports of DAPH. With regard to controlling the disease, the major concern is to keep *Salmonella* carrier status (i.e., *S.* Gallinarum and *S.* Pullorum) at a level less than 1% in poultry breeder flocks (Gunawardena *et al*., 2003). The programme consists of routine screening to detect carrier status, monitoring of hatcheries for *Salmonella* isolation and improvement of biosecurity measures in breeder farms.

For routine screening to detect carrier status, the WBAT is used as a simple and inexpensive method to screen a flock of birds (Quinn *et al.*, 2003; Grimes and Simmons, 1972). In order to perform WBAT, stained *S.* Pullorum antigen is produced in the VRI (Gunawardena *et al*., 2003) following the guidelines in OIE Terrestrial Manual 2010. Monitoring of hatcheries for isolation of *Salmonella* is carried out once in every three-month period in all hatcheries (DAPH Annual report, 2016). This is done by officers of relevant VICs visiting hatcheries on a hatch-date[4] for random sampling of fluff[5], dead-in-shell-eggs[6], dead chicks, meconium etc.

[4]Hatch-date: Estimated hatched out of approximately 90% eggs in an egg batch. Usually pull out in a hatchery is carried out after completion of incubation: between 504 to 512 hours (~21 days).

Isolation of *Salmonella* is performed using enrichment and selective *Salmonella* growth media, and identification is carried out by conventional biochemical tests at VICs, which are then followed by serotyping of confirmed isolates at VRI. Improving biosecurity measures of breeder farms and hatcheries is a recent addition to the control programme (DAPH Annual report, 2016). In this regard, all premises were categorised in to three groups, A, B and C, based on their prevalent biosecurity levels, with the objective being the provision of technical expertise to improve up to the next level of biosecurity.

Vaccination against *Salmonella* is another means of disease control. It is argued that birds immunised with live or killed *Salmonella* vaccines would possess antibodies which might interfere with WBAT, thus the use of *Salmonella* vaccine has been prohibited in breeder flocks to eliminate such possibilities (Medewewa *et al.*, 2012). However, in the face of disease outbreaks, *Salmonella* live vaccine SG 9R (Bouzoubaa, *et al*., 1989; Silva, *et al*., 1981) was allowed in 2012 for breeder flocks for a period of three years as a disease control measure (DAPH Annual report, 2012). Subsequently, an indirect ELISA study was conducted to assess the immunity development of breeder birds following vaccination (Liyanagunawardena *et al*., 2016b), which indicated a statistically significant difference in mean antibody titers in vaccinated versus unvaccinated groups. However, for a few years from 2015, no vaccination was permitted for breeder birds (DAPH Annual report, 2016), while killed *Salmonella* vaccine continues to be permitted for commercial layers in Sri Lanka (Priyantha, 2009). At present, breeder farms are allowed to use killed *Salmonella* vaccine as a control strategy.

[5]Fluff: debris remain after hatching (e.g., down feathers) in the hatcher of a hatchery

[6]Dead-in-shell eggs: Unhatched eggs with dead and/or live (weak) chicks or dead embryos, after completion of the usual incubation period (~21 days) in a hatchery

A national policy for salmonellosis in poultry was proposed in 2017 as a joint effort encompassing all stakeholders. The new policy puts forth new regulations to cover every aspect of poultry industry from farm to fork as well as to include motile or NTS into the control programme. New regulations comprise identification of persons, premises and products that are stakeholders in the poultry industry in Sri Lanka to establish and enforce good management practices which are necessary for the early detection, prevention and control of salmonellosis (DAPH *Salmonella* policy, 2017).

Chapter 3: Determination of *Salmonella* carriage in a sample of broiler farms and associated hatcheries in Sri Lanka

3.1 Abstract

Salmonellosis is one of the most significant conditions affecting poultry in Sri Lanka. Since previous work has been limited to outbreaks and occurrence of *Salmonella* in chicken meat/ eggs, there is a crucial need to broaden the knowledge base in Sri Lanka to include carriage of *Salmonella* in poultry. Therefore, the objective of this study was to estimate the prevalence of *Salmonella* carriage in broiler farms and to investigate associated hatcheries to determine the magnitude of the issue.

A pilot study provided baseline information upon which to design a cross-sectional survey where proportionate sampling was carried out from July to December 2017 in broiler farms (n=115) in the five highest poultry- dense districts in Sri Lanka. Sampling per flock was by two pairs of boot socks and collecting 25 cloacal swabs to make five pooled samples. Associated hatcheries (15) were also included to facilitate investigating a possible source of infection in the case of *Salmonella* positive broiler farms. Sampling per egg batch comprised pooled samples of fluff, eggshells, meconium, dead-in-shell eggs and dead chicks separately. Culture identification was followed by PCR confirmation.

There was an overall farm and adjusted flock prevalence of 32.2%, CI 95% [23.6-40.7] and 28.9%, CI 95% [21.7-37] respectively. Of the associated hatcheries, 66.7%, CI 95% [42.8-90.5] were *Salmonella* positive while adjusted prevalence at egg batch level was 42%, CI 95% [21.0-66.0]. Unexpectedly, all the isolates (128) obtained in the present study were motile *Salmonella*. The overall *Salmonella* recovery rate in farm sampling was 7%, CI 95% [5.5-8.4], which comprised an 18.7%, CI 95% [14.4-23.0] positive rate in boot socks compared to 2.5%,

CI 95% [1.4-3.5] in cloacal swabs. In the hatchery context, overall *Salmonella* recovery rate was 9.1%, CI 95% [6.7-11.5] with fluff and eggshells each having 13.3%, CI 95% [7.89-18.77] higher *Salmonella* recovery rate compared to all other samples. This study reports the first comprehensive analysis on *Salmonella* carriage in Sri Lankan poultry, which could be essential in widening the existing knowledge base as well as in implementing effective control strategies.

3.2 Introduction

Salmonellosis remains a significant threat affecting poultry and/or human health in many developing countries (Barrow *et al.*, 2012). Salmonellosis in humans is typically a food-borne zoonotic disease (Hohmann, 2001), and poultry meat and eggs are common vehicles of transmission (Threlfall *et al.*, 2014).

The poultry industry is the most developed among all livestock industries in Sri Lanka (DAPH Livestock Statistics, 2015), representing over 50% of the total contribution of the livestock sector to the country's GDP in 2016 (DAPH Poultry forecast, 2017). The broiler sector in particular displayed substantial growth over the past decade. Amongst the multi-ethnic population, chicken is the most popular source of animal protein (De Silva *et al*., 2010) and there is a thriving chicken meat production industry in the country (DAPH Poultry forecast, 2017). It already accounts for over two-thirds of the total meat industry in Sri Lanka, while an annual growth of 3-5% in the production capacity is also expected (DAPH Poultry forecast, 2017). There is, therefore, a timely need to secure a broad export market, and efforts are directed to such ends.

Salmonellosis is one of the most significant conditions affecting poultry in Sri Lanka. The number of cases[7] reported in commercial poultry in 2018 and 2020 were 78,928 and 50,642

[7]Salmonellosis cases: could comprise individual birds or several birds of a flock. These are largely based on clinical signs and post-mortem lesions rather than on laboratory confirmation.

respectively, out of which, majority of the cases were due to fowl typhoid (*Salmonella* Gallinarum) and pullorum disease (*Salmonella* Pullorum) (DAPH Annual report, 2018; 2020). Case studies of poultry outbreaks have reported occurrences of non-typhoidal *Salmonella* such as *S.* Enteritidis and *S.* Typhimurium, in addition to the predominant *S.* Gallinarum and *S.* Pullorum (Gunawardena *et al*., 2006; Priyantha *et al*., 2007; Liyanagunawardena *et al*., 2012). Although the figures are not available for Sri Lanka, the impact of fowl typhoid and pullorum disease generally translates to extensive economic loss. Additionally, the surviving birds are likely to become lifelong carriers, are immune-compromised and grow slowly, resulting in reduced production (Shivaprasad, 2000).

A control programme against poultry salmonellosis has been in place since 2001, with the aim of reducing *Salmonella* carrier status to a level less than 1% in poultry breeder flocks (Gunawardena *et al*, 2003). The programme employs routine screening with a whole blood agglutination test; a simple and inexpensive method for flock screening (Quinn *et al*., 2003; Grimes and Simmons, 1972) with removal of positive reactors. However, *Salmonella* has continued to infect commercial (Liyanagunawardena *et al*., 2012) and breeder flocks (Wijemanna *et al*., 2008; Liyanagunawardena *et al*., 2013) despite the remedial measures carried out through the years. In the face of disease outbreaks in 2012, an immunization programme was initiated by allowing SG 9R live *Salmonella* vaccine[8] (Silva *et al.*, 1981) for breeder flocks (DAPH Annual report, 2012). Nevertheless, there is no reliable information to assess vaccination efficacy.

Salmonella is considered one of the main bacterial pathogens causing foodborne illnesses in Sri Lanka (Ministry of Health, 2013). Nevertheless, the burden of foodborne salmonellosis in

[8]SG 9R vaccine: Rough strain *S*. Gallinarum 9R (Smith, 1956), which was developed initially for immunization against *S*. Gallinarum. Also being used for active immunization against *S*. Pullorum and to active cross protection against infections caused by other Salmonellae of group D such as *S*. Enteritidis.

Sri Lanka is not well understood and there are few publications. Recent studies provide evidence of the occurrence of *Salmonella* in retail chicken meat/eggs with known human pathogenic *Salmonella* (Kalupahana *et al.*, 2017; Tay *et al.*, 2019; Kulasooriya *et al.*, 2019) and 40 nontyphoidal *Salmonella enterica* were isolated from routine human clinical specimens from eight cities in Sri Lanka (Tay *et al.*, 2019). This evidence suggests, on the one hand, a considerable threat to public health given the high demand for chicken meat among Sri Lankans; and on the other, a significant challenge for the Sri Lankan broiler meat export trade. Since previous work has been limited to outbreaks and occurrence of *Salmonella* in chicken meat/eggs, there is a crucial need to broaden the knowledge base on salmonellosis in poultry in Sri Lanka to include carriage.

In this context, the present study is the first comprehensive attempt to study *Salmonella* carriage in Sri Lankan poultry. A pilot study to provide baseline data was followed by a cross-sectional survey in broiler farms in the five districts with the highest poultry density. The broiler hatcheries from which the chicks were brought in (referred to as "associated hatcheries" hereafter) were also included in the case of *Salmonella* positive farms[9], to investigate a possible source of infection. Throughout this chapter *Salmonella* identification is discussed up to species level, i.e., *Salmonella enterica*. Further, association of *Salmonella* within farms and/or farms and their corresponding hatcheries with regard to possible transmission will be discussed in chapter 6. Thus, the objective of this chapter is to estimate the prevalence of *Salmonella* carriage in broiler farms and to further investigate the associated hatcheries to determine the magnitude of the issue.

[9] *Salmonella* positive farm: a farm from which at least one confirmed *Salmonella* isolate was obtained in this study.

3.3 Materials and methods

3.3.1 Ethics

Ethical approval for this study was granted by the Committee for Ethical Clearance on Animal Research of the Faculty of Veterinary Medicine, University of Peradeniya, Sri Lanka. The pilot work carried out between April and June 2017, was conducted with provisional approval (reference VER-2017-004 in Appendix I), and full approval (reference VS/ERC/17/04 in Appendix I) was subsequently granted in July 2017.

3.3.2 Pilot study

To provide baseline information on *Salmonella* carriage and to assist in designing the main study, a pilot study was carried out in commercial broiler farms in Kurunegala district in North-Western Province of Sri Lanka. Kurunegala district is known to have the highest poultry density in Sri Lanka (Dept of Census and Statistics, 2015). Associated hatcheries were also noted while sampling farms, in order to explore a possible source of infection by identifying the origin of chicks.

Selection of broiler farms

A list of broiler farms with a capacity greater than or equal to 1000 chicken[10] (also known as farms with over 1000 chicken), situated in three Government Veterinary Ranges (GVR)[11] Wariyapola, Panduwasnuwara and Katupotha, in Kurunegala district was identified (through personal communications with government veterinarians in the area) as the sampling frame. Subsequently, 20 commercial farms were randomly selected using an online number generator (http://numbergenerator.org) and sampled once between February and April 2017.

[10]Commercial broiler farms are categorized into two: farms with greater than or equal to 1000 chicken (also called farms with over 1000 chicken) and farms with fewer than 1000 chicken (also called farms with below 1000 chicken).

[11]GVR: refers to a geographical area under the purview of one senior government veterinarian (who may have one or two veterinarians reporting to him/her). GVRs roughly correspond to the administrative divisions of the country. In a GVR, the senior veterinarian-in-charge is responsible for providing the required services (health, breeding, extension etc.) for government sector and small-scale private sector ventures as well as for households, though not for large-scale private enterprises that employ their own veterinarians.

Sampling broiler farms

The initial protocol was to sample two flocks[12] of birds from each farm and consider flock age; i.e., birds in first two weeks (day 1- 14) and last two weeks (day 28- 42), to investigate disease transmission between flocks and to select two flocks from the several flocks within a farm, respectively. However, small-scale farms with only one flock were also sampled regardless of age.

Cloacal swabs[13]: Ten cloacal swabs per flock were obtained to make two pooled samples. In the absence of assistance, the researcher attempted to select birds from different areas of the pen (i.e., from the four corners and the middle) for swabbing. However, this was not practical as the birds tend to huddle together or move to a corner as sampling progressed. Subsequently, with an assistant, a spinner (phone application) was used to point to birds at random, so that the particular bird could be caught while the researcher kept watch on it. Birds were excluded from sampling if they appeared injured or sick to the researcher. Birds thus selected were kept in a partitioned area within the pen and released after swabbing, to avoid sampling the same bird twice.

Boot socks[14] and drag swabs[15]: A pair of boot socks were worn over disposable polythene overshoes to walk inside the pen, after which the pair were cultured as one sample. Since the length of poultry houses differs from one farm to another, a count of approximately 100 steps was done while walking inside the pen through its perimeter. Simultaneously, two drag swabs were dragged through each poultry pen over the litter, to make one pooled sample.

[12]flock: birds of same age in a farm. Often a flock is housed together, however it can differ depending on the number of birds and space availability.

[13]Cloacal swabs: Cotton-wool buds (available for ear cleaning) purchased, made one-sided and autoclave sterilized. Collected as pooled samples in 1% buffered peptone water.

[14]Boot socks: Ordinary cotton socks were purchased, and autoclave sterilized. Pre-moistened with 1% buffered peptone water and worn over disposable polythene overshoes and packed in a sterile polythene bag after sampling.

[15]drag swabs: Prepared using gauze bandage and twine (string), and autoclave sterilized. Pre-moistened with 1% buffered peptone water and packed in a sterile polythene bag after sampling.

Sampling associated hatcheries

Hatchery samples were collected once from associated hatcheries between February and April 2017. Egg batch[16]-wise sampling was done for all the available egg batches on a given hatch-date[17]. Sampling per egg batch comprised of 10-20g of fluff[18] to make three pooled samples, 15 eggshells[19] to make three pooled samples, meconium from 10 chicks to make two pooled samples, 10 dead-in-shell eggs[20] to make two pooled samples and up to five dead chicks to make one pooled sample.

3.3.3 Cross-sectional study (main study)

Sample size calculation

The sample size calculation was based on the results of the pilot study into the prevalence of *Salmonella* in the Kurunegala district. To estimate the total number of farms to be sampled, a simple random sampling approach was taken, and calculations were performed in R-Studio (R version 3.5.3 https://www.r-project.org). The *Salmonella* positive percentage (25%) from the pilot study was used, with 95% certainty of the maximum relative difference between the estimate and the unknown population value being within +/-30% of the true population percentage, to determine the required sample size as 116 farms.

The main study was then extended to another four districts, so that five districts were included altogether, namely, Kurunegala, Puttalam, Kegalle, Gampaha and Kandy. These were the five highest poultry-dense districts in Sri Lanka, which comprised 68% (1269/1847) of the total commercial broiler farms with over 1000 chickens in 2015 (Dept of Census and Statistics, 2015). Accordingly, proportionate sampling from the selected districts formed the sampling frame comprising the required sample size of 116 farms as presented in Table 3.1.

[16]Egg batch: eggs in a hatchery, which is the offspring of same batch of parent birds.
[17]Hatch-date: Estimated hatched out of approximately 90% eggs in an egg batch. Usually pull out in a hatchery is carried out after completion of incubation: between 504 to 512 hours (~21 days).
[18] Fluff: debris remain after hatching (e.g., down feathers) in the hatcher of a hatchery
[19]Eggshells: eggshells remain after hatched chicks were collected in the hatcher of a hatchery
[20]Dead-in-shell eggs: Unhatched eggs with dead and/or live (weak) chicks or dead embryos, after completion of the usual incubation period (~21 days) in a hatchery

Table 3.1: Total broiler farms over 1000 chickens (Dept of Census and Statistics, 2015) and number to sample (from the five selected districts) in the cross-sectional study to estimate prevalence of *Salmonella* carriage in Sri Lanka

District	Total Farms	Number to sample
Kurunegala	571	47
Puttalam	257	23
Kegalle	183	18
Gampaha	178	18
Kandy	80	10
Total	1269	116

For the main study, a stratified approach was adopted instead of simple random sampling in the pilot study, to ensure that all available types of broiler farms were included in the sampling frame from the five districts concerned. Thus, farms within each selected district were then stratified based on the following features: a) their capacities[21]; b) operation types: buy-back[22], self-owned and company- owned; and c) housing types: simple open-house[23] pens and closed-house[24].

Sampling broiler farms and hatcheries

Ninety-five broiler farms were sampled once between July and December 2017. Sampling was performed in a similar manner to that of the pilot study except for the following adjustments:

- The age of the broiler flocks was not considered as it was not practical to estimate this at the farm level.

[21]Capacity of farms: small-scale (up to 5,000 birds), medium-scale (5,001-25,000 birds), large-scale (>25,000 birds).

[22]The buy-back system in the broiler industry is a unique feature where small-scale farmers are engaged in rearing for companies. Many leading broiler companies adopt this system, where they enter into a contract with small-scale farmers for rearing chickens.

[23]Open-house: poultry pen with half walls of approximately 0.5 m height, with a wire mesh to complete the walls up to roof.

[24] Closed-house: environmental controlled (optimal temperature, humidity etc) poultry house in large-scale farms, often company-owned. The flock capacity can be 10,000 birds upwards with automated facilities and managed by trained staff.

- The drag swab was not used as there were no *Salmonella* isolations from drag swabs in the pilot study.
- The number of boot socks and cloacal swabs were increased. Accordingly, sampling per flock was done by obtaining two pairs of boot socks (one pair analysed as one sample) and collecting 25 cloacal swabs to make five pooled samples.

Associated parent hatcheries were sampled following the identification of *Salmonella* positive flocks during this period, in the same way as the pilot study. Source of parent birds[25] was noted during hatchery sampling to further examine corresponding grandparent (GP) hatchery, in case of a *Salmonella* positive[26] parent hatchery. Levels of sampling are illustrated in Figure 3.1.

Sample dispatch and transportation

Collected samples were labelled and transported in refrigerated conditions, using a chilly bin (or cooler box) with sufficient ice packs and a thermometer, with the aim of keeping the inside temperature around 4^{o}C. Samples were sent to the Veterinary Research Institute, Peradeniya, Sri Lanka, for processing.

[25]Source of parents: Majority of the broiler parent requirement is supplied by three local grand-parent farms while the rest are imported.
[26]*Salmonella* positive hatchery: a hatchery from which, at least one confirmed *Salmonella* isolate was obtained in this study.

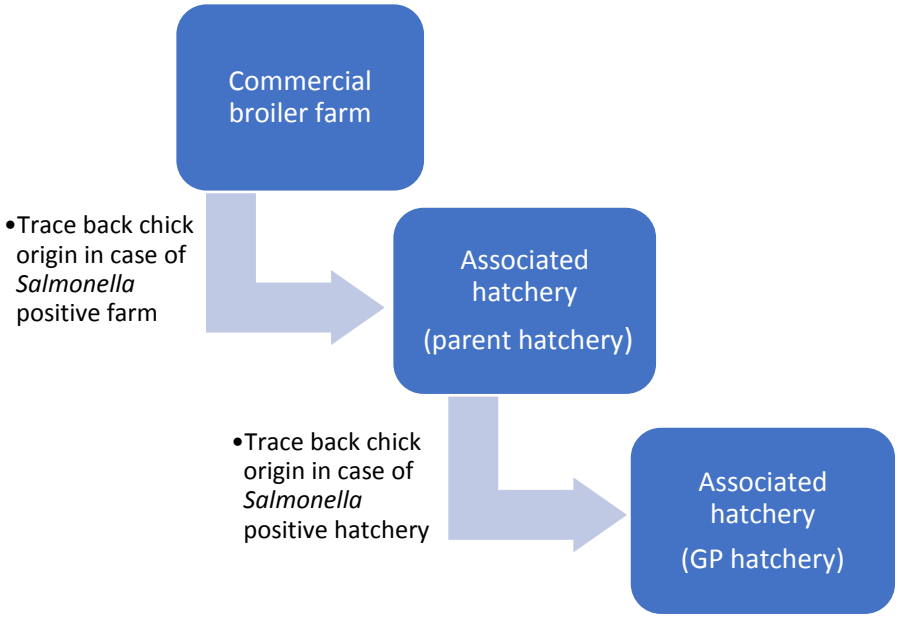


Figure 3.1: The levels of sampling carried out in the cross-sectional study to estimate prevalence of *Salmonella* carriage in Sri Lanka

3.3.4 Recording coordinate data of farms and hatcheries

Coordinate data were collected on farms and hatcheries sampled, using a GPS device (GPSMap 62, Garmin Ltd, Kansas, USA). This was accomplished during the visits for sampling. However, there were technical issues in gathering coordinate data due to low signal in some areas. This necessitated an extra visit to the same location to ensure the correct recording of the coordinate data. On several occasions coordinate data had to be collected at the nearest possible location to the entrance to the property as the farm or hatchery entry permission was not granted for a second time.

3.3.5 Microbiology

Culture Identification of Salmonella

Sample processing was commenced within one hour of reception. The laboratory procedure was based on (ISO-6579:2002/Amd.1:2007 (E), 2007) standard, which is the horizontal method for the detection of *Salmonella*. All culture media and biochemical reagents were from Oxoid, UK and was prepared within the laboratory following instructions provided by

the manufacturer. Buffered peptone water (1%) was used as a pre-enrichment medium after which incubation at 37^{o} C for 18-24 hours or overnight was carried out. Selective enrichment was then carried out in Rappaport-Vassiliadis soy peptone broth (RVS broth) for 24- 48 hours at 42^{o} C. This step was followed by spreading on selective agar plates, i.e., Xylose Lysine Desoxycholate (XLD) agar, Brilliant Green Agar (BGA) and *Salmonella* Chromogenic Agar, and incubation at 37^{o}C for 18-24 hours. Subcultivation of *Salmonella* suspected colonies was carried out on XLD or BGA. Later, the following biochemical tests were carried out with known positive (*Salmonella* Pullorum and *Salmonella* Enteritidis) and negative (*Escherichia coli* and *Pasteurella multocida*) cultures: Gram stained smear; Triple Sugar Iron agar; Urease; Citrate; Oxidase; Sulfide Indole Motility; and O-Nitrophenyl-β-D-Galactopyranoside. Two separate colonies (per positive sample) from 129 purified *Salmonella* isolates (labelled 'a' and 'b') were then stored in semisolid nutrient agar initially and subsequently in freeze dried ampules.

3.3.6 Isolate transfer

Documentation for material transfer was done in collaboration with the Molecular Epidemiology and Public Health Laboratory (^{m}EpiLab), Massey University and Veterinary Research Institute (VRI). This included a material transfer agreement between Department of Animal Production and Health (DAPH) and Massey University, authority to leave Sri Lanka, and a user permit from Ministry of Primary Industries to enter New Zealand (Appendix I).

3.3.7 Further identification of isolates

PCR confirmation

Revival of *Salmonella* isolates was done in the PC3 facility in the ^{m}EpiLab in adherence to PC3 protocols (protocols on culture, DNA extraction and disposal of *Salmonella* can be found in Appendix II), following which conventional PCR was performed on extracted DNA to confirm

Salmonella isolates using the primer pair Stn-101; 5'-CTTTGGTCGTAAAAT AAGGCG-3' and Stn-111; 5'-TGCCCAAAGCAGA GAGATTC-3' (Makino *et al.*, 1999).

3.3.8 Data management and statistical analysis

Sampling data and laboratory results were stored on a Microsoft SQL Database Cluster (Microsoft Corporation, Redmond WA, USA) as the back end, and Microsoft Access (Microsoft Corporation, Redmond WA, USA) as the front end. Descriptive statistical analysis of the results was performed in R-Studio (R version 3.5.3 https://www.r-project.org) and Microsoft Excel (Microsoft corporation, Redmond WA, USA). The R packages 'RODBC' (https://cran.r-project.org/web/packages/RODBC/index.html) and 'sqldf' (https://cran.r-project.org/web/packages/sqldf/index.html) were used for accessing data from the database.

Estimating Salmonella prevalence

A farm or hatchery from which at least one confirmed *Salmonella* isolate was obtained in this study was considered a positive property. The prevalence at the farm level was calculated as a proportion of *Salmonella* positive farms out of the total sampled within each district. The flock level prevalence was calculated adjusting for the clustering of flocks within farms using the R packages 'survey' (Lumley, 2020), 'purrr' (Henry and Wickham, 2020), 'tidyr' (Wickham and Henry, 2021) and 'dplyr' (Wickham *et al*., 2021). *Salmonella* prevalence at the hatchery level was calculated as a proportion of *Salmonella* positive hatcheries out of the total sampled while the egg batch level prevalence was calculated adjusting for the clustering, in the same way as for the flock level prevalence within farms. For creating bar charts with error bars, the R packages 'ggplot2' (Wickham, 2016), 'tidyverse' (Wickham *et al*., 2019), 'viridis' (Garnier et al., 2021) were used.

Mapping coordinate data

The coordinate data were extracted and checked for accuracy in Google Maps (https://www.google.co.nz/maps) using known precise addresses or nearest villages. Then the mapping of positive and negative broiler holdings in a map of Sri Lanka was done using the shapefiles downloaded from the Survey Department of Sri Lanka (Sri Lanka Administrative Boundary Common Operational Database) and some R packages. R packages used were 'epiR' (Stevenson *et al*., 2021), 'ggplot2' (Wickham, 2016), 'ggmap' (Kahle and Wickham, 2013), 'RColorBrewer' (Neuwirth, 2014), 'scales' (Wickham and Seidel 2020), 'grid' (R Core Team, 2020), 'plyr'(Wickham, 2011), 'sp' (Pebesma and Bivand, 2005), 'raster' (Hijmans, 2021), 'rgeos' (Bivand and Rundel, 2021), 'rgdal' (Bivand *et al*., 2021), 'classInt' (Bivand, 2020), 'maptools' (Bivand and Lewin-Koh, 2021) and 'spatstat'(Baddeley and Turner, 2015).

3.4 Results

3.4.1 Sampling Broiler farms

A total of 140 broiler farms with farm capacity of over 1000 chicken from the five selected districts were contacted and invited for participation in the study, out of which 118 farms consented. Proportionate sampling was done from 115 broiler farms in the five districts, however fewer than expected numbers were sampled from Kurunegala and Puttalam districts. Sampling covered 173 flocks in 115 farms. This included 30 flocks from 20 farms and 143 flocks from 95 farms in the pilot study and main study respectively, and all the farms were sampled once with no overlap. General features of the farms such as type of operation, type of housing, farm capacity and number of available flocks are described in Table 3.2. All the farms were managed under the deep-litter management system, with litter being either paddy husk, wood shavings or both. There were seven company-owned farms from five large-scale broiler chicken producers in Sri Lanka. Different farms that were sampled during the study are presented in Figure 3.2.

Table 3.2: General features of the 115 broiler farms sampled (from five selected districts) in the cross-sectional study to estimate prevalence of *Salmonella* carriage in Sri Lanka in 2017

	Kurunegala	**Puttalam**	**Gampaha**	**Kegalle**	**Kandy**
Operation Type					
Buy-back (64)	14	10	11	18	11
Self-owned (44)	21	10	9	2	2
Company-owned (7)	5	1	0	0	1
Housing Type					
Open-house (108)	35	20	20	20	13
Closed-house (7)	5	1	0	0	1
Farm Capacity					
Small-scale (87)	28	16	15	18	10
Medium-scale (21)	7	4	5	2	3
Large-scale (7)	5	1	0	0	1
Flocks Available					
One (74)	16	14	17	16	11
Two (26)	17	3	1	3	2
Three or more (15)	7	4	2	1	1
Total Farms	40	21	20	20	14

Figure 3.2: Examples of different broiler farms that were sampled in the cross-sectional study conducted in Sri Lanka in 2017 (Top left: large-scale, top right: medium-scale and below: small-scale)

3.4.2 Sampling hatcheries

Associated hatcheries were sampled to trace back possible source of infection, following the identification of *Salmonella* positive farms. Consequently, 50 egg batches from 15 associated hatcheries (13 parent hatcheries and two GP hatcheries) were sampled, following which *Salmonella* isolation and identification were carried out. These hatcheries represented different levels of DAPH biosecurity grading[27] in 2016. Their varied production capacities were evident from the number of day-old chicks produced per week. These traits, along with the source of parents (or grandparents if the hatchery is a GP hatchery) and number of parent farms providing eggs, are displayed in Table 3.3.

Local GP farms are denoted as GP1, GP2 and GP3 in the table. Sampling was carried out egg batch-wise and comprised pooled samples of fluff (150), eggshells (150), meconium (100), dead-in-shell eggs (100), and dead chicks (50).

[27]DAPH biosecurity Grading: All hatcheries are monitored annually and scaled from A to C (A- satisfactory, C- poor), based on their prevalent biosecurity levels, with the objective being the provision of technical expertise to improve to next level of biosecurity or to maintain the level if it is within the level of satisfactory.

Table 3.3: General features of the 15 associated hatcheries sampled in the cross-sectional study to estimate prevalence of *Salmonella* carriage in Sri Lanka in 2017

	Associated Hatcheries
DAPH biosecurity Grading	
A	7
B	4
C	4
Production of day-old chicks/ week	
15,000- 30,000	7
90,000- 150,000	5
150,000- 200,000	2
3 50,000	1
Source of parents	
GP1	8
GP2	1
GP3	1
Imported	3
Sources mixed	2
Parent farms providing eggs	
One	11
Two	3
More than two	1

3.4.3 *Salmonella* identification

Biochemical identification was followed by PCR, resulting in 128/129 isolates confirmed as *Salmonella* with the amplification of DNA fragments of 260 base-pairs (bp) in length. All isolates were motile *Salmonella* as per the motility testing, which was carried out under biochemical tests. The results of the biochemical tests and PCR can be found in Appendix III. The locations of *Salmonella* positive and negative broiler holdings (i.e., farms, hatcheries and GP hatcheries) are presented in Figure 3.3 and the data used for mapping is available in Appendix III.

3.4.4 *Salmonella* prevalence at broiler farms and hatcheries

Pilot work revealed 25% (5/20) *Salmonella* positive status at farm level and 23% (7/30) at flock level. Overall study prevalence i.e., both pilot and main studies, was 32.2%, CI 95% [23.6-40.7] at farm level; while adjusted prevalence at flock level was 28.9%, CI 95% [21.7-

37]. Inclusion of five districts compared to only one in the pilot study was a major alteration to the sampling of the main study. Stratification of farms was another major modification done in the main study's design. Simple random selection in the pilot study comprised only small-scale farms, whereas stratified sampling in the main study led to the representation of farms of all three capacities. A district-wise summary of the *Salmonella* prevalence by farm level and flock level is found in Table 3.4 and in Figure 3.4.

Table 3.4: *Salmonella* prevalence in the 115 broiler farms (from five selected districts) in the cross-sectional study carried out in Sri Lanka in 2017

	Farm level		**Flock level***	
District	**Prevalence %**	**CI 95%**	**Prevalence %**	**CI 95%**
Kurunegala	37.5 (15/40)	22.5 -52.5	34.3 (24/70)	22.3-49
Puttalam	33.3 (7/21)	9.3-47.9	26.7 (8/30)	13.2-46
Gampaha	15.0 (3/20)	0.00-30.6	14.3 (4/27)	3.6-42
Kegalle	35.0 (7/20)	18.5-61.5	30.4 (7/23)	13.5-55
Kandy	35.7 (5/14)	10.6-60.8	31.8 (7/23)	15.2-55
Total	32.2 (37/115)	23.6-40.7	28.9 (50/173)	21.7-37

*Adjusted for clustering at the farm level

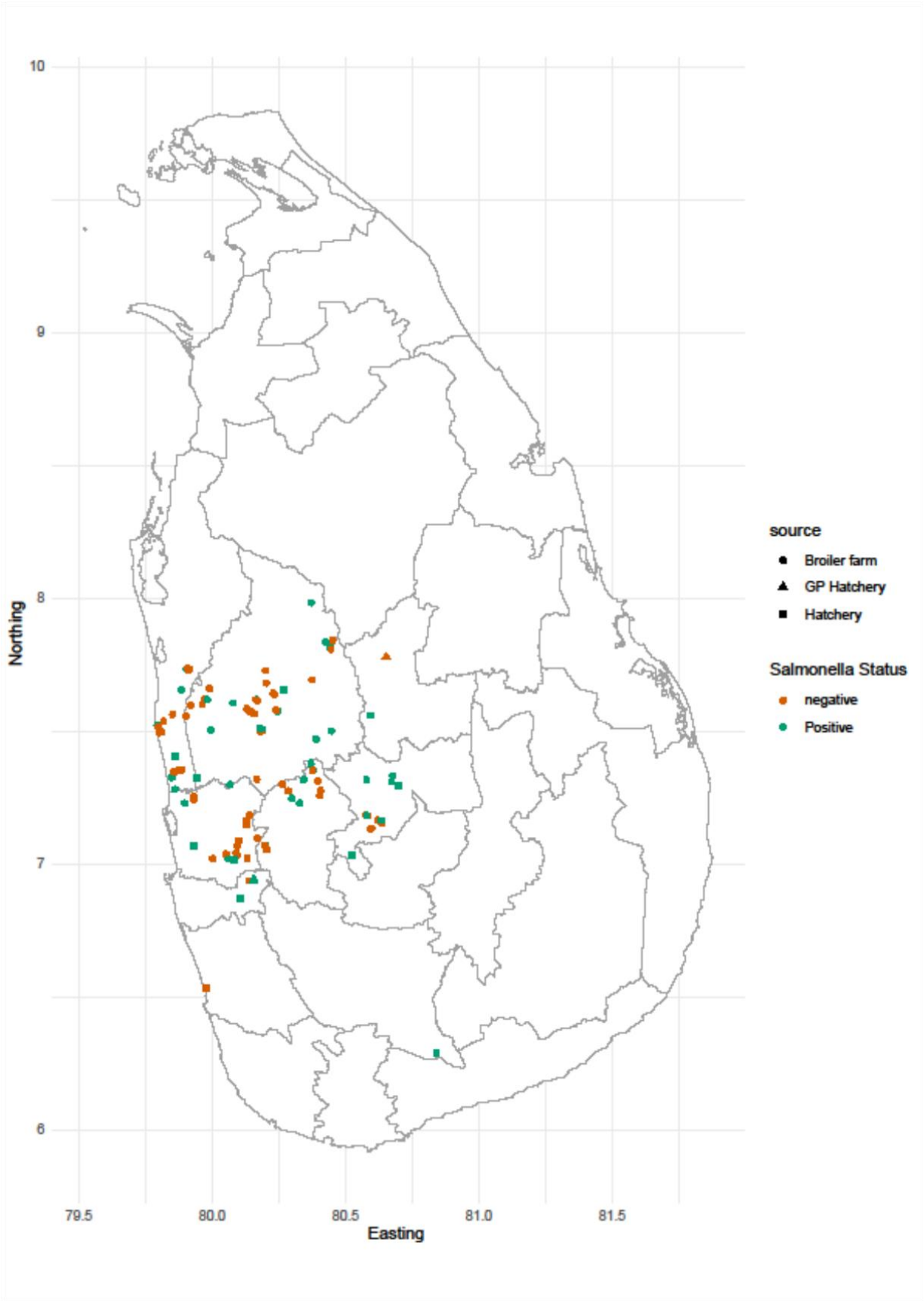


Figure 3.3: Map of Sri Lanka showing locations of *Salmonella* positive and negative broiler holdings sampled in the cross-sectional study conducted in 2017

Following the identification of *Salmonella* positive commercial farms (37/115, 32.2%), their source/s of chicks i.e., associated hatcheries, were retrieved. 34/37 (92%) farms reported a single source of chicks and the other three farms had multiple sources, adding up to a total of 14 associated hatcheries. All except one of these hatcheries were sampled, with the results indicating a *Salmonella* positive status of 9/13 (69%) at hatchery level. For the next level of sampling, the parent source for positive hatcheries was identified. One positive hatchery had an imported parent source, which therefore was out of reach of sampling; all other positive hatcheries were associated with two local GP hatcheries, which were then sampled.

Salmonella prevalence at the hatchery level was 66.7%, CI 95% [42.8-90.5], while adjusted prevalence at egg batch level was 42%, CI 95% [21.0-66.0]. A summary of the *Salmonella* status of hatcheries is presented in Table 3.5 and in Figure 3.4. Among the positive hatcheries, seven had more than one infected egg batch: five of them having two egg batches and two having four egg batches positive.

Table 3.5: *Salmonella* status in the 15 associated hatcheries in the cross-sectional study carried out in Sri Lanka in 2017

Type of Hatchery	**Hatchery level**		**Egg Batch level***	
	Proportion %	CI 95%	Proportion %	CI 95%
Parent	69.2 (9/13)	44.1-94.3	44.2 (19/43)	23.5-67.0
Grand Parent	50.0 (1/2)	0.0-100.0	28.6 (2/7)	0.0-NA
Total	66.7 (10/15)	42.8-90.5	42.0 (21/50)	21.0-66.0

*Adjusted for clustering at the hatchery level

3.4.*5 Salmonella* recovery from samples

In the farm setting, overall, the *Salmonella* recovery rate was 7%, CI 95% [5.5-8.4], which comprised a 18.7%, CI 95% [14.4-23.0] positive rate in boot socks compared to 2.5%, CI 95% [1.4-3.5] in cloacal swabs. Thus, the results are indicative of a higher *Salmonella* recovery rate from boot socks as shown in Table 3.6 and in Figure 3.4.

Table 3.6: Recovery of *Salmonella* from the 115 broiler farms (from five selected districts) in the cross-sectional study carried out in Sri Lanka in 2017

Sample	Positive Rate (%)	CI 95%
Boot Socks	18.7 (59/316)	14.4-23.0
Pooled Cloacal Swabs	2.5 (19/775)	1.4-3.5
Drag Swabs	0.0 (0/30)	0-0
Total	7 (78 / 1121)	5.5-8.4

In the hatchery context, overall *Salmonella* recovery rate was 9.1%, CI 95% [6.7-11.5] as illustrated in Table 3.7 and in Figure 3.4. Fluff and eggshells each had 13.3%, CI 95% [7.9-18.8] *Salmonella* recovery rate which was higher than that of other samples: meconium, dead-in-shell eggs and dead chicks.

Table 3.7: Recovery of *Salmonella* from the 15 associated hatcheries in the cross-sectional study carried out in Sri Lanka in 2017

Pooled Sample	Positive Rate (%)	CI 95%
Fluff	13.3 (20/150)	7.9-18.8
Egg Shells	13.3 (20/150)	7.9-18.8
Dead Chicks	6.0 (3/50)	0.0-12.6
Meconium	5.0 (5/100)	0.7-9.3
Dead-in-shell eggs	2.0 (2/100)	0.0-4.7
Total	9.1 (50/550)	6.7-11.5

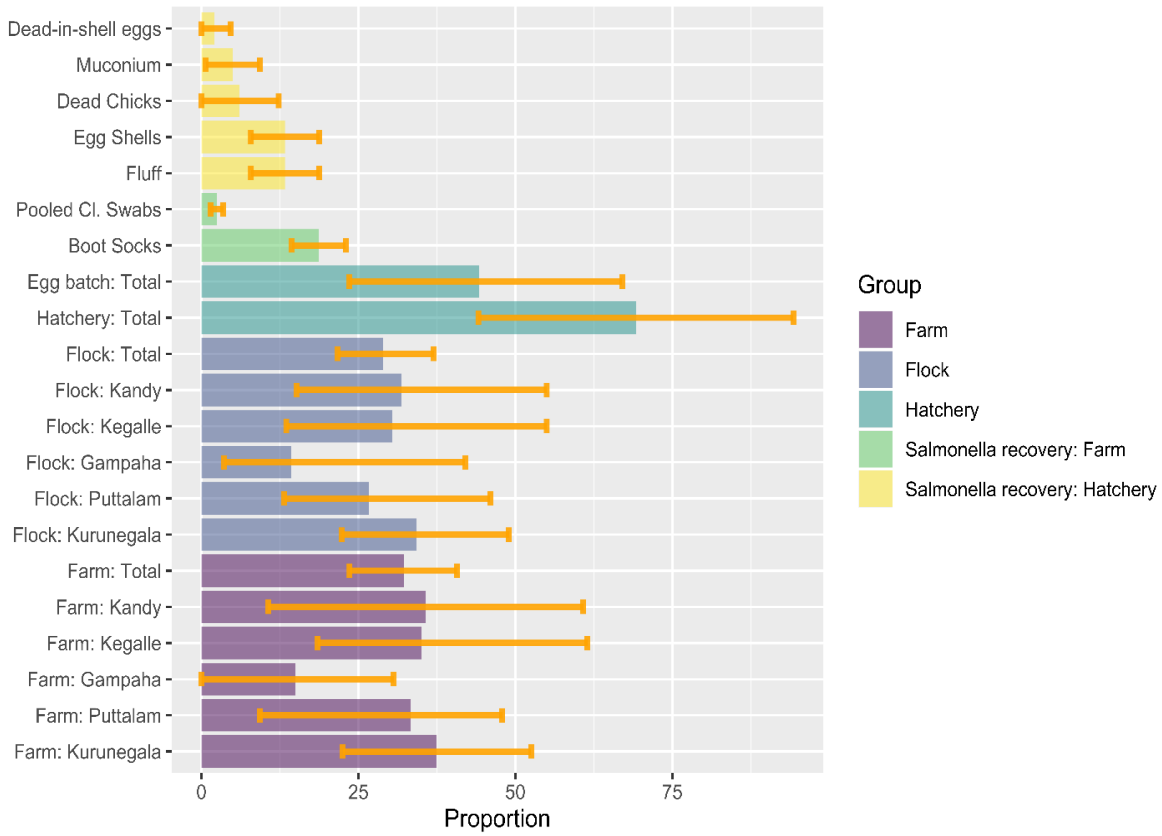


Figure 3.4: Bar chart of the apparent prevalence and recovery of *Salmonella* from broiler farms and hatcheries in the cross-sectional study carried out in Sri Lanka in 2017 (error bars represent 95% confidence intervals)

3.5 Discussion

The present study is the first comprehensive study on *Salmonella* carriage in Sri Lankan poultry. This study reports the prevalence of *Salmonella* in broiler farms of the five highest poultry-dense districts which comprised 1269/1847 (68.7%) of the total commercial broiler farms with over 1000 chickens in the country in 2015 (Dept of Census and Statistics, 2015). The cross-sectional survey covered 115/1269 (9%) of broiler farms in the selected districts. The researcher also examined associated hatcheries for *Salmonella* status. In 2016, day-old chicks for the commercial level farms were supplied by 27 parent hatcheries linked to 32 parent farms. Three GP farms (with hatchery in each) were in operation providing 76% of the parent bird requirement while the balance was imported as day-old chicks (DAPH Poultry

forecast, 2017). Thus, associated hatcheries included in this study comprised 13/27 (48%) of parent hatcheries and two thirds of GP hatcheries in Sri Lanka in 2016.

The results of the study indicated an overall farm and flock prevalence of 32.2%, CI 95% [23.6-40.7] and 28.9%, CI 95% [21.7-37] respectively in the five main poultry production districts of Sri Lanka. The absence of data on farm prevalence impedes any comparative analysis of these results in the Sri Lankan context. There are two studies of *Salmonella* prevalence in broiler carcasses in Sri Lanka, with which to compare the findings of the present study. Weerasooriya *et al* (2008) and Kottawatta *et al* (2014) revealed much lower incidence of 16.7% (24/144) and 9% (18/200) respectively from pooled caecal samples of broiler carcasses. The reasons for the lower prevalence in earlier studies could be attributable to the use of different methods with a lower sensitivity rather than an increase in prevalence over time. The sampling for the cross-sectional survey was conducted from July to December 2017. Being a tropical country, climatic conditions (such as temperature and humidity) in Sri Lanka are almost same throughout the year except for the high rainfalls from May to September due to monsoons. Further, there are two known festive seasons in Sr Lanka (i.e., the Sri Lankan New Year in April and Christmas in December) with high broiler production to cater for the high demand. These seasonal patterns may introduce bias due to the time in which sampling was conducted (i.e., July to December with Christmas season). Information on prevalence of *Salmonella* in India and other countries in the region is also fragmentary (Singh *et al.*, 2010). However, in other parts of the world such as Europe, infection rates in broilers varied from 1% in Scandinavia through 14.9% in Spain to 85% in Hungary (EFSA: European Food Safety Authority as quoted by Barrow *et al*., 2012). Overall, in Europe *Salmonella* was found in 3.31% of the flocks (or 11,730 flocks) in 2017 compared with 2.61% in 2016 (EFSA and ECDC, 2018).

Poor hygienic conditions are noticeable among the small-scale broiler farms with minimal infrastructure in Sri Lanka. In the present study 87/115 (75.6%) farms were small-scale. A study on 100 small-scale broiler farms across three districts in Sri Lanka reported 56% of the farms had clay floors as an inexpensive alternative for proper cemented floor (Rifky, 2016), where satisfactory cleaning and disinfection could not be accomplished between flocks. It is noteworthy that carryover infection in the same holding has been identified to increase the incidence of *Salmonella* prevalence in broilers (Snow *et al.*, 2008; Davies *et al.*, 2001). Rifky (2016) also stated that 52% of the farms had cadjan (mats woven from coconut palm leaves) as roofing material, thus contact with rodents that are known *Salmonella* carriers (Andrés-Barranco *et al.*, 2014) and/or their droppings was unavoidable. Nevertheless, *Salmonella* positivity rates among small (26/87, 29.9%), medium (6/21, 28.6%) and large-scale farms (5/7, 71.4%) in this study provides evidence of considerably higher prevalence in large-scale farms. A simple explanation for this is having higher number of susceptible birds housed together (Franz *et al.*, 2012) and sharing same litter, which could get contaminated with faecal shedding of *Salmonella* from infected birds.

Contrary to expectations, 66.7%, CI 95% [42.8-90.5] of associated hatcheries were *Salmonella* positive. Among those were one out of the two GP hatcheries sampled through investigating possible association to positive parent hatcheries. Since 2015, routine monitoring of all the hatcheries for *Salmonella* isolation has been carried out under the national *Salmonella* control programme, and none were reported to be positive (DAPH Annual report, 2016). On the other hand, earlier studies (Liyanagunawardena *et al*., 2012 and Liyanagunawardena *et al*., 2013) had reported *Salmonella* isolation from 2/12 (17%) and 5/20 (25%) conveniently sampled parent hatcheries respectively. Further, there has been no detectable *Salmonella* -carrier status between 2008 and 2016 in any of the GP farms through routine testing with whole blood agglutination testing (DAPH Annual report, 2016) while

similar findings were also reported from a study carried out on *Salmonella* carrier status of GP flocks (Liyanagunawardena *et al*., 2016a). *Salmonella* status of the hatcheries might be related to their poor biosecurity measures. All the hatcheries sampled had a DAPH biosecurity grading, and the *Salmonella* positive status of the hatcheries with DAPH gradings of A, B and C was 3/7 (43%), 3/4 (75%) and 4/4 (100%) respectively. There being a large proportion of Grade C hatcheries that were *Salmonella*-positive, suggests the need to improve biosecurity measures to a higher level. Further, after an initial contamination of hatcheries, possibly by eggs from *Salmonella*- carrier flocks, it is possible that these bacteria might continue to infect subsequent batches of eggs through environmental contamination (Sivaramalingam *et al.*, 2013). It is reported that some serovars, such as *Salmonella* Senftenberg, can survive for periods up to 30 months despite cleaning and disinfection (Pedersen *et al.*, 2008). Seventy percent of the positive hatcheries studied had more than one affected egg batch; five had two egg batches and two had all four egg batches positive. It was noted that some hatcheries had several egg batches set in one hatcher[28] (different hatcher trays with colour code for identification). Air movements (inside hatcher) during hatching can easily distribute bacteria from a few infected egg surfaces to contaminate all other eggs/ chicks throughout the hatcher (Kim and Kim, 2001). This could possibly be a reason for getting higher number of infected egg batches within a hatchery.

Significantly, all the isolates obtained in the present study were motile. This is in contradiction to common knowledge and earlier findings that poultry specific, non-motile *S*. Gallinarum and/ or *S*. Pullorum were predominant in poultry infection in Sri Lanka. Gunawardena *et al* (2006) and Priyantha *et al* (2007) reported 78% (78/100) and 87% (95/109), respectively, of *Salmonella* isolates from poultry post-mortem samples to be non-motile. Liyanagunawardena *et al* (2012) confirmed 28/56 (50%) and 23/56 (41%) of

[28]Hatcher: a cabinet used for egg incubation in the hatchery

suspected *Salmonella* outbreaks in commercial poultry were due to *S*. Gallinarum and *S*. Pullorum respectively, thus indicating a total of 91% (51/56) being poultry-adapted, non-motile *Salmonella*. However, an important difference is that, while previous studies were based on confirmed and/ or suspected salmonellosis outbreaks, the present study was a cross-sectional survey inapparently healthy broiler flocks.

Europe and North America had been successful in controlling both fowl typhoid and pullorum disease through 'test-and-cull' policies (Barrow *et al.*, 2012). Similarly, routine screening with whole blood agglutination test for detection and removal of *Salmonella* carrier status is being done extensively since last decade, in all breeder flocks in Sri Lanka. Consequently, the two GP farms (there were only two GP farms at that time) were reported to have *Salmonella* carrier status under 1%, affirming control of fowl typhoid and pullorum disease up to the level recommended by the DAPH (Liyanagunawardena, 2016a). Vaccination against *Salmonella* is another means of disease control, in which countries such as South Korea used SG 9R live vaccine to control fowl typhoid (Barrow *et al.*, 2012). Likewise, breeder flocks except GP in Sri Lanka were allowed to use SG 9R live *Salmonella* vaccine as an option in controlling disease (DAPH Annual report, 2012), so that a majority of parent farms had been carrying out vaccination from 2012 to 2015. Besides controlling fowl typhoid, SG 9R vaccine (with somatic antigens 1, 9 and 12 similar to that of other Salmonellae of group D) thus provides a cross protection against *S*. Pullorum and *S*. Enteritidis (Feberwee *et al.*, 2001). The acquisition of better control over non motile *Salmonella* through these interventions is another potential reason for obtaining only motile isolates in this study.

The pathogeneses of poultry-adapted (i.e., non-motile) and non-host-adapted (i.e., motile) serotypes are noteworthy to provide a reasonable explanation for obtaining only motile isolates in this study. Poultry-adapted serotypes characteristically cause severe systemic

‘‘typhoid-like’’ infections; fowl typhoid and pullorum disease, (Barrow, 2007) with involvement of the monocyte-macrophage series and rarely intestinal colonization (Van Immerseel *et al.*, 2005). This explains the difficulty of isolating host-adapted serovars from faeces (i.e., boot socks or cloacal swabs in this study) from sub clinically infected carrier birds, even if they were present at the time of sampling. The OIE Terrestrial Manual recommendation is to culture tissues from suspected birds instead where possible (OIE, 2018a). The non-host-adapted serovars (i.e. motile), conversely, can infect poultry but may appear largely asymptomatic (Van Immerseel *et al.*, 2005), although most are able to colonize the alimentary tract causing widespread contamination at slaughter and gain entry into the human food chain (Barrow, 2007). Therefore, it could be concluded that the apparently healthy flocks at the time of sampling had been harbouring motile *Salmonella* without producing any clinical illness.

There was a higher *Salmonella* recovery rate from boot socks (18.7%, CI 95% [14.4-23.0]) as compared to pooled cloacal swab samples (2.5%, CI 95% [1.36-3.54]). Boot socks are preferred for environmental sampling of litter, faeces and even dust in poultry houses, where floor housing is practiced. It is much more convenient than collecting litter/ faeces from a number of representative locations within a poultry pen and has increased sensitivity (Carrique-Mas and Davies, 2008). Isolation of *Salmonella* from a poultry pen using two pairs of boot socks (as one pooled sample), was reported to be at least as sensitive as collecting 60 faecal droppings to make one pooled sample (Gradel *et al.*, 2002). Accordingly, EU *Salmonella* baseline surveys of poultry had used a similar method of sampling using five pairs of boot socks, which were then analysed separately (EFSA, 2007). On the other hand, cloacal swabs are relatively insensitive in the flocks where prevalence is low and/ or faecal excretion of organisms is intermittent (Carrique-Mas and Davies, 2008). Environmental samples in a hatchery setting includes hatcher fluff, debris and macerated egg/chick waste

samples, and chick box liners (OIE, 2018b); out of which the present study sampled only fluff. Sampling hatcher fluff has been used by itself for hatchery monitoring (Sivaramalingam *et al.*, 2013), although eggshells and hatcher basket liners are considered more sensitive in *Salmonella* detection (Davies, 2005). All positive egg batches in this study had at least one positive sample of pooled fluff or eggshell, each revealed to be positive in equal numbers of egg batches, i.e., 13/21 (70%), representing their importance among other hatchery samples. Besides, fluff and eggshells each had 13.3%, CI 95% [7.89-18.77] higher *Salmonella* recovery rate compared to all other samples.

Limitations and recommendations

Limitations of this study included the cross-sectional study design; initially a simple random approach, then a district-wise proportionate sampling and finally a stratified approach within districts, which lacked calculating cluster sampling with a design effect.

Isolation of *Salmonella* can be highly dependent on the laboratory procedures adopted in a study. While recovering serovars such as *S*. Pullorum and *S*. Gallinarum in faeces is always difficult (Carrique-Mas and Davies, 2008), it should be noted that there is a discrepancy on the use of pre-enrichment stage in OIE terrestrial manual chapters: fowl typhoid and pullorum disease and salmonellosis. In the present study, following pre-enrichment, selective enrichment was carried out in RVS broth, one of the several media recommended (OIE, 2018b). However, it is preferable to avoid the pre-enrichment stage for isolating host-adapted serovars, as they are more likely to be overgrown by other organisms during pre-enrichment than Salmonellae that are not host-adapted. Thus, a direct enrichment is encouraged (OIE, 2018a). Furthermore, selenite-based broths are considered preferable for isolation of *S*. Pullorum and *S*. Gallinarum from faeces by direct enrichment (OIE, 2018a). There is further discrepancy in information about the incubation temperature of RVS broth. To achieve optimum *Salmonella* recovery, the manufacturer's (Oxoid, UK) recommendation

is to incubate RVS broth at 42 ± 1°C, according to which the study samples were incubated (at 42°C). Higher temperatures such as 41.5°C for RVS broth are recommended in order to provide *Salmonella* with an advantage over most competitive organisms (OIE, 2018a). However, there can be temperature-sensitive strains such as *S.* Dublin, with which the ideal would be to use of at least two enrichment broths, one incubated at 37°C and the other at a suitable higher temperature (OIE, 2018b). Nonetheless, promising applications for future work on *Salmonella* isolation from poultry - especially where host-adapted *Salmonella* is not yet controlled - would be to have three concurrent sets of samples; two direct enrichments in selenite-based broth and RVS broth (at 37°C) separately, and a pre-enrichment stage following subsequent selective enrichment in RVS broth at 42°C.

3.6 Conclusions

The study indicated a *Salmonella* prevalence of 32.2%, CI 95% [23.6-40.7] in the broiler farms across the five main poultry production districts of Sri Lanka. Poor hygienic conditions, especially owing to the infrastructure (such as clay floors and inexpensive roofing material), were observed in the small-scale broiler farms surveyed. Of the associated hatcheries, 66.7%, CI 95% [42.8-90.5] were revealed to be *Salmonella* positive. All the isolates obtained in the present study were motile *Salmonella*; a finding that is inconsistent with common knowledge and earlier findings. Boot socks could be considered ideal for environmental sampling with a *Salmonella r*ecovery rate of 18.7%, CI 95% [14.4-23.0] as compared to pooled cloacal swab samples (2.5%, CI 95% [1.36-3.54]).

Chapter 4: Estimation of risk factors for *Salmonella* carriage in poultry on Sri Lankan broiler farms

4.1 Abstract

The majority of human non-typhoidal *Salmonella* infections are believed to be transmitted via the food chain, thus interventions at the production level are needed to minimize risk factors for contamination. As chicken is the most popular meat among Sri Lankans, the objective of this study was to estimate putative risk factors for *Salmonella* infection in broiler farms.

A questionnaire and observation-based study was carried out from July to December 2017 in 115 broiler farms selected from the five districts with the highest poultry density in Sri Lanka. The questionnaire was verbally administered to farmers at their respective farm premises and the responses, as well as on-site observations, were manually recorded by the researcher. The information gathered comprise data on farm management, biosecurity, usage of vaccination and antibiotics, and recent disease history. These data were then matched with the laboratory results (the study in chapter 3) in the database to identify risk factors for *Salmonella* carriage using multivariate logistic regression modelling using two outcome variables. The initial model had binary outcome variable: '*Salmonella* status' at farm-level while subsequent model had 'proportion of samples' (proportion of *Salmonella* positive and negative samples) as the outcome variable with adjustments for sampling bias.

In the final model with a random effect for farm (mixed-effect sample-level model), litter management, rest period between flocks, feed storage, district and farmers' knowledge of sick birds were identified as risk factors. Specifically, raking litter, having a rest period of two weeks or more, proper feed storage, and being a farm located in Gampaha district were

associated with a reduced risk of *Salmonella* carriage. Contrary to expectation, lack of knowledge in managing sick birds was also associated with reduced risk for *Salmonella* carriage. In addition, through the observations made, better housing conditions are highly recommended for broiler farms of all scales while measures should be taken to implement basic biosecurity and hygienic measures.

4.2 Introduction

Salmonellosis continues to be one of the most important food-borne zoonotic diseases posing a potentially major public health threat (Hohmann, 2001; Antunes *et al.*, 2016). It was estimated that 95.1 million (95% CI 41.6–184.8) episodes of illness due to non-typhoidal *Salmonella* (NTS) had occurred in 2017, leading to 50,800 (95% CI 2820–130,000) deaths among humans worldwide (Stanaway *et al*. 2019). However, the lack of available information from some regions with a large proportion of the global population, such as parts of Africa, Asia, Latin America and the Caribbean, can have a significant impact on the accuracy of the global estimate (Majowicz *et al.*, 2010). In Sri Lanka, NTS is considered one of the main causes of foodborne illnesses (Ministry of Health, 2013), though the extent of its impact is not clearly understood. This could be partly due to the non-differentiating nature of surveillance on food and water borne diseases carried out in Sri Lanka (Ministry of Health, 2018) as well as the lack of published information. However, there is evidence of NTS occurrence in retail chicken (Kulasooriya *et al.*, 2019; Kalupahana *et al.*, 2017); while a recent study identified 40 NTS isolates from human clinical specimens in eight cities in the country (Tay *et al.*, 2019).

