PREVALENCE AND CHARACTERISTICS OF *SALMONELLA SPP.* ISOLATED DURING BROILER PRODUCTION, PROCESSING AND SALE IN TRINIDAD AND TOBAGO USING THE 'FARM TO FORK' APPROACH

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ABSTRACT

Prevalence and characteristics of *Salmonella spp.* isolated during broiler production, processing and sale in Trinidad and Tobago using the 'farm to fork' approach

Anisa Sarah Khan

Globally, *Salmonella* spp. is an important pathogen associated with foodborne diseases but there is a dearth on information on the occurrence and characteristics of *Salmonella* spp. in the country. The farm-to-fork investigation, using cross-sectional studies, determined the prevalence and characteristics of *Salmonella* in imported fertile hatching eggs, day-old chicks at hatcheries, broilers on farms, processing plants and chicken sold at retail outlets, using phenotypic methods and whole genome sequencing (WGS).

At the level of broiler production, the prevalence of *Salmonella* was 0.0%, 7.6% and 2.8% for imported fertile eggs, hatcheries, and farms, respectively ($p=0.006$). The highest frequency of isolation of *Salmonella* was 28.0% and 2.2% in stillborn chicks and cloacal swabs, respectively, and the predominant serovars isolated were Kentucky (83.3%) and Infantis (62.5%).

At the processing plants, the overall prevalence of *Salmonella* was 27.0%. *S.* Enteritidis, Javiana and Infantis were the predominant serotypes isolated, accounting for 20.8%, 16.7% and 12.5%, respectively, of the serotypes.

The prevalence of *Salmonella* spp. in chicken carcasses sampled from cottage poultry processors and supermarkets was 20.5% and 8.3% respectively ($p<0.001$); the predominant serotypes isolated were Kentucky (30.9%) and Javiana (22.7%). Overall, all isolates exhibited resistance to one or more of the 16 antimicrobial agents tested.

Whole genome sequencing (WGS) of 146 isolates that originated from the three levels of the industry was conducted. Antimicrobial resistance genes conferring resistance to aminoglycosides, cephalosporins, peptides, sulfonamides and antiseptics were detected. Overall, virulence factors associated with secretion system and fimbrial adherence determinants accounted for 69.3% and 29.2% of the counts, respectively. Analysis of the core genome phylogenies revealed reliable clustering among isolates of serovars detected within and between sampling levels. The use of WGS confirmed the genetic relatedness and transmission of *Salmonella* serovars contaminating chickens in broiler processing and retailing in the country, with zoonotic and food safety implications.

Keywords: broilers; broiler production; broiler processing; broiler retailing; Caribbean; molecular characterisation; phenotypic characterisation; *Salmonella*; Trinidad (Trinidad and Tobago); Trinidad and Tobago.

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List of Abbreviations

ACSSuT	Ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracyclines
AMR:	Antimicrobial resistance
AOAC:	Association of Official Analytical Chemists
ARO	Antibiotic Resistance Ontology
BAM	Bacteriological Analytical Manual
BGA	Brilliant Green agar
BPW	Buffered Peptone Water
BSA	Bismuth Sulphite agar
CARPHA	Caribbean Public Health Agency
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CRISPRs	Clustered Regularly Interspaced Short Palindromic Repeats
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
E test	Epsilometer test
ERIC-PCR	Enterobacterial repetitive intergenic consensus
ESBL/ EsβL	Extended-spectrum β-lactamases
ESI	Emergent *Salmonella* Infantis

EU	European Union
FAO	Food and Agriculture Organisation of the United Nations
FBD	Foodborne disease
FBO	Foodborne outbreaks
FDA	Food and Drug Administration
FDA-CFSAN	Food Drug Administration, Center for Food Safety and Applied Nutrition
FERN	Food Emergency Response Network
FSIS	Food Safety and Inspection Service
GMI	Global microbial identifier
HE	Hektoen enteric
ICC	Intra-cluster correlation coefficient
ISO	International Organisation for Standardisation
KW	Kauffman-White-Le-minor
LB	Lactose broth
LIA	Lysine iron agar
LMIC	Low- and Medium-Income Countries
LPS	Lipopolysaccharide
MDR	Multidrug resistance/resistant (resistance to 3 or more classes of antimicrobial agents)
MIC	Minimal inhibitory concentration
MKTTn	Muller-Kauffmann Tetrathionate Novobiocin broth
MLST	Multi loci sequence typing

MLVA	Multiple Locus Variable Number of Tandem Repeats Analysis
NGS	Next generation sequencing
NS	Neck skin maceration
NTS	Non-typhoidal *Salmonella*
ONPG	o-nitrophenyl-b-D-galactopyranoside
OR	Odds ratio
OECD	Organisation for Economic Co-operation Development
OIE	World Organisation for Animal Health
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PMQR	Plasmid mediated quinolone resistance
QAC	Quaternary ammonium compound
RAPD	Random amplified polymorphic DNA
REP-PCR	Repetitive extragenic palindromic sequences
RFLP	Ribotyping/ Restriction fragment length polymorphism
RT-PCR	Reverse transcriptase PCR
RV	Rappaport Vassiliadis
RVS	Rappaport Vassiliadis Soya broth
SIM	Sulfide-indole-motility
ST	Sequence type
T3SS	Type III secretion systems

TSI	Triple sugar iron agar
TT	Tetrathionate broth
US	United States
USA	United States of America
USDA	United States Department of Agriculture
USDA-FSIS	United States. Department of Agriculture Food Safety and Inspection Service
UWI	University of the West Indies
VFDB	Virulence Factor Database
WCE	Whole carcass enrichment
WCR	Whole carcass rinse
WGS	Whole genome sequencing
WHO	World Health Organisation
WKL	White–Kauffman–Le Minor
XLD	Xylose lysine deoxycholate
XLT-4	Xylose lysine tergitol 4

CHAPTER 1: INTRODUCTION

Salmonellosis can be defined as the disease caused by either typhoidal or non-typhoidal *Salmonella* strains. Typhoidal strains of *Salmonella* are host-specific/restricted, causing systemic disease in one host-species but are not pathogenic to other host species whereas non-typhoidal strains are either host-adapted (causing disease in one particular host-species, with potential to cause disease or act as carriers in other host species) or non-host-specific/un-restricted (causing systemic disease or self-limiting gastroenteritis in a broad range of unrelated host-species [1]. As such, non-typhoidal *Salmonella* (NTS) strains are zoonotic and are the primary focus of this thesis.

Salmonella is the most common bacterial agent responsible for foodborne illness globally. The bacterium is spread via direct ingestion of undercooked food or indirect contamination of food or water. The disease process elicited by *Salmonella* can be either subclinical or apparent, thus, making it difficult to control its spread from animals to humans. Additionally, the clinical signs in humans and animals are non-specific therefore making it difficult to diagnose thus leading to the underreporting of cases, affecting the potential of epidemiological studies. Animals are the most common source of *Salmonella* contamination, harboring the organism in their intestinal tract. Faecal contamination of surfaces or vegetation as well as improper slaughtering practices of infected animals are sources of *Salmonella* contamination.

Severity of clinical signs and disease vary with the host's immune system, the invading serotype together with its various virulence factors. Clinical signs in human salmonellosis can vary from self-limiting gastroenteritis (nausea, vomiting, diarrhoea and abdominal cramps), commonly seen in foodborne outbreaks with serotypes such as *S.* Kentucky and *S.* Infantis [2] to the less frequent but more severe invasive bacteremia leading to endocarditis, meningitis and pneumonia to name a few [3]. It should be noted that whilst symptoms usually subside within

seven days, however, risk groups such as children, the elderly and immunocompromised individuals may experience severe symptoms for several weeks [4]. Broilers infected with most *Salmonella* serovars do not show clinical signs of the disease, therefore making it difficult to diagnose at the farm. In broilers infected with host-specific serovars such as *S.* Pullorum and *S.* Gallinarum, clinical signs reported include stillborn chicks, severe mortality among newly hatched chicks, weak day-old chicks, drooping of wings, reduced feed intake, death of grower birds, ruffled feathers and diarrhoea [5]. Birds that survive initial infection may become carriers with or without the presence of clinical signs [6]. Whilst *S.* Pullorum and *S.* Gallinarum have been eradicated in commercial poultry in regions such as the U.S.A., Canada and Western Europe [7], serovars Enteritidis, Kentucky, Heidelberg and Typhimurium are most commonly associated with poultry [8].

There are several factors that affect the prevalence of *Salmonella* in low- and medium-income countries (LMIC) and high-income countries, notably the regulation and continuous testing of produce entering the food chain with adequate tracing methods in place. This is necessary should there be a need for a recall of produce, commonly practised in high-income countries. Poor sanitation practices, improper handling of raw meat and inadequate laboratory facilities for food safety measures to be enforced by authorities are some of the factors that affect the control of salmonellosis in developing countries.

Poultry is a common source of human salmonellosis due to improper slaughtering practices, improper handling of raw products or under-cooking of poultry. It is also one of the most economical sources of protein, globally, with a projected global consumption rate of 131,230 kt rtc (kilotonne, ready to cook) in 2020 according to the FAO [9]. In 1970, the most frequently isolated serovar from human and non-human sources was *S.* Typhimurium [10]. However, after the eradication of *S.* Pullorum and *S.* Gallinarum in 1975, this created a favourable niche for the zoonotic serotype, *S.* Enteritidis [11, 12]. As such, *S.* Enteritidis cases began increasing in the 1980s [13, 14] and by the 1990s, it was the most frequently reported serotype

[7]. In 2015 *S.* Enteritidis still remains the most frequently reported serotype isolated in human infections in the U.S.A. [15] with chicken meat and eggs being important sources of the serotype [16].

The emergence of antimicrobial resistance can be attributed to poor biosecurity and hygiene practices along the entire broiler production continuum (breeder flocks, hatchery, grow-out phase, and processing plant) [17], the improper use of antimicrobial agents in food-producing animals [18, 19], as well as the unregulated use of antibiotics in humans in LMIC countries [20, 21]. Pre-harvest antimicrobial resistance control strategies such as vaccination, testing, flock management and sanitation, and gastrointestinal colonisation are well-established [22]. Recent development in alternative methods affecting gastrointestinal colonisation, such as the addition of organic acids to feed and drinking water [23, 24], diet modification [25, 26] to reduce *Salmonella* susceptibility, as well as the use of bacteriophages [27], have been deemed effective in reducing *Salmonella* proliferation in poultry thus limiting its spread.

Over the years, there have been several studies done on *Salmonella* isolated at broiler and layer farms, processing plants and cottage processors in the Trinidad and Tobago. A study conducted in 2012 [28] followed the transmission of *Salmonella* from broiler farms through processing plants as well as retail outlets, however, sampling was not conducted at the hatcheries and imported fertile eggs thus, was insufficient for a thorough epidemiological study of the broiler continuum. Also, *Salmonella* strains were not characterised. An important finding in that study was the detection of a higher frequency of contamination of carcasses at the retail outlets, 77.1% (108/140) compared with 50.0% and 51.3% found on broiler farms and processing plants, respectively. *S.* Typhimurium was isolated from broiler farms in Trinidad and Tobago in 1994 but the isolation rate of *Salmonella* was only 0.5% from a sample size of 660 birds collected from 10 broiler farms, where no molecular characterisation was performed [29]. Studies in the country have been conducted on foodborne pathogens isolated from cottage poultry

processors (‘pluck shops’) [30, 31] where the serotypes and antibiograms of the isolates were determined and their relatedness reported using the pulse-field gel electrophoresis (PFGE). However, to date there are no published report on the molecular characterisation and diversity of *Salmonella* isolates recovered from the poultry production (hatcheries and farms) and processing (processing plants) chain in the country. Furthermore, there has been no documentation of the genetic relatedness of *Salmonella* isolates from poultry in the country or any of the English-speaking Caribbean countries.

With the limited information available, it is evident that the epidemiology of *Salmonella* in the broiler industry has not been fully elucidated in Trinidad and Tobago. Therefore, this study aims to investigate the epidemiology of *Salmonella* in the poultry chain (production, processing and retailing), utilising a ‘farm to fork’ approach. This approach will also investigate the role played by the importation of fertile hatching eggs, thus providing invaluable data to guide intervention strategies to prevent and monitor the entry and spread of *Salmonella* at each level in the poultry industry. Data obtained from this study would benefit health care professionals, as well as the poultry industry at various levels and the general public since chickens are widely consumed throughout our economic strata in the country.

This comprehensive study encompassing epidemiology, bacteriology, molecular approach, food safety, and public health, is anticipated to generate invaluable data with a potential to contribute to food safety issues at large. These data are also expected to be beneficial to humans and the poultry industry as they should form the basis to drive prevention and control measures, identification of risk factors for infection, and provision of guidance for the implementation of intervention measures.

1.1 Objectives

- To determine the prevalence of non-typhoidal *Salmonella* in imported fertile hatching eggs, broilers at hatcheries, farms, processing plants, and retail outlets ('farm to fork') in Trinidad and Tobago **(Chapter 3, Chapter 4 and Chapter 5)**.
- To determine the risk factors associated with non-typhoidal *Salmonella* infection and contamination of broilers **(Chapter 3, Chapter 4 and Chapter 5)**.
- To characterise *Salmonella* strains to determine the epidemiology of non-typhoidal *Salmonella* throughout the broiler production chain using phenotypic methods **(Chapter 3, Chapter 4 and Chapter 6)** and whole genome sequencing (WGS) **(Chapter 7 and Chapter 8)**.

CHAPTER 2: LITERATURE REVIEW

2.1 About the organism

*Salmonella*e are non-spore forming, Gram negative bacilli that are predominantly motile with non-motile variants belonging to the family of Enterobacteriaeceae [32, 33]. *Salmonella* is a ubiquitous and hardy bacterium that can survive several weeks to several months in dry and wet environments, respectively, but survives best in organic material [34, 35]. The optimal temperature range for growth is between 35-43^{o}C with growth rates drastically decreasing under 15^{o}C and almost ceasing under temperatures less than 7^{o}C for most *Salmonella* spp. [36]. *Salmonella* can persist in the digestive tract of both humans and animals, therefore its presence in the environment, food and water is due to faecal contamination [7]. Serovars of *Salmonella* vary based on host preferences and can be host-specific, host-adapted or non-host-specific. Host-specific serovars elicit systemic disease in one very specific host only whereas host-adapted serovars can cause severe systemic disease infection in their preferred host and are usually excreted without any clinical signs when they accidentally infect hosts other than their most preferred host. Non-host-specific serovars have a broad host range where they may elicit mild enteric disease and usually persists in the host without severe clinical signs. These serovars can invade different hosts without resistance therefore have greater zoonotic potential than the other categories of serovars [37]. Poultry can be infected by host-specific and non-host-specific *Salmonella* serovars. Whilst host-specific *S*. Gallinarum and *S.* Pullorum causes systemic disease in poultry, non-host-specific serovars are mainly asymptomatic but are associated with widespread human illness.

2.1.1 *Classification*

The Kauffman and White scheme of *Salmonella* classification classifies *Salmonella* by serotype based on three major antigenic determinants: somatic (O), capsular (K) and flagellar (H) [38]. The somatic (O) and flagellar (H) antigen determine the

group and serotype the *Salmonella* isolate belongs to [39]. Using this system, over 2600 serotypes have been identified based on the unique combinations of somatic (O) and flagellar (H) antigens [40]. This scheme will be discussed further in 2.3.1.1 Kauffmann-White-Le Minor scheme.

To date, the issue of nomenclature of *Salmonella* has been controversial and still evolving. The nomenclature system of *Salmonella* recommended by the World Health Organisation (WHO) Collaborating Centre is used by the Centers for Disease Control and Prevention (CDC) [41]. Based on this system, the genus *Salmonella* is classified into two species, *Salmonella enterica* and *Salmonella bongori* based on differences in their 16S rRNA sequence analysis [42]. *S. enterica* is further divided into six subspecies characterised by both a roman numeral and name: I, *S. enterica* subsp. enterica; II, *S. enterica* subsp. salamae; IIIa, *S.* enterica subsp. arizonae; IIIb, *S.* enterica subsp. diarizonae; IV, *S. enterica* subsp. houtenae and VI, *S. enterica* subsp. Indica based on their biochemical properties, and can be further subdivided into serotypes based on their interactions with antiserum [38, 43]. *S. enterica* consists of more than 2600 serotypes, where the most common subspecies is subsp. enterica which accounts for 99% of the infections in humans and warm-blooded animals [44, 45]. The other five *S. enterica* subspecies and *S. bongori* rarely infect humans but are mainly found in cold-blooded animals, where the genus is said to have originated [46], as well as in the environment [38].

2.1.2 *Epidemiology*

Globally, despite the implementation of several control measures, *Salmonella* infections continue to be problematic with millions of cases occurring annually world-wide, in both humans and animals [47]. According to the most recent European Food Safety Authority (EFSA) report, 91,857 confirmed human cases of salmonellosis from 1,580 foodborne outbreaks were documented in 2018 across 24 European Union (EU) member states [48]. An estimated 1.35 million *Salmonella*

infections occur annually in the United States with food as the main source of infection according to the Centers for Disease Control and Prevention (CDC) [49]. The economic burden of salmonellosis is substantial in both high-income and LMIC as the European Union estimates an annual expense of nearly €3 billion [50], while the United States Department of Agriculture (USDA) estimates the cost of *Salmonella* in the United States (US) at USD$3.67 billion [51-53]. Closer to home, it is estimated that 1 in 49 people (approximately 142,000 people) in the Caribbean acquire a foodborne illness because of ingesting contamination food or drink, with this incidence increasing to 1 in 11 during peak tourism periods since the Caribbean is a well-known tourist destination [54]. The economic cost estimated to be US $21 million a year, highlighting the extensive health and economic implications of foodborne illness to the region [54].

The source of human salmonellosis outbreaks varies extensively from fruits to processed meat and pets. In 2019 alone, the CDC reported several outbreaks of *Salmonella* contamination of food origin linked to cut fruit (*S.* Javiana), ground beef (*S.* Dublin), papayas (*S.* Uganda), tahini (*S.* Concord), frozen raw tuna (*S.* Newport), pre-cut melon (*S.* Carrau), and ground turkey (*S.* Schwarzengrund) [55]. Additionally, there were outbreaks where the sources of contamination were pet turtles, backyard poultry and pet hedgehogs [55].

Salmonella foodborne outbreaks (FBOs) reported in Europe were caused by eggs and egg products, bakery products and mixed foods whereas in food, poultry meat and other meat intended to be cooked before consumption contained the highest levels of *Salmonella* positive samples [48].

According to data retrieved from the US, European countries and LMIC, control programmes aimed at mitigating *Salmonella* contamination along the food chain have been unsuccessful since globally, salmonellosis is the most reported caused of bacterial foodborne disease (FBD) [56].

Salmonella infections in high-income countries are primarily due to contaminated food and cause self-limiting gastroenteritis in healthy adults [57], whereas in sub-Saharan Africa, non-typhoidal *Salmonella* are the most isolated bacterial cause of severe invasive disease in adults and children presenting as febrile illness [58]. Invasive disease caused by non-typhoidal *Salmonella* is more prevalent in immunocompromised individuals (young, old and HIV positive) and has the highest incidence worldwide in sub-Saharan Africa, estimated at 34.5 (26.6 to 45.0 cases per 100,000), whereas the incidence rate of other regions (Southeast Asia, East Asia, Oceania, Central Europe, Eastern Europe, Central Asia, Latin America, Caribbean, North Africa, the Middle East and South Asia) ranges from 1.1 to 2.7 cases per 100,000 humans [58]. It should be highlighted that the source of NTS in sub-Saharan Africa is not commonly associated with foodborne diseases but human-to-human transmission [59, 60].

This thesis focusses solely on enteric NTS transmitted along the food chain via poultry, which will therefore be reflected in proceeding chapters.

2.1.3 Virulence

Salmonella pathogenicity islands (SPIs) and its type III secretion systems (T3SS), flagella, fimbriae, toxins, capsule, adhesion systems and virulence plasmids constitute *Salmonella*'s virulence factors [61, 62]. Whilst all the aforementioned factors may not be present in all NTS serotypes, their presence or absence influences the virulence and host range of a serotype or isolate. The following highlights the various virulence factors of NTS.

Many of the major components responsible *Salmonella* virulence functions are directly linked to genes encoded within large regions of bacterial chromosomes called *Salmonella* pathogenicity islands (SPIs). Some SPIs are found in all *Salmonella* whilst others are associated with certain strains or absent in non-pathogenic species [63]. There has been 24 SPIs described to date which encode genes that facilitate (i) expression of secretion systems, fimbriae, flagella, and

capsules, (ii) serotype conversion and (iii) host colonisation and subsequent survival within the host [64, 65]. Amongst the 24 SPIs, SPI-1 and 2 are the best characterised genetically and phenotypically. SPI-1 is ubiquitous in all *Salmonella* species and subspecies whereas SPI-2 is only found in *S.* enterica [66]. Of the 24 SPIs, 20 SPIs are variably present among NTS serotypes [67, 68]. The manipulation of host cell function by *Salmonella* is accomplished by expression of type III secretion systems (T3SS) which injects bacterial or effector proteins directly into the host cell cytoplasm. SPI-1 and SPI-2 each encode a T3SS enabling invasion of epithelial cells and evasion of lysosome fusion followed by persistence in *Salmonella* containing vacuoles (SCV), respectively. SPI-3 and SPI-4 are less studied than the previously mentioned and are involved in intramacrophage survival [69, 70]. SPI-5 mediates an increase in intestinal fluid secretion therefore is involved in gastroenteritis-associated phenotypes [71]. Some SPIs are associated with certain serotypes such as SPI-7, encoding the Vi capsule was proposed to be only found in *S.* Typhi, however it has been found in strains of the NTS *S.* Dublin, with both serotypes associated with invasive disease in humans [72, 73].

Most NTS are motile due to the expression of flagella. Flagella synthesis, assembly and maintenance require more than 50 genes with the antigenic subunit, flagellin being encoded by three genes, *fliC* (phase 1), *fljB* (phase 2) and *flpA* (phase 3; rare and mostly plasmid encoded) [74, 75]. Flagella aid *Salmonella* cells in motility towards the host epithelial layers and host-derived nitrate and tetrathionate (used as alternate terminal electron acceptors) and are potent inducers of host innate immune response [76, 77]. Flagellar expression in NTS serotypes may play a role in intentionally triggering an inflammatory immune response allowing for propagation in the intestine or evading apoptosis [76].

Fimbriae or pili are thin appendages that aid in attachment and adhesion. *Salmonella* has 39 putative fimbrial operons, of which *agf* operon is found in both *S. enterica* and *S. bongori*, encoding the nucleator-dependent curli fimbriae, which are thin, aggregative fimbriae that may aid in bacterial adhesion and invasion [64, 78]. Thirty-six fimbrial operons encode chaperone-usher-dependent fimbrial

pathways of which 27 have been identified in NTS, typically containing 5-14 gene clusters per serotype [78]. Differences in fimbrial gene clusters among serotypes have been implicated in the ability of some serotypes to colonise and persist in different hosts/environments [78]. Yue et al. [78] demonstrated that serotypes such as Dublin and Gallinarum encode multiple non-functional fimbrial genes whereas, serotypes with wider host ranges such as Typhimurium, Enteritidis, Montevideo, Kentucky and others, have relatively few degraded fimbrial genes.

The genotoxin, typhoid toxin, the exotoxin secreted by *S.* Typhi has been recently identified in at least 41 NTS serotypes being absent in *S.* Typhimurium, Enteritidis and Newport, the serotypes responsible for most clinical infections worldwide [79]. In NTS, this toxin has been shown to contribute to systemic host colonisation by *S.* Javiana [80]. NTS serotypes such as Enteritidis, Indiana, Changa, Saintpaul, Typhimurium and Virchow reportedly produce heat-labile, trypsin-sensitive cytotoxins with the *stn* gene implicated [81, 82].

The presence of virulence plasmids is variable among serotypes, with nine NTS serotypes harbouring low copies of virulence plasmids varying in size and genetic content [83]. Isolates harbouring plasmids generally exhibit increased virulence. The effector proteins of the 7.8 kb *spvRABCD* (*Salmonella* plasmid virulence) operon alter host cell cytoskeleton to enhance bacterial survival [84]. *Spv* is common to all *Salmonella* virulence plasmids, however, other virulence factors or antimicrobial resistance genes may also be encoded. The *spv* operon encodes a toxin implicated in host cell cytoskeletal rearrangements and apoptosis of the host cell [85]. Isolates encoding the *spv* operon are often associated with invasive disease as *spv* genes aid in suppression of the innate immune response by attenuating the intestinal inflammatory response [86].

Multiple individual virulence genes have variable distribution in *Salmonella* serotypes therefore this complicated combination of genes contribute to the virulence diversity, which presents a challenge in virulence profiling and risk assessment [87].

2.1.4 Antimicrobial resistance

Selective pressures, proliferation of multiple resistant clones and the inability to detect emerging phenotypes play important roles in the development of antimicrobial resistance. Selective pressures include the use of antimicrobials in the treatment of diseases, agriculture and in-home disinfectants [88]. Antimicrobial resistance (AMR) occurs because of microbes changing to reduce or escape the effect of an antimicrobial it was once susceptible to. This can occur naturally (intrinsically, a universal trait always expressed in a species, independent on antimicrobial exposure or induced, where genes are naturally occurring in the bacteria but are only expressed after antimicrobial exposure) or acquired through exchange of DNA [89]. Acquired resistance occurs via horizontal gene transfer where the acquisition of new antimicrobial resistance genes harbored on mobile genetic elements, such as plasmids, transposons and integrons play a crucial role in the development and dissemination of antimicrobial resistance [90].

A comprehensive review regarding genes and mutations that confer antimicrobial resistance in *Salmonella* are well reported [91-93]. Tetracycline has been approved for use in animal feed since 1951 in the US, without need of a veterinary prescription for the treatment of coccidiosis, growth promotion and other purposes [94]. Thus far, five tetracycline resistance genes which all code for efflux pumps have been identified in *Salmonella*: *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(G) [95, 96]. The transposon-borne resistance genes *tet*(A) and *tet*(B) were most frequently detected whereas *tet*(G), was exclusively found within *Salmonella* Genomic Island 1 (SGI1) or 2 (SGI2) associated multidrug resistance (MDR) gene clusters [93].

Resistance to aminoglycosides occur due to decreased drug uptake, drug modification and modification of the ribosomsal target of the drug [97]. With the widespread use of penicillins (β-lactams), resistance to ampicillin, methicillin and other penicillin drugs is common [98]. The most common cause of resistance is the secretion of beta-lactamases by genes typically carried on plasmids [99]. At least ten different *aadA* genes coding for aminoglycoside-3”-O-adenly-transferases that confer resistance to the aminoglycosides, streptomycin and spectinomycin are

known to occur in *Salmonella*: *aadA1*, *aadA2*, *aadA5*, *aadA6*, *aadA7*, *aadA12*, *aadA21*, *aadA22*, *aadA23*, *aadA24*, *aadA26* and *aadA27* [110, 101]. Additionally, the aminoglycoside-2”-O-adenlytransferase gene *aadB* (resistance to gentamicin, kanamycin and tobramycin), aminoglycoside-N-acetyltransferase genes *aacC* and *aacA* (resistance to gentamicin) and genes *armA* and *rmtC* which code for 16S rRNA methylases (resistance to all clinically available aminoglycosides except streptomycin) have also been described in *Salmonella* [93, 102]. β-lactamases are responsible for resistant to β-lactam antibiotics, with over 13 different types including TEM, SHV, PSE, OXA, PER, CTX-M, CMY, ACC, DHA, KPC, SCO, NDM and VIM, known to occur in *Salmonella* [93, 103, 104]. Of importance is the presence of the blaCTX-M, which codes for cephalosporinases that hydrolyse most β-lactams except cephamycins and carbapenems, and the blaOXA-48/blaKPC/blaNDM/blaVIM genes that code for carbapenemases.

Combinations of trimethoprim and sulfonamides have been used in veterinary medicine since 1970 due to its broad spectrum of activity, clinical efficacy and low cost [105]. Resistance in *Salmonella* has been reported to both these drugs [99]. At least 17 different *dfrA* genes and one *dfrB* gene coding for trimethoprim resistance have been isolated in *Salmonella* isolates [93, 101]. Enterobacteriaceae occurring sulphonamide resistance genes, *sul1*, *sul2* and *sul3*, have also been detected in *Salmonella* [93].

After the approval of fluoroquinolone use in animals, the rate of fluoroquinolone-resistant *Salmonella* in food animals and subsequently in human infection rapidly increased in several countries [106]. Sarafloxacin and enrofloxacin, having previously been approved for use in poultry, was removed from the approval list due to increased antimicrobial resistance in *Campylobacter* and *Salmonella spp*. recovered in human disease [107]. Mutations in the *gyrA*, *gyrB*, *parC* and/or *parE* genes are responsible for resistance to quinolones/fluoroquinolones observed in *Salmonella* isolates. Additionally, various plasmid-mediated quinolone resistance (PMQR) genes such as the *qnr*D, *qnr*A, *qnr*B and *qnr*S variants, coding for DNA

topoisomerase protecting proteins amongst others, are known to occur in *Salmonella* isolates [108, 109].

Macrolide resistance genes *ere(A)*, *mph(A)*, *mph(E)* and *msr(E)*, lincosamide resistance gene *linG*, streptothricin acetyltransferase genes *sat* and *sat2*, fosfomycin resistance gene *fosA3* [104] and the transferable colistin resistance gene *mcr-1* have been detected in *Salmonella* isolates [110, 111].

Due to the rapid development of AMR in humans and animals, antimicrobial therapy is not recommended for the treatment of non-severe, non-typhoidal *Salmonella* in healthy adults or children. Antimicrobial therapy is recommended for cases of sepsis or extra-intestinal infection and cases at risk of developing bacteraemia and disseminated disease as seen in invasive non-typhoidal salmonellosis. *Salmonella* is described as being resistant to traditional first-line antibiotics such as ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole [112]. It was reported that the most common multidrug resistant (MDR) phenotype among *Salmonella* strains containing SGI1, was to five agents: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracyclines (ACSSuT) [113].

Ceftriaxone resistance in *Salmonella* is of public health significance since it is commonly used to treat severe *Salmonella* infections, especially in children [114]. Carbapenems and tigecycline could be potential alternatives should resistance to developed to all first- and second-line drugs [115]. However, combinations of fluoroquinolones, cephalosporins and azithromycin are used frequently in patients who fail to respond promptly with the hope of delaying resistance by broadening the spectrum of activity and using any synergistic effects between agents [116, 117].

2.2 **Detection, isolation, and identification of *Salmonella* spp.**

2.2.1 Conventional cultural methods

This traditional method of isolation entails the non-selective enrichment (pre-enrichment) of a specific weight or volume of sample followed by selective enrichment, plating onto selective agars, biochemical panel identification and serological confirmation of suspect colonies. Several regulatory agencies, such as the International Organisation for Standardisation (ISO), Association of Official Analytical Chemists (AOAC), Food and Drug Administration (FDA), Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) have different standardised methods of enrichment using the unique physical properties of the organism [118]. This method is widely used by many food safety and public health laboratories due to their ease of use, reliability of results, high sensitivity and low specificity and cost when compared to emerging molecular-based technologies [118]. Disadvantages to this method include the long period of time for complete isolation and confirmation as well as possible false-positive results due to competitive flora such as *Proteus* spp. [119].

2.2.1.1 Isolation: non-selective and selective enrichment and plating

The commonly used media in non-selective enrichment are Buffered Peptone Water (BPW) and lactose broth (LB) [118]. Following incubation, the non-selectively enriched sample is inoculated onto selective enrichment which further enhances growth, thereby increasing the sensitivity. This inhibition of the growth of other bacteria permits the continuous growth of *Salmonella* in the selective enrichment [120]. The FDA Bacteriological Analytical Manual (BAM) and Food Emergency Response Network (FERN) have approved the use of Rappaport Vassiliadis (RV) medium and tetrathionate (TT) broth as *Salmonella* enrichment media in their standardised methods. The current International Organisation for Standardisation (ISO), ISO 6579-1:2017 [121], specifies a horizontal method for

the detection of *Salmonella* spp. in the food production chain using non-selective pre-enrichment of sample in a 10-fold dilution with BPW followed by selective enrichment in Rappaport Vassiliadis Soya broth (RVS) and Muller-Kauffmann Tetrathionate Novobiocin broth (MKTTn). Other regulatory agencies have standard methods for *Salmonella* detection like, ISO 6579-1:2017.

Selective enriched media are subsequently streaked onto solid selective media to isolate presumptive *Salmonella* colonies whilst inhibiting proliferation of other bacteria [120]. Recovery and selectivity of *Salmonella* may be amplified by using two or more selective media with supplements such as thiosulphate, malachite green, novobiocin and sulphamethazine [120]. This is required since *Salmonella* colonies appear different to coliforms on these media [122]. Some of the selective agar commonly used include Bismuth Sulphite agar (BSA), Brilliant Green agar (BGA), Hektoen enteric (HE) and *Salmonella Shigella* Agar (SSA) [122, 123]. Different *Salmonella* serotype colonies appear differently on the respective selective plate used and some serotypes which are not distinctive may go unnoticed on some media. ISO 6579-1:2017 recommends the use of xylose lysine deoxycholate (XLD) agar in conjunction with another isolation agar [124].