The majority of human NTS infections, particularly in the developed world, are believed to be transmitted via the food chain (Majowicz *et al.*, 2010). The gastrointestinal tracts of livestock are often the primary reservoirs of food-borne NTS (Crump *et al.*, 2015; Barrow *et al.*, 2012). In order to decrease NTS in humans, it is essential to have interventions at the

farm level to minimize risk factors for contamination from farm to fork. One such example is the European regulation in 2003 obliging member states to monitor and set control measures in place for *Salmonella* in primary production, following a high level of poultry meat contamination (Van Immerseel and Ducatelle, 2005). However, risk factor and other epidemiological studies are scarce in most developing countries, and the diversity of NTS circulating in livestock industries is poorly understood (Odoch *et al.*, 2018). In Sri Lanka, the poultry industry is the most established among all livestock industries (DAPH Livestock Statistics, 2015), with commercial poultry farms (both broiler and layer) alone accounting for 43% (195,462/453,886) of the total livestock enterprises in Sri Lanka (Dept of Census and Statistics, 2015). There is a massive representation of chicken meat (70%) in the meat industry (De Silva *et al*., 2010), while eggs are high in demand as the cheapest source of animal protein (DAPH Livestock Statistics, 2015). It follows that, poultry is very likely a source of NTS in the country.

Several studies carried out in Europe and USA have identified risk factors for *Salmonella* infection in poultry farms. The main risk factors identified include: large flock[29] sizes (Franz *et al.*, 2012; Snow *et al.*, 2008; Mollenhorst *et al.*, 2005), carry-over infection between flocks in the same holding (Snow *et al.*, 2008; Davies *et al.*, 2001), the housing system (Mollenhorst *et al.*, 2005), farms having flocks of different ages (Mollenhorst *et al.*, 2005) and the feed company (Franz *et al.*, 2012). However, these findings can vary from region to region. As reported in a study in Malaysia, *Salmonella* positivity among chickens could be strongly predicted by storage of feeds i.e., uncovered feed (Jajere *et al.*, 2019). *Salmonella* status of the previous flock and of day-old chicks appeared to be important risk factors for *Salmonella* infection in Senegalese broiler-chicken flocks (Cardinale *et al.*, 2004). A study in Nigeria

[29]flock: birds of same age in a farm. Often a flock is housed together, however it can differ depending on the number of birds and space availability.

identified previous contamination by *Salmonella*, the presence of rodents, movement from one pen to the other by farm-handlers, parking trucks near the entrance to poultry farms and the use of untreated water to be among the risk factors (Agada *et al.*, 2014).

There is no available information from Sri Lanka on risk factors associated with *Salmonella* carriage in poultry farms; though it is clear that identifying risk factors for *Salmonella* at farm level is crucial, given the diversity of poultry farms. That is, poultry farms in Sri Lanka vary in bird capacity[30] and operation types; buy-back practices[31], in terms of whether they are self-owned or company-owned; and housing types, from simple open-house[32] pens to closed-house[33]. In this context, a questionnaire and observation-based study was carried out on a sample of broiler farms selected from the five districts with the highest poultry density in Sri Lanka. The information thus gathered through this study was aimed at estimating the putative risk factors for *Salmonella* infection in broiler farms. To the researchers' knowledge, the present study is the first of its kind carried out in Sri Lanka.

4.3 Materials and methods

4.3.1 Ethics

Ethical approval for the present study was granted by the Committee for Ethical Clearance on Animal Research of the Faculty of Veterinary Medicine, University of Peradeniya, Sri Lanka as described in 3.3.1 in chapter 3.

[30] bird capacity: categorised as small-scale (up to 5000 birds), medium scale (5001-25,000 birds), large-scale (>25,000 birds).

[31] buy-back system: a unique feature in the broiler industry, where small-scale farmers are engaged in rearing for companies. Many leading broiler companies adopt this system, where they enter into a contract with small-scale farmers for rearing chickens.

[32] open-house: poultry pen with half walls of approximately 0.5 m height, with a wire mesh to complete the walls up to roof.

[33] closed-house: environmental controlled (optimal temperature, humidity etc) poultry house in large-scale farms, often company-owned. The flock capacity is 10,000 birds or upwards with automated facilities and managed by trained staff.

4.3.2 Study location and data collection from farms

The cross-sectional study was conducted in commercial broiler farms with a capacity greater than or equal to 1,000 chickens[34] (also known as farms with over 1000 chickens), located in the five districts with the highest poultry-density in Sri Lanka, namely, Kurunegala, Puttalam, Kegalle, Gampaha and Kandy. Selection of farms was based on random proportionate sampling within districts, as described in 3.3.3 of chapter 3. Sampling for *Salmonella* isolation and data collection was performed by visiting each farm once between July and December 2017.

Questionnaire development and delivery

A 20-item questionnaire was developed to gather information on possible associations between the presence of *Salmonella* in a broiler farm and putative risk factors. The questionnaire explored themes such as general farm management, biosecurity, usage of vaccination and antibiotics, recent disease history and experience level of the farmer/owner. The questionnaire and its translation (local language, Sinhala) can be found in Appendix IV. Pretesting of questionnaire was done on ten farms. It was carried out by face-to-face interviewing of the respective farmer/owner or a person representing them, by verbal administration in the local language, Sinhala, at the farm premises. The content and wording of the questionnaire were reviewed in relation to the responses received during the pre-test, and necessary modifications were done. The modified questionnaire was then administered, and the responses were manually recorded by the researcher in the same way as during the pretest. Verbal administration of the questionnaire was found effective as it enabled the researcher to give further explanation and clarification where necessary to ensure the respondents comprehension.

[34] classification of the commercial broiler farms by the department of Census and statistics in Sri Lanka; farms with greater than or equal to 1000 chicken (also called farms over 1000 chicken) and farms with fewer than 1000 chicken (also called farms below 1000 chicken).

Recording observations

Following verbal administration of the questionnaire, on-site observations on ten themes comprising involving general farm management, cleanliness; inside poultry pen, outside poultry pen, feed storage etc., biosecurity measures; availability of footbath, facility for hand washing, changing boots etc. were made by the researcher. These were separately noted at the end of the questionnaire.

4.3.3 Data management

Questionnaire and observation-based data as well as sampling and laboratory results were then entered and stored on a Microsoft SQL Database Cluster (Microsoft Corporation, Redmond WA, USA) as the back end, and Microsoft Access (Microsoft Corporation, Redmond WA, USA) as the front end.

4.3.4 Statistical analysis

The questionnaire and observation-based data were initially kept as two separate datasets and the binary outcome variable: *Salmonella* status[35] at the farm level (also mentioned in detail under 4.3.6) was used for the analysis. Two preliminary logistic regression (Hosmer *et al*., 2013) models were based on questionnaire and observation data separately, and these were combined into a final model (referred to as the final farm-level model). Subsequently, using a new outcome variable; proportion of samples[36] at the farm level (also mentioned in detail under 4.3.7), a second multivariable logistic regression model was built (referred to as sample-level model). Finally, a random effect for farm was included (referred to as mixed effect sample-level model). The flowchart in Figure 4.1 presents the sequence of statistical analyses performed.

[35] *Salmonella* status: The 'positive' status was given to farms from which at least one sample from a flock was confirmed positive for *Salmonella* while all others were considered 'negative'

[36] Proportion of samples: Proportion of *Salmonella* positive and negative samples was considered herewith as an adjustment for sampling bias at the farm level

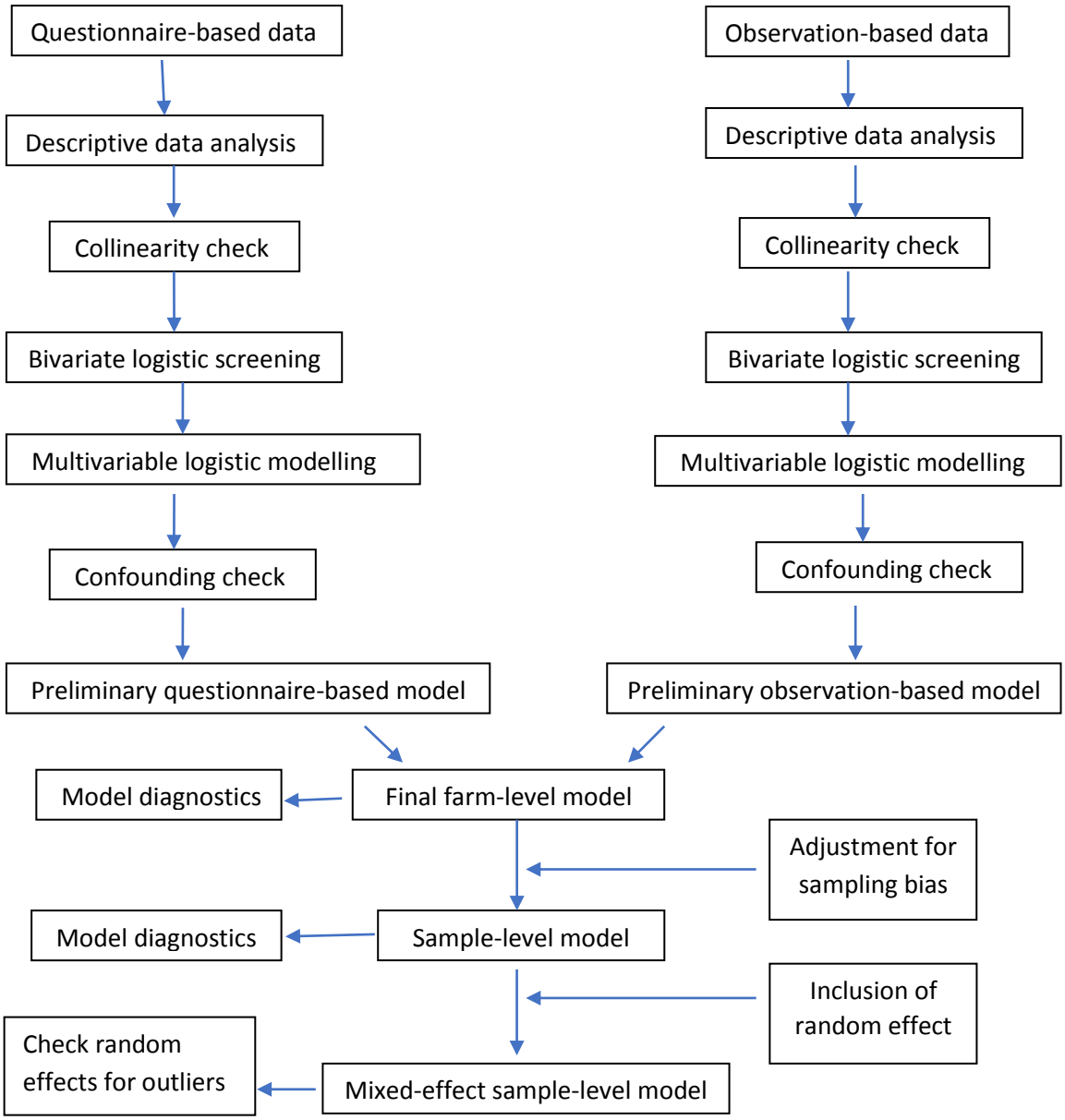


Figure 4.1: Sequence of statistical analyses performed in the questionnaire and observation-based data obtained from the cross-sectional study to identify risk factors for *Salmonella* carriage in broiler farms in Sri Lanka

Statistical analysis of data was performed in R-Studio (R version 3.6.3; https://www.r-project.org) and Microsoft Excel (Microsoft corporation, Redmond WA, USA). The R packages 'RODBC' (https://cran.r-project.org/web/packages/RODBC/index.html) and 'sqldf' (https://cran.r-project.org/ web/packages/sqldf/index.html) were used for accessing and analysis of data from the database. A number of packages available within the R-Studio

programme were used including 'epiR' (Stevenson *et al*, 2021), 'ggplot2' (Wickham, 2016), 'ResourceSelection' (Lele *et al.*, 2019), 'tidyr' (Wickham and Henry, 2021), 'foreign' (R Core Team, 2020), 'MASS' (Venables and Ripley, 2002), 'survey' (Lumley, 2020), 'faraway' (Faraway, 2016), 'lmtest' (Zeileis and Hothorn, 2002), 'ROCR' (Sing *et al*., 2005), 'lme4' (Bates *et al*., 2015) and 'lattice' (Sarkar, 2008).

4.3.5 Explanatory variables

All the explanatory (or the independent) variables in the datasets were factors; ordinal, categorical or binary. Each variable was manually checked and some were recoded to ensure levels had non-zero entries and/or to make categories that were biologically sensible. For example, some variables had nothing to do with the outcome variable. Also, the questions that didn't get any responses need to remain blank.

Questionnaire-based data:

The variables with more than two levels were district (in which the farm located), types of operation, bird capacity, number of flocks, bird strain, feed source, water source, litter disposal, carcass disposal, record keeping, farmer's experience on broiler farming (in years) and rest period[37] prior to new intake (in days). The following variables were recoded as binary variables: farm stage[38], litter management[39], availability of footbath, routine medication[40], prophylactic antibiotics to chicks, routine vaccination[41], farmer's knowledge in managing sick birds[42] (knowledge on sick birds), awareness of antibiotic withdrawal, high deaths in the available flocks[43], high deaths in the farm due to disease within last six

[37] rest period: lapse of time of having an empty poultry house after cleaning and disinfection, between two successive flocks on the same holding

[38] farm stage: single-stage/ one flock (i.e., all in all out) or multi-stage (more than one flock simultaneously)

[39] litter management: refers to litter raking with the optional use of disinfectants

[40] routine medication: refers to administration of probiotics, multivitamins, prophylactic antibiotics etc.

[41] routine vaccination: refers vaccines against viral diseases such as Infectious Bursal Disease and Newcastle Disease

[42] farmer's knowledge in managing sick birds: based on the responses for "What is the usual way of managing sick birds?" and then recoded as a binary variable

[43] high deaths in the available flocks: refers population wise mortality of 5% or more

months[44], training received by the farmer and assistance from veterinary or extension services.

Observation-based data:

All the variables were recoded as binary: location of the farm, restricted entry for visitors, type of housing, presence of other animals, functional footbath, changing of boots, hand washing facility, litter condition, rodent control, type of feeders, type of drinkers, cleanliness inside pen, cleanliness outside pen and feed storage.

4.3.6 Multivariable logistic regression at farm-level

Salmonella status as *outcome variable:*

The outcome (or the dependent) variable '*Salmonella* status at the farm level was coded as a binary variable: positive or negative, following laboratory test results matched by the farm identification number. The 'positive' status was given to farms from which at least one sample from a flock was confirmed positive for *Salmonella* while all others were considered 'negative'.

Descriptive data analyses

Descriptive data analyses were carried out for all the explanatory variables of interest. Variables were first described by one-way tables and subsequently two-way tables to determine the relationship between each explanatory variable and the outcome variable. The statistical significance of variables in two-way tables were then assessed by chi-squared (χ^2) test, p-values and odds ratios (OR).

Bivariate logistic screening

Bivariate associations between the outcome variable, i.e., *Salmonella* status in farms and the variables of interest were explored by logistic regression (Hosmer *et al*., 2013). All explanatory variables in the two datasets were analysed in this way and the variables where

[44] high deaths in the farm within last 6 months: refers population wise mortality of 5% or more

at least one factor (or level) in the variable had an associated p-value below or equal to 0.2 with the outcome variable, were selected for the next stage.

Collinearity check

Collinearity i.e., the presence of more than one variable that measures the same factor within the datasets, was also checked. Of the collinear variables, only one variable was selected based on the significance of the associated p value.

Confounder check

Variables that were not significant according to the pre-determined critical probability (i.e., ≤0.05) were excluded unless they were a confounder or the exposure of interest. Confounders were identified by adding variables stepwise into the regression model. Confounding was determined by a change of the coefficient of any other variable of 20% or more in the presence of the confounder and their association with both outcome and explanatory variables, as suggested by Dohoo et al (2003).

Multivariable logistic regression modelling

Multivariable models were built for the questionnaire and observation-based data separately. Variables selected by bivariate screening were initially added into the multivariable logistic regression model. The variables with a significant Wald test statistic (p value ≤0.05) for the variable as a whole and at least one factor with a significant p-value (i.e., p≤0.05) were retained in the model. The default baseline of some variables, such as bird capacity and rest period prior to new intake (in days), were changed in order to interpret the results in a more meaningful way.

In establishing a final logistic regression model, a stepwise backward elimination method was applied to confirm exclusion of variables from the model. Further consideration was given to identify and eliminate any collinearity and variables that reflect the possible consequences of outcome (i.e., could not be considered causally associated with the

outcome). Following the two logistic regression models that were based on questionnaire and observation data separately, a final model (i.e., final farm-level model) was built by combining them and considering a significance level of p≤0.05 as in the previous models. Additional measures, such as reducing the number of explanatory variables by removing those that essentially measured similar exposures (footbath), were taken to avoid over fitting of the model.

4.3.7 Multivariable logistic regression at sample-level

As a way of adjusting for sampling bias of the earlier model, a new outcome variable, proportion of *Salmonella* positive and negative samples at the farm level (i.e., modelling k successes in n Bernoulli trials where k is the number of samples positive and n is the number of samples taken), was used following laboratory test results matched by the farm identification number. The same explanatory variables used in the final farm-level model (mentioned in 4.3.6) were used here with the new outcome variable, sample proportion, and the final model (i.e., sample-level multivariable model) was established by considering confounding variables and associated p value significance level of p≤0.05.

4.3.8 Mixed-effect multivariable logistic regression

In addition to the fixed effects (i.e., explanatory variables) in the sample-level outcome multivariable model (mentioned in 4.3.7), a random effect for “farm” was added to create a mixed-effect model.

4.3.9 Model Diagnostics

Likelihood-ratio tests (LRT), Hosmer-Lemeshow goodness of fit (GOF) test (Hosmer *et al*., 2013), and the area-under the receiver operating characteristic (ROC) curve were used to evaluate the final model at the farm-level. Influential observations (Cook’s distance) and outliers (Pearson residuals) were assessed for the final farm-level model. Model evaluation for the sample-level model was done using LRT and Hosmer-Lemeshow GOF test, while

random effects of the mixed-effect model were checked for outliers. Additionally, p-values, residual deviance and Akaike information criterion (AIC) were also used in assisting the variable selection of the models.

4.4 Results

4.4.1 Data collection and management

Data were collected from 115 broiler farms with over 1000 chickens (the farms that consented, as mentioned in 3.4.1 in chapter 3), from the five selected districts: Kurunegala, Puttalam, Kegalle, Gampaha and Kandy. Following data entry, 17/115 (15%) randomly chosen questionnaires were subjected to quality checking, for confirmation of accuracy. There were seven errors including incorrect spellings, which were deemed acceptable after the corrections were made.

4.4.2 Explanatory variables

In the selection of variables, high deaths in the available flocks and high deaths in the farm due to disease within last six months were excluded, as these variables reflected the possible consequences of *Salmonella* infection rather than being a putative risk factor.

The number of farms recruited into the study was proportional to the total number of farms in each district: Kurunegala 40/115 (34.8%), Puttalam 21/115 (18.3%), Gampaha 20/115 (17.4%), Kegalle 20/115 (17.4%) and Kandy 14/115 (12.2%). All broiler strains available in Sri Lanka, namely, Cobb 500 (66/115, 57.4%), Indian River (28/115, 24.4%) and Hubbard (21/115, 18.3%), were found among the farms surveyed in the present study. Three types of operation: buy-back (64/115, 55.6%), self-owned (44/115, 38.3%) and company-owned (7/115, 6%), were identified among the farms recruited. In terms of bird capacity, the farms surveyed were predominantly small-scale (87/115, 75.6%) while medium and large-scale representation were 21/115 (18.5%) and 7/115 (6%) respectively. The number of flocks in the farms ranged from one to eight; and these were accordingly identified in the analysis as

farms with one flock (74/115, 64.3%), two (26/115, 22.6%) and three or more (15/115, 13%). Some of the descriptive statistics are as shown in the Table 4.1 and the details of explanatory variables (questionnaire and observation-based risk factors) are provided in the Appendix V.

Table 4.1: Descriptive statistics of the 115 broiler farms surveyed to estimate risk factors for *Salmonella* carriage in Sri Lanka

	Kurunegala	**Puttalam**	**Gampaha**	**Kegalle**	**Kandy**
Broiler strain					
Cobb 500 (66)	27	12	11	10	6
Hubbard (21)	7	6	1	1	6
Indian River (28)	6	3	8	9	2
Operation Type					
Buy-back (64)	14	10	11	18	11
Self-owned (44)	21	10	9	2	2
Company-owned (7)	5	1	0	0	1
Housing Type					
Open-house (108)	35	20	20	20	13
Closed-house (7)	5	1	0	0	1
Bird capacity					
Small-scale (87)	28	16	15	18	10
Medium-scale (21)	7	4	5	2	3
Large-scale (7)	5	1	0	0	1
Flocks Available					
One (74)	16	14	17	16	11
Two (26)	17	3	1	3	2
Three or more (15)	7	4	2	1	1
Flock size					
1,000- 1,500 (64)	22	12	11	13	6
1,501-3,000 (30)	10	4	6	5	5
3,001-10,000 (13)	3	4	3	2	1
>10,000 (8)	5	1	0	0	2
Total Farms	40	21	20	20	14

Bivariate analysis of risk factors for Salmonella positivity at the farm-level (N=115)

Results of bivariate screening revealed eight and six variables that had associated p value of ≤0.2 from questionnaire and observation-based data respectively and are presented in Tables 4.2 and 4.3.

Table 4.2: Descriptive statistics and bivariate analysis (p≤0.20) of the questionnaire-based risk factors associated with *Salmonella* status in broiler farms in Sri Lanka

	Descriptive Statistics	**Bivariate Analysis**			
Variable	***Salmonella* positive status n/N (%)**	**β-Coefficient**	**Crude OR**	**95% CI**	**P**
District					
Gampaha	3/20 (15.0)	Baseline			
Kandy	5/14 (35.7)	1.15	3.15	0.6-16.3	0.17
Kegalle	7/20 (35.0)	1.12	3.05	0.7-14.1	0.15
Kurunegala	15/40 (37.5)	1.22	3.40	0.9 - 13.6	0.08
Puttalam	7/21 (33.3)	1.04	2.83	0.6 - 13.0	0.18
Bird capacity					
Small-Scale	26/87 (29.9)	Baseline			
Medium-Scale	6/21 (28.6)	-0.06	0.94	0.3 - 2.7	0.9
Large-Scale	5/7 (71.4)	1.77	5.86	1.1 - 32.2	0.04
Bird strain					
Cobb500	26/66 (39.4)	Baseline			
Hubbard	5/21 (23.8)	-0.73	0.48	0.2 - 1.5	0.2
Indian River	6/28 (21.4)	-0.87	0.42	0.2 - 1.2	0.1
Litter management					
Yes	8/45 (17.8)	Baseline			
No	29/70 (41.4)	1.19	3.27	1.3 - 8.1	0.01
Rest period					
Days 14 or more	3/40 (7.5)	Baseline			
Days 10	8/30 (26.7)	1.50	4.48	1.1-18.7	0.04
Days 7	26/45 (57.8)	2.82	16.87	4.5-62.9	<0.01
Knowledge on sick birds					
No	18/73 (24.7)	Baseline			
Yes	19/42 (45.2)	0.93	2.52	1.1 - 5.7	0.02

Number of observations=115, OR = Odds Ratio, CI= Confidence Interval, P= P- value

Table 4.3: Descriptive statistics and bivariate logistic analysis (p≤0.20) of the observation-based risk factors associated with *Salmonella* status in broiler farms in Sri Lanka

	Descriptive Statistics	Bivariate Analysis			
Variable	***Salmonella* positive status n/N (%)**	**β-Coefficient**	**Crude OR**	**95% CI**	**P**
Litter condition					
Dry	18/71 (25.4)	Baseline			
Wet	19/44 (43.2)	0.81	2.24	1.0 - 5.0	0.05
Functional footbath					
No	29/101 (28.7)	Baseline			
Yes	8/14 (57.1)	1.2	3.31	1.1 - 10.4	0.04
Type of drinker					
Manual	17/65 (26.2)	Baseline			
Automatic	20/50 (40)	0.63	1.88	0.9 - 4.2	0.12
Cleanliness inside pen					
Yes	23/91 (25.3)	Baseline			
No	14/24 (58.3)	1.42	4.14	1.6 - 10.6	<0.01
Cleanliness outside pen					
Yes	18/70 (25.7)	Baseline			
No	19/45 (42.2)	0.75	2.11	1.0 - 4.7	0.07
Feed storage					
Yes	14/64 (21.9)	Baseline			
No	23/51 (45.1)	1.07	2.93	1.3 - 6.6	<0.01

Number of observations=115, OR = Odds Ratio, CI= Confidence Interval, P= P- value

Collinearity of variables

Bird capacity, flock size and number of flocks were identified as collinear variables in the questionnaire-based data as all three variables provided indices on the capacity of the farm. Bird capacity was selected for multivariable analyses based on this variable having the lowest p value (p≤0.20) on bivariate screening. Among the observation-based risk factors, restricted entry for visitors, functional footbath and changing of boots were identified as collinear, all of which reflected the availability of biosecurity resources/ infrastructure. Only the functional footbath variable was selected for the multivariable model based on this variable having the lowest p value on bivariate screening.

Similarly, cleanliness outside poultry house and feed storage were collinear, and only one i.e., feed storage was selected based on the bivariate p value. Collinearity was also identified when the two initial models were combined to make one final model. Litter management (questionnaire) and litter condition (observation) measured the same factor based on data collection procedures. Furthermore, bird capacity from the questionnaire based model and functional footbath from the observation-based model reflected the capacity or the availability of resources in the farm. In the final selection, litter management and bird capacity were selected as more appropriate, and thus included in the final combined model.

4.4.3 Multivariable logistic regression of risk factors for *Salmonella* positivity at the farm-level (N=115)

Preliminary questionnaire-based model

Of the six variables selected through bivariate analysis, five remained in the preliminary adjusted questionnaire-based model and had statistically significant Wald test statistic ($p \leq 0.05$) or were confounding variables. They were district, bird capacity, litter management, rest period and knowledge on sick birds. OR gained by exponentiation of the β-coefficients obtained from the multivariable logistic regression model are presented in Table 4.4.

Table 4.4: Multivariable preliminary questionnaire-based model of risk factors associated with *Salmonella* status in broiler farms in Sri Lanka

Variable	β-Coefficient	SE	OR (95% CI)	P
District				0.08 #
Gampaha	Baseline			
Kandy	3.25	1.26	25.90 (2.2-307.2)	<0.01
Kegalle	1.74	0.94	5.69 (0.9- 35.8)	0.06
Kurunegala	2.04	0.90	7.65 (1.3- 44.6)	0.02
Puttalam	1.24	0.92	3.46 (0.6-20.8)	0.18
Bird capacity				0.08 #
Small-Scale	Baseline			
Medium-Scale	0.56	0.86	1.75 (0.3-9.5)	0.51
Large-Scale	3.61	1.60	36.84 (1.6-852.3)	0.02
Litter management				
Yes	Baseline			
No	3.33	1.15	28.04 (2.9-268.5)	<0.01
Rest period				<0.01 #
Days 14 or more	Baseline			
Days 10	2.30	0.89	10.02 (1.7-57.8)	<0.01
Days 7	3.45	0.85	31.43 (6.0- 165.5)	<0.01
Knowledge on sick birds				
No	Baseline			
Yes	1.36	0.61	3.88 (1.2-13.0)	0.03

SE= Standard Error, OR = Odds Ratio, CI= Confidence Interval, P= P- value
# Wald test statistic for variable

Preliminary observation-based model

Of the six variables selected through bivariate analysis, five remained in the preliminary adjusted observation-based model. The variables comprised litter condition, functional footbath, type of drinker, cleanliness inside pen and feed storage, all of which had statistically significant associated p values ($p \leq 0.05$) and were confounding variables except litter condition. The β-coefficients and OR obtained are shown in Table 4.5.

Table 4.5: Multivariable preliminary observation-based model of risk factors associated with *Salmonella* status in broiler farms in Sri Lanka

Variable		β-Coefficient	SE	OR (95% CI)	P
Litter condition					
	Dry	Baseline			
	Wet	0.76	0.47	2.13 (0.9-5.4)	0.11
Functional footbath					
	No	Baseline			
	Yes	1.4	0.73	4.08 (1.0-17.0)	0.05
Type of drinker					
	Manual	Baseline			
	Automatic	0.96	0.54	2.62 (0.9-7.6)	0.08
Cleanliness inside pen					
	Yes	Baseline			
	No	1.7	0.55	5.47 (1.9-16.2)	<0.01
Feed storage					
	Yes	Baseline			
	No	1.43	0.49	4.17 (1.6-11.0)	<0.01

SE= Standard Error, OR = Odds Ratio, CI= Confidence Interval, P= P- value

Final model of risk factors for Salmonella positivity at the farm-level (N=115)

Collinearity was excluded in combining two preliminary models, thus the resulting final model had seven variables. The variables were district, bird capacity, litter management, rest period, knowledge on sick birds, cleanliness inside pen and feed storage, all of which were found to have one or more levels with statistically significant associated p values ($p \leq 0.05$) and were confounding variables. The β-coefficients and OR obtained are shown in Table 4.6.

Table 4.6: Multivariable farm-level model of risk factors associated with *Salmonella* status in broiler farms in Sri Lanka

Variable	β-Coefficient	SE	OR (95% CI)	P
District				0.03 #
Gampaha	Baseline			
Kandy	5.36	1.63	212.6 (8.7-5170.8)	<0.01
Kegalle	2.24	1.24	9.4 (0.8-107.0)	0.07
Kurunegala	3.01	1.21	20.3 (1.9- 218.7)	0.01
Puttalam	1.99	1.23	7.3 (0.7-80.4)	0.11
Bird capacity				0.04 #
Small-Scale	Baseline			
Medium-Scale	0.68	1.19	2.0 (0.2-20.2)	0.56
Large-Scale	4.63	1.85	103.0 (2.7-3894.3)	0.01
Litter management				
Yes	Baseline			
No	3.16	1.33	23.6 (1.7-317.7)	0.02
Rest period				<0.01 #
Days 14 or more	Baseline			
Days 10	2.72	1.08	15.2 (1.8-125.9)	0.01
Days 7	3.78	1.00	43.9 (6.2-309.6)	<0.01
Knowledge on sick birds				
No	Baseline			
Yes	2.37	0.80	10.7 (2.2-51.5)	<0.01
Cleanliness inside pen				
Yes	Baseline			
No	2.52	0.89	12.4 (2.2-70.8)	<0.01
Feed storage				
Yes	Baseline			
No	2.46	0.83	11.7 (2.3-59.2)	<0.01

SE= Standard Error, OR = Odds Ratio, CI= Confidence Interval, P= P- value
# Wald test statistic for variable

The final model at the farm-level fitted adequately with the data as identified by the model diagnostics: LRT (p-value <0.001), Hosmer-Lemeshow GOF test (p-value <0.001) and area under ROC curve (0.94). Performance of the final model was tested with and without outliers and influential observations, after which those were retained in the model. Plots of area under ROC curve, outliers and influential observations can be found in Appendix V.

4.4.4 Multivariable logistic regression analysis of risk factors for *Salmonella* positivity at the sample- level (N=1,121)

Farms varied in their bird capacity and/ or available flocks, hence in some instances, more than one flock per farm was sampled. To adjust for this sampling bias, the number of positive and negative samples from each farm was considered as the outcome in this analysis. A summary of *Salmonella* positivity at farm and sample-level is presented in Table 4.7.

Table 4.7: A district wise summary of *Salmonella* positivity at farm-level and sample-level in the 115 broiler farms sampled in Sri Lanka

	***Salmonella* positivity**			
	Farm-level		**Sample-level**	
District	**n/N (%)**	**CI 95%**	**n/N (%)**	**CI 95%**
Kurunegala	15/40 (37.5)	22.5 -52.5	33/400 (8.3)	5.6- 10.9
Puttalam	7/21 (33.3)	9.3-47.9	11/210 (5.2)	2.2- 8.3
Gampaha	3/20 (15.0)	0.00-30.6	6/196 (3.1)	0.6- 5.5
Kegalle	7/20 (35.0)	18.5-61.5	13/161 (8.1)	3.9- 12.3
Kandy	5/14 (35.7)	10.6-60.8	15/154 (9.7)	5.1- 14.4

The resulting sample-level model had five variables; district, litter management, rest period. knowledge on sick birds, and feed storage. Bivariate logistic analysis ($p<0.20$) of the variables and the β-coefficients and OR obtained through the multivariable logistic regression are shown in Table 4.8. The model fitted adequately with the data as described by the model diagnostics; LRT (p-value <0.001) and Hosmer-Lemeshow GOF test (p-value <0.001).

Table 4.8: Bivariate logistic analysis (p<0.20) of the variables and multivariable sample-level model of risk factors associated with proportion of positive and negative samples in broiler farms in Sri Lanka

	Bivariate Analysis			Multivariable Analysis			
Variable	**β-Coefficient**	**Crude OR (95% CI)**	**P**	**β-Coefficient**	**SE**	**OR (95% CI)**	**P**
District							<0.01[#]
Gampaha	Baseline						
Kandy	1.14	3.1 (1.2-8.3)	0.02	2.05	0.55	7.8 (2.6-23.2)	<0.01
Kegalle	0.99	2.7 (1.0-7.2)	0.05	1.70	0.55	5.5 (1.9-15.9)	<0.01
Kurunegala	1.01	2.7 (1.1-6.7)	0.03	1.30	0.47	3.7 (1.5-9.2)	<0.01
Puttalam	0.52	1.7 (0.6- 4.7)	0.31	0.45	0.53	1.6 (0.6-4.5)	0.39
Litter management							
Yes	Baseline						
No	0.58	1.8 (1.1-2.9)	0.02	0.54	0.32	1.7 (0.9-3.2)	0.09
Rest period							<0.01[#]
Days 14 or more	Baseline						
Days 10	1.16	3.2 (1.1-9.0)	0.03	0.88	0.54	2.4 (0.8-7.0)	0.10
Days 7	2.11	8.3 (3.3-20.8)	<0.01	2.06	0.48	7.9 (3.1-20.2)	<0.01
Knowledge on sick birds							
No	Baseline						
Yes	0.85	2.3 (1.5-3.8)	<0.01	0.97	0.28	2.6 (1.5-4.6)	<0.01
Feed storage							
Yes	Baseline						
No	0.48	1.6 (1.0-2.6)	<0.01	0.88	0.32	2.4 (1.3-4.5)	<0.01

SE= Standard Error, OR = Odds Ratio, CI= Confidence Interval, P= P- value
[#] Wald test statistic for variable

4.4.5 Mixed-effect multivariable logistic regression anaylsis of risk factors for *Salmonella* positivity at the sample-level (N=1,121)

In the mixed-effects model, unmeasured variation at the farm level was included as a random effect in addition to the known fixed effects. District, litter management, rest period, knowledge on sick birds and feed storage were all identified as significant risk factors for *Salmonella* carriage similar to that of the previous model (i.e., sample-level multivariable model in 4.4.4). The resulted β-coefficients, OR and random effect are presented in Table 4.9. Random effects were checked for outliers and the distribution was approximately normal and can be found in Appendix V.

Table 4.9: Multivariable mixed-effect sample-level model of risk factors associated with proportion of positive and negative samples in broiler farms in Sri Lanka

	Multivariable Analysis			
Variable	**β-Coefficient**	**SE**	**OR (95% CI)**	**P**
District				
Gampaha	Baseline			
Kandy	2.23	0.67	9.3 (2.5-34.6)	<0.01
Kegalle	1.70	0.61	5.5 (1.7-18.3)	<0.01
Kurunegala	1.40	0.55	4.1 (1.4-12.0)	0.01
Puttalam	0.50	0.60	1.7 (0.5-5.4)	0.40
Litter management				
Yes	Baseline			
No	0.76	0.41	2.1 (1.0-4.8)	0.06
Rest period				
Days 14 or more	Baseline			
Days 10	0.79	0.58	2.2 (0.7-6.9)	0.18
Days 7	2.08	0.51	8.0 (3.0-21.7)	<0.01
Knowledge on sick birds				
No	Baseline			
Yes	0.94	0.32	2.6 (1.4-4.8)	<0.01
Feed storage				
Yes	Baseline			
No	0.97	0.38	2.6 (1.3-5.5)	0.01

SE= Standard Error, OR= Odds Ratio, P= P value, CI= Confidence Interval
Random effect: Farm (Variance = 0.27, Standard deviation=0.52)

4.5 Discussion

The present study is the first attempt to study putative risk factors for *Salmonella* carriage in Sri Lankan poultry. The study was carried out in the five highest poultry-dense districts which comprised 1269/1847 (68.7%) of the farms with over 1000 chickens in Sri Lanka in 2015 (Dept of Census and Statistics, 2015). The survey covered 115/1269 (9%) of broiler farms in these districts and the risk factors were identified by logistic regression modelling using two outcome variables. In the final model (i.e., mixed-effect sample-level model), litter management, rest period, feed storage, district and farmers' knowledge on sick birds were identified as risk factors.

Litter management was found to be an important factor in the final models. All the farms surveyed were managed under the deep-litter management system. The litter type was observed as paddy husk (also known as rice hull), wood shavings or both and was replaced between successive flocks as reuse of litter is not practised in Sri Lanka. Among the other important functions of the litter is moisture absorption to provide a dry bedding for the birds throughout their grow-out period (Grimes *et al.*, 2002). Litter raking is the most common litter management practice in Sri Lanka, which facilitates moist litter drying thus preventing litter caking (i.e., formation of a thick wet layer of litter due to high moisture content and compression). Therefore, litter management in this study referred to litter raking, performed regularly (daily) or irregularly, with the optional use of disinfectants. Results revealed that farms where litter was raked had lower risk for *Salmonella* carriage in comparison to those where it was not performed. This could be explained through the concept of litter moisture content, which is better measured as water activity of the litter i.e., mass of water divided by mass of moist litter, expressed as a percentage (van der Hoeven-Hangoor *et al.*, 2014). Previous studies examining the water activity of litter have shown that lower activity has been demonstrated to correspond to lower growth of pathogens including *Salmonella* (Payne *et al.*, 2007). Thus, the association between litter raking in the present study and a lower risk of *Salmonella* may be explained by an effect of litter raking on reducing water activity and lower survival of *Salmonella* in the poultry-shed environment.

The rest period was another risk factor identified as significantly associated with *Salmonella* carriage in the present study. The rest period in this regard is the period of time between two successive flocks in the same holding, when the poultry house is empty after depopulation, litter removal, cleaning and disinfection. For single-stage (all in all out) farms it is a whole farm approach, while in multi-stage farms this is carried out poultry house-wise.

All the farms surveyed responded affirmatively to having a rest period prior to a new intake, however the duration of this rest period differed between farms. Results of the final logistic regression revealed the odds of a sample being positive from a farm where the rest period was seven days, was eight times (adj. OR 8.0, [95% CI 3.0-21.7]) the odds when the rest period was 14 days or more (baseline). A clean downtime (equivalent to rest period) of ideally two weeks between flocks is reported necessary to reduce bacterial and viral loads in the poultry house (Nespeca *et al.*, 1997) although no significant associations were identified between the length of downtimes and *Salmonella* carriage in poultry (Volkova *et al.*, 2011; Skov *et al.*, 1999). However, studies on *Campylobacter* carry over infections have provided evidence of the length of a downtime of fewer than 14 days being a significant risk factor (Hald *et al.*, 2000; Chowdhury *et al.*, 2012). There is no suggested rest period in Sri Lanka, yet two or three weeks are needed between batches (i.e., turnaround time[45]) to allow the premises to be cleared and disinfected (Bandara and Dassanayake, 2006).

Feed storage was another factor identified from the study; satisfactory storage was associated with a reduced likelihood of a sample being positive for *Salmonella*. Feed storage in the present study was determined by observations made by the interviewer. It was observed whether a farm had a separate area inside or out of the poultry house with adequate shelter and rodent-proof roof/walls, where feed bags could be stored on a shelf/ stage with no direct floor contact to avoid dampness. Farms with unsatisfactory storage and *Salmonella* carriage were more than twice as likely to be positive for *Salmonella* (adj. OR 2.6, [95% CI 1.3-5.5], baseline satisfactory storage) in the present study. However, a study in Malaysia reported that village chicken farms with uncovered feed storage were ten times more likely to test positive for *Salmonella* as compared to their counterparts with covered storage (Jajere *et al.*, 2019). It is noteworthy that rodents and wild birds are known as

[45]turnaround time: total time (including cleaning, disinfection and rest period) between two successive flocks on the same holding

Salmonella carriers (Andrés-Barranco *et al.*, 2014), thus uncovered feed is likely to get contaminated through them and/or their droppings.

The district where the farm was located was significantly associated with the outcome. In comparison with a farm in Gampaha district (baseline), samples taken from farms in any other district were associated with increased odds of being *Salmonella* positive. Particularly, the odds in Kandy were nine times (adj. OR 9.3, [95% CI 2.5-34.6]) the odds in Gampaha. The reasons for this are not clear, however it could be attributed to farm management practices, biosecurity measures, farmer awareness and the extension service offered by the relevant veterinary/ field officers/ buy-back companies. Further, Kandy has much cooler climate with frequent heavy rains compared to other districts including Gampaha.

Farmer's knowledge in managing sick birds (binary: yes or no) was also significantly associated with the *Salmonella* status in the final models. Contrary to expectation, lack of knowledge in managing sick birds was associated with reduced risk for *Salmonella* carriage in the present study. While this cannot be fully explained, it is arguable that farmers who were confident of their knowledge in this regard had considerable experience in managing birds with infections including *Salmonella* or were potentially less concerned about farm hygiene and biosecurity. Yet, among those who responded affirmatively to the question were 6/7 (86%) large-scale farms with all the general biosecurity measures such as restricted entrance, functioning water-bath, change of footwear and hand washing facilities, which were not available at the small or medium-scale holdings.

It should be noted that the same variables discussed above were included in the farm-level model along with two other variables; bird capacity (three levels: small, medium or large-scale) and cleanliness inside the pen (binary: yes or no). In the farm-level model, large-scale farms were found to be at a higher risk for *Salmonella* infection than the other two farm

categories. The reason for this in the present study is likely to be sampling bias, i.e., more samples were taken from larger farms, however earlier studies have identified large flock sizes (Franz *et al.*, 2012; Snow *et al.*, 2008; Mollenhorst *et al.*, 2005) as a main risk factor for *Salmonella* carriage in poultry. The flock size has been reported as a risk factor for *Salmonella* prevalence in broilers, the risk increasing with the number of birds per flock (Namata *et al.*, 2009; Snow *et al.*, 2008). This could be due to higher number of susceptible birds being housed together (Franz *et al.*, 2012) and the potential of *Salmonella* transmission through faecal shedding from infected birds that share the same litter. Cleanliness inside the pen in the present study was based on observations of the interviewer at the time of the visit, which suggested regular cleaning. Where there was no observable evidence of gross faecal contamination on equipment, feeders and drinkers, the pen was noted as clean. Accordingly, in the adjusted farm-level model, farms that were 'not clean' were found to be at a higher risk for *Salmonella* infection opposed to 'clean' (baseline), nevertheless there is no published evidence in this regard.

Salmonella control in a broiler farm environment is complicated, due to multiple potential contamination sources; chicks, feed, rodents, wild birds, insects, litter and the farm environment (Bailey *et al.*, 2001). Many factors related to chickens such as the age of the bird, health status, genetic susceptibility and stress; overcrowding or environmental conditions (temperature and humidity) can trigger their susceptibility to *Salmonella* colonization (Bailey, 1988). *Salmonella* control within the pre-harvest phase (i.e., breeding farms and broiler farms) involve reducing the number of *Salmonella* positive flocks, *Salmonella* positive birds within a flock, and colonization levels in the bird's gut or other tissues. Thus, it is important that general hygienic and biosecurity measures are put in place for the overall farm management, which include, but are not limited to, introducing *Salmonella*-free chicks, proper cleaning and disinfection, insect and rodent control,

decontamination of feed and water (Van Immerseel *et al.*, 2009). Within the pre-harvest phase, cleaning and disinfection in particular have been shown to significantly reduce contamination of *Salmonella* (Garber *et al.*, 2003) however the efficacy is often variable and depends on the accuracy of the cleaning procedure and potential effectiveness of disinfectants (Breslin, 2003).

The present study used mixed-effects logistic regression, one of the most widespread epidemiological methods in risk factor analysis. However, this is a classical statistical approach that considers only the association of variables with one specific outcome of interest, thus it could potentially limit the understanding of the complex relationships between risk factors. Studies on infections such as feline calicivirus (Kratzer *et al.*, 2020) and bovine viral diarrhoea virus (Han *et al.*, 2018) have successfully studied disease epidemiology using a combined approach of logistic regression and Bayesian network modelling (i.e., multivariate network modelling approach). Therefore, to reveal complex relationships including associations between variables of interest (i.e., broiler farm management practises) as well as with the outcome and causal pathway effects, Bayesian network modelling could be highly beneficial. Promising adaptation for future work would comprise considering the infrastructure of the poultry pen in particular when there is a high diversity among recruited farms. A number of small-scale farms surveyed had clay floors and cadjan (mats woven from coconut palm leaves) or thin plastic (polythene) as roofing material. The bird density i.e., calculated as the number of broilers per square meter (m^2), would be another important factor for consideration. Overcrowding stress is an imperative stressful conditions in poultry production (Gomes *et al.*, 2014), that can affect the immune system in broilers, thereby decreasing the ability to overcome potential bacterial infections (Humphrey, 2006).

Recommendations for broiler farms in Sri Lanka

Poor hygienic conditions are unavoidable in small-scale broiler farms with minimal infrastructure. In addition, proper cleaning and disinfection prior to new intake is not possible in the farms where the floor is of clay and/ or with crevices on cement floor. For example, a few farms that had clay floors reported a different cleaning protocol, where they use hot charcoal to heat/burn the pen floor instead of power washing and spraying of disinfectants after litter removal, followed by application of a new layer of clay. While the effectiveness of this procedure is arguable, a durable floor is one of the main requirements in deep litter system (Rifky, 2016). Further, quite a few earlier studies have demonstrated cleaning or cleaning and disinfection of farms/pens before a new intake to be a significant factor in reducing the risk of *Salmonella* carryover infection (Davies and Breslin, 2003; Davies *et al.*, 2001; Van Hoorebeke *et al.*, 2010; Wales *et al.*, 2006). Thus, better housing conditions are highly recommended for broiler farms of all scales. Further, measures should be taken to implement basic biosecurity and hygienic measures such as hand washing and change of footwear between flocks within the broiler farm to minimise possible *Salmonella* transmission.

Increased moisture in litter favours bacterial growth. Litter raking in this study was considered a means of reducing litter moisture. However, other factors such as ventilation, drinker design and litter type may also affect litter moisture (Shepherd and Fairchild, 2010; Cengiz *et al.*, 2017). Better housing conditions (ensuring adequate ventilation and sunlight) and timely replacement of leaky drinkers are two convenient management practices.

While a rest period of at least two weeks is recommended before a new intake, Bandara and Dassanayake (2006) point out that most small-scale broiler farmers in Sri Lanka tend to rear several batches with one-week gap or turnaround time between batches during seasons of high demand, such as the Sri Lankan New Year in April and Christmas in December. In contrast, the minimum rest period in the present study was seven days, which could mean

nearly two weeks of turnaround time. However, the potential negative impact of this practice needs to be addressed by raising farmers' awareness, as it could impede the effectiveness of cleaning, thereby enhancing the risk of carryover infection.

Relevant awareness of proper feed storage is required among farmers, not only to avoid risk of *Salmonella* infection, but also to prevent feed wastage due to fungi/ mould formation. In several small-scale farms, feed bags were left open rendering them potentially accessible to chickens, rodents or their droppings, while in some others unopened feed bags could be seen stacked inside the poultry pen where chickens pecked and scratched them. A satisfactory feed store could be made with the available resources by partitioning a corner within the poultry pen in small-scale farms which have no room for separate storage. Further, it is recommended to store feed on a stand/shelf (without direct contact with floor) to prevent their becoming damp especially during the rainy season.

4.6 Conclusions

The risk factors for *Salmonella* carriage in broiler farms were analysed by logistic regression modelling at different stages. In the final model, factors that associated with reduced risk for *Salmonella* carriage were litter management practices, rest period of two weeks or more, proper feed storage and being a farm located in Gampaha district. Further, lack of farmers' knowledge in managing sick birds was identified to have increased risk for *Salmonella* carriage, which was contrary to the expectation.

Chapter 5: Antibiotic usage in broiler production in Sri Lanka

5.1 Abstract

The use of antimicrobials is a common practice globally in intensively managed poultry farms. However, there is sparse data available on antibiotic usage in Sri Lanka. The present study was carried out to develop better understanding of the use of antibiotics and foster awareness on antibiotic withdrawal in broiler farms.

The present study formed one part of the questionnaire and observation-based study mentioned in chapter 4. Of the questions, those addressing the themes of general farm management, routine medication, antibiotic usage practices and awareness on antibiotic withdrawal were included for the purpose of the present study. A total of 115 broiler farms from the five districts with the highest poultry density in Sri Lanka participated in the study. Use of prophylactic antibiotics (i.e., without considering in-feed antibiotics) in the participating farms was 49/115 (42.6%, CI 95% [33.5-51.6]) while it was 11.3%, CI 95% [5.5-17] for therapeutic antibiotics. Commonly administered antibiotics in the participating farms were enrofloxacin, amoxycillin and sulpha-trimethoprim. The highest prophylactic antibiotics use was identified among small-scale farms (~45%). Further, it was revealed that some farmers (16) were unable to differentiate prophylactic antibiotics from other routinely used medicine such as probiotics and multivitamins. In agreement with the earlier studies, all the participants who responded (37) to the question on the flock age and the duration of prophylactic antibiotic use stated that they administered antibiotics for a duration of three to five days from day one of their broiler flock. Of the participants, 58.2%, CI 95% [49.2-67.3] were aware of antibiotic withdrawal periods and 85%, CI 95% [76.5-93.6] of them mentioned they were practising antibiotic withdrawal in their farms. Raising awareness of antibiotics among the wider broiler farming community, including resistance development, usage in

poultry and withdrawal periods, is highly necessary in order to reduce antibiotic usage at the farm level.

5.2 Introduction

The increased use of antimicrobials in food animals is identified as a consequence of the global dietary trend for high-protein diets and the subsequent expansion of intensive meat production (Van Boeckel *et al.*, 2019). Thus, the estimated global consumption of all antimicrobials in animals raised for food was 131,109 metric tonnes [CI 95% 100,812-190,492] in 2013 and is expected to reach 200,235 metric tonnes [CI 95% 150,848-297,034] by 2030 (Van Boeckel *et al.*, 2017). In Sri Lanka, the poultry industry has grown exponentially over the past few decades (DAPH Poultry forecast, 2017) and is well established throughout the country. Similar to elsewhere, the use of antimicrobials is considered a common practice in intensively managed poultry farms in Sri Lanka (FAO, 2013; Liyanage and Pathmalal, 2017).

Unlike in humans, the purpose of antimicrobial use in food animal production is not limited to therapy i.e., to treat sick individuals. Antimicrobials are widely applied in food animals for the following reasons: metaphylaxis, to treat sick animals while also medicating others in the herd/flock who are not clinically sick but to prevent infection spread; prophylaxis, to prevent infections at high-risk periods such as transport or weaning; and growth promotion, to enhance feed efficiency for high production (McEwen and Fedorka-Cray, 2002; Viola and DeVincent, 2006). In intensive chicken farming, antimicrobials are used to prevent or control several common infectious diseases. For example, broiler rations may contain a coccidiostat, i.e., antimicrobials such as ionophores and sulfonamides, to prevent coccidiosis, a common parasitic problem. Other antimicrobials such as bacitracin, chlortetracycline, virginiamycin and arsenical compounds maybe used as in-feed growth promotors (McEwen and Fedorka-Cray, 2002) in relatively low concentrations (known as a subtherapeutic or nontherapeutic

dose) for longer periods. Thus growth promoters have been the most controversial of all types of antimicrobial applications in livestock, largely attributed to selection for antimicrobial resistance (Viola and DeVincent, 2006). Often growth promotion and prophylaxis are not clearly distinguishable as certain antimicrobial agents are used for both purposes. Examples are bacitracin and virginiamycin, which are used mainly for growth promotion in broilers though they also control necrotic enteritis caused by *Clostridium perfringens* (McEwen and Fedorka-Cray, 2002).

Over the last decade, there is increased recognition of the need to regulate antimicrobial use, following the suggestion that indiscriminate use of antimicrobials in livestock is a crucial factor leading to antimicrobial resistance (Rosengren *et al.*, 2010). The WHO recommends an overall reduction of all antimicrobials in food-producing animals, as well as complete restriction of growth promotion and prevention of infectious diseases by using all classes of medically important antimicrobials (WHO, 2017b). At present, prophylactic antibiotics are allowed in all large broiler-producing countries/regions, namely USA, Brazil, the European Union (EU) and China, and the type of the antibiotics may differ as approved by the respective national or regional regulatory agencies (Roth *et al.*, 2019). The latest WHO categorization describes quinolones, cephalosporins (third, fourth and fifth generations), macrolides and ketolides, glycopeptides and polymyxins as “highest priority critically important” antibiotics for human medicine, and therefore not to be used for animal production (WHO, 2017a). Yet, the use of these are permitted by the largest global broiler-producers, with few exceptions such as for fluoroquinolones in the USA and cephalosporins in the EU (Roth *et al.*, 2019). However, the use of antibiotics for growth promotion in food animals has been completely banned in two of the largest global broiler- producers; EU in 2006 (Castanon, 2006) and the USA in 2017 (USFDA, 2018). Additionally, in 2018, a total of 118/153 (77%) responding countries (of OIE member countries) reported not having used

any antimicrobial agents for growth promotion in animals, either with or without national legislation or regulations (OIE, 2020). Unavailability of reliable quantitative data on antibiotic usage, not only in broiler production but also for other food animals, remains a major limitation in analysing the present global situation. On a more positive note, 139/153 (~91%) of OIE members that submitted country reports had also provided data on antibiotic usage in animals in the most recent round of data collection. Although these data range from baseline to different levels of detailed quantitative data, there is an increased participation in data reporting when compared to the previous rounds (OIE, 2020).

In many low-income countries, antibiotic use in food animal production remains unregulated (Glasgow *et al.*, 2019) and/or antibiotics are used inappropriately (Redding *et al.*, 2014). It was reported that antimicrobial agents are commonly used in livestock production in most ASEAN countries (Archawakulathep *et al.*, 2014). A systematic review (of publications from 2000 to 2016) related to antimicrobial usage and resistance in animal production in South East Asia reported that approximately 52–276 mg of antimicrobials were being administered per kilogram weight of live chicken produced, excluding in-feed antimicrobials (Nhung *et al.*, 2016). The same study found amoxicillin to be the most common antibiotic used in animal production in the region, followed by enrofloxacin, norfloxacin, doxycycline, ampicillin and colistin. In Mekong Delta chicken farms, penicillin, lincosamides, quinolones, and combinations of sulpha-trimethoprim were the most widely used compounds, and were used for prophylaxis in 84% of the cases (Nhung *et al.*, 2016). Another study in Thailand reported routine use of amoxicillin, colistin, doxycycline, oxytetracycline and tilmicosin, for prophylaxis, in three out of eight farms (medium-scale with 10,000-28,000 chickens) surveyed (Wongsuvan *et al.*, 2018).These findings are concerning as these routinely used antibiotics include several of the critically important antibiotics named by the WHO (WHO, 2017a). Additionally, colistin at present is considered the last defence against multidrug-

resistant bacterial infections in human medicine (Nation and Li, 2009). It is noteworthy that the previously mentioned study in Thailand reported that none of the farms surveyed had used antibiotic growth promoters (Wongsuvan *et al.*, 2018).

In Sri Lanka, antimicrobials are incorporated in commercial broiler feed. A recent study on 39 broiler farms owned by six leading broiler chicken producers in Sri Lanka, including six closed-house[46] farms directly under company management and 33 open-house[47] farms managed under a contract system known as buy-back[48], had used three commercial feed types, all which contain growth promoters as specified by the manufacturer (Lowe *et al*., 2019), although details of the type of antibiotics were not provided in the article. The use of antibiotics for prophylaxis is believed to be a common practice in commercial broiler operations in Sri Lanka, yet only few studies have investigated it to date. Lowe *et al* (2019) also reported (by interviewing the famers and/or the workers) the farms to be administering amoxicillin, enrofloxacin or tylosin for prophylaxis for the first few days of the broiler life cycle. Another study that surveyed 11 broiler farms (around 2,000 birds per flock) in Kandy district reported the use of prophylactic antibiotics during the first week/s of the broiler life cycle, among which tetracycline, enrofloxacin and amoxicillin were the most commonly used (Herath *et al.*, 2015). Notably, both these studies state that all the participating farms had been using prophylactic antibiotics from the age of day one of their birds, although the duration and the type of antibiotics used varied. Lowe *et al* (2019) also studied participants' awareness of antibiotic withdrawal periods. They found that 15/33 (45.5%) of the buy-back farm contractors (commonly referred as buy-back farmers) were not aware of withdrawal;

[46]Closed-house: environmental controlled (optimal temperature, humidity etc) poultry house in large-scale farms, often company-owned. The flock capacity can be 10,000 birds upwards with automated facilities and managed by trained staff.

[47]Open-house: poultry pen with half walls of approximately 0.5 m height, with a wire mesh to complete the walls up to roof.

[48]The buy-back system in the broiler industry is a unique feature where small-scale farmers are engaged in rearing for companies. Many leading broiler companies adopt this system, where they enter into a contract with small-scale farmers for rearing chickens.

while in the farms which were directly under company management, all (i.e., managers and supervisors) except workhands were aware of withdrawal periods. To the researcher's knowledge, the use of therapeutic antibiotics in broiler farms in Sri Lanka has not been studied.

Despite there being few studies that have looked at antibiotic usage in broiler production in Sri Lanka, not much is known about the wider broiler farming community. Lack of consistent data on antibiotic usage in broiler production systems is a major gap in many countries including Sri Lanka, and it is difficult to compare the qualitative data available from different studies. In this context, a study was carried out in a sample of broiler farms in the five districts with the highest poultry density in Sri Lanka to collect data and observe practices with regard to antibiotic use. Thus, it is aimed at providing a better understanding of the use of antibiotics, prophylactic and therapeutic, as well as awareness on antibiotic withdrawal at the farm level.

5.3 Materials and Methods

5.3.1 Ethics

Ethical approval for the present study, as a part of the study on *Salmonella* prevalence in poultry, was granted by the Committee for Ethical Clearance on Animal Research of the Faculty of Veterinary Medicine, University of Peradeniya, Sri Lanka. (Reference VS/ERC/17/04).

5.3.2 Study location and farm selection

The study was conducted in commercial broiler farms with over 1,000 chickens, located in the five districts with the highest poultry-density in Sri Lanka, namely, Kurunegala, Puttalam, Kegalle, Gampaha and Kandy. Selection of farms was done based on random proportionate sampling within districts, as described in 3.3.3 of chapter 3. Data collection was performed while visiting each farm once between July and December 2017.

5.3.3 Questionnaire development and delivery

A 20-item questionnaire was developed to gather the required information for the larger project on putative risk factors for *Salmonella* carriage (chapter 4), of which the present study forms one part. Out of the questions, those addressing the themes of general farm management, routine medication, antibiotic usage practices and awareness on antibiotic withdrawal were included for the purpose of the present study. The complete questionnaire can be found in Appendix IV. Pretesting the questionnaire by face-to-face verbal administration in the local language (Sinhala) was followed by modifying the content as required. The questionnaire was then verbally administered to farmers at their respective farm premises. Participants' responses were manually recorded by the researcher. (The process of questionnaire administration is explained in detail in 4.3 of chapter 4).

5.3.4 Observation

The verbal administration of the questionnaire was complemented by on-site observations on general farm management, particularly the presence of antibiotics on site; e.g. on a shelf inside poultry pen, office area (in medium or large-scale farms), feed stores etc. Observations were separately noted at the end of the questionnaire.

5.3.5 Data collection and assessment

Prophylactic antibiotics

Participants were initially asked whether they were using medication as a routine practice for the flocks. Only those who responded affirmatively were considered for further inquiry on the use of prophylactic antibiotics. The following question aimed to categorize the type of routine medication/s being used, as 'probiotics', 'multivitamins', 'prophylactic antibiotics' or 'other'. One or more of these options were ticked as per the farmer's response. There were a number of occurrences where a farmer was not aware of the type of medication that they used and therefore was unable to differentiate between the given options, in which case the final option 'other' was ticked. Whenever routine medication was categorised under 'other',

more information on the choice was noted as a clarification. For example, a few such cases were clarified as “coccidiostat”, “acidifier”, “unknown to farmer” etc. Subsequent questions were aimed at gathering data on the name of the antibiotic or antibiotics used, duration, method of administration etc. As a part of this procedure, farmers were requested to provide evidence i.e., labels or the empty packages if they could be found in the premises.

Accordingly, prophylactic antibiotic usage at farm level was determined at two stages as indicated below.

- Based on the questionnaire: Positive response (‘yes’) to use of routine medication, followed by selecting ‘prophylactic antibiotics’ as an option for the type of routine medication used.

- Based on the questionnaire and requested evidence: Initial positive response (‘yes’) to use of routine medication, with one or more options selected for the type of routine medication used (‘probiotics’, ‘multivitamins’, ‘prophylactic antibiotics’ and ‘other’). Then it was further augmented by being able to mention the name of the antibiotic and/ or able to provide a label or an empty package. This is illustrated in Figure 5.1.

Therapeutic antibiotics

Participants were asked whether there were any disease conditions in the available flocks that were treated or currently being treated with antibiotics. From those who affirmed (i.e., responded ‘yes’), further information was requested, such as the name/s of the therapeutic antibiotic/s used, duration and method of administration. These were noted down in a similar way as that of the data on prophylactic antibiotics. Additional evidence such as available labels or empty packages of the antibiotics used and any records on the disease or prescriptions by veterinarian were also recorded.

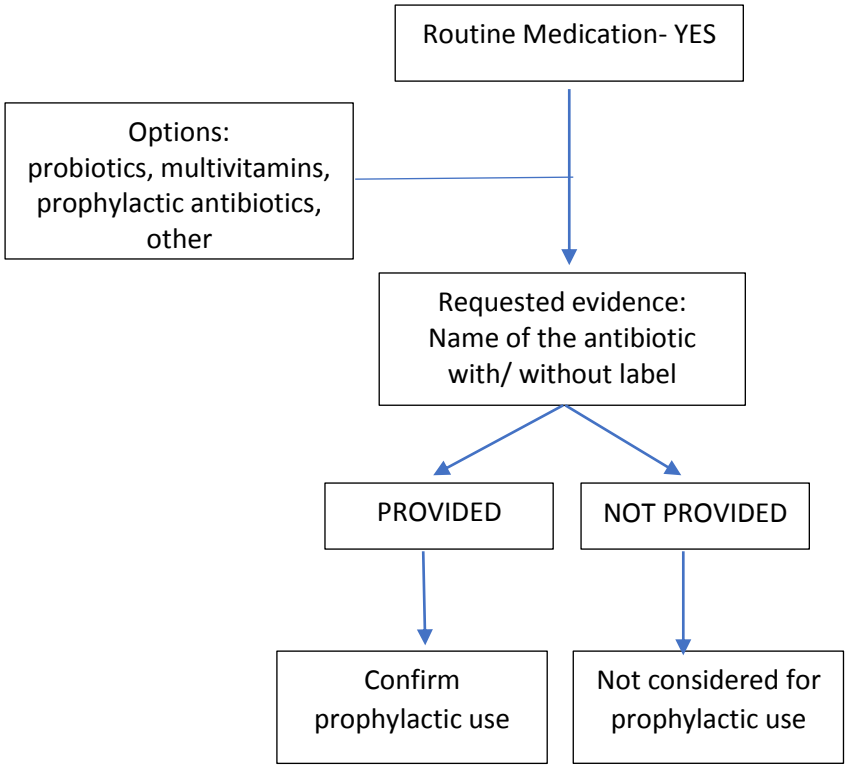


Figure 5.1: Assessment of questionnaire and requested evidence based on prophylactic antibiotic use in the participating broiler farms

Overall antibiotic usage

Overall antibiotic usage was determined by combining the data on prophylactic antibiotic use (based on both questionnaire and requested evidence), therapeutic antibiotic use, and the antibiotics present on-site that were not mentioned by the farmer for either prophylactic or therapeutic use. If the observed product was a specific antibiotic preparation for poultry/ livestock, it was counted for overall use with the assumption that the farmer had used it.

Antibiotic withdrawal

Farmers were asked about their awareness of antibiotic withdrawal (closed question with answers 'yes' and 'no'). The subsequent two questions were directed only to the farmers who were aware of the issue and aimed to further clarify whether they were abiding with withdrawal periods (closed question with answers 'yes' and 'no') and any reasons for their choice (opened question).

5.3.6 Data management

Questionnaire and observational data were stored on a Microsoft SQL Database Cluster (Microsoft Corporation, Redmond WA, USA) as the back end, and Microsoft Access (Microsoft Corporation, Redmond WA, USA) as the front end.

5.3.7 Statistical analysis

Descriptive analysis of questionnaire data was performed in R-Studio (R version 3.6.3 https://www.r-project.org) and Microsoft Excel (Microsoft corporation, Redmond WA, USA). The R packages "RODBC" (https://cran.r-project.org/web/packages/RODBC/index.html) and "sqldf" (https://cran.r-project.org/web/packages/sqldf/index.html) were used for accessing and analysing data from the database.

5.4 Results

5.4.1 Data collection in broiler farms

One hundred and forty broiler farms with the capacity greater than or equal to 1000 chicken[49] (also known as farms with over 1000 chicken), from five selected districts were contacted and invited for participation in the survey, out of which 118 farms consented and data were collected from 115. All the participating farms were managed under the deep-litter management system, though they varied in terms of their capacities[50], operation types

[49]Commercial broiler farms are categorized into two: farms with greater than or equal to 1000 chicken (also called farms with over 1000 chicken) and farms with fewer than 1000 chicken (also called farms with below 1000 chicken).

[50]Capacity of farms: small-scale (1000-5000 birds), medium scale (5001-25,000 birds), large-scale (>25000 birds).

(buy-back, self-owned and company-owned) and housing types (simple open-house pens to closed-house) as described in Table 5.1.

Table 5.1: General features of the broiler farms surveyed from five districts in Sri Lanka in 2017

	Kurunegala	**Puttalam**	**Gampaha**	**Kegalle**	**Kandy**
Operation Type					
Buy-back (64)	14	10	11	18	11
Self-owned (44)	21	10	9	2	2
Company-owned (7)	5	1	0	0	1
Farm Capacity					
Small: 1000-5000 (87)	28	16	15	18	10
Medium: 5001-25,000 (21)	7	4	5	2	3
Large: >25,000 (7)	5	1	0	0	1
Total Farms (115)	40	21	20	20	14

5.4.2 Prophylactic antibiotic use

Prophylactic antibiotic usage was determined at two stages: firstly, based on farmers' responses to the questionnaire, and secondly, on their responses and requested evidence. Responding to the questionnaire, a total of 101/115 (87.8%, CI 95% [81.8-93.8]) farmers affirmed the use of routine medication for their flocks (i.e., 'probiotics', 'multivitamins', 'prophylactic antibiotics' or 'other'), out of which 33 indicated use of prophylactic antibiotics with or without other medicine as routine medication. Therefore, based on the questionnaire alone, 33/115 (28.7%, CI 95% [20.4-37.0]) of the farms participated could be identified as using prophylactic antibiotics. When the questionnaire responses were supplemented by evidence (i.e., being able to mention the name of the antibiotic and/ or able to provide a label or empty package of the antibiotic), it was revealed that a total of 49/115 (42.6%%, CI 95% [33.5-51.6]) farms used prophylactic antibiotics. The results regarding the use of prophylactic antibiotics are shown in Table 5.2.

Table 5.2: Use of prophylactic antibiotics in the broiler farms surveyed (based on questionnaire only and on both questionnaire and evidence) in Sri Lanka in 2017

	Questionnaire only				Questionnaire and evidence
	No medicine	**Prophylactic Antibiotics**	**Medicine other than antibiotics**	**Unknown medicine**	**Prophylactic Antibiotics**
District					
Kurunegala (40)	8	14	13	5	20
Puttalam (21)	1	6	8	5	11
Gampaha (20)	4	3	7	6	4
Kegalle (20)	0	3	10	7	7
Kandy (14)	1	6	5	2	7
Operation Type					
Buy-back (64)	3	13	24	24	29
Self-owned (44)	10	18	15	1	18
Company-owned (7)	1	2	4	0	2
Farm Capacity					
Small (87)	8	24	31	23	39
Medium (21)	3	7	8	2	9
Large (7)	3	2	4	0	2
Total (115)	14	33	43	25	49

With regard to district, farm capacity and operation type, no statistically significant differences ($p<0.05$) were detected in the use of prophylactic antibiotics. However, district-wise prophylactic antibiotic use was around 50% except in the districts Gampaha (4/20, 20%) and Kegalle (7/20, 35%). When considering the operation type and farm capacity, ~45% of the buy-back and small-scale farms had used prophylactic antibiotics.

Thirty-seven participants responded on the flock age and the duration of prophylactic antibiotic use. All 37 respondents had administered antibiotics from day one of their broiler flocks, and the duration of use was three to five days. Of the respondents 27/37 (73%) were small-scale farmers. Only one farmer claimed a subsequent use at 21 days after initial antibiotic administration at the age of day one. The antibiotics used for prophylaxis were enrofloxacin (23), amoxycillin (11), doxycycline (4), sulpha-trimethoprim (4), neomycin-tetracycline (3), and a combination of enrofloxacin and amoxycillin (4).

5.4.3 Therapeutic antibiotic use

Of the participating broiler farms, 13/115 (11.3%, CI 95% [5.5-17]) reported using antibiotics to treat their flocks and they were all able to provide the requested evidence. The age of the broiler flocks at the time of the antibiotic treatment ranged from day 3 to 30, while the duration of treatment was three (5), five (7) and seven (1) days. The antibiotics used were enrofloxacin (5), sulpha-trimethoprim (4), tylosin (2), amoxycillin (1) and neomycin (1). Of the 13 respondents, eight stated the treatment provider: veterinarian or field officer (5), buy-back company (1) and fellow farmer (2). The other five respondents chose not to state the treatment provider.

5.4.4 Antibiotics present on-site

In six farms (two each from small, medium and large capacities) where the respective farmers did not state using any antibiotics, specific antibiotic preparations for poultry/ animals were observed. The said antibiotics were sulpha-trimethoprim (3), neomycin-tetracycline (2) and tilmicosin (1), and it was assumed that these preparations were being used or had been used in the farms.

5.4.5 Overall antibiotic usage

Overall antibiotic usage in this study was viewed as a combination of prophylactic antibiotic use (based on both questionnaire and requested evidence), therapeutic antibiotic use, and the antibiotics present on-site without having been mentioned by the farmer. Accordingly, 49/115, (42.6%%, CI 95% [33.5-51.6]) and 13/115 (11.3%, CI 95% [5.5-17]) of the participated farms used antibiotics for prophylactic and therapeutic purposes respectively, while three (2.6%, 95% CI [0.0-5.5]) of those farms used both prophylactic and therapeutic antibiotics. Together with the six (5.21%, 95% CI [1.2-9.5]) farms that had antibiotics on-site, it was concluded that a total of 65/115 (56.5%, CI 95% [47.5-65.6]) farms were using prophylactic and/or therapeutic antibiotics. It was further noted that all the farmers used an

oral form of antibiotics dissolved in drinking water for the entire flock, regardless of whether the purpose was prophylactic or therapeutic. Frequently administered antibiotics in the participant farms were enrofloxacin (32), amoxycillin (16) and sulpha-trimethoprim (11), while few other types were also found as shown in Table 5.3. This includes four farms that were using both enrofloxacin and amoxycillin simultaneously for prophylaxis. Some of the antibiotic packages that were encountered are presented in Figure 5.2.

Table 5.3: Types of antibiotics used in the broiler farms surveyed in Sri Lanka in 2017

	Purpose of the antibiotic			
	Prophylactic	Therapeutic	Observed on site	Total
Enrofloxacin	27	5	0	32
Amoxycillin	15	1	0	16
Sulpha-trimethoprim	4	4	3	11
Neomycin-tetracycline	3	0	2	5
Doxycycline	4	0	0	4
Tylosin	0	2	0	2
Neomycin	0	1	0	1
Tilmicosin	0	0	1	1

Figure 5.2: Examples of antibiotic packages that were encountered during the data collection in broiler farms in Sri Lanka in 2017

5.4.6 Antibiotic withdrawal

In responding to the questionnaire, 67/115 (58.2%, CI 95% [49.2-67.3]) of farmers reported being aware of antibiotic withdrawal periods. Based on the operating types of the participating farms, the lowest level of awareness was among buy-back farmers, with only 29/64 (45.3%) participants responding positively. When farm capacity was considered, the least knowledgeable were the small- scale farmers (45/87, 51.7% responding positively). Out of the participants who were aware of antibiotic withdrawal, 57/67 (85%, CI 95% [76.5-93.6]) mentioned that they were abiding by the withdrawal period. Further, 39/67 (58.2%, CI 95% [46.4-70]) participants chose to expand on their uptake of antibiotic withdrawal. Fifteen participants stated they would abide by the advice of veterinarian/ extension officer/ buy-back company; 20 knew that antibiotic residues could be harmful for human consumption; and four claimed it was not practical for them to withdraw antibiotics. Details are provided in Table 5.4.

Table: 5.4: Antibiotic withdrawal awareness, practise and views of broiler farmers participated in the survey in Sri Lanka in 2017

			Reasons or views on withdrawal		
	Aware of withdrawal	**Abide with withdrawal**	**As per advice**	**Harmful to humans/residues**	**Not practical**
District					
Kurunegala (40)	26	22	6	7	1
Puttalam (21)	8	7	1	3	1
Gampaha (20)	10	9	0	5	1
Kegalle (20)	12	11	7	2	0
Kandy (14)	11	8	1	3	1
Operation Type					
Buy-back (64)	29	24	9	7	2
Self-owned (44)	31	26	3	11	2
Company-owned (7)	7	7	3	2	0
Farm Capacity					
Small: 1000-5000 (87)	45	38	11	12	2
Medium: 5001-25,000 (21)	15	12	1	6	2
Large: >25,000 (7)	7	7	3	2	0
Total respondents	67	57	15	20	4

5.5 Discussion

The present study interviewed 115 participants on the antibiotic usage for prophylactic and therapeutic purposes as well as their awareness on antibiotic withdrawal in commercial broiler farms with over 1000 chickens. To our knowledge, this is the first comprehensive attempt, comprised of the five highest poultry-dense districts with the farms varying in capacity and operating systems, of exploring antibiotic use in broiler farms in Sri Lanka.

Requested evidence was a crucial step in this study in verifying routine medications, particularly those categorised under 'other' and/ or 'unknown' to the participants, as well as those which the farmers had misidentified medications as 'prophylactic antibiotics' which were actually 'multivitamins' and/or 'probiotics' and vice versa. The difference between the prophylactic antibiotic use proportion as obtained by the questionnaire response 33/115 (28.7%, CI 95% [20.4-37.0]) and the responses combined with requested evidence 49/115 (42.6%%, CI 95% [33.5-51.6]), suggests that a considerable proportion of farmers were unable to differentiate antibiotics from other medicine. Of the participants who claimed to have used medicine but did not know what it was, 24/25 (96%) were buy-back farmers and when the farm capacity was considered, 23/25 (92%) were small-scale farmers. Notably, buy-back farms in Sri Lanka are invariably small-scale, thus these two are often correlated. Most of the small-scale broiler producers, who manage their own business or engage in buy-back system, belong to the lowermost economy levels in the country (Bandara and Dassanayake, 2006). This could be explained through the lack of access to information as well as lack of English literacy (most of the labels being in English) among small-scale farmers.

With regard to operation type-wise and farm capacity-wise classifications, the highest prophylactic antibiotics use was identified among buy-back and small-scale farms respectively, each accounting ~45%. This could partially explain buy-back farmers' using

routine medication without being aware of the type of medicine. The lowest district-wise prophylactic antibiotic use was reported in Gampaha (4/20, 20%) followed by Kegalle (7/20, 35%), where the majority of farms 15/20 (75%) and 18/20 (90%) respectively were small-scale. The reason for this is not entirely understood, though could be attributed to farmer awareness and farm management practises of the relevant veterinary/ field officers and buy-back companies.

Based on both the questionnaire and requested evidence, it was concluded that 42.6%, CI 95% [33.5-51.6] of the farms were using prophylactic antibiotics. This is in contrast to previous studies done in Sri Lanka, which state all the participating farms as using prophylactic antibiotics. Specifically, Herath *et al* (2015) and Lowe *et al* (2019) studied 11 small-scale farms in Kandy district and 39 farms owned by six leading broiler chicken producers respectively. The present study, in contrast, interviewed more participants (115 broiler farms) covering a more diverse range of farms, as described in Table 5.1. All the participants who responded (37) to the question on the flock age and the duration of prophylactic antibiotic use stated that they administered antibiotics for a period of three to five days from day one of their broiler flock, though the type of antibiotics used varied. This is in agreement with the findings of earlier studies (Lowe *et al.,* 2019; Herath *et al.*, 2015), confirming that prophylactic antibiotic use in broiler farms start from day one of a new intake.

A possible explanation for administering antibiotics to new flocks could be prophylaxis for transport stress: day old chicks are often transported 100km or more from hatcheries (mostly in warm and humid weather, over few hours) to reach their destination. Another reason could be to prevent infections, which are more likely to occur due to poor hygienic conditions associated with minimal infrastructure in broiler farms. The quality of the poultry house or the material used was not considered in the present study, however this is

particularly true for some small-scale (farm capacity wise) and buy-back farms (operation type wise). A study on buy-back farms (100) across three districts in Sri Lanka reported 44% of the farms had cemented floors while rest had clay floors which was an inexpensive alternative, although it is important to have proper insulation and durable floors deep litter system (Rifky, 2016). The same study stated 52% of the farms had cadjan (mats woven from coconut palm leaves) as roofing material. Notably, 27/37 (73%) of the respondents in the present study were small-scale farmers. Thus, famers may treat the flock with antibiotics based on their perception of the likelihood of disease (Herath *et al.*, 2015). However, in the buy- back system, production practices are closely monitored and all the inputs (including veterinary services) are supplied by the contractor (Bandara and Dassanayake, 2006; Rifky, 2016). Hence it is likely that prophylactic antibiotic use, age of the flock at use, duration of treatment and the type of the drug, are decided and supplied by the buy-back company.

It was revealed 11.3%, CI 95% [5.5-17] farms had used antibiotics for treatment of disease. There is no other available information on therapeutic use of antibiotics in broiler farms in Sri Lanka for a comparison of present findings. Of the farmers reporting therapeutic antibiotic use, 8/13 identified who the treatment provider was. Five farmers had treated their flocks following veterinary advice, while others mentioned guidance from buy-back companies and fellow farmers. It is possible that the farmers who chose not to answer (5/13) may not have used antibiotics under veterinary advice, hence the reluctance to respond.

Antibiotics were observed onsite in six farms across all three farm capacities, in which the respective farmers did not acknowledge using antibiotics. This suggests a potential reluctance on the part of the respondents to admit their use; particularly in the two large-scale farms, where the staff including managers are likely to be well aware of the purpose and the importance of antibiotics. Accordingly, one may assume that the actual antibiotics

use among the farms studied could have been higher than reported. It can also be argued that presence of antibiotic preparations on-site does not provide sufficient evidence of their being used in the farm. However, as they were specific antibiotic preparations for poultry/ animal and often in large quantity of litres, it was assumed that these preparations were in fact being used for the flocks in the farm.

Commonly administered antibiotics in the participating farms were enrofloxacin (32), amoxycillin (16) and sulpha-trimethoprim (11), while neomycin, doxycycline, tylosin and tilmicosin were also used. Previous studies by Herath *et al* (2015) and Lowe *et al* (2019) indicated the use of enrofloxacin and amoxicillin for prophylaxis, which are recorded as the most frequently used antibiotics in the present study as well, signalling the use on farms of WHO defined "highest priority critically important" antibiotics intended to treat infections in humans (WHO, 2017a).

Of the participants, 58.2%, CI 95% [49.2-67.3] were aware of antibiotic withdrawal periods. The relevant awareness was at the lowest among buy-back farmers (29/64, 45.3%) when the farms were classified in terms of different operating types; and small-scale farmers (45/87, 51.7%) in terms of capacity. Previous findings indicate 54.4% awareness on antibiotic withdrawal among buy-back farmers (Lowe *et al.,* 2019), which corroborates the present findings. Accordingly, there is a timely need to address the persistent low levels of antibiotic withdrawal awareness among the buy-back farmers. This could potentially be achieved through their close supervision by the buy-back companies. Out of the participants who reported being aware of antibiotic withdrawal periods, 85%, CI 95% [76.5-93.6] mentioned they were abiding by their knowledge, which is a positive trend. In an earlier study, all (39) farms, which were owned by six leading broiler producers in Sri Lanka, had reported withholding antibiotics after 24 days of age (Lowe *et al.,* 2019). In the present study, out of the 39 participants who chose to explain their practice in this regard, 15 stated that they

followed the advice of the veterinarian/ buy-back companies, while only 20 mentioned antibiotic residues and/ or harmful effects through consumption of meat with residual antibiotics. This too suggests the need for increased awareness on antibiotic withdrawal among the wider broiler farming community.

The present study did not take into consideration any in-feed antibiotic growth promoters. However, according to Lowe *et al* (2019), all 39 broiler farms they surveyed (belonging to leading broiler producers in Sri Lanka), used commercial feed which contain antibiotic growth promoters. Thus, it could be presumed that a majority of the broiler farms in the present study, too, may have used in-feed antibiotics. The Global Action Plan on Antimicrobial Resistance was endorsed by the World Health Assembly, and it calls on all countries to adopt national strategies to regulate antibiotic use within two years (WHO, 2015). Accordingly, the national plan of Sri Lanka has introduced some important measures on antibiotic regulation (Ministry of Health, 2017). This comprises a complete ban on antibiotic growth promoters for animal production and restrictions for veterinary use of WHO categorized “highest priority critically important” (DAPH VDCA, 2018). Yet to reduce preventive and/or treatment medication, proper vaccination and biosecurity measures need to be implemented in broiler farms. In particular, small-scale farms need to adapt better housing and hygienic conditions.

Recommendations

Relevant awareness on antibiotics, such as resistance development, usage in animal production and withdrawal periods, is required to be conducted from grassroot level throughout the broiler production system. The adult literacy rate in Sri Lanka in 2019 was 92.3% according to UNESCO (http://uis.unesco.org/en/country/lk), which is quite high in comparison to its Asian counterparts. Although the education attainment and the language proficiency of the participant farmers were not recorded in this study, they were all literate

in their local language, Sinhala and/or Tamil. Therefore, raising antibiotic awareness in local languages among farmers would be an essential step towards regulating and reducing antibiotic usage in broiler production. Further, in addition to the available labels (in English) on the antibiotic preparations, use of local languages for product name and/ or the type of medication would be highly beneficial for the non-English literate farmers to identify them as antibiotics.

5.6 Conclusions

Use of prophylactic antibiotics ((i.e., without considering in-feed antibiotics) in the participating farms was 42.6%, CI 95% [33.5-51.6] and mostly for a duration of three to five days from day one of their broiler flock. Further, it was revealed that some farmers (16) were unable to differentiate prophylactic antibiotics from other medicine. Present study also revealed 11.3%, CI 95% [5.5-17] of therapeutic antibiotic use. Enrofloxacin, amoxycillin and sulpha-trimethoprim were found to be the most frequently administered antibiotics in the present study. Of the participants, 58.2%, CI 95% [49.2-67.3] were aware of antibiotic withdrawal periods and 85%, CI 95% [76.5-93.6] of them mentioned they were practising antibiotic withdrawal in their farms.

Chapter 6: Determination of whole-genome sequencing-based population structure and antimicrobial resistance of *Salmonella* in poultry in Sri Lanka

6.1 Abstract

The significant impact of *Salmonella* on poultry, together with its potential public health threat, underlie the critical need for control strategies for poultry salmonellosis in Sri Lanka. We used whole genome sequencing to understand the population structure of *Salmonella,* phylogenetic relationships and antimicrobial resistance of *Salmonella* to provide critical information to inform these strategies.

Altogether 164 pure *Salmonella* isolates; 128 from the cross-sectional survey (mentioned in chapter 3) and 36 from poultry outbreaks (from 2010 to 2018), were included following PCR. Subsequently, whole-genome sequencing (WGS) was performed and genetic relatedness between the isolates was examined using both allele-based and single nucleotide polymorphism-based methods. Phenotypic and genotypic antimicrobial resistance profiles were determined through the disc diffusion method and the resistome obtained through WGS analysis respectively.

Fifteen different serovars and eighteen different multi locus sequence types (MLST) of *Salmonella* enterica were identified. The majority of isolates comprised *S*. Kentucky ST314 (26.8%, CI 95% [20.0-33.6]) and *S*. Enteritidis ST11 (19.5%, CI 95% [13.4-25.6]) while serovars Typhimurium, Bareilly, Gallinarum, Corvallis, Braenderup, Butanatan, Senftenberg, Paratyphi B var.Java 1, Chester, Durban, Virchow, Weltevreden and Tennessee were present in low frequencies. Notably, Enteritidis ST11 Gallinarum (ST78 and ST92) and Typhimurium (ST36, ST19 and ST99) were responsible for more than 83% (30/36) of poultry outbreaks in the

present study while Tennessee ST319 and Gallinarum (ST78 and ST92) were seen only among the outbreak isolates. A strong association was observed between the clustering of serovar and MLST in both allele and single nucleotide polymorphisms-based analysis. Further, *Salmonella* transmission between farms could be inferred through the genetic relatedness of the isolates and available metadata including the proximity of farms and shared services or facilities such as feed distributor and buy-back provider. The present study also investigated the isolates from associated hatcheries, thus the available metadata could be linked in two instances to probable *Salmonella* transmissions from hatchery to farm. Notably high percentages of quinolone non-susceptibility were revealed in the present study, comprising resistance to nalidixic acid (41.5%, CI 95% [33.9-49.1]) and intermediate resistance to ciprofloxacin (45.1%, CI 95% [37.5-52.7]) and enrofloxacin (35.4%, CI 95% [28.0-42.7]). The concordance between resistance genotype and phenotype was observed to be over 98% for beta-lactams and tetracycline, while it was relatively low for quinolones i.e., 74% each for nalidixic acid and ciprofloxacin and 81% for enrofloxacin. The present study provides a significant baseline for the WGS-based population structure of *Salmonella* in poultry in Sri Lanka. Further, it is the first comprehensive investigation of *Salmonella* genomics to understand its diversity and genetic basis in Sri Lankan poultry.

6.2 Introduction

Whole-genome sequencing (WGS) yields the available genetic data of the entire genome of an organism, thus allowing a comprehensive, high-resolution analysis of the diversity, structure and composition of genes. In terms of resolution, WGS-based analyses can differentiate between isolates that were otherwise indistinguishable, allowing the highest possible discrimination (Pérez-Losada *et al.*, 2018). Over the last decade, next-generation sequencing (NGS), also known as high-throughput sequencing, has made WGS a realistic and efficient tool for studying population genetics and phylogenetic structures of the

pathogens found in epidemiological investigations (Deng *et al.*, 2016; Pérez-Losada *et al.*, 2018). NGS technologies such as Illumina sequencing, Ion Torrent sequencing, Oxford Nanopore technologies and Pacific Biosciences sequencing are the most popular platforms utilised in the field of genomic epidemiology. With far-reaching developments in the Illumina sequencing platforms in the recent past, WGS has become a feasible and cost-effective tool in investigating foodborne pathogens (Deng *et al.*, 2016). Consequently, WGS has become increasingly available for routine surveillance and outbreak analysis of significant foodborne pathogens of global public health concern (Didelot *et al.*, 2012; Franz *et al.*, 2014) such as *Salmonella* (Ford *et al.*, 2019; Mather *et al.*, 2015; Bloomfield *et al.*, 2017).

Salmonella enterica is a global public health concern associated with significant incidence of foodborne infection (Yachison *et al.*, 2017; Hohmann, 2001; Antunes *et al.*, 2016). In 2017, for example, enterocolitis due to non-typhoidal *Salmonella* (NTS) has resulted in an estimated 95.1 million (95% confidence interval (CI) 41.6-184.8) cases and 50,771 (95% CI 2,820–130,000) deaths (Stanaway *et al*. 2019). In Sri Lanka, although the scale of the issue is not well understood due to inadequacy of available statistics, *Salmonella* is considered the main bacterial pathogen causing foodborne illnesses followed by *Campylobacter jejuni* and *Escherichia coli* (Ministry of Health, 2013). Moreover, there are documented occurrences of NTS such as *S.* Mbandaka*, S.* Braenderup and *S.* Corvallis (Kalupahana *et al.*, 2017) and *S.* Agona (ST13), *S.* Kentucky (ST314), and *S.* Newport (ST31) (Tay *et al.*, 2019), in chicken eggs and meat respectively. Further, Tay *et al* (2019) also identified NTS serovars Enteritidis (ST11), Corvallis (ST1514), Chester (ST2063) and Mbandaka (ST1602) from human clinical specimens, providing insights into a potential public health threat. In addition to being a threat to public health, salmonellosis is also currently one of the most significant conditions affecting poultry in the country, second only to two viral infections: Infectious Bursal Disease

and Newcastle Disease. The annual number of salmonellosis cases[51] reported in commercial poultry was 65,716 and 78,928 in 2017 and 2018 respectively (DAPH Annual report, 2018, 2019). Case studies further indicate the predominance of *S.* Gallinarum and *S.* Pullorum, which cause fowl typhoid and pullorum disease respectively, as well as occurrences of NTS such as *S.* Enteritidis and *S.* Typhimurium (Gunawardena *et al*., 2006; Priyantha *et al*., 2007; Liyanagunawardena *et al*., 2012).

For nearly 100 years, epidemiological investigations on *Salmonella* relied solely on conventional serotyping, which serves as the basis for the present classification of *Salmonella* known as the White-Kauffmann-Le Minor (WKL) scheme (Grimont and Weill, 2007). This is widely considered the gold standard for phenotypic subtyping; a *Salmonella* isolate is identified by its antigenic makeup: somatic (O) and flagellar (H) antigens, through reactions with specific antisera. A major limitation of phenotypic subtyping is the deficiency of information on the phylogenetic relationships of strains at a taxonomic level (Wattiau *et al.*, 2011). In response, alternative methods, mostly DNA or molecular based, such as multi-locus sequence typing (MLST) were developed for subtyping (Maiden *et al.*, 1998). MLST assigns unique allele numbers to different alleles of seven housekeeping genes, thereby distinguishing genetically or evolutionarily related clusters of *Salmonella,* unlike conventional serotyping based on phenotypic typing (Achtman *et al.*, 2012).

With the advance of WGS, more comprehensive typing approaches, broadly categorised as single nucleotide polymorphisms (SNP) based and allele-based methods, were made available. In whole-genome SNP (wgSNP) and core-genome SNP (cgSNP) based analysis, SNPs are detected via reference-based mapping of assembled sequence reads, which is then followed by recording nucleotide differences (den Bakker *et al.*, 2011). Allele-based methods

[51]Salmonellosis cases: could comprise individual birds or several birds of a flock. These are largely based on clinical signs and post-mortem lesions rather than on laboratory confirmation.

such as core-genome MLST (cgMLST) (Ruppitsch *et al.*, 2015) and whole-genome MLST (wgMLST) (Maiden *et al.*, 2013), were developed as an upscaled approach to scheme-dependent 7-gene MLST initiated by Maiden et al (1998), where genome wide comparisons of thousands of gene loci within a species could be studied. Both SNP and allele-based analyses are reliable and frequently used in investigating *Salmonella* and other foodborne pathogens and related outbreaks (Pearce *et al.*, 2018). One limitation of methods that rely on mapping to a reference genome is that the choice of reference will affect the number of SNPs identified – the closer a reference is to the isolates in question, the greater the number of SNPs that will be identified and the greater the phylogenetic resolution. Similarly, the more diverse a set of samples mapped against a reference, the fewer the number of shared reference positions that will be identified, subsequently leading to less coverage of SNPs. Scheme-dependent allele-based methods have the advantage that they are easier to standardise: for example the cgMLST schemes use a consistent set of conserved loci and wgMLST is an extension to cgMLST (i.e., uses accessory loci in addition to core-genome loci) for an entire taxonomic group, such as a species (Uelze *et al.*, 2020). The commercially available programme Bionumerics[52] has cgMLST and wgMLST schemes available for *Salmonella*. In addition, ad-hoc wgMLST methods are available that are scheme-independent such as Fast-Genome Profiler (Fast-GeP) (Zhang *et al.*, 2018). Further, alleles from ad-hoc wgMLST can be concatenated, recombinant regions removed, and non-recombinant SNPs used as an alternative wgSNP method. Different methods and software commonly available in analysing WGS data are presented in Table 6.1.

Nevertheless, the use of serotyping continues to be important as an easily recognised form of nomenclature in maintaining communication among public health laboratories (Ashton *et al.*, 2016; Yachison *et al.*, 2017). To increase the backwards-compatibility of WGS-based

[52]https://www.applied-maths.com/sites/default/files/extra/Release-Note-Salmonella-enterica-schema.pdf

genome typing methods to old serotyping scheme, tools have also been developed to perform serovar prediction by geno-serotyping, such as the *Salmonella in silico* Typing Resource (Sistr) (Yoshida *et al.*, 2016) and SeqSero (Zhang *et al*., 2015).

Table 6.1: Different methods and software available for analysing WGS data

		Unit used for calculating genetic distance		Proportion of genome used			Reliance on pre-determined scheme	
Method	Software	SNP	Allele	Targeted gene set	Shared genome	Shared and non-shared genome	Scheme-dependent	Ad-hoc
7 gene MLST	PubMLST		■	■			■	
rMLST	PubMLST/BIGSdb		■	■			■	
cgMLST	PubMLST/BIGSdb		■		■		■	
cgSNP	Snippy/Nullabor	■			■			■
wgMLST	FastGEP		■		■			■
wgMLST	Bionumerics*		■			■	■	
wgSNP	Fast-GeP/concat**	■			■			■
wgSNP	Bionumerics	■				■		■

*Bionumerics: https://www.applied-maths.com/sites/default/files/extra/Release-Note-*Salmonella-enterica*-schema.pdf
**concatenated: following removal of recombinant regions using Gubbins

The resistome or the group of genes that resist the action of antibiotics, is another significant component of the genome available from WGS data. For decades, antibiotic susceptibility tests (AST) with phenotypic methods i.e., broth dilution and disk diffusion, alone have been used to detect resistance in bacterial isolates (Jorgensen and Ferraro, 2009). However, these methods have several recognised drawbacks, such as time delays and cost; particularly for organisms that are difficult to grow (Hendriksen *et al.*, 2009). Further, lack of harmonisation hinders data comparison between laboratories, as does limitations of the number of antibiotic agents that can be tested, different valid methods between organisms and different standards for interpretation of results (Kahlmeter, 2014). The ability of WGS to predict antimicrobial resistance (AMR) in *Salmonella* had been evaluated by

comparison with phenotypic procedures. Given the high degree of concordance, it has been recommended as a routine tool for surveillance (Zankari *et al.*, 2013; McDermott *et al.*, 2016).

At present, tens of thousands *Salmonella* isolates have been sequenced and many made publicly available. Public Health England (Ashton *et al.*, 2016) and National Surveillance in Canada (Yachison *et al.*, 2017) have implemented WGS as a routine typing tool for their public health surveillance of *Salmonella*. Further, SNP-based detection and phylogenetic analysis are used by US Food and Drug Administration Center for their surveillance on *Salmonella* (Pettengill *et al.*, 2014) as well as for routine surveillance (Ashton *et al.*, 2016; Yachison *et al.*, 2017). In Sri Lanka, characterising *Salmonella* is performed through conventional serotyping. Several such studies were carried out to broaden the understanding of common *Salmonella* serovar among poultry farms (Gunawardena *et al*., 2006; Priyantha *et al*., 2007; Liyanagunawardena *et al*., 2012), and retail chicken meat (Kottawatta *et al.*, 2014) and eggs (Kalupahana *et al.*, 2017). To date, only Tay *et al* (2019) has examined *Salmonella* isolates from Sri Lanka through WGS. This recent study used WGS to provide a molecular snapshot of genetic diversity among the 73 NTS isolates derived from chicken meat (33) and human clinical samples (40) from eight cities. Serovars Agona (ST13) and Enteritidis (ST11) were the most prevalent serovars that were observed among chicken meat and human isolates respectively, while Corvallis (ST1541) was the only serovar that occurred in both humans and chicken meat. No attempt was made to infer a correlation between human and chicken isolates in the Tay *et al* (2019) study.

The significant impact of *Salmonella* on poultry, together with its potential public health threat in Sri Lanka, call for a study of *Salmonella* genomics to understand its diversity and genetic basis. In this context, the present study is aimed at using WGS data to provide a better understanding of the population structure, phylogenetic relationships and AMR of

Salmonella in the poultry industry in Sri Lanka. To the best of investigators' knowledge, the present study is the second attempt of using WGS to study NTS in Sri Lanka and the first of its kind in primary production in the Sri Lankan poultry industry. Along with phenotypic and genotypic identification, phylogenetic relationships of *Salmonella* in carriage (i.e., broiler farms and hatcheries) and in outbreaks will provide insight into the genetic basis of the population structure of *Salmonella*, to inform control strategies.

6.3 Materials and methods

6.3.1 Ethics

Ethical approval for the present study was granted by the Committee for Ethical Clearance on Animal Research of the Faculty of Veterinary Medicine, University of Peradeniya, Sri Lanka, as described in 3.3.1 in chapter 3.

6.3.2 *Salmonella* isolates

The present study used *Salmonella* isolates sourced from poultry in Sri Lanka. This comprised isolates from the cross-sectional study, which were obtained from broiler farms and associated hatcheries[53], i.e., parent and grandparent (GP), in 2017, as described in chapter 3. Further, isolates from known *Salmonella* outbreaks were sourced from the Veterinary Research Institute (VRI) in Sri Lanka for inclusion in the study. The outbreak isolates in this regard consisted of *Salmonella* recovered from the samples received at VRI for confirmatory diagnosis from May 2017 to March 2018 as well as some older isolates (i.e., dating back to 2010-2012) revived from the culture bank at VRI. All the isolates were PCR confirmed (with the methods mentioned in 3.3.4 in chapter 3) prior to further analysis.

[53]Associated hatcheries: Parent and/ or GP hatchery were included into the study in the case of *Salmonella* positive farm/ hatchery, to investigate a possible source of infection. In case of a positive farm this refers to the parent hatchery from which the chicks were brought in and for a positive parent hatchery it is the GP hatchery.

6.3.3 Extraction of genomic DNA and whole-genome sequencing

Bacterial isolates were cultured on Columbia horse blood agar (Fort Richard Laboratories, Auckland, New Zealand) with overnight incubation at 37°C and then a single colony sub-cultured following the same conditions. Extraction of genomic DNA was done using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the bacterial colony protocol and PCR-grade water eluate was used. The protocols on culture and DNA extraction is available in Appendix II.

The quality of the extraction was assessed by fluorescent spectrometry (Qubit 2.0, Invitrogen, Carlsbad CA, U.S.A.), after which genomic DNA was stored at -20°C prior to library preparation. DNA was then normalised to a concentration of 0.3ug/mL in order to prepare libraries (Nextera XT DNA library preparation kits, Illumina Inc., San Diego, U.S.A.). A library was prepared for each isolate, and the information on the protocol used for library preparation can be found in Appendix II. The sequencing libraries were assessed for quality and fragment size distribution by an automated gel fragment analyser [Labchip GX Touch HT (PerkinElmer, PerkinElmer, Waltham, U.S.A.); or 2100 BioAnalyzer (Agilent, Santa Clara, U.S.A.)] prior to pooling and then being sent by Massey Genome Services to Novagene Ltd., China for Illumina sequencing (2x150 bp on HiSeq^{TM} X platform). (Relevant supplementary data can be found in Appendix VI).

6.3.4 Bioinformatics

The pair-end sequencing reads were used as input into the Nullarbor bioinformatics pipeline (version: 2.0.20181010; https://github.com/tseemann/nullarbor). The results obtained by the Nullarbor pipeline with default settings comprised a quality control check of the reads, sequence data, species Identification, MLST, genome assembly, resistome (Abricate version: 0.8.10 + ResFinder (Zankari *et al*., 2012), virulome and core SNP phylogeny (Snippy version 4.3.6). Serovars were predicted for the genome assemblies with the *Salmonella in Silico*

Typing Resource (Sistr) (Yoshida *et al.*, 2016). High quality assemblies, with mean read coverage ≥ 50X, full length in million base pairs (Mb) ≥ 4.5 and number of contigs ≤ 500, were included for further analysis.

The programme Fast-GeP (Zhang *et al*., 2018) was used to perform *ad hoc* whole-genome MLST (wgMLST) using the reference genome *S. enterica* strain CT18 (Parkhill *et al.*, 2001). The phylogenetic relationships of the isolates based on allele differences were visualised as a minimum spanning tree using the online tool Grape Tree (version 1.5.0) (Zhou *et al.*, 2018) and were further studied with associated spatial coordinate data using the online tool Microreact (Argimón *et al.*, 2016).

The shared-gene sequences found in the wgMLST analyses were concatenated and further analysed using the software tool Gubbins (version 2.3.2) (Croucher *et al.*, 2015). The final recombination-free Maximum Likelihood phylogeny inferred by Gubbins was visualised by uploading the tree files to Interactive Tree Of Life (iTOL: https://itol.embl.de/) (Letunic and Bork, 2007) for annotating and labelling. The created figures were further amended using Inkscape open software version 0.92.4 (https://inkscape.org). To study the detailed relationships within MLSTs, the genome assemblies of the same MLST that had at least four sequences (minimum of four different sequences are needed to create a phylogeny) were grouped to run several independent *ad hoc* wgMLST analyses using one of the genome sequences in the group as reference (i.e., sequence with the highest quality of assemblies). Relationships inferred by Gubbins tree outputs were further supported by obtaining bootstrap values using the software Molecular Evolutionary Genetic Analysis (MEGA X) version 10.2.2 (https://www.megasoftware.net) (Kumar *et al.*, 2018). All available metadata of the isolates- recorded at the time of collection (such as location and source), obtained from Nullarbor report (i.e., MLST and resistome) and through Sistr (i.e., predicted serovars) -

were marked on the phylogenetic trees. The sequence of bioinformatics analyses performed in the study is presented in Figure 6.1.

6.3.5 Data management and Statistical analysis

Metadata and all the results (i.e., laboratory and bioinformatics-based analysis) of the isolates were stored on a Microsoft SQL Database Cluster (Microsoft Corporation, Redmond WA, USA) as the back end, and Microsoft Access (Microsoft Corporation, Redmond WA, USA) as the front end. Descriptive statistical analysis of the results was performed in R-Studio (R version 3.6.3 https://www.r-project.org) and Microsoft Excel (Microsoft corporation, Redmond WA, USA). The R packages 'RODBC' (https://cran.r-project.org/web/packages/RODBC/index.html) and 'sqldf' (https://cran.r-project.org/web/packages/sqldf/index.html) were used for accessing data from the database.

6.3.6 Diversity indices

Diversity of *Salmonella* isolates were determined based on their source, MLST types (from the Nullarbor report) and predicted serovars (from Sistr), matched by the isolate identification number. Simpson's and Shannon's diversity indices (Gorelick, 2006) were estimated and rarefaction curves of MLST diversity were plotted using the R packages 'rgl' (Murdoch and Adler, 2021), 'dplyr' (Wickham *et al*., 2021), 'sparr' (Davies *et al*, 2018), 'tidyr' (Wickham and Henry, 2021), 'ggplot2' (Wickham, 2016), 'misc3d' (Feng and Tierney, 2008), 'vegan' (Oksanen *et al*, 2020), 'vegetarian' (Charney and Record, 2012) and 'patchwork' (Pedersen, 2020).

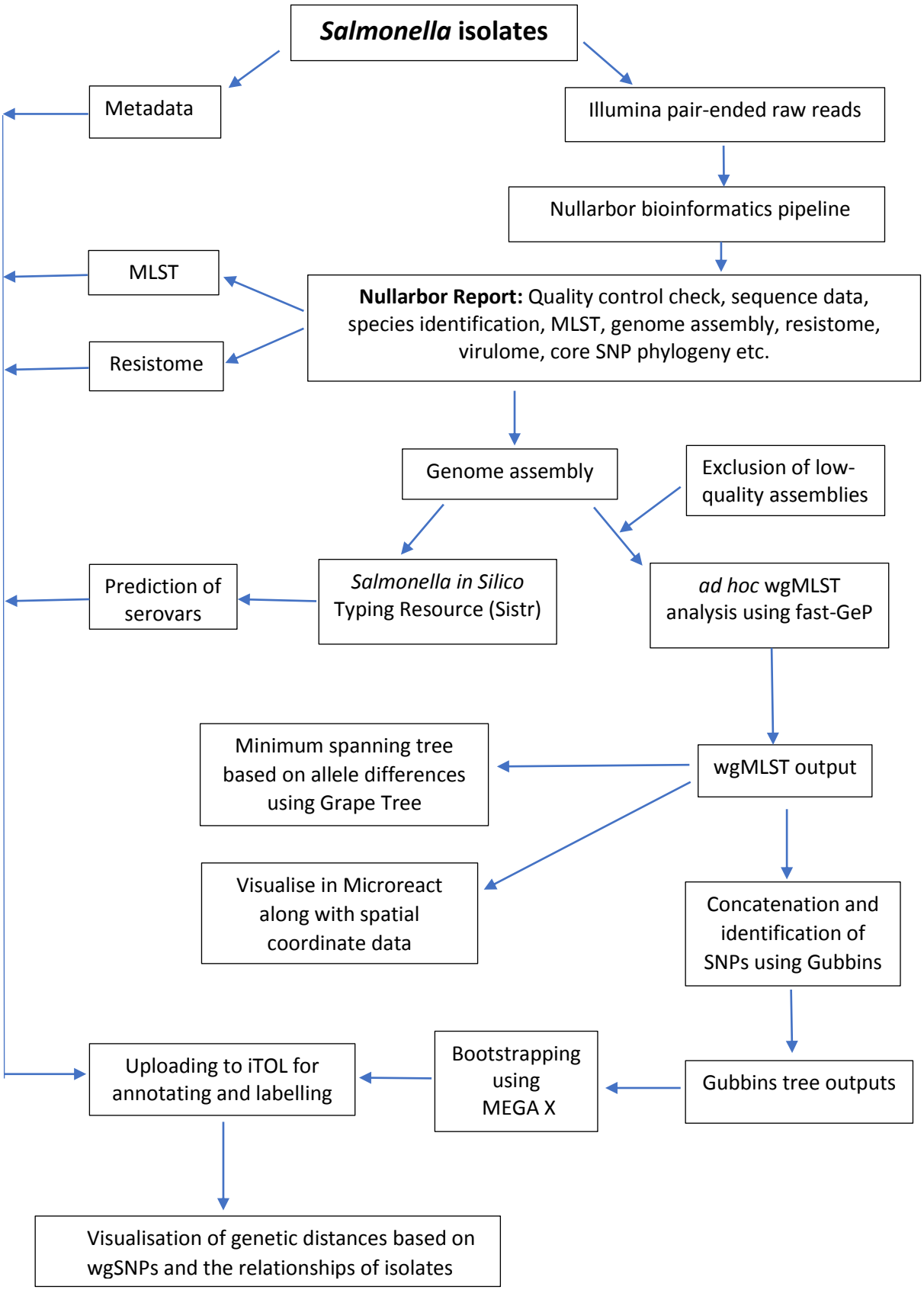


Figure 6.1: Sequence of bioinformatics analyses performed in the study on the WGS-based population structure of *Salmonella* isolates sourced from poultry in Sri Lanka in 2017

6.3.7 Antibiotic Susceptibility Testing

Selection of antibiotics

Selection of antibiotics was based on several factors:

- Their importance in Sri Lankan poultry determined from recent publications (Kalupahana *et al.*, 2017; Kottawatta *et al.*, 2017) on AST for poultry isolates
- Personal communication with the Sri Lankan National Veterinary Investigation Centre for Poultry about the use of therapeutic antibiotics
- Information gathered on antibiotic usage at farm level, through the administration of a questionnaire (as described in chapter 5 of the thesis)
- Antibiotics of interest to human health identified through the Fleming Fund programme[54], as relevant to Sri Lanka (details available in Appendix VI)
- World Health Organisation (WHO) guidelines on important antimicrobials for human medicine (WHO, 2017a) and use of antimicrobials in food producing animals (WHO, 2017b).
- World Organisation for Animal Health (OIE) list of antimicrobial agents of veterinary importance (OIE, 2018c).

AST protocol in the PC3 facility

AST by disc diffusion was selected over other methods to avoid culture and incubation of *Salmonella* in liquid media in accordance with the biosafety measures in the PC3. A protocol for the laboratory procedures was prepared and approval was gained, details of which can be found in Appendix II. AST was carried out with Oxoid antibiotic discs (Oxoid, UK) according to the manufacturer's instructions and the Clinical and Laboratory Standards Institute (CLSI) guidelines (https://clsi.org/standards/products/free-resources/access-our-free-resources/).

[54]Fleming Fund programme: builds partnerships across Sub-Saharan Africa, South and South-East Asia that support countries to collect, analyse, share and use high-quality data on antimicrobial resistance. In this regard Sri Lanka is one of the 24 countries to receive a country grant (July 2019 to July 2021) to strengthen One Health laboratory capacity and antimicrobial resistance surveillance.

The antibiotic discs used in this study and their breakpoints for Enterobacteriaceae are shown in Table 6.2. The reference strains *E.coli* ATTC®25922 and *P. aeruginosa* ATTC®27853 (only for carbapenems) were used for routine quality control (QC) according to CLSI guidelines and the zone ranges for QC can be found in Appendix VI.

Table 6.2: AST zone diameters for Enterobacteriaceae under CLSI guidelines

Antibiotic	Product code	Disc dose (µg)	Breakpoint diameter[¥] (mm)		
			S	I	R
Cefotaxime	CTX	30	≥26	23- 25	≤22
Meropenem	MEM	10	≥23	20- 22	≤19
Gentamicin	CN	10	≥15	13- 14	≤12
Trimethoprim-sulphamethoxazole	SXT	25[*]	≥16	11- 15	≤10
Ampicillin	AMP	10	≥17	14- 16	≤13
Tetracycline	TE	30	≥15	12- 14	≤11
Nalidixic acid	NA	30	≥19	14- 18	≤13
Ciprofloxacin	CIP	5	≥31	21- 30	≤20
Chloramphenicol	C	30	≥18	13- 17	≤12
Enrofloxacin	ENR	5	≥23	17- 22	≤16

[¥] Breakpoint diameter: categorised to 3 levels; S: Sensitive, I: Intermediate and R: Resistant

∗ Dose combination of SXT: Trimethoprim (1.25 µg) and sulphamethoxazole (23.75 µg)

Reference: CLSI VET 08- ED 4: 2018 for Enrofloxacin and
CLSI M100- ED 29: 2019 for all other antibiotics

Salmonella isolates were cultured on Colombia horse blood agar (Fort Richard Laboratories, Auckland, New Zealand) with overnight incubation at 37°C and then a single colony subcultured following the same conditions. Three colonies were transferred using a sterile cotton swab into a sterile saline solution, in order to prepare a suspension with an even turbidity that reached the density of a 0.5 McFarland standard (Remel™, Lenexa, Kansas). Mueller-Hinton agar (Fort Richard Laboratories, Auckland, New Zealand) plates were then inoculated by swabbing in three directions using a cotton swab. Antimicrobial discs were applied to the inoculated agar plate. After incubation at 35°C for 18 hours, the inhibition zone diameters were measured to the nearest millimetre with a digital calliper (150mm).

AST zone reading and interpretation

Salmonella isolates were tested against ten antibiotics from different classes, in duplicate, while a third round was done for those that showed a difference of 3mm or more between the first two readings of the zone diameter. Finally, the average of the readings was taken as the final zone diameter, which then was used to categorise as sensitive, intermediate and resistant as per CLSI breakpoint.

Multidrug resistance (MDR)

MDR was defined as non-susceptibility/resistance to at least three classes of antimicrobials (Magiorakos *et al.*, 2011). Hence, the isolates that were either resistant or intermediate against three or more of the following antimicrobial classes were considered as MDR: beta-lactam (CTX, MEM, AMP), aminoglycoside (CN), trimethoprim/sulphonamide (SXT), tetracycline (TE), chloramphenicol (C) and quinolone (NA, CIP, ENR).

UpSet plots were created to visualise individual antimicrobial non-susceptibility/resistance rates and MDR based on isolate source. The following R packages were used: BiocManager (Morgan, 2021), ComplexHeatmap (Gu *et al*., 2016), UpSetR (Gehlenborg, 2019); in addition to two packages - 'ggplot2' and 'patchwork' - that were previously used.

6.3.8 Resistance genes

Resistance genes in *Salmonella* isolates against different classes of antibiotics were examined using the Nullarbor pipeline (version: 2.0.20181010; https://github.com/tseemann/nullarbor). The concordance between phenotypic and genotypic AMR was then determined by using a Chi-squared test based on AST and resistome (from the Nullarbor report), matched by the isolate identification number. Chromosomal point mutations in important genes (such as *gyr*A, *gyrB, parC* and *parE*) were further studied with two different approaches: firstly, by uploading contigs into ResFinder 4.1 (Zankari *et al.*, 2017); and secondly, a custom BLAST database was set up using a

reference fasta file for the relevant gene (e.g. *gyrA*) extracted from the reference genome (NC003197). All contigs were queried against the reference gene, returning regions with BLAST matches to the database. Custom functions were created in R (R script files provided by Dr. David Wilkinson) to extract DNA regions that best matched the provided reference sequence from all sequences, and then the regions were aligned using MAFFT (Katoh *et al*., 2002). The alignments were then queried in Geneious (https://www.geneious.com) to identify allele variants known to be associated with AMR.

6.4 Results

6.4.1 *Salmonella* isolates

A total of 164 *Salmonella* isolates were used, comprising the following: 128 sourced from the cross-sectional study (sampled in 2017) on broiler farms (78) and associated hatcheries (50), and one isolate each from 36 known *Salmonella* outbreaks that occurred in poultry in the periods of 2010- 2012 (23) and 2017- 2018 (13).

6.4.2 *Salmonella* genomes

The *de novo* assembled genomes were 4.6 - 4.8 Mb in length, similar to the range of typical *S. enterica* genomes, and their species identification revealed to be *S. enterica* through the Nullarbor pipeline. One assembly of poor quality was detected and excluded from wgMLST analysis, however all the 164 genome assemblies were used in other analyses.

6.4.3 Serovar and MLST types

The results revealed eighteen different MLST types and two possibly new MLST types (i.e., not assigned with a corresponding MLST) within fifteen different serovars belonging to diverse serogroups such as B, C1, C2-4, D1, E1 and E4 of *S. enterica* as presented in Table 6.3. Relevant metadata of the individual isolates can be found in Appendix VI. Accordingly, Kentucky ST314 (26.8%, CI 95% [20.0-33.6]), Enteritidis ST11 (19.5%, CI 95% [13.4-25.6]) and Typhimurium ST36 (9.8%, CI 95% [5.2-14.3]) were the most prevalent serovars observed.

MLST diversity of Salmonella isolates

An increase in both the richness and the evenness of the MLST diversity is represented by Simpson's and Shannon's indices, however Shannon's index places more weight on taxonomic richness than evenness when compared to Simpson's index. In the present study, broiler farms showed the greatest MLST richness (n=13), followed by hatchery (n=9) and outbreaks (n=8). Simpson's and Shannon's indices for *Salmonella* diversity are presented in Table 6.4. Consistent with the diversity indices, rarefaction curves indicated a greater taxonomic diversity among broiler farms than for other sources, allowing for differences in sample size, as shown in Figure 6.2.