There have been improvements made to the conventional methods reducing cost and labour by using chromogenic and fluorogenic growth media (e.g., SM-ID agar, Rambach agar and BBL CHROMagar *Salmonella*) for detection, enumeration, and identification directly on the isolation plate. These have been reported to be more convenient, reliable, and specific than the traditional conventional media [125], with results are typically available 1 day earlier, therefore not substantially faster, thereby limiting their usage in situations of outbreaks and recalls requiring fast results.

2.2.1.2 Biochemical tests

Suspected *Salmonella* colonies identified based on their appearance on the selective agars are subjected to biochemical panel screening after purification on blood agar

or nutrient agar. The colonies are streaked onto triple sugar iron agar (TSI), Urea agar, lysine iron agar (LIA), β-galactosidase (ONPG), Voges Proskauer and Indole tests in this order in accordance with ISO- 6579 [126]. Detection of the presence of the enzyme urease and β-galactosidase by using the compound o-nitrophenyl-beta-D-galactopyroniside (ONPG) have been published elsewhere [118].

2.2.1.3 Polyvalent antisera

Serological tests are based on the antibodies in serum produced in response to bacterial antigen exposure, which will agglutinate with bacteria carrying homologous antigens. Serological confirmation tests typically utilise polyvalent antisera for flagellar (H) and somatic (O) antigens. Serological tests must be performed with biochemical analysis since serological testing alone only suggests presumptive identification [127]. Latex agglutination test utilises antibody-coated latex particles which react with antigens on the surface of *Salmonella* forming visible aggregates, thus confirming the presence of *Salmonella*. This test reduces time and resources, allowing negative results to be reported at least 24 h earlier than by conventional cultural methods. This slide agglutination test is based exclusively on phenotypic characteristics where false-positive reactions may occur due to weak, non-specific agglutination [128]. Several kits that tests for the identification of *Salmonella* serogroups A, B, C, D, E and G and the V_i antigen are commercially available.

2.2.2 Immunology based methods

Immunology-based assays include enzyme- linked immunosorbent assay (ELISA), latex agglutination tests, immunodiffusion and immunochromatography. These methods utilise specific mono or polyclonal antibodies that bind with somatic (O) or flagella (H) antigens. These assays are used routinely and form a valuable option based on the ability to detect viable non-culturable *Salmonella* cells [129].

2.2.2.1 Enzyme linked immunosorbent assay (ELISA)

ELISA is the most used immunological assay for the detection of antigens or antibodies against foodborne pathogens [118]. This assay utilises a *Salmonella* antibody in a solid matrix whereby once a specific antigen is bound to the antibody an antigen-antibody complex is formed, a colour change is elicited due to enzymatic cleavage of a chromogenic substrate to detect antigens [120, 130]. Detection of *Salmonella* antibodies in a sample is also possible with this assay, where antigens are coupled to the solid phase of ELISA [131]. ELISA (immunology based) and PCR (nucleic acid-based, to be discussed later) methods show comparable specificity and sensitivity to conventional methods, able to detect *Salmonella* at a concentration of $10^4 – 10^5$ per ml (ELISAs) and 10^4 per ml after enrichment (PCR). The background microflora, sample matrix, presence of non-culturable cells and inhibitory substances such as fats, proteins, polysaccharides, heavy metal, antibiotics, and organic compounds have been reported to affect the sensitivity and specificity of these methods [119, 125]. However, additional sample preparation and modification of pre-enrichment and enrichment media, dilution, centrifugation, filtration, flow injection, chromatography, organic solvent extract and fluorescence hybridisation have been reported to improve their sensitivity and detection levels [132]. This method is used as a confirmatory analysis technique since it is very sensitive, specific, uncomplicated, reliable, and available commercially from several manufacturers [133, 134].

2.2.2.2 Latex agglutination tests

This technique utilises antibody coated latex particles which react with antigens on the surface of *Salmonella* forming visible aggregates thus confirming the presence of *Salmonella*. This assay was described earlier in 2.2.1.3 Polyvalent antisera.

2.2.2.3 Lateral Flow Immunoassay

On-site immunological techniques based on lateral flow immunoassays such as immunodiffusion, dipstick and immunochromatography are becoming popular methods of pathogen (antigen) detection in the food industry [135]. A sandwich type ELISA is typically used in these immunoassays with polyclonal antibodies being used as a capture antibody with a monoclonal antibody as the detection antibody [123]. The principles and methodologies for these assays have been described [136]. False positives may be observed due to denaturation or degradation of the capture antibody or the non-specific binding of enzyme-conjugated antibody to denatured capture antibody. This method has been shown to be equivalent to the cultural method with modifications in the enrichment stage before inoculation and increased incubation times further improving the detection rate of *Salmonella* spp. [137].

2.2.3 Molecular based methods

Nucleic acid or molecular based assays have been the most extensively explored and developed *Salmonella* detection techniques in the past decade due to the advantages of sensitivity, specificity, inclusivity over other methods, and being able to rapidly identify *Salmonella* without obtaining pure cultures [138, 139]. Other notable advantages are that these assays can detect very low numbers of organisms in the sample and the high throughput capability of large number of samples for routine analysis [138, 140]. Two major techniques of molecular assays are amplification methods commonly known as polymerase chain reaction (PCR) and nucleic acid hybridisation (DNA probe) methods. Over the years, there have been improvements made to PCR methods for *Salmonella* detection such as multiplex PCR and real-time quantitative PCR (qPCR).

2.2.3.1 Polymerase chain reaction (PCR)

PCR assay is the preferred non-cultural technique for *Salmonella* detection due to its ability to detect low concentrations of *Salmonella* and shorter enrichment times required for reliable detection when compared to other assays [141].

The presence of PCR-inhibiting compounds in samples such as organic chemicals, sucrose and denatured proteins may bind to the DNA template or alter DNA polymerase activity thereby decreasing the sensitivity of the assay [142]. Also, the presence of cells and DNA, other than the target organism, can decrease the sensitivity of this method [143].

2.2.3.2 Multiplex PCR (mPCR)

This is a modified PCR assay that allows for the simultaneous detection of multiple target sequences within a single PCR reaction. The use of multiple primer sets (more than five to six primer pairs) in a single reaction can be challenging since there have been reported difficulties in optimisation of reaction conditions such as annealing temperature, cross-reaction among primer pairs as well as difficulty in discriminating between prominent PCR product sizes on traditional agarose gel electrophoresis [144, 145].

2.2.3.3 Real time PCR (qPCR)

Real-time detection of PCR products is enabled by the inclusion of a fluorescent reporter molecule in each reaction well that yields increased fluorescence with an increasing amount of target/product DNA [146]. Using quantitative PCR, it is possible to determine the initial number of copies of template DNA (quantitative) or the presence of absence of a sequence (qualitative). This technique has been widely used to quantify *Salmonella* from poultry samples [147, 148]. The use of fluorescence technology (SYBR Green or Taqman) allows for detection of targets with increased sensitivity thereby overcoming the limitations of conventional PCR

such as errors associated with end-point analyses and lack of quantification capabilities [149].

2.2.3.4 DNA microarray

This is one of the more recent developments in molecular based detection methods being applied to foodborne pathogens. It involves using selected single stranded oligonucleotide probes attached to a solid surface of glass slides or fluorescently encoded beads which hybridises with target DNA isolated from samples that are labelled with a fluorophore [150].

2.3 Methods used for characterisation of *Salmonella* spp.

2.3.1 Phenotypic typing methods

Serotyping (Kauffmann-White-Le Minor scheme), phage typing and antibiogram are traditional methods used for discriminating between bacteria from a single species based on phenotypes.

2.3.1.1 Serotyping- Kauffmann-White-Le Minor scheme

Serotypes are groups within a single species of microorganisms which share distinctive surface antigens, which in *Salmonella* are the O and H antigens. Conventional or antigenic classification is based upon the Kauffman-White-Le Minor scheme which designates *Salmonella* serotypes based on antibody reaction with three types of surface antigens: somatic (O), flagellar (H) and capsular (Vi) antigens. Capsular antigens only occur in *S.* Typhi, *S.* Paratyphi C and *S.* Dublin [151]. Reactions to specific antisera of the somatic (O) antigen, the polysaccharide component of the lipopolysaccharide (LPS) exposed on the bacterial surface and flagellar (H) antigen, slender thread like structures which are parts of the flagella determine the group and serovar the *Salmonella* isolate, respectively [39]. The

Kauffman-White-Le minor (KW) scheme recognizes 64 somatic (O) and 114 flagellar (H) variants whereby over 2600 serotypes have been identified based on unique combinations of O and H antigens [40]. Most *Salmonella* spp. possess two flagellar genes *fliC* and *fljB*, which encode the *Salmonella* phase 1 or H1 and phase 2 or H2 antigens respectively and are considered as the *Salmonella* serotype determinant genes. Even though one H antigen can be expressed at a given time, both H1 and H2 can be detected in pure cultures due to expression of the two phases by different bacterial populations within the same culture. The antigenic formula is reported as the subspecies name written in Roman numerals followed by their O, H1 and H2 antigens separated by colons (e.g., I 1,4,[5],12:r:1,2, Figure 1). The antigenic classification system used today, the Kauffmann-White-Le Minor scheme, was accumulated over 80 years and is maintained and updated by the World Health Organisation Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France. Due to the amount of data generated, it remains as the gold standard for *Salmonella* serotyping.

Traditional serotyping of *Salmonella* requires more than 150 specific antisera [152]. This method of typing is labour intensive, time consuming since it only allows for detection of a single antibody-antigen reaction at a time [153], can be imprecise, and requires trained technicians [154].

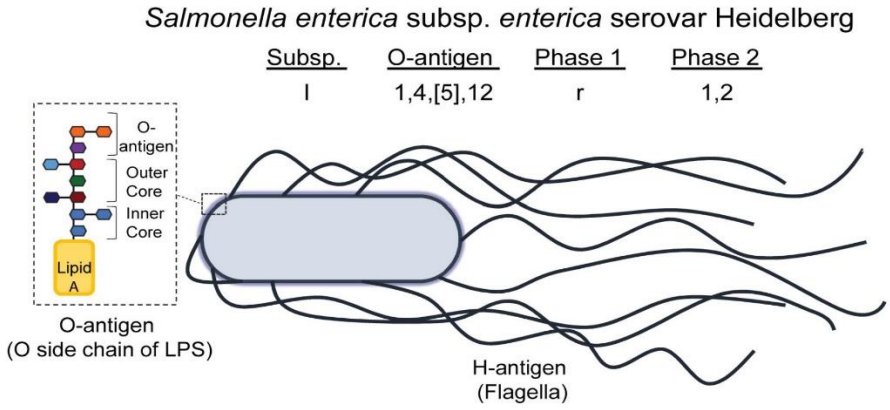

Figure 1.1. Illustration showing O- and H-antigens, comprising the serotyping scheme defined by Kauffman-White-Le Minor [155].

2.3.1.2 Phage typing

This method was used to discriminate between *Salmonella* strains belonging to the same serovar. Initially used in *S.* Typhi, Paratyphi A and Typhimurium, the system was then extended to *S.* Enteritidis [156] amongst other serovars [157]. A lawn of inoculum of the target bacteria is grown on a suitable culture medium and subjected to attack by a series of different known phages. Some phages will kill the bacteria and lyse the colony, which can be visualised (as plaques) and measured, but others won't be able to kill a given bacteria. Bacteria are given a number, corresponding to a phage-type based on the ability of a given phage to lyse the investigated strain [158]. Phage typing has the advantage of producing results easily, since it requires basic laboratory equipment and rapidly (less than 24 h) once the system is set up. It also has a high throughput where 60 to 75 strains can be typed per day, requiring minimal personnel time [157].

2.3.1.3 Antimicrobial resistance typing

This technique determines the profile of resistance of *Salmonella* strains towards a panel of antimicrobial agents. Whilst commonly used in the past as a subtyping method to determine correlation between isolates, antibiogram has poor discriminatory power since AMR occurs due to selective pressure, is often associated with mobile genetic elements and strains that are deemed to be epidemiologically related may have different AMR profiles due to their genetic structure (loss of plasmids or acquisition of new genetic material) [159, 160]. The two most used methods in veterinary laboratories are the agar disc-diffusion method and the broth microdilution method.

2.3.1.3.1 Disc diffusion

Disc diffusion is one of the oldest approaches to antimicrobial susceptibility testing and remains one of the most widely used methods in routine clinical microbiology laboratories [161]. The standardised disc diffusion method was introduced by

Bauer and Kirby in 1956 and to date is widely used. Mueller–Hinton agar plates (90 mm diameter) are inoculated with a standardized inoculum of the test micro-organism (corresponding to 0.5 McFarland turbidity standard). Up to 12 commercially prepared paper discs (approximately 6 mm in diameter) with desired concentrations of the tested agent are placed on the inoculated agar surface. Agar plates are incubated under suitable conditions, typically for 16–24 h at 35–37°C [162, 163]. The diameter of the growth inhibition zones around each antibiotic disc is then measured in millimetres. Results are reported qualitatively as susceptible, intermediate, or resistant using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards, NCCLS) [164] or other international societies such as the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST) [163]. This method has the potential for routine testing since it is widely accepted, simple to conduct, cost effective and ease of interpreting results categorically [165]. Disadvantages include no automation of the test, insufficient data available for many bacteria and poor performance when analysing slow growing and fastidious bacteria [166]. Physiochemical factors such as evaporation, solubility, pH, temperature, and requirement of nutrient media restrict its suitability for accurate diagnostics [167].

2.3.1.3.2 Broth microdilution method

Minimum inhibitory concentration (MIC) values achieved via the broth dilution method serves to provide a quantitative result (in μg/mL) along with a categorical interpretation (susceptible, intermediate, or resistant) that can guide antimicrobial therapy more precisely. The broth dilution method (macrodilution) was one of the earliest antimicrobial susceptibility testing methods [168], however due to the miniaturisation the broth microdilution method is more practical and the standard method used in most reference laboratories in the United States and abroad. A small sterile disposable polystyrene microtitration plate usually containing 96 wells is used. Each well containing a volume between 0.1 and 0.2 ml therefore allowing

testing of approximately 12 antibiotics over a range of 8 twofold dilutions on one plate [169]. The tray is filled with serial two-fold dilutions of the antimicrobial agents (expressed as μg ml^{-1}), and then the plate is inoculated with a diluted microbial suspension standardized to an optical density of 0.5 McFarland scale to achieve a final microbial concentration of 1–5 x 10^5 CFU per ml (colony forming unit per ml). [170, 171]. The wells are mixed and the plate is incubated under suitable conditions. The MIC, the lowest concentration of antibiotic preventing visible growth is determined visually or by automated viewing device, a photometric analysis device at 620 nm [172].

The small amount of sample required, low costs and reproducibility are some of the advantages of this technique, however, difficulty detecting contamination and inoculum viability are notable disadvantages [173].

Conventional methods as discussed previously require a defined quantity of inoculum of an isolated bacterial culture, the evaluation of the growth of bacteria visually or automatically as well as being incubated for 16-20h, according to breakpoints calibrated by CLSI and EUCAST [174]. These limitations are resolved by the utilisation of commercial semi-automated or automated instruments.

2.3.1.3.3 Commercial semi-automated and automated instruments

Commercial semi-automated or automated instruments such as the MicroScan WalkAway System (Beckman Coulter, Franklin Lakes, NJ), the novel and more modern VITEK version—VITEK 2 (bioMérieux, Marcy-l'Étoile, France), Sensititre AutoReader and Sensititre ARIS2X (Trek Diagnostic Systems, Cleveland, OH) and the newest system BD Phoenix 100 (BD Diagnostic Systems, Sparks, MD) [175, 176] are some of the instruments used for antimicrobial sensitivity testing that reduce incubation and hands-on time. These devices utilize optical systems for measuring subtle changes, determine bacterial growth and antimicrobial sensitivity [177] and can produce results ranging from 6-12h, a shorter time period than conventional manual methods [178]. Whilst these instruments have their benefits, the lack of reproducibility, sensitivity, and

reliability compared with the existing traditional methods and the high cost of instruments and consumables, are all significant issues that restrict these systems from frequent analysis [179].

2.3.2 Molecular typing and characterisation methods

Over the years, various rapid molecular based subtyping methods have been developed to overcome the limitations of traditional serotyping, enabling faster and more accurate subtyping of *Salmonella*. These include nucleotide banding pattern-based techniques such Pulse-Field Gel Electrophoresis (PFGE) where restriction enzymes recognize specific restriction sites along the genomic DNA and fragment the DNA to sizes normally ranging from 20 to 800 kb (up to 2,000 kb) [180]. These large fragments are separated in a flat agarose gel by constantly changing the direction of the electric current (pulsed field), causing the DNA to separate by size, generating a specific "fingerprint pattern" for a given isolate [181]. Several limitations such as the technique being time consuming, lack of automation potential and lack of genetic information such virulence potential, antimicrobial resistance genes and phylogenetic relatedness due to the fragments being separated by size and not sequence hamper its efficiency as an epidemiological tool [182-184].

The second major genotyping tool after PFGE used in the PulseNet network, multiple locus variable number of tandem repeats analysis (MLVA) was primarily used in public health surveillance and outbreak investigation of *Salmonella* in Europe [183, 185]. This method is used as a complementary to conventional serotyping or PFGE for routine surveillance since PFGE is unable to differentiate genetically homogenous serotypes [183]. MLVA assays simultaneously measure the length of variable number of tandem repeat loci by PCR amplification and electrophoresis whereby this information is used to create a genotype the distinguish between isolates of the same serovar. MLVA offers a cheaper, faster, simpler technique than other molecular methods, however, MLVA protocols are

serovar specific thereby requiring prior serotyping before selection of a specific MLVA subtyping scheme [186, 187].

Whilst DNA fragment analysis-based methods such as PFGE and MLVA and other methods have been useful in the past epidemiological investigations, these methods are being replaced with multi-locus sequence typing (MLST) and whole-genome sequencing (WGS).

Multi-locus sequence typing (MLST) is another molecular typing method used to differentiate isolates based on nucleotide sequence differences in sets of gene fragments, each 450–500 bp in length [188]. For *Salmonella* typing, seven housekeeping genes aroC (chorismite synthase), dnaN (DNA polymerase III beta subunit), hemD (uroporphyrinogen III cosynthase), hisD (histidinol dehydrogenase), thrA (aspartokinase+homoserine dehydrogenase), sucA (alpha ketoglutarate dehydrogenase) and purE (phosphoribosylaminoimidazole carboxylase) is one of the most popular genotyping methods [188]. Housekeeping genes are involved in primary metabolism of the organism and are present in all bacteria within a species [189]. MLST entails PCR amplification and sequencing of these seven loci whereby each locus is assigned an allele based on finding a match in a central database and each strain is assigned an allelic profile of seven numbers designated as sequence type (ST) [190]. MLST is the preferred method of *Salmonella* serotyping of Public Health England since 2015 and is mainly used in research studies to assess population genetics and evolution of *Salmonella* [191]. MLST data is maintained on an online publicly available databases called Enterobase or the global MLST database (https://pubmlst.org/organisms/salmonella-spp). MLST data can be used to subtype bacteria as well as study the structure of the population and evolution of bacterial pathogens [188]. Results from MLST is obtained faster than PFGE and results are highly reproducible and exchangeable due to publicly available databases and query system [154, 192]. However, the high cost of reagents, laborious and time-consuming method with the potential of insufficient discriminatory power for routine use make this a challenge [193].

2.3.2.1 Whole genome sequencing (WGS)

WGS, unlike Sanger sequencing, allows for the sequencing of the entire genome in a cost-effective manner as opposed to a single DNA fragment at a time. As such, WGS is a valuable tool capable of identifying and characterising bacteria based on subtle differences between genomes with numerous applications such as outbreak investigations and source attribution [194, 195] and microbiology and diagnostics identification, typing, resistance, detection and virulence gene detection [196, 197]. With the decrease in cost over the years this approach is becoming more accessible [198, 199].

WGS platforms can be categorised as long-read or short-read sequencers, with the latter being more popular. Output from short-read platforms result in genome assemblies that are nearly complete since some gaps are expected because of the shorter length of the sequencing reads used, typically between 50 and 400 base pairs [200]. Together with low error rates and adequate coverage, the short-read platform, Illumina Miseq is well suited to pathogen sequencing. The long-read length of Pacific Biosciences (PacBio) suite, 20 kilobases, offers an important and distinct advantage of single-molecule real-time sequencing (SMRT) sequencing, in that the reads are able to produce a high-quality genomic sequence that typically captures all of the genetic material seen (e.g. closed genome). However, use of long read runs can sacrifice length for accuracy with error rates as high as 15%. As such, use of long-reads is useful to regulatory authorities where there is a need to have a genomic sequence that is as complete as possible.

The most widely used WGS sequencing platform is the Illumina system (MiSeq) which produces DNA sequence reads of 50-300 bp in length using sequencing by synthesis (SBS) [201]. Bioinformatic analyses comprise guiding data files generated by sequencers through a series of data transformations, called a pipeline or workflow. Initially, raw sequence data are subjected to quality control and

quality assurance measures to ensure consistent, high quality comparable genomic data. Software such as FASTQC [202] and SAMStat [203] enable analyses such as per base sequence quality, nucleotide composition, read-length distribution, base quality distribution that can be used to estimate the sequence quality and to identify possible errors before proceeding with further analyses. Following quality checks, reads undergo a pre-processing step in which they are "trimmed" to remove low quality data including adapter sequences inserted during library preparation. Software such as TRIMMOMATIC [204], designed to handle pair-end data, is the program commonly used for bacteria. After trimming, species information can be determined using several software packages such as Kaiju [205], where each read is assigned to a taxon in the NCBI taxonomy by comparing it to a reference database containing microbial protein sequences.

In silico determination of serotypes is based on two main approaches: indirect determination using genetic markers associated with serotypes and direct determination using genes responsible for the expression of the somatic O (*rfb* gene cluster) and flagellar H (*fljB* and *fliC*) antigens. WGS *in silico* direct serotype determination has become the most used approach with at least two *Salmonella* serotype pipelines available and routinely used, namely, SeqSero [206] and SISTR [207].

SeqSero can potentially identify 2,389 of the 2,577 serotypes described in the White-Kauffmann-Le minor scheme using its database of 473 alleles representing 56 *fliC* antigenic types and 190 alleles representing 18 *fljB* antigenic types in a combined H-antigen database. SeqSero database has an accuracy of 91.5-92.6% for serotype prediction [206]. SISTR includes the *Salmonella* Genoserotyping Array (SGSA) tool among other resources. SGSA depends on the allelic differences found within the *rfb* gene cluster for determination of 18 of the 46 somatic O-antigens, and *flj*B and *fli*C for determination of 41 flagellar H antigens, targeting 2,190 *Salmonella* serotypes. If SGSA is unable to determine the serotype, SISTR has the option of using the core genome MLST (cgMLST) scheme to infer serotype based

on phylogenetic context. SISTR has an accuracy close to 95% in serotype prediction [208, 209].

Another possibility offered with WGS is predicting the antimicrobial resistance and virulence status of strains of bacteria using various tools and databases. These databases can be accessed using tools such as CARD (The Comprehensive Antibiotic Resistance Database) [210], VFDB (Virulence Factor Database) [211], CGE (Center for Genomic Epidemiology) ResFinder [212], Resistance Gene Identifier (RGI) [210] or the BLAST (Basic Local Alignment Search Tool) algorithm.

Unlike MLST, the advantage of WGS in microbial typing is the possibility to assess variation in hundreds or thousands of targets in the genome simultaneously, thus providing high through-put and high-resolution genotyping. Genomic information (phylogenetic inference) can be extracted from reads through different allele-calling strategies such as, *de novo* assembly-based analyses (eg. gene-by-gene approach), reference-based mapping (e.g. SNP calling) and de novo allele calling (e.g. k-mer) [213, 214].

De novo assembly requires short DNA reads to be aligned into longer sequences called contigs before being subjected to data analysis using various pipelines. Depending on the platform used to produce the reads and the result, draft or complete assembly, several software packages are available for *de novo* genome assembly. Velvet [215] and SPAdes [216] are two assemblers used for the *de novo* assembly of Illumina reads. *De novo* assembled reads can subsequently be analysed using two methodologies: core genome alignment and gene-by-gene analysis, using its respective software. Reference-based mapping (Single Nucleotide Polymorphism, SNP) compares processed reads to a reference or high-quality finished complete genome, as a means for the discovery of SNPs that can be used to infer phylogenetic relationships [214]. The GenomeTrakr network (provided by the FDA's Center for Food Safety and Applied Nutrition, CFSAN) is the first distributed network of laboratories to collect, sequence and share genomes of over 150,000 foodborne pathogens collected from various sources. The database, housed

at the National Center for Biotechnology Information (NCBI), uses NCBI's pathogen detection pipeline [217] to produce daily phylogenetic trees that can be accessed by researchers and public health officials for real time comparison and analysis.

Complex data analysis requires expertise in bioinformatics to deploy and run software programmes [218, 219].

2.4 Prevalence, serotypes, and risk factors for *Salmonella* spp.

2.4.1 Production of broilers

2.4.1.1 Fertile hatching eggs

Vertical transmission also called the trans-ovarian route occurs as a result of *Salmonella* infection of the reproductive organs (ovaries and oviduct) whereby the egg yolk membrane or albumen surrounding the egg are directly contaminated [220]. Whilst natural vertical transmission is common, several studies showed that *Salmonella* contaminated eggs can be produced by artificially inoculating the hen [221-223]. Vertical transmission is commonly seen with host restricted *Salmonella* serovars such as *S.* Gallinarum and *S.* Pullorum but has also been demonstrated in un-restricted *Salmonella* serovars such as *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg [224]. Transmission via this route is directly related to the affinity of certain serovars for the reproductive tract of the hens [225].

Horizontal transmission also called the trans-shell route occurs when *Salmonella* penetrates through the eggshell during or following oviposition (expulsion of egg through oviduct to environment) [226]. Various *Salmonella* serovars can be found in the egg contents following penetration through the eggshell. Trans-shell transmission is more likely to occur in the first few minutes after oviposition when the egg's cuticle is immature and offers less protection against penetration of bacteria. Eggshell quality [227], pH [228] and number of pores on an eggshell [229] are some factors that affect the penetration ability of bacteria. Horizontal

transmission from infected pen mates and the subsequent laying of *Salmonella*-positive eggs (vertical transmission) have been reported [230]. On sampling naturally contaminated hens, the incidence of *S.* Enteritidis in eggs is low, with a reported 0.065% (2/16,000) of eggs tested being positive [231]. Other studies [232, 233] also reported very low isolation rates of naturally contaminated *S.* Enteritidis flocks in egg contents, however, *S.* Enteritidis, *S.* Typhimurium, and *S.* Hadar were isolated from eggshells.

2.4.1.2 Hatcheries

Broiler hatcheries are known to be reservoirs of *Salmonella* and source of dissemination across the farm to fork continuum [234, 235]. The prevalence of *Salmonella* is very low in fertile hatching eggs, however a study done by Cox et al. [234] isolated *Salmonella* in 75, 91 and 67% of samples taken at three hatcheries in the U.S.A., respectively.

Contaminated fertile eggs hatch at a rate of 86%, despite high *Salmonella* contamination, therefore chicks in contaminated eggs are likely to hatch and contaminate other chicks in the same hatcher cabinet and environment [236]. At the hatchery, chicks are capable of self-infecting from pipping *Salmonella* positive exterior or in shell membranes [237]. It is reported that more salmonellae are spread and chicks are contaminated in the hatcher after pipping than through the eggshells or in the incubator [236, 238].

2.4.1.3 Farms

Zomora-Sanabria et al. [239] addressed the importance of determining the source of *Salmonella* infection at the farm level since there has been a direct association with human food-borne salmonellosis and the development of the food industry. The authors highlighted factors such as hygiene, biosecurity procedures, animal feed surveillance, cleaning and disinfection programs amongst others that should form part of food safety programs at the farm level. Additionally, the authors

suggested that since *Salmonella* spp. easily survive in the environment it can easily find other sources of contamination such as pests (rodents, flies, mites, cockroaches), wild birds, water (biofilm), humans, manure, transportation coops, tractors or vehicles and farm environment.

The presence of *Salmonella* on broiler farms depends not only on-farm management practices but also on the day-old chicks and feed being *Salmonella*-free. On-farm surveillance programs have been proven to effectively control and eradicate foodborne pathogens [240] and to significantly reduce the pressure on the food safety management systems implemented at subsequent steps in the poultry chain such as at processing plants [241].

Salmonella prevalence of 1.02%, 2.5% and 16.05% in broiler flocks in Spain [242], Malaysia [243] and Korea [244], respectively have been reported. Additionally, the reported broiler farm prevalence was 3.4%, 9.2%, 2.7% and 0.1% in France, Italy, Germany and Sweden, respectively [245].

2.4.2 Poultry slaughterhouses

It has been reported that the slaughtering of chicken at commercial slaughterhouses contributes significantly to the contamination of dressed chicken carcasses with *Salmonella* [242, 246]. Unhygienic carcass handling, soiled slaughter equipment [247, 248], contaminated water (scalding and immersion chiller water) and waste generated from evisceration and de-feathering processes have been implicated as major sources of *Salmonella* contamination during broiler processing [249-251].

2.4.3 Retail outlets: supermarkets, 'wet markets' 'pluck shops'

In developing countries, the wet markets or cottage poultry processors serve as important sources of chickens consumed by members of the population. Prevalence rate for *Salmonella* spp. from raw chickens have varied from countries and outlets as documented in Korea, 42.7% [252], Vietnam, 45.9% [253], China, 52.2%, [254],

India, 65.0% [255], Thailand, 72.0% [256], Malaysia, 100.0% [257]. Poor sanitary conditions have been attributed to practices at these outlets where hardly any regulatory measures are in place which may have contributed to a high frequency of *Salmonella*-contaminated carcasses reported [254-257].

Commercial outlets such as supermarkets also serve as sources, by selling chickens previously contaminated with *Salmonella*. Reported prevalence of *Salmonella* spp. have ranged from 4.0% to 20.0% in developed countries [258-262]. Prevalence of *Salmonella* in chickens at supermarkets have been reported to range from 43.0% to 62.5% in developing countries [253, 263-265].

2.5 Farm to fork approach to study salmonellosis and *Salmonella* spp.

The farm to fork approach is an important perspective when studying foodborne pathogens because each level in the continuum plays a role in the eventual microbial status of the final product consumed by humans.

The ‘starting point’ of the broiler supply chain is the primary breeding companies which supply grandparent stock from which the parent stock is grown at pullet and breeder farms [266]. Primary production operations such as hatcheries and broiler farms have been reported as an origin of *Salmonella* contamination [267]. *Salmonella* is commonly detected on poultry carcasses in processing plants and has the potential to lead to cross-contamination, if proper measures are not taken [268, 269].

The isolation of *Salmonella* in broiler meat after leaving the processing plant may vary with the different distribution channels due to fluctuations in storage temperatures, worker sanitation, rate of worker turn-over and lack of training of workers which are all common issues faced by local vendors when compared to larger retail outlets [270].

The cross-contamination and recontamination of poultry from contaminated kitchen surfaces and fresh ingredients due to a lack of personal hygiene, play more

important roles in the spread of *Salmonella* than undercooked poultry meat [271]. Undercooking of poultry meat was another contributor in causing *Salmonella* infections with suboptimal cooking temperatures and cooking time, increasing the survival of *S.* Typhimurium by 34% during further processing and in commercial kitchens [272]. Cooking of poultry to an internal temperature of 165 °F or 74 °C is required to kill *Salmonella* and ensure consumer safety as outlined in the USDA guidelines [273].

2.6 Prevention and control of salmonellosis

The OIE recommends *Salmonella* surveillance programmes in different poultry flocks (flock of origin at trade of live poultry, breeding flock of origin and the hatchery at trade of day-old birds and in the breeding flock of origin at trade of hatching eggs) where the absence of *S.* Enteritidis and *S.* Typhimurium is required. Similar conditions are required for *S.* Pullorum and *S.* Gallinarum for international trade of poultry and hatching eggs [274]. Monitoring of poultry feed, use of competitive exclusion in day-old chicks, vaccination as part of control programme, culling of infected breeder flocks and use of organic acids are some measures mentioned in the OIE report that pertain to poultry production [274].

At the farm level effective biosecurity and hygiene practices such as disinfection practices, pest controls, animal, person and vehicle movements, feed and water sanitation should complement other control measures. The following have shown to not only improve animal health but can also protect birds from *Salmonella* infections. Acidic compounds or organic acids have been shown to directly reduce colonisation and shedding of *Salmonella* [275]. Prebiotics (non-digestible feed ingredients) have been shown to stimulate the growth of one or a limited number of resident beneficial bacterial species in chickens [276]. Competitive exclusion products (gastrointestinal contents of adult poultry) given to chicks to establish an adult microflora that is resistant to *Salmonella* colonisation [277]. Probiotics (living

microbial feed ingredients) have shown anti-inflammatory effects and the potential to modify the microbiological ecosystem within the poultry gut to control *Salmonella* [278]. Phytobiotics have shown the ability to modulate the chicken microbiome similar to antimicrobials [279], prevent the biofilm production [280], and prevent gastrointestinal diseases in broilers [281], without causing selection pressure to antimicrobial resistance in bacteria [282]. Potential feed additives, such as flavophospholipol, could have the beneficial effect of reducing both *Salmonella* shedding and the number of animals carrying *Salmonella* at slaughter [283]. Bacteriophages (live viruses that infect and kill bacteria) have shown promise in reducing colonisation in experimental trials [27, 284, 285], but field trial studies are limited [275].