Table 6.3: MLST types and *in silico* serovars of *Salmonella* sourced from poultry in Sri Lanka in 2017

Serovar	Serogroup	MLST	Isolates[¥]				Percentage (CI 95%)
			BF	BH	OB	Total	
Kentucky	C2-C3	314	30	12	2	44	26.8 (20.0-33.6)
Enteritidis	D1	11	8	13	11	32	19.5 (13.4-25.6)
Typhimurium	B	36	11	5	0	16	9.8 (5.2-14.3)
		19	2	0	2	4	2.4 (0.1-4.8)
		99	0	0	2	2	1.2 (0.0-2.9)
Bareilly	C1	203	0	6	2	8	4.9 (1.6-8.2)
		909	3	4	0	7	4.3 (1.2-7.4)
Gallinarum	D1	78	0	0	11	11	6.7 (2.9-10.5)
		92	0	0	4	4	2.4 (0.1-4.8)
Corvallis	C2-C3	1541	6	2	0	8	4.9 (1.6-8.2)
Chester	B	2063	5	0	0	5	3.0 (0.4-5.7)
		NA-2[*]	1	0	0	1	0.6 (0.0-1.8)
Virchow	C1	16	0	6	0	6	3.7 (0.8-6.5)
Senftenberg	E4	14	6	0	0	6	3.7 (0.8-6.5)
Braenderup	C1	22	1	1	0	2	1.2 (0.0-2.9)
Tennessee	C1	319	0	0	2	2	1.2 (0.0-2.9)
Durban	D1	2330	2	0	0	2	1.2 (0.0-2.9)
Butantan	E1	NA-1[*]	2	0	0	2	1.2 (0.0-2.9)
Paratyphi B var. Java	B	1588	1	0	0	1	0.6 (0.0-1.8)
Weltevreden	E1	365	0	1	0	1	0.6 (0.0-1.8)

[¥]Isolates: Categorised according to their source; BF: Broiler Farm, BH: Broiler Hatchery, OB: Outbreak, and total
[*]NA: not assigned with a corresponding MLST

Table 6.4: Simpson's and Shannon's indices of MLST diversity of *Salmonella* sourced from poultry in Sri Lanka in 2017

Source	Simpson's index (CI 95%)	Shannon's index (CI 95%)
Broiler farm	0.20 (0.17-0.23)	2.02 (1.91-2.13)
Hatchery	0.18 (0.16-0.20)	1.89 (1.78-2.00)
Outbreak	0.21 (0.17-0.25)	1.77 (1.63-1.91)

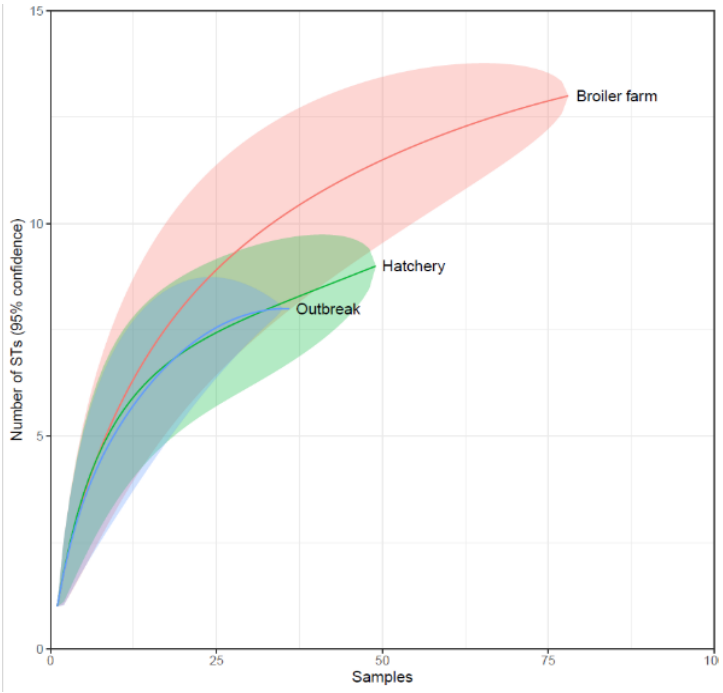


Figure 6.2: Rarefaction curves of the broiler farm, hatchery and outbreak *Salmonella* MLSTs sourced from poultry in Sri Lanka in 2017. The shaded areas represent 95% CI

6.4.4 Results of AST

All *Salmonella* isolates (164) were tested against ten different antibiotics for antimicrobial resistance. Importantly, all the isolates were found to be 100% sensitive for cefotaxime, meropenem and gentamicin. Comparatively high non-susceptibility (i.e., resistance and/ or intermediate in resistance) was revealed for all three quinolones tested. Resistance for nalidixic acid was 41.5%, CI 95% [33.9-49.1] while 18.3%, CI 95% [12.4-24.2] of the isolates were intermediate in resistance. In contrast, there was no evidence of resistance to ciprofloxacin, however as many as 45.1%, CI 95% [37.5-52.7] of the isolates were found to be

intermediate in resistance. For enrofloxacin, 9.1%, CI 95% [4.7-13.6] and 35.4%, CI 95% [28.0-42.7] of isolates showed evidence of resistance and intermediate in resistance, respectively. Individual antimicrobial non-susceptibility/resistance rates (other than for cefotaxime, meropenem and gentamicin) for *Salmonella* are shown in Table 6.5. Detailed results on AST on final zone diameters and categorisation as sensitive, intermediate and resistant for as per CLSI breakpoint can be found in Appendix VI.

Table 6.5: Antimicrobial resistance revealed through AST results of *Salmonella* isolates sourced from poultry in Sri Lanka in 2017 (CLSI M100- ED 29: 2019 and CLSI VET 08- ED 4: 2018)

Antibiotic	Disc dose (µg)	Isolates resistant (total=164)	Resistant % (CI 95%)	Isolates intermediate (total=164)	Intermediate % (CI 95%)
Nalidixic acid (NA)	30	68	41.5 (33.9-49.1)	30	18.3 (12.4-24.2)
Ampicillin (AMP)	10	33	20.0 (14.0-26.3)	0	0.0 (0.0-0.0)
Tetracycline (TE)	30	28	17.1 (11.3-22.8)	0	0.0 (0.0-0.0)
Trim-sulpha (SXT)∗	25	18	11.0 (6.2-15.8)	0	0.0 (0.0-0.0)
Enrofloxacin (ENR)	5	15	9.1 (4.7-13.6)	58	35.4 (28.0-42.7)
Chloramphenicol (C)	30	7	4.3 (1.2-7.4)	2	1.2 (0.5-2.9)
Ciprofloxacin (CIP)	5	0	0.0 (0.0-0.0)	74	45.1 (37.5-52.7)

∗ SXT: Trimethoprim-sulphamethoxazole, dose combination trimethoprim (1.25 µg) and sulphamethoxazole (23.75 µg)

MDR phenotypes

MDR phenotypes were observed in 32/164 (19.5%) of the isolates, all of which had resistance for beta-lactam (i.e., ampicillin) and quinolone (nalidixic acid and/or enrofloxacin). Additionally, 14/23 MDR isolates were also resistant for trimethoprim-sulphamethoxazole (14/23), tetracycline (19/23) and both trimethoprim-sulphamethoxazole and tetracycline (1/23). MDR phenotypes were observed in Kentucky ST314 (27), Chester ST2063 (5) and Corvallis ST1541 (1).

UpSet plots in Figure 6.3 present individual antimicrobial non-susceptibility/ resistance rates (other than for cefotaxime, meropenem and gentamicin) and MDR based on isolate source.

The relatively high proportion of isolates resistant to five or more antibiotics in broiler farms is evident in this plot (combinations to the left of each plot).

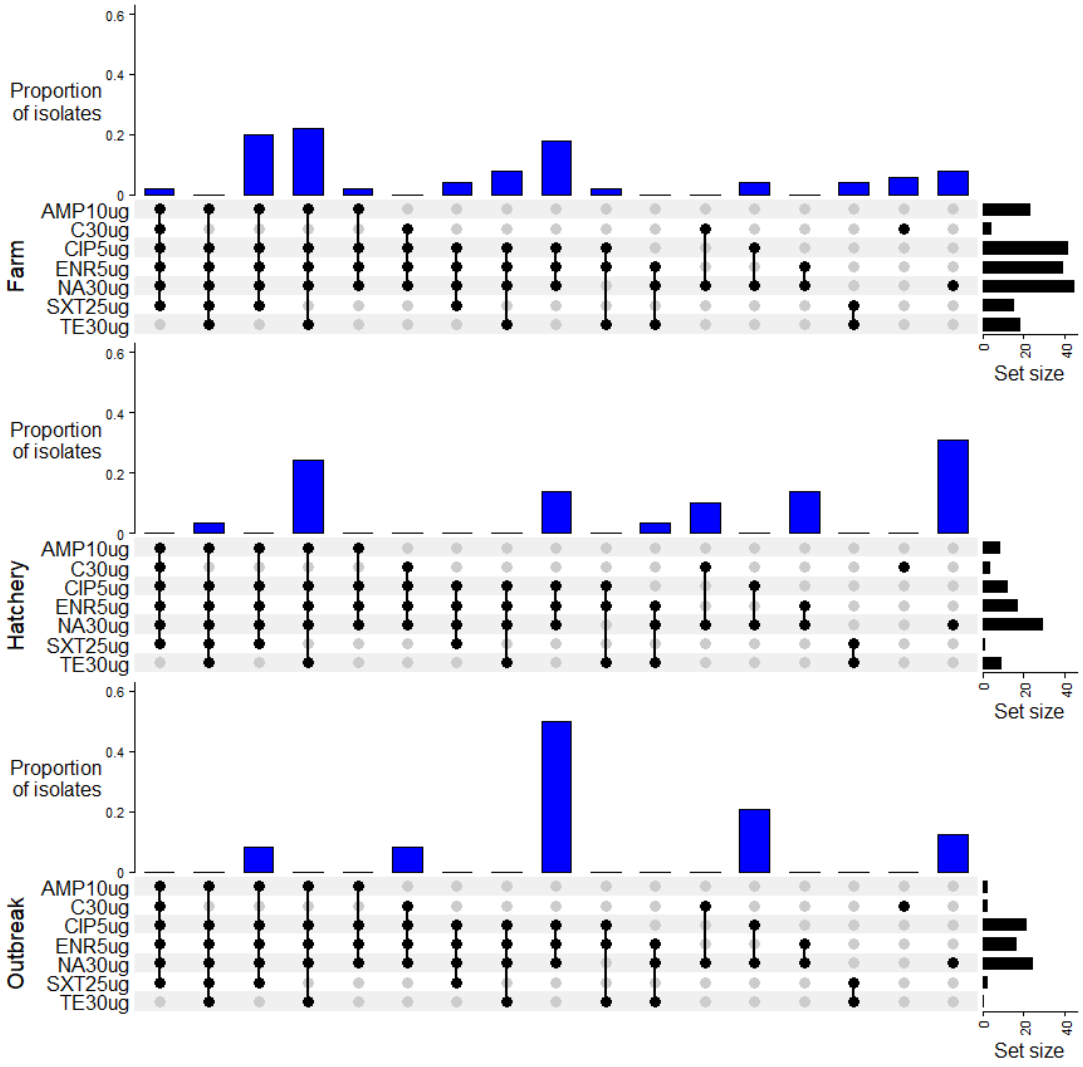


Figure 6.3: Antimicrobial non-susceptibility/resistance proportions and MDR based on isolate source of *Salmonella* isolates sourced from poultry in Sri Lanka in 2017. Each plot shows the frequency of each combination of non-susceptibility (resistant and intermediate isolates) for isolates from broiler farms (Farm), hatcheries (Hatchery) and flock outbreaks (Outbreak), with each combination indicated by the black dots. The isolates with most resistances/intermediates are to the left of each plot, and the single resistances to the right of the plot. 'Set size' in each plot corresponds to row total (i.e., number of isolates).

6.4.5 Resistance genes and their association with phenotypic AMR

The most detected resistance genes (through the Nullarbor report) were associated with quinolone resistance, which comprised 45.1%, CI 95% [37.5-52.7] of the isolates. In addition, resistance genes for aminoglycoside and sulphonamide were present in 37.8%, CI 95% [30.4-45.2] and 32.3%, CI 95% [25.2-39.5] of the isolates respectively. A summary of the presence of resistance genes against eight classes of antibiotics are shown in the Table 6.6, the details of which are available in Appendix VI.

Table 6.6: Resistance genes against eight classes of antibiotics (as per Nullarbor report) from 164 *Salmonella* sourced from poultry in Sri Lanka in 2017

Antibiotic Class	Resistance genes	Isolates (total=164)	Proportion (CI 95%)
Quinolone	*qnrB4*, *qnrD1, qnrS1*	74	45.1 (37.5-52.7)
Aminoglycoside	*aadA, aph (3")*, *aph (6)*	62	37.8 (30.4-45.2)
Sulphonamide	*sul2, sul3*	53	32.3 (25.2-39.5)
Macrolide	*mef(B)*	43	26.2 (19.5-33.0)
Beta-lactam	bla_{TEM-1}	37	22.6 (16.2-29.0)
Tetracycline	*tet(A)*	30	18.3 (12.4-24.2)
Trimethoprim	*dfrA1*, *dfrA14*	17	10.4 (5.7-5.0)
Phenicol	*cmlA1*	7	4.3 (1.2-7.4)

There was over 98% concordance between phenotypic and genotypic (i.e., presence of genes) resistance for beta-lactam and tetracycline (p^{55}<0.005 Chi-squared test). AST phenotypes for trimethoprim-sulphamethoxazole were 99% and 78% (p<0.005; Chi-squared test) in agreement with resistance genotypes for trimethoprim and sulphonamide respectively. Concordances between phenotypic and genotypic non-susceptibility/resistance for quinolones tested were 74% each for nalidixic acid and ciprofloxacin and 81% for enrofloxacin. While the concordance between quinolone resistance genes and phenotype was high there was a relatively high level of discordance for isolates identified as Enteritidis ST11 or Gallinarum ST78. This was further examined using the customised BLAST approach

[55] p: pre-determined critical probability (i.e.,<0.05)

in section 6.4.9. AMR profiles (genotypic and phenotypic) of *Salmonella* isolates are presented in the Gubbins tree alongside heat maps in Figure 6.6.

6.4.6 Phylogenies of *Salmonella* isolates

Overall, there were 3148 shared-loci between the 163 isolates, of which 22 were identical across all isolates. After excluding 42 shared-loci that were identified as hypothetical gene duplicates, 3106 were used to construct the distance matrix for further analysis, of which 3084 were polymorphic. There were 110,995 core SNPs identified across 5,133,713 bp.

6.4.7 Allele-based phylogenies of *Salmonella* isolates

Minimum spanning tree

The allelic differences of wgMLST between and within serovars as well as different sources are visualised by the top and bottom minimum spanning trees respectively in Figure 6.4 using a pairwise distance matrix. In the minimum spanning trees, isolates with no allele differences represent the same node while the connecting lines and the number of loci mentioned on them reflect the distance between nodes. There was a strong correlation between the clustering of serovar and MLST. Although there was some grouping by serogroup (e.g. D1), there were large allele differences between serovars in the same serogroup; e.g. the allele difference between Enteritidis ST11 and Gallinarum ST78 of serogroup D1 is 1684.

Four of the outbreak isolates showed no allele differences with that of a few farm and hatchery isolates, namely, OB28 and F071 (ST19), OB23 and F044 (ST314), OB21 and H014 (ST203) and OB1, F009 and F015 (ST314). Single-locus variants between farms such as F051 and F031 (ST2063) and F064 and F010 (ST314) were also revealed. Further, five isolates- H005, OB24, OB31, OB34 and OB36- belonging to *S*. Enteritidis (ST11) show few allele differences between one another. All of the above-mentioned observations could suggest relatively recent direct transmission.

Phylogeny with associated spatial coordinate data

The geographical distributions of farms sampled were within the five districts; Kurunegala, Puttalam, Gampaha, Kegalle and Kandy, while associated hatcheries were found throughout the country. Coordinate data (can be found in Appendix III) of *Salmonella* positive farms (37) and hatcheries (10) were mapped and presented in Figure 6.5 along with the phylogenetic tree based on wgMLST of *Salmonella* isolates sourced from corresponding locations (i.e., farms and hatcheries) as well as outbreaks, for the latter coordinate data not being available. There was no obvious spatial pattern of serovars; and the results suggest widespread distribution of *S*. Kentucky and *S*. Enteritidis, the two most abundant serovars followed by *S*. Typhimurium.

6.4.8 SNP-based phylogenies of *Salmonella* isolates

Relatedness of the isolates was studied with maximum-likelihood phylogeny generated from wgSNPs across the 163 *Salmonella* isolates alongside isolate metadata as presented in Figure 6.6. There was complete concordance between the maximum-likelihood phylogeny and MLST types while high concordances were observed between phenotypic and genotypic resistance profiles.

Separation of isolates into their origin depicted that only two of the MLSTs (Kentucky ST314 and Enteritidis ST11) occurred among all sources, while other MLSTs were restricted to one or two sources. For example, some MLSTs such as Chester ST2063 and Senftenberg ST14 were associated only with farms while Virchow ST16 was isolated only from hatcheries. Further, two MLSTs of Gallinarum (ST78 and ST92), Typhimurium ST99 and Tennessee ST319 were only isolated from outbreaks.

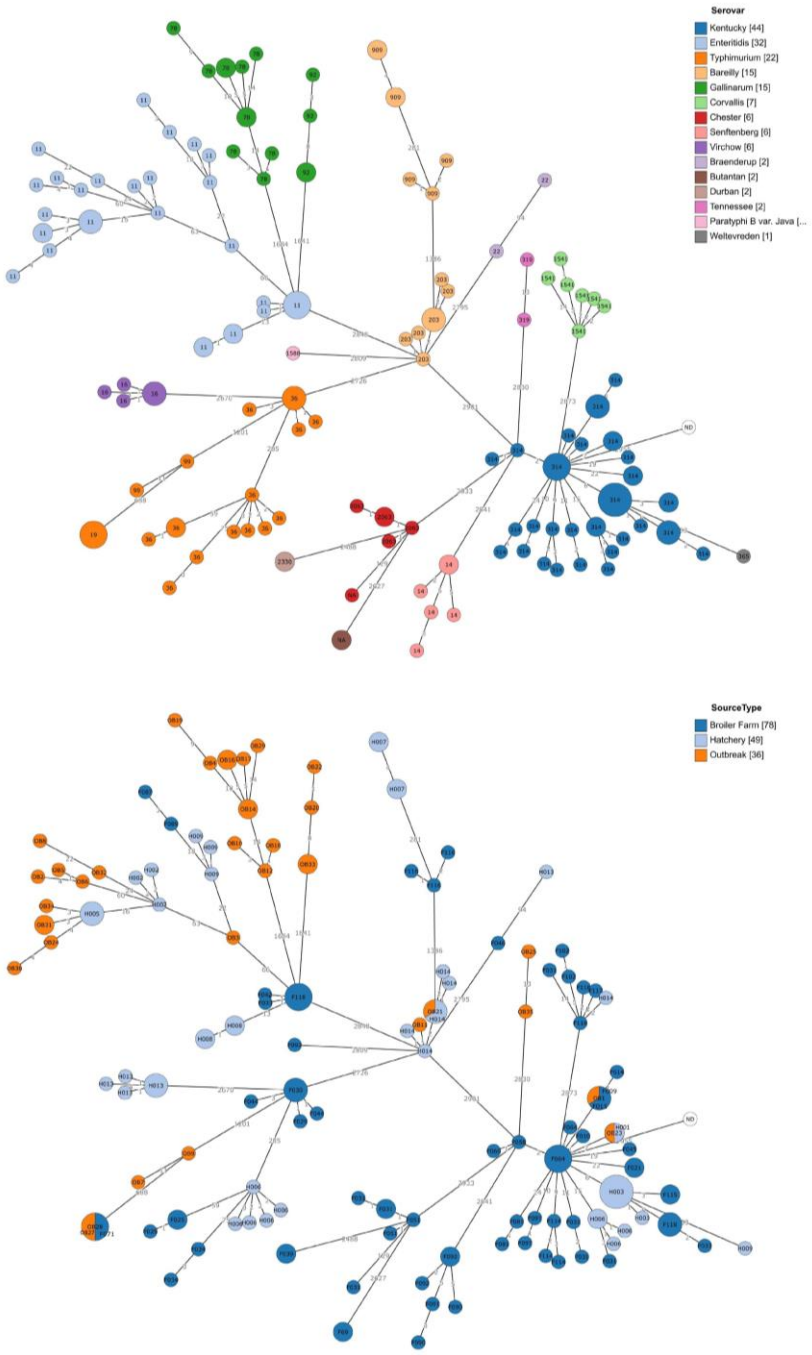


Figure 6.4: Minimum spanning tree of *Salmonella* isolates; top: coloured by serovar and bottom: coloured by source (farm (F), hatchery (H) or outbreak (OB), from poultry in Sri Lanka in 2017. Nodes are labelled with 7 gene STs in the figure on top and farm name or outbreak number in the figure on bottom. Node size is proportional to the number of isolates. Node representing the reference genome (*S*. Typhi CT18) is labelled as ND. The allele differences (number of loci) are mentioned on the connecting lines. Figure created using Grape Tree (Zhou *et al.*, 2018).

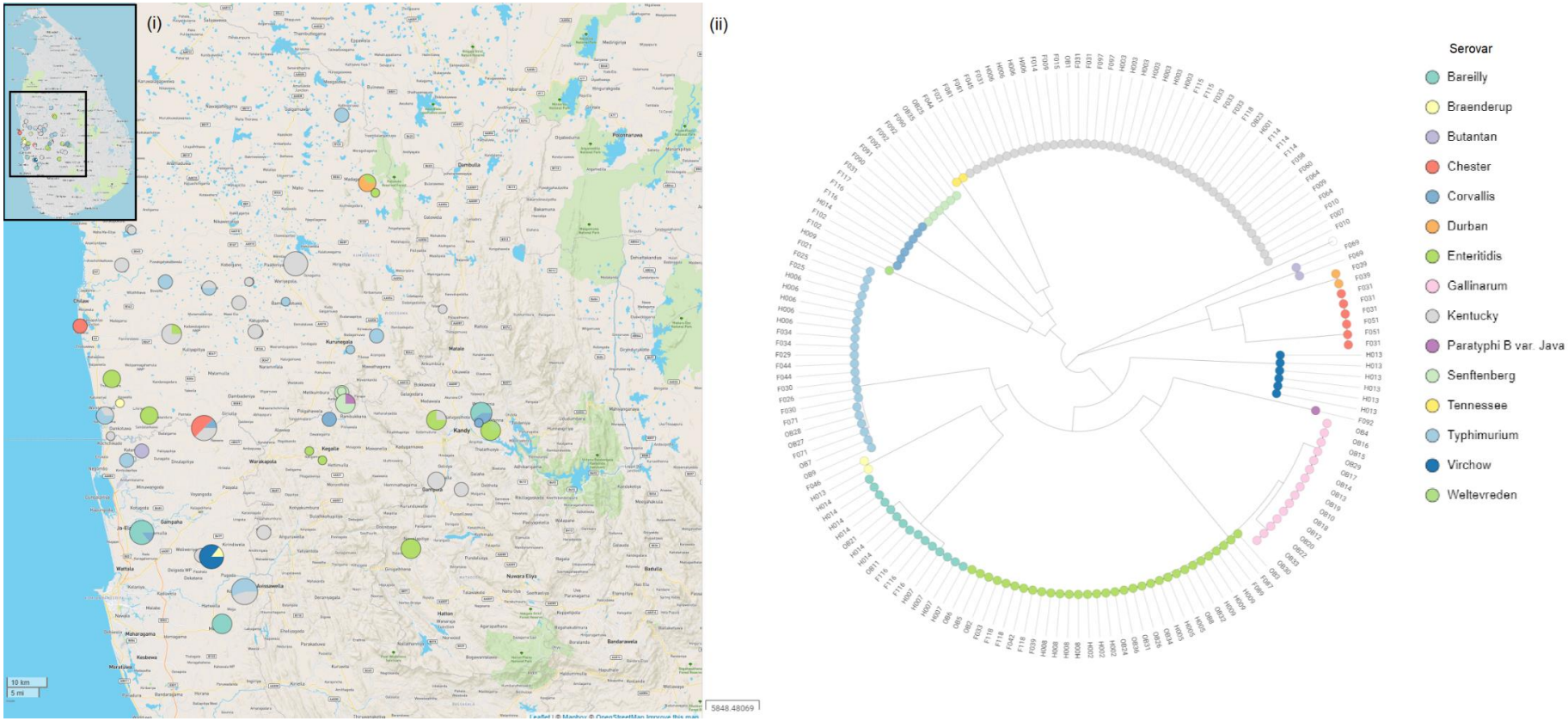


Figure 6.5: (i) Map showing the geographical distribution of *Salmonella* positive farms and hatcheries in Sri Lanka coloured by serovar as discovered in 2017 (size of the circle is proportional to the number of isolates). (ii) Phylogeny was generated from wgMLST allelic profiles of 163 *S. enterica* sourced from farms, hatcheries and outbreaks in poultry in Sri Lanka. The terminal tree nodes of farm (F) and hatchery (H) correspond to locations shown in the map. Figure created using Microreact (Argimón *et al.*, 2016)

6.4.9 MLST-based analysis

The fine-scale difference between isolates within the clades (i.e., MLST types) is concealed when all clades are considered together due to the comparatively large genetic distances between clades as evident in Figure 6.6. Therefore, detailed relationships between the isolates within MLSTs were independently studied and maximum-likelihood phylogeny of higher resolution was created. Traced back data of the isolates (i.e., associated hatchery) were observed where necessary for genetic relatedness. Association of positive farms and their associated hatcheries as well as positive parent hatcheries and GP hatcheries can be found in Appendix VI.

MLSTs that had a minimum of four isolates i.e., Kentucky (ST314), Enteritidis (ST11), Typhimurium (ST36), Bareilly (ST203 and ST909), Gallinarum (ST78 and ST92) and Corvallis (ST1541) were able to create a phylogeny. Bootstrap values from 500 replicates were generated to measure the association between the isolates within these groups and only those that had statistical support of 50% or more are indicated as branch labels. However, four of the MLSTs with four or more isolates- Senftenberg (ST14), Virchow (ST16), Chester (ST2063) and Typhimurium (ST19)- could not be used to build a phylogeny because they lacked a minimum of four different sequences.

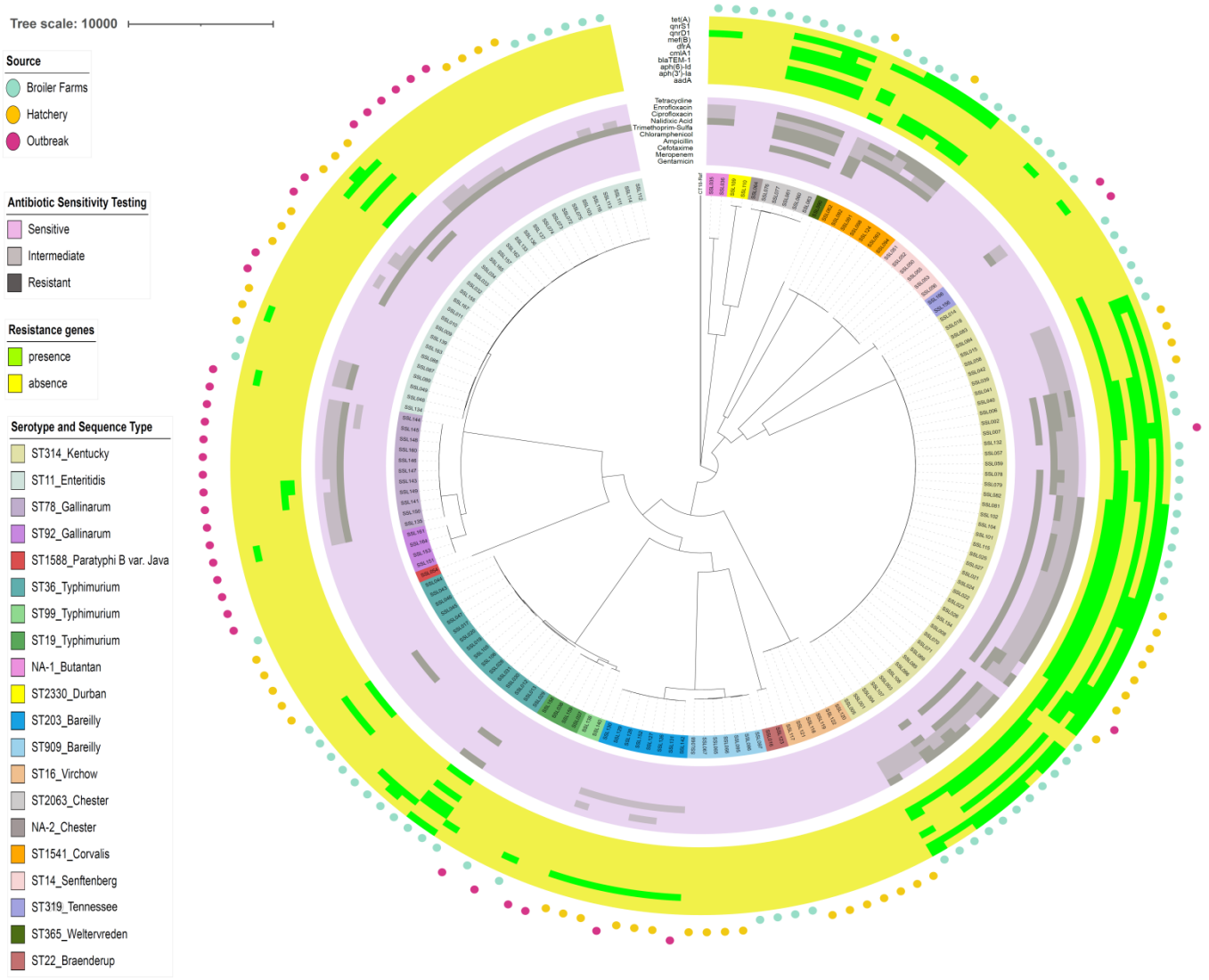


Figure 6.6: Gubbins tree of 163 *Salmonella* isolates (reference genome *S*. Typhi CT18) sourced from poultry in Sri Lanka in 2017, annotated with serovar, MLST types (coloured ranges), source and antimicrobial resistance profiles (heatmaps). Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007) and edited with Inkscape version 0.92.4 (https://inkscape.org)

- *S*. Kentucky ST314

This forms the largest subset identified in the present study (n=44) overlapping between broiler farm, hatchery and outbreaks. A maximum-likelihood phylogeny for *S*. Kentucky ST314 with available metadata is presented in Figure 6.7. There is high bootstrap support (99%) for a distinct subclade (bounded by SSL104 and SSL107 in the lower half of the plot). There is evidence of Kentucky ST314 being isolated from broiler farms in all five districts, three hatcheries and from two outbreaks that occurred between 2010 to 2012.

Isolates that are genetically identical between farms suggest either a common source or transmission of Kentucky ST314 between farms. For example, in two adjacent government veterinary ranges (GVR)[56] in Kurunegala district, the isolates were identical: SSL002 (F009) in Katupotha, SSL006 (F014) and SSL007 (F015) in Panduwasnuwara. Interestingly, the farms F014 and F015 are located within 400m distance of one another and owned by the same person. Further, identical sequences are found between districts: SSL014 and SSL018 from Puttalam and Kurunegala respectively, SSL101 and SSL115 from Kurunegala and Kandy respectively, and SSL007 from Puttalam with that of three isolates (SSL001, SSL003, SSL005) from Kurunegala. When traced back to respective associated hatcheries, it was evident that SSL101 and SSL115 were linked to the same hatchery i.e., hatchery 1. It is noteworthy that *S*. Kentucky ST314 was also isolated from hatchery 1 (SSL008), though the strain was distantly related to SSL101 and SSL115. It is noteworthy that hatchery 6, which was also found to be infected with Kentucky ST314, is a GP hatchery. However, hatcheries supplied by this GP hatchery (i.e., hatchery 2 and 7) were positive for *Salmonella* with different strains. The two outbreak isolates of Kentucky ST314 in the present study show close genetic association to

[56]GVR: refers to a geographical area under the purview of one senior government veterinarian (who may have one or two veterinarians reporting to him/her). GVRs roughly correspond to the administrative divisions of the country. In a GVR, the senior veterinarian-in-charge is responsible for providing the required services (health, breeding, extension etc.) for government sector and small-scale private sector ventures as well as for households, though not for large-scale private enterprises that employ their own veterinarians.

that of isolates from the cross- sectional study, indicating impending threat of future outbreaks. The outbreak isolate SSL132 is identical (0 SNP difference) to SSL002 and SSL007 (from broiler farms in Kurunegala) while SSL154, also sourced from an outbreak which occurred sometime from 2010 to 2012, is highly related to SSL008 (from hatchery 1) with only a few SNPs difference. However, due to lack of fine-level data on outbreaks, these isolates could not be related epidemiologically.

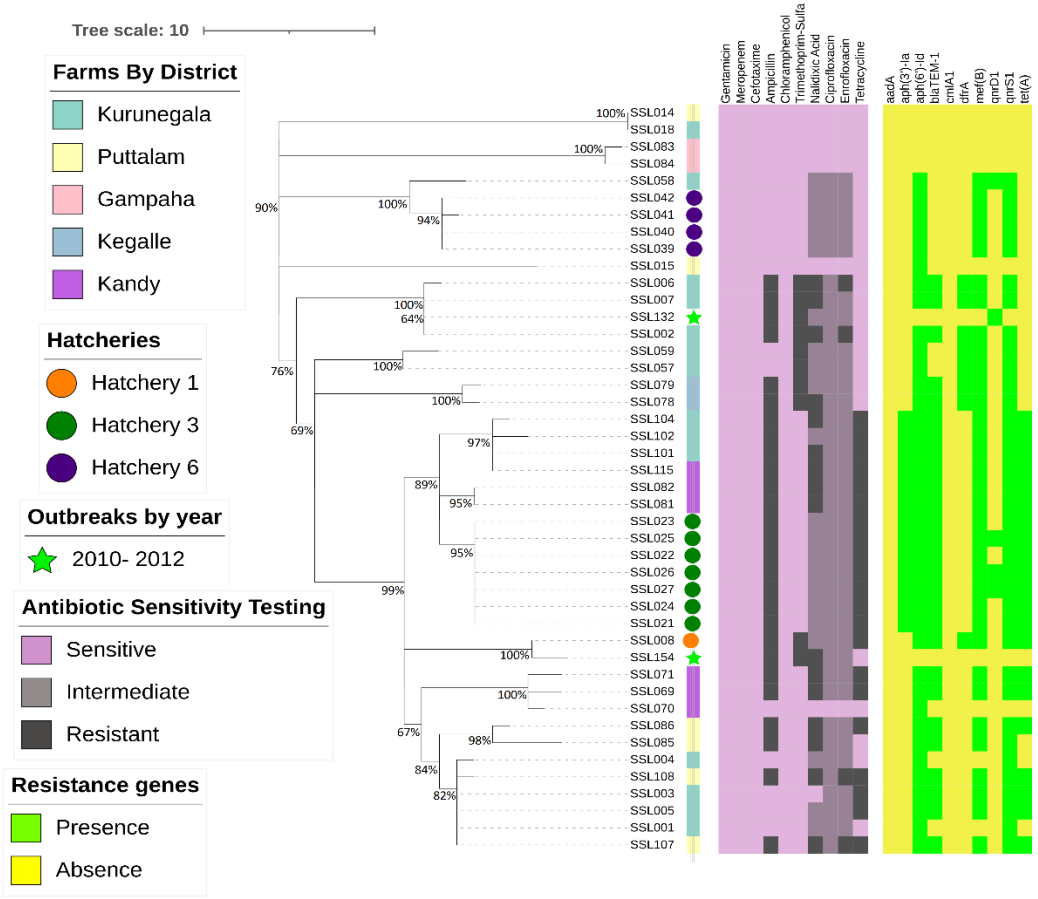


Figure 6.7: Gubbins tree of 44 *S*. Kentucky ST314 (reference genome SSL005), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

Comparatively higher non-susceptibility/resistance for quinolones was observed among Kentucky sequences, which was followed by resistance for ampicillin and tetracycline.

Significantly, 27/33 (82%) MDR phenotypes of all *Salmonella* in the present study were Kentucky ST314, which comprised isolates from broiler farms (17), hatcheries (8) and outbreaks (2). Further, it is noteworthy that none of the other MLSTs were found to have MDR isolates sourced from hatcheries and/ or outbreaks.

- *S*. Enteritidis ST11

Enteritidis ST11 is the second largest group with 32 isolates overlapping between all three sources. A maximum-likelihood phylogeny for *S*. Enteritidis ST11 with available metadata is presented in Figure 6.8. In the present study, Enteritidis ST11 was isolated from broiler farms in the three districts, four hatcheries and outbreaks occurred between 2010 to 2012 (5) and 2017 to 2018 (6). There is high bootstrap support (100%) for several distinct sub-clades within these sequences.

Very closely related SSL048 (F087) and SSL049 (F089) sequences from Kegalle could further be linked through metadata: the farms' geographical location was in the same GVR (i.e., Galigamuwa) with approximately 6km distance via roads (2-3 km aerial distance) from one another and having the same buy-back[57] provider. Similarly, identical sequences SSL111 (F039) and SSL112 (F042) were sourced from two farms in Polpithigama (7km distance via roads) in Kurunegala district, both farms being under one buy-back provider. Monophyletic relationships with bootstrap support of 100% for the isolates SSL103, SSL111 and SSL112 (from Kurunegala) and SSL113, SSL114 and SSL116 (from Kandy), provide evidence of the spread of Enteritidis ST11 between farms within the two districts. Of those, all except SSL103 were linked to a single hatchery, thus suggesting hatchery to farm spread. However, *Salmonella* was not isolated upon tracing back from the particular hatchery. Three isolates

[57]The buy-back system in the broiler industry is a unique feature where small-scale farmers are engaged in rearing for companies. Many leading broiler companies adopt this system, where they enter into a contract with small-scale farmers for rearing chickens.

(SSL133, SSL136 and SSL137) of the outbreak cluster sourced from 2010-2012 were closely related to one another, yet due to lack of metadata the sequences could not be epidemiologically linked. Another outbreak cluster which was sourced from 2017-2018 (SSL155, SSL157, SSL162, SSL165, and SSL167) is closely associated, though the locations of which are in different areas. Further, these isolates from outbreaks in 2017-2018 are associated with hatchery 5 (SSL032, SSL033 and SSL034), although an epidemiological link between the sources is not evident.

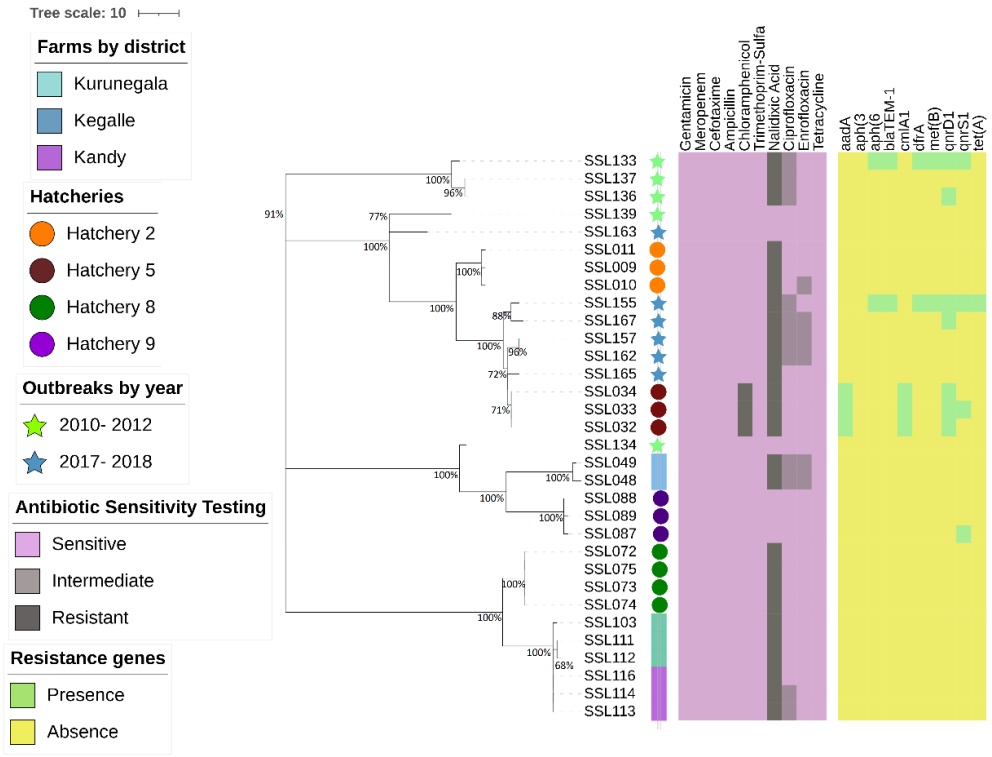


Figure 6.8: Gubbins tree of 32 *S*. Enteritidis ST11 (reference genome SSL157), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

Comparatively high non-susceptibility/resistance for quinolones (i.e., resistance to nalidixic acid and intermediate resistance to ciprofloxacin and/ or enrofloxacin) was observed,

although few isolates possessed genes associated with quinolone resistance (e.g. *qnr*D1 and *qnr*S1) according to the Nullarbor report. Chromosomal point mutations in relevant resistance genes (such as *gyr*A, *gyr*B, *par*C and *par*E) were not detected by ResFinder 4.1. However, the customised BLAST approach detected well-known allele variants in the *gyr*A gene in 24/32 isolates, all of which displayed resistance to nalidixic acid and varied susceptibility (i.e., intermediate or sensitive) to ciprofloxacin and/ or enrofloxacin. These comprised a change at codon Asp-87 to Tyr (11), Gly (10) or Asn (3). However, none was found as MDR.

- *S*. Typhimurium ST36

Sixteen *S*. Typhimurium (ST36) isolates were sourced from broiler farms in two districts and a hatchery (i.e., hatchery 6) in the present study. A maximum-likelihood phylogeny for *S*. Typhimurium ST36 with available metadata is presented in Figure 6.9.

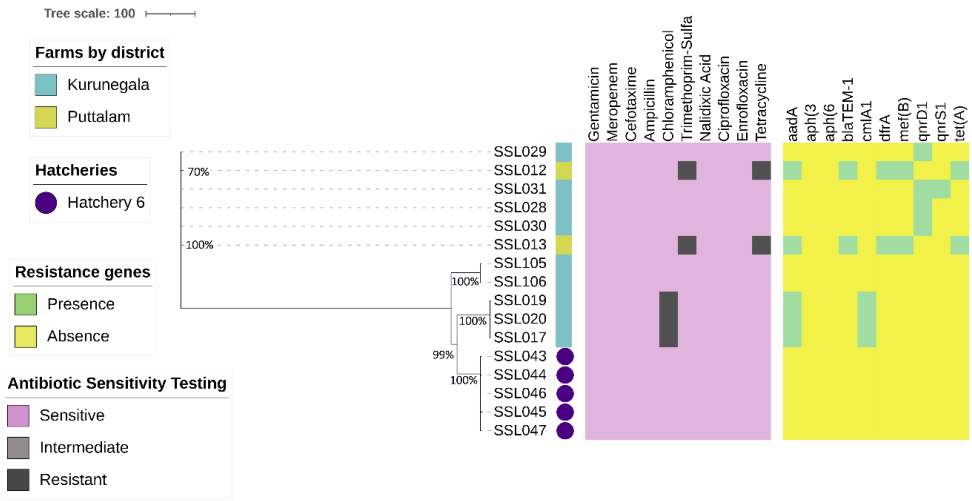


Figure 6.9: Gubbins tree of 16 *S*. Typhimurium ST36 (reference genome SSL019), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

Phylogenetic data suggest *Salmonella* transmission between farms within the two districts, Puttalam and Kurunegala, i.e., sequences SSL012 and SSL013 (from the same farm in

Puttalam) show no SNP difference with SSL028, SSL029, SSL030 and SSL031 (from three different farms in Kurunegala). Further, sequences SSL029 and SSL030 were sourced from two farms (i.e., F029 and F030 respectively) in Kurunegala which are situated approximately 9km (road distance) from one another and share the same feed distributor. The above farm isolates from Kurunegala could be linked to two hatcheries from where the chicks had been supplied. However, the respective hatcheries were positive for *Salmonella* strains other than *S*. Typhimurium ST36. There is some AMR which may be correlated to the presence of specific genes.

- *S*. Bareilly ST203

The sequences of *S*. Bareilly ST203 indicate close phylogenetic association between its isolates sourced from outbreaks in 2012 and from hatchery 14. However, lack of data on outbreaks limits the identification of possible epidemiological relationships. No AMR patterns were associated with *S*. Bareilly ST203 and the maximum- likelihood phylogeny along with metadata is presented in Figure 6.10.

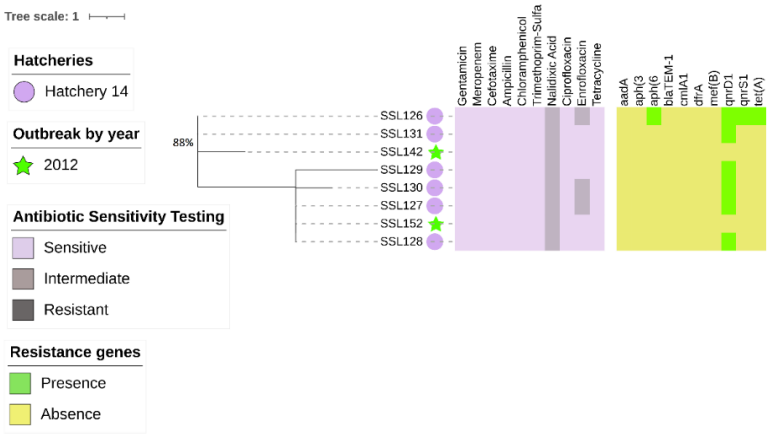


Figure 6.10: Gubbins tree of eight *S*. Bareilly ST203 (reference genome SSL130), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

- *S*. Bareilly ST909

The farm and hatchery isolates of *S*. Bareilly ST909 were clustered separately with no phylogenetic or epidemiological association. No AMR was observed. Maximum-likelihood phylogeny is presented in Figure 6.11.

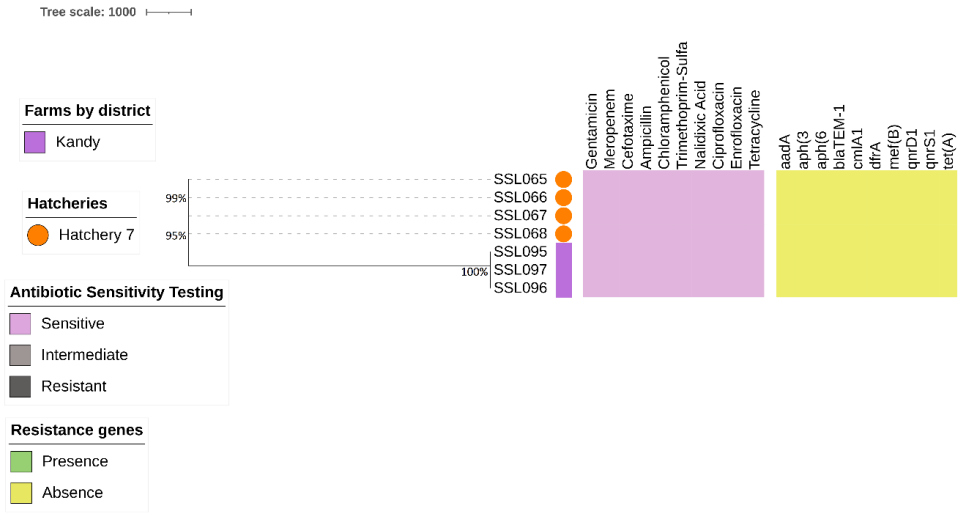


Figure 6.11: Gubbins tree of seven *S*. Bareilly ST909 (reference genome SSL095), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

- *S*. Gallinarum ST78

S. Gallinarum ST78 is the causative agent of fowl typhoid in chickens. All the isolates within this group were associated with outbreaks that occurred in 2010, 2012 or 2017. It is evident that the outbreaks in 2012 were caused by isolates that had no or only a few SNPs difference, such as SSL145 and SSL144 (bootstrap support of 92%), SSL146 and SSL147 (bootstrap support of 96%) and SSL143 and SSL149 (bootstrap support of 92%). However, there is no epidemiological evidence due to the lack of metadata i.e., the location and the date of outbreaks. Further, there is only one Gallinarum ST78 isolate sourced from 2017, which is relatively distant from all other sequences.

All the isolates showed non-susceptibility for quinolones (i.e., resistance to nalidixic acid and intermediate resistance to ciprofloxacin and/or enrofloxacin) while they were susceptible to other classes of antibiotics. The quinolone resistance was found to be associated with Ser-83 to Phe mutation in *gyr*A, which was detected by customised BLAST. Maximum-likelihood phylogeny is presented in Figure 6.12.

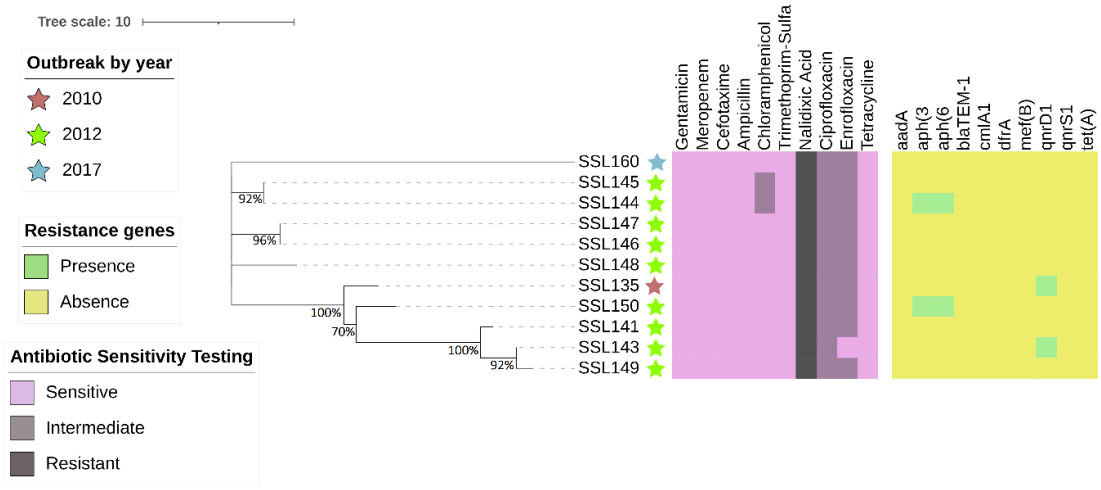


Figure 6.12: Gubbins tree of 11 *S*. Gallinarum ST78 (reference genome SSL135), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

- *S*. Gallinarum ST92

S. Gallinarum ST92 commonly known as *S*. Pullorum, is the causative agent of pullorum disease in chickens. All the isolates within this group are associated with outbreaks that occurred in 2012 or 2017. The isolates from 2017 (SSL161 and SSL164) showed no SNPs difference and were clustered separately (with bootstrap support of 100%) with no close association to those of 2012. The isolates from 2017 were sourced from two outbreaks that occurred within five weeks in two GVRs in Kandy: Galaha and Gampola. Although these two GVRs have a maximum aerial distance of 20km from one another, on some sides they are adjacent. Without coordinate data, proximity of the geographic locations could not be

determined. However, given the location and date of outbreaks, it is highly suggestive of *Salmonella* transmission between areas. No AMR phenotypes or genotypes were observed from Gallinarum ST92. A maximum-likelihood phylogeny is presented in Figure 6.13.

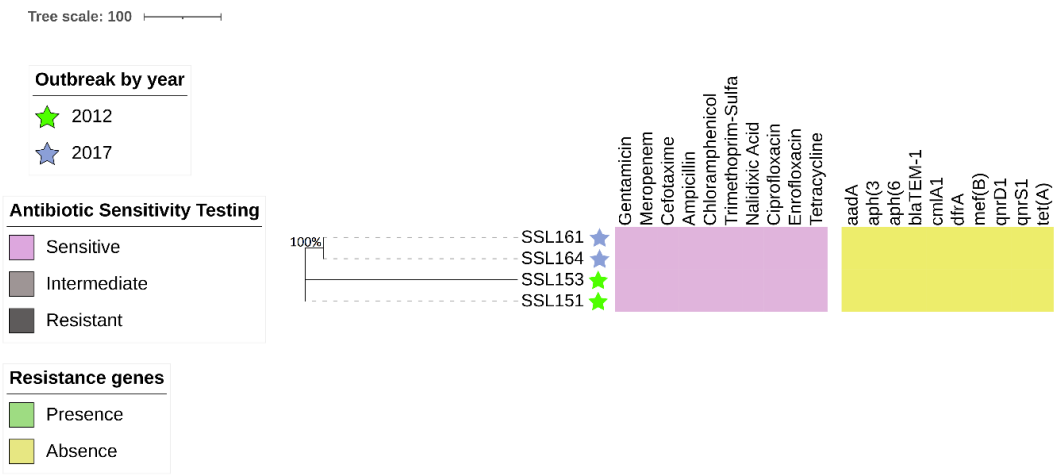


Figure 6.13: Gubbins tree of four *S*. Gallinarum ST92 (reference genome SSL164), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

- *S*. Corvallis ST1541

S. Corvallis (ST1541) isolates were sourced from broiler farms of three districts and a hatchery (i.e., hatchery 14) in the present study and their maximum-likelihood phylogeny is presented in Figure 6.14. The isolates from farm F102 in Kegalle (SSL091 and SSL092) and farm F116 in Kandy (SSL093, SSL094 and SSL098) were closely related to that of hatchery 14 (SSL124) with few SNPs difference. In addition to the genetic association, available metadata revealed hatchery 14 to be the associated hatchery of the farm F102 from where SSL091 and SSL092 were sourced. This signifies probable transmission from hatchery to farm. All the isolates showed non-susceptibility for quinolones (i.e., resistance to nalidixic acid and intermediate resistance to ciprofloxacin and/or enrofloxacin) and one isolate was defined as MDR.

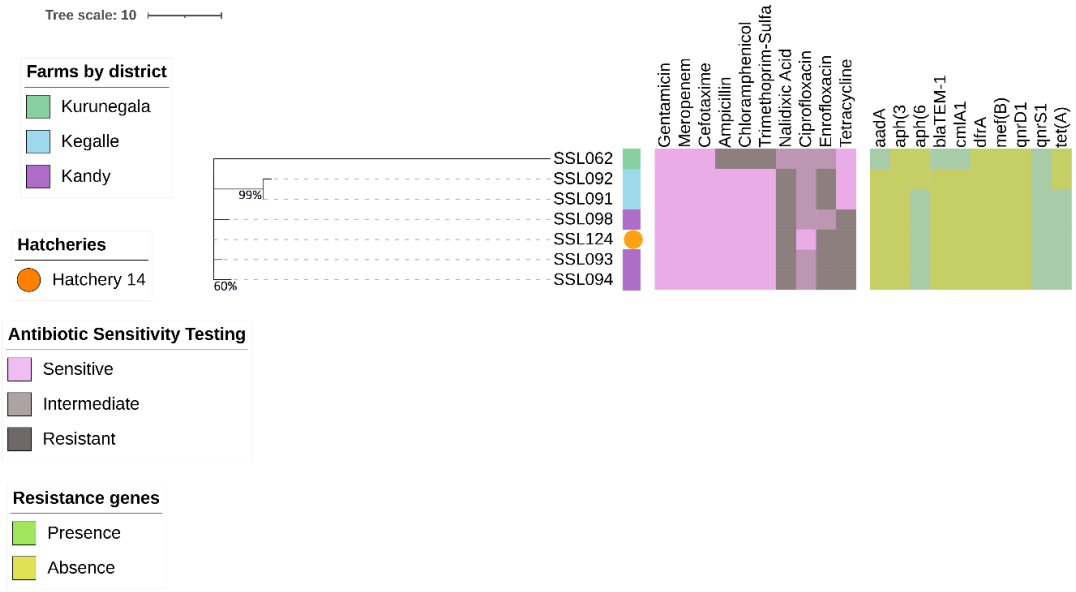


Figure 6.14: Gubbins tree of seven *S*. Corvallis ST1541 (reference genome SSL092), annotated with isolate origin and antimicrobial resistance profiles in the form of heatmaps. Branch labels indicate bootstrap support values (50% or more) for 500 replicates. Figure created using iTOL (https://itol.embl.de/) (Letunic and Bork, 2007). The tree was rooted using mid-point root and the scale bar represents SNPs.

- *S*. Senftenberg ST14

All six isolates were sourced from three broiler farms (F090, F091 and F092) in a single GVR (Rambukkana) in Kegalle. Of these, two isolates were identical while others had few allele differences as presented in the minimum spanning tree in Figure 6.4. Coordinate data shows approximately 500m distance between F090 and F091 while F092 is located around 4km (road distance) away from F090 and F091. Further, all three farms are managed under the same buy-back provider, suggesting possible means of spread of *Salmonella* between farms or of a common source. All these farms on the other hand were associated to hatchery 15, however, *Salmonella* was not detected there when sampled. None of these isolates had evidence of phenotypic or genotypic AMR.

- *S*. Virchow ST16

All the six isolates were sourced from hatchery 13, all of which were closely related with three identical sequences and others with a difference of few alleles. The isolates were susceptible for all the classes of antibiotics tested and no resistant genes were present.

- *S*. Chester ST2063

Five isolates of *S*. Chester ST2063 were identified from two broiler farms in Kurunegala (3) and Puttalam (2) districts, all of which were closely related with identical sequences and with a difference of few alleles. All these were found to have same MDR phenotypes.

- *S*. Typhimurium ST19

Four isolates of *S*. Typhimurium ST19, two each sourced from a farm in Gampaha district and outbreaks in 2017, had no phenotypic or genotypic AMR profiles.

6.5 Discussion

The present study reports the first comprehensive analysis using WGS data to study *Salmonella* in Sri Lankan poultry. The study investigated the phenotypic and genetic diversity and the phylogenetic relationships of *Salmonella* in carriage, based on isolates from a cross-sectional survey carried out in 2017 on broiler farms and associated hatcheries in poultry dense districts, and in outbreaks, based on poultry salmonellosis outbreaks in 2010- 2012 and 2017-2018. To date, one WGS-based analysis has been carried out in non-typhoidal *Salmonella* in Sri Lanka (Tay *et al.*, 2019), thus a comparison of the results of the two studies could be made.

To the best of the investigators' knowledge, nine of the MLST types in the present study are reported for the first time in Sri Lankan poultry. These comprise two MLSTs of Bareilly (ST203 and ST909), Virchow ST16, Senftenberg ST14, Tennessee ST319, Durban ST2330, Typhimurium ST19 and 99 and Paratyphi B var. Java ST1588. Further, isolates of serovars Butantan and Chester with no corresponding MLST types, which could potentially be two

new MLST types, were identified. *S. enterica* MLSTs reported by Tay *et al* (2019) were also noted: Enteritidis (ST11), Typhimurium (ST36), Chester (ST2063) and Weltevreden (ST365) from human clinical samples, Kentucky (ST314) from raw chicken meat, and Corvallis (ST1541) from both human clinical samples and chicken meat. In addition, *S. enterica* serovars, which have so far been identified only through conventional serotyping, were also reported in the present study. These include Kentucky (Kottawatta *et al.*, 2014), Braenderup (Kalupahana *et al.*, 2017) and Gallinarum (Gunawardena *et al*., 2006; Priyantha *et al*., 2007; Liyanagunawardena *et al*., 2012).

The most widespread strain in the present study was *S*. Kentucky ST314 (26.8%, CI 95% [20.0-33.6]) followed by *S*. Enteritidis ST11 (19.5%, CI 95% [13.4-25.6]), both of which overlapped among all sources. Though Tay *et al* (2019) reported *S*. Agona ST13 as the commonest strain among raw chicken meat (16/33), it was not isolated in the present study, while *S*. Enteritidis ST11 (21/40) was only isolated from human clinical samples by Tay *et al* (2019). Additionally, *S*. Newport ST31 (4/33), which was sourced from raw chicken by Tay *et al* (2019), was not found in the present study. While the reason behind the difference in the distribution of poultry sourced *Salmonella* strains is not very clear, it could possibly be due to sampling locations: all the raw chicken samples in the Tay *et al* (2019) study were collected from Colombo (i.e., the capital of Sri Lanka), which was not a location examined in the present study. Apart from widespread *S*. Kentucky and *S*. Enteritidis, most of the other strains were associated with one or two sources i.e., *S*. Bareilly ST909 was isolated from a farm in Kandy and a hatchery (H007), *S*. Senftenberg ST14 from three broiler farms (F090, F091 and F092) in Kegalle and *S*. Virchow ST16 from a hatchery (H0013), suggesting restricted spread.

The common knowledge and earlier findings on poultry holdings in Sri Lanka indicated the predominance of host-adapted, non-motile *S*. Gallinarum and *S*. Pullorum; causative agents

of fowl typhoid and pullorum disease respectively (Gunawardena *et al*., 2006; Priyantha *et al*., 2007; Liyanagunawardena *et al*., 2012). Nevertheless, present findings revealed *S*. Gallinarum ST78 and *S*. Gallinarum ST92 (*S*. Pullorum), were associated only with outbreaks. Notably, earlier studies on poultry holdings in Sri Lanka were based on confirmed and/ or suspected salmonellosis cases, while the present study is the only one to date that looked at *Salmonella* carriage through a cross-sectional study in broiler flocks and their associated hatcheries. Acquisition of better control over non-motile *Salmonella* in breeder flocks, through the nation-wide immunisation programme with SG 9R live *Salmonella* vaccine[58] (Silva *et al.*, 1981) between 2012 to 2015 (DAPH Annual report, 2012), could be another potential reason for obtaining only motile isolates in this study. (This is further discussed in 3.5 of chapter 3).

The present study revealed quite a few different NTS, some of which are known human pathogens. Amongst the few available publications, there is evidence of NTS such as Enteritidis (ST11), Corvallis (ST1514) and Chester (ST2063), being isolated from 40 human clinical specimens from eight cities in Sri Lanka (Tay *et al.*, 2019). Further, *S*. Enteritidis and *S*. Corvallis have been identified as causing bacteraemia in immunocompromised humans (Mubarak and Chandrasiri, 2013). Thus, the findings are suggestive of a substantial threat to public health given the high demand for chicken meat among Sri Lankans. As early as 1970s, there had been a protracted outbreak (i.e., over 27 months) of infections with *S*. Bareilly in a maternity hospital in Colombo (Mendis *et al*., 1976). The present study also revealed two strains of *S*. Bareilly (i.e., ST203 and ST909), which could possibly be an impending public health threat.

[58]SG 9R vaccine: Rough strain *S*. Gallinarum 9R (Smith, 1956), which was developed initially for immunisation against *S*. Gallinarum. Also being used for active immunisation against *S*. Pullorum and to active cross protection against infections caused by other Salmonellae of group D such as *S*. Enteritidis.

The phylogenetic relationships of the isolates were studied based on allele as well as SNP differences. A strong association was observed between the clustering of serovar and MLST in both allele and SNP based analysis. The association between the isolates within MLST were further studied with bootstrap support, thus, sequences with identical or few alleles/SNPs differences were identified as due to probable transmission or a common source. While *Salmonella* transmission between farms is multifactorial, the present study could link available metadata to identify possible causes. The metadata thus used were the proximity of farms (geographical location and road access) and shared services or facilities such as feed distributor and buy-back provider. For example, Kentucky ST314 infected two farms (Kurunegala) were under the same owner; two farms with Enteritidis ST11 (Kegalle) were linked to the same buy-back provider; and Typhimurium ST36-infected farms (Kurunegala) were supplied by the same feed distributor. However, genetic evidence of *Salmonella* (i.e., Kentucky ST314, Enteritidis ST11, Typhimurium ST36 and Corvallis ST1541) transmission between districts were not supported by other epidemiological evidence.

The present study used Gubbins on a concatenate alignment of alleles, similar to that of Roary (the pan genome pipeline). However, unlike Roary, the concatenations of the gene sequences in fast-GeP *ad hoc* wgMLST analysis were ordered according to the gene order of the reference genome. Instead of mapping the sequencing reads, fast-GeP mapped the contigs sequences to the ordered CDSs of the reference genome. It is similar to the analysis using SNIPPY. Although “core genome alignments” produced by SNIPPY are not real whole-genome alignments they are recommended by the author as inputs for Gubbins. Therefore, fast-GeP generated gene concatenations can also be seen as approximations of whole-genome alignments and thus, in principle, could be used as input for Gubbins. In a few previous studies on the genomics epidemiology of *E. coli*, this approach was compared with the two complementary evolutionary inference methods (Bai *et al*., 2019; Yang *et al*., 2020).

No significant differences in tree topology were detected between the trees generated by the three methods, although there were some differences in estimated branch lengths.

The present study also investigated the associated hatcheries, parent hatchery and GP hatchery, in the case of *Salmonella* infected farms and parent hatcheries, respectively, to study possible sources of infection. However, only in two instances the genetic association and the available metadata of the sequences could be linked to a probable transmission: one being Kentucky ST314 from farms in Kurunegala and Kandy with hatchery 1, and the other, Corvallis ST1541 from farms in Kegalle and Kandy with hatchery 14. Further, one GP hatchery (i.e., hatchery 6) became positive when traced back, though the strains isolated (Kentucky ST314 and Typhimurium ST36) were different to that of its corresponding hatcheries (hatchery 2 and 7) and could not be linked to any possible transmission. Nevertheless, the present study reports 10/15 associated hatcheries; nine parent hatcheries and one GP hatchery, infected with one or more *Salmonella* strains. These parent hatcheries supply day old chicks to broiler farms throughout the country, thus providing insights into possible transmission of *Salmonella* and subsequent outbreaks in poultry. More importantly, a *Salmonella* infected GP hatchery could be an impending threat to the poultry industry as a whole, as the majority of the broiler parent requirement is supplied by three GP hatcheries in Sri Lanka, while the rest are imported.

Thirty six known poultry *Salmonella* outbreaks which occurred in 2010, 2012 and 2017-2018, were studied using one isolate from each outbreak. On several occasions phylogenetic associations between the sequences were highly suggestive of *Salmonella* transmission; although the available metadata is not sufficient (i.e., no data on the locations and the dates of outbreaks) to make an epidemiological link. For example, in 2010 and 2012 Enteritidis ST11 had a distinct cluster of identical sequences (SSL133, SSL136 and SSL137), and Gallinarum ST78 had sequences with few SNPs difference (SSL 143 and SSL149, SSL144 and

SSL145, SSL146 and SSL147). Further, there could be probable re-emergence of *Salmonella* outbreaks as some of the previous outbreak isolates (2010 and 2012) show identical phylogenetic associations to that of *Salmonella* sourced from farms and hatcheries in 2017. Examples for such associations are: Kentucky ST314 outbreak isolates SSL132 with SSL002 and SSL007 isolated from two farms in Kurunegala; and Bareilly ST203 outbreak isolates SSL152 with SSL127 and SSL128 sourced from hatchery 14.

AMR findings suggested a notably high percentage of non-susceptibility/resistance for quinolones, which comprised 41.5% (CI 95% [33.9-49.1]) resistance for nalidixic acid as well as intermediate resistance of 45.1%, CI 95% [37.5-52.7] to ciprofloxacin and 35.4%, CI 95% [28.0-42.7] to enrofloxacin. This is in contrast to previous studies done in Sri Lanka, where all *Salmonella* isolates (n=18) of poultry origin were found to be susceptible (Kottawatta *et al.*, 2014) and only one isolate (n=15) was resistant (Kalupahana *et al.*, 2017) to enrofloxacin. The present study is arguably more comprehensive in its scope, with more isolates (164) from a more diversified range of poultry sources. The primary target of quinolone action in *Salmonella* is DNA gyrase, thus quinolone-resistant *Salmonella* frequently contain a mutation in the quinolone resistance-determining region of *gyr*A (Piddock, 2002; Eaves *et al.*, 2004) while mutations in *gyr*B, and topoisomerase IV genes *par*C, and *par*E are considered relatively rare. In the present study, some well-known allele variants in the *gyr*A gene were associated with quinolone resistance. Examples are Enteritidis ST11 (24/32); Asp-87 to Tyr, Gly and Asn as well as all 11 sequences of Gallinarum ST78; Ser-83 to Phe. Such point mutations in the *gyr*A gene can mediate resistance to nalidixic acid while it confers decreased susceptibility to ciprofloxacin (Turner *et al.*, 2006). Accordingly, all the above mentioned quinoline-resistant Enteritidis ST11 and Gallinarum ST78 presented resistance for nalidixic acid and intermediate resistance for ciprofloxacin and enrofloxacin. Further, it is

considered that complete resistance to ciprofloxacin is mediated by two or more mutations in the *gyr*A gene or other topoisomerase genes (Turner *et al.*, 2006).

With regard to beta-lactams, phenotypic ampicillin resistance was revealed as 20% (CI 95% [14.0-26.3]) and was in 99% concordance with the possession of extended-spectrum beta-lactamase (ESBL) gene, bla_{TEM-1}. This is supported by Kottawatta *et al* (2014), who reported 33.3% (6/18) resistance in poultry *Salmonella* against ampicillin. In addition to the antibiotics tested, colistin was considered an important antibiotic of interest to human health identified through the Fleming Fund programme as relevant to Sri Lanka (Appendix VI). However, with the compliances of the PC3 laboratory, it was not feasible to handle liquid media, thus *Salmonella* isolates in the present study could not be tested against colistin. The present study reports MDR (i.e., defined as resistance to at least three classes of antimicrobials) in 20% (33/164) of the isolates, all including resistance for beta-lactam (i.e., ampicillin) and quinolone (nalidixic acid and/or enrofloxacin). Significantly, 82% (27/33) of the MDR phenotypes in the present study belonged to *S*. Kentucky ST314 followed by *S*. Chester ST2063 (5/33) and *S*. Corvallis ST1541 (1/33). This is in agreement with a recent study, where 15.2% (5/33) of the isolates sourced from chicken meat, including two *S*. Kentucky ST314 strains as well as 5% (2/40) of human origin isolates belonging to *S*. Chester ST2063, were observed to be MDR (Tay *et al.*, 2019). Further, 94% (31/33) MDR phenotypes in the present study were isolated from *Salmonella* in carriage (i.e., broiler farms and hatcheries).

In the present study, over 98% concordance between resistance genotype and phenotype was observed for beta-lactams and tetracycline, while concordance was relatively low for quinolones i.e.,74% each for nalidixic acid and ciprofloxacin and 81% for enrofloxacin. The discordances could be due to an isolate being phenotypically susceptible while resistance determinants were present in the resistome (Neuert *et al.*, 2018). Such a mismatch could be

associated with the breakpoints used for phenotypic testing, absence of the genes (such as plasmid-encoded) detected during sequencing when phenotypic testing is performed on a different colony (Neuert *et al.*, 2018) and presence of silent genes such as bla_{CMY-2} and *tet* variants (Heider *et al*., 2009). Conversely, the discordances could also occur when an isolate that is genotypically predicted to be susceptible, displays phenotypic resistance (Neuert *et al.*, 2018). Some of the possible reasons for such disparities are the presence of resistance determinants not included in the reference database, unknown/ novel resistance mechanisms and resistance mechanisms that are not frequently detected (such as impermeability or efflux pump) (Neuert *et al.*, 2018). Of the quinolone resistant phenotypes in this study, only some possessed genes associated with quinolone resistance (e.g. *qnr*D1 and *qnr*S1) while chromosomal point mutations in *gyr*A, *gyr*B, *par*C and *par*E were not detected through the Nullarbor bioinformatics pipeline (version: 2.0.20181010; https://github.com/tseemann/nullarbor) with default settings.

The global increase of resistance for fluoroquinolones and beta-lactams may potentially be associated with the use of these as growth promoters in food-producing animals (Crump *et al.*, 2015), though they are banned for growth promotion purposes in Sri Lanka. However, the use of antibiotics for prophylaxis is believed to be a common practice, particularly enrofloxacin and amoxicillin being recorded as the most frequently used antibiotics in commercial broiler farms in Sri Lanka (Herath *et al.*, 2015; Lowe *et al*., 2019). (This is also revealed in the study in chapter 5 and discussed in 5.4.5). It is noteworthy that as both fluoroquinolones and beta-lactams are used to treat human *Salmonella* infection in Sri Lanka (Tay *et al.*, 2019), resistance to these can have severe impact on treatment options. On a positive note, all the isolates in the present study were found to be susceptible to other beta-lactams (cephalosporins and carbapenems) tested: namely, cefotaxime and meropenem.

The present study marks the first comprehensive investigation of the genetic diversity, phylogenetic relationships and AMR of *Salmonella* in Sri Lankan poultry. Consequently, this chapter provides a significant baseline for the WGS- based population structure of *Salmonella* in poultry. Furthermore, the data derived through WGS will also be useful in future studies to inform *Salmonella* control strategies as well as to determine any potential zoonotic transmissions associated with the known human pathogenic NTS revealed, such as Enteritidis ST11 and Kentucky ST314.

6.6 Conclusions

The present study reported the presence of some MLST types of *Salmonella enterica* for the first time in Sri Lankan poultry. The study revealed quite a few different NTS, however the most prevalent strains were *S*. Kentucky ST314 (26.8%, CI 95% [20.0-33.6]) and *S*. Enteritidis ST11 (19.5%, CI 95% [13.4-25.6]). *Salmonella* transmission between farms and hatchery to farm could be inferred through the genetic relatedness of the isolates and available metadata. Further, notably high percentage of quinolone non-susceptibility were revealed in the present study, which included resistance to nalidixic acid (41.5%, CI 95% [33.9-49.1]) and intermediate resistance to ciprofloxacin (45.1%, CI 95% [37.5-52.7]) and enrofloxacin (35.4%, CI 95% [28.0-42.7]).

Chapter 7: General Discussion

This chapter draws the major research findings of the thesis into a final discussion. It explores how the findings of the different chapters are interrelated, along with their potential impact, recommendations and how they inform existing knowledge and practices involving *Salmonella* in Sri Lankan poultry. Potential avenues for future research are also suggested.

7.1 Introduction

Centred on the cross-sectional study in broiler farms in the five highest poultry-dense districts and associated hatcheries[59], this thesis aimed to study different aspects of poultry salmonellosis. Thus, the first three analytical chapters (i.e., chapters 3, 4 and 5) are based on *Salmonella* carriage in broiler farms and associated hatcheries, risk factors for *Salmonella* carriage and antibiotic usage at the farm level. Using all the *Salmonella* isolates which were sourced from known poultry outbreaks as well as from the cross-sectional study, chapter 6 focuses on whole-genome sequence (WGS) based analysis to understand the population structure of *Salmonella,* phylogenetic relationships and antimicrobial resistance of *Salmonella*.

The research described in this thesis was based on sampling and data collection carried out from April to December 2017 and initial laboratory isolation of *Salmonella* performed in the Veterinary Research Institute (VRI), Sri Lanka. These were followed by material transfer from Sri Lanka to PC3 facility (Massey University) in New Zealand, a lengthy process (January to November 2018) involving substantial paperwork as well as multi-institutional collaboration between the two countries. The next stage consisted of further identification of *Salmonella*

[59]Associated hatcheries: In the case of *Salmonella* positive farms, the broiler hatcheries from which the chicks were brought in were also included in to investigate a possible source of infection.

through WGS-based analysis, antimicrobial resistance, use of bioinformatics and statistical analysis.

The initial part of the research carried out in Sri Lanka (field visits for sampling and data collection and laboratory isolation) was quite similar to the routine work I perform as a veterinary researcher at VRI. However, in the subsequent stages of this study, such as the preparation work for material transfer, working in the PC3 environment and library preparation for WGS analysis, I used tools and techniques that were completely new to me, some of which I found challenging. In addition, I learnt how to use R and R studio for the purpose of performing statistical analyses (such as logistic regression) in the thesis and used several different software and tools for bioinformatics. In this regard I was fortunate in getting to work alongside other technically sound and highly skilled researchers, who enabled me to learn and apply new techniques where necessary.

All research questions (under 1.3 in Chapter 1) were effectively addressed in the discussion of each research chapter and under 7.2 in this chapter. Further, each analytical chapter consists of novel and valuable data generated through the study. Chapter 3 presents the first study on *Salmonella* carriage in Sri Lankan broiler farms while chapter 4 marks the first of its kind to identify putative risk factors for *Salmonella* carriage in Sri Lankan poultry. Further, chapter 5 describes the first comprehensive study on exploring antibiotic use (i.e., both prophylactic and therapeutic) in broiler farms with varying capacity and operating systems in Sri Lanka. The last analytical chapter i.e., chapter 6, reports the first WGS-based analysis on genetic diversity and phylogenetic relationships of *Salmonella* in Sri Lankan poultry.

7.2 Potential impacts of the significant findings

In this section, the important findings are discussed with regard to their relevance to the poultry industry as well as their public health aspects, following a brief overview on Sri Lankan poultry and salmonellosis.

In Sri Lanka, the poultry industry is at the forefront of all livestock industries (DAPH Livestock Statistics, 2015). Unlike other meat types, consumption of chicken is not at odds with any major ethno-religious beliefs in Sri Lanka (Alahakoon *et al*., 2016; De Silva *et al*., 2010). Accordingly, chicken is reported as the most popular type of meat in the country, whereas eggs are also widely consumed due to their greater affordability when compared to other sources of animal protein (DAPH Livestock Statistics, 2015). Poultry meat production is a thriving industry in Sri Lanka, fulfilling the domestic demand for chicken in its entirety and exporting a minute surplus at present (DAPH Poultry forecast, 2017). However, the future prospects could hardly be realised unless local meat products conform to the international hygienic standards. The high prevalence of foodborne pathogens of public health relevance would prove a significant threat in this regard.

Poultry salmonellosis remains one of the major causes of economic loss in the Sri Lankan poultry industry (DAPH Annual report, 2017; 2018) despite the control measures undertaken. Studies on poultry salmonellosis in Sri Lanka have so far been limited to confirmed and/ or suspected salmonellosis cases so that *Salmonella* prevalence in poultry holdings have remained undetermined. Non-typhoidal *Salmonella* (NTS) have been identified as important foodborne zoonotic pathogens in Sri Lanka (Ministry of Health, 2013). However, the severity of their threat has often been overlooked and/or undermined, due to lack of data as well as the high and more immediate impact of other infectious diseases such as dengue (Ministry of Health, 2018).

7.2.1 *Salmonella* carriage (Related to research questions 1, 2 and 3 in 1.3 of chapter 1)

The cross-sectional study carried out in this thesis revealed *Salmonella* carriage in broiler farms to be as high as 32.2%, CI 95% [23.6-40.7]. A comparison could not be made with India and other countries in the region due to fragmented data on farm-level prevalence of *Salmonella*. However, present findings are alarming in comparison to those from the European union, where *Salmonella* was found in 3.31% of broiler flocks (or 11,730 flocks) in 2017 (EFSA and ECDC, 2018). Often the causes for *Salmonella* carriage in a broiler farm are not clearly understood as there can be multiple potential sources simultaneously, such as hatchery-borne infection, feed, rodents and carry-over infection from previous flock in the same holding. The risk factors identified for *Salmonella* carriage along with the recommendations for broiler farms are presented in chapter 4 (under 4.5).

High *Salmonella* carriage in poultry is concerning as there can be numerous destructive impacts on the sustainability of the industry. It is usual for motile serovars to colonise the alimentary tract of birds without producing any clinical illness, so that the infected birds appear healthy (Van Immerseel *et al.*, 2005). However, an immune suppression following overcrowding, environmental change (temperature and humidity), or a concurrent viral infection (such as infectious bursal disease) can trigger poultry's susceptibility to *Salmonella* colonisation (Bailey, 1988), thus developing into clinical illness and potential outbreaks. High morbidity and mortality mean huge production losses to the poultry industry, as well as an economic loss to the farmer due to there being no proper compensation system. Additionally, the cost of veterinary services and medicine following an illness may significantly impact on broiler farming (Rifky, 2016; Bandara and Dassanayake, 2006). Use of one or more antibiotic preparations when faced with an early outbreak (i.e., low mortality and/or few sick birds) is believed to be a common practice in order to sustain the production cycle until 38-42 days. Further, antibiotics for prophylaxis, particularly frequent use of

enrofloxacin and amoxicillin being recorded in this study (in chapter 5 and discussed in 5.4.5) in agreement with the previous studies (Herath *et al.*, 2015; Lowe *et al*., 2019).

Thus, while the impact of the disease - such as production loss - could be concealed, it leads to prolonged antibiotic use and consequences thereafter. Further, broilers with high *Salmonella* carriage can cause extensive contamination at slaughter and/ or processing plants and gain entry into the human food chain (Barrow, 2007). This, understandably, is a serious public health concern.

7.2.2 Non-motile serovars (Related to research question 4 in 1.3 of chapter 1)

Based on earlier findings, it was considered that poultry-adapted, non-motile *S.* Gallinarum and *S.* Pullorum (causing fowl typhoid and pullorum disease respectively) were predominant in Sri Lankan poultry (Gunawardena *et al*., 2006; Priyantha *et al*., 2007; Liyanagunawardena *et al*., 2012). However, only motile serovars were found from the cross-sectional survey (broiler farms and their associated hatcheries) carried out in this thesis, which, incidentally, is the first study on *Salmonella* carriage in Sri Lanka. While this finding cannot be explained in full, potential reasons for not obtaining non-motile isolates include the implementation of better control programmes for non-motile *Salmonella* in breeder flocks, and/ or evidence that the pathogenesis of these serotypes rarely involves intestinal colonisation - thus reducing the likelihood of isolating them from faeces using boot socks or cloacal swabs. This is discussed in detail in chapter 3 (under 3.5).

As revealed in the thesis, two multi-locus sequence typing (MLST) types of serovar Gallinarum, i.e., Gallinarum ST78 (*S*. Gallinarum) and Gallinarum ST92 (*S*. Pullorum), were identified as linked to outbreaks. The present study examined a total of 36 isolates sourced from poultry salmonellosis outbreaks (i.e., one isolate each from an outbreak), out of which fifteen (eleven Gallinarum ST78 and four Gallinarum ST92) isolates were non-motile.

However, in the absence of a systematic surveillance programme, it cannot be concluded that non-motile *Salmonella* predominate among outbreaks in Sri Lanka.

7.2.3 Motile serovars (Related to research question 4 in 1.3 of chapter 1)

The present study provided insight into the diversity of NTS in poultry by identifying nine MLST types reported for the first time in Sri Lankan poultry. Of those, *S*. Kentucky ST314, *S*. Enteritidis ST11 and *S*. Typhimurium ST36 were the most prevalent serovars reported, all of which are known human pathogens. Additionally, there are other reports of NTS in eggs (Kalupahana *et al.*, 2017) and chicken meat (Tay *et al.*, 2019). Further, Tay *et al* (2019) also reported NTS serovars (such as *S*. Enteritidis ST11, *S*. Corvallis ST1514, and *S*. Mbandaka ST1602) from human clinical specimens. Thus, collating the findings of the present study with those of previous studies, insights can be gained into an impending public health threat, one that is further exacerbated given the high demand for chicken meat among Sri Lankans.

In this situation of known human pathogenic NTS predominating in poultry carriage, strict hygienic and biosecurity measures need to be implemented to minimise contamination from farm to fork. Although the present findings were from broiler farms, it is highly likely that a similar situation exists in the layer farm settings as well. Thus, the entire poultry industry should focus more on consumer-safety. This need has been identified and included in the recently developed national policy on salmonellosis in poultry (DAPH *Salmonella* policy, 2017) to be fully implemented by 2025. As such, it would be highly pertinent to explore the possibility of conforming to international hygienic standards to secure the Sri Lankan export market share.

7.3 Furthering existing knowledge

The economic and technological constraints of Sri Lanka as a developing country have severely impacted the research efforts on salmonellosis. Most of the available findings have

been generated through case studies and/ or serological studies while actual *Salmonella* prevalence remained unknown. Further, the reliance on conventional methods (i.e., culture identification followed by biochemical tests) for diagnosis as well as the inadequacy of facilities have even hindered the identification of common serotypes.

In this context, the data generated from the present research are of immense importance in broadening and strengthening the knowledge base on salmonellosis in poultry in Sri Lanka. Thus, this thesis will inform the national control programme, increase awareness (such as on risk factors for *Salmonella* carriage and antibiotic use) among stakeholders and provide reliable baseline data for future research efforts.

7.3.1 Upgrade control programme and the National Policy

The *Salmonella* control programme in Sri Lanka dates back to 2001 (Gunawardena *et al*., 2003), when there were massive outbreaks in commercial poultry owing to fowl typhoid (caused by S. Gallinarum) and pullorum disease (caused by S. Pullorum). In addition to causing high mortality, the birds that survive from these diseases often become lifelong carriers, causing both vertical and horizontal transmission (Shivaprasad, 2000). Thus, *Salmonella*-carrier status plays a significant role in fowl typhoid and pullorum disease transmission. With this disease epidemiology, continents such as Europe and North America have achieved success in controlling these two diseases through 'test-and-cull' policies in the mid-20th century (Barrow *et al.*, 2012). Similarly, the major concern of the control programme initiated in Sri Lanka was to keep *Salmonella*-carrier status at a level less than 1% in poultry breeder flocks (Gunawardena *et al*., 2003) through routine screening with Whole Blood Agglutination Test (WBAT). Subsequently, monitoring of hatcheries for *Salmonella* isolation and inspection of breeder farms for improvement of biosecurity measures were also added as control measures, and the programme was established as a national control programme by the government regulatory body, the Department of Animal

Production and Health (DAPH). Another means of control is vaccination, and countries with developing industries such as South Korea have used SG9R live vaccine to control fowl typhoid (Barrow *et al*., 2012). Likewise, breeder flocks in Sri Lanka were permitted to use SG9R live *Salmonella* vaccine as an option in controlling disease for three years from 2012 (DAPH Annual report, 2012).

A National Policy on Salmonellosis in poultry is a long-required need for the poultry industry in Sri Lanka. In 2017 DAPH along with all the stakeholders proposed a national policy to be implemented within the next few years (DAPH *Salmonella* policy, 2017). The national control programme at present covers only the breeder flocks (i.e., breeder farms and hatcheries) whereas new regulations have identified every aspect of poultry industry from farm to fork. Thus, an appropriate regulatory system to monitor *Salmonella* in commercial poultry and processing plants is also proposed to be implemented (DAPH *Salmonella* policy, 2017). In this regard, recommendations for commercial broiler farms based on the risk factors identified in this thesis (mentioned in pages 85- 87) could be of immense importance and be incorporated into the regulations step wise.

The control programme to date is designed to control fowl typhoid and pullorum disease in breeder farms/hatcheries. However, focusing on *Salmonella*-carrier status in breeder flocks and possible vertical transmission might not be adequate for controlling motile *Salmonella*. In my experience with WBAT in the field, *Salmonella*-carrier status could only be detected for serogroup D (i.e., *S.* Gallinarum, *S*. Pullorum and *S*. Enteritidis). It is considered that having originated from *S*. Pullorum/Gallinarum, *S*. Enteritidis possesses the same group D somatic antigen (Thomson *et al.*, 2008). The WBAT and related tests can be used for the diagnosis of *S*. Enteritidis infection, however the sensitivity is low (OIE, 2018a). The timely need of including motile or NTS into the control programme, which I have highlighted, has

received priority in the recently developed National Policy on Salmonellosis in poultry (DAPH *Salmonella* policy, 2017).

A lack of systematic surveillance or research-based evidence is another drawback in directing the national control programme. In this regard, the present thesis, with its in-depth analyses of different aspects of *Salmonella* in Sri Lankan poultry, would be of immense importance. The knowledge thus generated can inform the implementation of more appropriate prevention and control strategies in order to strengthen the national control programme.

7.3.2 Awareness among stakeholders

The important findings and knowledge gained through this research, especially in terms of risk factors for *Salmonella* carriage, public health significance of motile serovars and data related to antibiotic use, need to be disseminated among the government and industry stakeholders through the relevant platforms. As a veterinary researcher from VRI, I am well placed to open up communication pathways in this regard: through direct communication with the higher management of the DAPH, as well as through poultry review meetings (where all the stakeholders meet) held on regular basis with the patronage of the DAPH. Potential risk factors for *Salmonella* carriage (recommendations mentioned under 4.5) and raising antibiotic awareness (recommendations mentioned under 5.5) are some of the relevant topics for such discussion. With successful two-way communication, these farmers/ companies might have insights or questions to inform further research directions.

7.3.3 As a reliable baseline for future research

As the most recent and the most comprehensive work related to different aspects of poultry salmonellosis, this thesis will be very informative in designing future research. One such example is the prospective research collaboration proposed between the Medical Research Institute and VRI on “Integrated laboratory surveillance of foodborne pathogens in Sri

Lanka", funded by the World Health Organisation. *Salmonella* and *Campylobacter* have been identified as the foodborne pathogens of interest to be studied in this project. While the present findings will be extremely valuable to inform the project on *Salmonella*, I, as a veterinary researcher from VRI, am also directly involved in the project and contributing my expertise towards the success of this effort.

7.4 Future perspectives

Additional insights can be gained through further analysis of the data generated through the studies described in this thesis. One such example is a more detailed analysis of WGS data produced, and comparisons with other publicly available data. The broad molecular-epidemiological perspective presented in this thesis (chapter 6), can be further analysed and utilised in future research. *Salmonella* data derived from this research (i.e., from poultry) could be used to determine any potential zoonotic transmission within the country by analysing the relationship with that of human derived *Salmonella* in Sri Lanka. This is particularly relevant with regard to *Salmonella* strains such as Enteritidis ST11, which was identified as widespread in the present study (chapter 6), and which is also the most frequently reported strain causing human infections around the world. A global genomic comparison of common *Salmonella* strains identified in the present study (Enteritidis ST11, Kentucky 314 and Typhimurium 36) could be made by analysing genetic similarity in the international data. Additionally, there is potential for further study with regard to antimicrobial resistance determinants of *Salmonella* and important genes for quinolone resistance and beta lactamase resistance (i.e., observed through phenotypic resistance).

As a part of the cross-sectional study, a questionnaire-based study (similar to that of broiler farms) was conducted in all the associated hatcheries (15), with the participation of 13 parent hatcheries and two grandparent hatcheries. This comprised more than 50% of broiler hatcheries in Sri Lanka. Consequently, a rich data set was gathered and secured in a

database in ^{m}EpiLab, however it was not used in the formation of this thesis. Analysing these data would be very important in identification of biosecurity practices in hatcheries in Sri Lanka.

There are potential avenues for future research by furthering the knowledge gained from this thesis. The risk factor analysis done in chapter 4 (risk factors for *Salmonella* carriage in broiler farms) could be further expanded with Bayesian network modelling (Han *et al.*, 2018; Kratzer *et al.*, 2020) to understand causal pathway effects and to reveal complex relationships between risk factors and with the outcome.

A systematic surveillance on poultry salmonellosis outbreaks in Sri Lanka, including identification of the isolate (up to serovar/ MLST) along with all required metadata, would be highly beneficial towards determining the status of field outbreaks, as well as to successfully control non-motile *Salmonella* and recommending better control strategies.

7.5 Concluding Remarks

Given the depth and breadth of the present study, this thesis makes a rich contribution towards enriching the knowledge base on *Salmonella* in Sri Lankan poultry. All the analytical chapters present significant novel data, most of which are generated for the first time with regard to Sri Lankan poultry. Thus, the thesis is of importance in providing valuable insights on salmonellosis which are specifically applicable to Sri Lankan poultry, and, at the same time, highly relevant to other countries with developing poultry industries. In particular, the thesis could be informative in implementing better prevention and control strategies to strengthen the national *Salmonella* control programme in Sri Lanka. Additionally, the sampling, laboratory techniques, data analysis and bioinformatics-based analysis performed in this thesis may be readily applied to any other context in a similar molecular epidemiological investigation.

In conclusion, I draw on the perceptive words of Dr. K. V. Nagaraja from his historical lecture at the annual meeting of The American Association of Avian Pathologists held on August 2019: "*The pullorum disease control/elimination story serves as a prime example of what can be achieved when scientists, government, and industry cooperate to solve health issues*" (Schat *et al.*, 2021). Just as cooperation among experts and stakeholders had been key to eliminating a disease that had devastated poultry industry in early 20th century, it would, I believe, be a critical factor that determines the future success of *Salmonella* control programme in Sri Lanka.

Appendix I: Ethics and Permission

A 1.1 Provisional Ethical Clearance

A diamond thus far and beyond
University of Peradeniya
Faculty of Veterinary Medicine & Animal Science
Committee for Ethical Clearance on Animal Research
Peradeniya 20400
Sri Lanka

7 April 2017

Provisional Ethical Clearance Certificate

Faculty of Veterinary Medicine and Animal Science

University of Peradeniya

Committee for Ethical Clearance on Animal Research of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya is in the process of reviewing your proposal and protocol titled **"Phenotypic and Molecular characterization of Salmonella isolates in the chain of broiler industry in Sri Lanka."**

Proposal ID: VER - 2017- 004

Investigators of the project:

Principal Investigator: Nilukshi Liyanagunawardena

Co- Investigators:

Dr. Palika Fernando - Head/Bacteriology, VRI

Dr. Ruwani Kalupahana - Senior Lecturer, FVMAS, Uni Peradeniya

Dr. Chinthana Karunaratne - Veterinary Investigation Officer/Wariyapola

Sampling methods in the study: Boot socks- litter and cloacal swabs from birds

I would like to grant provisional approval for your proposal at this point and you will receive full approval of the committee in our next meeting.

Thank you,

Dr. R.A.D. Eranda Indrajith (BVSc, MS, PhD, DACVB, DACAW)
Chairperson
Committee for Ethical Clearance on Animal Research
Faculty of Veterinary Medicine and Animal Science
University of Peradeniya

A 1.2 Ethical Clearance

A diamond thus far and beyond
University of Peradeniya
Faculty of Veterinary Medicine & Animal Science
Committee for Ethical Clearance on Animal Research
Peradeniya 20400
Sri Lanka

Ethical Clearance Certificate

Faculty of Veterinary Medicine and Animal Science

University of Peradeniya

Committee for Ethical Clearance on Animal Research of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya has reviewed the research proposal and protocol titled **"Phenotypic and Molecular characterization of *Salmonella* isolates in the chain of broiler industry in Sri Lanka"** submitted by **Dr. N. Liyanagunawardena** on **01st of April 2017.**
The committee has decided to approve the referenced protocol subjected to the following conditions.

Your proposal ID is **VS/ERC/17/04** and any change or amendment to the submitted protocol should not be adopted without prior approval from the same committee.

This certificate is valid until **25/07/2018** and for further extension of the certificate an extension must be submitted to the committee one month before the termination date.

Any unexpected or serious event occurred during the trial must be informed to the committee.

A consent should be obtained from all participating subjects/ owners of animals.

A copy of the final report of the study must be submitted to Committee for Ethical Clearance on Animal Research.

Failing to comply with the conditions of the ethics review committee, the approval may terminate.

Dr. R.A.D. Eranda Indrajith (BVSc, MS, PhD, DACVB, DACAW)
Chairperson
Committee for Ethical Clearance on Animal Research
Faculty of Veterinary Medicine and Animal Science
University of Peradeniya

A 1.3 Material Transfer Agreement

Department of Animal Production and Health

P.O. Box: 13, Peradeniya 20400, Sri Lanka.

Reference: 300003300

Material Transfer agreement

Recipient full name	Massey University acting through its mEpi Lab
Recipient address	School of Veterinary Sciences, Massey University Tennent Drive Palmerston North 4410 New Zealand.

DAPH investigator	Dr. Palika Fernando
Recipient investigator	Nilukshi Liyanagunawardena (PhD Candidate)

Department of Animal Production and Health (DAPH) owns certain biological material described below (the "Material"). A student of the Recipient, the Recipient's Investigator, wishes to use the Material to conduct certain experiments using or relating to the Material, in the Recipient's laboratories, described below (the "Experiments"). DAPH is willing to supply the Recipient with the Material for the Recipient's Investigator to conduct the Experiments. Both parties agree to be bound by the attached *Conditions of Engagement*.

Name of the recipient Laboratory where Materials are stored and research will be carried out.	mEpi Lab
Address of the recipient Laboratory	School of Veterinary Sciences, Massey University Palmerston North 4410 New Zealand

Material type	*Salmonella* isolates
Material number	167 X 3 ampoules
If bacterial isolates	~~Culture plates/Semi solid/Freeze dried vials~~/ Freeze dried ampoules/ ~~C~~ ~~ryovials of bacterial dissolved broth~~
Labeling system	~~Printed on sticker papers and pasted~~ / ~~Printed in stationery paper and pasted~~ /~~Printed and pasted in special papers and pasted~~/ written on ampule or vials/other please specify.
Unit volume	0.5ml
Date of samples are filled	13/09/2018

Experiment
Antimicrobial sensitivity testing (disc diffusion) and whole genome sequencing

Publication
Submission to an international high ranking peer reviewed journals such as Applied and Environmental Microbiology is planned.

Authorized signatory-DAPH

Name: Dr. NihalWedasinghe

Title/Post: Director General, DAPH

Signature:

14.09.2018

Dr. Nihal Wedasingha
Director General
Dept. of Animal Production & Health
P. O. Box 15, Gatambe
Peradeniya

Authorized signatory-Massey University

Name: Dr. Viv Smith

Title/Post: Director Research Operations

Signature:

Dr Viv Smith
Director Research Operations
Research and Enterprise
Massey University

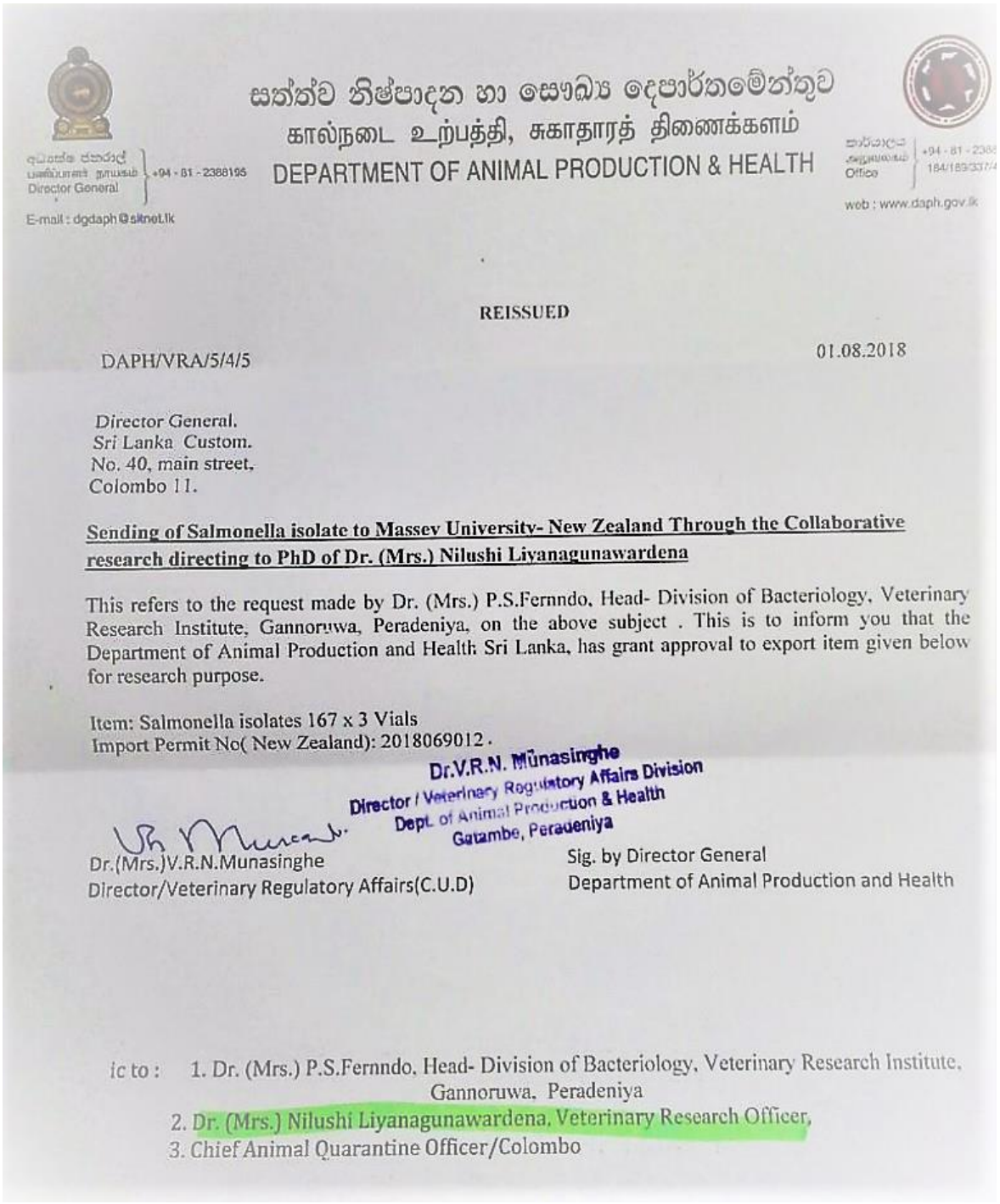
20 Oct 2018

Date: Date:

Conditions of Engagement – Material Transfer Agreement

1. Department of Animal Production and Health, Sri Lanka will supply a sample of the material to the recipient, at the recipient expense, after this agreement is signed.
2. For the purpose of this agreement, "Material" includes all organism and other biological material included in the sample, all progeny of that biological material and all cell, protein, nucleic acid, chemical or other component derived from that biological material or its progeny.
3. The material remains the property ofDepartment of Animal Production and Health,Sri Lanka. There is no transfer or licence, or implied transfer or licence, of right in the material from Department of Animal Production and Health,Sri Lanka to the recipient other than limited rights set out in this agreement.
4. The recipient will retain the material on its premises and will not permit the material; or any part of it to come into the possession or control of any other organization or any individual other that its employees.
5. The recipient will use the material only to carry out the experiments. The experiments must be for research purposes only and not subject to any licensing or other commercial obligations.
6. The recipient will not use the material in any experiments involving humans and will not use the material in contact with any other material that are to be infused into humans. The recipient warrants that it will comply with all relevant laws,regulations and guideline in handling material and conducting the experiments. If the experiments involve animals studies, the recipient warrants that the recipient's investigators has considered in vitro approaches to the research and has followed the appropriate guideline for animals experimentation regarding such work. The recipient confirms that any infectious or potentially infectious material will be handled only in suitable containment facilities, which meet all necessary regulatory requirements by fully trained and competent employees of the recipient.
7. The recipient will keep Department of Animal Production and Health, Sri Lanka informed of the experiments (including all intellectualproperty rights that arise directly from use the material in the experiments)("Results").The recipient will submit a written report to Department of Animal Production and Health, Sri Lanka at the end of the experimentswhich disclose all results.
8. Department of Animal Production and Health, Sri Lanka will keep the results in confidence, provided that this obligation will not apply in relation to any results to the extent:
    (a) they were already known to Department of Animal Production and Health, Sri Lanka; or
    (b) they are in or become part of the public domain or are otherwise independently developed or acquired by Department of Animal Production and Health, Sri Lanka without a breach of this agreement.

A 1.4 Permission for Material Transfer



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கால்நடை உற்பத்தி, சுகாதாரத் திணைக்களம்
DEPARTMENT OF ANIMAL PRODUCTION & HEALTH

අධ්‍යක්ෂ ජනරාල් කාර්යාලය / பணிப்பாளர் நாயகம் / Director General +94 - 81 - 2388195
E-mail : dgdaph@sltnet.lk

කාර්යාලය / அலுவலகம் / Office +94 - 81 - 2388 184/189/337/4
web : www.daph.gov.lk

REISSUED

DAPH/VRA/5/4/5 01.08.2018

Director General,
Sri Lanka Custom.
No. 40, main street,
Colombo 11.

Sending of Salmonella isolate to Massey University- New Zealand Through the Collaborative research directing to PhD of Dr. (Mrs.) Nilushi Liyanagunawardena

This refers to the request made by Dr. (Mrs.) P.S.Fernndo, Head- Division of Bacteriology, Veterinary Research Institute, Gannoruwa, Peradeniya, on the above subject . This is to inform you that the Department of Animal Production and Health Sri Lanka, has grant approval to export item given below for research purpose.

Item: Salmonella isolates 167 x 3 Vials
Import Permit No(New Zealand): 2018069012 .

Dr.V.R.N. Munasinghe
Director / Veterinary Regulatory Affairs Division
Dept. of Animal Production & Health
Gatambe, Peradeniya

Dr.(Mrs.)V.R.N.Munasinghe
Director/Veterinary Regulatory Affairs(C.U.D)

Sig. by Director General
Department of Animal Production and Health

ic to : 1. Dr. (Mrs.) P.S.Fernndo, Head- Division of Bacteriology, Veterinary Research Institute, Gannoruwa, Peradeniya
2. Dr. (Mrs.) Nilushi Liyanagunawardena, Veterinary Research Officer,
3. Chief Animal Quarantine Officer/Colombo

A 1.5 Permit to Import- MPI

Ministry for Primary Industries
Manatū Ahu Matua

PERMIT TO IMPORT RESTRICTED BIOLOGICAL PRODUCTS OF ANIMAL ORIGIN

This permit is issued under The Biosecurity Act, 1993. Any queries, please contact Animal Imports & Exports Group, MPI, PO Box 2526, Wellington, (Phone 64 4 8940100, Facsimile 64 4 8940733)

Authorising Officer : **Katherine Hall** on **25 Jun 2018**

for Director General, Ministry for Primary Industries, acting under delegated authority.

Permit for : AgResearch Hopkirk Research Institute
Cnr University Ave & Library Road
Massey University, Tennet Drive
Palmerston North
New Zealand
Attention : Roy Meeking
roy.meeking@agresearch.co.nz

Permit No : 2018069012
Replaces Permit No : 2018068715
Expiry Date : 25 Jun 2019
No of Consignments : Multiple
Import Purpose :

End Users :

Client Refs : 1015596

Shipped From : All Countries

Description of Items	Supplier	Quantity	Measure
Bifidobacterium choerinum (as per HSNO approval NOC002470)		Unlimited	Units
Clostridium bowmanii (as per HSNO Approval NOC002287)*		Unlimited	Units
Clostridium frigoris (as per HSNO Approval NOC002288)*		Unlimited	Units
Clostridium lacusfryxellense (as per HSNO Approval NOC002289)*		Unlimited	Units
Clostridium psychrophilum (as per HSNO Approval NOC002290)*		Unlimited	Units
Clostridium tagluense (as per HSNO Approval NOC100152)*		Unlimited	Units
Escherichia fergusonii (as per HSNO Approval NOC100010)*		Unlimited	Units

Description of Items	Supplier	Quantity	Measure
FASTPlaque TB kit (containing Mycobacteriophage D29, as per HSNO approval NOC100152, and Mycobacterium smegmatus)*	Biotek Laboratories	Unlimited	Units
Genetically modified Risk Group 1 microorganisms (including Bacteria, Viruses, Bacteriophages, Micro-eukaryotes, Algae, Fungi, Yeasts, Phytoplankton, Zooplankton, Protozoa & Microinvertebrates) (HSNO Approval GMC100216)		Unlimited	Units
Genetically modified Risk Group 2 microorganisms (including Bacteria, Archaea, Viruses, Bacteriophages, Microeukaryotes, Algae, Fungi, Yeasts, Phytoplankton, Zooplankton, Protozoa, & Microinvertebrates) (HSNO Approval GMC 100219) *		Unlimited	Units
Genetically modified non-mycobacterial Mycobacterium avium subspecies paratuberulosis (as per HSNO approval GMC000112)*		Unlimited	Units
Genetically modified Mycobacterium smegmatis (as per HSNO approval GMC000113)*		Unlimited	Units
Genetically modified Mycobacterium tuberculosis complex species (M. tuberculosis, M. bovis and M. pinnipedii (as per HSNO approval GMC000114)**		Unlimited	Units
Genetically modified Salmonella enterica subspecies enterica serovars Typhimurium, Brandenburg and Enteritidis (as per HSNO approval GMC 100169)*		Unlimited	Units
Genetically modified non-pathogeniic Escherichia coli (as per HSNO approval GMC 001340)		Unlimited	Units
Genetically modified animal cell lines (including immortalized and primary cell lines) from organisms within the Kingdom Animalia, Phylum Arthropoda, and Phylum Chordata as per HSNO approval GMC100227		Unlimited	Units

Description of Items	Supplier	Quantity	Measure
Genetically modified Campylobacter jejuni (as per HSNO approval GMC100219)*	School of Molecular Biosciences, Washington State University	Unlimited	Units
M13 phage display peptide library (HSNO approval GMD003576)		Unlimited	Units
Meat samples and meat drip (as per HSNO approval NOC100152)*		Unlimited	Units
Mycobacteriophages (as per HSNO approval NOC100152)*	Various	Unlimited	Units
Pure Salmonella enterica cultures (all subspecies and serotypes; includes unwanted organisms)***		Unlimited	Units
Risk Group 1 microorganisms (as cultures or within samples, derived from animals (not humans), plants and the environment, where those plants and animals are not diseased or displaying signs of disease or illness) as per HSNO approval NOC100151		Unlimited	Units
Risk Group 2 microorganisms (as cultures or within samples, derived from animals (not humans), plants and the environment, where those plants and animals are not diseased or displaying signs of disease or illness) as per HSNO approval NOC100152*		Unlimited	Units

Import Health Standard

MICROIC.ALL, 31 Jan 2010, Microorganisms from All Countries

Post Entry Type : Containment

Standard Reference : 154.03.02 (Facilities for Microorganisms and Cell Cultures: 2007a)

Containment Facility : AgResearch - Hopkirk Research Institute
Massey University
University Avenue
Palmerston North

Operator : Roy Meeking

Ref : 14207

Special Conditions : You must comply with the import health standard(s) listed in this permit. For further information contact MPI at the top of the permit or via the MPI website; http://mpi.govt.nz/law-and-policy/requirements/import-health-standards/

* Must be kept in a containment facility operating to Physical Containment Level 2 (PC2

** Must be kept in a containment facility operating under PC2/PC3 as per HSNO approval GMC000114

*** Must be imported into a containment facility operating at Physical Containment Level 3 (PC3) and must comply with the ss52/53 permission signed by Brendan Gould on 10 May 2018 (permission is valid until 30 June 2022).

All new organisms (including GMOs) must be accompanied by a letter from the exporter confirming that the organism imported is that described in the appropriate EPA approval.

A clerical error by EPA has an import approval listed as a development approval. This is GMD05025 (GMD003576), used by the Hopkirk Research Institute.

IMPORTANT INFORMATION FOR PERMIT HOLDERS AND AGENTS

1 This permit, and compliance with the provisions of the specific import health standard(s) and/or entry conditions, does not guarantee that the goods you import will be given biosecurity clearance. There are other restrictions in sections 27 and 28 of the Biosecurity Act 1993 which apply to the giving of biosecurity clearance. The Biosecurity Act may be accessed at www.legislation.govt.nz

2 You will need to ensure that the goods you import comply with the provisions of the specific import health standard(s) and/or entry conditions that are applicable. The import health standard may be amended during the course of your permit. Import Management will notify you of any significant changes to the import health standard and will re-issue the permit to accommodate these changes.

3 There are a number of other provisions in the Biosecurity Act 1993 which may affect you. If you commit an offence against the Biosecurity Act 1993, heavy penalties under section 157 of the Act might apply.

4 Apart from the Biosecurity Act 1993, there are other laws relating to or prohibiting the importation of goods. This permit, and compliance with the provisions of the specific import health standard(s) and/or entry conditions, does not absolve you of the need to comply with these laws.

5 Unless specifically identified in 'Description of Items' or 'Special Conditions' of the permit, NO new organisms, including genetically modified organisms, are permitted entry under this permit.

6 All cultures must be:
- clearly labelled with the scientific strain and name
- consigned in leakproof packaging
- free from contaminants

7 All new organisms must not be opened until received by the nominated containment facility.

Appendix II: Laboratory Protocols

A 2.1 Protocols on Culture, DNA Extraction and Disposal of *Salmonella* in the PC3

The *Salmonella* being handled in the PC3 lab are imported. The conditions of the CTO permission (20172020) from MPI state that the organisms must be unpacked, held, cultured, DNA extracted from, and stored in the PC3 laboratory. Only purified *Salmonella* DNA may leave the PC3 lab.

Salmonella can cause diarrhoea, fever and cramps. Users should be aware of the symptoms and should inform their general practitioner or healthcare provider of the possibility of *Salmonella* infection or person-to-person transmission through family exposure if presenting diarrhoeal symptoms themselves or with household contacts.

The imported *Salmonella* may be a risk to New Zealand's wildlife and/or livestock.

To avoid the potential for aerosols or spills, do not culture *Salmonella* in liquid culture.

Personal Protective Equipment

- A lab coat and disposable gown shall be worn for all manipulations involving *Salmonella*.
- Double gloves shall be worn when working in the Class II Biological Safety cabinet; the outermost layer of gloves shall be discarded as Dry Waste prior to exiting the Class II Biological Safety cabinet.
- Disposable overshoes shall be worn for all manipulations involving the use of *Salmonella*.

Biohazard Waste Disposal

- Before undertaking work each day the autoclaving/disposal requirements should be discussed with the Laboratory Services personnel.
- All dry waste (e.g. masks, paper, gloves, plastic loops) used within a Class II cabinet shall be collected in a plastic bag within a container in the Class II cabinet. When full, the top of the bag should be folded over to create a loose seal and sprayed with 5% SteriGENE Clear disinfectant.
- After 10 minutes, the discard bag should then be removed from the Class II cabinet and placed within an autoclave bag labelled "Biological Hazard" within the closed waste bins provided in each laboratory.
- All waste from the QiaAmp DNA extraction kit produced within a Class II cabinet shall be collected a labelled blue-capped Schott bottle. The bottle should be kept in the Class II cabinet. At the end of each day the exterior of the capped bottle shall be sprayed thoroughly with 5% SteriGENE Clear disinfectant. After 10 minutes the

Schott bottle should then be removed from the Class II cabinet and placed within the closed waste bins provided in each laboratory prior to removal from the laboratory for autoclaving. Lab services staff shall be informed of the presence of this waste in the autoclave load.

- General laboratory waste shall be collected in biohazard bags and closed using tape. The exterior of the bags shall be sprayed thoroughly with 5% SteriGENE Clear disinfectant and held in the closed waste bins provided in each laboratory.
- The closed waste bin shall then be taken out of the laboratory on a trolley to await autoclaving; Lab Services personnel shall be informed that waste is available for autoclaving. All waste should be autoclaved as soon as possible after bags/containers are placed in the Restricted Corridor 2.07 – preferably the bags should go straight into the autoclave.
- No sharps or anything that may puncture the bags are to be disposed of in the autoclave bags. Sharps are to be collected in the specialised containers provided. At the end of each culturing session the sharps containers are sprayed with 5% SteriGENE and removed for autoclaving.
- Where possible all work involving the inoculation and culture of *Salmonella* shall be undertaken using disposable plastic ware such as inoculating loops, pipettes/pipette tips, culture vessels, etc. The use of glass items is discouraged.

Spillage of microorganisms

- Full details of procedures to take if a spillage of microorganisms occurs can be found in the Hopkirk Institute PC3 Containment Manual.
- In every case of a spillage of organisms or infectious materials of any risk group a 'Bacterial/Infectious Material Spillage Report Form' must be completed and handed to a PC3 Manager or the Facility Manager. All report forms shall be retained by the Facility Manager.
- Refer to PC3 Containment Manual Appendix 2B: Personal Safety & the Procedure for Decontaminating Laboratories and Equipment following Accidental Spillage of Hazardous Micro-organisms

- *Refer to PC3 Containment Manual Appendix 8: Notice of Procedures for Dealing with Accidental Spillage and Contamination by Pathogenic Organisms*
- *Refer to PC3 Containment Manual Appendix 13: Bacterial / Infectious Material Spillage Report Form*

Spills Outside a Class II Biosafety Cabinet

- In the event of spillage of *Salmonella*, staff must follow the procedures specified for decontamination.
- Generally the greatest risk from a spill of infectious material is from contamination through aerosol generation, particularly from liquid cultures. In the PC3 laboratories in the Hopkirk there is continuous air movement and aerosols can be dispersed rapidly. It is therefore important to leave the

contaminated areas as quickly and as safely as possible.

- If clothing and footwear, or exposed body areas have possibly been contaminated, measures must be taken to get rid of the bulk of the infectious material before exiting the airlock. A change of clothing is provided in the airlock to each PC3 laboratory.
- Only when you are in a position of safety and the laboratory is contained should steps be taken to decontaminate.
- Do not enter the contaminated area for at least an hour. This allows aerosols to be diluted or removed through continuous air changes and liquid spillages to dry. This will substantially reduce the contamination risk. All contaminated or potential contaminated material must be decontaminated, preferably by autoclaving.
- If a breach in secondary containment has occurred within the PC3 laboratory (such as a break in the outer biohazard bag, but not the inner vessel/bag and there has been no spill of the bacteria) fix the containment problem by placing all containers affected by the breach in a third container/bag and report this to one of the PC3 Laboratory Managers.

Spills Inside a Class II Biosafety Cabinet

- Spills of *Salmonella* up to 1 ml may be treated by wiping or flooding with a suitable disinfectant solution.
- In the event of a larger spill, staff shall ensure the cabinet remains operating and, with gloved hands, lay down absorbent material wetted with disinfectant such as 5% SteriGENE Clear and allow a minimum time of 10 minutes to effect disinfection. Absorbent material shall then be discarded in the biohazard plastic-lined waste bins provided in every laboratory.

Resuscitation of *Salmonella* from freeze dried ampoules

- Pre-label blood agar plates
- Open up Class II Biological Safety cabinet, spray and wipe with 5% SteriGENE Clear and/or treat interior of cabinet with UV light for 20 minutes. Allow 5 minutes contact time with disinfectant before wiping off.
- Ensure disposable inoculating loops, disposable forceps, 70% ethanol, paper towels or tissues, glass cutter/file, and sharps container, broth, sterile transfer pipettes, discard container and sharps container are available within the Class II Biological Safety cabinet.
- Disinfect the outer surface of the ampoule by cleaning it with 70% ethanol and allow to dry. Tap the ampoule gently on the work bench to loosen the frieze dried material from the glass. Open the glass ampoule by scoring the glass just above the cotton plug with an ampoule cracker. Remove the cotton plug gently with sterile disposable forceps (discard cotton and forceps in discard container). With a sterile disposable transfer pipette add a few drops (0.1 – 0.5 ml) of sterile broth such as Brain Heart Infusion broth or Lysogeny broth to the ampoule. Using the transfer pipette carefully

mix the contents by gentle aspiration and transfer two or three drops to the surface of the appropriately labelled blood agar plate. Discard all the ampoule fragments plus the ampoule cracker in the sharps container. Discard the pipette into the discard container.

- Using disposable loops, streak the plate for single colonies
- Incubate plates at 37°C for 18-24 hrs
- Record on data-sheet.
- If growth appears pure after 18-24 hrs, subculture a single colony to a fresh blood agar for making DNA preps and/or freezing.
- If growth does not appear pure, record on datasheet (for MPI notification) and discard the agar plate.

Freezing of *Salmonella* for long term storage on Microbank beads

- Pre-label (using a permanent marker), Microbank vials.
- Open up Class II Biological Safety cabinet, spray and wipe with 5% SteriGENE Clear and/or treat interior of cabinet with UV light for 20 minutes. Allow 5 minutes contact time with disinfectant before wiping off.
- Ensure disposable inoculating loops, sterile transfer pipettes and discard containers (including a blue-capped Schott bottle containing 5% Sterigene) are available within the Class II Biological Safety cabinet. Double glove.
- Aseptically inoculate the cryopreservative fluid with 18-24 hr growth using a disposable loop. Aim for a visibly turbid suspension (approximately a 3-4 McFarland standard).
- Close vial tightly and invert 4-5 times to emulsify organism. DO NOT VORTEX! Allow vial to sit for at least 2 mins for binding to the beads
- Remove the cryopreservative liquid as completely as possible with a sterile transfer pipette discarding liquid and pipette into the provided disinfectant.
- Once the Microbank vials have been inoculated, the outer pair of gloves shall be removed and be discarded as Dry Waste. The Microbank vials and rack shall then be sprayed with 5% SteriGENE Clear and removed from the Class II Biological Safety cabinet. One further disposable glove shall be removed and this ungloved hand used to open the door of the PC3 lab and store the Microbank vials in the -20°C freezer in the PC3 lab annex.
- Record on data-sheet
- Upon completion of work in the Class II Biological Safety cabinet, the interior work surfaces shall be sprayed and wiped down with 5% SteriGENE Clear. Allow 5 minutes contact time with disinfectant before wiping off. The Class II hood shall also be sterilized using the built-in UV lamp for 20 minutes. Prior to exiting of the PC3 lab, all personal protective equipment shall be removed and hands washed and dried thoroughly.