As for the processing component of broiler production, several interventions exist along the processing line that aim to reduce cross contamination from carcass to equipment as well as to reduce bacteria on the carcass by using sprays or dips. A study conducted by Cason et al. [286] detected *Salmonella* in 60% (42/70) feather samples collected from broilers pre-slaughter in addition to being isolated from the crop, head and feet, colon and caeca and defeathered carcass. Pre-scald bird brushes are used to reduce the organic load in eventual processing steps (scalding tank, carcass sprays and immersion chiller tank) since organic matter reduces the disinfectant capacity of chlorine, a commonly used, economically friendly disinfectant used in the poultry industry. *Salmonella* reduction intervention at the scalder level involves the use of counter current scalding tanks [287, 288] and use of organic acids [289, 290]. These interventions have been shown to significantly impact bacterial load on carcasses during scalding. Use of carcass and machine sprays throughout the processing line, with the addition of chemical agents or chlorine has also been used. Interventions at the immersion chiller level are deemed to be the most significant step for controlling *Salmonella* on broiler carcasses [291]. A counter current flow, adequate freshwater input with a chlorine concentration of 50 ppm, water temperature below 40 °F/ 4 °C, low organic matter content, control

of the chlorine level to ensure a free available chlorine concentration of 1 to 5 ppm with an adjusted pH ranging from 5 to 6.5 (achieved by using citric acid, sodium acid sulfate or carbon dioxide) as well as adequate contact time are all parameters essential to reducing *Salmonella* in immersion chillers [291]. Air chilling as opposed to immersion chilling, has the potential to reduce cross contamination experienced with an improperly controlled immersion chiller. However, with air chilling, it is not possible to use chemicals to reduce *Salmonella*, thus being counterproductive as an intervention [292-294]. Post-chill dips and sprays, with the addition of chemical agents are also useful in reducing bacteria on the carcass [295].

A novel approach to elimination of *Salmonella* showed that use of bacteriophages could be a practical method of *Salmonella* reduction on chicken carcasses [296, 297] in addition to live birds alluded to earlier in this section.

2.7 Prevalence of *Salmonella* spp. isolated from poultry in the Caribbean region

2.7.1 Trinidad and Tobago

Whilst there were some *Salmonella* studies done several years ago on broiler chickens [29, 298, 299], conducted at specific sampling stages along the farm to fork continuum, some of these studies were void of serotyping, antimicrobial sensitivity analyses and molecular characterisation, unlike more recent studies [263-266]. Most recently, a study conducted by Kumar et al. [30] reported a prevalence of 6.1% (91/1503) for *Salmonella* spp. In caecal of chickens and ducks slaughtered at ‘pluck shops’ in Trinidad and Tobago. Apart from the study done in 1994 on *Salmonella* isolation in contract broiler farms in Trinidad and Tobago [29], there is a dearth of information, not only from broiler farms but the entire farm to fork continuum, which this study intends to explore.

2.7.2 Barbados

There are two published reports on the prevalence and exposure factors for *Salmonella* on commercial layer farms in Barbados [304, 305], but there are no known published papers on the prevalence of *Salmonella* on broiler farms. Notably, Hull-Jackson et al. [306] reported on foodborne disease outbreaks involving *Salmonella* spp., over a 12-year period, as well as the bacteriological quality and prevalence of *Salmonella* in ready-to- eat foods in the country. These studies provide valuable information on the "fork" aspect and can be useful in legislative framework in Barbados to limit the occurrence of foodborne diseases.

2.7.3 Jamaica

Vailliant et al. [307] isolated *S.* Typhimurium, *S.* Montevideo and *S.* Yeerongpilly from egg yolks of chickens, quails, geese and pigeons in Jamaica. This study is of public health significance since eggs of these birds are consumed in large amounts in Jamaica. The microbial assessment of processed poultry in Jamaica was determined in 1992 by Hamilton and Ahmad [308]. Most recently, a study conducted by Curtello et al. [309] reported an overall prevalence of *Salmonella* to be 1% (79/6693) on poultry farms where a higher prevalence was observed in organic poultry farms. In that study, *S.* Augustenborg and *S.* Kentucky were identified for the first time in Jamaica.

2.7.4 Other countries

Adesiyun et al. [310] surveyed chicken layer farms for *Salmonella* contamination in St. Lucia (15 farms) and Grenada (10 farms), where the overall prevalence of *Salmonella* spp. was 26.7% and 40.0%, respectively. In that study, the only risk factor reported to be significantly associated with the frequency of isolation of *Salmonella* was the farm size ($p= 0.031$).

CHAPTER 3: OCCURRENCE, RISK FACTORS, SEROTYPES AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* STRAINS ISOLATED FROM IMPORTED FERTILE HATCHING EGGS, HATCHERIES, AND BROILER FARMS IN TRINIDAD AND TOBAGO

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3.1 Abstract

This cross-sectional study was conducted to determine the occurrence, risk factors, and characteristics of *Salmonella* isolates recovered from imported fertile broiler hatching eggs, hatcheries, and broiler farms in Trinidad and Tobago. Standard methods were used to isolate and characterise *Salmonella* isolates from two broiler hatcheries and 27 broiler farms in the country. The frequency of isolation of *Salmonella* was 0.0% for imported fertile hatching eggs (0 of 45 pools of 10 eggs each, i.e., 450 eggs), 7.6% for hatcheries (12 of 158 samples), and 2.8% for broiler farms (24 of 866 samples) ($P = 0.006$). Stillborn chicks at hatcheries had the highest prevalence of *Salmonella* (7 of 28 samples; 28.0%), whereas on broiler farms the cloacal swabs had the highest prevalence of *Salmonella* (15 of 675 samples; 2.2%). None of the 15-farm management and production practices investigated was significantly associated ($P > 0.05$) with the isolation of *Salmonella*. The predominant *Salmonella* serotypes were Kentucky (83.3%) and Infantis (62.5%) among hatchery and farm isolates, respectively. The disk diffusion method revealed frequencies of antimicrobial resistance (i.e., resistance to one or more agents) of 44.0% (11 of 25 isolates) and 87.5% (35 of 40 isolates) at hatcheries and broiler farms, respectively ($P = 0.0002$). Antimicrobial resistance among hatchery isolates was highest (28.0%) to doxycycline and kanamycin and was very high (.65%) among farm isolates to sulfamethoxazole-trimethoprim, gentamicin, ceftriaxone, kanamycin, and doxycycline. Multidrug resistance (MDR; i.e., resistance to antimicrobial agents from three or more classes) was exhibited by 4.0 and 85.7% of *Salmonella* isolates recovered from several environmental and animal sources at the hatcheries and farms, respectively ($P < 0.0001$). The high level of antimicrobial resistance and the presence of MDR among *Salmonella* isolates from broiler farms highlight the therapeutic implications and the potential for MDR strains to enter the food chain.

3.2 Introduction

Salmonella is one of the foremost pathogens in the poultry industry [311]. It is also a major cause of bacterial foodborne gastroenteritis in humans. Poultry has been attributed as an important source of *Salmonella* infections in consumers of improperly cooked contaminated chicken and chicken products [312]. Salmonellosis in broiler flocks poses severe economic loss due to a reduction in chicken production as morbidity and mortality can be extremely high and vary widely [313].

Fertile imported hatching eggs can be a source of *Salmonella* introduction and transmission through the poultry production chain. Vertical (trans-ovarial) transmission occurs when *Salmonella*-infected breeder hens with contaminated reproductive tracts lay fertile broiler eggs that harbour *Salmonella* [236]. Broiler eggs may be contaminated at the breeder farm [314] and hatching chicks may be infected with *Salmonella* by vertical transmission via the infected hen or horizontal transmission when *Salmonella*-positive eggs contaminate *Salmonella*-free eggs at the breeder farm. *Salmonella* associated with poultry has been shown to persist in the hatchery environment, contaminating subsequent batches of eggs and birds [315]. A study conducted by Adesiyun et al. [316] reported that 91% of the 27 Caribbean countries (including Trinidad and Tobago) imported egg and egg products from foreign sources. The increasing international trade poses an increased risk of transfer of microbes from one country to another [317].

Hatcheries are considered major sources of *Salmonella* infection in young chicks since *Salmonella* negative chicks can be exposed to *Salmonella*-positive chicks in a confined environment [318]. It has been documented that broiler chicks are most susceptible to pathogen colonisation during the first few days of hatching since their immune system and intestinal flora are immature [319] following vertical transmission from infected breeders and horizontal transmission during handling and transportation [320]. Several risk factors have been associated with contamination of hatcheries by *Salmonella* [321].

The presence of *Salmonella* on broiler farms depends not only on-farm management practices but also on the day-old chicks and feed being *Salmonella*-free. On-farm surveillance programs have been proven to effectively control and eradicate foodborne pathogens [240] and to significantly reduce the pressure on the food safety management systems implemented at subsequent steps in the poultry chain such as at processing plants [241]. A vertical integration model established for producing chicken meat has been recommended to act as one of the main tools to reduce the presence of *Salmonella* in the final product [322]. This is because the control measures and interventions established at each integration sector contribute to the reduction in *Salmonella*. Water quality (microbial, physical, and chemical), presence of pests (rodents, wild birds) and insects/larvae, inappropriate disposal of dead *Salmonella*-positive birds, feed/drinker management, cleanliness of feathers, and litter condition have all been associated with *Salmonella* isolation at broiler farms [240].

The broiler industry in Trinidad and Tobago is operated primarily by vertically integrated companies that have contractual agreements with farmers who are paid to produce grow-out chicks to processing age. Operating costs, such as repairs, and maintenance of the broiler houses are the farmers' responsibility. However, the integrated companies take responsibility for the chicks, the supply and the distribution of feed, the provision of veterinary and management services, and the transportation of birds to processing plants for processing, and subsequent marketing. Additionally, the integrated broiler companies are responsible for hatching of imported fertile hatching eggs at their hatcheries. Therefore, there is a holistic control of the introduction, propagation and dissemination of *Salmonella* from the hatchery to the country's retail outlets. The management and veterinary services provided by the integrated poultry companies to the contracted farmers and hatcheries are independent of government agencies, and their intervention takes place only when notifiable diseases are suspected. All fertile hatching broiler eggs are imported into the country. Broiler production in Trinidad and Tobago is estimated to be 42 million chickens per year where approximately 1 million chickens are consumed weekly, of which 80% is produced locally [323]. Broiler

production is solely for the local market as chickens produced locally are not exported.

The objectives of this cross-sectional study were to determine the occurrence of *Salmonella* in imported fertile hatching broiler eggs, hatcheries and broiler farms, to identify the risk factors/management practices associated with *Salmonella* contamination, and to determine the antimicrobial resistance profiles of the isolates to commonly used agents in the poultry industry in Trinidad and Tobago.

3.3 Materials and methods

3.3.1 Sampling site and sources of samples

The study was conducted in Trinidad and Tobago, the twin-island Caribbean country located in the southern Caribbean. The poultry industry in Trinidad is vertically integrated. Each company imports fertile hatching eggs for their respective hatchery. The companies also provide day-old chicks to their contracted farmers for regular supply of broilers for slaughter at their processing plants. Imported fertile hatching eggs imported weekly into the country originate from two foreign companies based outside the Caribbean region. The breeds of chickens imported belong to Cobb x Cobb, Ross x Ross or Hubbard x Cobb. There are four commercial broiler hatcheries and approximately 400 contracted broiler farms in Trinidad, where three integrated poultry companies own most farms. In this study, the hatcheries and farms owned and operated by the two largest poultry integrated companies, representing 11.2% (27/242) of the farms owned by both integrators, were sampled during the following periods: Farms: February-July 2019; Hatcheries: August-September 2019; Imported fertile hatching eggs: July-August 2019. The 27 farms sampled shown in Figure 3.1 were dispersed across the country.

3.3.2 Determination of sample size for the study

The study estimated the sample size for an infinite population using, n_o=$Z_u^2 P_{ex}$ (1-p_{ex})/d^2 [324], where:

n_o= Estimated sample size; Z_u= Degree of confidence= 1.96, P_{ex} = Expected prevalence= 50%; d= Desired absolute precision

For the imported fertile hatching eggs, since pooled eggs were to be processed, a convenience sampling approach was used. The study design was to collect a crate consisting of 30 eggs during each of 15 sampling visits to the airport, the port of entry. Thereafter, from each crate of 30 eggs, 10 eggs were pooled to constitute a composite sample for processing.

For the hatcheries, n_o = Estimated sample size; Z_u= Degree of confidence= 1.96, P_{ex} = Expected prevalence= 50%; d= Desired absolute precision = 8%, n_o = [1.96^2 x 0.5 (1 - 0.5)]/0.08^2 = 150.

For the study, a total of 158 samples were collected from both hatcheries.

For the broiler farms, n_o = Estimated sample size; Z_u= Degree of confidence= 1.96, P_{ex} = Expected prevalence= 50%; d= Desired absolute precision = 3.5%, n_o = [1.96^2 x 0.5 (1 - 0.5)]/0.035^2 = 784.

For the study, a total of 866 samples were collected from the 27 broiler farms.

Fertile hatching eggs imported by two (50%) of the integrated poultry companies in Trinidad and Tobago destined for their respective hatcheries were collected on 15 sampling visits on the day they arrived at the airport. During each visit, one crate (30 eggs) was collected from the shipment using sterile gloves, transported in a cooler with ice packs to the laboratory and processed within 3 h of collection. Ten eggs were pooled to constitute a composite sample from which eggshell and egg content samples were separately processed as previously described [310, 325].

Overall, 45 eggshell and 45 egg content samples, each consisting of 10 eggs, were processed.

Samples were collected from the destination broiler hatcheries (A and B) during 5 sampling visits (A-3 and B-2 visits). The samples collected were representative of each step of the hatching process. Table 3.1 shows the types and number of samples collected at both hatcheries. All samples were collected using methods previously reported [310], with slight modifications, where a pool of 10 eggs were used instead of the reported 6 eggs.

Twenty-seven broiler farms (Figure 3.1) owned by the two integrated poultry companies sampled at the hatchery level were sampled to determine the presence of *Salmonella* in selected individual broilers and environment (drag swab of litter, feed, water and boot swabs). Farms were selected proportionally based on their geographical location, the total number of contract farms owned by the respective company, the throughputs and the number of pens. Of the 27 farms sampled, four and 23 farms were supplied by Hatchery A and B, respectively. Table 3.2 shows the type and number of samples collected from the broiler farms. Sample collection was conducted using methods previously reported [310].

Table 3.1. Type and number of samples collected at two broiler hatcheries

	No. of samples collected from hatcheries:		
Type of sample collected	**A**	**B**	**Total**
Swabs of eggs in incubator[a]	7	5	12
Swabs of eggs in hatcher[a]	8	4	12
Swabs of the interior of incubator[b]	7	5	12
Swabs of the interior of hatcher[b]	8	4	12

Stillborn chicks	15	10	25
Broken eggshells[c]	15	11	26
Hatcher fluff[c]	15	10	25
Incubator air unit	7	4	11
Hatcher exhaust fans	2	4	6
Meconium samples[c]	0	10	10
Egg transfer machine	1	0	1
Chick conveyor belt	2	0	2
Drain	2	2	4
Total	89	69	158

[a] One sample comprised pooling swabs of 10 eggs in 1 crate from adjacent eggs at each of the four corners (2 eggs/corner) and the centre (2 eggs) of each crate/tray

[b] One sample comprised four 12.7 x 12.7 cm surface area swabs on each side of the incubator/ hatcher, i.e., a total of 100 cm^2 area was sampled

[c] Each sample collected as a composite sample representative of each hatcher

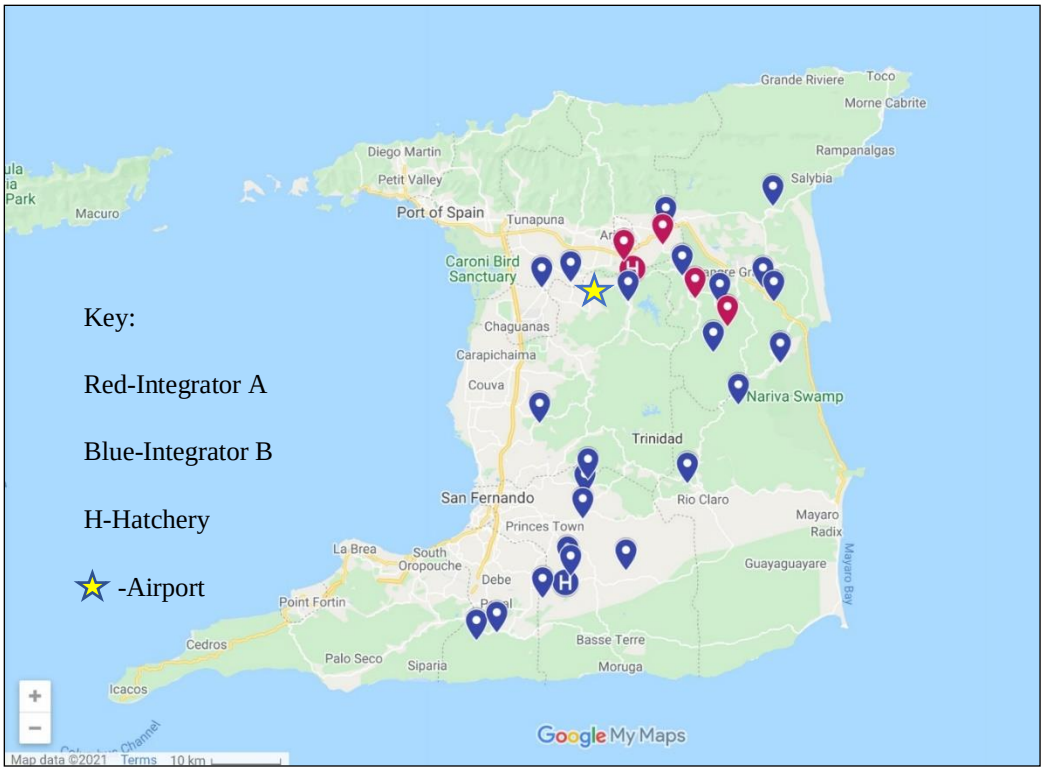

Figure 3.1. Map of Trinidad and Tobago showing farm and hatchery locations sampled

Table 3.2. Type and number of samples collected at 27 broiler farms in Trinidad and Tobago

Type of sample	**No. of samples collected per farm[a]**	**Total No. of samples collected**
Cloacal swabs	25	675[b]
Feed[c]	1-2[d]	53
Water	2	54
Boot swabs	1-3[d]	27
Drag swabs	1-3[d]	57
Total		866

[a]Of a total of 27 broiler farms sampled
[b] Collected from a total of 675 individual broilers

[c] Two feed samples were collected from all except one farm from feeding containers and newly opened bags but only was sample was collected from one farm due to limited available containers and bags
[d] Samples collected varied due to the number of active pens and frequency of use of boots on different farms

3.3.3 Administration of questionnaires at hatcheries and broiler farms

Questionnaires were administered at each hatchery and broiler farm sampled to obtain information on the demographic data, operational information, and risk factors for *Salmonella* contamination. Some of the questions included the number of workers employed, the types of pests encountered, the average mortality rates at broiler farms, the sources of hatching eggs, the types of sanitizers used for cleaning, and the vaccines administered at hatcheries. The questionnaires administered are displayed as supplemental materials (Appendix 3.1: Questionnaire administered at the Hatcheries; Appendix 3.4: Questionnaire administered at the broiler farms).

3.3.4 Processing of samples collected

Published standard procedures were used to process the samples collected at the airport for imported fertile hatching eggs, the hatcheries and the broiler farms. To process hatching eggs, the procedures earlier described for eggs [325, 326] were used. The only modification made was the pooling of 10 eggs to constitute a composite sample. Briefly, for the 10 pooled eggs, the egg contents and eggshells were processed separately to detect the presence of *Salmonella.* Overall, 45 pools each of eggshells (450 eggs) and egg contents (450 eggs) were processed in the study. To process samples (eggs, chicks, and environmental) from the hatcheries, the procedures earlier described were used [310]. To process broiler farm samples (water, feeds, cloacal swabs, boot swabs, drag swabs of litter), the procedure used to process layer farms in three Caribbean countries was used [310].

3.3.5 Isolation and identification of Salmonella

For the isolation of *Salmonella*, the procedures earlier described were used [300, 310] with slight modifications. All samples collected from the three sources (airport, hatcheries and farms) were initially pre-enriched (1:10) in buffered peptone water (BPW) (Oxoid Ltd., Hampshire, U.K.) incubated at 37ºC for 18-24 h and then selectively enriched in tetrathionate (TT) broth (Oxoid Ltd., Hampshire, England) and Rappaport-Vassiliadis Soya (RVS) broth (Oxoid Ltd., Hampshire, England) and incubated for 18-24 h at 37 °C and 42 °C, respectively.

Samples enriched in selective broths were sub-cultured onto xylose-lysine tergitol 4 (XLT-4; Oxoid Ltd., Hampshire, England) and brilliant green agar (BGA; Oxoid Ltd.) and incubated at 37 °C for 18-24 h. Suspected *Salmonella* colonies, based on their phenotypic appearance on the respective agar, were subjected to a panel of biochemical tests that included triple sugar iron (TSI) agar, lysine iron agar (LIA), urea, citrate, methyl red, sulfide-indole-motility (SIM) medium, and o-nitrophenyl-b-D-galactopyranoside (ONPG; Oxoid Ltd.) for preliminary identification of *Salmonella* spp. using standard methods. Isolates biochemically confirmed as *Salmonella* were then subjected to a slide agglutination test using *Salmonella* polyvalent antiserum (A-I and Vi, Difco Laboratories, Detroit, MI). Initial complete confirmation and serotyping of *Salmonella* isolates, representative of those recovered by RVS/TT and BGA/XLT-4 combination of media, were performed using the phase reversal technique, and the results were interpreted according to the Kauffman-White scheme [43] at the Public Health Laboratory, Ministry of Health, St. Michael, Barbados.

3.3.6 Determination of antimicrobial resistance

The antimicrobial resistance of 65 *Salmonella* isolates recovered from the hatcheries and farms was determined using the disk diffusion method according to

the Clinical and Laboratory Standards Institute (CLSI) guidelines [162]. Eight antimicrobial agents commonly available and used in the local market and frequently used in the country's poultry industry were included in the panel. Chloramphenicol was included in the panel primarily for comparison and for research purposes only. The antimicrobial agents and concentrations used were as follows: amoxycillin-clavulanic acid (AMC, 30 µg); doxycycline (DO, 30 µg); ceftriaxone (CRO, 30 µg); gentamicin (CN, 10 µg); kanamycin (K, 30 µg); chloramphenicol (C, 30 µg); sulphamethoxazole-trimethoprim (SXT, 23.75 and 1.25 µg); and ciprofloxacin (CIP, 5 µg; BD Difco). The tests were performed on Mueller-Hinton agar (Difco), followed by aerobic incubation at 37 °C for 24 h. The zones of inhibition were interpreted as recommended by the disk manufacturer and CLSI [162]. For this investigation, isolates that exhibited intermediate and resistance zones of inhibition were classified as resistant.

3.3.7 Statistical analyses

Chi-square analyses were conducted using the Statistical Product and Service Solution, SPSS (version 27, IBM Corp., Somers, NY) to determine statistically significant associations in the frequency of isolation of *Salmonella* spp. among: (i) the risk factors associated with *Salmonella* contamination on broiler farms and hatcheries, (ii) the types of samples collected, (iii) the antimicrobial resistance among the type of sample, and (iv) the antimicrobial resistance among the serotypes. The Fisher's exact test was used for 2 x 2 tables with expected frequencies of <5. The level of significance was set at an alpha level of 0.05.

3.4 Results

3.4.1 Overview of demographic and risk factors

Of a total of 37 demographic and risk factors investigated at both hatcheries (Appendix 3.1: Questionnaire administered at the Hatcheries), none were

statistically significantly (P>0.05) associated with the frequency of isolation of *Salmonella*.

For the broiler farms, of the 15 factors (demographic data and management practices) investigated for their association with the isolation of *Salmonella*, none were statistically significant (P>0.05) (Table 3.3).

Table 3.3. Demographic data and risk factors associated with *Salmonella* isolation at broiler farms

Factor	**No. of farms sampled**	**No. (%) of farms[a] positive for *Salmonella***	***p- value***
Farm management			
Number of workers			0.25
0-5	24	7 (29.2)	
>5	3	2 (66.7)	
Management system			1
Automatic/Semi-automatic	12	4 (33.3)	
Manual	15	5 (33.3)	
Pen type			1
Open-sided	25	8 (32.0)	
Tunnel ventilated	2	1 (50.0)	
Pen capacity/birds			0.406
1000- 15,000	17	7 (41.2)	
>15,000	10	2 (20.0)	
Number of pens/farms			0.582
1-5 pens	23	7 (30.4)	
>5 pens	4	2 (50.0)	
Vaccines given on farm			0.582
Yes	23	7 (30.4)	
No	4	2 (50.0)	
Goal weight of bird[b]			0.683
3.8-4.5 lb.	15	6 (40.0)	
> 4.5 lb.	12	3 (25.0)	
Water supply			0.377
Municipal only	14	5 (35.7)	
Municipal/Pond	7	1 (14.3)	
Pond and River	3	2 (66.7)	
Others[c]	3	1 (33.3)	

Type of feed used			1
Marsh	2	1 (50.0)	
Pelleted	25	8 (32.0)	
Use of artificial lighting			0.676
Yes	8	2 (25.0)	
No	19	7 (36.8)	
Pests on farm			0.103
Yes	14	7 (50.0)	
No	13	2 (15.4)	
Type of pests present			0.115
Rats	5	3 (60.0)	
Mongooses	7	1 (14.3)	
Birds	8	6 (75.0)	
Other[d]	5	2 (40.0)	
Type of pest control			0.54
Poison	6	3 (50.0)	
Bait	5	3 (60.0)	
Other[e]	12	5 (41.7)	
Biosecurity measures			0.992
All-in all-out	27	9 (33.3)	
Restricted access	23	8 (34.8)	
Foot dip	5	1 (20.0)	
One-way traffic	5	2 (40.0)	
Vehicle dips/wheel baths	4	2 (50.0)	
Closed system- bird proofed	2	1 (50.0)	
Fenced property	2	1 (50.0)	
Protective clothing for workers	2	1 (50.0)	
None	3	1 (33.3)	
Mortality rate (%)			1
0 - 5.0	20	7 (35.0)	
>5.0	7	2 (28.6)	

[a] Farms were deemed positive once *Salmonella* was detected in any sample collected

[b] Each farm aimed to achieve a specific weight per bird at the end of the grow-out period

[c] Comprised the following: Municipal/Pond/Rain (1), Municipal/River (1), Spring (1)

[d] Others include pest are cats and mattes (lizards)

[e] Other types of pest control methods used included music, dogs, insecticidal and herbicidal use around the pens

3.4.2 Frequency of isolation by source and serotypes

All the 45 composite samples of imported fertile hatching eggs were negative for *Salmonella*. In effect, *Salmonella* was not detected in 45 composite samples each consisting of a pool of 10 eggs, i.e., a total of 450 eggs processed separately as eggshells and egg contents.

The frequency of isolation of *Salmonella* from a total of 158 samples comprising 13 types of samples collected from the two hatcheries was 7.6% (12/158) (Table 3.4). The frequency of isolation ranged from 0.0% (seven types of samples: swabs of interior of incubator, chick take-off, drain, hatcher exhaust fans, incubator air unit, egg transfer machine and meconium samples) to 28.0% (stillborn chicks) across the 13 types of samples. The frequency of isolation of *Salmonella* from stillborn chicks, 28.0% (7/25) was statistically significantly ($p<0.001$) higher than found for all other hatchery samples, 3.8% (5/133). Serotype Westhampton was isolated from 3.8% (1/26) of the broken eggshell samples while serotype Kentucky was predominantly isolated and responsible for 83.3% (10/12) of the isolates recovered from *Salmonella*-positive samples. The isolates obtained from stillborn chicks (2 isolates) and hatcher fluff (1 isolate) at Hatchery A were all serotype Kentucky. In Hatchery B, serotype Kentucky was isolated from 5 samples obtained from stillborn chicks and one isolate each from eggs in the incubator and the interior of the hatchers.

For the two hatcheries, the overall frequency of isolation of *Salmonella* was 3.4% (3/89) and 13.0% (9/69) in Hatchery A and Hatchery B, respectively. The difference was statistically significant ($p=0.023$). For the 13 types of samples tested, 15.4% (2/13) yielded *Salmonella* at a frequency of 6.7% (1/15) (hatcher fluff) and 13.3% (2/15) (stillborn chicks) in positive samples in Hatchery A ($p=1$). In Hatchery B, 30.8% (4/13) sample types yielded *Salmonella* at a frequency ranging from 9.1% (1/11) (broken eggshells) to 50.0% (5/10) (stillborn

chicks) but the differences in the isolation of *Salmonella* by sample type was not statistically significant (p=0.064).

From a total of 27 farms sampled, the overall frequency of isolation of *Salmonella* from 866 samples, where 5 different types were collected, was 2.8% (24/866) (Table 4). The farm prevalence of *Salmonella* spp. was 33.3% (9/27). The frequency of isolation of *Salmonella* was 2.2% (15/675) for cloacal swabs, and 4.7% (9/191) for environmental samples (feeds, water, workers' boots, and drag swabs of litter). For the environmental samples, the frequency of isolation of *Salmonella* ranged from 1.9% (1/53) in feeds to 11.1% (3/27) in boot swabs. The differences in the frequency of isolation of *Salmonella* among the different types of samples were statistically significant (p=0.049). Serotype Infantis was the predominant serotype isolated, having been recovered from 4 (80.0%) of the 5 types of samples and accounted for 15 (62.5%) of the 24 isolates recovered from *Salmonella*-positive samples. Serotypes Albany, Othmarschen and *Salmonella* spp., were isolated from 4 (16.7%), 1 (4.2%) and 4 (16.7%) isolates, respectively.

Regarding the hatchery sources of chicks supplied to farms, for the farms provided chicks by Hatchery A, all farm isolates (7/7; 100%) comprising 4 cloacal swabs, 1 drag swab, 1 feed sample and 1 boot swab were serotype Infantis. However, for the farms supplied with chicks from Hatchery B, serotypes Albany (4 cloacal swabs), Infantis (7 cloacal swabs and 1 boot swab), Othmarschen (1 drag swab) and *Salmonella* spp. (3 water samples, 1 boot swab) were isolated from various types of samples.

Overall, of a total of 1024 samples collected from 2 hatcheries and 27 broiler farms, 36 (3.5%) were positive for *Salmonella*, and the frequency of isolation was statistically significantly (p=0.0025) higher for the samples from the hatcheries, 7.6% (12/158) than for broiler farm samples, 2.8% (24/866).

Table 3.4. Frequency of isolation of *Salmonella* serotypes from imported fertile hatching eggs, hatcheries and broiler farms in Trinidad and Tobago

Source of samples	**Type of sample**	**No. of samples collected**	**No. (%) of samples positive for *Salmonella***	**Serotype (No., %)**
Imported fertile hatching eggs	Egg shells and albumen	45[a]	0 (0.0)	NA[b]
Hatcheries	Stillborn chicks	25	7 (28.0)	Kentucky (7, 100.0)
	Broken eggshells	26	1 (3.8)	Westhampton (1, 100.0)
	Swabs of interior of incubator	12	0 (0.0)	NA
	Swabs of interior of hatcher	12	1 (8.3)	Kentucky (1, 100.0)
	Swabs of eggs in incubator	12	1 (8.3)	Kentucky (1, 100.0)
	Swabs of eggs in hatcher	12	1 (8.3)	Group D (1, 100.0)
	Chick take-off	2	0 (0.0)	NA
	Hatcher fluff	25	1 (4.0)	Kentucky (1, 100.0)
	Drain	4	0 (0.0)	NA
	Hatcher exhaust fans	6	0 (0.0)	NA
	Incubator air unit	11	0 (0.0)	NA
	Egg transfer machine	1	0 (0.0)	NA
	Meconium samples	10	0 (0.0)	NA
	p-value		0.075[c]	
	Sub-total	158	12 (7.6)	
Farms	Cloacal swab	675	15 (2.2)	Infantis (11, 73.3) Albany (4, 26.7)
	Feed	53	1 (1.9)	Infantis (1, 100.0)

Water	54	3 (5.6)	*Salmonella* spp. (3, 100.0)
Boot swabs	27	3 (11.1)	Infantis (2, 66.7)
			Salmonella spp. (1, 33.3)
Drag swabs	57	2 (3.5)	Othmarschen (1, 50.0)
			Infantis (1, 50.0)
p-value		0.049	
Sub-total	866	24 (2.8)	
p-value		0.0025	
Total	1024	36 (3.5)	

[a] Forty-five composite samples (eggshell and egg content) comprised of 45 pools containing 10 eggs each, i.e., a total of 450 eggs
[b] NA: Not applicable
[c] Frequency of isolation of *Salmonella* from stillborn samples, 28.0% (7/25) was statistically significantly ($p<0.001$) higher than found for all other hatchery samples, 3.8% (5/133)

3.4.3 Frequency of antimicrobial resistance among Salmonella isolates

The frequencies of resistance to eight antimicrobial agents by *Salmonella* isolates recovered from the hatcheries and broiler farms are shown in Table 3.5. Among the 25 isolates of *Salmonella* from six types of samples collected from the hatcheries, 11 (44.0%) exhibited resistance to one or more antimicrobial agents tested. The differences in the frequency of resistance in *Salmonella* isolates across the type of samples were not statistically significant (p=0.10). Resistance was exhibited by *Salmonella* isolates to four of the eight antimicrobial agents, specifically to, amoxycillin-clavulanic acid (4.0%), gentamycin (4.0%), doxycycline (28.0%), and kanamycin (28.0%). For the six types of samples tested, the frequency of resistance among the *Salmonella* isolates was statistically significant only to kanamycin (p=0.043).