Resuscitation of *Salmonella* from Microbank beads

- Pre-label blood agar plates
- Open up Class II Biological Safety cabinet, spray and wipe with 5% SteriGENE Clear and/or treat interior of cabinet with UV light for 20 minutes. Allow 5 minutes contact time with disinfectant before wiping off.
- Ensure disposable inoculating loops, straight wires and discard container are available within the Class II Biological Safety cabinet.
- While wearing gloves remove the required Microbank vials from the -20°C freezer (2.09 PC3 lab foyer) and place in the Mr Frosty box that is kept within the -20°C freezer. Transport the Mr Frosty box in a Polystyrene box.
- To enter the PC3 lab (2.09) remove one disposable glove use the ungloved hand to activate and open the inner door. Upon entry to the PC3 lab, place the Polystyrene box in the Class II Biological Safety cabinet and put on a disposable gown and double gloves.
- Using a disposable straight wire, aseptically remove a single bead and gently streak onto the plate (discard bead and wire into discard), streak for single colonies.
- Once all inoculations have been completed, remove and discard the outer pair of gloves. Spray the Microbank vials, Mr Frosty and polystyrene box with 5% SteriGENE Clear, wait 10 minutes and remove from the Class II Biological Safety cabinet. Remove one glove and open the door of the PC3 lab with the ungloved hand. Replace the Microbank vials and Mr Frosty in the -20°C freezer in the PC3 lab foyer (2.09). Gloves shall be discarded in the dry waste for autoclaving and hands washed and dried thoroughly.
- Incubate plates at 37°C for 18-24 hrs before subculturing a single colony to a fresh blood agar and incubating at 37°C for a further 18-24 hrs.

DNA Extraction using Spin Columns

Preparation

- Turn shaking heating block to 56°C and other block to 70°C
- Place bullet rack filled with water in 37°C incubator
- Freshly prepared (18-24 hr) single isolates on blood agar plates

Tips

- Always have pop-stoppers on tubes when vortexing and incubating
- Briefly centrifuge between each incubation and vortexing step (in Class II hood) to remove moisture and drops from lids.

Method

1. Dispense 180 uL of ATL buffer (briefly incubate at 37°C to dissolve precipitate) into Eppendorfs
    a. In Class II hood, remove 1 loop (half of red loop, or full yellow loop) of growth from plate and suspend in buffer
2. Add 20 uL of Proteinase K, pulse vortex (in Class II hood)

    a. Incubate at 56°C for 1-2 hours, in shaking heat block
3. Add 4-20 uL (4 µl = kit recommendation) of RNase A (100mg/ml), pulse vortex (in Class II hood) and briefly centrifuge
    a. Incubate at 37°C for 40 minutes (or at 4-20°C overnight)
        i. Pulse vortex at 20 minutes (in Class II hood)
4. Add 200uL of AL buffer, pulse vortex (in Class II hood)
    a. Incubate at 70°C for 10 minutes
5. Add 200 uL of ethanol (96%-100%), pulse vortex (in Class II hood)
6. Pipet mixture (with precipitate…that's the DNA) to labelled spin column placed in 2ml collection tube
    a. Do not "wet the rim"…be precise to place liquid below cap
    b. Centrifuge at 6000 g for 1 minute
    c. Change collection tube (discard old tube with filtrate into waste bottle)
7. Add 500 uL of AW1 buffer (don't wet rim)
    a. Centrifuge at 6000 g for 1 minute
    b. Empty collection tube into waste bottle
8. Add 500 uL of AW2 buffer (don't wet rim)
    a. Centrifuge at 14,000 rpm for 3 minutes
    b. Empty collection tube
    c. Centrifuge at 14,000 rpm for 1 minute
9. Replace collection tube with new one (discard old tube into waste bottle)
    a. Incubate spin column and tube at 37°C for 10 minutes
    b. Incubate sterile commercial DNA-ase free water at 37°C for 10 minutes
10. Add 25-75uL of pre-warmed DNA-ase free water to top of spin column
    a. Incubate another 10 minutes at 37°C
11. Centrifuge at 6000 g for 1 minute
12. Transfer eluted DNA to labelled Lo-bind Eppendorf tubes.

DNA-containing tubes and rack or box should be wiped with 5% SteriGENE Clear before being removed from the PC3 lab.
Upon completion of work in the Class II Biological Safety cabinet, the interior work surfaces shall be sprayed and wiped down with 5% SteriGENE Clear.

Allow 10 minutes contact time with disinfectant before wiping off. The Class II hood shall also be sterilized using the built-in UV lamp for 20 minutes. Prior to exiting of the PC3 lab, all personal protective equipment shall be removed and hands washed and dried thoroughly.

PC3 Laboratory and Deputy Laboratory Managers

- Responsibilities: The PC3 Managers for Room 2.09 shall provide support to the Operator and Facility Manager in the preparation and review of documentation, implementation of procedures and ensuring compliance with the Hopkirk Research Institute PC3 Containment Manual. They will also assist with fumigations (actual or test), with the training and instruction of laboratory

personnel in good work practices in the PC3 areas and with internal and external audits.

- Responsibilities: The PC3 Deputy Managers for Room 2.09 shall provide support to the Facility Manager and PC3 Manager in the review and implementation of procedures and ensuring compliance with the PC3 Containment Manual. They will also assist with fumigations (actual or test), with the training and instruction of laboratory personnel in good work practices in the PC3 areas and with internal and external audits.

Hours of operation

- For safety reasons, people should work within the Room 2.09 only during standard working hours. If personnel occasionally need to use Room 2.09 outside the hours of 0800 and 1700 they must have authorisation from a PC3 Manager or Deputy Manager for each such instance.
- Such authorisations will be recorded in a log book located in the Restricted Corridor 2.07.

A 2.2 Protocol on Library Preparation: *Salmonella*

In brief:

1) Normalise DNA: prepare genomic DNA to working concentrations – 0.3 ng/ul
2) Fragmentation and tagging of DNA – referred to as "tagmentation"
3) Clean up

Things to know before you begin:

-Determine which combination of indexes you will be using BEFORE you begin
-We want fragments between 250-2000 bp
-Have your DNA at working concentrations BEFORE you begin the tagmentation step
-Book RT-PCR machine (1st Step) & shaking heat-block (2nd step)
- Use a fresh tip for each transfer
-Mix or pipette mix EVERYTHING, this will improve the efficiency of your protocol
-If you are doing the clean-up step, prepare FRESH 80% ethanol that day

About Reagents:

- Storage of reagents:

-NT = fridge
-TD, ATM, NPM, RSB (Library prep kit) & indexes (index kit) = freezer

- Pre-warm/ thaw your reagents just before you begin

-Warm at room temp: RSB, AmPure XP beads, NT
-Warm on ice: ATM, TD, NPM & index box (put on something plastic first so the box doesn't get wet)

Protocol

1). Normalise DNA: Prepare genomic DNA to working concentration of 0.3 ng/ul

- ✓ Use PCR grade water for dilutions
- ✓ Store DNA at working concentrations in safelock/PCR tubes

2). "Tagmentation" protocol

- ✓ Pre-warm to room temperature:
  - Genomic DNA (at working concentrations)
  - NT (quenching)
- ✓ Thaw on ice:
  - ATM (amplicon tagment mix)
  - TD (tagment DNA buffer)
  - Index Adaptors
    - Index 1 primers – (S5XX)
    - Index 2 primers—(N7XX)
  - NPM (Nextera PCR master mix)
- ✓ PCR tubes (labelled)- one tube per sample

1). To each PCR tube,

a. 10ul of TD
b. 5ul of ATM

c. 5uL of normalised genomic DNA. Pipette mix thoroughly

NB: it is IMPORTANT to do this in the following order to reduce contamination of your reagents

2). Place tubes into thermocycler and run tagmentation program [SPRINGDOG -> Tagment 10].

a. IMPORTANT: as soon as the last tube is mixed IMMEDIATELY run PCR run, ATM is an enzyme cocktail and will fragment you DNA, this is a time sensitive step.
b. IMPORTANT: DO NOT leave your products sitting in the thermocycler too long after the program has finished, the enzymes are STILL fragmenting your DNA

3). Add 5ul NT, pipette to mix. Leave couple of minutes.

4). Arrange the index primers in the "TruSeq index plate fixture'

a. Place index 1 primers down the COLUMN of the plate
b. Place index 2 primers across the ROWS of the plate

5). Add 5ul of index 1 primer to each tube along each ROW

6). Add 5ul of index 2 primer to each tube along each COLUMN

ie:

	Index 2 -A	Index 2 - B	Index 2- C	
Index 1 – a				
Index 1 – b				
Index 1 - c				

*Use replacement caps (discard existing index adaptor caps after use)

7). Add 15ul NPM to each tube, Pipette to mix (NPM is viscous, use 20ul tips)

8). Place tubes into the thermocycler and run program [SPRINGDOG -> library amp]

THIS IS A SAFE STOPPING POINT:

if you are stopping here, can leave the PCR product overnight in the thermocycler or store at 2-8 °C.

3). Clean-up protocol

- ✓ Pre-warm to room temp
  - RSB (resuspension buffer)
  - AmPure XP Beads
- ✓ Ethanol
  - Make fresh 80% ethanol each day for clean-up. Use ethanol for WGS (red box) and DNAse free water (orange box labelled in fridge)
  - Use 200µL per wash, make 500µL per tube. Ex: Ethanol for 12 samples= 6ml (4.8ml Ethanol: 1.2ml water)
- ✓ 1.5ml safelock tubes (labelled)- one tube per sample
- ✓ 0.5ml safelock LoBind tubes (fully labelled for storage)- one tube per sample

1) Mix thoroughly AmPure XP beads and pour into 1.5 Eppendorf for use.

2) Add 40ul of AmPure XP beads Into clean 1.5ml safelock tubes (mix well between every second pipetting)
3) 50uL of PCR product
4) Pipette to mix
5) Put tubes on the shaking heating block for 5-6 min, 25C at 1500 rpm.
6) Place onto magnetic stand, with caps opening away from you
7) Wait for the suspension to become clear and a visible pellet forms. Approx. 5-10 min
8) Carefully open the tubes, take care not to shift the tubes as the pellet will not stay intact.
9) Without touching the pellet, remove the supernatant (adjust to 110µL) slowly.
10) Wash the pellet 2 times with 80% ethanol. DO NOT pipette directly on to the pellet. (First wash add 200µL and pipette out 200µL, second wash add 195µL & pipette out 200µL)
11) Make sure to remove all the residual ethanol on second wash. Any Ethanol (including small drops on the side) will reduce the yield.
12) In a biological safety cabinet, with open cap in the magnetic strip, air dry the pellet.
    a. In a cabinet it will dry in about 10 mins, take care not to over dry the pellet.
    b. Keep watching tubes as they may dry a lot quicker. Dry when small cracks appear and beads turn a lighter brown colour.
13) Keep the tubes in the magnetic stand.
14) Add 45 uL of RSB to the tube, and close the caps.
15) Gently flick the tube to mix pellet with RSB
16) Shake at 1500rpm for 5 mins at 25 °C
17) Place the tubes back on to the magnetic strip.
18) Without touching the pellet, transfer the supernatant (40 ul) to a clean safelock LoBind tube for storage.

These products can be stored at -20C

A 2.3 Protocol for Antibiotic Sensitivity Testing- disk diffusion of *Salmonella* in the PC3

The *Salmonella* being handled in the PC3 lab are imported. The conditions of the CTO permission (20172020) from MPI (see attached) state that the organisms must be unpacked, held, cultured, DNA extracted from, and stored in the PC3 laboratory. Only purified *Salmonella* DNA may leave the PC3 lab.

Salmonella can cause diarrhoea, fever and cramps. Users should be aware of the symptoms and should inform their general practitioner or healthcare provider of the possibility of *Salmonella* infection or person-to-person transmission through family exposure if presenting diarrhoeal symptoms themselves or with household contacts.

The imported *Salmonella* may be a risk to New Zealand's wildlife and/or livestock.

To reduce the potential for aerosols or spills, avoid culture of *Salmonella* in liquid broth.

Personal Protective Equipment

- A lab coat and disposable gown shall be worn for all manipulations involving *Salmonella*.
- Double gloves shall be worn when working in the Class II Biological Safety cabinet; the outermost layer of gloves shall be discarded as Dry Waste prior to exiting the Class II Biological Safety cabinet.
- Disposable overshoes shall be worn for all manipulations involving the use of *Salmonella*.

Biohazard Waste Disposal

- Before undertaking work each day the autoclaving/disposal requirements should be discussed with the Laboratory Services personnel.
    - All dry waste (e.g. masks, paper, gloves, plastic loops) used within a Class II cabinet shall be collected in a plastic bag within a container in the Class II cabinet. When full, the top of the bag should be folded over to create a loose seal and sprayed with 5% SteriGENE Clear disinfectant.
- After 10 minutes, the discard bag should then be removed from the Class II cabinet and placed within an autoclave bag labelled "Biological Hazard" within the closed waste bins provided in each laboratory.
- General laboratory waste shall be collected in biohazard bags and closed using tape. The exterior of the bags shall be sprayed thoroughly with 5% SteriGENE Clear disinfectant and held in the closed waste bins provided in each laboratory.
- The closed waste bin shall then be taken out of the laboratory on a trolley to await autoclaving; Lab Services personnel shall be informed that waste is available for autoclaving. All waste should be autoclaved as soon as possible after bags/containers are placed in the Restricted Corridor 2.07 – preferably the bags should go straight into the autoclave.

- No sharps or anything that may puncture the bags are to be disposed of in the autoclave bags. Sharps are to be collected in the specialised containers provided. At the end of each culturing session the sharps containers are sprayed with 5% SteriGENE and removed for autoclaving.
- Where possible all work involving the inoculation and culture of *Salmonella* shall be undertaken using disposable plastic ware such as inoculating loops, pipettes/pipette tips, culture vessels, etc. The use of glass items is discouraged.

Spillage of microorganisms

- Full details of procedures to take if a spillage of microorganisms occurs can be found in the Hopkirk Institute PC3 Containment Manual.
- In every case of a spillage of organisms or infectious materials of any risk group a 'Bacterial/Infectious Material Spillage Report Form' must be completed and handed to a PC3 Manager or the Facility Manager. All report forms shall be retained by the Facility Manager.
- Refer to PC3 Containment Manual Appendix 2B: Personal Safety & the Procedure for Decontaminating Laboratories and Equipment following Accidental Spillage of Hazardous Micro-organisms
- *Refer to PC3 Containment Manual Appendix 8: Notice of Procedures for Dealing with Accidental Spillage and Contamination by Pathogenic Organisms*
- *Refer to PC3 Containment Manual Appendix 13: Bacterial / Infectious Material Spillage Report Form*

Spills Outside a Class II Biosafety Cabinet

- In the event of spillage of *Salmonella*, staff must follow the procedures specified for decontamination.
- Generally the greatest risk from a spill of infectious material is from contamination through aerosol generation, particularly from liquid cultures. In the PC3 laboratories in the Hopkirk Research Institute there is continuous air movement and aerosols can be dispersed rapidly. It is therefore important to leave the contaminated areas as quickly and as safely as possible.
- If clothing and footwear, or exposed body areas have possibly been contaminated, measures must be taken to get rid of the bulk of the infectious material before exiting the airlock. A change of clothing is provided in the airlock to each PC3 laboratory.
- Only when you are in a position of safety and the laboratory is contained should steps be taken to decontaminate.
- Do not enter the contaminated area for at least an hour. This allows aerosols to be diluted or removed through continuous air changes and
- liquid spillages to dry. This will substantially reduce the contamination risk. All contaminated or potential contaminated material must be decontaminated, preferably by autoclaving.

- If a breach in secondary containment has occurred within the PC3 laboratory (such as a break in the outer biohazard bag, but not the inner vessel/bag and there has been no spill of the bacteria) fix the containment problem by placing all containers affected by the breach in a third container/bag and report this to one of the PC3 Laboratory Managers.

Spills Inside a Class II Biosafety Cabinet

- Spills of *Salmonella* up to 1 ml may be treated by wiping or flooding with a suitable disinfectant solution.
- In the event of a larger spill, staff shall ensure the cabinet remains operating and, with gloved hands, lay down absorbent material wetted with disinfectant such as 5% SteriGENE Clear and allow a minimum time of 10 minutes to effect disinfection. Absorbent material shall then be discarded in the biohazard plastic-lined waste bins provided in every laboratory.

Resuscitation of *Salmonella* from Microbank beads

- Pre-label blood agar plates
- Open up Class II Biological Safety cabinet, spray and wipe with 5% SteriGENE Clear and/or treat interior of cabinet with UV light for 20 minutes. Allow 5 minutes contact time with disinfectant before wiping off.
- Ensure disposable inoculating loops, straight wires and discard container are available within the Class II Biological Safety cabinet.
- While wearing gloves remove the required Microbank vials from the -20°C freezer (2.09 PC3 lab foyer) and place in the Mr Frosty box that is kept within the -20°C freezer. Transport the Mr Frosty box in a Polystyrene box.
- To enter the PC3 lab (2.09) remove one disposable glove use the ungloved hand to activate and open the inner door. Upon entry to the PC3 lab, place the Polystyrene box in the Class II Biological Safety cabinet and put on a disposable gown and double gloves.
- Using a disposable straight wire/ 10 μl extra-long pipette tip, aseptically remove a single bead and gently streak onto the plate (discard bead and wire/tip into discard), streak for single colonies.
- Once all inoculations have been completed, remove and discard the outer pair of gloves. Spray the Microbank vials, Mr Frosty and
- polystyrene box with 5% SteriGENE Clear, wait 10 minutes and remove from the Class II Biological Safety cabinet. Remove one glove and open the door of the PC3 lab with the ungloved hand. Replace the Microbank vials and Mr Frosty in the -20°C freezer in the PC3 lab foyer (2.09). Gloves shall be discarded in the dry waste for autoclaving and hands washed and dried thoroughly.
- Incubate plates at 37°C for 18-24 hrs before subculturing a single colony to a fresh blood agar and incubating at 37°C for a further 18-24 hrs.

Antibiotic Sensitivity Testing- Disk diffusion

Consumables and equipment:
Mueller-Hinton agar plates
Saline (0.85%) 2.5ml (sterile)
Cotton swabs (sterile)
Antibiotic disks
Disk dispenser/ forceps (sterile)
Ruler/ caliper/ automated zone reader
0.5 McFarland turbidity standard
Dark background for reading

Preparation:

- Open up Class II Biological Safety cabinet, spray and wipe with 5% SteriGENE Clear and/or treat interior of cabinet with UV light for 20 minutes. Allow 5 minutes contact time with disinfectant before wiping off.
- Ensure consumables, equipment (mentioned above) and discard containers (including a blue-capped Schott bottle containing 5% Sterigene) are available within the Class II Biological Safety cabinet
- Double gloves.
- Pre-label Mueller-Hinton plates (3 replicates for each isolate and antibiotic disk).
- Make sure that agar plates are at room temperature and well-dried prior to inoculation.
- Use 2 separate cotton swabs to pick colonies and inoculate plate.
- Allow disks to reach room temperature before opening cartridges or containers used for disk storage.
- Adjust disk dispenser height between 5-7 scale

Protocol:

1. Preparation of inoculum:

Use a cotton swab to pick colonies from an overnight culture on blood agar. Use 1-3 morphologically similar colonies, gently resuspend in saline and carefully mix without aerosol production to an even turbidity to reach density of a 0.5 McFarland turbidity standard. Use the adjusted inoculum suspension within 15 min of preparation.

2. Inoculation of agar plates

Dip a sterile cotton swab into the saline suspension. Remove excess fluid (to avoid over-inoculation) by pressing and turning the swab against the inside of the tube. Discard saline suspension into the provided disinfectant.

Mueller-Hinton plates are inoculated by swabbing in three directions. Spread the inoculum evenly over the entire agar surface ensuring that there are no gaps between streaks. ‘

3. Application of antimicrobial disks

Apply disks firmly using a disk dispenser or sterile forceps to the surface of the inoculated agar plate within 15 min of inoculation (maximum 6 disks per plate- depending on the trials done)

4. Incubation of plates

Invert agar plates and make sure disks do not fall off the agar surface. Incubate plates within 15 min of disk application. Incubation conditions: 35 °C for 18 hours, maximum 4-6 plates per stack

5. Examination of plates after incubation

A correct inoculum and satisfactorily streaked plates should result in a confluent lawn of growth. If individual colonies can be seen, the inoculum is too light and the test must be repeated.

6. Measurement of zones

Remove agar plates from the incubator and ensure that there is no excessive condensation associated with the plates that represents a risk of contamination. In the class II biosafety cabinet, antimicrobial inhibition test zone edge should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye. Read plates from the back with reflected light and the plate held above a dark background.

Measure the inhibition zone diameters to the nearest millimeter with a ruler or a caliper.

7. Recording results and transfer

Record results on the appropriate data sheet, which will remain in PC3 but can later be transferred out of the lab as a digital photograph.

The photographs are to be taken using a mobile phone from 2.09 PC3 lab foyer once the data sheets are completed and will be taped facing outwards on to the PC3 lab door window (from inside).

8. Quality Control

Perform quality control (each time along with the tests) of working supplies to control that the antimicrobial disks have not lost potency during storage. In addition to routine QC testing, test each new batch of Mueller-Hinton agar to ensure that all zones are within range.

Upon completion of work in the Class II Biological Safety cabinet, discard agar plates in to discard container and the interior work surfaces shall be sprayed and wiped down with 5% SteriGENE Clear (5 minutes contact time). The Class II hood shall also be sterilized using the built-in UV lamp for 20 minutes. Prior to exiting of the PC3 lab, all personal protective equipment shall be removed and hands washed and dried thoroughly.

PC3 Laboratory and Deputy Laboratory Managers

- Responsibilities: The PC3 Managers for Room 2.09 shall provide support to the Operator and Facility Manager in the preparation and review of documentation, implementation of procedures and ensuring compliance with the Hopkirk Research Institute PC3 Containment Manual. They will also assist with fumigations (actual or test), with the training and instructions of laboratory personnel in good work practices in the PC3 areas and with internal and external audits.
- Responsibilities: The PC3 Deputy Managers for Room 2.09 shall provide support to the Facility Manager and PC3 Manager in the review and implementation of procedures and ensuring compliance with the PC3
- Containment Manual. They will also assist with fumigations (actual or test), with the training and instruction of laboratory personnel in good work practices in the PC3 areas and with internal and external audits.

Hours of operation

For safety reasons, people should work within the Room 2.09 only during standard working hours. If personnel occasionally need to use Room 2.09 outside the hours of 0800 and 1700 they must have authorisation from a PC3 Manager or Deputy Manager for each such instance. Such authorisations will be recorded in a log book located in the Restricted Corridor 2.07.

Appendix III: Supplementary Data for Chapter 3

Table A 3.1: Biochemical Tests and PCR results of isolates

Isolate ID	Gram smear	TSI reaction[60]			Urease	Oxidase	Citrate	SIM[61]		ONPG[62]	PCR confirm
		Alk slant, acid butt	H2S	gas				Indole	Motility		
SSL001	neg rod	yes	no	yes	neg	neg	pos	neg	yes	neg	Yes
SSL002	neg rod	yes	no	yes	neg	neg	pos	neg	yes	neg	Yes
SSL003	neg rod	yes	no	yes	neg	neg	pos	neg	yes	neg	Yes
SSL004	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL005	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL006	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL007	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL008	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL009	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL010	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL011	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL012	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL013	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL014	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL015	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL016	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL017	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL018	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL019	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL020	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL021	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL022	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL023	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL024	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL025	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL026	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL027	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL028	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL029	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL030	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL031	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL032	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL033	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL034	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes

[60] TSI: Triple Sugar Iron agar

[61] SMI: Sulfide Indole Motility

[62] ONPG: O-Nitrophenyl-β-D-Galactopyranoside

Isolate ID	Gram smear	TSI reaction			Urease	Oxidase	Citrate	SIM		ONPG	PCR confirm
		Alk slant, acid butt	H2S	gas				Indole	Motility		
SSL035	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL036	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL037	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL038	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL039	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL040	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL041	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL042	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL043	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL044	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL045	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL046	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL047	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL048	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL049	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL050	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL051	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL052	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL053	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL054	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL055	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL056	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL057	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL058	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL059	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL060	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL061	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL062	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL063	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL064	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL065	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL066	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL067	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL068	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL069	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL070	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes

Isolate ID	Gram smear	TSI reaction			Urease	Oxidase	Citrate	SIM		ONPG	PCR confirm
		Alk slant, acid butt	H2S	gas				Indole	Motility		
SSL071	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL072	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL073	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL074	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL075	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL076	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL077	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL078	neg rod	yes	no	no	neg	neg	pos	neg	yes	neg	Yes
SSL079	neg rod	yes	no	no	neg	neg	pos	neg	yes	neg	Yes
SSL080	neg rod	yes	no	no	neg	neg	pos	neg	yes	neg	No
SSL081	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL082	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL083	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL084	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL085	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL086	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL087	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL088	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL089	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL090	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL091	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL092	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL093	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL094	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL095	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL096	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL097	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL098	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL101	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL102	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL103	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL104	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL105	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL106	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL107	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL108	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes

Isolate ID	Gram smear	TSI reaction			Urease	Oxidase	Citrate	SIM		ONPG	PCR confirm
		Alk slant, acid butt	H2S	gas				Indole	Motility		
SSL109	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL110	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL111	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL112	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL113	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL114	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL115	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL116	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL117	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL118	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL119	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL120	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL121	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL122	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL123	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL124	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL125	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL126	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL127	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL128	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL129	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL130	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL131	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL132	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL133	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL134	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL135	neg rod	yes	no	no	neg	neg	pos	neg	no	neg	Yes
SSL136	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL137	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL138	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL139	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL140	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL141	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes
SSL142	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL143	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes
SSL144	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes

Isolate ID	Gram smear	TSI reaction			Urease	Oxidase	Citrate	SIM		ONPG	PCR confirm
		Alk slant, acid butt	H2S	gas				Indole	Motility		
SSL145	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes
SSL146	neg rod	yes	yes	yes	neg	neg	pos	neg	no	neg	Yes
SSL147	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes
SSL148	neg rod	yes	no	no	neg	neg	pos	neg	no	neg	Yes
SSL149	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes
SSL150	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes
SSL151	neg rod	yes	no	yes	neg	neg	pos	neg	no	neg	Yes
SSL152	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL153	neg rod	yes	no	yes	neg	neg	pos	neg	no	neg	Yes
SSL154	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL155	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL156	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL157	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL158	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL159	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL160	neg rod	yes	yes	no	neg	neg	pos	neg	no	neg	Yes
SSL161	neg rod	yes	no	yes	neg	neg	pos	neg	no	neg	Yes
SSL162	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL163	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL164	neg rod	yes	no	yes	neg	neg	pos	neg	no	neg	Yes
SSL165	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes
SSL166	neg rod	yes	yes	yes	neg	neg	pos	neg	yes	neg	Yes
SSL167	neg rod	yes	yes	no	neg	neg	pos	neg	yes	neg	Yes

Table A 3.2: *Salmonella* status of broiler holdings with coordinate data

Source ID	Source	Salm Status[63]	latitude	longitude
F001	Broiler farm	negative	7.727265	80.20026
F002	Broiler farm	negative	7.680504	80.20233
F003	Broiler farm	negative	7.584605	80.13088
F004	Broiler farm	negative	7.564938	80.15882
F005	Broiler farm	negative	7.573624	80.14238
F006	Broiler farm	negative	7.499739	80.18689
F007	Broiler farm	Positive	7.507192	80.1881
F008	Broiler farm	negative	7.499569	80.18066
F009	Broiler farm	Positive	7.509826	80.17976
F010	Broiler farm	Positive	7.57178	80.1438
F011	Broiler farm	negative	7.580384	80.13483
F012	Broiler farm	negative	7.570275	80.14682
F013	Broiler farm	negative	7.567291	80.15261
F014	Broiler farm	Positive	7.618218	80.16699
F015	Broiler farm	Positive	7.618269	80.16626
F016	Broiler farm	negative	7.617234	80.16477
F017	Broiler farm	negative	7.616167	80.16878
F018	Broiler farm	negative	7.614485	80.16814
F019	Broiler farm	negative	7.636808	80.23368
F020	Broiler farm	negative	7.64204	80.22967
F021	Broiler farm	Positive	7.605197	80.07854
F022	Broiler farm	negative	7.599422	79.96352
F023	Broiler farm	negative	7.619086	79.97263
F024	Broiler farm	negative	7.659289	79.99069
F025	Broiler farm	Positive	7.618066	79.98228
F026	Broiler farm	Positive	7.574638	80.24671
F028	Broiler farm	negative	7.577558	80.23998
F029	Broiler farm	Positive	7.46978	80.38909
F030	Broiler farm	Positive	7.50032	80.4467
F031	Broiler farm	Positive	7.297853	80.06702
F032	Broiler farm	negative	7.318079	80.16758
F033	Broiler farm	Positive	7.503886	79.99588
F034	Broiler farm	Positive	7.982868	80.37059
F035	Broiler farm	negative	7.556545	79.90322
F037	Broiler farm	negative	7.595316	79.91993
F038	Broiler farm	negative	7.692724	80.37563
F039	Broiler farm	Positive	7.834877	80.42617
F040	Broiler farm	negative	7.840635	80.45238
F042	Broiler farm	Positive	7.812609	80.44406
F043	Broiler farm	negative	7.807759	80.44601
F044	Broiler farm	Positive	7.325131	79.84868

[63] *Salmonella* status

Source ID	Source	Salm Status[63]	latitude	longitude
F045	Broiler farm	Positive	7.28061	79.86079
F046	Broiler farm	Positive	7.352555	79.88128
F047	Broiler farm	negative	7.347089	79.85461
F048	Broiler farm	negative	7.351529	79.87203
F049	Broiler farm	negative	7.348765	79.86292
F050	Broiler farm	negative	7.353697	79.88573
F051	Broiler farm	Positive	7.521439	79.79403
F052	Broiler farm	negative	7.514705	79.79674
F053	Broiler farm	negative	7.493758	79.80288
F054	Broiler farm	negative	7.495426	79.81135
F055	Broiler farm	negative	7.537158	79.81806
F056	Broiler farm	negative	7.562907	79.85236
F057	Broiler farm	negative	7.733038	79.90397
F058	Broiler farm	Positive	7.734603	79.90376
F059	Broiler farm	negative	7.730013	79.90781
F060	Broiler farm	Positive	7.730917	79.90758
F061	Broiler farm	negative	7.729911	79.91196
F062	Broiler farm	negative	7.732664	79.91676
F063	Broiler farm	negative	7.736059	79.90852
F064	Broiler farm	Positive	7.654725	79.88577
F065	Broiler farm	negative	7.245811	79.93285
F066	Broiler farm	negative	7.243396	79.93235
F067	Broiler farm	negative	7.254197	79.93175
F068	Broiler farm	negative	7.254023	79.9311
F069	Broiler farm	Positive	7.248207	79.93002
F070	Broiler farm	negative	7.248258	79.92999
F071	Broiler farm	Positive	7.227995	79.89738
F072	Broiler farm	negative	7.087301	80.09977
F073	Broiler farm	negative	7.087406	80.09985
F074	Broiler farm	negative	7.087721	80.10021
F075	Broiler farm	negative	7.09618	80.16882
F076	Broiler farm	negative	7.021598	80.13174
F077	Broiler farm	negative	7.067361	80.09477
F078	Broiler farm	negative	7.031817	80.09374
F079	Broiler farm	negative	7.042706	80.0903
F080	Broiler farm	negative	7.036865	80.05218
F081	Broiler farm	Positive	7.018451	80.06148
F082	Broiler farm	negative	7.019471	80.0022
F083	Broiler farm	negative	7.1623	80.1272
F084	Broiler farm	negative	7.184926	80.14103
F085	Broiler farm	negative	7.276375	80.40608
F086	Broiler farm	negative	7.276385	80.4061
F087	Broiler farm	Positive	7.228208	80.32835
F088	Broiler farm	negative	7.247569	80.29967

Source ID	Source	Salm Status[63]	latitude	longitude
F089	Broiler farm	Positive	7.247565	80.29941
F090	Broiler farm	Positive	7.375834	80.37056
F091	Broiler farm	Positive	7.379203	80.37014
F092	Broiler farm	Positive	7.351558	80.37881
F093	Broiler farm	negative	7.352492	80.37704
F094	Broiler farm	negative	7.256929	80.40446
F095	Broiler farm	negative	7.070215	80.19848
F096	Broiler farm	negative	7.070006	80.19847
F097	Broiler farm	Positive	7.07002	80.19848
F098	Broiler farm	negative	7.070022	80.19817
F099	Broiler farm	negative	7.053234	80.20543
F100	Broiler farm	negative	7.053564	80.20575
F101	Broiler farm	negative	7.298749	80.26318
F102	Broiler farm	Positive	7.317263	80.34251
F103	Broiler farm	negative	7.31165	80.39564
F104	Broiler farm	negative	7.274781	80.28582
F105	Broiler farm	negative	7.181969	80.58286
F106	Broiler farm	negative	7.183129	80.57683
F107	Broiler farm	negative	7.182605	80.57741
F108	Broiler farm	negative	7.130877	80.59477
F109	Broiler farm	negative	7.134523	80.59738
F110	Broiler farm	negative	7.163408	80.62363
F111	Broiler farm	negative	7.15292	80.63577
F112	Broiler farm	negative	7.164863	80.6222
F113	Broiler farm	negative	7.164863	80.6222
F114	Broiler farm	Positive	7.182872	80.57946
F115	Broiler farm	Positive	7.163527	80.63394
F116	Broiler farm	Positive	7.331607	80.67742
F117	Broiler farm	Positive	7.309454	80.67273
F118	Broiler farm	Positive	7.31673	80.57925
H001	Hatchery	Positive	7.558946	80.59328
H002	Hatchery	Positive	7.406018	79.864
H003	Hatchery	Positive	7.65725	80.26875
H004	GP Hatchery	negative	7.777225	80.65271
H005	Hatchery	Positive	7.3253	79.94552
H006	GP Hatchery	Positive	6.939848	80.15637
H007	Hatchery	Positive	6.869248	80.10692
H008	Hatchery	Positive	7.033582	80.52277
H009	Hatchery	Positive	7.292671	80.69903
H010	Hatchery	negative	6.93685	80.13862
H011	Hatchery	negative	6.531924	79.97739
H012	Hatchery	negative	7.14832	80.12999
H013	Hatchery	Positive	7.016551	80.08348
H014	Hatchery	Positive	7.069542	79.92923
H015	Hatchery	Positive	6.28855	80.84087

Appendix IV: Questionnaires

A 4.1 Questionnaire

Farm No: ……

District: ……

Broiler Farm Questionnaire

Farm contact details

- Name of the Company/ Owner: ……
- Person to whom the questionnaire delivered to: ……
- Name and address of the Farm: ……
- Contact No: ……
- Government Veterinary Range: ……

General Information

1. Type of operation: Buy- back ☐ self-owned ☐
2. Single age/ multi age: ..... No. of flocks available: ……..
3. Flock size: .....
4. Total No. of poultry houses: .....
5. Maximum bird capacity of the farm:

   1000-5000 ☐ 5000-25,000 ☐ >25,000 ☐
6. Source of chicks/ Hatchery: …… Strain: ……

Housing and Management

7. Feed supply:

   Self-mixed ☐ Commercial feed ☐ Both ☐ Other……
8. Water supply: Well ☐ Tube well ☐ Municipal water ☐

   Stream ☐ Other ……
9. Evidence of record keeping:

   Electronic records ☐ Book/flock card ☐ No records ☐
10. Litter management practice: Yes ☐ No ☐ NA ☐

    If yes,

    Raking once a day ☐ Raking but not regular ☐

    Use disinfectants ☐ Other ……

    If use disinfectants, please specify name, dose and frequency

    ……

11. Footbath maintenance: Yes ☐ No ☐ NA ☐

If yes,

Replace water once a day ☐ Replace water but not regular ☐

Use disinfectants ☐ Other .....

If use disinfectants, please specify name, dose and frequency:

……..

12. Waste Disposal:

i. Litter:

Burn ☐ Use as fertilizer by the farmer ☐ Sell ☐ Other …….

ii. Carcasses:

Burn ☐ Bury on the ground ☐ Other……

13. Preparation of the poultry house before new intake:

i. Cleaning: Yes ☐ No ☐

If yes, specify procedure .....

ii. Disinfection: Yes ☐ No ☐

If yes, names of the disinfectants and dose:

……

iii. Resting period practiced: Yes ☐ No ☐

If yes, how many days:……..

Vaccination, Usage of drugs and diseases

14. Do you practice routine vaccination for birds? Yes ☐ No ☐

If yes, what are the vaccines used

……

15. Do you use routine medications for your flocks? Yes ☐ No ☐

If yes, what are the types of routine medications used?

Probiotics ☐ Multi vitamins ☐ Prophylactic Antibiotics ☐

Other……

If using Prophylactic Antibiotics,

Age of bird	Name of the drug	Duration	Method delivered	Purchase drugs from/provided by

Any labels or any empty packages? .....

16. a). What do you do if you see a sick bird?

Treat ☐ destroy ☐ sell ☐

b). If you treat,

Is it the particular bird ☐ whole flock ☐

c). What is the usual way of managing sick birds?

I have a fair knowledge on disease conditions ☐
Use antibiotics which I have used in previous occasions ☐
Get advice from veterinarian/ Livestock field officers ☐
Other: ……

17. a). Any diseases or high deaths occurred in the available flocks?

Yes ☐ No ☐

If yes, what was it?

Disease ☐ Heat stress ☐ No idea ☐ Other/specify……

If Disease, what was the suspected cause? .....
Diagnosed by? .....
Treatment given?

Age of bird	Name of the drug	Duration	Method delivered	Purchase drugs from/provided by

Any labels or any empty packages?.....

b). Any conditions causing more than 5% mortality within last 6 months?

Yes ☐ No ☐

If yes, what was it?

Disease ☐ Heat stress ☐ No idea ☐ Other …….

If Disease, what was the suspected cause? .....

Diagnosed by? .....

18. Are you aware of antimicrobial withdrawal periods? Yes ☐ No ☐

If yes, do you abide to that? Yes ☐ No ☐

Any reasons ……..……..……..

Experience, knowledge and extension services

19. a). Your experience on broiler management?

Within last year ☐ 1-5 years ☐ More than 5 years ☐ More than 10 years ☐

b). Any training received? Yes ☐ No ☐

If yes, please mention…….

20. Any assistance from an extension officer? Yes ☐ No ☐

If yes, please mention

……..……..

Observations

21. Location of the farm:

Isolated area ☐

Residential area ☐

Same premises with farmers house ☐

Presence of nearby poultry farms ☐

Other…….

22. Restricted entry for visitors: Yes ☐ No ☐

23. Housing: Environment controlled house ☐ Opened house ☐

24. Management system:

Deep litter ☐ Battery cage ☐ Both ☐ Other……..

If deep litter,

i. What is the type of litter? Paddy husk ☐ Wood shavings ☐

Other……

ii. Litter condition: Wet ☐ Dry ☐

25. Presence of functional foot bath:

i at farm entrance: Yes ☐ No ☐

ii at each poultry house: Yes ☐ No ☐

26. a). Change of boots/shoes at each poultry house: Yes ☐ No ☐

b). Hand washing facility for workers between sheds: Yes ☐ No ☐

27. a). Evidence of Rodent control:

Baits for rodents seen ☐ rodents seen ☐ rodent droppings seen ☐

Other……

b). Presence of other animals in the farm: Yes ☐ No ☐

If yes, specify ……

28. a). Type of feeder: Manual ☐ Automated ☐

b). Type of drinkers: Manual ☐ Automated ☐

29. Cleanliness/condition of the farm*

i Cleanliness of feeder: Satisfactory ☐ No ☐

ii Cleanliness of drinkers: Satisfactory ☐ No ☐

iii Cleanliness inside poultry house: Satisfactory ☐ No ☐

iv Cleanliness outside poultry house: Satisfactory ☐ No ☐

v Feed storage**: Satisfactory ☐ No ☐

30. Evidence of antibiotics usage?

.....

Any special note

.....

.....

*Cleanliness: Satisfactory

Inside pen: No evidence of gross faecal contamination (other than minor flecks) on equipment, feeders and drinkers, prior to entry into buildings etc.

Outside: No garbage, clear zone around the pen (land free of all vegetation)

**Feed storage: Satisfactory

Separate area with shelter (could be inside poultry pen) rodent proof and feed stored on a shelf/ stage (with no direct floor contact

A 4.2. Translation (Sinhala) of the questionnaire for broiler Farms

ගොවිපල අංකය:.....

දිස්ත්‍රික්කය: .....

බ්‍රොයිලර් ගොවිපල ප්‍රශ්නාවලිය

ගොවිපල සම්බන්ධ විස්තර

- අයිතිකරුගේ/ සමාගමේ නම: .....
- ප්‍රශ්නාවලිය යොමු කළ අයගේ නම: .....
- ගොවිපලේ නම හා ලිපිනය: .....
- දුරකතන අංකය: .....
- පශු වෛද්‍ය කොට්ඨාශය: .....

සාමාන්‍ය තොරතුරු

1. ක්‍රියාත්මක වන ආකාරය: බයි බැක් ☐ පෞද්ගලික අයිතිය ☐

2. එකම වයස/ විවිධ වයස: ..... කණ්ඩායම් ගණන .....

4. කණ්ඩායමේ ප්‍රමාණය:.....

4 .මුළු කුකුල් නිවාස ගණන: .....

5. ගොවිපලේ උපරිම සතුන් ධාරිතාව: 1000-5000 ☐ 5000-25000 ☐ 25000 වැඩි ☐

6. පැටවු ලබා ගන්නා ආකාරය: ..... ප්‍රභේදය .....

නිවාස හා කළමනාකරණය

7. ආහාර සැපයුම: තමන්ම සකස් කරයි☐ වාණිජ ආහාර ☐ දෙවර්ගයම ☐ වෙනත් ☐

8. ජල මූලාශ්‍රය: ළිඳ ☐ නළ ළිඳ☐ නල ජලය ☐ ඇළ ☐ වෙනත් ☐

9. වාර්තා ලබා ගැනීම පරිගණක දත්ත ☐ බෙහෙත් කාඩ් ☐ වාර්තා නැත ☐

10. අතරණුව කළමනාකරණ ක්‍රමවේදය: ඔව් ☐ නැත ☐ අදාල නැත.☐

ඔව් නම්,

දිනකට වරක් පේක්ක තිරීම ☐ පේක්ක කළද ක්‍රමානුකූල නැත ☐ විශබීජනාශක භාවිතා තිරීම ☐

වෙනත් .....

විශබීජනාශක භාවිතා කරයි නම් කරුණාකර නම, ප්‍රමාණය හා වාර ගණන සඳහන් කරන්න.

.....

11. පාදෝවනය නඩත්තුව: ඔව් ☐ නැත. ☐ අදාල නැත.☐

ඔව් නම්,

දිනපතා වතුර මාරු කරයි ☐ වතුර මාරු කරන නමුත් විධිමත් නැත☐ විෂබීජ නාශක භාවිතා කරයි☐

වෙනත් .....

විශබීජනාශක භාවිතා කරයි නම්, කරුණාකර නම, ප්‍රමාණය හා වාර ගණන සඳහන් කරන්න.

.....

12. අපද්‍රව්‍ය බහැර කිරීම

i අතරණුව: පුළුස්සයි ☐ ගොඩියා විසින් පොහොර ලෙස භාවිතා කරයි. ☐ අලවි කරයි ☐ වෙනත් ☐

ii මලසිරුරු: පුළුස්සයි ☐ පොළවේ වල දමයි ☐ වෙනත් .....

13. අලුතින් පැටවු දැමීමට පෙර කුකුල් කුඩු සූදානම් කිරීම

i පිරිසිදු කිරීම: ඔව් ☐ නැත ☐

ඔව් නම් ක්‍රමවේදය .....

ii විෂබීජහරණය: ඔව් ☐ නැත ☐

ඔව් නම් කරුණාකර නම, ප්‍රමාණය .....

iii නිදහස් කාලය ක්‍රියාත්මක කරයි. ඔව් ☐ නැත ☐

ඔව් නම් දින ගණන .....

එන්තත්කරණය, ඖෂධ භාවිතා කිරීම හා ළෙඩ රෝග

14. ඔබ සාමාන්‍ය එන්තත්කරණය සිදුකරන්නේද ඔව් ☐ නැත ☐

ඔව් නම් භාවිතා කරන එන්තත් මොනවාද? .....

15.ඔබ සාමාන්‍ය ඖෂධ භාවිතා කරන්නේද? ඔව් ☐ නැත ☐

ඔව් නම් ඔබ සාමාන්‍යයෙන් භාවිතා කරන ඖෂධ මොනවාද? ප්‍රෝබයෝටික්ස් ☐ මල්ටි විටමින්, ☐ රෝග නිවාරක ප්‍රතිජීවක ☐ වෙනත් ☐

රෝග නිවාරක ප්‍රතිජීවක භාවිතා කරන්නේ නම් . . . .

සතුන්ගේ වයස	ඖෂධයේ නම	ලබාදෙන කාලය	ලබාදෙන ක්‍රමය	මිලදී ගන්නා ආකාරය

ගිස් ඇසුරුම් හෝ ලේබල් ඔබ සතුව ඇතිද? .....

16. a. සතකු ලෙඩ වූ විට ඔබ කුමක් කරන්නේද? ප්‍රතිකාර කරමි ☐ විනාශ කරමි ☐ අලවි කරමි ☐

b. ඔබ ප්‍රතිකාර කරන්නේ නම්

ඒ රෝගී සතා පමනකි ☐ මුළු රැලටම ☐

c. රෝගී සතෙකු බලාගන්නා සාමාන්‍ය ක්‍රමය

- මට රෝග තත්වයක් ගැන සාමාන්‍ය අවබෝධයක් ඇත. ☐
- මීට පෙර භාවිතා කළ ප්‍රතිජීවක ඖෂධ භාවිතා කරමි. ☐
- පශු වෛද්‍යවරුන්ගෙන් හෝ පශු සම්පත් ක්ෂේත්‍ර නිලධාරීන්ගෙන් උපදෙස් ගනිමි. ☐
- වෙනත් .....

17. a. දැනට සිටින රැල වල ලෙඩ රෝග හෝ විශාල ලෙස සතුන් මිය යාම සිදුවී ඇතිද? ඔව් ☐ නැත ☐

ඔව් නම් ඊට හේතුව: ලෙඩ රෝග ☐ උෂ්ණත්වය ☐ අදහසක් නැත ☐ වෙනත්.....

රෝගයක් නම් ඔබ සැකකරන හේතුව.....

රෝග විනිශ්චය කරන ලද්දේ? .....

ලබා දුන් ප්‍රතිකාර

සතුන්ගේ වයස	ඖෂධයේ නම	ලබාදෙන කාලය	ලබාදෙන ක්‍රමය	මිලදී ගන්නා ආකාරය

හිස් ඇසුරුම් හෝ ලේබල් ඔබ සතුව ඇතිද? .....

b. පසුගිය මාස හය තුල 5% කට වඩා සතුන් මරණයට පත්කල අවස්ථාවක් අත්දුටුවේද? ඔව් ☐ නැත ☐

ඔව් නම් ඊට හේතුව: ලෙඩ රෝග ☐ උෂ්ණත්වය ☐ අදහසක් නැත ☐ වෙනත් .....

රෝගයක් නම් ඔබ සැකකරන හේතුව.....

රෝග විනිශ්චය කරන ලද්දේ? .....

18. ප්‍රතිජීවක ඖෂධ නතර කිරීමේ කාලය ගැන ඔබ දැනුවත්ද? ඔව් ☐ නැත ☐

ඔව් නම් ඔබ එය පිළිපදිනවාද? ඔව් ☐ නැත ☐

හේතුව .....

ප්‍රාදේශීය පළාත්පාලන ආයතන සේවා

19. a. මොඩලර් පාලනය ගැන ඔබගේ අත්දැකීම්

වසරට අඩු ☐ වසර 1 - 5 ☐ වසර 5ට වැඩි ☐ වසර 10ට වැඩි ☐

b. ප්‍රතිලාභයක් ලබා තිබේද? ඔව් ☐ නැත ☐

ඔව් නම් ඒ කුමක්ද? .....

20. ව්‍යාපෘති නිලධාරියෙකුගේ උදව් ලැබී තිබේද? ඔව් ☐ නැත ☐
ඔව් නම් ඒ කුමක්ද?

නිරීක්ෂණ

21. ගොවිපලේ පිහිටීම.

තුදෙකළා ප්‍රදේශයකි ☐ නේවාසික ප්‍රදේශයකි ☐ ලඟපාත කුකුල් ගොවිපල ඇත. ☐
ගොඩියාගේ නිවස තියන ඉඩමීම ඇත. ☐ වෙනත් .....

22. බාහිර අයට ඇතුල්වීම සීමා කර ඇත : ඔව් ☐ නැත ☐

23. නිවාස: පාරිසරික තත්ව කළමනාකරණය කර ඇත ☐ විවෘත නිවාස ☐

24. කළමනාකරණය ක්‍රමය්

ගැඹුරු ආස්තරණය ක්‍රමය ☐ බැටරි කූඩු ☐ දෙකම ☐ වෙනත් .....

- ගැඹුරු ආස්තරණය නම් භාවිතා කරන ආස්තරණය දහයියා ☐ ළි කූඩු ☐ වෙනත්
  .....
- ආස්තරණයක් ස්වභාවය: වියළිය ☐ තෙතය ☐

25. ක්‍රියාත්මක පාලෝවන

ගොවිපල ඇතුලු වන තැන: ඔව් ☐ නැත ☐

එක් එක් කූඩුවට ඇතුලු වන තැන: ඔව් ☐ නැත ☐

26. a. එක් එක් කූඩුව ළඟදී බුරිස් සපන්තු මාරු කරයි: ඔව් ☐ නැත ☐

b. වැඩ කරන අය සඳහා කූඩු අතර යාමේදී අත් සේදීමේ පහසුකම: ඔව් ☐ නැත ☐

27. a. මියන් මර්ධන කටයුතු : මී උගුල් දුටුවා ☐ මියන් දුටුවා ☐ මී බෙට් දුටුවා ☐ වෙනත් .....

b. වෙනත් සතුන් දුටුවා: ඔව් ☐ නැත ☐

ඔව් නම් ඒ සඳහන් කරන්න .....

28. a. ආහාර භාජන: අතින් ☐ යාන්ත්‍රික ☐

b. වතුර භාජන: අතින් ☐ යාන්ත්‍රික ☐

29. පිරිසිදු බව/ හොඳපලේ තත්වය

1.සෑම භාජන වල පිරිසිදුකම: සතුටුදායකයි ☐ නැත ☐

2.වතුර භාජන වල පිරිසිදුකම: සතුටුදායකයි ☐ නැත ☐

3.කුඩු ඇතුළත පිරිසිදුකම: සතුටුදායකයි ☐ නැත ☐

4.කුඩු වලින් පිට පිරිසිදුකම: සතුටුදායකයි ☐ නැත ☐

5.ආහාර ගබඩා වල පිරිසිදුකම: සතුටුදායකයි ☐ නැත ☐

29. ප්‍රතිපීවක භාවිතය ගැන නිරීක්ෂණ .....

31. විශේෂ සටහන්

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* පිරිසිදු බව සතුටුදායක නම්

කුඩුව ඇතුළත : උපකරණ සෑම භාජන ජල භාජන වල කුකුල් බෙට් වලින් අපිරිසිදු වී නැත.

කුඩුව පිටත : - කුනු නොමැති අතර කුඩුව වටා පිරිසිදු සීමාවක් ඇත.(කිසිඳු ශාක වර්ග වලින් තොර බව)

* ආහාර ගබඩාව සතුටුදායක නම්

වෙනම ප්‍රදේශයක් (කුඩුව ඇතුලේම විය හැක) මියන්ගෙන් තොරව / ආහාර තට්ටුවක් මත තැන්පත් කර ඇත.