For the 40 farm isolates of *Salmonella* recovered from five types of samples from the farms, the overall frequency of resistance to antimicrobial agents was 87.5% (35/40) with a range from 40.0% (water) to 100.0% (feeds, litter drag swabs, and boot swabs). The differences were statistically significant (p= 0.02). Resistance was exhibited to six of the eight antimicrobial agents with a range in frequency from 2.5% (1/40) (ciprofloxacin) to 85.0% (34/40) (doxycycline) ($p<0.001$). The frequency of resistance of *Salmonella* isolates to antimicrobial agents for the types of samples varied significantly for doxycycline (p=0.044), ceftriaxone (p=0.001), gentamycin (p=0.002), kanamycin (p=0.01), and sulphamethoxazole-trimethoprim (p=0.017).

Table 3.5. Frequency of antimicrobial resistance of *Salmonella* isolated at hatcheries and broiler farms based on type of samples

Source of samples	Type of sample	No. of isolates tested	No. (%) of isolates resistant[a]	No. (%) isolates resistant: AMC[b]	DO	CRO	CN	K	C	SXT	CIP
Hatcheries	Stillborn chicks	17	6 (35.3)	1 (5.9)	6 (35.3)	0 (0.0)	1 (5.9)	3 (17.6)	0 (0.0)	0 (0.0)	0 (0.0)
	Broken eggshells[d]	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Swab of eggs in hatcher[d]	1	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Swab of eggs in incubator[d]	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Swab of hatcher walls[d]	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Hatcher fluff[d]	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	p-value		0.1	0.993	0.591	NA[c]	0.993	0.043	NA	NA	NA
	Sub-total	25	11 (44.0)	1 (4.0)	7 (28.0)	0 (0.0)	1 (4.0)	7 (28.0)	0 (0.0)	0 (0.0)	0 (0.0)
Farms	Cloacal swab	27	25 (92.6)	0 (0.0)	25(92.6)	24 (88.9)	23 (85.2)	24 (88.9)	0 (0.0)	21 (77.8)	1 (3.7)
	Feed	1	1 (100.0)	0 (0.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)
	Water	5	2 (40.0)	0 (0.0)	2 (40.0)	0 (0.0)	0 (0.0)	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Drag swab	2	2 (100.0)	0 (0.0)	2 (100.0)	1 (50.0)	2 (100.0)	2 (100.0)	0 (0.0)	1 (50.0)	0 (0.0)
	Boot swabs	5	5 (100.0)	0 (0.0)	4 (80.0)	4 (80.0)	3 (60.0)	5 (100.0)	0 (0.0)	3 (60.0)	0 (0.0)
	p-value		0.02	NA	0.044	0.001	0.002	0.01	NA	0.017	0.974
	Sub-total	40	35 (87.5)	0 (0.0)	34 (85.0)	30 (75.0)	29 (72.5)	33 (82.5)	0 (0.0)	26 (65.0)	1 (2.5)
	p-value		<0.001	0.203	<0.001	<0.001	<0.001	<0.001	NA	<0.001	0.426
	Total	65	46 (70.8)	1 (1.5)	41 (63.1)	30 (46.2)	30 (46.2)	40 (61.5)	0 (0.0)	26 (40.0)	1 (1.5)

[a] Resistance to one or more agents tested
[b] AMC:Amoxycillin-clavulanic acid, DO: Doxycycline; CRO: Ceftriaxone; CN: Gentamicin; K: Kanamycin; C: Chloramphenicol; SXT: Sulphamethoxazole-trimethoprim and CIP: Ciprofloxacin
[c] NA: Not applicable
[d] Pooled/composite sample representative of individual hatcher/incubator/pen

3.4.4 Resistance of Salmonella isolates based on serotype

From the study conducted at the hatcheries, the frequency of resistant *Salmonella* in serotypes Kentucky and Westhampton was 36.4% and 100%, respectively (Table 3.6). For the predominant serotype Kentucky, the overall frequency of resistance to antimicrobial agents was 36.4% (8/22), and the highest frequency of resistance was exhibited to doxycycline (36.4%).

For the isolates of *Salmonella* from broiler farms, the overall frequency of resistance to antimicrobial agents was 100.0%, 50.0%, and 100.0% for serotypes Infantis, Albany and Othmarschen, respectively. All (100.0%) isolates of serotype Infantis exhibited resistance to doxycycline.

Table 3.6. Frequency of antimicrobial resistance of *Salmonella* isolates based on serotype

Sources of samples	Serotype	No. of isolates tested	No. (%) of isolates resistant[a]	No. (%) isolates resistant: AMC[b]	DO	CRO	CN	K	C	SXT	CIP
Hatcheries	Kentucky	22	8 (36.4)	1 (4.5)	8 (36.4)	0 (0.0)	1 (4.5)	4 (18.2)	0 (0.0)	0 (0.0)	0 (0.0)
	Westhampton	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	p-value		0.163	1	0.536	NA[c]	1	0.054	NA	NA	NA
	Subtotal	24	10 (41.7)	1 (4.2)	8 (33.3)	0 (0.0)	1 (4.2)	6 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)
Farms	Infantis	16	16 (100.0)	0 (0.0)	16 (100.0)	16 (100.0)	16 (100.0)	15 (93.8)	0 (0.0)	16 (100.0)	0 (0.0)
	Albany	4	2 (50.0)	0 (0.0)	2 (50.0)	1 (25.0)	0 (0.0)	2 (50.0)	0 (0.0)	0 (0.0)	1 (25.0)
	Othmarschen	1	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	p-value		0.01	NA	0.009	<0.001	<0.001	0.075	NA	<0.001	0.107
	Subtotal	21	19 (90.5)	0 (0.0)	19 (90.5)	17 (90.0)	17 (90.0)	18 (85.7)	0 (0.0)	16 (76.2)	1 (4.8)

[a] Resistance to one or more agents tested
[b] AMC:Amoxycillin-clavulanic acid, DO: Doxycycline; CRO: Ceftriaxone; CN: Gentamicin; K: Kanamycin; C: Chloramphenicol; SXT: Sulphamethoxazole-trimethoprim and CIP: Ciprofloxacin
[c] NA: Not applicable

3.4.5 Resistance patterns exhibited by isolates of Salmonella from hatcheries and broiler farms

Only 4.0% (1/25) of the *Salmonella* isolates from the hatcheries exhibited multidrug resistance i.e., resistant to antimicrobial agents belonging to 3 or more classes. Among the 11 isolates from hatcheries, four different patterns were observed consisting of DO (36.4%), DO-K (27.3%), K (27.3%) and AMC-DO-CN-K (9.1%). Among the 35 resistant isolates from broiler farms, 30 (85.7%) were MDR and seven resistance patterns were observed. DO-CRO-CN-K-SXT was the predominant pattern, exhibited by 68.6% (24/35) isolates while other patterns observed were DO-K (8.6%; 3/35), DO-CRO-CN-SXT (5.7%; 2/35), DO-CRO-K (5.7%; 2/35), DO-CRO-CN-K (5.7%; 2/35), DO-CN-K (2.9%; 1/35) and K (2.9%; 1/35).

3.5 Discussion

Our current study is part of cross-sectional studies conducted at three levels of the broiler processing industry in Trinidad and Tobago, using a 'farm to fork' approach. We had earlier reported on the prevalence and characteristics of *Salmonella* isolated from retail outlets (supermarkets and cottage poultry processors 'pluck shops') [300, 327] and processing plants [328] in the country. The current study reports the occurrence of *Salmonella*, risk factors and characteristics of *Salmonella* isolated from imported fertile hatching eggs, hatcheries and poultry farms in Trinidad and Tobago. Of significance is the finding that all 45 eggshell and 45 egg content samples comprising 450 pooled fertile hatching broiler eggs that were randomly collected at the airport were negative for *Salmonella*. This is because imported fertile hatching eggs have the potential to introduce new and existing serotypes of *Salmonella* into the country [329]. However, based on our data it appears that the risk is minimal or non-existent. Our failure to isolate *Salmonella* from imported fertile hatching eggs in the current study agrees with the findings on imported table eggs and hatching layer eggs in a previous study in Trinidad, Grenada, and St. Lucia

[310]. However, to fully understand the risk posed by imported fertile hatching eggs to serve as a vehicle to introduce *Salmonella* into the country, it may be prudent to conduct a longitudinal study with a larger sample size. This is due to the fact that the current study is a cross-sectional study where a small sample size was used. Fertile hatching broiler eggs from *Salmonella* -infected breeder flocks have been reported to serve as a source of the pathogen for hatched day-old chicks, the contamination of hatchery environments and the exposure of broiler flocks on farms to the *Salmonella* [330]. The possibility of movement of *Salmonella* from the hatcheries to the farms and then to the processing plants therefore creates an opportunity for the pathogen to enter the food chain directly through the consumption of *Salmonella* -contaminated broiler meat contaminated during broiler production, processing and retailing [331].

The finding that 6 (46.2%) of 13 sample types collected from the hatcheries were positive for *Salmonella* , even though all the imported fertile hatching eggs were negative for the pathogen, is of interest. This finding may be explained, in part, by factors such as the prior horizontal contamination of the hatchery environment [332], the low numbers of fertile hatching eggs sampled, and the fact that the hatching eggs sampled at the airport may not directly represent the eggs already in the incubators and hatchers which were sampled in the study. Another possible explanation for cross-contamination is that both hatcheries did not practise eggs' sanitation pre-incubation which is a common practice to reduce contamination [333].

Generally, in our study, the hatchery practices and the findings at both integrated companies were similar, except for the mortality rates (0.01% versus 0.05%), production system (semi-automated versus manual), vaccination method (in-ovo vaccination versus subcutaneous injection), and the frequency of isolation of *Salmonella* (3.4% and 13.0%). Some of the practices common to both hatcheries included the lack of restriction of workers' movement, candle eggs pre-hatcher placement, and removal of chicks via a machine, practices that have been

implicated in *Salmonella* contamination [333]. Variable frequencies of isolation of *Salmonella* from hatcheries have been reported and attributed to the differences in the enforcement of regulations by authoritative bodies. For example, the isolation rate of *Salmonella* from hatcheries was reported to be 0.3% in the Netherlands [334], 4.3% in China [335], and 34.0% in Korea [322].

It is important to have detected that the frequency of isolation of *Salmonella* was statistically significantly higher in stillbirth chicks (28.0%) compared to the 3.8% found in all the other 12 types of samples collected from the hatcheries. These findings indicate that vertical transmission of *Salmonella* to the fertile hatching eggs may have occurred considering that the overall frequency of horizontal contamination at the hatcheries was low at 3.8% (average frequency of isolation for all types of samples except stillbirth chicks). These findings suggest the involvement of *Salmonella* as potential cause of the stillbirths tested for the pathogen. A considerably higher frequency of isolation of *Salmonella* (34%) was reported for hatcheries in Korea [322]. Although our study demonstrates a low frequency of environmental contamination by *Salmonella* at the two hatcheries, considerably higher frequencies of isolation of *Salmonella* from hatchery environments have been reported by others. *Salmonella* was isolated from eggshell fragments (71%), in the swabs of conveyor belt (80%) and in chick liners (74%) in the USA [234], and the recovery of the pathogen from hatcher interiors (75%), chick sorting/dispatch area (100%), meconium (50%), ventilation outlets (50%) and waste area samples (75%) in Korea [330]. Also, unlike our study where all the meconium samples were negative for *Salmonella,* Byrd et al. [336] reported that 12% of chick tray liners (meconium) samples were positive for *Salmonella* .

The potential aetiologic role of serotype Kentucky in the causation of stillbirth chicks was evident because all (100.0%) seven *Salmonella*-positive stillbirth chicks yielded only the serotype, in addition to its accounting for 83.3% of the isolates recovered from both hatcheries. It has been reported that *Salmonella* spp., including serotype Kentucky, have been responsible for stillbirth chicks in hatcheries and it

has emerged as the most important serovar in poultry processing plants [337]. Additionally, it is also important to have noted that serotype Kentucky was also recovered from other environmental sources (swabs of the interior of hatcher, swabs of eggs in the incubator and hatcher fluff), albeit at lower frequencies. This is not surprising because Kentucky is one of the serotypes commonly associated with hatcheries and broilers [336]. Recent studies conducted at broiler processing plants [328] and retail outlets [300] in the country also detected Kentucky, confirming the local circulation of this serotype. However, variable predominant serotypes of *Salmonella* have been isolated from hatcheries in other countries., such as serotypes Pullorum, Enteritidis, Indiana, and Thompson in China [338], and serotypes Enteritidis, Heidelberg and Senftenberg in Korea [330]. The differences in the serotypes of *Salmonella* isolated from hatcheries in various countries were not unexpected because this may be multi-factorial, including the prevalent serotypes infecting breeder flocks and contaminating hatchery environments, management, and sanitary practices in the respective countries [321].

In our study, the farm prevalence of *Salmonella* was 33.3% (9/27) compared with the reported 36.7% in Algeria [339], 47.9% in Nigeria [340] and 27.3% in Ireland [341]. For all the samples from the 27-broiler farms tested in our study, only 2.8% (24/866) yielded *Salmonella,* a finding in agreement with similarly low frequencies reported on broiler farms in Spain, 1.0% [242], in Algeria, 1.7% [339], in Sweden, 2.3% [342] and in Columbia, 2.8% [343]. However, considerably higher overall prevalence of *Salmonella* have been reported elsewhere, such as, 15.9% in Nigeria [340], 19.8% in Ireland [341], 26.6% in Bangladesh [344] and 59.1% in Nigeria [345].

In the current study, the prevalence of *Salmonella* in the cloacal swabs of broilers reared on conventional broiler farms was considered low at 2.2% (15/675), but the possibility of cross contamination of carcasses with the pathogen during processing cannot be ignored. This is supported by the findings in a most recent cross-sectional study conducted in four processing plants where broilers from the 27 farms are

slaughtered, reported an overall frequency of *Salmonella* from the cloacal swabs (pre-slaughter) was 2.2% (2/90) while 44.4% (20/45) of chilled whole carcasses were contaminated with *Salmonella* [328]. The prevalence of 2.2% for *Salmonella* in cloacal swabs in our study is lower than reported, also on conventional farms, by researchers in the Netherlands, 5.9% (85), 14.8% in Ethiopia [346], 38.8% in the USA [347], and 55.6% in Korea [330].

Serotype Infantis was unquestionably the predominant serotype circulating in the 27 broiler farms sampled across the country, having been detected in 75.0% of the 20 isolates recovered from *Salmonella* -positive samples that were serotypeable and isolated in 80.0% of the five types of samples tested. These findings indicate that the serotype may be important in the epidemiology of *Salmonella* infections on broiler farms in the country, considering that it has also been isolated from table eggs [325], humans [348] and most recently chickens processed at broiler processing plants [328] in the country. Like our study, serotype Infantis has been reported to be the predominant serotype isolated from broiler farms in South America [349] and Japan [350]. However, unlike our findings, other predominant serotypes of *Salmonella* have been reportedly isolated from broiler farms in other countries. These include the most frequently detected serovars of *Salmonella* being Enteritidis in Brazil [351], Indiana in Zimbabwe [352] and Typhimurium in Bangladesh [344].

In this study, among the environmental samples collected from the farms which have the potential to serve as vehicles for the transmission of *Salmonella* and other pathogens within the farms, the boots of workers (11.1%) pose the highest risk, followed by water in drinkers (5.6%), the litter, based on drag swab samples (3.5%) and then feeds (1.9%). A slightly lower frequency of isolation (5.9%) was reported for plastic boot covers/fresh droppings in Dutch broiler flocks [334]. The need to use disposable boot covers along with regular changes between pens cannot be over-emphasized. Regarding the contamination of drinking water (storage tanks or drinkers in pens) of broilers, a considerably lower frequency of *Salmonella*

contamination has been reported by Bailey et al. [353] who documented that it was affected by several factors such as the types of drinkers (trough, plastic bell and nipple drinkers), the methods of disposal of dead birds and the infestation of farms by pests such as rats and wild birds [354, 355].

The frequency of isolation of *Salmonella* (3.5%) in litter drag swab samples detected in the current study is lower than the 26.7% reported for drag swab samples of layer farms in an earlier study in the country [310]. Also, at variance with our findings on broiler farms, is the study conducted in Korea [330], where 30% of drag swab samples were positive for *Salmonella*. Multi-use of litter, which is practised on broiler farms in the country, where 'dirty' or 'caked' litter is removed, and fresh litter is added and treated before placement of chicks, has been known to increase pathogen contamination in the litter [356].

In our study, only 1.9% of the feed samples collected from the 27 farms were contaminated with *Salmonella*, a finding which agrees with a similarly low frequency (2.8%) reported for feed samples collected from 23 layer farms in the country [310]. The risk of pests contaminating the feeds can, however, not be ignored since in the current study, 14 (51.9%) of the 27 farms sampled reported experiencing pest problems, including infestation by rats (60%), mongooses (14.3%) and wild birds (75%) which are potential carriers or shedders of *Salmonella* . These pests have been shown to play some role in the transmission of *Salmonella* on poultry farms [240].

Our detection of an overall frequency of resistance to antimicrobial agents among hatchery isolates of *Salmonella* was moderate at 44.0%, and it is of interest that resistance was exhibited to only four of the eight agents ranging from 4.0% to 28.0%. These findings may reflect the exposure of *Salmonella* isolates to these antimicrobial agents, particularly to kanamycin and doxycycline, on the breeder farms from where the hatching eggs originated. The possibility of development of resistance by *Salmonella* isolates recovered from environmental hatchery sources

resulting from the overuse of antimicrobial agents in the local poultry industry cannot be ignored. Some reports on the frequency of resistance to antimicrobial agents are at variance with our findings [335, 357]. A low frequency of antimicrobial resistance (<10%) to CRO, CN, K, C, CIP and SXT among *Salmonella* isolated from hatcheries was reported in China [335] agrees with the 0% to 28.0% prevalence of resistance in the *Salmonella* isolates in the current study. In agreement with our study where all our *Salmonella* isolates were susceptible to ciprofloxacin, Shang et al. [358] reported a frequency of resistance (0.0%) to CIP, CN and AMC in *Salmonella* isolated at hatcheries in Korea. On the other hand, the authors detected a high frequency (52.8%) of MDR compared to only 4.0% found in our study. These differences in the occurrence of AMR and MDR may reflect the types and spectra of antimicrobial agents used in the poultry industry across countries.

It was not a surprise that the frequency of resistance to antimicrobial agents in *Salmonella* isolates in farm isolates was statistically significantly higher than found in hatchery isolates (87.5% versus 44.0%), as well as the occurrence of MDR (85.7% versus 4.0%). These findings can be explained, in part, by the fact that broilers on the farms are also exposed to antimicrobial agents through their use for prophylaxis, therapy, and growth promotion as indicated by the questionnaire data in the current study. This is important in Trinidad and Tobago where, although regulations exist on the use of antimicrobial agents in the livestock industry, they are not routinely enforced.

The frequency of resistance to antimicrobial agents found in isolates of *Salmonella* from broiler farms (87.5%) agrees with the 100% detected in China [359], 100% in Ethiopia [346] and 100% in Colombia [360] but considerably lower frequency of resistance (73.1%) was documented on broiler farms in Algeria [339]. The considerably high frequencies of resistance (77.8% - 92.6%) to five antimicrobial agents (doxycycline, ceftriaxone, gentamycin, kanamycin, and sulphamethoxazole-trimethoprim), exhibited by *Salmonella* isolates recovered from the cloacal swabs

of individual broilers could therefore have therapeutic implications since agents such as tetracycline and sulphamethoxazole-trimethoprim are most readily available and used locally in the poultry industry. The need for a prudent use and regulation of antimicrobial agents on broiler farms can therefore not be over-emphasized [361]. Resistance to these six antimicrobial agents by *Salmonella* have similarly been reported in isolates recovered from chicken from retail outlets [327], layer farms [362] and table eggs [362] in the country.

Overall, serotype Kentucky, the predominant serotype isolated from the hatcheries exhibited a moderate level (36.4%) of resistance to antimicrobial agents, particularly to doxycycline (36.4%) and kanamycin (36.4%). The importance is that antimicrobial-resistant isolates of serotype Kentucky may be transferred to the broiler farms in the country. Although the occurrence of MDR is low (4.0%) in serotype Kentucky in our study, the serotype has been identified as a potential emerging MDR human pathogen in Europe, North Africa, and Asia because of the increase in the number of cases over the last decade where the source of infection was poultry [363, 364].

It is of potential therapeutic significance that all (100.0%) isolates of serotype Infantis were resistant to doxycycline, ceftriaxone, gentamycin, and sulphamethoxazole-trimethoprim, and therefore may compromise therapeutic interventions. Resistant serotypes of *Salmonella* have been isolated from poultry products by others [30, 365], and it has been suggested that the broad AMR patterns displayed by the strains of serotype Infantis are cause for concern [366].

A limitation of the study is that only two of the four functional hatcheries in the country agreed to participate thereby reducing the number of samples available for the study.

3.6 Conclusions

In conclusion, the fact that serotype Kentucky was isolated at an overwhelming frequency from stillbirth chicks compared to the environmental samples is indicative of vertical transmission of *Salmonella* at the breeder farms from where the hatched eggs originated. The failure to isolate *Salmonella* from the imported fertile hatching eggs may be partly attributed to the small sample size, coupled with the fact that the study was cross sectional and not longitudinal in design, may have limited the inferences that could be drawn from the findings of our study. Future investigations should therefore use larger sample sizes and be longitudinal in design. The low frequency of isolation of *Salmonella* in the cloacal swabs of broilers does not reduce the risk posed to food safety when slaughtered considering that *Salmonella* -positive broilers may cross-contaminate several carcasses during processing.

The finding that different serotypes of *Salmonella* were predominant at the hatcheries (Kentucky) and at the broiler farms (Infantis) supplied with chicks from the hatcheries is an indication that other sources of contamination of farm environments may be important, considering that 51.9% of the farms experienced pest problems (rats and wild birds).

To reduce or prevent the on-farm spread of *Salmonella* within and across pens, it is concluded that intervention measures such as the mandatory use of disposable workers' boots may be effective.

Finally, the high prevalence of resistance (85.7%) to commonly used antimicrobial agents (sulphamethoxazole-trimethoprim, gentamicin, ceftriaxone, kanamycin, and doxycycline) detected in the *Salmonella* isolates on the farms may pose therapeutic and food safety risks. Therefore, it is imperative to enforce existing policy on the

use of antimicrobial agents, through the institution of a surveillance system in the country.

Connecting statement to the next chapter

The relatively low prevalence of *Salmonella* isolated in imported fertile broiler hatching eggs (0.0%), at hatcheries (7.6%) and broiler farms (2.8%) in Trinidad and Tobago, determined using a cross-sectional study (Chapter 3), a cross-sectional study was conducted at the four operational broiler processing plants in Trinidad and Tobago (Chapter 4) to assess the processing practices and risk factors associated with *Salmonella* contamination during processing, sequentially along the processing lines.

CHAPTER 4: CHARACTERISATION OF *SALMONELLA* ISOLATES RECOVERED FROM STAGES OF THE PROCESSING LINES AT FOUR BROILER PROCESSING PLANTS IN TRINIDAD AND TOBAGO

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4.1 Abstract

This cross-sectional study determined the prevalence, characteristics, and risk factors for contamination of chicken with *Salmonella* at four operating broiler processing plants in Trinidad and Tobago. Standard methods were used to isolate and characterise the *Salmonella* isolates. The overall prevalence of *Salmonella* at the four processing plants was 27.0% (107/396). The whole carcass enrichment (WCE) method yielded a statistically significantly ($p = 0.0014$) higher frequency of isolation (53.9%; 97/180) than the whole carcass rinse (35.0%; 63/180) and neck skin methods (42.2%; 38/90). *S.* enterica serotypes Enteritidis, Javiana, and Infantis were the predominant serotypes isolated accounting for 20.8%, 16.7% and 12.5%, respectively, of the serotyped isolates. Risk factors included the use of over 100 contract farmers (OR 4.4), pre-chiller (OR 2.3), addition of chlorine to chiller (OR 3.2), slaughtering sick broilers (OR 4.4), and flocks with >0.50% mortality. Multi-drug resistance was detected in 12.3% (14/114) of the isolates of *Salmonella* . Resistance was high to kanamycin (85.7%) and doxycycline (74.6%) but low to amoxicillin-clavulanic acid (2.4%) and sulphamethoxazole-trimethoprim (0.8%). The occurrence of resistant *Salmonella* in chickens processed at commercial broiler processing plants has implications for salmonellosis and therapeutic failure in consumers of improperly cooked contaminated chickens from these plants in the country.

Keywords: broiler processing plants; *Salmonella;* serotypes; risk factors; antimicrobial resistance; Trinidad and Tobago.

4.2 Introduction

Salmonellosis is the third leading cause of death among food transmitted diseases [367] with an estimated global *Salmonella* enterocolitis incidence of 95.1 million cases [368], accounting for 50,771 deaths in 2017 [369]. In the Caribbean, *Salmonella* is the most common laboratory-confirmed cause of foodborne diseases since 2005 [370]. Poultry has been reported to be the main carrier of *Salmonella*

infections to humans [371], more common than any other animal species [372]. Broiler meat is an economical source of protein and estimated to be the most widely consumed meat, globally.

The human population of the twin-island Republic of Trinidad and Tobago is 1,366,725 [373] with a reported 58.3 kg per capita poultry consumption rate; 800,000 broilers are produced weekly, of which 20% is imported [323]. Consumers purchase chicken from cottage poultry processors, where they are freshly slaughtered and from supermarkets, which offer chilled and frozen locally processed as well as imported frozen chicken. Broiler processing plants are responsible for 50% of local broiler processing [374] where supermarkets and the franchised foodservice sector are supplied with chilled chickens as well as further-processed products [374].

Several studies have reported the high frequency of contamination with *Salmonella* of chicken meat sold at the informal and formal outlets in developed and developing countries with *Salmonella* [375, 376]. It has also been reported that the processing of chicken at commercial processing plants contributes significantly to the contamination of dressed chicken carcasses with *Salmonella* before they reach the retail outlets [242, 246]. Unhygienic carcass handling, soiled slaughter equipment [247, 248], contaminated water (scalding and immersion chiller water) and waste generated from evisceration and de-feathering processes have been implicated as major sources of *Salmonella* contamination during broiler processing [249-251]. *Salmonella*-free broilers leaving farms may potentially become contaminated by the pathogen during processing through contact with immersion chiller water contaminated with *Salmonella* originating from the positive broilers [377, 378]. These can occur, should there be improper pH and chemical agents' concentrations, as well as a failure to maintain good sanitary practices throughout processing [379]. With the increase in production and consumption of broiler meat over the years, the use, misuse and overuse of veterinary drugs for prophylaxis, therapeutic and growth promotion purposes [380, 381] are common in countries, such as Trinidad and

Tobago. In the country, although regulations on the use of veterinary drugs in livestock exist, they are not routinely enforced. The increase in the isolation of *Salmonella* in humans, and the resistance of *Salmonella* strains to antimicrobial agents commonly used in food-producing animals is a major health concern [382, 383]. Worldwide, of a greater concern is the emergence of multidrug-resistant (MDR) *Salmonella* [384], which have been implicated in foodborne outbreaks due to contaminated meat [385, 386].

To isolate *Salmonella* from poultry processing plants, different approaches have been reported and recommended. In the European Union, the use of neck skin (NS) maceration [124] is most frequent whereas, in the United States, the U.*S.* Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) [387] recommends the use of whole carcass rinse (WCR) method. Whilst the WCR is the most commonly used method for isolation of *Salmonella* in broiler carcasses [388-390], the whole carcass enrichment (WCE) and neck skin (NS) methods have been shown to be just as effective [391] or even more than the WCR [392]. However, the large space required for incubating whole carcasses makes the WCE method impractical for routine testing, but it is valuable for research purposes [393].

In Trinidad and Tobago and the Caribbean, there is a dearth of comprehensive up-to-date data on the role played by the commercial broiler processing plants in the contamination of processed chicken carcasses with *Salmonella* . The only available recent published data were from studies conducted at the outlets of cottage poultry processors (‘wet market’) where the slaughtering and retailing of dressed chicken were practiced [30] and at supermarkets where retailing of chicken from the commercial processing plants occurs [300] and the antimicrobial resistance profiles of *Salmonella* isolates from both sources were determined [327].

Considering the limited current information on the status and dynamics of *Salmonella* contamination of chicken carcasses at the commercial broiler processing plants, the present study with the following objectives was conducted:

(i) to determine the frequency of isolation of *Salmonella* longitudinally from the different stages of processing, from pre-slaughter broilers to chilled carcasses, (ii) to evaluate the efficacy of three isolation methods for *Salmonella* , (iii) to identify the risk factors associated with *Salmonella* contamination of chicken carcasses at the plants and finally, (iv) to determine the serotypes and antimicrobial resistance profiles of the isolates of the pathogen recovered from the four plants operating in Trinidad and Tobago.

4.3 Materials and methods

4.3.1 Sampling site

The study was conducted in Trinidad and Tobago, the twin-island Caribbean country located in the southern Caribbean, north-east of the South American country of Venezuela, northwest of Guyana, and south of Grenada in the Lesser Antilles. There are currently four commercial broiler processing plants in Trinidad and Tobago. These plants process only broiler chickens and supply supermarkets and food outlets with dressed chilled and/or frozen chicken. Each processing plant packages whole dressed chickens, various packaged chicken parts (legs, thighs, breasts, wings and mixed parts), offal (liver, gizzard), feet and necks all of which are available for sale at their retail outlet (at the respective plant) or supplied to supermarkets or food outlets. The similarities and differences in the operations which may impact on the bacteriological quality of broilers at the four processing plants studied are shown in a flow chart (Appendices 4.1-4.4).

The number of samples to be collected for this study was estimated using the formula:

Estimated sample size for an infinite population, $n_o = Z_u^2 P_{ex} (1-p_{ex})/d^2$ [324] where:

n_o= Estimated sample size; Z_u= Degree of confidence= 1.96

P_{ex} = Expected prevalence= 50%; d= Desired absolute precision = 5%

$n_o = [1.96^2 \times 0.5(1-0.5)]/0.05^2 = 384$

A total of 396 samples were collected comprising swabs of pre-slaughter cloacae, pre-evisceration carcasses, post-evisceration carcasses, chilled whole chickens (dressed) and chilled chicken parts (dressed), as well as neck skins and chiller water. Sample collection was conducted during the period January to September 2019. The total number of samples collected at each plant was determined using proportional sampling based on their throughputs. Therefore, two, four, one and two sampling visits were made to plants A, B, C and D, respectively. Plant A and D received chicken from their 210 and 98 contract farms, respectively, whereas Plants B and C were owned by the same parent company that controlled 32 farms. Samples were collected in individual sterile bags and bottles and transported on ice to the laboratory of the Veterinary Public Health Unit, School of Veterinary Medicine, for processing within 4-6 h after collection. Standardised, pre-tested questionnaires were administered at each broiler processing plant to obtain information about demography, operational information, and risk factors for carcass contamination with *Salmonella*. Some of the questions were designed to elicit information on the average number of contract farmers, the average waiting period between arrival of chickens to slaughter, disposal of waste material and source of water supply (Appendix 4.5).

4.3.2 Processing of samples collected from processing plants

During each visit to the broiler processing plant the following samples were collected in sterile bottles/bags: 10 cloacal swabs, 5 pre-evisceration carcasses (post- defeathering), 5 post-evisceration carcasses, 10 neck skins, 4 immersion chiller water samples, 5 chilled whole carcasses (after removal from immersion chiller) and 5 packs of chilled chicken parts each of legs, thighs, breast, wings and mixed parts.

The WCR method, described by the USDA-FSIS [353] for *Salmonella* isolation was used. Each carcass was rinsed in 430 ml of buffered peptone water (BPW)

(Oxoid, Hampshire, England), rotated for no less than 30 times and 30 ml of the rinsate was removed and incubated.

Each carcass with the remaining 400 ml BPW in the WCR process above, was incubated in accordance with the WCE method as described by Cox et al. [360] and constituted the WCE sample. Neck skin (NS) samples were processed as recommended by the Commission Regulation (EC) No 2073/2005 [361] with the following modification. Each neck skin was collected in a sterile bag from which approximately 10-15 g was aseptically excised and added to BPW in a 1: 9 ratio and incubated at 37 °C for 18-24 h. Each excised neck skin was treated as one (1) sample as performed in other studies [391, 396].

Each cloacal swab sample was added to 9 ml BPW and subsequently incubated [397]. During each sampling visit to the plants, 400 ml of immersion chiller water was collected four (4) times, at an interval of 1.5 h to provide representative samples of potential contamination over a 6 h period. In the laboratory, 100 ml were aseptically removed from each 400 ml sample and centrifuged at 4470 x g for 20 min after which 1 ml of sediment was removed and transferred to 9 ml BPW and incubated [398].

All pre-enriched BPW samples were incubated at 37 °C for 18-24 h. Samples were then selectively enriched in 9 ml tetrathionate (TT) broth (Oxoid, Hampshire, England) and 9 ml Rappaport-Vassiliadis Soya (RVS) broth (Oxoid, Hampshire, England) and incubated at 37 and 42 °C, respectively.

4.3.3 Isolation and identification of Salmonella

Samples enriched in selective broths were sub-cultured onto Xylose-lysine tergitol 4 (XLT-4; Oxoid, Hampshire, England) and Brilliant green agar (BGA; Oxoid) and incubated at 37 ° C for 18-24 h. Suspected *Salmonella* colonies that displayed characteristic colonies on both selective agar plates were then purified on blood

agar plates (Oxoid) and incubated at 37 °C for 18 - 24 h. Pure cultures were subjected to a panel of biochemical tests that included triple sugar iron agar, lysine iron agar, urea, citrate, methyl red, sulfide-indole-motility medium, and o-nitrophenyl-b-D-galactopyranoside (Oxoid) [327, 399]. Isolates biochemically confirmed as *Salmonella* were then subjected to a slide agglutination test using *Salmonella* polyvalent antiserum (A-I & Vi, Difco, Detroit, MI). Complete confirmation and serotyping of *Salmonella* isolates representative of those recovered by the WCR/WCE/NS, RVS/TT and BGA/XLT-4 methods were performed using the phase reversal technique, and the results interpreted according to the Kauffman-White scheme [43] at the Public Health Laboratory, Ministry of Health, St. Michael, Barbados. Molecular confirmation of tentatively identified *Salmonella* was conducted using conventional polymerase chain reaction (PCR). Initially, DNA was extracted from the *Salmonella* isolates by the boiling method [400], followed by the use of conventional PCR to detect the *inv*A gene as described earlier [400]. The following primer sequences were used to amplify a 284 bp fragment of the *inv*A gene, Forward: 5' GTGAAATTATCGCCACGTTCGGGCAA 3' and Reverse: 5' TCATCGCACCGTCAAAGGAACC 3' as described by Oliviera et al. [401].

4.3.4 Determination of antimicrobial resistance

The antimicrobial resistance of 126 *Salmonella* isolates recovered from the samples obtained at the four broiler processing plants was determined using the disk diffusion method according to the Clinical and Laboratory Standards Institute [162, 402] guidelines. Eight antimicrobial agents commonly available in the local market and frequently used in the poultry industry in Trinidad and Tobago formed the panel of antimicrobial agents. The antimicrobial agents, concentrations and classes (Difco, Becton Dickinson, Sparks, MD, USA) used comprised the following: amoxicillin-clavulanic acid (AMC, 30 µg, β-lactam); doxycycline (DO, 30 µg, Tetracycline); ceftriaxone (CRO, 30 µg, Cephalosporin); gentamicin (CN, 10 µg, Aminoglycosides); kanamycin (K, 30 µg, Aminoglycosides); chloramphenicol (C,

30 µg, Phenicol); sulphamethoxazole-trimethoprim (SXT, 23.75 and 1.25 µg, Sulphonamides); and ciprofloxacin (CIP, 5 µg, Fluoroquinolones). The tests were performed on Mueller-Hinton agar (Difco), followed by aerobic incubation at 37 °C for 24 h. The zones of inhibition were interpreted as recommended by the disk manufacturer and Clinical and Laboratory Standards Institute [162].

4.3.5 Statistical analysis of data

Chi-square analyses were conducted using the Statistical Package for Social Sciences, SPSS (version 27, IBM Corp., Somers, NY) to determine statistically significant associations in the frequency of isolation of *Salmonella* amongst (i) the three different sampling methods, (ii) the risk factors associated with *Salmonella* contamination, (iii) the types of samples collected and iv) the plants sampled. The Fisher's exact test was used for 2 x 2 contingency tables with expected frequencies of <5. The level of significance was set at an alpha ≤ 0.05. Univariate analysis of associations was conducted using the *Salmonella* status of the sample as a binary outcome (positive or negative). The predictor variables were the average number of farmers, number of workers directly involved in processing, waiting period, the mortality rate on arrival, treatment of diseased birds, use of a pre-chiller, agents used in chiller, temperature of chiller water and segregation of workers. Each predictor variable was tested for significant associations with the *Salmonella* status using the chi-square test of association. Significant variables ($p<0.05$) in the univariate analysis were assessed for collinearity using the chi-square statistic and were considered collinear if $p<0.05$. A forward stepwise regression model where entry of $p < 0.5$ and removal of $p < 0.10$ was used in the regression analysis. Hosmer-Lemeshow chi-square was used as a goodness of fit test. Statistical analysis was done using SPSS (version 27) at an alpha level of 0.05.

4.4 Results

4.4.1 Overview of management and production data

In Trinidad and Tobago, the poultry industry is vertically integrated, where each company controls its respective hatcheries, contracted farms, feed mills and processing plant. However, because of the limited supply of broilers to the smaller integrated companies, broilers often originated from competitor farms. A summary of the management and production data on the four processing plants is shown in Table 4.1.

Table 4.1. Management and production data from four broiler processing plants in Trinidad and Tobago.

	Processing plant:			
Parameter	**Plant A**	**Plant B**	**Plant C**	**Plant D**
Total installed capacity of the processing plant (birds/week)	160,000	250,000	<100,000	100,000
Average number of broilers processed daily	32,000	50,000	15,000	20,000
Number of days operational weekly	5	5	4	5
Average number of contract farmers used	210	32	32	100
Number of workers directly involved in processing[a]	150	400	75	150
Number of workers indirectly involved in processing[b]	100	1000	1000	75
Waiting period (h) between arrival of birds at plant and slaughter	2-6	0.5-3	1-3	12
Average mortalities (%) or broilers dead on arrival at plant	0.7	0.02	0.94	0.50
Disposal of solid waste (faecal materials) from broilers	Rendered[c]	External Company	Rendered	Rendered

Disposal of wastewater	River	Settling ponds	Settling ponds	Settling ponds
Treatment of water at the plant[d]	No	Yes	No	No

[a] Workers who have contact with the birds/carcass at one point during processing
[b] Workers involved in the management of the plant but not having contact with the birds/carcass during processing
[c] Rendering (in-house) to convert animal tissue waste to useable by-product meal
[d] All plants utilised municipal water supply as their source.

4.4.2 Comparison of sampling methods

Salmonella was isolated from 35.0%, 53.9%, and 42.2% of samples subjected to the WCR, WCE, and neck skin methods, respectively (p= 0.0013) (Figure 4.1). Significant differences in the frequency of isolation of *Salmonella* by sampling method were found in pre-evisceration carcasses (p<0.001), post-evisceration carcasses (p<0.001), and all samples (p<0.001). Chilled whole carcasses subjected to the WCE method yielded a higher frequency of isolation (60%; 27/45) when compared to the WCR method (31.1%; 14/45) (p=0.01). Selective enrichment in tetrathionate broth plated onto XLT-4 agar yielded the highest frequency of *Salmonella* positive samples among the three methods (p<0.001). Overall, 8.9% (40/450), 29.8% (134/450), 1.8% (8/450) and 3.6% (16/450) of the samples were isolated on RVS/XLT-4, TT/XLT-4, RVS/BGA and TT/BGA, respectively (p<0.001).

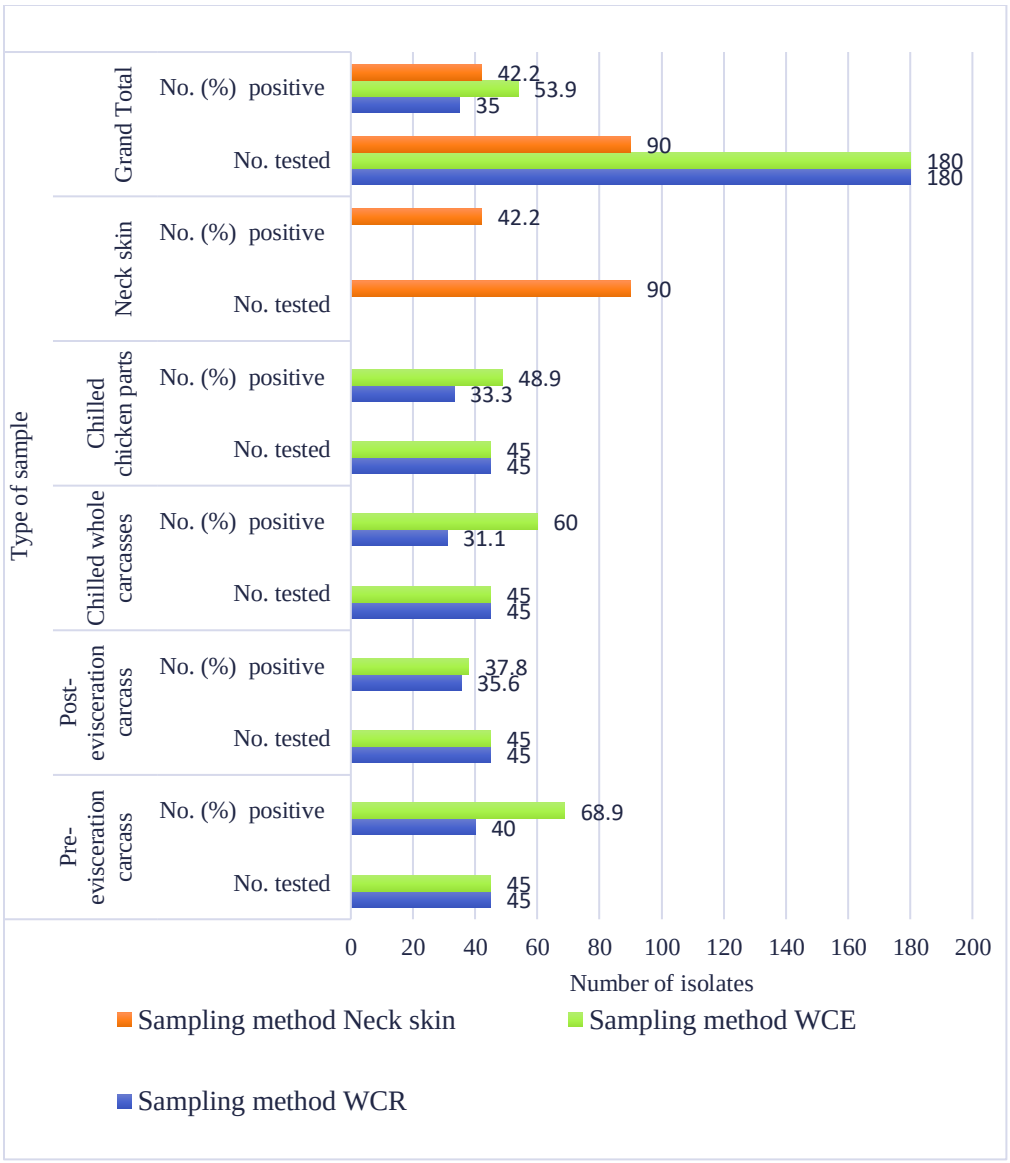


Figure 4.1. Recovery of *Salmonella* based on the method used

4.4.3 Risk factors associated with Salmonella contamination during broiler processing

The association of risk factors with the frequency of contamination of chickens processed is shown in Table 4.2. Of the 14 risk factors investigated, 10 (71.4%) were determined to be statistically significantly associated with the contamination with *Salmonella* during processing.

Table 4.2. Risk factors associated with *Salmonella c*ontamination of carcasses

Risk factor	**Total No. samples tested**	**Total No. (%) positive for *Salmonella***	**p-value**	**Odds Ratio**	**CI (95%)**
Size of plant[a]			p<0.001		
Small	44	5 (11.4)		Ref	
Medium	176	70 (39.8)		5.1	1.94-13.71
Large	176	32 (18.2)		1.7	0.63-4.74
Average number of contract farmers			p<0.001		
≤ 100 farmers	308	61 (19.8)		Ref	
> 100 farmers	88	46 (52.3)		4.43	2.68-7.34
Number of workers directly involved in processing operation			p= 0.001		
≤ 150 workers	220	75 (34.1)		Ref	
> 150 workers	176	32 (18.2)		0.43	0.27-0.70
Average waiting period from arrival at plant to processing			p= 0.95		
≤ 10 h	308	83 (26.9)		Ref	
> 10 h	88	24 (27.3)		1.01	0.60-1.73
Average mortality rate (%) of birds on arrival at plant			p= 0.001		
< 0.50	176	32 (18.2)		Ref	
≥ 0.50	220	75 (34.1)		2.32	1.45-3.74
Handling of sick/diseased birds			p<0.001		
Rejected at farm	308	61 (19.8)		Ref	
Processed last	88	46 (52.3)		4.43	2.68-7.34
Use of pre-chiller			p= 0.001		
Yes	220	75 (34.1)		Ref	
No	176	32 (18.2)		2.32	1.45-3.74
Agents used in pre-chiller[b]			p= 0.11		
Citric acid + chlorine	88	24 (27.3)		Ref	
No agents added	132	51 (38.6)		1.67	0.94-3.02
Temperature of pre-chiller[b]			p< 0.001		
Room temperature	88	46 (52.3)		Ref	
10 °C	44	5 (11.4)		0.11	0.04-0.33
20 °C	88	24 (27.3)		0.34	0.18-0.64
Agents used in chiller			p= 0.01		
Chlorine	352	102 (29.0)		3.18	1.22-8.30
No agents added[c]	44	5 (11.4)		Ref	
Concentration of chlorine used in chiller[c]			p= 0.79		
20 ppm	88	24 (27.3)		Ref	

21- 50 ppm	264	78 (29.5)		1.11	0.65-1.92
Temperature of chiller			p= 0.14		
< 1 °C	132	29 (22.0)		Ref	
1- 4 °C	264	78 (29.5)		1.49	0.91-2.43
Agents used for general cleaning of plant during processing			p= 0.01		
Sanitizer	352	102 (29.0)		Ref	
Hot water only	44	5 (11.4)		0.31	0.12-0.82
Worker segregation[d]			p= 0.01		
Yes	352	102 (29.0)		3.18	1.22-8.30
No	44	5 (11.4)		Ref	

[a] Based on weekly throughput, small< 100,000 birds; medium 101,000- 160,000 birds; large > 161,000 birds.
[b] Only 3 plants use pre-chillers.
[c] Only 3 plants add additional chlorine to chiller water. Chlorine concentration ranged from 1-5 ppm in the municipal water supply
[d] Colour coding of workers was used to limit movement of workers to prevent cross-contamination of dirty and clean work areas.

4.4.4 Multivariate logistic regression of risk factors for isolation of Salmonella

Of the nine variables included in the initial logistic regression model, only the average number of contract farmers, the number of workers directly involved in the processing, and the waiting period were retained in the final model. Processing plants with more than 100 contract farms were significantly associated with increased odds of *Salmonella* isolation (OR= 8.5; $\chi 2$= 16.968, p< 0.001) (Table 4.3). Similarly, plants, where the waiting period between arrival and slaughter was more than 10 h, were significantly associated with *Salmonella* isolation (OR= 2.9; $\chi 2$= 4.072, p=0.044). Plants, where there were more than 150 workers directly involved in processing, were included in the model but were not a significant predictor in the equation (p=0.284). The Hosmer-Lemeshow test of goodness-of-fit test was not significant ($\chi 2$= 0.00, p=1), showing that the final logistic regression model fitted the data well.

Table 4.3. Results of a multivariate logistic regression of risk factors for *Salmonella* isolation from carcasses sampled at broiler processing plants in Trinidad and Tobago

						95.0% CI	
Variable	**Coef.**	**Standard Error[a]**	**Chi-Square**	**p-value**	**Odds Ratio**	Lower	Upper
>100 versus ≤ 100 farmers	2.145	0.521	16.968	<0.001	8.543	3.078	23.707
>150 versus ≤ 150 workers	0.55	0.514	1.147	0.284	1.733	0.633	4.744
> 10 h versus ≤ 10 h waiting period	1.073	0.532	4.072	0.044	2.925	1.031	8.296
Constant	-2.054	0.475	18.7	<0.001	0.128		

[a] Standard error of the coefficient

4.4.5 Isolation from different broiler processing plants and types of samples

Overall, the isolation rate of *Salmonella* in carcasses sampled at broiler processing plants was 27.0% (107/396) (Table 4.4). Among all the samples collected during broiler processing, the isolation rate of *Salmonella* was highest in pre-evisceration carcasses (51.1%; 23/45) followed by chilled whole carcasses (44.4%; 20/45), chilled chicken parts (40.0%; 18/45) and post-evisceration carcasses (37.8%; 17/45) (Table 4.4). *Salmonella* was detected only in 2.2% (2/90) and 5.6% (2/36) cloacal swabs and immersion chiller water samples, respectively.

Table 4.4. Frequency of isolation of *Salmonella* by type of samples tested at each plant

Stage in Processing	Type sample collected	Plant A		Plant B		Plant C		Plant D		Total No. tested	Total No. (%) positive for *Salmonella*
		No. of samples tested	No. (%) positive	No. of samples tested	No. (%) positive	No. of samples tested	No. (%) positive	No. of samples tested	No. (%) positive		
Pre-evisceration	Cloacal swab	20	2 (10.0)	40	0 (0.0)	10	0 (0.0)	20	0 (0.0)	90	2 (2.2)
	De-feathered carcass	10	7 (70.0)	20	9 (45.0)	5	1 (20.0)	10	6 (60.0)	45	23 (51.1)
	p-value		0.002		<0.001		0.333		0.0004		<0.001
	Subtotal	30	9 (30.0)	60	9 (30.0)	15	1 (6.7)	30	6 (20.0)	135	25 (18.5)
Post- evisceration	Eviscerated carcass	10	7 (70.0)	20	5 (25.0)	5	0 (0.0)	10	5 (50.0)	45	17 (37.8)
	Neck skin	20	14 (70.0)	40	9 (22.5)	10	0 (0.0)	20	2 (10.0)	90	25 (27.8)
	p-value		0.656		0.535		NA		0.026		0.972
	Subtotal	30	21 (70.0)	60	14 (23.3)	15	0 (0.0)	30	7 (23.3)	135	42 (31.1)
Chiller water and carcasses	Chilled water	8	0 (0.0)	16	2 (12.5)	4	0 (0.0)	8	0 (0.0)	36	2 (5.6)
	Chilled whole carcass	10	7 (70.0)	20	5 (25.0)	5	3 (60.0)	10	5 (50.0)	45	20 (44.4)
	Chilled- parts	10	9 (90.0)	20	2 (10.0)	5	1 (20.0)	10	6 (60.0)	45	18 (40.0)
	p-value		0.0004		0.391		0.123		0.024		0.0003
	Subtotal	28	16 (57.1)	56	9 (16.1)	14	4 (28.6)	28	11 (39.3)	126	40 (31.7)
Total		88	46 (52.3)	176	32 (18.2)	44	5 (11.4)	88	24 (27.3)	396	107 (27.0)

p-value									<0.001
Pre-evisceration	30	9 (30.0)	60	9 (30.0)	15	1 (6.7)	30	6 (20.0)	135	25 (18.5)
Post- evisceration	30	21 (70.0)	60	14 (23.3)	15	0 (0.0)	30	7 (23.3)	135	42 (31.1)
Chiller water and carcasses	28	16 (57.1)	56	9 (16.1)	14	4 (28.6)	28	11 (39.3)	126	40 (31.7)
p-value		0.007		0.439		0.041		0.215		0.023

4.4.6 Serotypes of Salmonella isolates

S. enterica serotype Enteritidis (20.8%; 15/72), Javiana (16.7%; 12/72) and Infantis (12.5%; 9/72) were the most prevalent among a total of 16 different serotypes isolated at broiler processing plants (Table 4.5). Serotypes Kentucky, Anatum, Schwarzengrund and Albany were found in less than 10% of the isolates. Only 1 isolate each of serotypes Hindmarsh, Madjorio, Mbandaka, *S. enterica* subspecies Houtenae, Virchow, Weltevrden, Aberdeen, Alachua and Ayinde were detected among all the isolates. Serotype Enteritidis was found primarily (14/15 samples: 93.3%) in chilled whole and chicken parts as well as neck skins.

Table 4.5. *Salmonella* serotypes isolated from different types of samples

Stage of processing	**No. of samples positive for *Salmonella***	**No. (%)[a] of isolates serotyped**	**Serotypes (No., %)**
Cloacal swabs	2	0 (0.0)	Not applicable
Pre-evisceration carcass	23	2 (8.7)	Weltevreden (1, 50.0)
			Enteritidis (1, 50.0)
Post-evisceration carcass	17	5 (29.4)	Javiana (3, 60.0)
			Virchow (1, 20.0)
			Infantis (1, 20.0)
Neck skins	25	25 (100.0)	Javiana (7, 28.0)
			Schwarzengrund (5, 20.0)
			Albany (4, 16.0)
			Anatum (3, 12.0)
			Infantis (2, 8.0)
			Group C2[b] (2, 8.0)
			Madjorio (1, 4.0)
			Enteritidis (1, 4.0)

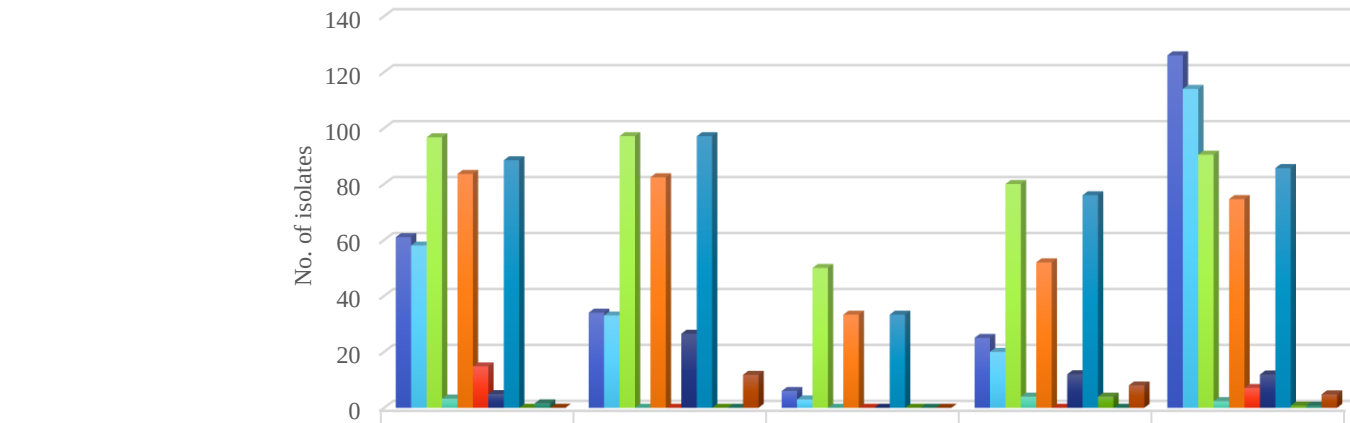
Chiller water	2	2 (100.0)	*Salmonella* spp. (1, 50.0) subspecies Houtenae IV (1, 50.0)
Chilled whole carcass	20	20 (100.0)	Enteritidis (7, 35.0) Infantis (4, 20.0) Anatum (2, 10.0) Albany (1, 5.0) Mbandaka (1, 5.0) Schwarzengrund (1, 5.0) Aberdeen (1, 5.0) Javiana (1, 5.0) Kentucky (1, 5.0) Ayinde (1, 5.0)
Chilled chicken parts	18	18 (100.0)	Enteritidis (6, 33.3) Kentucky (6, 33.3) Infantis (2, 11.1) Hindmarsh (1, 5.6) Javiana (1, 5.6) Anatum (1, 5.6) Alachua (1, 5.6)
Total	107	72 (67.3)	

[a]Of the number of randomly selected *Salmonella* serotypes from each source
[b]Serogroup (Group C2) could not be determined to the serotype level

4.4.7 Frequency of resistance of Salmonella isolates to eight antimicrobial agents at different processing plants

The prevalence of resistance to antimicrobial agents among *Salmonella* isolates tested was 90.5% (114/126) as resistance was exhibited to one or more of the 8 antimicrobial agents tested (Figure 4.2). Overall, resistance was relatively high to K (85.7%) and DO (74.6%) but relatively low to SXT (0.8%), C (0.8%), and AMC

(2.4%). The differences were statistically significant ($p<0.05$). The overall prevalence of resistance to antimicrobial agents by *Salmonella* isolates was 96.7% (58/61), 97.1% (33/34), 50.0% (3/6) and 80.0% (20/25) at plant A, B, C and D, respectively and these differences were statistically significant ($p<0.05$).



	A	B	C	D	Total
No. isolates tested	61	34	6	25	126
No. of resistant isolates	58	33	3	20	114
No. (%) of resistant isolates	96.7	97.1	50	80	90.5
No. (%) isolates resistant to: AMC*	3.3	0.0	0.0	4.0	2.4
No. (%) isolates resistant to: DO	83.6	82.4	33.3	52.0	74.6
No. (%) isolates resistant to: CRO	14.8	0.0	0.0	0.0	7.1
No. (%) isolates resistant to: CN	4.9	26.5	0.0	12.0	11.9
No. (%) isolates resistant to: K	88.5	97.1	33.3	76.0	85.7
No. (%) isolates resistant to: C	0.0	0.0	0.0	4.0	0.8
No. (%) isolates resistant to: SXT	1.6	0.0	0.0	0.0	0.8
No. (%) isolates resistant to: CIP	0.0	11.8	0.0	8.0	4.8

Plant

*AMC, amoxicillin-clavulanic acid (30 µg); DO, doxycycline (30 µg); CRO, ceftriaxone (30 µg); CN, gentamicin (10 µg); K, kanamycin (30 µg); C, chloramphenicol (30 µg); SXT, sulfamethoxazole-trimethoprim (23.75 and 1.25 µg); CIP, ciprofloxacin (5 µg).

Figure 4.2. Antimicrobial resistance of *Salmonella* isolates isolated from four processing plants

4.4.8 Frequency of antimicrobial resistance of Salmonella isolates based on the type of sample

The frequencies of resistance to antimicrobial agents (Table 4.6) were similar amongst the various types of samples, ranging from 86.2% to 100% in chilled chicken parts, post-evisceration carcasses, chilled whole carcasses, neck skins, pre-evisceration carcasses, chiller water and cloacal swabs. The frequency of resistance to DO was significantly ($p=0.045$) higher for isolates of *Salmonella* that originated from pre-evisceration carcasses (23/25, 92.0%) compared with isolates from other types of samples. The differences in the frequency of resistance were not statistically significant ($P>0.05$) for *Salmonella* isolated from the other types of samples other than from chiller water samples.

Table 4.6. Antimicrobial resistance of *Salmonella* isolated from various stages of processing

Stage in Processing	Type of sample Collected	No. of isolates tested	No. (%) of isolates resistant[a]	No. (%) resistant to[b]: AMC	DO	CRO	CN	K	C	SXT	CIP
Pre-evisceration	Cloacal swab	3	3 (100.0)	0 (0.0)	1 (33.3)	0 (0.0)	1 (33.3)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Defeathered carcass	25	24 (96.0)	0 (0.0)	23 (92.0)	1 (4.0)	2 (8.0)	23 (92.0)	0 (0.0)	0 (0.0)	0 (0.0)
	p-value		1	NA	0.045	1	0.298	1	NA	NA	NA
	Subtotal	28	27 (96.4)	0 (0.0)	24 (85.7)	1 (3.6)	3 (10.7)	26 (92.9)	0 (0.0)	0 (0.0)	0 (0.0)
Post- evisceration	Eviscerated Carcass	17	15 (88.2)	0 (0.0)	12 (70.6)	0 (0.0)	2 (11.8)	14 (82.4)	0 (0.0)	0 (0.0)	0 (0.0)
	Neck skin	25	23 (92.0)	0 (0.0)	18 (72.0)	2 (8.0)	6 (24.0)	21 (84.0)	0 (0.0)	0 (0.0)	3 (12.0)
	p-value		1	NA	1	0.506	0.439	1	NA	NA	0.260
	Subtotal	42	38 (90.5)	0 (0.0)	30 (71.4)	2 (4.8)	8 (19.0)	35 (83.3)	0 (0.0)	0 (0.0)	3 (7.1)
Chiller water and carcasses	Chiller water	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	1 (50.0)
	Chilled -whole carcass	29	25 (86.2)	0 (0.0)	20 (69.0)	4 (13.8)	4 (13.8)	24 (82.8)	0 (0.0)	0 (0.0)	2 (6.9)
	Chilled- parts	25	22 (88.0)	3 (12.0)	20 (80.0)	2 (8.0)	0 (0.0)	21 (84.0)	1 (4.0)	1 (4.0)	0 (0.0)
	p-value		0.846	0.140	0.050	0.698	0.135	0.814	0.532	0.532	0.009
	Subtotal	56	49 (87.5)	3 (5.4)	40 (71.4)	6 (10.7)	4 (7.1)	47 (83.9)	1 (1.8)	1 (1.8)	3 (5.4)
Total		126[c]	114 (90.5)	3 (2.4)	94 (74.6)	9 (7.1)	15 (11.9)	108 (85.7)	1 (0.8)	1 (0.8)	6 (4.8)

Pre-evisceration	28	27 (96.4)	0 (0.0)	24 (85.7)	1 (3.6)	3 (10.7)	26 (92.9)	0 (0.0)	0 (0.0)	0 (0.0)
Post- evisceration	42	38 (90.5)	0 (0.0)	30 (71.4)	2 (4.8)	8 (19.0)	35 (83.3)	0 (0.0)	0 (0.0)	3 (7.1)
Chiller water and carcasses	56	49 (87.5)	3 (5.4)	40 (71.4)	6 (10.7)	4 (7.1)	47 (83.9)	1 (1.8)	1 (1.8)	3 (5.4)
p-value		0.422	0.147	0.310	0.373	0.193	0.471	0.533	0.533	0.374

[a] Resistance to one or more agents tested.
[b] AMC, amoxicillin-clavulanic acid (30 µg); DO, doxycycline (30 µg); CRO, ceftriaxone (30 µg); CN, gentamicin (10 µg); K, kanamycin (30 µg); C, chloramphenicol (30 µg); SXT, sulfamethoxazole-trimethoprim (23.75 and 1.25 µg); CIP, ciprofloxacin (5 µg).
[c] A total of 126 isolates may have included duplicates of isolates obtained from TT/XLT-4, TT/BGA, RVS/XLT-4 and RVS/BGA media, solely of phenotypes.
NA: Not applicable.

4.4.9 Resistance of Salmonella isolates based on serotype

Sixteen different serotypes of *Salmonella* were identified from the 72 isolates subjected to conventional serotyping. Serotypes Enteritidis and Javiana were the most prevalent serotypes with 60.0% (9/15) and 83.3 % (10/12) exhibiting resistance to one or more agents, respectively (Table 4.7). All isolates (100.0%) belonging to serotypes Albany, Anatum and Kentucky; 88.9% for Infantis, 83.3% for Javiana and 83.3% for Schwarzengrund exhibited resistance to antimicrobial agents. Amongst the different serotypes, the differences in the resistance exhibited were only statistically significant to DO ($p<0.001$).

4.4.10 Antimicrobial resistance patterns

Fourteen (12.3%) of 114 isolates of *Salmonella* exhibited multidrug resistance i.e., resistance to antimicrobial agents belonging to 3 or more classes. Overall, a total of 12 different patterns were observed consisting of DO-K, which was the predominant pattern, with 54.4% isolates exhibiting the resistance pattern. Resistance to K alone was exhibited by 15 (13.2%) isolates, 12 (10.5%) isolates exhibited resistance to DO-CN-K, 8 (7.0%) exhibited resistance to DO-CRO-K and 6 (5.3%) were resistant to DO alone. Other patterns observed ranged from 0.9% to 2.6% of resistant isolates.

Table 4.7. Resistance exhibited by different serotypes isolated at four processing plants

Serotype[a]	No. of isolates tested	No. (%) of isolates resistant[b]	No. (%) isolates resistant to[c]: AMC	DO	CRO	CN	K	C	SXT	CIP
Albany	5	5 (100.0)	0 (0.0)	5 (100.0)	2 (40.0)	0 (0.0)	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)
Anatum	6	6 (100.0)	0 (0.0)	6 (100.0)	2 (33.3)	0 (0.0)	4 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)
Enteritidis	15	9 (60.0)	1 (6.7)	1 (6.7)	0 (0.0)	0 (0.0)	9 (60.0)	1 (6.7)	0 (0.0)	2 (13.3)
Infantis	9	8 (88.9)	0 (0.0)	6 (66.7)	0 (0.0)	2 (22.2)	8 (88.9)	0 (0.0)	0 (0.0)	0 (0.0)
Javiana	12	10 (83.3)	0 (0.0)	10 (83.3)	0 (0.0)	4 (33.3)	10 (83.3)	0 (0.0)	0 (0.0)	3 (25.0)
Kentucky	7	7 (100.0)	2 (28.6)	7 (100.0)	1 (14.3)	0 (0.0)	6 (85.7)	0 (0.0)	1 (14.3)	0 (0.0)
Schwarzengrund	6	5 (83.3)	0 (0.0)	4 (66.7)	0 (0.0)	2 (33.3)	5 (83.3)	0 (0.0)	0 (0.0)	0 (0.0)
p value			0.813	<0.001	0.252	0.238	0.745	1	0.996	0.192
Total	60	50 (83.3)	3 (5.0)	39 (65.0)	5 (8.3)	8 (13.3)	46 (76.7)	1 (1.7)	1 (1.7)	5 (8.3)

[a] In addition, 2 (100.0%) of 2 Group C2 isolates exhibited resistance to one or more of the eight antimicrobial agents tested; 1 (100.0%) of 1 of the following serotypes Aberdeen, Alachua, Ayinde, Hindmarsh, Madjorio, Mbandaka, *Salmonella* sp. (untypable), *S.* Houtenae, Virchow and Weltevreden were resistant, i.e., a total of 12 isolates

[b] Exhibited resistance to one or more antimicrobial agents.