Appendix V: Supplementary Data for Chapter 4

Table A 5.1: Descriptive Statistics of the 115 farms (Questionnaire-based)

Farm ID	District	Operation Type	No. Flocks	Farm Bird Capacity	Source Strain	Litter Mgt	Rest period	Antibiotic on day 1	Farmer's Sick Bird Knowledge	High Deaths	Experience yrs
F001	Kurunegala	buy-back	2	5000-25,000	IndianRiver	Yes	14days	yes	No	No	1-5
F002	Kurunegala	buy-back	1	1000-5000	Cobb500	Yes	14days	No	Yes	Yes	> 10
F003	Kurunegala	buy-back	2	1000-5000	Cobb500	No	7days	yes	No	No	> 5
F004	Kurunegala	self-owned	2	1000-5000	Cobb500	Yes	14days	yes	No	Yes	1-5
F005	Kurunegala	buy-back	4	5000-25,000	Cobb500	Yes	7days	No	No	No	> 5
F006	Kurunegala	self-owned	2	1000-5000	Cobb500	No	14days	yes	Yes	No	> 10
F007	Kurunegala	self-owned	2	1000-5000	Cobb500	No	7days	No	Yes	No	> 5
F008	Kurunegala	self-owned	2	1000-5000	Cobb500	No	7days	No	No	No	last year
F009	Kurunegala	buy-back	2	1000-5000	Cobb500	No	14days	yes	No	No	> 5
F010	Kurunegala	self-owned	2	1000-5000	Cobb500	No	7days	No	No	Yes	> 5
F011	Kurunegala	self-owned	1	1000-5000	Cobb500	No	14days	No	Yes	No	> 10
F012	Kurunegala	self-owned	1	1000-5000	Cobb500	No	10days	yes	No	No	1-5
F013	Kurunegala	self-owned	1	1000-5000	Cobb500	Yes	14days	yes	No	No	1-5
F014	Kurunegala	self-owned	1	1000-5000	Cobb500	No	14days	No	No	Yes	1-5
F015	Kurunegala	self-owned	1	1000-5000	Cobb500	No	10days	No	No	Yes	1-5
F016	Kurunegala	self-owned	1	5000-25,000	Cobb500	Yes	14days	yes	Yes	No	> 5
F017	Kurunegala	self-owned	2	1000-5000	IndianRiver	Yes	14days	No	No	Yes	> 5
F018	Kurunegala	self-owned	1	1000-5000	Cobb500	No	7days	No	No	Yes	1-5
F019	Kurunegala	self-owned	2	1000-5000	IndianRiver	Yes	14days	yes	Yes	Yes	> 5
F020	Kurunegala	self-owned	3	1000-5000	IndianRiver	Yes	14days	yes	Yes	Yes	> 5
F021	Kurunegala	self-owned	2	5000-25,000	Cobb500	No	7days	yes	Yes	Yes	> 10
F022	Kurunegala	buy-back	3	1000-5000	Cobb500	Yes	14days	yes	No	No	last year
F023	Kurunegala	buy-back	2	1000-5000	Cobb500	No	10days	No	No	Yes	1-5
F024	Kurunegala	buy-back	1	1000-5000	Cobb500	No	14days	yes	Yes	No	> 5
F025	Kurunegala	buy-back	1	1000-5000	Cobb500	No	14days	yes	No	Yes	1-5
F026	Kurunegala	self-owned	2	1000-5000	Cobb500	No	7days	No	Yes	No	> 10
F028	Kurunegala	self-owned	2	1000-5000	Cobb500	No	10days	yes	No	No	> 5
F029	Kurunegala	self-owned	2	1000-5000	IndianRiver	No	10days	No	Yes	No	1-5
F030	Kurunegala	self-owned	2	1000-5000	Cobb500	No	10days	yes	Yes	Yes	> 5
F031	Kurunegala	company-owned	6	>25,000	IndianRiver	Yes	7days	yes	Yes	Yes	> 10
F032	Kurunegala	company-owned	6	>25,000	Cobb500	Yes	14days	No	Yes	No	> 10
F033	Kurunegala	company-owned	8	>25,000	Hubbard	Yes	7days	No	Yes	Yes	> 10
F034	Kurunegala	company-owned	6	>25,000	Cobb500	Yes	7days	No	Yes	No	> 10
F035	Kurunegala	company-owned	1	>25,000	Cobb500	Yes	14days	No	Yes	No	1-5
F037	Kurunegala	self-owned	1	5000-25,000	Hubbard	Yes	14days	No	Yes	Yes	> 5
F038	Kurunegala	buy-back	1	5000-25,000	Hubbard	Yes	7days	yes	No	No	1-5

Farm ID	District	Operation Type	No. Flocks	Farm Bird Capacity	Source Strain	Litter Mgt	Rest period	Antibiotic on day 1	Farmer's Sick Bird Knowledge	High Deaths	Experience yrs
F039	Kurunegala	buy-back	1	1000-5000	Hubbard	No	10days	yes	No	Yes	last year
F040	Kurunegala	buy-back	1	1000-5000	Hubbard	No	14days	yes	No	No	last year
F042	Kurunegala	buy-back	2	5000-25,000	Hubbard	No	7days	No	Yes	No	> 10
F043	Kurunegala	buy-back	1	1000-5000	Hubbard	No	7days	No	No	No	last year
F044	Puttalam	self-owned	4	5000-25,000	Cobb500	No	10days	yes	Yes	No	> 10
F045	Puttalam	buy-back	1	1000-5000	Cobb500	No	7days	yes	No	Yes	last year
F046	Puttalam	buy-back	2	1000-5000	Cobb500	No	7days	yes	No	Yes	1-5
F047	Puttalam	buy-back	1	1000-5000	IndianRiver	No	7days	No	No	No	1-5
F048	Puttalam	self-owned	1	1000-5000	Cobb500	No	14days	yes	Yes	No	> 5
F049	Puttalam	self-owned	1	1000-5000	Cobb500	No	21days	No	Yes	No	> 10
F050	Puttalam	self-owned	8	5000-25,000	Cobb500	Yes	10days	No	No	Yes	> 5
F051	Puttalam	self-owned	3	1000-5000	Cobb500	No	7days	yes	Yes	No	1-5
F052	Puttalam	self-owned	2	1000-5000	IndianRiver	No	21days	yes	Yes	No	> 5
F053	Puttalam	self-owned	1	5000-25,000	Cobb500	No	10days	No	No	Yes	> 5
F054	Puttalam	self-owned	1	5000-25,000	Cobb500	Yes	7days	yes	Yes	No	> 5
F055	Puttalam	self-owned	1	1000-5000	Cobb500	No	7days	No	Yes	No	
F056	Puttalam	self-owned	2	1000-5000	IndianRiver	Yes	10days	No	Yes	No	> 10
F057	Puttalam	buy-back	1	1000-5000	Hubbard	No	14days	yes	No	No	last year
F058	Puttalam	buy-back	1	1000-5000	Cobb500	No	7days	yes	No	Yes	1-5
F059	Puttalam	buy-back	1	1000-5000	Hubbard	No	14days	No	No	Yes	last year
F060	Puttalam	buy-back	1	1000-5000	Hubbard	No	7days	No	No	Yes	1-5
F061	Puttalam	buy-back	1	1000-5000	Hubbard	No	10days	No	No	Yes	1-5
F062	Puttalam	buy-back	1	1000-5000	Hubbard	No	10days	No	No	Yes	last year
F063	Puttalam	buy-back	1	1000-5000	Hubbard	Yes	14days	yes	Yes	Yes	1-5
F064	Puttalam	company-owned	6	>25,000	Cobb500	Yes	7days	yes	Yes	Yes	> 10
F065	Gampaha	buy-back	1	1000-5000	Cobb500	No	7days	yes	No	No	1-5
F066	Gampaha	buy-back	1	1000-5000	IndianRiver	No	14days	No	No	No	last year
F067	Gampaha	buy-back	1	1000-5000	Cobb500	No	10days	yes	No	Yes	1-5
F068	Gampaha	self-owned	1	1000-5000	IndianRiver	No	7days	No	No	No	1-5
F069	Gampaha	self-owned	1	1000-5000	Cobb500	No	7days	No	No	No	> 5
F070	Gampaha	self-owned	1	1000-5000	Cobb500	Yes	10days	No	No	Yes	> 5
F071	Gampaha	self-owned	1	1000-5000	IndianRiver	No	7days	No	Yes	No	> 10
F072	Gampaha	self-owned	1	5000-25,000	IndianRiver	No	7days	No	No	No	1-5
F073	Gampaha	buy-back	1	1000-5000	IndianRiver	Yes	14days	No	No	No	last year
F074	Gampaha	buy-back	1	1000-5000	IndianRiver	Yes	14days	No	No	No	last year
F075	Gampaha	buy-back	1	1000-5000	IndianRiver	No	10days	No	Yes	No	> 10
F076	Gampaha	buy-back	2	5000-25,000	Cobb500	Yes	14days	No	No	Yes	1-5
F077	Gampaha	buy-back	1	1000-5000	Cobb500	No	7days	No	No	No	1-5
F078	Gampaha	buy-back	1	1000-5000	Cobb500	No	10days	No	No	No	last year
F079	Gampaha	self-owned	6	5000-25,000	Cobb500	Yes	7days	No	Yes	No	> 10
F080	Gampaha	self-owned	3	5000-25,000	Cobb500	Yes	14days	No	No	No	> 10
F081	Gampaha	self-owned	1	1000-5000	Cobb500	No	7days	No	Yes	No	> 5
F082	Gampaha	self-owned	1	5000-25,000	Cobb500	Yes	10days	No	No	No	> 10

Farm ID	District	Operation Type	No. Flocks	Farm Bird Capacity	Source Strain	Litter Mgt	Rest period	Antibiotic on day 1	Farmer's Sick Bird Knowledge	High Deaths	Experience yrs
F083	Gampaha	buy-back	1	1000-5000	IndianRiver	No	7days	yes	No	No	last year
F084	Gampaha	buy-back	1	1000-5000	Hubbard	No	14days	yes	Yes	No	> 5
F085	Kegalle	buy-back	2	1000-5000	Cobb500	Yes	14days	No	No	Yes	> 5
F086	Kegalle	buy-back	1	1000-5000	IndianRiver	Yes	10days	yes	No	No	1-5
F087	Kegalle	buy-back	1	1000-5000	Cobb500	No	10days	No	No	No	1-5
F088	Kegalle	buy-back	2	1000-5000	Cobb500	Yes	14days	No	No	No	1-5
F089	Kegalle	buy-back	1	1000-5000	Cobb500	No	10days	No	No	No	1-5
F090	Kegalle	buy-back	1	1000-5000	Cobb500	No	7days	No	No	Yes	1-5
F091	Kegalle	buy-back	1	1000-5000	Cobb500	No	10days	No	No	No	1-5
F092	Kegalle	buy-back	1	1000-5000	Cobb500	No	7days	No	Yes	No	> 10
F093	Kegalle	self-owned	1	1000-5000	Cobb500	No	21days	yes	Yes	No	> 5
F094	Kegalle	self-owned	1	1000-5000	Cobb500	Yes	7days	No	No	Yes	> 5
F095	Kegalle	buy-back	1	1000-5000	IndianRiver	No	7days	yes	No	Yes	1-5
F096	Kegalle	buy-back	1	1000-5000	Cobb500	No	7days	No	No	No	1-5
F097	Kegalle	buy-back	1	5000-25,000	IndianRiver	No	7days	yes	No	Yes	1-5
F098	Kegalle	buy-back	1	1000-5000	IndianRiver	No	7days	yes	No	No	last year
F099	Kegalle	buy-back	1	1000-5000	IndianRiver	Yes	14days	No	No	No	last year
F100	Kegalle	buy-back	4	5000-25,000	IndianRiver	No	14days	No	No	No	> 10
F101	Kegalle	buy-back	1	1000-5000	IndianRiver	Yes	14days	yes	No	No	> 5
F102	Kegalle	buy-back	1	1000-5000	IndianRiver	No	7days	yes	No	No	> 5
F103	Kegalle	buy-back	1	1000-5000	IndianRiver	No	10days	No	No	No	last year
F104	Kegalle	buy-back	2	1000-5000	Hubbard	No	10days	No	Yes	No	> 10
F105	Kandy	buy-back	1	1000-5000	Cobb500	Yes	10days	yes	No	No	> 5
F106	Kandy	buy-back	1	1000-5000	Hubbard	Yes	10days	yes	No	No	> 5
F107	Kandy	buy-back	1	1000-5000	Hubbard	Yes	10days	yes	No	No	> 5
F108	Kandy	buy-back	2	5000-25,000	Hubbard	Yes	10days	yes	No	No	> 5
F109	Kandy	buy-back	1	1000-5000	IndianRiver	Yes	10days	No	No	Yes	last year
F110	Kandy	buy-back	1	1000-5000	Cobb500	No	14days	No	Yes	Yes	> 5
F111	Kandy	buy-back	1	1000-5000	Cobb500	Yes	10days	No	No	No	last year
F112	Kandy	buy-back	1	1000-5000	Hubbard	No	14days	No	No	Yes	> 10
F113	Kandy	buy-back	1	1000-5000	Hubbard	No	14days	No	No	No	1-5
F114	Kandy	self-owned	1	1000-5000	Cobb500	No	7days	yes	Yes	Yes	> 10
F115	Kandy	company-owned	8	>25,000	Cobb500	Yes	7days	No	No	No	1-5
F116	Kandy	buy-back	2	5000-25,000	Cobb500	Yes	7days	No	Yes	No	> 10
F117	Kandy	buy-back	1	1000-5000	IndianRiver	Yes	7days	yes	Yes	No	1-5
F118	Kandy	self-owned	1	5000-25,000	Hubbard	Yes	7days	yes	Yes	Yes	> 10

Table A 5.2: Descriptive Statistics of the 115 farms (Observation-based)

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
F001	Isolated	No	Paddy	Wet	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	Yes
F002	Same Premises	No	Wood	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	No	Yes
F003	Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	No	No	Yes	Yes	Yes
F004	Same Premises	No	Wood	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	No	No
F005	Same Premises	No	Wood	Wet	No	No	No	No	No	Manual	No	No	No	Yes	Yes
F006	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	Yes
F007	Same Premises	No	both	Dry	No	No	No	Dropping	No	Manual	Yes	Yes	Yes	No	No
F008	Same Premises	No	both	Wet	No	No	No	No	No	Manual	No	No	No	Yes	No
F009	Same Premises	No	both	Dry	No	No	No	No	No	Manual	Yes	Yes	No	No	No
F010	Same Premises	No	Paddy	Wet	No	No	Yes	Dropping	No	Manual	Yes	Yes	No	No	No
F011	Residential& Same Premises	No	Wood	Dry	No	No	Yes	No	No	Manual	No	No	Yes	Yes	Yes
F012	Same Premises	No	both	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	Yes	No
F013	Residential& Same Premises	No	both	Wet	No	No	Yes	No	No	Manual	No	No	No	No	Yes
F014	Same Premises	No	both	Dry	No	No	No	No	No	Manual	Yes	Yes	No	Yes	No
F015	Same Premises	No	both	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	No	No

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
F016	Same Premises	No	Wood	Wet	No	No	Yes	Bait	No	Automatic	No	No	Yes	Yes	Yes
F017	Same Premises	No	Paddy	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	Yes	No
F018	Same Premises	No	Wood	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	No	No
F019	Same Premises	No	Wood	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	No	No
F020	Same Premises	No	Wood	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	No	No
F021	Isolated	No	Paddy	Wet	No	No	No	No	No	Manual	Yes	Yes	No	Yes	No
F022	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	Dropping	No	Manual	No	No	No	No	No
F023	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	Yes	No	Yes	Yes	Yes
F024	Same Premises	No	Paddy	Wet	No	No	Yes	No	No	Manual	Yes	Yes	Yes	Yes	No
F025	Same Premises	No	Paddy	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	No	Yes
F026	Residential& Same Premises	No	Paddy	Wet	No	No	Yes	Bait	No	Manual	Yes	Yes	Yes	Yes	Yes
F028	Same Premises	No	Wood	Dry	No	No	No	No	Yes	Automatic	Yes	No	Yes	Yes	Yes
F029	Same Premises	No	Wood	Dry	No	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	No	No
F030	Residential& Same Premises	No	Paddy	Wet	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	No
F031	Isolated	Yes	both	Wet	Yes	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F032	Isolated	Yes	Paddy	Wet	Yes	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F033	Isolated	Yes	Paddy	Wet	Yes	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F034	Isolated	Yes	Paddy	Dry	Yes	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
F035	Isolated	Yes	Paddy	Dry	No	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F037	Same Premises	No	both	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	No	No
F038	Isolated	Yes	Wood	Wet	No	No	Yes	Bait	No	Automatic	No	No	No	Yes	Yes
F039	Same Premises	No	Paddy	Dry	No	No	No	No	Yes	Manual	Yes	Yes	Yes	Yes	No
F040	Isolated	No	Paddy	Wet	No	No	Yes	No	No	Manual	Yes	Yes	Yes	No	Yes
F042	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Automatic	Yes	No	No	No	Yes
F043	Isolated	Yes	Paddy	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	No	Yes
F044	Same Premises	No	Wood	Wet	No	No	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	No
F045	Same Premises	No	Wood	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	No	No
F046	Residential& Same Premises	No	Wood	Wet	No	No	No	No	No	Manual	No	No	No	No	No
F047	Same Premises	No	Wood	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	Yes	No
F048	Same Premises	No	Wood	Wet	No	No	Yes	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F049	Same Premises	No	Wood	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	No	Yes
F050	Same Premises	No	Wood	Dry	No	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F051	Isolated	No	Wood	Dry	No	No	Yes	Bait	No	Automatic	Yes	Yes	Yes	No	No
F052	Isolated	No	Wood	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F053	Isolated	No	Wood	Dry	No	No	No	Bait	No	Automatic	Yes	Yes	Yes	No	Yes
F054	Isolated	Yes	both	Wet	Yes	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	Yes
F055	Isolated	Yes	Paddy	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	Yes	Yes
F056	Same Premises	No	Paddy	Dry	No	No	No	No	No	Automatic	No	Yes	Yes	Yes	Yes

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
F057	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	Yes	Manual	Yes	Yes	Yes	No	No
F058	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	No	No	No	Yes	Yes
F059	Same Premises	No	Paddy	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F060	Same Premises	No	Paddy	Dry	No	No	No	Dropping	No	Manual	Yes	No	No	No	No
F061	Residential& Same Premises	No	Paddy	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	No
F062	Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	Yes	No	Yes	Yes	Yes
F063	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	Yes	No
F064	Isolated	Yes	Wood	Wet	Yes	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	No	No
F065	Residential& Same Premises	No	Paddy	Wet	No	No	Yes	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F066	Residential& Same Premises	No	Wood	Dry	No	No	No	No	No	Manual	No	No	Yes	No	Yes
F067	Residential& Same Premises	No	Wood	Dry	No	No	Yes	No	No	Manual	Yes	No	Yes	No	No
F068	Residential& Same Premises	No	Wood	Wet	No	No	No	Dropping	No	Manual	Yes	Yes	Yes	Yes	Yes
F069	Residential& Same Premises	No	Wood	Wet	No	No	Yes	No	No	Manual	Yes	Yes	Yes	No	No
F070	Residential& Same Premises	No	Wood	Wet	No	No	No	No	No	Manual	No	No	No	Yes	Yes

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
F071	Residential& Same Premises	No	Wood	Wet	No	No	No	Dropping	No	Manual	Yes	Yes	No	No	No
F072	Residential& Same Premises	No	Wood	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	No	No
F073	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F074	Residential& Same Premises	No	Wood	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	No
F075	Residential& Same Premises	No	Paddy	Wet	No	No	No	No	No	Automatic	No	No	Yes	Yes	No
F076	Residential& Same Premises	No	Paddy	Wet	No	No	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F077	Residential& Same Premises	No	Paddy	Wet	No	No	No	No	No	Manual	No	No	Yes	Yes	No
F078	Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	No	No
F079	Isolated	Yes	Wood	Dry	Yes	Yes	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F080	Isolated	Yes	Paddy	Dry	No	No	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F081	Isolated	Yes	Paddy	Wet	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	No
F082	Isolated	Yes	Paddy	Dry	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	No	No
F083	Residential& Same Premises	No	Paddy	Wet	No	No	Yes	No	No	Manual	No	No	No	Yes	No
F084	Residential& Same Premises	No	Paddy	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F085	Residential& Same	No	Paddy	Dry	No	No	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
	Premises														
F086	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	No	No
F087	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Automatic	Yes	Yes	No	Yes	No
F088	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	Yes
F089	Residential& Same Premises	No	Paddy	Wet	No	No	No	No	No	Automatic	Yes	Yes	Yes	No	No
F090	Residential& Same Premises	No	both	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	Yes	No
F091	Residential& Same Premises	No	both	Dry	No	No	Yes	No	No	Automatic	Yes	Yes	No	Yes	No
F092	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	No	Automatic	Yes	Yes	No	No	Yes
F093	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	Dropping	No	Manual	No	No	No	Yes	Yes
F094	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F095	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Manual	No	No	No	Yes	Yes
F096	Same Premises	No	both	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	No	Yes
F097	Same Premises	No	Paddy	Wet	No	No	No	Dropping	Yes	Automatic	No	No	No	Yes	No
F098	Same Premises	No	Paddy	Dry	No	No	No	No	Yes	Manual	Yes	Yes	Yes	Yes	No

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
F099	Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	No	No	No	No	No
F100	Isolated	Yes	both	Dry	No	No	Yes	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes
F101	Same Premises	Yes	Paddy	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F102	Isolated	No	Paddy	Wet	No	No	No	No	No	Manual	No	No	No	No	Yes
F103	Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Manual	No	Yes	Yes	No	No
F104	Same Premises	No	Paddy	Dry	No	No	Yes	No	Yes	Automatic	Yes	Yes	Yes	Yes	Yes
F105	Residential& Same Premises	No	Paddy	Dry	No	No	Yes	No	No	Manual	Yes	Yes	Yes	No	Yes
F106	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	No	Automatic	Yes	No	Yes	Yes	Yes
F107	Same Premises	No	Paddy	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	No	Yes
F108	Isolated	No	Paddy	Dry	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	Yes
F109	Residential& Same Premises	No	Paddy	Dry	No	No	No	No	No	Automatic	Yes	Yes	Yes	No	No
F110	Isolated	No	Paddy	Wet	No	No	No	Dropping	No	Automatic	Yes	Yes	Yes	No	Yes
F111	Same Premises	No	Paddy	Dry	No	No	No	No	No	Manual	No	No	Yes	Yes	Yes
F112	Same Premises	No	both	Dry	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F113	Residential& Same Premises	No	Paddy	Wet	No	No	No	No	Yes	Manual	No	No	Yes	Yes	No
F114	Isolated	No	Paddy	Wet	No	No	No	No	No	Manual	Yes	Yes	Yes	Yes	Yes
F115	Isolated	Yes	Paddy	Dry	Yes	No	Yes	Bait	No	Automatic	Yes	Yes	Yes	No	Yes
F116	Isolated	No	Paddy	Dry	No	No	No	Bait	No	Automatic	Yes	Yes	Yes	Yes	Yes

Farm ID	Farm Location	Entry Restrict	Litter Type	Litter Cond	Foot bath	Boot Change	Hand Wash	Rodent Evidence	Other Poultry	Drinker Type	Clean Feeder	Clean Drinker	Clean Inside pen	Clean Outside pen	Feed Storage
F117	Isolated	No	Paddy	Wet	No	No	No	No	No	Automatic	Yes	Yes	Yes	No	Yes
F118	Residential& Same Premises	Yes	Paddy	Dry	No	No	Yes	No	No	Automatic	Yes	Yes	Yes	Yes	Yes

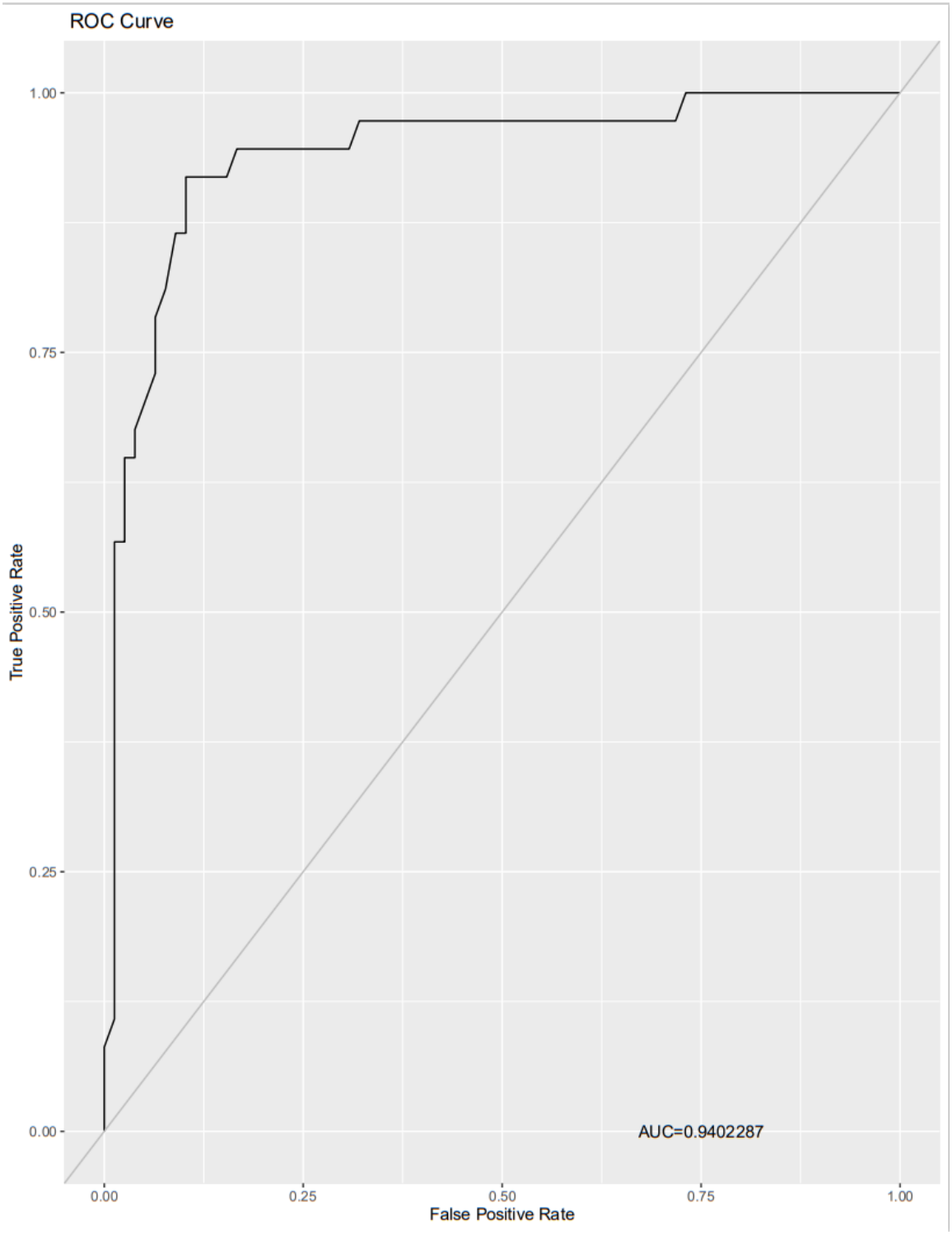

Figure A 5.1: ROC curve of multivariable farm-level model

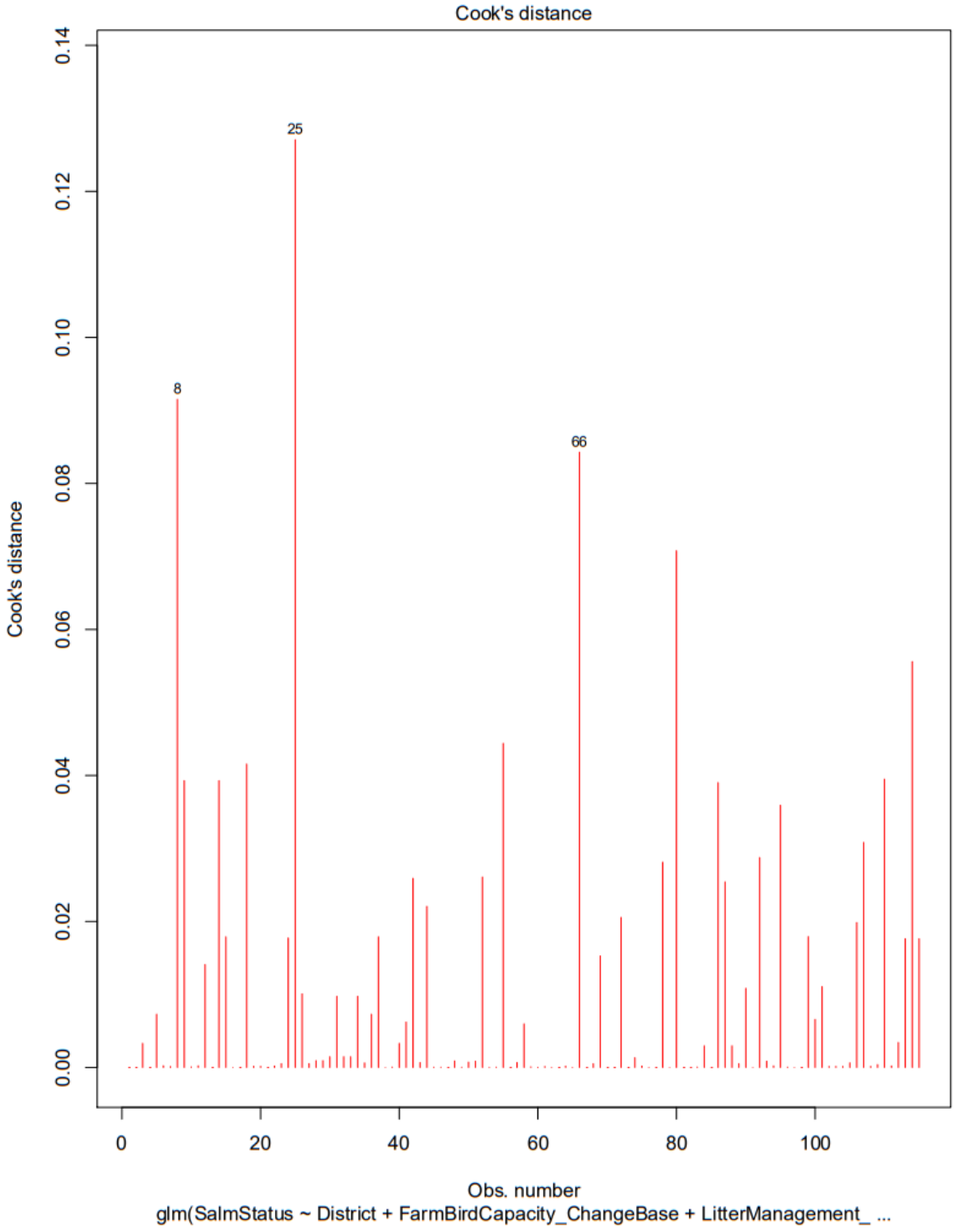

Figure A 5.2: Cook's distance of multivariable farm-level model

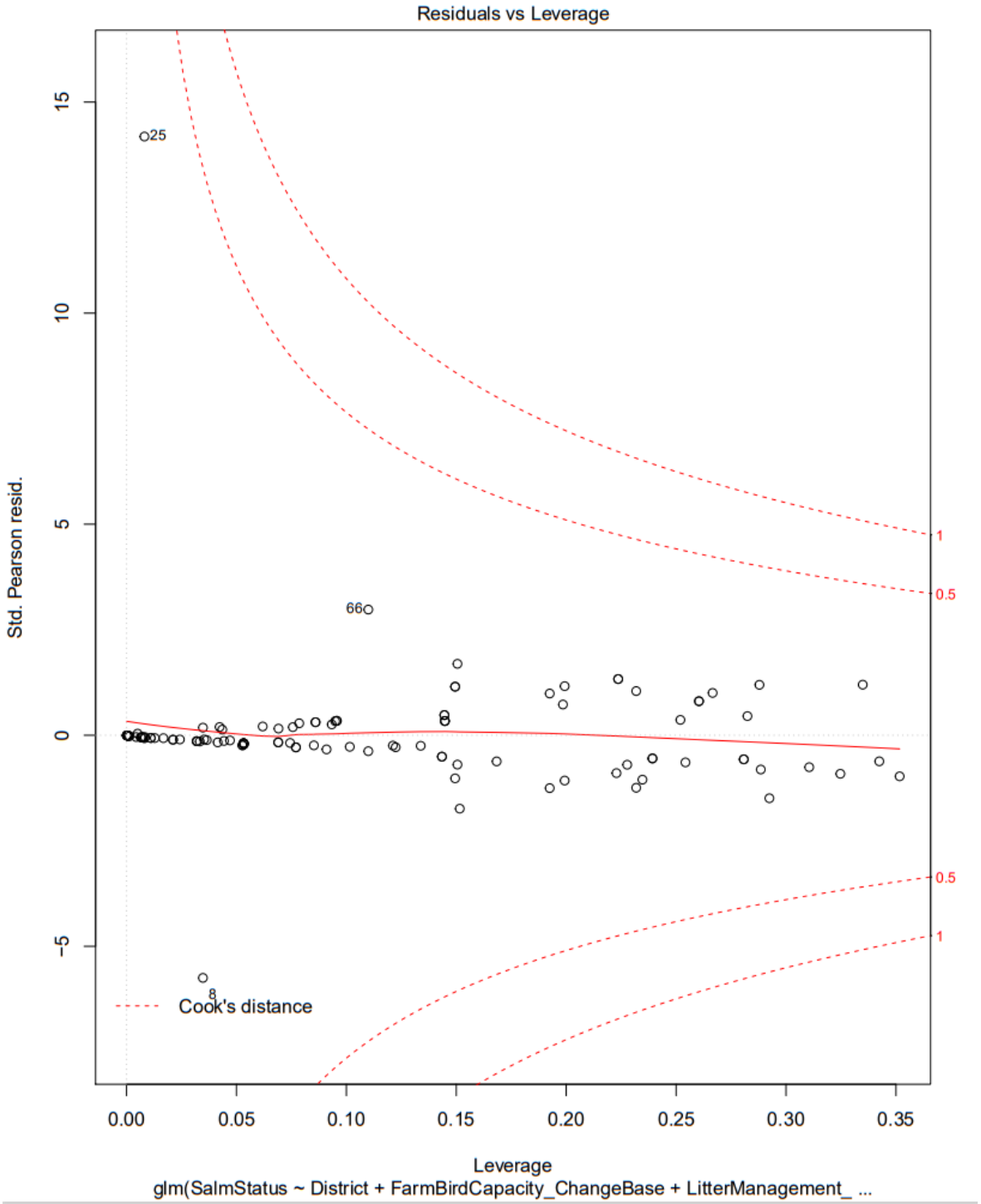

Figure A 5.3: Pearson residuals of multivariable farm-level model

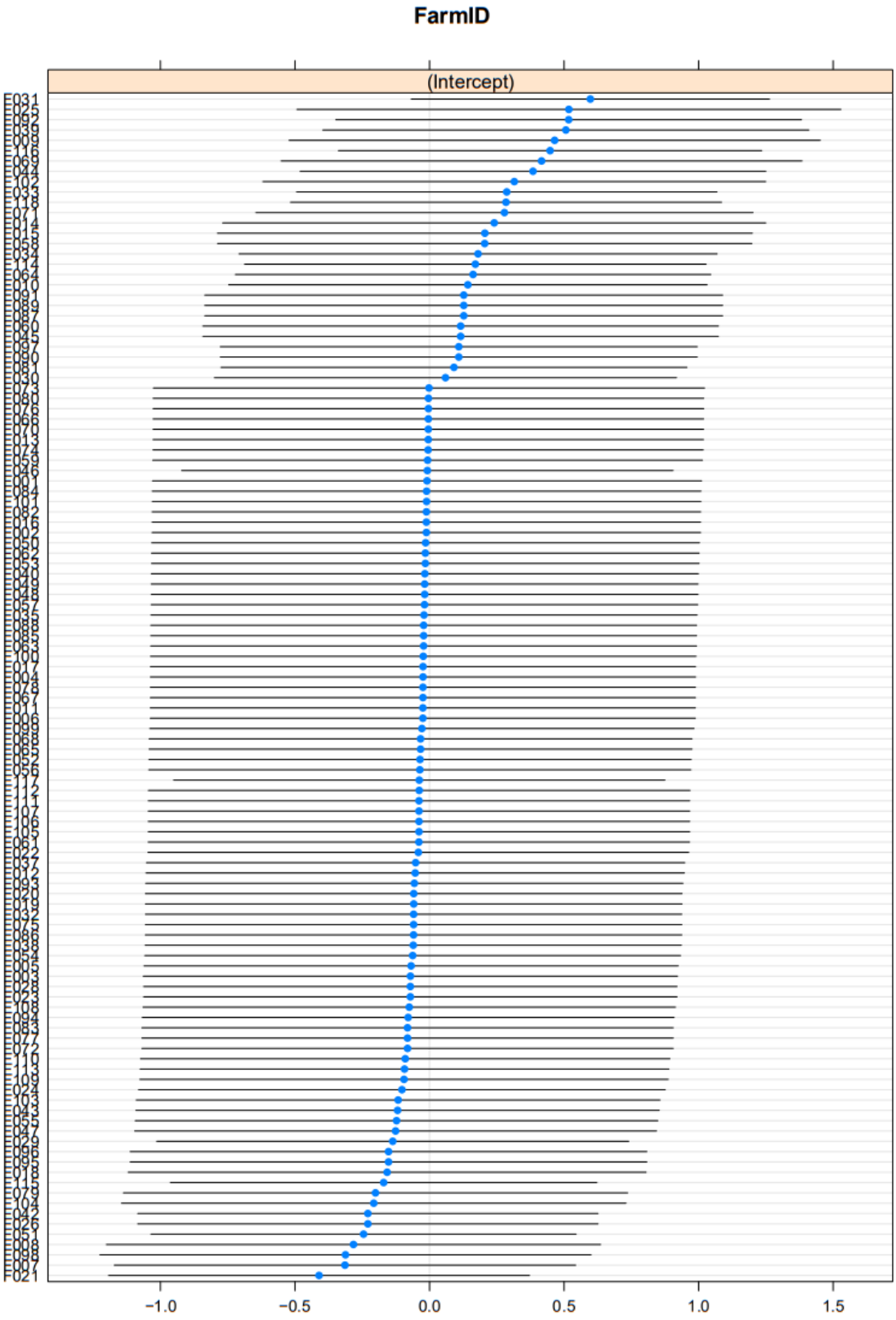

Figure A 5.4: Random effects of multivariable mixed-effect sample-level model

Appendix VI: Supplementary Data for Chapter 6

Table A 6.1: DNA, library and lab chip data of the isolates

Isolate ID	DNA conc (ng/uL)	Library Conc (ng/ul)	Labchip: fragment size (bp)
SSL001	3.2	9.16	1045
SSL002	2.37	9.68	958
SSL003	2.23	10.1	793
SSL004	1.74	20	937
SSL005	2.98	7.8	865
SSL006	5.52	15.9	926
SSL007	6.82	4.68	811
SSL008	3.83	16.6	772
SSL009	2.9	5.6	991
SSL010	9.54	13.9	709
SSL011	3.06	17.4	744
SSL012	7.37	20.8	556
SSL013	3.18	10.2	489
SSL014	1.67	15.1	609
SSL015	1.42	11.3	740
SSL016	0.413	6.16	619
SSL017	7.66	2.82	742
SSL018	0.396	20.8	856
SSL019	6.82	9.16	767
SSL020	2.26	13.8	1031
SSL021	2.81	5.24	797
SSL022	3.45	7.12	595
SSL023	0.98	5.08	548
SSL024	1.29	7.88	569
SSL025	2.66	3.74	557
SSL026	0.566	3.61	545
SSL027	0.72	4.6	608
SSL028	0.996	3.04	492
SSL029	1.27	3.15	582
SSL030	1.93	6.44	730
SSL031	1.43	13.9	688
SSL032	3.91	9.64	712
SSL033	1.23	22	720
SSL034	2.18	5.36	659
SSL035	2.98	9.2	777
SSL036	2.83	6.08	821
SSL037	6.51	15.8	868
SSL038	0.531	5.72	559

Isolate ID	DNA conc (ng/uL)	Library Conc (ng/ul)	Labchip: fragment size (bp)
SSL039	19.3	14.7	657
SSL040	5.41	18	867
SSL041	6.06	7.16	669
SSL042	8.99	12.6	676
SSL043	2.71	9.2	759
SSL044	2.89	14	778
SSL045	1.73	17	620
SSL046	0.683	9.72	727
SSL047	6.8	8.76	748
SSL048	2.47	12.6	626
SSL049	3.17	11.1	807
SSL050	0.834	2.36	570
SSL051	0.723	18.5	700
SSL052	0.635	16.9	644
SSL053	0.453	10.8	658
SSL054	2.7	2.88	602
SSL055	0.306	5.08	723
SSL056	0.602	8.72	822
SSL057	3.49	5.72	703
SSL058	1.38	7.04	549
SSL059	3.3	7.8	616
SSL060	1.75	8.28	688
SSL061	2.82	3.9	719
SSL062	2.22	4.16	853
SSL063	3.92	19.2	645
SSL064	1.6	6.92	592
SSL065	0.71	22.4	675
SSL066	0.71	4.04	602
SSL067	0.62	11.6	623
SSL068	0.476	7.12	633
SSL069	5.63	7.36	797
SSL070	4.68	12.5	889
SSL071	0.811	6.96	664
SSL072	7.49	5.68	699
SSL073	1.57	22	700
SSL074	2.88	2.5	615
SSL075	4.29	23.2	568
SSL076	4.68	11	839
SSL077	1.21	3.01	789
SSL078	3.73	16.4	669
SSL079	0.791	4	740
SSL081	6.41	11	585
SSL082	1.76	8.32	546

Isolate ID	DNA conc (ng/uL)	Library Conc (ng/ul)	Labchip: fragment size (bp)
SSL083	3.81	20	740
SSL084	6.28	13.9	706
SSL085	5.89	9.96	718
SSL086	4.22	5.76	700
SSL087	3.23	16.3	669
SSL088	3.41	22.4	913
SSL089	3.52	5.36	823
SSL090	1.06	6	685
SSL091	7.14	3.42	704
SSL092	12.2	2.66	592
SSL093	6.39	2.74	758
SSL094	7.18	16.2	747
SSL095	2.99	4.88	593
SSL096	4.74	3.74	558
SSL097	0.577	2.94	800
SSL098	0.674	17.9	743
SSL101	3.17	19.3	808
SSL102	1.23	7.36	739
SSL103	0.683	4.36	669
SSL104	1.69	17.1	742
SSL105	1.96	2.39	767
SSL106	1.87	2.58	715
SSL107	1.96	20.8	714
SSL108	2.76	2.95	793
SSL109	0.608	3.53	890
SSL110	1.4	3.24	709
SSL111	3.75	3.7	892
SSL112	1.27	3.69	522
SSL113	3.67	10.5	657
SSL114	3.28	3.05	653
SSL115	4.97	13.2	697
SSL116	1.79	3.39	701
SSL117	0.601	18.5	776
SSL118	0.556	3.58	760
SSL119	0.743	2.77	635
SSL120	Out of range	15	708
SSL121	1.19	6.44	613
SSL122	0.955	11	574
SSL123	0.712	15.8	695
SSL124	1.24	3.83	813
SSL125	1.6	1.86	360
SSL126	0.465	16	729

Isolate ID	DNA conc (ng/uL)	Library Conc (ng/ul)	Labchip: fragment size (bp)
SSL127	0.779	18.2	864
SSL128	0.53	4.04	735
SSL129	0.448	22.8	824
SSL130	0.82	4	724
SSL131	0.585	4.12	799
SSL132	2.01	4.4	740
SSL133	2.32	3.51	788
SSL134	0.875	19.2	773
SSL135	4.47	2.57	763
SSL136	1.57	22.4	857
SSL137	2.2	22.8	884
SSL138	2.39	16.8	793
SSL139	1.96	15.4	846
SSL140	2.28	4.72	851
SSL141	4.57	20.4	647
SSL142	0.342	5.76	731
SSL143	3.93	21.6	791
SSL144	5.64	18.1	961
SSL145	4.57	20.4	757
SSL146	5.15	11.6	803
SSL147	1.7	19.9	715
SSL148	1.73	19	704
SSL149	1.59	20.4	632
SSL150	1.78	14	760
SSL151	2.36	4.92	724
SSL152	0.933	22.4	681
SSL153	3.55	4.64	818
SSL154	1	18.7	679
SSL155	2.19	19.4	637
SSL156	0.617	20.8	792
SSL157	0.943	12	661
SSL158	4.39	4.8	833
SSL159	1.57	22	795
SSL160	2.99	4.2	719
SSL161	8.68	20.4	795
SSL162	Out of range	19.2	876
SSL163	2.55	23.6	751
SSL164	8.02	4	746
SSL165	3.13	5	857
SSL166	0.373	4	929
SSL167	3.32	4.28	758

Table A 6.2: Antibiotics identified through the Fleming Fund programme as relevant to Sri Lanka

Antibiotic Class/antibiotic	*E. coli*	*Salmonella spp.*	*Campylobacter spp.*	*Enterococcus spp.*
Aminoglycosides	Gentamicin		Gentamicin Streptomycin	
Amphenicol	Chloramphenicol	Chloramphenicol		
Carbapenem	Meropenem Ertapenem	Meropenem Ertapenem		
Cephalosporins III	Ceftriaxone	Ceftriaxone		
Cephalosporins IV	Cefepime			
Quinolones	Ciprofloxacin	Ciprofloxacin Pefloxacin	Ciprofloxacin	
Glycopeptides				Vancomycin
Glycylcyclines				Tigecycline
Oxazolidinones				Linezolid
Penicillins	Ampicillin	Ampicillin	Ampicillin	Ampicillin
Polymixins	Colistin[a]	Colistin[a]		
Synercids				Quinupristin-dalfopristin
Tetracyclines	Tetracycline	Tetracycline	Tetracycline	
Sulphonamides/Trimethoprim	Co-trimoxazole	Co-trimoxazole		

Table A 6.3: AST QC zone ranges for non-fastidious organisms under CLSI guidelines

Antibiotic	Product code	Disc dose (µg)	Zone range (mm)
Cefotaxime	CTX	30	15-22
Meropenem	MEM	10	29-35
Gentamicin	CN	10	21-27
Trimethoprim-sulphamethoxazole	SXT	25*	29-37
Ampicillin	AMP	10	21-28
Tetracycline	TE	30	19-26
Nalidixic acid	NA	30	27-33
Ciprofloxacin	CIP	5	18-25
Chloramphenicol	C	30	22-28
Enrofloxacin	ENR	5	32-40

∗ Dose combination of SXT: Trimethoprim (1.25 µg) and sulphamethoxazole (23.75 µg)
Reference: CLSI VET 08- ED 4: 2018 for Enrofloxacin and
CLSI M100- ED 29: 2019 for all other antibiotics

Table A 6.4: Metadata of Individual Isolates

SampleID	IsolateID	SourceID	SourceType	SampleType	Date of collection	Age	AgeType	MLST	Serovar
SSL001	SSL001a	F007	Broiler farm	Boot Socks	28/02/2017	13	days	314	Kentucky
SSL002	SSL002a	F009	Broiler farm	Boot Socks	28/02/2017	15	days	314	Kentucky
SSL003	SSL003a	F009	Broiler farm	Boot Socks	28/02/2017	33	days	314	Kentucky
SSL004	SSL004a	F010	Broiler farm	Boot Socks	28/02/2017	9	days	314	Kentucky
SSL005	SSL005a	F010	Broiler farm	Boot Socks	07/03/2017	32	days	314	Kentucky
SSL006	SSL006a	F014	Broiler farm	Boot Socks	14/03/2017	15	days	314	Kentucky
SSL007	SSL007a	F015	Broiler farm	Boot Socks	14/03/2017	26	days	314	Kentucky
SSL008	SSL008a	H001	Hatchery	fluff	04/04/2017	45	weeks	314	Kentucky
SSL009	SSL009a	H002	Hatchery	eggshell	26/04/2017	60	weeks	11	Enteritidis
SSL010	SSL010a	H002	Hatchery	eggshell	26/04/2017	50	weeks	11	Enteritidis
SSL011	SSL011a	H002	Hatchery	dead chick	26/04/2017	50	weeks	11	Enteritidis
SSL012	SSL012a	F044	Broiler farm	Boot Socks	16/05/2017	43	days	36	Typhimurium
SSL013	SSL013a	F044	Broiler farm	Cloacal swab	16/05/2017	43	days	36	Typhimurium
SSL014	SSL014a	F044	Broiler farm	Boot Socks	16/05/2017	43	days	314	Kentucky
SSL015	SSL015a	F045	Broiler farm	Boot Socks	16/05/2017	12	days	314	Kentucky
SSL016	SSL016a	F046	Broiler farm	Cloacal swab	16/05/2017	22	days	22	Braenderup
SSL017	SSL017a	F021	Broiler farm	Boot Socks	28/05/2017	10	days	36	Typhimurium
SSL018	SSL018a	F021	Broiler farm	Boot Socks	28/05/2017	28	days	314	Kentucky
SSL019	SSL019a	F025	Broiler farm	Boot Socks	28/05/2017	22	days	36	Typhimurium
SSL020	SSL020a	F025	Broiler farm	Boot Socks	28/05/2017	22	days	36	Typhimurium
SSL021	SSL021a	H003	Hatchery	fluff	01/06/2017	52	weeks	314	Kentucky
SSL022	SSL022a	H003	Hatchery	fluff	01/06/2017	52	weeks	314	Kentucky
SSL023	SSL023a	H003	Hatchery	dead chick	01/06/2017	52	weeks	314	Kentucky

SampleID	IsolateID	SourceID	SourceType	SampleType	Date of collection	Age	AgeType	MLST	Serovar
SSL024	SSL024a	H003	Hatchery	fluff	01/06/2017	35	weeks	314	Kentucky
SSL025	SSL025a	H003	Hatchery	eggshell	01/06/2017	35	weeks	314	Kentucky
SSL026	SSL026a	H003	Hatchery	DIS	01/06/2017	35	weeks	314	Kentucky
SSL027	SSL027a	H003	Hatchery	DIS	01/06/2017	35	weeks	314	Kentucky
SSL028	SSL028a	F026	Broiler farm	Boot Socks	06/06/2017	39	days	36	Typhimurium
SSL029	SSL029a	F029	Broiler farm	Boot Socks	06/06/2017	35	days	36	Typhimurium
SSL030	SSL030a	F030	Broiler farm	Boot Socks	06/06/2017	42	days	36	Typhimurium
SSL031	SSL031a	F030	Broiler farm	Boot Socks	06/06/2017	42	days	36	Typhimurium
SSL032	SSL032a	H005	Hatchery	dead chick	03/07/2017	50	weeks	11	Enteritidis
SSL033	SSL033a	H005	Hatchery	muconeum	03/07/2017	50	weeks	11	Enteritidis
SSL034	SSL034a	H005	Hatchery	eggshell	03/07/2017	50	weeks	11	Enteritidis
SSL035	SSL035a	F069	Broiler farm	Boot Socks	14/09/2017	24	days	NA	Butantan
SSL036	SSL036a	F069	Broiler farm	Boot Socks	14/09/2017	24	days	NA	Butantan
SSL037	SSL037a	F071	Broiler farm	Boot Socks	14/09/2017	40	days	19	Typhimurium
SSL038	SSL038a	F071	Broiler farm	Boot Socks	14/09/2017	40	days	19	Typhimurium
SSL039	SSL039a	H006	Hatchery	fluff	20/09/2017	52	weeks	314	Kentucky
SSL040	SSL040a	H006	Hatchery	fluff	20/09/2017	52	weeks	314	Kentucky
SSL041	SSL041a	H006	Hatchery	eggshell	20/09/2017	52	weeks	314	Kentucky
SSL042	SSL042a	H006	Hatchery	eggshell	20/09/2017	52	weeks	314	Kentucky
SSL043	SSL043a	H006	Hatchery	fluff	20/09/2017	36	weeks	36	Typhimurium
SSL044	SSL044a	H006	Hatchery	eggshell	20/09/2017	36	weeks	36	Typhimurium
SSL045	SSL045a	H006	Hatchery	eggshell	20/09/2017	36	weeks	36	Typhimurium
SSL046	SSL046a	H006	Hatchery	muconeum	20/09/2017	36	weeks	36	Typhimurium
SSL047	SSL047a	H006	Hatchery	muconeum	20/09/2017	36	weeks	36	Typhimurium
SSL048	SSL048a	F087	Broiler farm	Cloacal swab	21/09/2017	28	days	11	Enteritidis
SSL049	SSL049a	F089	Broiler farm	Cloacal swab	21/09/2017	24	days	11	Enteritidis

SampleID	IsolateID	SourceID	SourceType	SampleType	Date of collection	Age	AgeType	MLST	Serovar
SSL050	SSL050a	F090	Broiler farm	Boot Socks	21/09/2017	8	days	14	Senftenberg
SSL051	SSL051a	F090	Broiler farm	Boot Socks	21/09/2017	8	days	14	Senftenberg
SSL052	SSL052a	F091	Broiler farm	Cloacal swab	21/09/2017	8	days	14	Senftenberg
SSL053	SSL053a	F092	Broiler farm	Cloacal swab	21/09/2017	18	days	14	Senftenberg
SSL054	SSL054a	F092	Broiler farm	Cloacal swab	21/09/2017	18	days	1588	Paratyphi B var. Java
SSL055	SSL055a	F092	Broiler farm	Boot Socks	21/09/2017	18	days	14	Senftenberg
SSL056	SSL056a	F092	Broiler farm	Boot Socks	21/09/2017	18	days	14	Senftenberg
SSL057	SSL057a	F031	Broiler farm	Boot Socks	25/09/2017	19	days	314	Kentucky
SSL058	SSL058a	F031	Broiler farm	Boot Socks	25/09/2017	19	days	314	Kentucky
SSL059	SSL059a	F031	Broiler farm	Cloacal swab	25/09/2017	19	days	314	Kentucky
SSL060	SSL060a	F031	Broiler farm	Boot Socks	25/09/2017	40	days	2063	Chester
SSL061	SSL061a	F031	Broiler farm	Boot Socks	25/09/2017	40	days	2063	Chester
SSL062	SSL062a	F031	Broiler farm	Cloacal swab	25/09/2017	40	days	1541	Corvallis
SSL063	SSL063a	F031	Broiler farm	Boot Socks	25/09/2017	45	days	2063	Chester
SSL064	SSL064a	F031	Broiler farm	Boot Socks	25/09/2017	40	days	NA	Chester
SSL065	SSL065a	H007	Hatchery	eggshell	28/09/2017	63	weeks	909	Bareilly
SSL066	SSL066a	H007	Hatchery	eggshell	28/09/2017	63	weeks	909	Bareilly
SSL067	SSL067a	H007	Hatchery	eggshell	28/09/2017	44	weeks	909	Bareilly
SSL068	SSL068a	H007	Hatchery	eggshell	28/09/2017	44	weeks	909	Bareilly
SSL069	SSL069a	F114	Broiler farm	Boot Socks	03/10/2017	8	days	314	Kentucky
SSL070	SSL070a	F114	Broiler farm	Cloacal swab	03/10/2017	8	days	314	Kentucky
SSL071	SSL071a	F114	Broiler farm	Cloacal swab	03/10/2017	8	days	314	Kentucky
SSL072	SSL072a	H008	Hatchery	eggshell	10/10/2017	54	weeks	11	Enteritidis
SSL073	SSL073a	H008	Hatchery	fluff	10/10/2017	54	weeks	11	Enteritidis
SSL074	SSL074a	H008	Hatchery	eggshell	10/10/2017	54	weeks	11	Enteritidis
SSL075	SSL075a	H008	Hatchery	fluff	10/10/2017	54	weeks	11	Enteritidis

SampleID	IsolateID	SourceID	SourceType	SampleType	Date of collection	Age	AgeType	MLST	Serovar
SSL076	SSL076a	F051	Broiler farm	Boot Socks	11/10/2017	28	days	2063	Chester
SSL077	SSL077a	F051	Broiler farm	Boot Socks	11/10/2017	28	days	2063	Chester
SSL078	SSL078a	F097	Broiler farm	Boot Socks	13/10/2017	31	days	314	Kentucky
SSL079	SSL079a	F097	Broiler farm	Boot Socks	13/10/2017	31	days	314	Kentucky
SSL081	SSL081a	F115	Broiler farm	Boot Socks	13/10/2017	6	days	314	Kentucky
SSL082	SSL082a	F115	Broiler farm	Boot Socks	13/10/2017	6	days	314	Kentucky
SSL083	SSL083a	F081	Broiler farm	Cloacal swab	24/10/2017	33	days	314	Kentucky
SSL084	SSL084a	F081	Broiler farm	Cloacal swab	24/10/2017	33	days	314	Kentucky
SSL085	SSL085a	F058	Broiler farm	Boot Socks	25/10/2017	14	days	314	Kentucky
SSL086	SSL086a	F060	Broiler farm	Boot Socks	25/10/2017	14	days	314	Kentucky
SSL087	SSL087a	H009	Hatchery	fluff	30/10/2017	43	weeks	11	Enteritidis
SSL088	SSL088a	H009	Hatchery	fluff	30/10/2017	43	weeks	11	Enteritidis
SSL089	SSL089a	H009	Hatchery	fluff	30/10/2017	53	weeks	11	Enteritidis
SSL090	SSL090a	H009	Hatchery	eggshell	30/10/2017	53	weeks	365	Weltevreden
SSL091	SSL091a	F102	Broiler farm	boot socks	30/10/2017	11	days	1541	Corvallis
SSL092	SSL092a	F102	Broiler farm	boot socks	30/10/2017	11	days	1541	Corvallis
SSL093	SSL093a	F116	Broiler farm	boot socks	31/10/2017	11	days	1541	Corvallis
SSL094	SSL094a	F116	Broiler farm	boot socks	31/10/2017	11	days	1541	Corvallis
SSL095	SSL095a	F116	Broiler farm	Cloacal swab	31/10/2017	11	days	909	Bareilly
SSL096	SSL096a	F116	Broiler farm	Cloacal swab	31/10/2017	11	days	909	Bareilly
SSL097	SSL097a	F116	Broiler farm	Cloacal swab	31/10/2017	11	days	909	Bareilly
SSL098	SSL098a	F117	Broiler farm	boot socks	31/10/2017	9	days	1541	Corvallis
SSL101	SSL101a	F033	Broiler farm	boot socks	06/11/2017	17	days	314	Kentucky
SSL102	SSL102a	F033	Broiler farm	boot socks	06/11/2017	17	days	314	Kentucky
SSL103	SSL103a	F033	Broiler farm	boot socks	06/11/2017	24	days	11	Enteritidis
SSL104	SSL104a	F033	Broiler farm	boot socks	06/11/2017	24	days	314	Kentucky

SampleID	IsolateID	SourceID	SourceType	SampleType	Date of collection	Age	AgeType	MLST	Serovar
SSL105	SSL105a	F034	Broiler farm	boot socks	14/11/2017	15	days	36	Typhimurium
SSL106	SSL106a	F034	Broiler farm	boot socks	14/11/2017	15	days	36	Typhimurium
SSL107	SSL107a	F064	Broiler farm	boot socks	17/11/2017	25	days	314	Kentucky
SSL108	SSL108a	F064	Broiler farm	boot socks	17/11/2017	25	days	314	Kentucky
SSL109	SSL109a	F039	Broiler farm	boot socks	22/11/2017	21	days	2330	Durban
SSL110	SSL110a	F039	Broiler farm	boot socks	22/11/2017	21	days	2330	Durban
SSL111	SSL111a	F039	Broiler farm	Cloacal swab	22/11/2017	21	days	11	Enteritidis
SSL112	SSL112a	F042	Broiler farm	Cloacal swab	22/11/2017	21	days	11	Enteritidis
SSL113	SSL113a	F118	Broiler farm	boot socks	12/12/2017	32	days	11	Enteritidis
SSL114	SSL114a	F118	Broiler farm	boot socks	12/12/2017	32	days	11	Enteritidis
SSL115	SSL115a	F118	Broiler farm	Cloacal swab	12/12/2017	32	days	314	Kentucky
SSL116	SSL116a	F118	Broiler farm	Cloacal swab	12/12/2017	32	days	11	Enteritidis
SSL117	SSL117a	H013	Hatchery	muconeum	11/12/2017	88	weeks	16	Virchow
SSL118	SSL118a	H013	Hatchery	eggshell	11/12/2017	88	weeks	16	Virchow
SSL119	SSL119a	H013	Hatchery	fluff	11/12/2017	68	weeks	16	Virchow
SSL120	SSL120a	H013	Hatchery	eggshell	11/12/2017	45	weeks	16	Virchow
SSL121	SSL121a	H013	Hatchery	muconeum	11/12/2017	35	weeks	16	Virchow
SSL122	SSL122a	H013	Hatchery	eggshell	11/12/2017	35	weeks	16	Virchow
SSL123	SSL123a	H013	Hatchery	eggshell	11/12/2017	35	weeks	22	Braenderup
SSL124	SSL124a	H014	Hatchery	fluff	14/12/2017	58	weeks	1541	Corvallis
SSL125	SSL125a	H014	Hatchery	fluff	14/12/2017	58	weeks	1541	Corvallis
SSL126	SSL126a	H014	Hatchery	fluff	14/12/2017	72	weeks	203	Bareilly
SSL127	SSL127a	H014	Hatchery	eggshell	14/12/2017	72	weeks	203	Bareilly
SSL128	SSL128a	H014	Hatchery	eggshell	14/12/2017	72	weeks	203	Bareilly
SSL129	SSL129a	H014	Hatchery	fluff	14/12/2017	42	weeks	203	Bareilly
SSL130	SSL130a	H014	Hatchery	fluff	14/12/2017	42	weeks	203	Bareilly

SampleID	IsolateID	SourceID	SourceType	SampleType	Date of collection	Age	AgeType	MLST	Serovar
SSL131	SSL131a	H014	Hatchery	fluff	14/12/2017	51	weeks	203	Bareilly
SSL132	SSL132a	OB1	outbreak	post mortem	01/01/2010	NA		314	Kentucky
SSL133	SSL133a	OB2	outbreak	post mortem	01/01/2010	NA		11	Enteritidis
SSL134	SSL134a	OB3	outbreak	post mortem	01/01/2010	NA		11	Enteritidis
SSL135	SSL135a	OB4	outbreak	post mortem	01/01/2010	NA		78	Gallinarum
SSL136	SSL136a	OB5	outbreak	post mortem	01/01/2010	NA		11	Enteritidis
SSL137	SSL137a	OB6	outbreak	post mortem	01/01/2012	NA		11	Enteritidis
SSL138	SSL138a	OB7	outbreak	post mortem	01/01/2012	NA		99	Typhimurium
SSL139	SSL139a	OB8	outbreak	post mortem	01/01/2012	NA		11	Enteritidis
SSL140	SSL140a	OB9	outbreak	post mortem	01/01/2012	NA		99	Typhimurium
SSL141	SSL141a	OB10	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL142	SSL142a	OB11	outbreak	post mortem	01/01/2012	NA		203	Bareilly
SSL143	SSL143a	OB12	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL144	SSL144a	OB13	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL145	SSL145a	OB14	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL146	SSL146a	OB15	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL147	SSL147a	OB16	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL148	SSL148a	OB17	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL149	SSL149a	OB18	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL150	SSL150a	OB19	outbreak	post mortem	01/01/2012	NA		78	Gallinarum
SSL151	SSL151a	OB20	outbreak	post mortem	01/01/2012	NA		92	Gallinarum
SSL152	SSL152a	OB21	outbreak	post mortem	01/01/2012	NA		203	Bareilly
SSL153	SSL153a	OB22	outbreak	post mortem	01/01/2012	NA		92	Gallinarum
SSL154	SSL154a	OB23	outbreak	post mortem	01/01/2012	NA		314	Kentucky
SSL155	SSL155a	OB24	outbreak	post mortem	20/05/2017	NA		11	Enteritidis
SSL156	SSL156a	OB25	outbreak	post mortem	02/06/2017	NA		319	Tennessee

SampleID	IsolateID	SourceID	SourceType	SampleType	Date of collection	Age	AgeType	MLST	Serovar
SSL157	SSL157a	OB26	outbreak	post mortem	25/07/2017	NA		11	Enteritidis
SSL158	SSL158a	OB27	outbreak	post mortem	31/08/2017	NA		19	Typhimurium
SSL159	SSL159a	OB28	outbreak	post mortem	04/09/2017	NA		19	Typhimurium
SSL160	SSL160a	OB29	outbreak	post mortem	23/10/2017	NA		78	Gallinarum
SSL161	SSL161a	OB30	outbreak	post mortem	20/11/2017	NA		92	Gallinarum
SSL162	SSL162a	OB31	outbreak	post mortem	24/11/2017	NA		11	Enteritidis
SSL163	SSL163a	OB32	outbreak	post mortem	22/12/2017	NA		11	Enteritidis
SSL164	SSL164a	OB33	outbreak	post mortem	28/12/2017	NA		92	Gallinarum
SSL165	SSL165a	OB34	outbreak	post mortem	10/01/2018	NA		11	Enteritidis
SSL166	SSL166a	OB35	outbreak	post mortem	25/01/2018	NA		319	Tennessee
SSL167	SSL167a	OB36	outbreak	post mortem	03/02/2018	NA		11	Enteritidis

** Date of collection was set as 1st January, when only the year is available (without exact date)

NA: Not Available

Table A 6.5: AST Zone Readings for individual isolates

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
SSL001	15/08/2019	34	34	27	24	19	23	13	27	26	16
	22/08/2019	33	34	29	24	20	22	14	26	28	18
	Average	34	34	28	24	20	23	14	27	27	17
SSL002	15/08/2019	36	36	6	6	20	24	13	27	28	16
	22/08/2019	36	37	6	6	21	24	13	29	28	16
	Average	36	37	6	6	21	24	13	28	28	16
SSL003	15/08/2019	37	37	24	25	6	24	13	29	28	16
	22/08/2019	34	37	22	24	6	23	14	24	28	18
	18/09/2019								25		
	Average	36	37	23	25	6	24	26	26	28	17
SSL004	15/08/2019	36	37	29	24	21	24	15	27	29	17
	22/08/2019	33	36	30	24	22	24	13	25	28	18
	Average	35	37	30	24	22	24	14	26	29	18
SSL005	15/08/2019	34	37	23	25	6	24	14	26	30	18
	22/08/2019	33	36	23	23	6	22	14	25	28	17
	Average	34	37	23	24	6	23	14	26	29	18
SSL006	15/08/2019	34	37	6	6	20	24	13	27	28	16
	22/08/2019	33	38	6	6	20	23	13	24	27	16
	Average	34	38	6	6	20	24	13	26	28	16
SSL007	15/08/2019	35	38	6	6	21	24	13	26	28	16
	22/08/2019	32	38	6	6	19	23	13	25	26	17
	Average	34	38	6	6	20	24	13	26	27	17
SSL008	15/08/2019	34	36	6	6	6	23	13	27	28	16
	22/08/2019	34	37	6	6	6	24	14	29	30	18
	Average	34	37	6	6	6	24	14	28	29	17
SSL009	15/08/2019	33	33	25	22	21	24	6	32	28	22
	22/08/2019	32	35	27	22	23	23	6	31	29	24
	Average	33	34	26	22	22	24	6	32	29	23
SSL010	15/08/2019	34	32	25	23	20	23	6	32	26	20
	22/08/2019	34	37	26	23	22	23	6	31	29	23
	18/09/2019		33								23
	Average	34	34	26	23	21	23	6	32	28	22
SSL011	15/08/2019	31	33	26	22	20	23	6	30	27	22
	22/08/2019	32	35	28	24	23	24	6	31	29	23