[c] AMC, amoxicillin-clavulanic acid (30 µg); DO, doxycycline (30 µg); CRO, ceftriaxone (30 µg); CN, gentamicin (10 µg); K, kanamycin (30 µg); C, chloramphenicol (30 µg); SXT, sulfamethoxazole-trimethoprim (23.75 and 1.25 µg); CIP, ciprofloxacin (5 µg).

4.5 Discussion

This is considered the first cross-sectional study conducted in the broiler processing plants in Trinidad and Tobago which documented the frequency of isolation of *Salmonella* along the processing lines. The study also characterised the isolates regarding their serotypes and antimicrobial resistance to currently used antimicrobial agents in the poultry industry. The food safety importance of the study cannot be underestimated because the four processing plants operational in the country supply the majority of local chickens and chicken products sold at supermarkets.

Of food safety concern, is the high level of contamination found in pre-packaged chilled whole carcasses (44.4%) and chilled chicken parts (40.0%) across the four processing plants. Salmonellosis has been reported in humans who consume inadequately cooked *Salmonella*-contaminated chicken meat [403, 404]. Our findings agree with the prevalence of *Salmonella* found in chilled chicken carcasses in abattoirs elsewhere, where 48.0% [396], 45.2% [405] and 50.0% [406] were reported in the United States, China and Brazil, respectively. These findings were higher than the 8.3% [407] and 3.75% [408] reported in Iran and the Czech Republic, respectively. It is interesting to note that the most recent study on the prevalence of *Salmonella* in chickens that originated from commercial processing plants in Trinidad and Tobago was 8.3% [300]. The differences in the prevalence have been reported to be affected by the carriage of *Salmonella* during de-feathering [408], evisceration and spray washing steps [409] as well as by contaminated chiller water [410].

The strategy used in our study which included the collection of samples from the time of reception of live chickens to the finished chilled chickens longitudinally, from the pre-evisceration samples to chilled carcasses during each visit provided evidence of statistically significant ($p=0.023$) increased levels of contamination along the stages of processing. The differences in the frequencies of isolation of *Salmonella* in the samples between and within the four processing plants, could be

due in part, to the different management, production and risk factors at these plants. These findings were not surprising because other studies have reported progressive increases in the frequency of contamination with *Salmonella* during processing [411, 412].

It is significant that the frequency of isolation of *Salmonella* from the cloacal swabs pre-slaughter across the four plants was 2.2% ranging from 0.0% to 10.0%. This is an indication that the prevalence of *Salmonella* was relatively low on the poultry farms from where the slaughtered birds originated. Our findings agree with the prevalence of *Salmonella* in cloacal swabs of broilers pre-slaughter reported in Trinidad and Tobago, 3.95% (3/76) [28], Brazil, 7.0% (7/100) [378] and Colombia, 12.5% (8/64) [413].

It was of epidemiological relevance to have detected that 71.4% of the 14 risk factors investigated demonstrated statistically significant association with the contamination of chicken carcasses during processing at the plants. Significantly higher frequencies of isolation of *Salmonella* were detected among the following factors including medium-sized plants, use of more than 100 contract farmers, employment of less than 150 workers directly involved in processing, the average mortality rate of over 0.5% in broilers on arrival at the plant, i.e., dead on arrival, the use of pre-chillers and the use of sanitizers in chiller water, used sanitizers for general cleaning of plants, among other factors. Many of these risk factors have been documented to be associated with the isolation of *Salmonella* in processing plants by others [248, 269, 414-416]. Standardised sanitation protocols with surveillance to monitor the efficacy and the development of resistance is suggested. In addition, frequent training programs for processing plant workers and farmers to educate them on the current best- practices will be beneficial in reducing cross-contamination along the continuum. Interestingly, further regression analyses and the odds ratio (OR) revealed that *Salmonella* was 4.4 more likely (95% CI: 2.68-7.34) to be isolated from chickens in plants that received birds from more than 100 farmers. This risk could be attributed to the increased possibility of slaughtering

broilers from *Salmonella*-infected farms. Similarly, it was detected that plants which allowed the slaughter of chickens from batches with mortality rates of over 0.5% on arrival at the plants were 2.3 times more likely (95% CI: 1.45-3.74) to lead to the isolation of *Salmonella* from chickens at those plants. Although the specific pathogens responsible for deaths experienced during transportation to the plant were not known, the possibility exists that *Salmonella* may be involved. The contamination of feathers of chickens from direct contact with faeces of infected broilers shedding *Salmonella* and exposure to the pathogen in the transport vehicle on its way to the plant has been documented [417, 418]. Similarly, the risk of contamination of chickens increased considerably by 4.4 times (95% CI: 2.68-7.34) in plants that permitted the slaughter of sick birds, albeit being processed last instead of being rejected at farms. The possibility of seeding the plant environment with pathogens, including *Salmonella*, is pertinent, if the cleaning of the plant is inadequate. *Salmonella* was isolated at a significantly higher frequency in plants that used chlorine (29.0%) than those that used hot water (11.4%). This is because *Salmonella* has been reported to develop resistance to sanitizers [268, 419, 420]. Additionally, our study noted that plants that used pre-chillers but did not add chemical agents were found to be 1.7 times more likely (95% CI: 0.94-3.02) to result in the recovery of *Salmonella.* The proper use of chillers and sanitizers in processing plants can therefore not be ignored [421, 422].

In our study, the WCE method yielded a statistically significant higher (53.9%) frequency of isolation of *Salmonella* than either the WCR (35.0%) or the NS (42.2%) methods, making it the most sensitive method for *Salmonella* detection as reported by others [396, 423]. Berrang et al. [393] attributed this increased sensitivity to the ability of the WCE method to facilitate the proliferation of *Salmonella* in low quantities or those firmly attached to the skin of the chicken. However, the challenges associated with WCE method, particularly the considerably larger incubator space requirement compared with the use of WCR and NS methods, cannot be disregarded thereby making it an impractical method for routine surveillance testing but applicable as a research tool. It has been reported

that the types of samples and the methods of enrichment affect their sensitivities to detect *Salmonella* in chickens [424, 425].

The predominant serotypes of *Salmonella* isolated were Enteritidis, Javiana and Infantis. These serotypes have similarly been isolated from chicken-associated samples in the country, such as chickens sampled from supermarkets that originated from broiler processing plants and outlets of cottage poultry processors [300] and chicken layers [310]. In the current study, it was found that the serotypes were detected at different frequencies from the types of samples tested in the processing plants, a finding that agrees with published reports [29, 426]. Of food safety and public health, the significance is the fact that some of these predominant serotypes were determined in the Caribbean Public Health Agency (CARPHA) State of Public Health report [348], to be amongst the top 15 human *Salmonella* serotypes detected in the region. Similarly, the predominant serotypes in our study were also reported to be the most commonly *Salmonella* serotypes associated with human salmonellosis in Trinidad and Tobago between 2005-2012 [314]. It cannot be underestimated that serotype Enteritidis has globally been associated with poultry meat and eggs, and responsible for human cases and epidemics of salmonellosis [427, 428].

The high prevalence of resistance (90.5%) to antimicrobial agents by the 126 isolates of *Salmonella* recovered from the four processing plants, has both zoonotic and therapeutic implications. It is important to have detected that the high prevalence of resistance was exhibited to antimicrobial agents routinely used in the poultry industry in the country. It has been reported that zoonotic spread of *Salmonella* to workers at the commercial processing plants may occur [429, 430] and as well as therapeutic failure in consumers of improperly cooked chickens contaminated by antimicrobial resistant-*Salmonella* [426]. Similarly, a high prevalence of resistance to antimicrobial agents (100.0%) has been reported in chilled chickens from supermarkets and cottage poultry processors [327]. Although the current study was not farm-based, the prevalence of resistant *Salmonella* in

chickens processed at the plants may be indicative of the level of resistance of *Salmonella* on the contract farms from where they originated. It has been documented that the misuse or over-use of antimicrobial agents by farmers may result in the development of resistance to antimicrobial agents [431]. This is a common practice particularly in developing countries, including Trinidad and Tobago, where although laws governing the type and use of antimicrobial agents for prophylaxis, growth promotion, and therapy exist, prevailing challenges limit or prevent their enforcement [432, 433].

With regard to the eight antimicrobial agents tested, it was important that the overall prevalence of resistance was comparatively low (0.8%- 11.9%) to six (amoxicillin-clavulanic acid, ceftriaxone, gentamicin, chloramphenicol, sulphamethoxazole-trimethoprim and ciprofloxacin) of the antimicrobial agents, while significantly higher prevalence was exhibited to doxycycline (74.6%) and kanamycin (85.7%). Furthermore, the study found that the prevalence of resistance to the antimicrobial agents varied significantly across the processing plants from where the *Salmonella* isolates originated. These findings reflect the differences in the types and the frequency of use of antimicrobial agents on the contract farms that supplied live broilers to the plants. The high prevalence of resistance exhibited to doxycycline and kanamycin has been documented in chickens in the country [327]. The detection of a high prevalence of resistance (60.0% to 88.9%) among the top three detected serotypes (Enteritidis, Javiana, and Infantis) may also be therapeutic significance to infected broilers or humans. Differences in the prevalence of resistance to antimicrobial agents by *Salmonella* have been reported to vary among serotypes of *Salmonella* from chickens by others [420, 434]. Therefore, there is a need to monitor the use of the two antimicrobial agents on broiler farms in the country.

4.6 Conclusions

It is concluded that the high prevalence of *Salmonella* (27.0%) including antimicrobial-resistant strains (90.5%), along with the predominance of three

serotypes (Enteritidis, Javiana and Infantis) among the isolates has implications for human salmonellosis in the country. The relative risk of salmonellosis posed by consumption of under-cooked *Salmonella*-contaminated chicken meat from these plants needs to be emphasized. The fact that 10 of the 14 risk factors investigated were statistically significantly associated with the contamination of chicken in the processing lines along with the odds ratio (OR) generated provides critical control points where interventions may be successfully applied. Our study reveals that the WCE method, which is not used for routine surveillance of *Salmonella* in chickens, demonstrated its significantly higher sensitivity when compared with either the WCR or NS methods, a finding which may be indicative of the potential under-reporting of the prevalence of antimicrobial resistant *Salmonella* in chickens in the country. The high prevalence of antimicrobial resistance exhibited by *Salmonella* isolates in this study poses both zoonotic and therapeutic implications to humans exposed to infected chickens. It is imperative to control the use of antimicrobial agents on poultry farms to reduce the development of antimicrobial resistance among *Salmonella* .

Connecting statement to the next chapter

Based on the high prevalence of *Salmonella* on chickens sampled at the four operational processing plants in Trinidad and Tobago, which was determined through longitudinal sampling along the processing lines (Chapter 4), a cross-sectional study was conducted at retail outlets (Chapter 5) to directly assess the risk of human salmonellosis posed to consumers of chicken meat purchased at supermarkets and cottage processors in the country.

CHAPTER 5: PREVALENCE OF *SALMONELLA* SPP. ON CHICKENS SOLD AT RETAIL OUTLETS IN TRINIDAD

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5.1 Abstract

This cross-sectional study determined the prevalence of *Salmonella* spp. and their serotypes on dressed chickens sold at retail outlets comprising cottage poultry processors and supermarkets in Trinidad and Tobago. The study also investigated the risk factors for contamination of dressed carcasses by *Salmonella* spp. at cottage poultry processors' outlets where birds are slaughtered and processed for sale. A total of 133 dressed, whole, chickens and 87 chicken parts were randomly collected from 44 cottage poultry processors and 36 dressed, whole, chickens and 194 chicken parts from 45 supermarket outlets, in the country. Isolation and identification of *Salmonella* spp. were performed using standard bacteriological techniques. Serotyping was done at a regional reference laboratory. The prevalence of *Salmonella* spp. in chickens sampled from cottage poultry processors and supermarket was 20.5% and 8.3% respectively ($p<0.001$). The frequency of isolation of *Salmonella* spp. at cottage poultry processors was 22.4%, 23.0%, 7.1%, and 10.0% for non-chilled whole chicken, non-chilled chicken parts, chilled whole chicken and chilled chicken parts respectively. Fresh, non-chilled chickens (22.6%) yielded a higher frequency of isolation of *Salmonella* spp. than chilled chickens (8.3%). For supermarket samples, the frequency of isolation of *Salmonella* spp. was 19.0%, 8.1%, 0.0% and 7.6% for chilled whole chicken, chill chicken parts, frozen whole chicken and frozen chicken parts respectively. The swab method yielded a statistically significantly ($p=0.029$) higher frequency (3.2 %) of *Salmonella* spp. than the rinse method (1.6%). The predominant serotypes isolated were Kentucky (30.9%) and Javiana (22.7%). Use of chilled water bath to cool carcasses was the only risk factor significantly ($p=0.044$) associated with isolation of *Salmonella* spp. Raw chickens purchased from cottage poultry processors pose a significantly higher risk of contamination by *Salmonella* spp. than those sold at supermarkets.

5.2 Introduction

Globally, despite the institution of several control measures, *Salmonella* infections continue to be problematic with millions of cases occurring annually, both in

humans and animals [47]. The annual incidence of human salmonellosis in the world has been estimated to be 93.8 million cases [435].

Poultry products have particularly served as vehicles of *Salmonella* spp. which have caused human and animal diseases as well as economic losses. Worldwide, foodborne infections are underreported but the problem is exacerbated in developing countries where infections and diseases are grossly under-reported making it difficult to assess the magnitude of the problem [436]. It has however been documented in the literature that the occurrence of chicken borne salmonellosis has varied from 15.7% [437] to 85.0% [438] in humans.

The under-reporting of salmonellosis, particularly in developing countries where facilities for diagnostics and qualified personnel to conduct investigations are inadequate [348, 436]. This has posed challenges to tracing the sources and causative agents implicated in epidemics.

In developing countries, the wet markets or cottage poultry processors serve as important sources of chickens consumed by members of the population. Prevalence rate for *Salmonella* spp. from raw chickens have varied from countries and outlets as documented in Korea, 42.7% [252], Vietnam, 45.9% [253], India, 65.0% [255], Malaysia, 100.0% [418]. Poor sanitary conditions have been attributed to practices at these outlets where hardly any regulatory measures are in place which may have contributed to a high frequency of isolation of *Salmonella* spp. from chicken carcasses [254, 418].

Many risk factors have been reported to be associated with the isolation of *Salmonella* spp. at the retail outlets, particularly at the wet markets or cottage poultry processors. These include the size such as wet markets, supermarkets and

independent poultry stores [253, 254, 439], sources of chickens such as integrated and non-integrated systems [439], storage temperature (ambient, chilling and freezing) [253, 439], region of the country [440] and type of rearing of chickens, conventional and organic [441] amongst other factors.

Commercial outlets such as supermarkets, also serve as source of chickens contaminated by *Salmonella* spp. Reported prevalence of *Salmonella* spp. have ranged from 4.0% to 20.0% in developed countries [246, 261, 262] where stringent measures have been instituted to control salmonellosis on poultry farms and good sanitary practices at poultry processing plants and sale outlets [242, 442] and from 43.0% to 62.5% in developing countries [253, 263-265].

Reported prevalence for *Salmonella* spp. vary globally but these rates are affected by the isolation or detected procedures, therefore when comparing frequencies of detection used, the methodologies used must be considered because they have different sensitivity and specificity [443]. Conventional methods have varied by the strategy used for processing samples such as the rinse method [444-447], whole carcass enrichment [392, 396, 423] and swab method [418, 448], types of non-selective enrichment media [31, 449] and selective enrichment media [450].

The serotypes of *Salmonella* spp. isolated from chickens sampled from retail outlets have varied by country [451, 452] but some serotypes such as *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Newport, and *S.* Derby have repeatedly been recovered from chickens and also associated with poultry-borne infections or outbreaks in humans [246, 348, 442]. Some reports have also documented the changes in the predominant serovars from *S.* Enteritidis to *S.* Heidelberg and *S.* Kentucky and *S.* Derby in the USA [453] and *S.* Enteritidis to *S.* Derby in Uruguay [454], respectively.

Since 1989, *S.* Enteritidis had emerged in the Caribbean as a pathogen of public health concern, causing sporadic cases and outbreaks of diarrhea in both local and tourist populations [348]. There is a dearth of information on the risk of salmonellosis posed by chicken sold at cottage chicken processors as well as chicken carcasses sold by supermarkets. A study conducted in 2006 [31] reported a prevalence of 7.3% for *Salmonella* spp. from chicken at selected cottage processing outlets and another study reported a prevalence of 71.4% for *Salmonella* spp. in chicken from cottage processors in one region of the country [455]. However, both studies did not evaluate the risk factors for *Salmonella* contamination at these outlets. In 2012, Dookeran et al. [28] observed that broiler carcass contamination with *Salmonella* spp. increased during transportation from the farm to the processing plant, during processing and at retail outlets. Moreover, there are no data on the prevalence of *Salmonella* contamination of chicken meat sold by supermarkets in the country.

In Trinidad and Tobago, cottage poultry processors have been reported to be sources of 55% of chickens consumed in the country while supermarkets are sources of 45% [455]. To date, there is a lack of current information on the prevalence and characteristics of *Salmonella* spp. from both types of retail outlets (cottage poultry processors and supermarkets). Therefore, the objectives of this study are to determine the prevalence and serotypes of *Salmonella* spp. in dressed broiler meat sold at retail outlets in Trinidad and Tobago. The study also assesses the risk factors for *Salmonella* contamination of broiler carcasses at the cottage processing outlets.

5.3 Materials and methods

5.3.1 Retail outlets for broiler chickens in Trinidad and Tobago

These retail outlets comprise primarily the cottage poultry processors or ‘pluck shops’ and supermarkets.

Cottage poultry processors are roadside establishments that slaughter and process poultry for customers on request, providing fresh whole chickens or chicken parts (cut up whole chickens). Most of these outlets offer for sale recently slaughtered chickens while a few sell chilled chickens. At the supermarkets, only chilled and frozen chicken carcasses or parts are sold.

The ‘pluck shops’ are conveniently located throughout each county across the country. The operations at these outlets comprise a pen or area where birds are kept prior to slaughter, plastic or galvanize cones that hold the birds prior to and after severing of the jugular vein, large pots/vessels with hot water for scalding birds prior to defeathering, defeathering machine/drums, counter-tops where carcasses are eviscerated and sink/container where carcasses are rinsed with tap water. Some establishments utilise a chilled/iced water bath to cool carcasses before they packaged for sale. Practices at these outlets are often not monitored by the designated authority due to manpower shortage.

Supermarkets are outlets of various sizes that retail poultry meat along with other products. Chicken carcasses originate from local commercial chicken processing plants, where meat inspectors from the Veterinary Public Health Unit conduct routine inspection. Some supermarkets also retail chicken carcasses from other countries.

5.3.2 Location of broiler chicken retail outlets

Retail outlets were located and identified, including the sizes of the supermarkets, using the information provided by the Poultry Surveillance unit, Veterinary Public Health Unit in the Ministry of Health and Association of Supermarket owners. Overall, a total of 265 cottage poultry processor outlets and 125 supermarkets and their locations were identified.

5.3.3 Sources and number of samples

The number of samples collected for this study was estimated using the formula for estimating prevalence from an infinite population size [324] and the estimated minimum sample size was determined to be 384. However, a total of 450 samples were collected. They were represented by 220 samples from outlets of cottage poultry processors and 230 samples from supermarkets. The sampling unit at each retail outlet was five dressed chickens, comprising whole carcasses and chicken parts.

5.3.4 Selection of retail outlets for the study

The cottage processor outlets were selected based on proportional sampling from each of the seven counties in Trinidad and Tobago using simple random sampling methods. Overall, a total of 44 cottage processing outlets were selected. Supermarkets were categorized into sizes based on the number of cashiers at each establishment and the selection from each group were done using proportional sampling and simple random sampling method within each group of supermarkets.

For the study, 46 supermarkets comprising 18, 14, 9 and 5 outlets classified as chain supermarkets (more than 1 outlet), large supermarkets (4 or more cash registers),

medium supermarkets (2-3 cash registers) and small supermarkets (1 cash register only) were included in the sampling plan.

5.3.5 Administration of questionnaire

At each selected cottage processor outlet, consenting owners, after being briefed about the project, were administered a questionnaire to elicit information on demographic data and risk factors for carcass contamination with *Salmonella* spp. These included the number of workers employed, levels of workers' training, experience of workers, source of live birds, sale activity, sanitary practices (scored by a sanitation score sheet: S1 Appendix), and presentation of the carcasses at outlets. The detailed questionnaire is available in S2 Appendix. At supermarkets, information obtained included the source of chickens (processing plants) and presentation (chilled or frozen) for sale.

5.3.6 Processing of samples

At each retail outlet, the five chicken carcasses were collected in separate sterile bags and transported on ice to the laboratory for processing within 3 h. In the laboratory, the initial processing of the carcasses or carcass parts followed the procedure described by Rodrigo et al. [31]. Subsequently the chicken samples were each processed using both the carcass swabs and carcass rinse water.

5.3.6.1 Swab method

A slight modification of earlier reported carcass swab-sampling method [456] was used. Internal and external swabs of each carcass was taken using sterile cotton swabs and placed in 9 ml Buffered Peptone Water (BPW) (Oxoid, Hampshire, England).

5.3.6.2 Rinse method

The same carcasses that were swabbed were then processed by a carcass rinse method [31, 446], a process different from the whole carcass enrichment method [396]. Six milliliters of Phosphate Buffered Saline (PBS, pH 7.2) per gram of carcass weight was placed in a sterile bag and the carcass was massaged and rotated no less than 25 times. From the resulting rinsate, 25 ml were aseptically collected and centrifuged at 4470 g for 20 minutes after which 1 ml of sediment was removed and transferred to 9 ml BPW.

5.3.7 Non-selective and selective enrichments of samples

Both pre- enriched BPW samples (swab and rinse method) were incubated at 37 ^{o}C for 18-24 h. The samples in enrichment media were then selectively enriched in tetrathionate (TT) broth (Oxoid, Hampshire, England) and Rappaport-Vassiliadis Soya (RVS) broth (Oxoid, Hampshire, England) and incubated for 18-24 h at 37 and 42 ^{o}C respectively.

5.3.8 Isolation and identification of Salmonella serotypes

Samples enriched in the selective media (TT and RVS) were sub-cultured onto xylose lysine tergitol 4 (XLT-4; Oxoid, Hampshire, England) and brilliant green agar (BGA; Oxoid) and incubated aerobically at 37 ^{o}C for 18-24 h. Suspected *Salmonella* colonies (pink isolated colonies on BGA, red colonies with black centers on XLT-4) were subjected to biochemical tests for identification of *Salmonella* spp. using standard methods [399]. All isolates biochemically confirmed to be *Salmonella* spp. were subjected to serological typing using *Salmonella* polyvalent antiserum (A-I and Vi, Difco, Detroit, MI). Complete confirmation and serotyping of *Salmonella* isolates representative of those recovered by the carcass rinse/swab, RVS/TT and BGA/XLT-4 methods were done at the Public Health Laboratory, Ministry of Health, Barbados.

5.3.9 Statistical analyses

Chi-square analyses were conducted using the Statistical Package for Social Sciences, SPSS (version 23, IBM Corp., Somers, NY) to determine whether there were statistically significant differences in the frequency of isolation of *Salmonella* spp. amongst (i) risk factors associated with *Salmonella* contamination, (ii) types of outlets (cottage poultry processors/supermarkets), (iii) carcass presentation (whole chicken/chicken parts), (iv) temperature at point of sale (ambient/chilling/freezing temperatures), and (v) method of bacterial isolation (carcass swab/ carcass rinse).

The level of significance was determined at an alpha level of 0.05. Additionally, a mixed effects logistic regression model was constructed with the presence of *Salmonella* in carcasses sampled as the outcome variable and supermarket size (large *versus* others) and whether it was a chain or not, as covariates. The supermarket identification was set a random variable to determine the degree of clustering. For cottage processors, county was used as covariates and cottage processor identification was set as a random variable. The intra-cluster correlation coefficient (ICC) was calculated to measure the proportion of variation between groups as a measure of the total between and with group variation. Data was analysed using STATA 12 (STATA Corporation, College Station Texas).

5.4 Results and Discussion

The relationship of the frequency of isolation of *Salmonella* and eight risk factors studied at the outlets of cottage operators are shown in Table 5.1. The only factor that had statistically significant association with isolation of *Salmonella* spp. was the use of chilled water baths to cool carcasses post-slaughter. In this study, carcasses purchased from outlets that used chilled water had significantly higher prevalence of *Salmonella* spp. (66.7%) compared to outlets that did not (25.7%). It is significant that the chilling water-bath often contained stagnant water, which if

not frequently changed, may result in a buildup of contamination and cross-contamination of carcasses with *Salmonella* spp. and other pathogens. This agrees with published results particularly in road-side poultry processing outlets as reported by others [254, 263, 457, 458]. Infrequent change of chilling water may also lead to an increase of the temperature of the chilling water tank as warm carcasses are constantly added. It has been reported that high temperature of the water bath for chilling may also lead to multiplication of bacteria [459].

Although the frequency of isolation of *Salmonella* spp. across broiler farm sources ranged from 18.2% (Source A) to 40.0% (Source C), the differences were however not statistically significant ($P>0.05$; X^2). This study is not in agreement with Donado et al. [460] who reported that the source (integrated *versus* non-integrated) of live chickens from retail outlets in Columbia significantly affected the frequency of isolation of *Salmonella* spp. from chicken carcasses. This may be explained, in part, by the apparent low prevalence of *Salmonella* spp. in live chickens from broiler farms in the country, ranging from 0.0% to 5.0% [29]. In our study the practices observed at the outlets resulting in cross-contamination, due to the use of chilled water-baths, may be more important contributors to carcass contamination than sources of live chickens. Nidaullah et al. [418] reported a high prevalence of *Salmonella* spp. in carcasses due to cross-contamination throughout the various stages of processing at wet markets. Rivera-Perez et al. [409] also investigated the risk points during broiler carcass processing and reported that *Salmonella* contamination increased from 10% to 40% during evisceration and subsequent spray washing.

Table 5.1. Risk factors for contamination of chicken carcasses by *Salmonella* spp. at outlets of cottage poultry processors in Trinidad and Tobago

Risk Factor	**No. of cottage poultry processor outlets sampled**	**No. (%) positive for *Salmonella* spp.**	**P-value**
Sale activity[a]: Weekday			1
Medium	25	9 (36.0)	
Large	18	6 (33.3)	
Sale activity: Weekends			1
Medium	24	8 (33.0)	
Large	20	7 (35.0)	
Source of live birds			0.801
Source A	11	2 (18.2)	
Source B	21	6 (28.6)	
Source C	10	4 (40.0)	
Source D	21	7 (33.3)	
Source Others	13	5 (38.5)	
Frequency of changing of rinse water			1
Every 20 birds or less	27	9 (33.3)	
Every 21 birds and greater	17	6 (35.3)	
Frequency of general cleaning of work area			1
Once daily	32	11 (34.4)	

More than once daily	12	4 (33.3)	
Frequency of thorough cleaning of work area			1
At least once a week	29	10 (34.5)	
Less than once a week	15	5 (33.3)	
Frequency of cleaning pens			0.761
At least once every 3 weeks	15	5 (33.3)	
Every 1-6 months	13	6 (46.2)	
Use of chilled water bath			0.044
Yes	9	6 (66.7)	
No	35	9 (25.7)	
Chicken sale from counter			0.976
Yes	16	6 (37.5)	
No	28	9 (32.1)	

[a]Medium; <350 birds; Large, 351 or more birds.

Of a total of 44 cottage processor outlets sampled across the seven counties, the frequency of isolation of *Salmonella* spp. was 34.1% (15/44). In each county (7/7; 100.0%) samples positive for *Salmonella* were found. (Table 5.2). The range of outlet positivity for *Salmonella* spp. was from 21.4% (Victoria) to 75.0% (Nariva/Mayaro) (p= 0.650). A study conducted in 2006 in the county of Mayaro [455] reported a prevalence of 71.4% for *Salmonella* spp. at broiler retail outlets, which agrees with our study.

At supermarkets, there was no difference in the odds of isolating *Salmonella* from large supermarkets *versus* other types of establishments or chain outlets. An ICC of 0.22 < 95% CI, 0.18- 0.737> was calculated, which indicates that 22% of the variation in detecting *Salmonella* is due to between supermarket factors and 78% is explained by differences within supermarkets.

As for cottage chicken processors, there was no difference in the odds of detecting *Salmonella* according to county in which the cottage processor outlet was located. An ICC of 0.80 <95% CI, 0.63- 0.91> was calculated which indicates that 80% of the variation in the detection of *Salmonella* in chickens sampled is explained between cottage chicken outlets and 20% is within samples from these cottage chicken outlets. This indicates a high level of clustering in the isolation of *Salmonella* at the level of the outlets.

Of a total of 220 chickens sampled from cottage poultry processors, 45 (20.5%) were positive for *Salmonella* spp. using both the carcass rinse and carcass swab methods. In a study conducted in 2006 using only the rinse method, Rodrigo et al. [31] reported a considerably higher prevalence of *Salmonella* spp. (7.3%) for broilers sampled from cottage poultry processors (‘pluck shops’) compared to a prevalence of only 2.3% (5/220) in the current study using a similar rinse method. Cox et al. [461] reported that there may be an increase or decrease in prevalence based on sampling technique and methods used.

In the current study, the frequency of isolation of *Salmonella* spp. from carcasses ranged from 6.7% (county St. Andrew/St. David) to 55.0% (county Nariva/Mayaro). The differences were statistically significant (P<0.05). However, different results were obtained by Rodrigo et al. [13] that reported a prevalence of 25.0% in county St. Andrew/St. David and 0.0% in counties Caroni and Victoria and 8.3% in county Nariva/Mayaro. The differences between the two studies could

be due, in part, to a true difference in prevalence or differences in methodologies. There have been several reports of higher sensitivity for the isolation of *Salmonella* spp. with buffered peptone water (BPW), Rappaport Vassiliadis Soya broth (RVS) and xylose-lysine decarboxylate- 4 (XLT-4) [428-430] compared to the media used in the study by Rodrigo et al. [31]. The implication of our finding is that chicken purchased at cottage poultry processors in county Nariva/Mayaro pose a higher risk of exposure to *Salmonella* spp. to consumers if improperly cooked. The frequency of isolation of *Salmonella* spp. from broilers from cottage poultry processors in Trinidad and Tobago is lower than those reported for wet markets and similar outlets in Nepal, 38.0% [465], Vietnam, 48.9% [253], China, 52.2% [254], Bangalore- India, 65.0% [255] and Malaysia; 100% [418].

Of a total of 46 supermarkets visited, 13 samples (28.3%) yielded *Salmonella* spp. with a range of 11.1% (Medium-sized outlets) to 50% (Large-sized outlets) ($p= 0.107$). Nineteen (8.3%) of 230 chicken carcasses sampled were positive for *Salmonella* spp. ($p= 0.023$). Studies conducted at supermarket outlets elsewhere have documented higher prevalence of *Salmonella* spp. as in Vietnam, 43.0% [466] and in China, 51.2% [254] where large supermarkets accounted for 50.3% and small supermarkets, 52.1% of the positive samples, respectively. The differences may be accounted for by many factors including high prevalence of *Salmonella* spp. in live broilers at slaughter, hygienic practices during slaughter at the wet markets [418, 466] and sensitivity of the laboratory techniques used in the isolation of the organism [425].

It is important for food safety to have detected that the prevalence (20.5%) of *Salmonella* spp. from cottage processor outlets was statistically significantly higher ($P<0.05$) than the prevalence found in supermarkets (8.3%). Therefore, chicken carcasses purchased from cottage poultry processors in Trinidad and Tobago pose a higher risk of salmonellosis than those that originate from supermarkets. On the contrary, studies conducted by Yang et al. [254] in China and Ta et al. [253] in

Vietnam reported no significant differences in the isolation rates from chickens sampled at wet markets and supermarkets. The reason for the higher isolation rate at cottage poultry processors could be due to cross-contamination resulting from poor hygiene and manual handling of the chicken during processing thereby exacerbating cross-contamination [418]. Additionally, the lower isolation rate from supermarkets in our study may be because most of the broiler chickens sold in supermarket outlets originated from commercial processing plants where sanitary practices are enforced by personnel of the Veterinary Public Health unit while no such quality control occurs at the cottage processor outlets.

Overall, 64 (14.2%) of 450 chicken samples from retail outlets (cottage poultry processors and supermarkets) were positive for *Salmonella* spp., a prevalence at the lower end of the 12-85% range for isolation of the pathogen from retail outlets worldwide [467].

Table 5.2. Frequency of isolation of *Salmonella* spp. from broilers sold at retail outlets across Trinidad and Tobago

Type of retail outlet	**County**	**Total No. of Cottage poultry processors[a]**	**No. (%) of Cottage poultry processors sampled**	**No. (%) of Cottage poultry processors positive for *Salmonella* spp.[b]**	***P-value***	**No. of chickens tested**	**No. (%) of chickens positive for *Salmonella* spp.[b]**	**P-value**
Cottage poultry processors	St George Central	17	3 (17.6)	1 (33.3)	0.65	15	5 (33.3)	<0.05
	St George East	27	5 (18.5)	2 (40.0)		25	4 (16.0)	
	St Andrew/St David	19	3 (15.8)	1 (33.3)		15	1 (6.7)	
	Nariva/Mayaro	23	4 (17.4)	3 (75.0)		20	11 (55.0)	
	Caroni	40	7 (17.5)	2 (28.6)		35	3 (8.6)	
	Victoria	91	14 (15.4)	3 (21.4)		70	10 (14.3)	
	St Patrick	48	8 (16.7)	3 (37.5)		40	11 (27.5)	
	Sub-Total	265	44 (16.6)	15 (34.1)		220	45 (20.5)	
Supermarkets	**Group of supermarket**	**Total No. of supermarkets[c]**	**No. (%) of supermarkets sampled**	**No. (%) of supermarkets positive for *Salmonella* spp.[b]**	**P value**	**No. of chickens tested**	**No. (%) of chickens positive for *Salmonella* spp.[b]**	**P value**
	Chain	74	18 (24.3)	3 (16.7)	0.107	90	4 (4.4)	0.023

	Large	18	14 (77.8)	7 (50.0)		70	11 (15.7)	
	Medium	17	9 (52.9)	1 (11.1)		45	1 (2.2)	
	Small	16	5 (31.3)	2 (40.0)		25	3 (12.0)	
	Subtotal	125	46 (36.8)	13 (28.3)		230	19 (8.3)	
	Total	390	90 (23.1)	28 (31.1)		450	64 (14.2)	

[a]Total number of cottage poultry processors in Trinidad and Tobago.
[b]Based on the use of both rinse and swab methods.
[c]Group of supermarkets: Chain- supermarket having more than one (1) outlet; Large- supermarket having 4 or more cash registers; Medium- supermarket having 2-3 cash registers; Small- supermarket having only 1 cash register.

The effects of the temperature (fresh/non-chilled, chilled and frozen) and type (whole and parts) of chicken carcasses presented for sale at the retail outlets on the frequency of isolation of *Salmonella* spp. are shown in Table 5.3. The range of isolation of *Salmonella* spp. based on temperature was from 0.0% for frozen chickens to 22.6% for fresh, non-chilled chickens (P>0.05). Of 190 fresh, non-chilled carcasses sampled, 43 (22.6%) were positive for *Salmonella* spp. whereas only 2 (8.3%) of 24 chilled carcasses sampled were contaminated by the pathogen (P>0.05). A possible reason for the lower isolation rate with a decrease in temperature could be because low temperatures have been reported to have bacteriostatic potential thereby causing injury to the bacterial cell wall, limiting its proliferation in non-selective and selective media [468]. In a review of 18 studies done worldwide, Huda et al. [467] reported that the highest contamination potential with *Salmonella* for chicken stored at room temperature was 37% with a lower and upper average of 24-29%. This is slightly higher than the 22.6% isolation rate detected in our un-chilled carcasses. Additionally, the authors reported that contamination of chicken stored at chilled and frozen temperatures were 34 and 35%, respectively. These findings are however higher than those detected in our study, where chilled and frozen chickens yielded *Salmonella* spp. at a rate of 8.3% and 0.0%, respectively. The differences in the duration of storage of chicken carcasses at ambient and chilling temperatures, which were not investigated in the current study, may also be responsible, in part, for our findings. Ta et al. [253] also failed to detect significant differences in the isolation rate of the organism with respect to storage temperatures (room temperature and chilled products), in agreement with our findings.

A comparison of the type of presentation (whole and part) of carcasses sampled from cottage processor outlets, revealed *Salmonella* isolation rates of 20.3% (27/133) and 20.7% (18/87) from whole carcasses and carcass parts, respectively (P>0.05). This suggests that the practice of cutting whole chickens into parts did not significantly increase *Salmonella* contamination.

For the samples collected from supermarket outlets where fresh, non-chilled chickens were not sold, the frequency of isolation of *Salmonella* spp. was 12.1% (7/58) for chilled carcass samples compared with 7.0% (12/172) for frozen carcasses but the difference was not statistically significant (p=0.097). Considering the possible effect of temperature on types of presentation, for whole carcass samples the frequency of isolation from chilled chicken was 19.0% (4/21) compared to 0.0% (0/15) for frozen whole chicken but the difference was not statistically significant (p=0.125). As for chicken parts, the frequency of isolation of *Salmonella* spp. was 8.1% (3/37) and 7.6% (12/157) for chilled chicken and frozen chicken respectively and the difference was not statistically significant (p=1). *Salmonella* was isolated from 4 (11.1%) of the 36 whole carcasses and from 15 (7.7%) of the 194 frozen part samples but the difference was not statistically significant (p= 0.510). Since the minimum growth temperature for *Salmonella* on poultry meat is 5^{o}C, with an optimum temperature ranging between 35-43^{o}C [469], this could have contributed to the difference in isolation rates based on storage conditions. Morris and Wells [470] reported an increase in isolation rate from chilled carcasses rotated in an ice slush and suggested that extensive contact between carcasses may have contributed to cross-contamination. Additionally, Ahmed et al. [468] found that freezing below -18^{o}C of *Salmonella*-positive carcasses eliminated its presence and suggested this was due to the low temperature damaging the bacterial cell wall leading to the death of the pathogen. Contrary to the previously mentioned studies and similar to our results, Yang et al. [254] found no significant difference between storage temperature and the isolation of *Salmonella* on raw poultry in China.

Of the 203 chicken samples of local origin collected from supermarket outlets, 18 (8.9%) were positive for *Salmonella* spp. while of the 27 samples of foreign origin, only 1 (3.7%) was positive (p=0.707). The difference in frequency of isolation of *Salmonella* spp. from chicken obtained from local processing plants compared with

those from plants in the USA sources, although not statistically significant, may reflect the fact that local chickens offered for sale at supermarkets originate from processing plants where thorough poultry inspection and sanitary practices are enforced, similar to that in the USA. The majority of imported poultry products to Trinidad and Tobago originate from the USA.

Table 5.3. Frequency of isolation of *Salmonella* spp. by presentation and temperature conditions at which chicken carcasses are kept at cottage poultry processors and supermarket outlets

Non-chilled				**Chilled**			**Frozen**			**All types**	
Type of retail outlet	**Type of chicken presentation**	**No. of samples tested**	**No. (%) positive for *Salmonella* spp.[a]**	**No. of samples tested**	**No. (%) positive for *Salmonella* spp.[a]**	***P- value***	**No. of samples tested**	**No. (%) positive for *Salmonella* spp.[a]**	***P-value***	**No. of samples tested**	**No. (%) positive for *Salmonella* spp.[a]**
Cottage poultry processor	Whole	116	26 (22.4)	14	1 (7.1)	0.327	3	0 (0.0)	NA	133	27 (20.3)
	Parts	74	17 (23.0)	10	1 (10.0)	0.635	3	0 (0.0)		87	18 (20.7)
	Total	190	43 (22.6)	24	2 (8.3)	0.176	6	0 (0.0)		220	45 (20.5)
	P-value		1		0.804			NA			1
Supermarket	Whole	NA	NA	21	4 (19.0)	NA	15	0 (0.0)	0.125	36	4 (11.1)
	Parts	NA	NA	37	3 (8.1)	NA	157	12 (7.6)	1	194	15 (7.7)
	Total	NA	NA	58	7 (12.1)	NA	172	12 (7.0)	0.097	230	19 (8.3)
	P-value	NA	NA		0.241			0.603			0.51

NA: Not applicable.

[a]Based on the use of both rinse and swab methods.

The frequency of isolation of *Salmonella* spp. from chickens reared under the conventional production system was 8.1% (17/209), therefore similar (p=0.687) to that observed for organically reared chickens (9.5%; 2/21). The low sample size of organically-reared chickens available for sampling in the current study was primarily because this is not a common practice in the country.

For chicken samples collected from supermarket outlets, the frequency of isolation of *Salmonella* spp. was similar for the rinse method (3.9%) and the swab method (3.5%) and the difference was not statistically significant (p=1) as shown in Table 5.4. However, for chicken samples from cottage poultry processors, the swab method (9.5%) was more sensitive (p=0.002) than the rinse method (2.3%). Overall, the swab method was determined to be a more sensitive method, 3.2% (29/450) than the rinse method, 1.6% (14/450, p=0.029).

In addition, the swab method yielded significantly a higher isolation rate for *Salmonella* spp. in whole chicken sampled at cottage poultry processors (11.3%; 15/133) *versus* the rinse method (2.3%; 3/133). Overall, for whole chicken samples (supermarkets and cottage poultry processors), 10.5% (20/191) were positive for *Salmonella* spp. by the swab method compared to 2.6% (5/191) by the rinse method (p=0.004). The finding that there was no statistically significant difference in the isolation rate of *Salmonella* spp. by the two methods for the samples collected at the supermarkets, together with the lower frequency of isolation, may suggest differences in the quantitative level of contamination between meats sold at supermarkets and cottage poultry processors. Swab method is used for environmental sampling [418] and large animal studies where the entire carcass cannot be used for testing. Whilst the swab techniques are commonly reported to focus on one site, there have been reports where two or three sites and whole swab techniques were compared and it was found that multi-site swabbing yielded significantly higher isolation rates of bacteria compared with the swabbing of one site only [448, 471]. Additionally, whole carcass sampling via rinsing or swabbing

was deemed necessary for optimum *Salmonella* recovery [448]. The findings in our study are however at variance with other studies which suggest that the swab method is not as sensitive as other methods such as excision of skin/muscle tissue or rinsing [456, 472].

Table 5.4. Frequency of isolation of *Salmonella* spp. by the rinse and swab methods

Source of sample	**Type of samples processed**	**No. of samples tested**	**No. (%) positive for *Salmonella* spp. by:**			
			Rinse method	**Swab method**	***P-value***	**Total**
Supermarkets	Whole chicken	58	2 (3.4)	5 (8.6)	0.438	7 (6.0)
	Parts	172	7 (4.1)	3 (1.7)	0.336	10 (2.9)
	Sub total	230	9 (3.9)	8 (3.5)	1	17 (3.7)
	P-value				0.057	
Cottage poultry processors	Whole chicken	133	3 (2.3)	15 (11.3)	0.007	18 (6.8)
	Parts	87	2 (2.3)	6 (6.9)	0.278	8 (4.6)
	Sub total	220	5 (2.3)	21 (9.5)	0.002	26 (5.9)
	P-value				0.195	
Total	Whole chicken	191	5 (2.6)	20 (10.5)	0.004	25 (6.5)
	Parts	259	9 (3.5)	9 (3.5)	1	18 (3.5)
	Sub total	450	14 (1.6)	29 (3.2)	0.029	43 (4.8)
	P-value				0.004	

The serotypes of *Salmonella* spp. isolated from the retail outlets were compared based on the type of samples collected (Table 5.5). Of a total of 68 isolates of *Salmonella* spp. from cottage processor outlets, 10 different serotypes were identified while 6 serotypes were identified among the 29 isolates from supermarket outlets. Whole carcasses accounted for 58.8% and 17.2% of the serotypes recovered from samples collected from the cottage poultry processors and supermarkets, and 38.2% and 82.8% for chicken parts respectively. The predominant serotypes

detected at cottage poultry processors were *S.* Javiana (30.9%; 21/68), *S.* Kentucky; 23.5%; 16/68) and *S.* Manhattan (16.2%; 11/68) whereas at supermarkets *S.* Kentucky, *S.* subspecies enterica I and *S.* San Diego accounted for 47.3%, 27.6% and 10.3% of the serotypes, respectively.

At cottage poultry processors, serotypes *S.* subspecies enterica I, *S.* Group D, *S.* Bloomsbury and *S.* Group C were detected in whole carcasses but not in chicken parts, whereas serotypes *S.* Aberdeen and *S.* Schwarzengrund were detected in chicken parts and not in whole carcasses.

At supermarkets, the serotypes detected differed between the types of sample, with *S.* Javiana being detected in whole carcasses but not in chicken parts. Serotypes detected in chicken parts but not in whole carcasses included *S.* subspecies enterica I, *S.* San Diego, *S.* Montevideo and *S.* Westhampton. Overall, serotypes detected in whole carcasses alone included *S.* Group D, *S.* Bloomsbury and *S.* Group C while serotypes being detected in chicken parts alone comprised *S.* Aberdeen, *S.* Schwarzengrund, *S.* San Diego, *S.* Montevideo and *S.* Westhampton. The differences in the serotypes of *Salmonella* spp. between the different type of sample could have been due to differences in origin and source of broiler chickens, for example importation, which were not determined in the current study.

Previous studies conducted in the country and the Caribbean region on broilers, layers and eggs have detected several serotypes. Notably Rodrigo et al. [13] reported isolation of serotypes *S.* Kiambu, *S.* Kentucky and *S.* Mbandaka in broilers, whereas Adesiyun et al. [29] detected *S.* Typhimurium in Trinidad and Tobago. In Jamaica, *S.* Austenborg and *S.* Kentucky were isolated from broilers [309]. Studies conducted on eggs and layer farms detected *S.* Enteritidis, *S.* Mbandaka, *S.* Javiana, *S.* Caracas, *S.* Ohio, *S.* Braenderup and *S.* Georgia in Trinidad and Tobago [473] and *S.* Enteritidis in Barbados [305].

According to the Caribbean Public Health Agency (CARPHA) State of Public Health report [348], *S.* Kentucky, *S.* Montevideo, *S.* Javiana and *S.* subspecies enterica, which were found in the current study, were amongst the top 15 human *Salmonella* serotypes detected in the region as well as the most common *Salmonella* serotypes associated with human salmonellosis in Trinidad and Tobago between 2005-2012 and are therefore of public health significance. This is of importance since the most recent study in the country, prior to this project, was performed in 2006 [31] where only three serotypes (Kiambu, Kentucky and Mbandaka) were detected from the cottage poultry processors. In the current study, 10 serovars (*S.* Javiana, *S.* Kentucky, *S.* Manhattan, *S.* Aberdeen, *S.* Bloomsbury, *S.* Schwarzengrund, *S.* Molade, *S.* Montevideo, *S.* San Diego, *S.* Westhampton) were recovered. These serotypes may therefore have public health implications for consumers of improperly cooked chicken from the outlets studied.

Table 5.5. Serotypes of *Salmonella* spp. isolated from retail outlets and by type of sample

Type of outlet	**Serotype**	**No. (%) of isolates**	**Type of sample**
Cottage poultry processor	Javiana	11 (16.2)	Whole carcass
	Kentucky	10 (14.7)	Whole carcass
	Manhattan	7 (10.3)	Whole carcass
	Warragul[a]	5 (7.4)	Whole carcass
	subspecies enterica I	2 (2.9)	Whole carcass
	Group D	2 (2.9)	Whole carcass
	Bloomsbury	2 (2.9)	Whole carcass
	Group C	1 (1.5)	Whole carcass
	Javiana	10 (14.7)	Chicken parts
	Kentucky	6 (8.8)	Chicken parts

	Manhattan	4 (5.9)	Chicken parts
	Aberdeen	3 (4.4)	Chicken parts
	Warragul[a]	2 (2.9)	Chicken parts
	Schwarzengrund	1 (1.5)	Chicken parts
	Subtotal	68 (70.1)[b]	Chicken parts
Supermarket	Kentucky	4 (13.8)	Whole carcass
	Javiana	1 (3.4)	Whole carcass
	Kentucky	10 (34.5)	Chicken parts
	subspecies enterica I	8 (27.6)	Chicken parts
	San Diego	3 (10.3)	Chicken parts
	Montevideo	2 (6.9)	Chicken parts
	Westhampton	1 (3.4)	Chicken parts
	Subtotal	29 (29.9)[c]	Chicken parts
	Total	97	

[a] Possible *Salmonella* Warragul
[b] Of a total of 68 isolates
[c] Of a total of 29 isolates

5.5 Conclusions

Data from this study indicate the extent of contamination by *Salmonella* spp. in the selected outlets of cottage processors and supermarkets and the risk of salmonellosis occurring in consumers of contaminated, undercooked chicken meat sold at retail outlets in the country**.**

Connecting statement to the next chapter

This chapter focussed primarily on the serotypes and risk factors associated with *Salmonella* isolation at retail outlets. The following chapter (Chapter 6) further characterises these *Salmonella* isolates using phenotypic antimicrobial sensitivities.

CHAPTER 6: ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATES RECOVERED FROM CHICKENS SOLD AT RETAIL OUTLETS IN TRINIDAD

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6.1 Abstract

This study determined the frequency of resistance of 135 isolates of *Salmonella*, including 15 serotypes recovered from chickens purchased from retail outlets (cottage processors and supermarkets) across Trinidad and Tobago. Resistance to 16 antimicrobial agents was determined by using the disk diffusion method. Resistance among the isolates was related to the type of retail outlet, location of outlets, type of sample, and isolate serotype. Overall, all isolates exhibited resistance to one or more of the 16 antimicrobial agents tested. All isolates were sensitive to cefoxitin and norfloxacin, with the overall frequency of resistance ranging from 1.1% (sulfamethoxazole-trimethoprim) to 100.0% (ceftiofur and doxycycline). The frequency of resistance to tetracycline, ampicillin, ceftriaxone, amoxycillin–clavulanic acid, and chloramphenicol was significantly ($P < 0.05$) higher in isolates recovered from cottage processor outlets compared with those from supermarkets. The frequency of resistance to antimicrobial agents was significantly different only to kanamycin ($P= 0.046$) and enrofloxacin ($P= 0.000$) across seven counties in Trinidad and Tobago). Regarding sample presentation (whole versus parts), the frequency of resistance was only significantly higher to gentamicin ($P= 0.039$) for chicken part isolates from cottage processor and to only tetracycline ($P= 0.034$) for isolates from whole carcasses from supermarkets. All the 135 *Salmonella* isolates exhibited multidrug resistance patterns. The high frequency of resistance to seven antimicrobial agents (erythromycin, streptomycin, ceftiofur, doxycycline, kanamycin, tetracycline, and ampicillin), some used in the poultry industry, coupled with the occurrence of multidrug resistance, may have potential therapeutic implications for broiler farmers in Trinidad and Tobago.

6.2 Introduction

Salmonella infections in humans and animals continue to be a major issue across the globe [474]. Although vaccination against salmonellosis in poultry has been useful [475], it may be ineffective with more pathogenic, nonhost-adapted *Salmonella* serovars [476]. In countries in which vaccination against salmonellosis

is practiced, there is a reliance on good farm management and sanitary practices, as well as prophylactic use of antimicrobial agents in feed and water [362]. As a consequence of poor preventive measures, the improper use of antimicrobial agents in food-producing animals [18, 19], as well as unregulated use of antibiotics in humans in developing countries [20, 21], has contributed to the increase in antimicrobial resistance. Food contaminated with antibiotic-resistant *Salmonella*e constitutes a major threat to public health because it has been established that resistant genes can be transferred to other pathogenic bacteria [447, 448] that potentially could compromise therapy and result in mortalities.

Salmonella spp. isolated from eggs, layers, and environmental samples on poultry farms in three Caribbean countries: Trinidad and Tobago, Grenada, and Saint Lucia found the frequency of resistance of *Salmonella* spp. and *Escherichia* coli isolates from table eggs to be 22.9 and 88.1%, respectively [365]. In studies conducted in Grenada, the antimicrobial sensitivity of *E.* coli strains recovered from free-range chickens [479], as well as broiler and layer chickens [480], was determined and found that free-range chickens exhibited resistance (71 of 202, 35.1%) to one or more agents. In Trinidad and Tobago, 97.8% of *Campylobacter* spp. recovered from chickens sampled from cottage processors exhibited resistance to one or more antimicrobial agents tested [481]. In Trinidad and Tobago, cottage processors have been reported to serve as 55% of chickens consumed in the country, while supermarkets are sources of 45% [299].

Cottage poultry processors are roadside establishments that slaughter and process poultry for consumers on request, providing fresh whole chickens or fresh chicken parts (cutup whole chickens). They are conveniently located throughout each county across the country. The practices and operations at these outlets are not monitored by meat inspectors as done for formal poultry chicken processing plants. Supermarkets in Trinidad and Tobago sell chilled or frozen chickens to consumers that are slaughtered and processed at formal poultry chicken processing plants.

Additionally, in Trinidad and Tobago, there is no legislative requirement regarding the presence or absence of *Salmonella* spp. in food or raw chicken meat; however, food in general must be safe and unadulterated.

There is paucity of information on antimicrobial resistance of *Salmonella* obtained from broiler meat in this country. Therefore, the objective of this study was to determine the antibiograms of *Salmonella* isolates recovered from processed chickens sampled from retail outlets in Trinidad and Tobago and to relate the frequency of resistance to the type of retail outlets, location of outlets, the type of sample, and the serotypes of isolates.

6.3 Materials and methods

6.3.1 Sources and number of samples

The number of samples collected for this study was estimated [324], and the estimated sample size was determined to be 384. However, considering the number of operating cottage poultry processors in the country at the time the current study was conducted, a total of 450 samples were collected at retail outlets, including 220 from cottage processors and 230 from supermarkets. The sampling unit at each retail outlet was five chickens. Samples were collected once from each of the selected outlets and included three whole carcasses and two parts (whole carcass cut into smaller pieces, approximately 40 pieces).

6.3.2 Selection of retail outlets for the study

Retail outlets (cottage poultry processors and supermarkets) were randomly selected by using information provided by the Poultry Surveillance Unit, Veterinary Public Health Unit in the Ministry of Health, and the Association of Supermarket owners. Overall, a total of 44 cottage processors and 46 supermarkets were sampled.

The cottage processor outlets were selected on the basis of the proportional sampling from each of the seven counties in Trinidad and Tobago by using stratified random sampling methods using a lottery method [482]. For supermarket outlets, these were classified based on the number of cashiers at each establishment, and the outlets from each class were selected by using proportional sampling for the different classes and stratified random sampling method [482] within each class of supermarket. The supermarkets were classified as chain supermarkets (with more than one outlet), large supermarkets (with four or more cash registers), medium supermarkets (with two to three cash registers), and small supermarkets (with only one cash register).

6.3.3 Processing of samples

A modification of the previously reported swab-sampling method [456] and the rinse method [31, 446] were used to process the samples, and the isolation technique for *Salmonella* spp., which was reported earlier was used [362]. Samples were pre-enriched in buffered peptone water (Oxoid, Ltd., Hampshire, UK) for 18 to 24 h at 37 °C, then selectively enriched in tetrathionate broth (Oxoid) and in Rappaport-Vassiliadis soya broth (Oxoid), and thereafter were incubated for 18 to 24 h at 37 and 42 °C, respectively.

6.3.4 Isolation, identification, and serotyping of Salmonella spp.

Samples enriched in selective broths (tetrathionate and Rappaport Vassiliadis soya) were then subcultured onto xylose lysine Tergitol 4 (Oxoid) and brilliant green agar (Oxoid) and were incubated aerobically at 37 °C for 18 to 24 h. Suspected *Salmonella* colonies that displayed characteristic colonies on both selective agar plates were then purified on blood agar plates (Oxoid) and incubated at 37 °C for 18 to 24 h. Pure cultures were subjected to a panel of biochemical tests that included triple sugar iron agar, lysine iron agar, urea, citrate, methyl red, sulfide-indole-

motility medium, and o-nitrophenyl-β-D-galactopyranoside (Oxoid) [399]. Biochemically confirmed isolates were then subjected to serological typing by using *Salmonella* polyvalent antiserum (A-I and Vi, Difco, Detroit, MI). Complete confirmation and serotyping of *Salmonella* isolates were performed by using the phase reversal technique, and the results interpreted according to the Kauffman-White scheme [43] at the Public Health Laboratory, Ministry of Health, St. Michael, Barbados.

6.3.5 Determination of resistance to antimicrobial agents

The antimicrobial resistance of 135 isolates of *Salmonella* recovered from retail outlets across Trinidad and Tobago (Fig. 6.1) was determined by using the disk diffusion method according to the Clinical and Laboratory Standards Institute [162] guidelines. Sixteen antimicrobial agents commonly available in the local market and frequently used in the poultry industry in Trinidad and Tobago were included in the following panel and disc concentration: ceftiofur (EFT, 30 µg); erythromycin (E, 15 µg); streptomycin (S, 10 µg); doxycycline (DO, 30 µg); kanamycin (K, 30 µg); tetracycline (TE, 30 µg); ampicillin (AM, 10 µg); ceftriaxone (CRO, 30 µg); amoxycillin–clavulanic acid (AMC, 30 µg); chloramphenicol (C, 30 µg); enrofloxacin (ENR, 5 µg); ciprofloxacin (C, 5 µg); gentamicin (CN, 10 µg); sulfamethoxazole- trimethoprim (SXT, 23.75 and 1.25 µg); norfloxacin (NOR, 10 µg); and cefoxitin (FOX, 30 µg; Oxoid). Three agents belonged to each of the classes quinolones, aminoglycosides, and cephalosporins, two agents each belonged to the beta-lactam and tetracycline classes, whereas one agent belonged to the macrolide, sulfonamide, and phenicol classes, respectively. The tests were performed on Mueller-Hinton agar (Difco), followed by aerobic incubation at 35 °C for 24 h. The zones of inhibition were interpreted as recommended by the disk manufacturer and Clinical and Laboratory Standards Institute [162]. For this study, isolates that exhibited intermediate and resistance zones of inhibition were classified as resistant.

6.3.6 Statistical analyses

Data were analysed by using the SPSS (Version 23, IBM Corp., Armonk, NY). Chi-square analysis was conducted to determine whether there were statistically significant differences in the frequency of resistance to antimicrobial agents among *Salmonella* isolates recovered between retail outlets (cottage processors versus supermarkets), counties across the country, presentation of carcasses (whole carcass and carcass parts), and the serotypes of *Salmonella* spp. The level of significance was determined at an alpha level of 0.05.

6.4 Results

6.4.1 Frequency of resistance of Salmonella isolates to antimicrobial agents

All 135 isolates were resistant to one or more of the 16 antimicrobial agents tested and to EFT, E, and S. However, all isolates were sensitive to FOX and NOR (Figure 6.1). Overall, for the retail outlets, resistance was relatively high to EFT (99.3%), DO (99.3%), and K (91.1%), but relatively low to CIP (3.7%), CN (3.0%), and SXT (1.5%), and these differences were statistically significant ($P < 0.05$). For 11 of the 12 antimicrobial agents to which resistance was exhibited, frequency of resistance was higher for isolates from cottage processor outlets than for those from supermarkets. Of these, significantly higher

frequency of resistance was detected to TE ($P= 0.003$), AM ($P= 0.011$), CRO ($P= 0.000$), AMC ($P= 0.026$), and C ($P = 0.005$). All isolates were sensitive to FOX (30 µg) and NOR (10 µg). All isolates were resistant to E (15 µg) and S (10 µg).

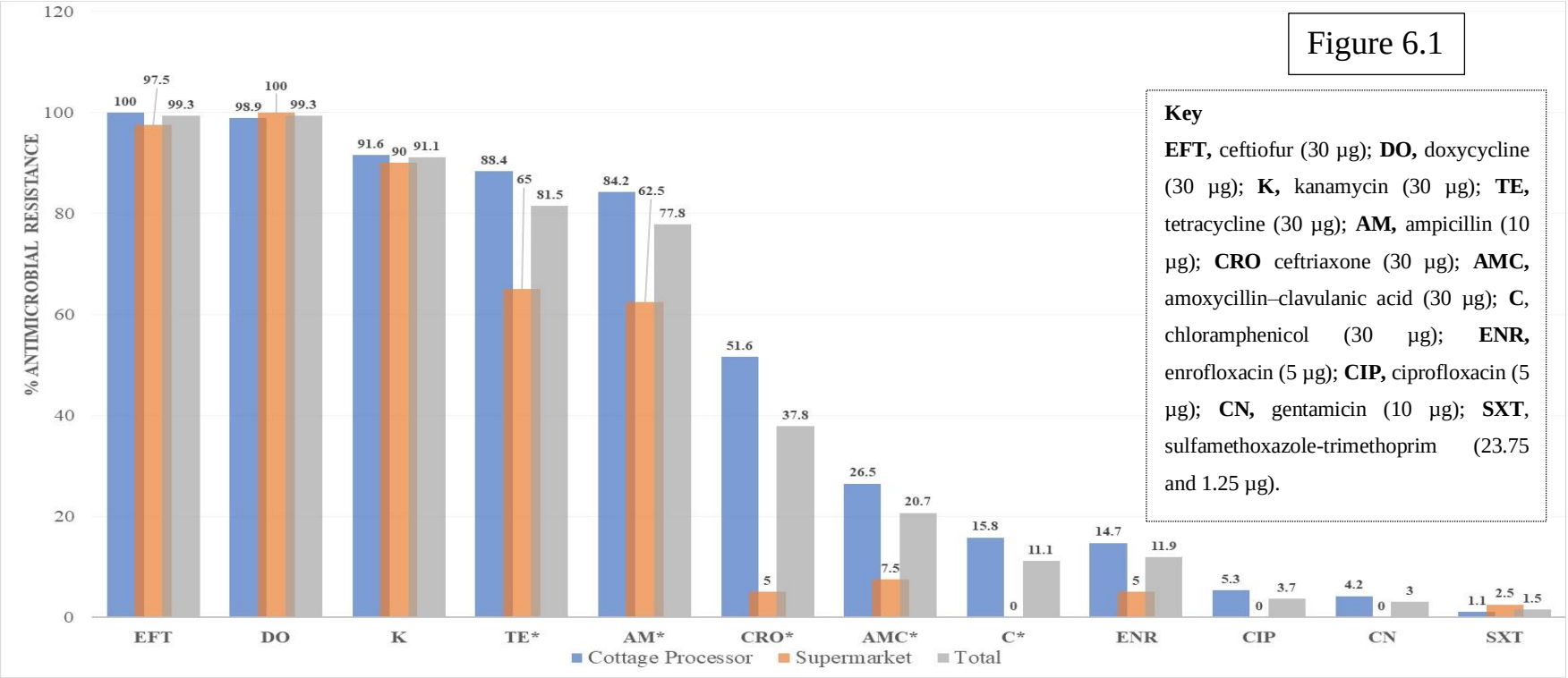

Figure 6.1. Frequency of resistance to antimicrobial agents among *Salmonella* isolates recovered from chickens sold at retail outlets in Trinidad and Tobago

* $P < 0.05$, significant differences between isolates obtained from cottage processors and supermarkets.

6.4.2 Frequency of resistance of Salmonella isolated at cottage poultry processors

All 95 isolates of *Salmonella* spp. from the seven counties were resistant to one or more antimicrobial agents (Table 6.1). Of the 14 antimicrobial agents to which the isolates exhibited resistance, the differences in the frequency of resistance for isolates of *Salmonella* spp. across the seven counties were statistically significant for K (P , 0.05), with a range from 50.0% (Caroni) to 100.0% (Saint Andrew–Saint David and Saint Patrick) and for ENR (P , 0.05), ranging from 0.0% (Saint George East, Saint Andrew–Saint David, Caroni, Victoria, and Saint Patrick) to 52.4% (Nariva-Mayaro). Across the seven counties, the frequency of resistance to DO (96.2 to 100.0%), TE (73.1 to 100.0%), and AM (70.8 to 100.0%) was relatively high but comparatively low to CIP (0.0 to 20.8%), CN (0.0 to 8.3%), and SXT (0.0 to 4.2%).

Table 6.1. Frequency of antimicrobial resistance of *Salmonella* isolates obtained from cottage poultry processors in various counties in Trinidad and Tobago

County	No. of isolates tested	No. (%) of resistant isolates[a]	No. (%) resistant[b, c] to:										
			DO	K	TE	AM	CRO	AMC	C	ENR	CIP	CN	SXT
St George Central	10	10 (100.0)	10 (100.0)	8 (80.0)	9 (90.0)	9 (90.0)	7 (70.0)	2 (20.0)	1 (10.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)
St George East	6	6 (100.0)	6 (100.0)	5 (83.3)	5 (83.3)	5 (83.3)	4 (66.7)	2 (33.3)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
St Andrew/St David	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nariva/Mayaro	24	24 (100.0)	24 (100.0)	23 (95.8)	23 (95.8)	17 (70.8)	11 (45.8)	4 (16.7)	0 (0.0)	13 (54.2)	5 (20.8)	0 (0.0)	0 (0.0)
Caroni	4	4 (100.0)	4 (100.0)	2 (50.0)	4 (100.0)	3 (75.0)	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Victoria	26	26 (100.0)	25 (96.2)	23 (88.5)	19 (73.1)	24 (92.3)	7 (26.9)	5 (19.2)	7 (26.9)	0 (0.0)	0 (0.0)	2 (7.7)	0 (0.0)
St Patrick	24	24 (100.0)	24 (100.0)	24 (100.0)	23 (95.8)	21 (87.5)	16 (66.7)	12 (50.0)	6 (25.0)	0 (0.0)	0 (0.0)	2 (8.3)	1 (4.2)
Total	95	95 (100.0)	94 (98.9)	86 (90.5)	84 (88.4)	80 (84.2)	49 (51.6)	25 (26.3)	15 (15.8)	14 (14.7)	5 (5.3)	4 (4.2)	1 (1.1)
P-value			0.848	0.046	0.153	0.493	0.051	0.091	0.140	0.000	0.140	0.708	0.810

[a] Resistant to one or more antimicrobial agents.

[b] DO, doxycycline- 30 µg; K, kanamycin- 30 µg; TE, tetracycline- 30 µg; AM, ampicillin- 10 µg; CRO, ceftriaxone- 30 µg; AMC, amoxycillin- clavulanic acid- 30 µg; C, chloramphenicol- 30 µg; ENR, enrofloxacin- 5 µg; CIP, ciprofloxacin- 5 µg; CN, gentamicin- 10 µg; SXT, sulphamethoxazole-trimethoprim- 23.75/1.25 µg.

[c] All isolates were sensitive to Cefoxitin (30 µg) and Norfloxacin (10 µg); All isolates were resistant to Ceftiofur (30 µg), Erythromycin (15 µg) and Streptomycin (10 µg).