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	18/09/2019					22					
	Average	32	34	27	23	22	24	6	31	28	23
SSL012	15/08/2019	33	34	6	24	6	25	22	33	27	30
	22/08/2019	33	34	6	23	6	22	23	34	28	29
	18/09/2019						23				
	Average	33	34	6	24	6	23	23	34	28	30
SSL013	15/08/2019	33	33	6	23	6	23	22	38	28	30
	22/08/2019	34	34	6	24	6	23	24	36	29	30
	Average	34	34	6	24	6	23	23	37	29	30
SSL014	15/08/2019	33	35	28	24	20	24	22	35	26	30
	22/08/2019	33	35	28	23	20	22	23	35	28	30
	Average	33	35	28	24	20	23	23	35	27	30
SSL015	15/08/2019	30	33	26	24	20	23	24	34	27	28
	22/08/2019	32	30	29	25	21	23	24	35	27	30
	18/09/2019			25							
	Average	31	32	27	25	21	23	24	35	27	29
SSL016	15/08/2019	31	32	25	22	18	22	22	34	27	30
	22/08/2019	34	34	26	23	21	22	24	34	25	31
	18/09/2019	32				20					
	Average	32	33	26	23	20	22	23	34	26	31
SSL017	15/08/2019	33	35	21	23	22	24	24	38	9	32
	22/08/2019	34	36	23	24	24	23	25	37	9	33
	Average	34	36	22	24	23	24	25	38	9	33
SSL018	15/08/2019	33	35	27	24	20	24	22	36	27	30
	22/08/2019	32	37	29	25	23	22	24	36	29	31
	18/09/2019					22					
	Average	33	36	28	25	22	23	23	36	28	31
SSL019	15/08/2019	35	38	22	27	23	24	25	37	8	33
	22/08/2019	33	36	22	25	24	26	24	38	8	31
	Average	34	37	22	26	24	25	25	38	8	32
SSL020	15/08/2019	33	36	20	24	21	24	24	35	8	31
	22/08/2019	32	36	22	23	22	23	24	36	8	31
	Average	33	36	21	24	22	24	24	36	8	31
SSL021	15/08/2019	32	35	20	6	6	22	13	25	26	16
	22/08/2019	34	36	23	6	6	24	15	27	28	18
	18/09/2019			21							
	Average	33	36	21	6	6	23	14	26	27	17
SSL022	15/08/2019	32	37	22	6	6	22	14	26	27	18
	22/08/2019	34	38	22	6	6	22	13	26	26	17

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	Average	33	38	22	6	6	22	14	26	27	18
SSL023	15/08/2019	33	36	21	6	6	22	14	27	28	17
	22/08/2019	32	36	21	6	6	24	15	27	28	17
	Average	33	36	21	6	6	23	15	27	28	17
SSL024	15/08/2019	31	36	21	6	6	24	14	27	28	17
	22/08/2019	30	37	21	6	6	23	13	25	28	17
	Average	31	37	21	6	6	24	14	26	28	17
SSL025	15/08/2019	34	36	22	6	6	24	14	28	28	17
	22/08/2019	32	36	21	6	6	22	13	29	28	19
	Average	33	36	22	6	6	23	14	29	28	18
SSL026	15/08/2019	33	34	21	6	6	23	13	26	27	17
	23/08/2019	33	36	22	6	6	23	14	27	28	17
	Average	33	35	22	6	6	23	14	27	28	17
SSL027	16/08/2019	30	33	19	6	6	22	13	25	27	17
	23/08/2019	33	36	21	6	6	23	14	26	26	17
	18/09/2019	31	32								
	Average	31	34	20	6	6	23	14	26	27	17
SSL028	16/08/2019	32	34	27	23	19	22	22	33	26	29
	23/08/2019	34	36	28	25	21	23	23	36	28	30
	18/09/2019								35		
	Average	33	35	28	24	20	23	23	35	27	30
SSL029	16/08/2019	32	35	27	22	19	22	22	34	27	29
	23/08/2019	33	36	28	24	20	23	23	35	28	30
	Average	33	36	28	23	20	23	23	35	28	30
SSL030	16/08/2019	32	35	27	25	20	22	23	32	27	29
	23/08/2019	33	35	26	22	20	22	22	36	28	29
	18/09/2019	23							33		
	Average	29	35	27	24	20	22	23	34	28	29
SSL031	16/08/2019	29	32	25	23	19	22	23	34	27	29
	23/08/2019	30	35	26	23	20	23	22	34	26	29
	Average	30	34	26	23	20	23	23	34	27	29
SSL032	16/08/2019	32	35	20	23	20	23	6	30	8	23
	23/08/2019	35	37	22	23	22	24	6	30	6	23
	18/09/2019	33							32		
	Average	33	36	21	23	21	24	6	31	7	23
SSL033	16/08/2019	33	26	20	24	21	23	6	31	8	24
	23/08/2019	34	36	21	23	22	24	6	31	7	23

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	18/09/2019		33								
	Average	34	32	21	24	22	24	6	31	8	24
SSL034	16/08/2019	35	37	23	24	21	24	6	29	8	23
	23/08/2019	34	36	21	24	24	25	6	32	7	24
	18/09/2019								32		
	Average	35	37	22	24	23	25	6	31	8	24
SSL035	16/08/2019	30	34	25	21	19	21	6	25	26	19
	23/08/2019	33	37	28	24	24	24	6	28	29	22
	18/09/2019	31	35	24	21	20	22		29	31	22
	Average	31	35	26	22	21	22	6	27	28	21
SSL036	16/08/2019	34	37	27	23	21	23	6	27	28	22
	23/08/2019	33	37	26	24	23	24	6	27	28	21
	Average	34	37	27	24	22	24	6	27	28	22
SSL037	16/08/2019	31	35	32	23	21	23	21	37	29	33
	23/08/2019	33	36	32	22	22	23	23	36	28	31
	Average	32	36	32	23	22	23	22	37	29	32
SSL038	16/08/2019	34	36	32	24	23	23	22	35	29	32
	23/08/2019	34	37	33	24	23	24	21	38	30	32
	18/09/2019								35		
	Average	34	37	33	24	23	24	22	36	30	32
SSL039	16/08/2019	32	36	27	25	22	23	14	26	27	17
	23/08/2019	34	37	28	25	21	24	15	27	27	18
	Average	33	37	28	25	22	24	15	27	27	18
SSL040	16/08/2019	31	35	28	24	19	23	15	25	25	16
	23/08/2019	33	36	27	25	22	24	16	25	27	18
	18/09/2019					20					
	Average	32	36	28	25	20	24	16	25	26	17
SSL041	16/08/2019	30	32	24	25	20	24	13	25	27	17
	23/08/2019	34	37	28	24	22	23	15	25	28	19
	18/09/2019	32	32	25							
	Average	32	34	26	25	21	24	14	25	28	18
SSL042	16/08/2019	32	34	27	25	21	24	14	25	27	16
	23/08/2019	34	37	29	25	22	24	16	26	27	18
	18/09/2019		33								
	Average	33	35	28	25	22	24	15	26	27	17
SSL043	16/08/2019	34	35	27	23	20	21	23	37	28	30
	23/08/2019	33	36	29	23	21	23	24	34	27	31
									35		
	Average	34	36	28	23	21	22	24	35	28	31
SSL044	16/08/2019	33	34	25	23	20	21	24	32	27	30
	23/08/2019	33	35	28	24	22	23	24	36	27	32

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	18/09/2019			25					35		
	Average	33	35	26	24	21	22	24	34	27	31
SSL045	16/08/2019	32	33	27	21	20	22	22	32	24	30
	23/08/2019	32	33	28	24	23	24	24	37	30	31
	18/09/2019				20	20			37	25	
	Average	32	33	28	22	21	23	23	35	26	31
SSL046	16/08/2019	29	32	25	22	20	22	23	30	24	28
	23/08/2019	32	33	28	22	21	22	24	36	26	30
	18/09/2019	31		25					34		
	Average	31	33	26	22	21	22	24	33	25	29
SSL047	16/08/2019	32	34	27	24	22	21	24	36	28	30
	23/08/2019	33	35	28	23	22	22	24	35	27	31
	Average	33	35	28	24	22	22	24	36	28	31
SSL048	16/08/2019	35	36	28	25	23	24	6	27	29	21
	23/08/2019	34	38	29	24	26	24	6	27	30	22
	18/09/2019					25					
	Average	35	37	29	25	25	24	6	27	30	22
SSL049	16/08/2019	35	36	29	25	24	25	6	28	28	21
	23/08/2019	32	34	27	22	25	24	6	26	29	20
	18/09/2019	33									
	Average	33	35	28	24	25	25	6	27	29	21
SSL050	16/08/2019	34	36	30	24	21	24	23	39	26	32
	23/08/2019	31	35	28	23	21	24	23	35	25	30
	18/09/2019	33							32		
	Average	33	36	29	24	21	24	23	35	26	31
SSL051	16/08/2019	33	36	28	24	21	24	23	34	24	31
	23/08/2019	33	33	28	24	22	23	23	34	24	30
	19/09/2019		33								
	Average	33	34	28	24	22	24	23	34	24	31
SSL052	16/08/2019	34	38	28	23	22	23	22	34	28	32
	23/08/2019	33	37	28	24	25	24	24	35	29	31
	Average	34	38	28	24	24	24	23	35	29	32
SSL053	16/08/2019	32	34	27	25	19	24	22	33	24	31
	23/08/2019	33	34	29	25	20	23	22	32	26	32
	Average	33	34	28	25	20	24	22	33	25	32
SSL054	16/08/2019	35	37	28	24	21	25	24	37	28	30
	23/08/2019	35	35	28	24	22	23	23	36	28	30
	Average	35	36	28	24	22	24	24	37	28	30
SSL055	16/08/2019	34	37	30	25	20	24	22	33	27	32
	23/08/2019	33	35	29	24	21	23	23	31	25	32

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	Average	34	36	30	25	21	24	23	32	26	32
SSL056	16/08/2019	31	35	26	24	17	21	21	31	35	31
	23/08/2019	35	35	27	25	20	23	22	32	25	32
	19/09/2019					16					
	Average	33	35	27	25	18	22	22	32	30	32
SSL057	16/08/2019	34	38	6	25	22	24	15	27	29	18
	23/08/2019	34	37	6	25	22	23	14	25	27	19
	Average	34	38	6	25	22	24	15	26	28	19
SSL058	16/08/2019	32	36	28	24	18	23	14	26	28	19
	23/08/2019	35	35	28	25	22	24	15	25	28	18
	19/09/2019					19					
	Average	34	36	28	25	20	24	15	26	28	19
SSL059	16/08/2019	33	38	6	24	21	24	15	26	28	18
	23/08/2019	32	36	6	24	23	23	15	26	29	19
	Average	33	37	6	24	22	24	15	26	29	19
SSL060	16/08/2019	32	34	6	6	18	23	13	25	27	13
	23/08/2019	32	35	6	6	20	23	13	33	32	16
	19/09/2019								28	28	14
	Average	32	35	6	6	19	23	13	29	29	14
SSL061	04/09/2019	31	34	6	6	18	21	12	27	27	14
	11/09/2019	34	33	6	6	19	22	12	28	26	15
	19/09/2019	33									
	Average	33	34	6	6	19	22	12	28	27	15
SSL062	04/09/2019	31	34	11	6	23	22	12	28	8	16
	11/09/2019	33	35	6	6	25	23	15	31	10	18
	19/09/2019	32							29		
	Average	32	35	9	6	24	23	14	29	9	17
SSL063	04/09/2019	33	35	6	6	20	22	12	28	27	15
	11/09/2019	34	34	6	6	20	23	13	28	28	16
	Average	34	35	6	6	20	23	13	28	28	16
SSL064	04/09/2019	33	35	28	23	19	23	22	35	27	30
	11/09/2019	35	37	28	24	21	22	23	36	28	31
	Average	34	36	28	24	20	23	23	36	28	31
SSL065	04/09/2019	31	33	25	23	20	22	22	38	25	31
	11/09/2019	35	33	28	24	20	23	25	40	27	33
	19/09/2019	33									
	Average	33	33	27	24	20	23	24	39	26	32
SSL066	04/09/2019	30	30	24	22	19	22	21	38	26	30
	11/09/2019	32	32	27	23	19	22	23	39	27	31

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	19/09/2019			25							
	Average	31	31	25	23	19	22	22	39	27	31
SSL067	04/09/2019	31	33	25	21	22	22	22	38	27	31
	11/09/2019	31	33	25	22	19	21	23	38	25	32
	19/09/2019					20					
	Average	31	33	25	22	20	22	23	38	26	32
SSL068	04/09/2019	33	34	27	24	20	22	24	39	26	31
	11/09/2019	35	35	29	25	23	23	25	40	28	34
	19/09/2019					22					33
	Average	34	35	28	25	22	23	25	40	27	33
SSL069	04/09/2019	31	33	21	6	6	23	12	27	26	18
	11/09/2019	31	34	22	6	6	23	12	26	28	19
	Average	31	34	22	6	6	23	12	27	27	19
SSL070	04/09/2019	31	35	27	24	20	23	21	34	27	30
	11/09/2019	33	36	29	24	21	23	24	34	27	31
	Average	32	36	28	24	21	23	23	34	27	31
SSL071	04/09/2019	33	34	21	6	6	22	12	25	27	16
	11/09/2019	32	36	23	6	6	23	13	26	28	17
	Average	33	35	22	6	6	23	13	26	28	17
SSL072	04/09/2019	31	33	25	23	22	23	6	34	26	24
	11/09/2019	33	35	25	23	23	23	6	33	26	25
	Average	32	34	25	23	23	23	6	34	26	25
SSL073	04/09/2019	31	33	25	24	22	24	6	32	27	23
	11/09/2019	35	36	26	24	23	23	6	34	29	26
	19/09/2019	32									
	Average	33	35	26	24	23	24	6	33	28	25
SSL074	04/09/2019	32	33	24	24	22	24	6	32	27	25
	11/09/2019	34	36	26	23	21	23	6	34	28	25
	Average	33	35	25	24	22	24	6	33	28	25
SSL075	04/09/2019	32	34	25	24	22	24	6	33	28	24
	11/09/2019	34	36	28	23	22	24	6	33	29	26
	19/09/2019			25							
	Average	33	35	26	24	22	24	6	33	29	25
SSL076	04/09/2019	34	35	6	6	21	23	12	28	27	14
	11/09/2019	31	34	6	6	20	23	13	28	28	16
	Average	33	35	6	6	21	23	13	28	28	15
SSL077	04/09/2019	32	35	6	6	21	22	12	27	26	15
	11/09/2019	34	35	6	6	21	23	13	29	28	16

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	Average	33	35	6	6	21	23	13	28	27	16
SSL078	04/09/2019	35	37	6	6	20	23	12	24	28	17
	11/09/2019	32	35	6	6	21	23	13	26	28	18
	19/09/2019										
	Average	34	36	6	6	21	23	13	25	28	18
SSL079	04/09/2019	31	37	6	6	21	22	13	25	26	16
	11/09/2019	32	34	6	6	22	23	14	26	27	17
	19/09/2019		34								
	Average	32	35	6	6	22	23	14	26	27	17
SSL081	04/09/2019	31	33	19	6	6	22	13	25	26	16
	11/09/2019	31	34	20	6	6	22	13	25	27	17
	Average	31	34	20	6	6	22	13	25	27	17
SSL082	04/09/2019	32	35	21	6	6	22	13	27	28	17
	11/09/2019	30	34	20	6	6	22	13	25	26	18
	Average	31	35	21	6	6	22	13	26	27	18
SSL083	04/09/2019	31	34	26	25	20	23	23	34	27	30
	11/09/2019	31	34	27	24	20	22	23	33	27	31
	Average	31	34	27	25	20	23	23	34	27	31
SSL084	04/09/2019	32	35	28	25	20	22	23	34	28	29
	11/09/2019	30	35	26	23	21	21	22	32	27	30
	Average	31	35	27	24	21	22	23	33	28	30
SSL085	04/09/2019	33	35	21	6	19	22	13	25	27	16
	11/09/2019	33	36	21	6	20	22	13	25	27	18
	Average	33	36	21	6	20	22	13	25	27	17
SSL086	04/09/2019	33	34	20	6	6	22	12	24	26	16
	11/09/2019	32	33	20	6	6	22	13	24	27	17
	Average	33	34	20	6	6	22	13	24	27	17
SSL087	04/09/2019	33	35	27	25	21	24	24	36	27	33
	11/09/2019	35	35	28	24	23	24	25	36	28	33
	Average	34	35	28	25	22	24	25	36	28	33
SSL088	04/09/2019	33	33	27	23	23	24	23	35	27	30
	11/09/2019	32	34	25	25	24	24	24	35	28	30
	Average	33	34	26	24	24	24	24	35	28	30
SSL089	04/09/2019	32	32	25	24	23	23	23	32	26	30
	11/09/2019	35	33	27	24	23	25	25	33	29	31

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	Average	34	33	26	24	23	24	24	33	28	31
SSL090	04/09/2019	32	33	26	23	21	22	23	39	28	30
	11/09/2019	32	33	24	23	22	22	24	34	29	31
	19/09/2019								35		
	Average	32	33	25	23	22	22	24	36	29	31
SSL091	05/09/2019	32	34	22	24	16	22	12	28	29	15
	12/09/2019	33	33	27	24	18	22	12	27	28	15
	19/09/2019			26							
	Average	33	34	25	24	17	22	12	28	29	15
SSL092	05/09/2019	34	36	26	25	23	22	12	29	28	16
	12/09/2019	34	36	28	25	24	23	12	28	30	16
	Average	34	36	27	25	24	23	12	29	29	16
SSL093	05/09/2019	33	34	21	25	6	22	12	27	29	16
	12/09/2019	33	34	20	24	6	23	13	29	29	16
	Average	33	34	21	25	6	23	13	28	29	16
SSL094	05/09/2019	31	33	20	24	6	22	12	28	29	16
	12/09/2019	34	35	22	25	6	23	13	28	29	16
	19/09/2019	32									
	Average	32	34	21	25	6	23	13	28	29	16
SSL095	05/09/2019	33	34	27	23	21	22	24	39	27	34
	12/09/2019	33	34	29	25	23	22	26	39	27	34
	Average	33	34	28	24	22	22	25	39	27	34
SSL096	05/09/2019	33	34	27	23	19	22	23	38	26	30
	12/09/2019	32	33	26	24	21	22	24	37	27	31
	Average	33	34	27	24	20	22	24	38	27	31
SSL097	05/09/2019	42	42	28	31	25	27	33	42	32	40
	12/09/2019	40	39	29	28	24	26	33	41	33	41
	Average	41	41	29	30	25	27	33	42	33	41
SSL098	05/09/2019	32	34	22	24	6	22	12	28	28	16
	12/09/2019	34	35	23	25	6	24	13	30	31	17
	19/09/2019									29	
	Average	33	35	23	25	6	23	13	29	29	17
SSL101	05/09/2019	31	35	20	6	6	22	12	26	27	16
	12/09/2019	33	35	22	6	6	23	13	27	28	19
	19/09/2019										16
	Average	32	35	21	6	6	23	13	27	28	17
SSL102	05/09/2019	33	36	22	6	6	24	14	25	26	16
	12/09/2019	32	34	22	6	6	24	16	29	28	20

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	19/09/2019								26		16
	Average	33	35	22	6	6	24	15	27	27	18
SSL103	05/09/2019	32	34	24	23	21	24	6	31	26	24
	12/09/2019	34	35	27	24	23	25	6	31	28	25
	19/09/2019			25							
	Average	33	35	25	24	22	25	6	31	27	25
SSL104	05/09/2019	30	33	19	6	6	22	12	25	26	16
	12/09/2019	32	34	22	6	6	22	14	25	28	18
	19/09/2019			20							
	Average	31	34	20	6	6	22	13	25	27	17
SSL105	05/09/2019	30	34	26	23	20	22	22	33	25	29
	12/09/2019	31	35	27	23	22	22	23	34	25	30
	Average	31	35	27	23	21	22	23	34	25	30
SSL106	05/09/2019	30	32	24	22	20	21	23	34	25	30
	12/09/2019	32	33	27	23	21	21	23	34	26	31
	19/09/2019			24							
	Average	31	33	25	23	21	21	23	34	26	31
SSL107	05/09/2019	30	33	19	6	6	21	11	24	26	15
	12/09/2019	32	34	20	6	6	21	13	25	26	17
	19/09/2019										16
	Average	31	34	20	6	6	21	12	25	26	16
SSL108	05/09/2019	31	33	20	6	6	22	12	24	26	15
	12/09/2019	31	33	19	6	6	22	13	25	26	18
	19/09/2019										16
	Average	31	33	20	6	6	22	13	25	26	16
SSL109	05/09/2019	33	34	25	23	19	22	22	35	28	29
	12/09/2019	32	33	26	23	20	21	22	35	31	29
	19/09/2019									29	
	Average	33	34	26	23	20	22	22	35	29	29
SSL110	05/09/2019	31	33	25	23	20	22	22	34	28	29
	12/09/2019	33	36	28	25	22	22	23	37	29	31
	19/09/2019			26					34		
	Average	32	35	26	24	21	22	23	35	29	30
SSL111	05/09/2019	33	37	26	22	21	23	6	32	27	23
	12/09/2019	31	33	25	23	22	22	6	29	26	24
	19/09/2019		34								
	Average	32	35	26	23	22	23	6	31	27	24
SSL112	05/09/2019	32	33	35	22	23	24	6	33	27	24
	12/09/2019	31	33	24	23	23	23	6	30	25	25
	19/09/2019								31		
	Average	32	33	30	23	23	24	6	31	26	25
SSL113	05/09/2019	33	33	27	22	21	23	6	31	26	23
	12/09/2019	33	34	25	22	21	23	6	29	26	24

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	19/09/2019								27		
	Average	33	34	26	22	21	23	6	29	26	24
SSL114	05/09/2019	34	32	25	22	21	22	6	30	26	23
	12/09/2019	31	33	26	25	24	24	6	30	25	25
	19/09/2019	33			25						
	Average	33	33	26	24	23	23	6	30	26	24
SSL115	05/09/2019	32	34	20	6	6	22	13	24	27	17
	12/09/2019	32	34	20	6	6	22	13	24	26	17
	Average	32	34	20	6	6	22	13	24	27	17
SSL116	05/09/2019	32	33	24	22	21	23	6	31	26	23
	12/09/2019	32	35	26	23	23	24	6	32	27	24
	19/09/2019					21					
	Average	32	34	25	23	22	24	6	32	27	24
SSL117	05/09/2019	31	31	25	22	20	21	22	35	25	29
	12/09/2019	33	34	26	26	21	23	23	35	26	32
	19/09/2019		32		25						31
	Average	32	32	26	24	21	22	23	35	26	31
SSL118	05/09/2019	33	31	26	23	21	22	23	33	26	29
	12/09/2019	35	34	29	25	21	24	24	35	28	32
	19/09/2019		32	27							30
	Average	34	32	27	24	21	23	24	34	27	30
SSL119	05/09/2019	32	31	26	23	19	22	22	32	25	28
	12/09/2019	35	34	28	25	21	23	24	35	24	31
	19/09/2019	32	32			20			33		29
	Average	33	32	27	24	20	23	23	33	25	29
SSL120	05/09/2019	32	31	26	23	20	22	23	34	26	30
	12/09/2019	33	33	28	23	22	23	23	32	25	30
	19/09/2019		30								
	Average	33	31	27	23	21	23	23	33	26	30
SSL121	06/09/2019	32	32	26	24	19	22	23	34	26	30
	13/09/2019	34	32	27	25	21	23	25	36	26	31
	Average	33	32	27	25	20	23	24	35	26	31
SSL122	06/09/2019	33	32	26	24	19	22	22	33	25	29
	13/09/2019	33	32	26	24	20	23	24	35	25	30
	Average	33	32	26	24	20	23	23	34	25	30
SSL123	06/09/2019	32	31	25	23	18	23	23	32	24	31
	13/09/2019	34	33	25	23	20	24	23	34	25	31
	Average	33	32	25	23	19	24	23	33	25	31
SSL124	06/09/2019	34	36	22	26	6	23	11	32	29	15
	13/09/2019	34	37	23	26	6	24	12	31	29	16

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	Average	34	37	23	26	6	24	12	32	29	16
SSL125	06/09/2019	34	35	21	25	6	22	12	33	28	15
	13/09/2019	34	36	23	25	6	24	12	31	29	16
	Average	34	36	22	25	6	23	12	32	29	16
SSL126	06/09/2019	32	32	25	23	18	22	16	34	27	22
	13/09/2019	33	33	20	24	18	23	18	32	26	21
	20/09/2019			20							
	Average	33	33	22	24	18	23	17	33	27	22
SSL127	06/09/2019	33	33	27	24	19	22	16	34	26	22
	13/09/2019	32	33	27	23	19	22	16	34	26	22
	Average	33	33	27	24	19	22	16	34	26	22
SSL128	06/09/2019	32	31	25	23	19	22	17	34	26	22
	13/09/2019	34	33	27	23	20	23	17	34	28	23
	Average	33	32	26	23	20	23	17	34	27	23
SSL129	06/09/2019	32	32	24	24	19	22	16	34	26	24
	13/09/2019	34	33	26	25	21	23	18	34	28	23
	Average	33	33	25	25	20	23	17	34	27	24
SSL130	06/09/2019	34	32	25	24	20	21	15	33	26	20
	13/09/2019	35	34	27	24	21	23	17	34	28	23
	Average	35	33	26	24	21	22	16	34	27	22
SSL131	06/09/2019	33	33	26	23	19	22	17	35	25	22
	13/09/2019	34	34	28	25	22	24	18	36	28	23
	20/09/2019					20					
	Average	34	34	27	24	20	23	18	36	27	23
SSL132	06/09/2019	32	34	6	6	18	22	17	24	25	21
	13/09/2019	33	37	6	6	20	24	18	31	26	20
	20/09/2019		33						24		
	Average	33	35	6	6	19	23	18	26	26	21
SSL133	06/09/2019	35	33	26	26	22	24	6	31	28	26
	13/09/2019	37	35	30	25	22	23	6	28	26	24
	20/09/2019			27					29		
	Average	36	34	28	26	22	24	6	29	27	25
SSL134	06/09/2019	34	34	26	24	22	24	22	36	27	31
	13/09/2019	34	35	27	23	22	24	23	36	29	32
	Average	34	35	27	24	22	24	23	36	28	32
SSL135	06/09/2019	40	40	29	27	24	26	6	23	26	19
	13/09/2019	40	42	26	27	23	26	6	24	24	19

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	20/09/2019			28							
	Average	40	41	28	27	24	26	6	24	25	19
SSL136	06/09/2019	33	35	26	24	22	22	6	29	27	23
	13/09/2019	33	34	26	23	21	23	6	28	27	23
	Average	33	35	26	24	22	23	6	29	27	23
SSL137	06/09/2019	35	35	27	24	24	24	6	29	28	23
	13/09/2019	36	36	28	25	23	24	6	31	29	24
	Average	36	36	28	25	24	24	6	30	29	24
SSL138	06/09/2019	35	36	30	24	21	24	30	38	29	38
	13/09/2019	36	38	32	24	22	25	32	37	31	40
	Average	36	37	31	24	22	25	31	38	30	39
SSL139	06/09/2019	32	33	26	24	22	23	22	33	26	32
	13/09/2019	33	33	26	24	20	23	22	32	25	31
	Average	33	33	26	24	21	23	22	33	26	32
SSL140	06/09/2019	34	36	29	27	24	25	27	41	29	36
	13/09/2019	37	37	30	28	25	26	28	37	30	36
		36							39		
	Average	36	37	30	28	25	26	28	39	30	36
SSL141	06/09/2019	38	38	25	25	20	29	6	23	19	19
	13/09/2019	36	37	26	25	20	29	6	24	20	19
	Average	37	38	26	25	20	29	6	24	20	19
SSL142	06/09/2019	32	33	28	24	21	22	17	31	27	23
	13/09/2019	33	32	26	23	20	22	18	33	27	22
	Average	33	33	27	24	21	22	18	32	27	23
SSL143	06/09/2019	39	40	19	27	24	30	6	26	20	22
	13/09/2019	41	42	20	27	24	31	6	26	22	23
	Average	40	41	20	27	24	31	6	26	21	23
SSL144	06/09/2019	37	39	22	24	18	32	6	24	14	21
	13/09/2019	39	40	22	24	19	32	6	24	15	21
	Average	38	40	22	24	19	32	6	24	15	21
SSL145	06/09/2019	38	39	23	23	19	31	6	22	15	21
	13/09/2019	37	40	24	24	20	32	6	24	16	20
	Average	38	40	24	24	20	32	6	23	16	21
SSL146	06/09/2019	39	40	26	26	22	30	6	24	21	20
	13/09/2019	37	40	25	27	23	29	6	23	18	19

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	Average	38	40	26	27	23	30	6	24	20	20
SSL147	06/09/2019	39	39	25	26	24	30	6	23	20	19
	13/09/2019	40	41	27	25	24	30	6	23	20	19
	Average	40	40	26	26	24	30	6	23	20	19
SSL148	06/09/2019	40	41	28	26	23	30	6	23	19	20
	13/09/2019	40	41	26	26	24	30	6	24	20	20
	Average	40	41	27	26	24	30	6	24	20	20
SSL149	06/09/2019	39	41	18	26	23	29	6	24	22	21
	13/09/2019	38	40	20	27	22	30	6	26	21	21
	Average	39	41	19	27	23	30	6	25	22	21
SSL150	06/09/2019	36	40	26	25	23	29	6	23	19	19
	13/09/2019	36	40	25	25	23	29	6	23	19	19
	Average	36	40	26	25	23	29	6	23	19	19
SSL151	06/09/2019	34	36	34	28	28	29	28	39	33	36
	13/09/2019	35	36	34	28	28	30	28	38	31	36
	Average	35	36	34	28	28	30	28	39	32	36
SSL152	06/09/2019	33	34	26	24	20	23	18	33	26	22
	13/09/2019	35	34	28	25	21	23	18	33	27	24
	Average	34	34	27	25	21	23	18	33	27	23
SSL153	06/09/2019	36	38	37	28	29	31	30	42	34	39
	13/09/2019	34	39	38	28	29	31	29	40	33	38
	Average	35	39	38	28	29	31	30	41	34	39
SSL154	06/09/2019	32	34	6	6	20	23	13	26	27	17
	13/09/2019	34	35	6	6	21	24	13	28	27	17
	Average	33	35	6	6	21	24	13	27	27	17
SSL155	06/09/2019	33	35	25	23	21	22	6	27	27	22
	13/09/2019	33	36	27	24	22	23	6	28	28	23
	Average	33	36	26	24	22	23	6	28	28	23
SSL156	06/09/2019	33	32	27	24	20	24	24	35	24	29
	13/09/2019	32	32	25	24	20	22	23	33	24	28
	Average	33	32	26	24	20	23	24	34	24	29
SSL157	06/09/2019	31	30	24	23	21	23	6	27	27	21
	13/09/2019	33	33	27	23	21	22	6	27	26	21

Isolate ID	Date Read	CTX	MEM	SXT	AMP	TE	CN	NA	CIP	C	ENR
	Average	32	32	26	23	21	23	6	27	27	21
SSL158	06/09/2019	34	33	30	25	22	22	20	35	28	30
	13/09/2019	36	35	31	25	23	23	22	36	29	31
	Average	35	34	31	25	23	23	21	36	29	31
SSL159	06/09/2019	32	34	31	24	22	23	21	35	27	30
	13/09/2019	33	34	32	25	23	23	22	35	28	31
	Average	33	34	32	25	23	23	22	35	28	31
SSL160	06/09/2019	38	39	24	26	20	30	6	22	20	19
	13/09/2019	40	41	26	26	19	30	6	23	19	19
	Average	39	40	25	26	20	30	6	23	20	19
SSL161	06/09/2019	33	34	34	29	29	29	28	38	32	36
	13/09/2019	38	36	35	31	29	29	27	38	34	35
		33									
	Average	35	35	35	30	29	29	28	38	33	36
SSL162	06/09/2019	32	31	25	23	21	21	6	27	26	21
	13/09/2019	33	32	24	23	21	22	6	26	26	22
	Average	33	32	25	23	21	22	6	27	26	22
SSL163	06/09/2019	34	34	28	24	22	23	24	34	28	30
	13/09/2019	33	33	28	25	22	23	24	32	28	31
	Average	34	34	28	25	22	23	24	33	28	31
SSL164	06/09/2019	34	35	34	30	28	28	27	38	34	37
	13/09/2019	36	37	34	29	29	29	26	38	31	34
	Average	35	36	34	30	29	29	27	38	33	36
SSL165	06/09/2019	35	35	28	25	25	25	6	31	28	23
	13/09/2019	33	34	26	23	23	24	6	30	27	22
	Average	34	35	27	24	24	25	6	31	28	23
SSL166	06/09/2019	34	33	27	26	20	22	12	29	25	18
	13/09/2019	33	34	29	25	20	24	14	28	27	18
	Average	34	34	28	26	20	23	13	29	26	18
SSL167	06/09/2019	31	33	25	23	22	23	6	29	26	22
	13/09/2019	33	35	27	24	21	23	6	28	25	21
	Average	32	34	26	24	22	23	6	29	26	22

Table A 6.6: AST Final Results

S- Sensitive, I- Intermediate, R-Resistant

Isolate ID	CTX	MEM	CN	SXT	AMP	TE	NA	CIP	C	ENR
SSL001	S	S	S	S	S	S	I	I	S	I
SSL002	S	S	S	R	R	S	R	I	S	R
SSL003	S	S	S	S	S	R	S	I	S	I
SSL004	S	S	S	S	S	S	I	I	S	I
SSL005	S	S	S	S	S	R	I	I	S	I
SSL006	S	S	S	R	R	S	R	I	S	R
SSL007	S	S	S	R	R	S	R	I	S	I
SSL008	S	S	S	R	R	R	I	I	S	I
SSL009	S	S	S	S	S	S	R	S	S	S
SSL010	S	S	S	S	S	S	R	S	S	I
SSL011	S	S	S	S	S	S	R	S	S	S
SSL012	S	S	S	R	S	R	S	S	S	S
SSL013	S	S	S	R	S	R	S	S	S	S
SSL014	S	S	S	S	S	S	S	S	S	S
SSL015	S	S	S	S	S	S	S	S	S	S
SSL016	S	S	S	S	S	S	S	S	S	S
SSL017	S	S	S	S	S	S	S	S	R	S
SSL018	S	S	S	S	S	S	S	S	S	S
SSL019	S	S	S	S	S	S	S	S	R	S
SSL020	S	S	S	S	S	S	S	S	R	S
SSL021	S	S	S	S	R	R	I	I	S	I
SSL022	S	S	S	S	R	R	I	I	S	I
SSL023	S	S	S	S	R	R	I	I	S	I
SSL024	S	S	S	S	R	R	I	I	S	I
SSL025	S	S	S	S	R	R	I	I	S	I
SSL026	S	S	S	S	R	R	I	I	S	I
SSL027	S	S	S	S	R	R	I	I	S	I
SSL028	S	S	S	S	S	S	S	S	S	S
SSL029	S	S	S	S	S	S	S	S	S	S
SSL030	S	S	S	S	S	S	S	S	S	S
SSL031	S	S	S	S	S	S	S	S	S	S
SSL032	S	S	S	S	S	S	R	S	R	S
SSL033	S	S	S	S	S	S	R	S	R	S
SSL034	S	S	S	S	S	S	R	S	R	S
SSL035	S	S	S	S	S	S	R	I	S	I
SSL036	S	S	S	S	S	S	R	I	S	I
SSL037	S	S	S	S	S	S	S	S	S	S

Isolate ID	CTX	MEM	CN	SXT	AMP	TE	NA	CIP	C	ENR
SSL038	S	S	S	S	S	S	S	S	S	S
SSL039	S	S	S	S	S	S	I	I	S	I
SSL040	S	S	S	S	S	S	I	I	S	I
SSL041	S	S	S	S	S	S	I	I	S	I
SSL042	S	S	S	S	S	S	I	I	S	I
SSL043	S	S	S	S	S	S	S	S	S	S
SSL044	S	S	S	S	S	S	S	S	S	S
SSL045	S	S	S	S	S	S	S	S	S	S
SSL046	S	S	S	S	S	S	S	S	S	S
SSL047	S	S	S	S	S	S	S	S	S	S
SSL048	S	S	S	S	S	S	R	I	S	I
SSL049	S	S	S	S	S	S	R	I	S	I
SSL050	S	S	S	S	S	S	S	S	S	S
SSL051	S	S	S	S	S	S	S	S	S	S
SSL052	S	S	S	S	S	S	S	S	S	S
SSL053	S	S	S	S	S	S	S	S	S	S
SSL054	S	S	S	S	S	S	S	S	S	S
SSL055	S	S	S	S	S	S	S	S	S	S
SSL056	S	S	S	S	S	S	S	S	S	S
SSL057	S	S	S	R	S	S	I	I	S	I
SSL058	S	S	S	S	S	S	I	I	S	I
SSL059	S	S	S	R	S	S	I	I	S	I
SSL060	S	S	S	R	R	S	R	I	S	R
SSL061	S	S	S	R	R	S	R	I	S	R
SSL062	S	S	S	R	R	S	I	I	R	I
SSL063	S	S	S	R	R	S	R	I	S	R
SSL064	S	S	S	S	S	S	S	S	S	S
SSL065	S	S	S	S	S	S	S	S	S	S
SSL066	S	S	S	S	S	S	S	S	S	S
SSL067	S	S	S	S	S	S	S	S	S	S
SSL068	S	S	S	S	S	S	S	S	S	S
SSL069	S	S	S	S	R	R	R	I	S	I
SSL070	S	S	S	S	S	S	S	S	S	S
SSL071	S	S	S	S	R	R	R	I	S	I
SSL072	S	S	S	S	S	S	R	S	S	S
SSL073	S	S	S	S	S	S	R	S	S	S
SSL074	S	S	S	S	S	S	R	S	S	S
SSL075	S	S	S	S	S	S	R	S	S	S
SSL076	S	S	S	R	R	S	R	I	S	R
SSL077	S	S	S	R	R	S	R	I	S	R
SSL078	S	S	S	R	R	S	R	I	S	I

Isolate ID	CTX	MEM	CN	SXT	AMP	TE	NA	CIP	C	ENR
SSL079	S	S	S	R	R	S	I	I	S	I
SSL081	S	S	S	S	R	R	R	I	S	I
SSL082	S	S	S	S	R	R	R	I	S	I
SSL083	S	S	S	S	S	S	S	S	S	S
SSL084	S	S	S	S	S	S	S	S	S	S
SSL085	S	S	S	S	R	S	R	I	S	I
SSL086	S	S	S	S	R	R	R	I	S	I
SSL087	S	S	S	S	S	S	S	S	S	S
SSL088	S	S	S	S	S	S	S	S	S	S
SSL089	S	S	S	S	S	S	S	S	S	S
SSL090	S	S	S	S	S	S	S	S	S	S
SSL091	S	S	S	S	S	S	R	I	S	R
SSL092	S	S	S	S	S	S	R	I	S	R
SSL093	S	S	S	S	S	R	R	I	S	R
SSL094	S	S	S	S	S	R	R	I	S	R
SSL095	S	S	S	S	S	S	S	S	S	S
SSL096	S	S	S	S	S	S	S	S	S	S
SSL097	S	S	S	S	S	S	S	S	S	S
SSL098	S	S	S	S	S	R	R	I	S	I
SSL101	S	S	S	S	R	R	R	I	S	I
SSL102	S	S	S	S	R	R	I	I	S	I
SSL103	S	S	S	S	S	S	R	S	S	S
SSL104	S	S	S	S	R	R	R	I	S	I
SSL105	S	S	S	S	S	S	S	S	S	S
SSL106	S	S	S	S	S	S	S	S	S	S
SSL107	S	S	S	S	R	R	R	I	S	R
SSL108	S	S	S	S	R	R	R	I	S	R
SSL109	S	S	S	S	S	S	S	S	S	S
SSL110	S	S	S	S	S	S	S	S	S	S
SSL111	S	S	S	S	S	S	R	S	S	S
SSL112	S	S	S	S	S	S	R	S	S	S
SSL113	S	S	S	S	S	S	R	I	S	S
SSL114	S	S	S	S	S	S	R	I	S	S
SSL115	S	S	S	S	R	R	R	I	S	I
SSL116	S	S	S	S	S	S	R	S	S	S
SSL117	S	S	S	S	S	S	S	S	S	S
SSL118	S	S	S	S	S	S	S	S	S	S
SSL119	S	S	S	S	S	S	S	S	S	S
SSL120	S	S	S	S	S	S	S	S	S	S
SSL121	S	S	S	S	S	S	S	S	S	S
SSL122	S	S	S	S	S	S	S	S	S	S

Isolate ID	CTX	MEM	CN	SXT	AMP	TE	NA	CIP	C	ENR
SSL123	S	S	S	S	S	S	S	S	S	S
SSL124	S	S	S	S	S	R	R	S	S	R
SSL125	S	S	S	S	S	R	R	S	S	R
SSL126	S	S	S	S	S	S	I	S	S	I
SSL127	S	S	S	S	S	S	I	S	S	I
SSL128	S	S	S	S	S	S	I	S	S	S
SSL129	S	S	S	S	S	S	I	S	S	S
SSL130	S	S	S	S	S	S	I	S	S	I
SSL131	S	S	S	S	S	S	I	S	S	S
SSL132	S	S	S	R	R	S	I	I	S	I
SSL133	S	S	S	S	S	S	R	I	S	S
SSL134	S	S	S	S	S	S	S	S	S	S
SSL135	S	S	S	S	S	S	R	I	S	I
SSL136	S	S	S	S	S	S	R	I	S	S
SSL137	S	S	S	S	S	S	R	I	S	S
SSL138	S	S	S	S	S	S	S	S	S	S
SSL139	S	S	S	S	S	S	S	S	S	S
SSL140	S	S	S	S	S	S	S	S	S	S
SSL141	S	S	S	S	S	S	R	I	S	I
SSL142	S	S	S	S	S	S	I	S	S	S
SSL143	S	S	S	S	S	S	R	I	S	S
SSL144	S	S	S	S	S	S	R	I	I	I
SSL145	S	S	S	S	S	S	R	I	I	I
SSL146	S	S	S	S	S	S	R	I	S	I
SSL147	S	S	S	S	S	S	R	I	S	I
SSL148	S	S	S	S	S	S	R	I	S	I
SSL149	S	S	S	S	S	S	R	I	S	I
SSL150	S	S	S	S	S	S	R	I	S	I
SSL151	S	S	S	S	S	S	S	S	S	S
SSL152	S	S	S	S	S	S	I	S	S	S
SSL153	S	S	S	S	S	S	S	S	S	S
SSL154	S	S	S	R	R	S	R	I	S	I
SSL155	S	S	S	S	S	S	R	I	S	S
SSL156	S	S	S	S	S	S	S	S	S	S
SSL157	S	S	S	S	S	S	R	I	S	I
SSL157	S	S	S	S	S	S	S	S	S	S
SSL159	S	S	S	S	S	S	S	S	S	S
SSL160	S	S	S	S	S	S	R	I	S	I
SSL161	S	S	S	S	S	S	S	S	S	S
SSL162	S	S	S	S	S	S	R	I	S	I
SSL163	S	S	S	S	S	S	S	S	S	S

Isolate ID	CTX	MEM	CN	SXT	AMP	TE	NA	CIP	C	ENR
SSL164	S	S	S	S	S	S	S	S	S	S
SSL165	S	S	S	S	S	S	R	S	S	S
SSL166	S	S	S	S	S	S	R	I	S	I
SSL167	S	S	S	S	S	S	R	I	S	I

Table A 6.7: Presence of resistance genes (as per Nullarbor report)

IsolateID	aadA	aph(3')-Ia	aph(6')-Id	blaTEM-1	cmlA1	dfrA	mef(B)	qnrD1	qnrS1	tet(A)
SSL001	No	No	Yes	No	No	No	No	No	Yes	No
SSL002	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL003	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL004	No	No	Yes	No	No	No	No	No	Yes	No
SSL005	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL006	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL007	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL008	No	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes
SSL009	No	No	No	No	No	No	No	No	No	No
SSL010	No	No	No	No	No	No	No	No	No	No
SSL011	No	No	No	No	No	No	No	No	No	No
SSL012	Yes	No	No	Yes	No	Yes	Yes	No	No	Yes
SSL013	Yes	No	No	Yes	No	Yes	Yes	No	No	Yes
SSL014	No	No	No	No	No	No	No	No	No	No
SSL015	No	No	Yes	No	No	No	No	No	No	No
SSL016	No	No	No	No	No	No	No	No	No	No
SSL017	Yes	No	No	No	Yes	No	No	No	No	No
SSL018	No	No	No	No	No	No	No	No	No	No
SSL019	Yes	No	No	No	Yes	No	No	No	No	No
SSL020	Yes	No	No	No	Yes	No	No	No	No	No
SSL021	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL022	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL023	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL024	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL025	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes
SSL026	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes
SSL027	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes
SSL028	No	No	No	No	No	No	No	Yes	No	No
SSL029	No	No	No	No	No	No	No	Yes	No	No
SSL030	No	No	No	No	No	No	No	Yes	No	No
SSL031	No	No	No	No	No	No	No	Yes	Yes	No
SSL032	Yes	No	No	No	Yes	No	No	Yes	No	No
SSL033	Yes	No	No	No	Yes	No	No	Yes	Yes	No
SSL034	Yes	No	No	No	Yes	No	No	Yes	No	No
SSL035	No	No	No	No	No	No	No	Yes	No	No
SSL036	No	No	No	No	No	No	No	Yes	No	No
SSL037	No	No	No	No	No	No	No	Yes	No	No
SSL038	No	No	No	No	No	No	No	No	No	No
SSL039	No	No	Yes	No	No	No	Yes	No	Yes	No
SSL040	No	No	Yes	No	No	No	Yes	No	Yes	No
SSL041	No	No	Yes	No	No	No	Yes	No	Yes	No

IsolateID	aadA	aph(3')-Ia	aph(6')-Id	blaTEM-1	cmlA1	dfrA	mef(B)	qnrD1	qnrS1	tet(A)
SSL042	No	No	Yes	No	No	No	Yes	No	Yes	No
SSL043	No	No	No	No	No	No	No	No	No	No
SSL044	No	No	No	No	No	No	No	No	No	No
SSL045	No	No	No	No	No	No	No	No	No	No
SSL046	No	No	No	No	No	No	No	No	No	No
SSL047	No	No	No	No	No	No	No	No	No	No
SSL048	No	No	No	No	No	No	No	No	No	No
SSL049	No	No	No	No	No	No	No	No	No	No
SSL050	No	No	No	No	No	No	No	No	No	No
SSL051	No	No	No	No	No	No	No	No	No	No
SSL052	No	No	No	No	No	No	No	No	No	No
SSL053	No	No	No	No	No	No	No	No	No	No
SSL054	No	No	No	No	No	No	No	No	No	No
SSL055	No	No	No	No	No	No	No	Yes	No	No
SSL056	No	No	No	No	No	No	No	No	No	No
SSL057	No	No	Yes	No	No	Yes	Yes	No	Yes	No
SSL058	No	No	Yes	No	No	No	Yes	Yes	Yes	No
SSL059	No	No	Yes	No	No	Yes	Yes	No	Yes	No
SSL060	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No
SSL061	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL062	Yes	No	No	Yes	Yes	No	No	No	Yes	No
SSL063	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL064	No	No	No	No	No	No	No	No	No	No
SSL065	No	No	No	No	No	No	No	No	No	No
SSL066	No	No	No	No	No	No	No	No	No	No
SSL067	No	No	No	No	No	No	No	No	No	No
SSL068	No	No	No	No	No	No	No	No	No	No
SSL069	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL070	No	No	Yes	No	No	No	No	No	No	No
SSL071	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL072	No	No	No	No	No	No	No	No	No	No
SSL073	No	No	No	No	No	No	No	No	No	No
SSL074	No	No	No	No	No	No	No	No	No	No
SSL075	No	No	No	No	No	No	No	No	No	No
SSL076	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL077	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL078	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL079	No	No	Yes	Yes	No	Yes	Yes	No	Yes	No
SSL081	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL082	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL083	No	No	No	No	No	No	No	No	No	No
SSL084	No	No	No	No	No	No	No	No	No	No
SSL085	No	No	Yes	Yes	No	No	Yes	No	Yes	No

IsolateID	aadA	aph(3')-Ia	aph(6')-Id	blaTEM-1	cmlA1	dfrA	mef(B)	qnrD1	qnrS1	tet(A)
SSL086	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL087	No	No	No	No	No	No	No	No	Yes	No
SSL088	No	No	No	No	No	No	No	No	No	No
SSL089	No	No	No	No	No	No	No	No	No	No
SSL090	No	No	No	No	No	No	No	No	No	No
SSL091	No	No	Yes	No	No	No	No	No	Yes	Yes
SSL092	No	No	No	No	No	No	No	No	Yes	No
SSL093	No	No	Yes	No	No	No	No	No	Yes	Yes
SSL094	No	No	Yes	No	No	No	No	No	Yes	Yes
SSL095	No	No	No	No	No	No	No	No	No	No
SSL096	No	No	No	No	No	No	No	No	No	No
SSL097	No	No	No	No	No	No	No	No	No	No
SSL098	No	No	Yes	No	No	No	No	No	Yes	Yes
SSL101	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL102	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL103	No	No	No	No	No	No	No	No	No	No
SSL104	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL105	No	No	No	No	No	No	No	No	No	No
SSL106	No	No	No	No	No	No	No	No	No	No
SSL107	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL108	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL109	No	No	No	No	No	No	No	No	No	No
SSL110	No	No	No	No	No	No	No	No	No	No
SSL111	No	No	No	No	No	No	No	No	No	No
SSL112	No	No	No	No	No	No	No	No	No	No
SSL113	No	No	No	No	No	No	No	No	No	No
SSL114	No	No	No	No	No	No	No	No	No	No
SSL115	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes
SSL116	No	No	No	No	No	No	No	No	No	No
SSL117	No	No	No	No	No	No	No	No	No	No
SSL118	No	No	No	No	No	No	No	No	No	No
SSL119	No	No	No	No	No	No	No	No	No	No
SSL120	No	No	No	No	No	No	No	No	No	No
SSL121	No	No	No	No	No	No	No	No	No	No
SSL122	No	No	No	No	No	No	No	No	No	No
SSL123	No	No	No	No	No	No	No	No	No	No
SSL124	No	No	Yes	No	No	No	No	No	Yes	Yes
SSL126	No	No	Yes	No	No	No	No	Yes	Yes	Yes
SSL127	No	No	No	No	No	No	No	Yes	No	No
SSL128	No	No	No	No	No	No	No	Yes	No	No
SSL129	No	No	No	No	No	No	No	Yes	No	No
SSL130	No	No	No	No	No	No	No	Yes	No	No
SSL131	No	No	No	No	No	No	No	Yes	No	No

IsolateID	aadA	aph(3')-Ia	aph(6')-Id	blaTEM-1	cmlA1	dfrA	mef(B)	qnrD1	qnrS1	tet(A)
SSL132	No	No	No	No	No	No	No	Yes	No	No
SSL133	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	No
SSL134	No	No	No	No	No	No	No	No	No	No
SSL135	No	No	No	No	No	No	No	Yes	No	No
SSL136	No	No	No	No	No	No	No	Yes	No	No
SSL137	No	No	No	No	No	No	No	No	No	No
SSL138	No	No	No	No	No	No	No	No	No	No
SSL139	No	No	No	No	No	No	No	No	No	No
SSL140	No	No	No	No	No	No	No	No	No	No
SSL141	No	No	No	No	No	No	No	No	No	No
SSL142	No	No	No	No	No	No	No	No	No	No
SSL143	No	No	No	No	No	No	No	Yes	No	No
SSL144	No	Yes	Yes	No	No	No	No	No	No	No
SSL145	No	No	No	No	No	No	No	No	No	No
SSL146	No	No	No	No	No	No	No	No	No	No
SSL147	No	No	No	No	No	No	No	No	No	No
SSL148	No	No	No	No	No	No	No	No	No	No
SSL149	No	No	No	No	No	No	No	No	No	No
SSL150	No	Yes	Yes	No	No	No	No	No	No	No
SSL151	No	No	No	No	No	No	No	No	No	No
SSL152	No	No	No	No	No	No	No	No	No	No
SSL153	No	No	No	No	No	No	No	Yes	No	No
SSL154	No	No	No	No	No	No	No	No	No	No
SSL155	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
SSL156	No	No	No	No	No	No	No	No	No	No
SSL157	No	No	No	No	No	No	No	No	No	No
SSL158	No	No	No	No	No	No	No	No	No	No
SSL159	No	No	No	No	No	No	No	No	No	No
SSL160	No	No	No	No	No	No	No	No	No	No
SSL161	No	No	No	No	No	No	No	No	No	No
SSL162	No	No	No	No	No	No	No	No	No	No
SSL163	No	No	No	No	No	No	No	No	No	No
SSL164	No	No	No	No	No	No	No	No	No	No
SSL165	No	No	No	No	No	No	No	No	No	No
SSL166	No	No	No	No	No	No	No	No	No	No
SSL167	No	No	No	No	No	No	No	Yes	No	No

Table A 6.8: Association of positive farms and associated hatcheries

Isolate ID	Fram ID	Associated Hatchery	*Salmonella* Status of associated hatchery
SSL001a	F007	H001	Positive
SSL002a	F009	H002	Positive
SSL003a	F009	H002	Positive
SSL004a	F010	H002	Positive
SSL005a	F010	H002	Positive
SSL006a	F014	H001	Positive
SSL007a	F015	H001	Positive
SSL012a	F044	H012	Negative
SSL013a	F044	H012	Negative
SSL014a	F044	H012	Negative
SSL015a	F045	H001	Positive
SSL016a	F046	H005	Positive
SSL017a	F021	H003	Positive
SSL018a	F021	H003	Positive
SSL019a	F025	H003	Positive
SSL020a	F025	H003	Positive
SSL028	F026	H008	Positive
SSL029	F029	H007	Positive
SSL030	F030	H008, H007	Positive
SSL031	F030	H008, H007	Positive
SSL035	F069	H005	Positive
SSL036	F069	H005	Positive
SSL037	F071	H007	Positive
SSL038	F071	H007	Positive
SSL048	F087	H015	Negative
SSL049	F089	H015	Negative
SSL050	F090	H015	Negative
SSL051	F090	H015	Negative
SSL052	F091	H015	Negative
SSL053	F092	H015	Negative
SSL054	F092	H015	Negative
SSL055	F092	H015	Negative
SSL056	F092	H015	Negative
SSL057	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011
SSL058	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011
SSL059	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011
SSL060	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011
SSL061	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011
SSL062	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011
SSL063	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011
SSL064	F031	H008, H007, H011	Positive-H008 and H007, Negative- H011

SSL069	F114	H009	Positive
SSL070	F114	H009	Positive
SSL071	F114	H009	Positive
SSL076	F051	H005	Positive
SSL077	F051	H005	Positive
SSL078	F097	H011	Negative
SSL079	F097	H011	Negative
SSL081	F115	H008	Positive
SSL082	F115	H008	Positive
SSL083	F081	H013	Positive
SSL084	F081	H013	Positive
SSL085	F058	H009	Positive
SSL086	F060	H010	Negative
SSL091	F102	H014	Positive
SSL092	F102	H014	Positive
SSL093	F116	H002	Positive
SSL094	F116	H002	Positive
SSL095	F116	H002	Positive
SSL096	F116	H002	Positive
SSL097	F116	H002	Positive
SSL098	F117	H011	Negative
SSL101	F033	not sampled	
SSL102	F033	not sampled	
SSL103	F033	not sampled	
SSL104	F033	not sampled	
SSL105	F034	H008	Positive
SSL106	F034	H008	Positive
SSL107	F064	H012	Negative
SSL108	F064	H012	Negative
SSL109	F039	H010	Negative
SSL110	F039	H010	Negative
SSL111	F039	H010	Negative
SSL112	F042	H010	Negative
SSL113	F118	H010	Negative
SSL114	F118	H010	Negative
SSL115	F118	H010	Negative
SSL116	F118	H010	Negative

Table A 6.9: Association of positive parent hatcheries and GP hatcheries

Isolate ID	Hatchery ID	Associated GP Hatchery	*Salmonella* Status of associated GP hatchery
SSL008a	H001	H004	Negative
SSL009a	H002	H004, H006	Negative- H004, Positive- H006
SSL010a	H002	H004, H006	Negative- H004, Positive- H006
SSL011a	H002	H004, H006	Negative- H004, Positive- H006
SSL021a	H003	H004	Negative
SSL022	H003	H004	Negative
SSL023	H003	H004	Negative
SSL024	H003	H004	Negative
SSL025	H003	H004	Negative
SSL026	H003	H004	Negative
SSL027	H003	H004	Negative
SSL032	H005	H004	Negative
SSL033	H005	H004	Negative
SSL034	H005	H004	Negative
SSL039	H006	Imported asGPs	
SSL040	H006	Imported asGPs	
SSL041	H006	Imported asGPs	
SSL042	H006	Imported asGPs	
SSL043	H006	Imported asGPs	
SSL044	H006	Imported asGPs	
SSL045	H006	Imported asGPs	
SSL046	H006	Imported asGPs	
SSL047	H006	Imported asGPs	
SSL065	H007	H006	Positive
SSL066	H007	H006	Positive
SSL067	H007	H006	Positive
SSL068	H007	H006	Positive
SSL072	H008	H004	Negative
SSL073	H008	H004	Negative
SSL074	H008	H004	Negative
SSL075	H008	H004	Negative
SSL087	H009	H004	Negative
SSL088	H009	H004	Negative
SSL089	H009	H004	Negative
SSL090	H009	H004	Negative
SSL117	H013	H004	Negative
SSL118	H013	H004	Negative
SSL119	H013	H004	Negative
SSL120	H013	H004	Negative
SSL121	H013	H004	Negative
SSL122	H013	H004	Negative

Isolate ID	Hatchery ID	Associated GP Hatchery	*Salmonella* Status of associated GP hatchery
SSL123	H013	H004	Negative
SSL124	H014	H004	Negative
SSL125	H014	H004	Negative
SSL126	H014	H004	Negative
SSL127	H014	H004	Negative
SSL128	H014	H004	Negative
SSL129	H014	H004	Negative
SSL130	H014	H004	Negative
SSL131	H014	H004	Negative

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