6.4.3 Frequency of resistance of Salmonella isolates by type of sample

For both types of retail outlets (cottage processor and supermarkets), the frequency of resistance to antimicrobial agents among *Salmonella* spp. were similar for most antimicrobial agents for isolates from whole carcasses and carcass parts (Table 6.2). The frequencies ranged from 88.4 to 100.0% to EFT, DO, K, and TE and were similar across both retail outlets, except for AM, where the frequency of resistance detected was 84.2 and 62.5% for isolates recovered from cottage poultry processors and supermarkets, respectively. However, for isolates of *Salmonella* spp. from cottage processors, the frequency of resistance to CN was significantly (P = 0.039) higher for isolates recovered from chicken parts (4 of 43, 9.3%) than for those from whole carcasses (0 of 52, 0.0%). For isolates of *Salmonella* that originated from supermarkets, the frequency of resistance to TE was significantly (P = 0.034) higher for whole carcass isolates (8 of 8, 100.0%) than for carcass parts (18 of 32, 56.3%).

Overall, for both retail outlets, a significantly (P = 0.005) higher frequency of resistance to CRO among *Salmonella* spp. was detected for isolates from whole chickens (31 of 60, 51.7%) than for those from carcass parts (20 of 75, 26.7%).

Table 6.2. Frequency of antimicrobial resistance of *Salmonella* isolates based on the presentation of broilers at retail outlets

Parts or carcasses tested at retail outlets	**No. of *Salmonella* isolates tested**	**No. (%) of resistant isolates[a]**	**No. (%) resistant to[b,d]:**											
			EFT	**DO**	**K**	**TE**	**AM**	**CRO**	**AMC**	**C**	**ENR**	**CIP**	**CN**	**SXT**
Cottage Processors														
Whole	52	52 (100.0)	52 (100.0)	52 (100.0)	49 (94.2)	45 (86.5)	43 (82.7)	30 (57.7)	13 (25.0)	8 (15.3)	5 (9.6)	1 (1.9)	0 (0.0)	0 (0.0)
Parts	43	43 (100.0)	43 (100.0)	42 (97.7)	38 (88.4)	39 (90.7)	37 (86.0)	19 (44.2)	12 (27.9)	7 (16.3)	9 (20.9)	4 (9.3)	4 (9.3)	1 (2.3)
Subtotal	95	95 (100.0)	95 (100.0)	94 (98.9)	87 (91.6)	84 (88.4)	80 (84.2)	49 (51.6)	25 (26.3)	15 (15.8)	14 (14.7)	5 (5.3)	4 (4.2)	1 (1.1)
P value			NA[c]	0.453	0.461	0.749	0.87	0.269	0.931	1	0.208	0.172	0.039	0.453
Supermarkets														
Whole	8	8 (100.0)	8 (100.0)	8 (100.0)	8 (100.0)	8 (100.0)	7 (87.5)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Parts	32	32 (100.0)	31 (96.9)	32 (100.0)	28 (87.5)	18 (56.3)	18 (56.3)	1 (3.1)	3 (9.4)	0 (0.0)	2 (6.3)	0 (0.0)	0 (0.0)	1 (3.1)
Subtotal	40	40 (100.0)	39 (97.5)	40 (100.0)	36 (90.0)	26 (65.0)	25 (62.5)	2 (5.0)	3 (7.5)	0 (0.0)	2 (5.0)	0 (0.0)	0 (0.0)	1 (2.5)
P value			1	NA[c]	0.566	0.034	0.219	0.364	1	NA[c]	1	NA[c]	NA[c]	1
Overall														
Whole	60	60 (100.0)	60 (100.0)	60 (100.0)	57 (95.0)	53 (88.3)	50 (83.3)	31 (51.7)	13 (21.7)	8 (13.3)	5 (8.3)	1 (1.7)	0 (0.0)	0 (0.0)
Parts	75	75 (100.0)	74 (98.7)	74 (98.7)	66 (88.0)	57 (76.0)	65 (86.7)	20 (26.7)	15 (20.0)	7 (9.3)	11 (14.7)	4 (5.3)	4 (5.3)	2 (2.7)
Total	135	135 (100.0)	134 (99.3)	134 (99.3)	123 (91.1)	110 (81.5)	115 (85.2)	51 (37.8)	28 (20.7)	15 (11.1)	16 (11.9)	5 (3.7)	4 (3.0)	2 (1.5)
P value			1	1	0.265	0.107	0.766	0.005	0.981	0.646	0.388	0.381	0.129	0.502

[a] Resistant to one or more antimicrobial agents.
[b] EFT, ceftiofur-30 µg; DO, doxycycline- 30 µg; K, kanamycin- 30 µg; TE, tetracycline- 30 µg; AM, ampicillin- 10 µg; CRO, ceftriaxone- 30 µg; AMC, amoxycillin- clavulanic acid- 30 µg; C, chloramphenicol- 30 µg; ENR, enrofloxacin- 5 µg; CIP, ciprofloxacin- 5 µg; CN, gentamicin- 10 µg; SXT, sulphamethoxazole-trimethoprim- 23.75/1.25 µg.
[c] NA: Not applicable.
[d] All isolates were sensitive to Cefoxitin (30 µg) and Norfloxacin (10 µg); All isolates were resistant to Erythromycin (15 µg) and Streptomycin (10 µg).

6.4.4 Frequency of resistance of Salmonella isolates to antimicrobial agents by serotype

Twelve of the 15 serotypes isolated were obtained at cottage poultry processors and were *Salmonella enterica*, predominantly serotypes Javiana (30.9%), Kentucky (23.5%), and Manhattan (16.2%; Table 6.3). The frequency of resistance among these serotypes was relatively high, ranging from 75.0 to 100.0% to EFT, DO, K, TE, and AM. *Salmonella* Javiana isolates were susceptible only to SXT, whereas *Salmonella* Kentucky and *Salmonella* Manhattan were susceptible to ENR, CIP, and SXT. The three most commonly detected serotypes among isolates from supermarkets were *Salmonella* Kentucky, group F, and *Salmonella* Sandiego, exhibiting a high frequency of resistance to EFT, DO, and K, ranging from 75.0 to 100.0%.

Table 6.3. Prevalence of resistance to antimicrobial agents amongst serotypes of *Salmonella* isolates recovered from chickens sold at retail outlets in Trinidad and Tobago

Serotypes isolated from retail outlets	**No. of *Salmonella* isolates tested**	**No. (%) of resistant isolates[a]**	**No. (%) resistant to[b,c]:**											
			EFT	**DO**	**K**	**TE**	**AM**	**CRO**	**AMC**	**C**	**ENR**	**CIP**	**CN**	**SXT**
Cottage processors														
Salmonella Javiana 9,12:l,z28:1,5	21	21 (100.0)	21 (100.0)	20 (98.9)	21 (100.0)	21 (100.0)	21 (100.0)	14 (66.7)	8 (38.1)	4 (19.0)	8 (38.1)	2 (9.5)	2 (9.5)	0 (0.0)
Salmonella Kentucky 8,20;I,z6	16	16 (100.0)	16 (100.0)	16 (100.0)	14 (87.5)	12 (75.0)	16 (100.0)	7 (43.8)	3 (18.8)	3 (18.8)	0 (0.0)	0 (0.0)	2 (12.5)	0 (0.0)
Salmonella Manhattan 6,8;d;1,5	11	11 (100.0)	11 (100.0)	11 (100.0)	11 (100.0)	10 (90.9)	10 (90.9)	5 (45.5)	4 (36.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (9.1)
Salmonella Aberdeen 11;I;1,2	3	3 (100.0)	3 (100.0)	3 (100.0)	3 (100.0)	3 (100.0)	2 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella Bloomsbury 3,10:g,t:1,5	2	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella subspecies enterica I 4,5,12:i:	2	2 (100.0)	2 (100.0)	2 (100.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella group D	2	2 (100.0)	2 (100.0)	2 (100.0)	1 (50.0)	2 (100.0)	1 (50.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella Schwarzengrund 4,12,27;d;1,7	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella group C2	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella Molade 8,20:z10,z6	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Salmonella Montevideo 6,7;gms:	1	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Possible *Salmonella* Warragul	7	7 (100.0)	7 (100.0)	7 (100.0)	7 (100.0)	6 (85.7)	1 (14.3)	2 (28.6)	2 (28.6)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Subtotal	68	68 (100.0)	68 (100.0)	67 (98.5)	63 (92.6)	61 (89.7)	58 (85.3)	35 (51.5)	19 (27.9)	12 (17.6)	9 (13.2)	2 (2.9)	4 (5.9)	1 (1.5)
Supermarkets														
Salmonella Kentucky 8,20;i;z6	14	14 (100.0)	13 (93.0)	14 (100.0)	12 (85.7)	14 (100.0)	8 (57.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella Group F	8	8 (100.0)	8 (100.0)	8 (100.0)	6 (75.0)	0 (0.0)	3 (38.5)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella San Diego *4,5 12:eh;enx/enz15*	3	3 (100.0)	3 (100.0)	3 (100.0)	3 (100.0)	1 (33.3)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella Montevideo 6,7;gms:	2	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella Javiana 9,12:l,z28:1,5	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Salmonella Westhampton 3,10:g,s,t	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)
Subtotal	29	29 (100.0)	28 (96.6)	29 (100.0)	25 (86.2)	18 (62.1)	14 (48.3)	0 (0.0)	1 (3.4)	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	1 (3.4)
Total	97	97 (100.0)	96 (99.0)	96 (99.0)	88 (90.7)	79 (81.4)	72 (74.2)	35 (36.1)	20 (20.6)	12 (12.4)	10 (10.3)	2 (2.1)	4 (4.1)	2 (2.1)

[a] Resistant to one or more antimicrobial agents.
[b] EFT, ceftiofur-30 µg; DO, doxycycline- 30 µg; K, kanamycin- 30 µg; TE, tetracycline- 30 µg; AM, ampicillin- 10 µg; CRO, ceftriaxone- 30 µg; AMC, amoxycillin- clavulanic acid- 30 µg; C, chloramphenicol- 30 µg; ENR, enrofloxacin- 5 µg; CIP, ciprofloxacin- 5 µg; CN, gentamicin- 10 µg; SXT, sulphamethoxazole-trimethoprim- 23.75/1.25 µg.
[c] All isolates were sensitive to Cefoxitin (30 µg) and Norfloxacin (10 µg); All isolates were resistant to Erythromycin (15 µg) and Streptomycin (10 µg).

6.4.5 Frequency of multidrug resistance patterns of Salmonella isolates to antimicrobial agents

All 135 isolates of *Salmonella* spp. tested exhibited multidrug resistant patterns, with a total of 43 different patterns observed (Table 6.4). The predominant pattern included EFT-DO-TE-E-K-AM-S (27.5%) and EFT-DO-TE-E-CRO-K-AM-S (18.1%) at supermarkets and cottage poultry processors, respectively.

For isolates of *Salmonella* spp. recovered from the outlets of cottage processors, there were a total of 33 multidrug resistance patterns. Of these patterns, 17 different patterns were displayed by more than one isolate, and 16 different patterns were exhibited by one isolate each. The most frequent patterns were EFT-DO-TE-E-K-AM-S (17.8%), EFT-DO-TE-E-CRO-K-AM-S (12.6%), EFT-DO-TE-E-K-S (8.1%), and EFT-DO-E-K-AM-S (8.1%). In addition, the following patterns were expressed by one isolate each: EFT-DO-TE-E-CRO-S; EFT-DO-E-CRO-K-AM-S; EFT-DO-TE-ENR-E-K-AM-C-S; EFT-DO-TE-E-AM-AMC-S; EFT-DO-TE-E-CRO-K-AM-C-S; EFT-DO-E-K-AMC-S; EFT-DO-TE-E-CRO-AMC-S; EFT-DO-TE-ENR-E-CRO-K-AM-AMC-S; EFT-DO-TE-SXT-E-CRO-K-AM-S; EFT-DO-TE-E-K-AM-AMC-C-S; EFT-DO-TE-CRO-K-AM-C-S; EFT-DO-TE-E-K-AM-GM-S; EFT-DO-E-K-S; EFT-E-AM-S; EFT-DO-TE-E-CRO-K-AMC-C-S; and EFT-DO-TE-E-K-C-S.

For isolates from supermarkets, a total of 13 resistance patterns were exhibited, consisting of 7 shown by multiple isolates and 6 by one isolate each. The predominant patterns were EFT-DO-TE-E-K-AM-S (27.5%), EFT-DO-TE-E-K-S (15.0%), and EFT-DO-E-K-AM-S (15.0%). The additional patterns exhibited by one isolate each are the following: EFT-DO-TE-E-S, EFT-DO-TE-ENR-E-S; EFT-

DO-TE-SXT-E-K-AM-S; EFT-DO-TE-ENR-E-K-AM-S, DO-TE-E-K-AM-S; and EFT-DO-E-K-AM-AMC-S.

Table 6.4. Antimicrobial resistance patterns among *Salmonella* isolates obtained from retail outlets in Trinidad and Tobago

Resistance patterns detected[a]	**No. (%) of *Salmonella* with resistance pattern from:**	
	Cottage poultry processors[b]	**Supermarket**[c]
EFT-DO-TE-E-CRO-K-AM-S	17 (18.1)	0 (0.0)
EFT-DO-TE-E-K-AM-S	13 (13.8)	11 (27.5)
EFT-DO-TE-E-CRO-K-AM-AMC-S	6 (6.4)	0 (0.0)
EFT-DO-TE-E-K-S	5 (5.3)	6 (15.0)
EFT-DO-E-K-AM-S	5 (5.3)	6 (15.0)
EFT-DO-E-K-S	0 (0.0)	5 (12.5)
EFT-DO-E-S	0 (0.0)	2 (5.0)
EFT-DO-TE-ENR-E-K-AM-S	4 (4.3)	0 (0.0)
EFT-DO-TE-E-CRO-K-AM-AMC-C-S	4 (4.3)	0 (0.0)
EFT-DO-TE-E-K-AM-AMC-S	3 (3.2)	0 (0.0)
EFT-DO-TE-E-K-AM-AMC-S	0 (0.0)	2 (5.0)
EFT-DO-TE-E-CRO-K-S	3 (3.2)	0 (0.0)
EFT-DO-TE-ENR-E-CRO-K-AM-CIP-S	3 (3.2)	0 (0.0)
EFT-DO-TE-ENR-E-CRO-K-AM-S	3 (3.2)	0 (0.0)
EFT-DO-TE-E-CRO-K-AM-AMC-C-GM-S	3 (3.2)	0 (0.0)
EFT-DO-E-K-AM-AMC-S	2 (2.1)	0 (0.0)
EFT-DO-TE-ENR-E-K-AM-CIP-S	2 (2.1)	0 (0.0)
EFT-DO-TE-E-CRO-K-AM-S	0 (0.0)	2 (5.0)
EFT-DO-TE-E-CRO-AM-S	2 (2.1)	0 (0.0)
EFT-DO-TE-E-K-AM-C-S	2 (2.1)	0 (0.0)
EFT-DO-TE-E-AM-S	2 (2.1)	0 (0.0)
Others	16 (17.0)	6 (15.0)

[a] EFT, ceftiofur- 30 µg; DO, doxycycline- 30 µg; TE, tetracycline- 30 µg; E, erythromycin- 15 µg; CRO, ceftriaxone- 30 µg; K, kanamycin- 30 µg; AM, ampicillin- 10 µg; ENR, enrofloxacin- 5 µg; CIP, ciprofloxacin- 5 µg; AMC, amoxycillin- clavulanic acid- 30 µg; C, chloramphenicol- 30 µg; CN, gentamicin- 10 µg; S, streptomycin- 10 µg; SXT, sulphamethoxazole-trimethoprim- 23.75/1.25 µg.

[b] Of a total of 94 isolates of *Salmonella* spp.

[c] Of a total of 40 isolates of *Salmonella* spp.

6.5 Discussion

To date, there has been no antimicrobial sensitivity testing done on *Salmonella* spp. isolated from chickens sold at retail outlets across Trinidad and Tobago. All the isolates of *Salmonella* spp. tested in the current study were resistant to one or more of the 16 antimicrobial agents, with a frequency of resistance ranging from 77.8 to 100.0% to seven of the antimicrobial agents (EFT, DO, K, TE, AM, E, and S). This could have therapeutic implications for the poultry industry primarily because these antimicrobial agents are used in the livestock industry, including on poultry farms (broilers and layers) in the country. Similar findings were reported in studies done in Colombia that found the frequency of resistance to EFT (44.7%) [460] and TE (60.8%) [483], as well as a study conducted in Malaysia [484], where the authors reported a high frequency of resistance to E (100.0%). In the current study, low frequencies of resistance (< 15.8%) were observed to C, ENR, CIP, CN, SXT, FOX, and NOR, similar to the study done in Malaysia [484], where susceptibilities to AMC (100.0%), CN (100.0%), and trimethoprim (100.0%) were reported.

For *Salmonella* spp. isolated from cottage processors, the frequency of resistance to TE (88.4%) and AM (84.2%) are at variance from the results obtained in studies conducted in Myanmar [457] and Vietnam [466], where the frequency of resistance to TE was 54.3 and 58.7% and to AM was 47.1 and 41.3%, respectively. The range of frequency of resistance against CRO, AMC, and C was 15.8 to 51.6% for isolates from cottage processors, similar to the findings of studies done in Myanmar [457] and China [263], where resistance to these antimicrobial agents ranged from 17.4 to 29.7% but differed from the isolates obtained from chickens sold at ‘‘wet markets’’ in Vietnam [466].

The isolates of *Salmonella* spp. from supermarkets exhibited high frequency of resistance to EFT, DO, and K that ranged from 90.0 to 100.0%, which was quite different from the range of resistance to these antimicrobial agents (2.1 to 27.3%) reported for *Salmonella* isolates from chickens sold at supermarkets in Guatemala [439] and Vietnam [466]. Low levels of resistance were exhibited by these

supermarket isolates against C, ENR, CIP, and CN, ranging from 0.0 to 5.0%, and were like studies done in Japan [485] and Vietnam [466].

The high frequency of resistance to antimicrobial agents detected in *Salmonella* isolates in the current study is in agreement with the findings reported for *Salmonella* spp. recovered from broilers sampled from retail outlets (combination of supermarkets and wet markets) elsewhere that documented similarly high levels of resistance to TE, AM, K, and S: 88.0 to 94.0% in Japan [486], 93.0 to 100.0% in China [487], 89.3 to 91.7% in Turkey [488], and 74.0 to 93.0% in South Africa [489]. The high frequency of resistance detected in these countries may be reflective of the use or misuse of these agents in the poultry industry. It has been reported that sulfonamides, fluoroquinolones, and tetracyclines are the most common antimicrobials used in the poultry industry globally [490], similar to the practice in Trinidad and Tobago.

Note that across the seven counties, the frequency of resistance to antimicrobial agents was significantly different for K and for ENR. These findings may reflect the differences in the use of antimicrobial agents on the farms that supplied chickens to the outlets sampled in these counties. It cannot be overemphasized that chickens sampled from cottage processors in a particular county do not necessarily originate from that county because there is widespread movement of live chickens from broiler farms across the country [481]. It has been established that the use and overuse of antimicrobial agents in the poultry industry [18, 362] have been associated with the increase in antimicrobial resistance in other countries; however, husbandry practices on chicken farms were not determined in this study, as this was not one of its objectives. It is, therefore, not possible to directly associate the occurrence of resistance of *Salmonella* spp. isolated and the use of antimicrobial agents on farms from which they originated.

The high level of susceptibility of *Salmonella* isolates is also of therapeutic significance, as this offers therapeutic options. In the current study, it is significant that the *Salmonella* isolates tested exhibited high susceptibility to SXT (98.5%),

ENR (88.1%), CN (97.0%), and CIP (96.3%), similar to the findings of other studies [491-493]. A recent study [362] conducted on *Salmonella* isolates recovered from layer farms in three Caribbean countries (Trinidad and Tobago, Saint Lucia, and Grenada), reported SXT to be one of the most effective among eight antimicrobial agents on isolates from Trinidad and Tobago, with a frequency of resistance of 20.9%. In another study conducted on table eggs in the country [365], all *Salmonella* isolates were susceptible to SXT. The low prevalence of resistance to SXT (1.5%) in the current study could be because SXT is not as widely used on broiler farms compared with layer farms. This low prevalence of resistance, however, is at variance with other studies done in Spain (40.3%) [242], Egypt (76.0%) [494], and China (97.0%) [487].

Across retail outlets, statistically significant differences in frequency of resistance were detected to TE, AM, CRO, AMC, and C compared with the other antimicrobial agents used in the current study. Note that while the use of C is banned in food-producing animals, it was included in the panel of antimicrobial agents for further characterisation of resistance. The significant differences in prevalence of resistance could be owing to favorable conditions for resistant bacteria on the meat matrix and/or to secondary contamination with resistant strains [260], especially when whole chickens are manually cut into parts at cottage processors. Chickens sold at supermarkets undergo a more stringent processing procedure at large processing plants, where better sanitary practices are followed, and the cutting of whole chickens is automated. To date, there have been very few observational studies done in Trinidad and Tobago on the frequency of resistance of *Salmonella* isolates obtained from broilers sold at retail outlets across the country. Cottage poultry processors are supplied by one or more independent broiler farms at any given time; therefore, information on the agents used at these farms was unavailable. The farm source of chickens that are sold at supermarkets is more regulated because integrated companies are the regular suppliers, and information on the agents used during the grow-out phase of these chickens is kept confidential.

Of the 15 *Salmonella* serotypes tested, *Salmonella enterica* serovar Kentucky isolates exhibited resistance ranging from 7.1% (to ENR) to 100.0% (to EFT, DO, AM, E, and S), having 100.0% sensitivity to CIP, SXT, FOX, and NOR. The resistance of *Salmonella* Kentucky isolates obtained from chickens in the United States was at variance to our findings where the frequency of resistance was 14.3 and 82.0% to AM and S, respectively [495]. However, a study done in Ghana [491] reported frequencies of resistance in agreement with our study to CN, C, and AMC, ranging from 12.5 to 18.8%, and this study reported resistance ranging from 0.0 to 23.5%.

Of the 30 *Salmonella enterica* serovar Javiana isolates tested, the range of frequency of resistance was from 9.5% (to CN and CIP) to 100.0% (to EFT, K, TE, AM, E, and S), with cottage processor isolates having 100.0% sensitivity to SXT, NOR, and FOX. This finding is of therapeutic significance because the most prevalent serotypes confer the highest prevalence of resistance. Also, quinolones, such as CIP, ENR, and NOR are sold over the counter without restrictions or prescriptions in Trinidad and Tobago, thereby leading to its overuse in human medicine [496]. Low frequencies of resistance of *Salmonella* Javiana isolates to SXT, NOR, and FOX have also been reported in Gambia and Senegal [497] and agree with the findings of this current study.

Salmonella enterica serovar Schwarzengrund isolated from chicken meat in Denmark [498] exhibited resistance of 75.0 and 85.0% to AM and TE, while isolates from Thailand [498] exhibited 47.9% to the respective agents, as well as 81.3 and 75.0% to S and SXT, respectively. Additionally, *Salmonella* Schwarzengrund isolated in Japan [485] exhibited 100% susceptibility to ENR and C. Note that only one *Salmonella* Schwarzengrund isolate was subjected to sensitivity testing in this study, therefore making a comparison unrealistic.

It is equally of therapeutic significance as all 135 isolates of *Salmonella* spp. tested exhibited multiresistance to two or more antimicrobial agents and more importantly, the detection of a total of 43 distinct resistance patterns. It is also

important that some isolates of *Salmonella* spp. were resistant to as many as seven antimicrobial agents and is a source of concern. Multidrug resistance patterns have been known to vary and reflect the use of agents in the respective countries [352, 485]. The occurrence of a high frequency of multidrug resistance (100.0%), together with the different patterns observed in this study, highlight the possibility for therapeutic failure associated with the agents used. High multidrug resistance in *Salmonella* isolates from broilers have also been reported in Colombia [483], Japan [486], Romania [499], and Spain [246] and agree with the findings of this study. Note that AM, AMC, CIP, NOR, DO, CN, SXT, and CRO are currently used in human medicine in Trinidad and Tobago, an indication that the results from this study can have public health significance. To date, in Trinidad and Tobago, no published report exists on the antimicrobial resistance of *Salmonella* isolates of human origin; therefore, no comparison can be made between isolates from foods and humans.

6.6 Conclusions

It is concluded that the high prevalence of resistance to a wide spectrum of antimicrobial agents, with an equally high frequency of multidrug resistance, has the potential to lead to therapeutic failure in chickens with associated economic losses, resulting from the high cost of treatment, morbidities, and mortalities. The findings also pose zoonotic risk to consumers of improperly cooked chickens and cross-contaminated surfaces, contaminated by the resistant *Salmonella* spp. The results of the current study offer useful information for clinicians in the treatment of human salmonellosis because CIP, SXT, CRO, and azithromycin (not used in our study) are commonly used to treat human cases of gastroenteritis in Trinidad and Tobago. The absence of a legislative framework to limit the use and overuse of antimicrobial agents in food-producing animals in this country is considered an important contributor to the high frequency of resistance of *Salmonella* to antimicrobial agents. This study therefore provides up-to-date information and highlights the need for the enforcement of effective regulations to control the

availability and use of antimicrobial agents. The need for public health awareness campaigns on proper handling and cooking of raw chickens is also apparent.

Connecting statement to the next chapter

This chapter completed the phenotypic characterisation of *Salmonella* isolated from the retail outlets. It also concluded our phenotypic characterisation of the *Salmonella* isolates using a 'farm to fork' approach, where Chapter 3 (imported fertile eggs, hatcheries and farms), Chapter 4 (processing plants) and Chapters 5 and 6, focussed on retail outlets. The following chapter characterised *Salmonella* from the 'farm to fork' continuum using whole genome sequencing.

CHAPTER 7: MOLECULAR CHARACTERISATION OF *SALMONELLA* ISOLATED FROM THE BROILER INDUSTRY IN TRINIDAD USING A 'FARM TO FORK' APPROACH: DETECTION OF RESISTANCE GENES AND VIRULENCE FACTORS

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7.1 Abstract

This cross-sectional study determined the serovars, antimicrobial resistance genes, and virulence factors of *Salmonella* isolated from hatcheries, broiler farms, processing plants, and retail outlets in Trinidad and Tobago. *Salmonella* in silico serotyping detected 23 different serovars where Kentucky 20.5% (30/146), Javiana 19.2% (28/146), Infantis 13.7% (20/146), and Albany 8.9% (13/146) were the predominant serovars. There was a 76.0% (111/146) agreement between serotyping results using traditional conventional methods and whole-genome sequencing (WGS) in *in silico* analysis. *In silico* identification of antimicrobial resistance genes conferring resistance to aminoglycosides, cephalosporins, peptides, sulfonamides, and antiseptics were detected. Multidrug resistance (MDR) was detected in 6.8% (10/146) of the isolates of which 100% originated from broiler farms. Overall, virulence factors associated with secretion systems and fimbrial adherence determinants accounted for 69.3% (3091/4463), and 29.2% (1302/4463) counts, respectively. Ten of 20 isolates of serovar Infantis (50.0%) showed MDR and contained the $bla_{CTX\text{-}M\text{-}65}$ gene. This is the first molecular characterisation of *Salmonella* isolates detected along the entire broiler production continuum in the Caribbean region using WGS. The availability of these genomes will help future source tracking during epidemiological investigations associated with *Salmonella* foodborne outbreaks in the region and worldwide.

7.2 Introduction

Since the 1950s, *Salmonella* has been highlighted as an economically important zoonotic pathogen by the World Health Organisation (WHO) and Food and Agriculture Organisation of the United Nations (FAO) [500]. The ability of *Salmonella* to cause foodborne diseases coupled with high mortality rates in humans are cause for public health concern [48, 49]. While many animals serve as reservoirs for *Salmonella*, poultry and poultry products are considered major sources of salmonellosis in humans. Therefore, the possibility of transmission from reservoirs to other animals and humans is concerning. This is compounded by

antimicrobial-resistant *Salmonella* strains within the environment, necessitating surveillance and control measures among suspected reservoirs such as chickens. In addition, *S.* Typhimurium and *S.* Enteritidis are of public health significance due to their ability to cause disease in humans and animals in developed and developing countries. However, variations in *Salmonella* serovar distribution have been reported in different countries and are said to be a function of geographic location [501, 502].

The use of antimicrobial agents in food-producing animals has been implicated in developing multidrug-resistant (MDR) microorganisms and spreading them through the food chain [503, 504]. Of importance to human health, some cephalosporins (β-lactams), quinolones, and aminoglycosides have been classified by the World Health Organisation (WHO) as critically important agents since they are used in the treatment of extra-intestinal salmonellosis [505]. The use of ciprofloxacin as the established therapy protocol for human salmonellosis could be jeopardized as genetic mechanisms promoting MDR isolates have been reported [506]. β-lactamases constitute the primary mechanism of cephalosporin resistance via enzymatic modification, where different genes are implicated. The extended spectrum β-lactamases (ESβL) include certain alleles of bla_{TEM}, and all alleles of $bla_{CTX\text{-}M}$ and bla_{SHV} genes. Extended-spectrum cephalosporins can also be hydrolyzed by the AmpC β-lactamases, of which bla_{CMY} is the most common of particular importance. Quinolone resistance was initially known to develop through chromosomal mutations [507]. However, the recent emergence of plasmid-mediated quinolone resistance (PMQR) mechanisms has been reported. These include qnr genes: *qnrA, qnrB, qnrS, qnrC*, and *qnrD*, that encode pentapeptide repeat proteins that bind to and protect DNA topoisomerase IV from inhibition by quinolones, the *aac (60)-Ib-cr* (modified acetyltransferase) and *qepA* (efflux pump) genes, respectively [508, 509]. Plasmids are traditionally known to carry antimicrobial-resistant genes and several virulence-associated traits; however, other resistance mechanisms have been reported in *Salmonella* elsewhere [510,

511]. Increasing trends of resistance to quinolones and 3rd generation cephalosporins such as ciprofloxacin and ceftiofur in clinical isolates have led to the introduction of carbapenems and colistin as critical antibiotics of last resort in human salmonellosis [505]. However, the use of colistin to treat both humans and animals has resulted in the emergence of mobilized colistin resistance (*mcr*) genes [512, 513]. To date, nine variants of mcr genes have been detected in *Salmonella* isolated from humans and animals [513-515].

Similar genetic determinants conferring resistance to aminoglycosides, tetracyclines, beta-lactams, and fluoroquinolones have been detected in *Salmonella* strains isolated from livestock and humans, concluding that food and environmental contamination from livestock are carriers of antimicrobial-resistant (AMR) *Salmonella* and are sources of infection to humans [516-518]. Thus, it is critical to investigate the resistance profiles and phenotypes they exhibit, and the mutations responsible for resistance using molecular analysis methods.

Therefore, the objectives of this study were to determine the genotypic profiles (serovar, antimicrobial resistance, and virulence factors) of *Salmonella* isolated from various stages of the broiler production–processing–retailing chain in Trinidad and Tobago.

7.3 Materials and methods

7.3.1 Sample selection

A total of 146 isolates of *Salmonella* used in this study originated from prior studies conducted on hatcheries and broiler farms [519], broiler processing plants [328] and retail outlets (pluck shops and supermarkets) [300, 327]. The type of samples collected from the various studies are as follows, hatcheries: broken eggshells, eggs in the hatcher, eggs in the incubator, hatcher environmental swabs, hatcher fluff, and stillborn chicks; broiler farms: boot swabs, cloacal swabs, litter drag swabs, feed, and water samples- in-house supply and storage tank; processing plants:

chilled chicken parts, chilled whole carcasses, neck skins, pre-evisceration carcasses, and post-evisceration carcasses; retail outlets: chicken carcasses. From a total of 207 duplicate isolates (from different enrichment and selective media) of *Salmonella*, which represented 23 serovars from the aforementioned sources, the selected 146 isolates were representatives of the serovars recovered from all *Salmonella*-positive samples. Briefly, samples were processed to isolate *Salmonella* using two enrichments broths, Rappaport-Vassiliadis Soya (RVS) and tetrathionate (TT) (Oxoid, Hampshire, England), and two selective agar, brilliant green agar (BGA) and xylose lysine tergitol 4 (XLT-4) selective media (Oxoid, Hampshire, England) [31]. Suspected *Salmonella* colonies (pink isolated colonies on BGA, red colonies with black centers on XLT-4) were subjected to biochemical tests for identification of *Salmonella* spp. using standard methods [399]. Isolates of *Salmonella* recovered from the four combinations of media (RVS/BGA, RVS/XLT-4, TT/BGA, and TT/XLT-4) were initially screened using the conventional slide agglutination test. Thereafter, 146 non-duplicate isolates of *Salmonella*, randomly selected to represent the serovars and positive samples were subjected to whole-genome sequencing. The following is a summary of the number of isolates included from earlier studies: hatcheries (n = 10), farms (n = 20), processing plant (n = 61), and retail outlets (n = 55). Five additional human clinical isolates of *Salmonella* obtained from the Caribbean Public Health Agency (CARPHA) were included in our panel of isolates subjected to WGS.

7.3.2 DNA extraction and sequencing

DNA was extracted using the Maxwell RSC cultured cells DNA kit with a Maxwell RSC instrument (Promega, Madison, WI) following the manufacturer's protocols for Gram- negative bacteria with additional RNase treatment. DNA concentrations were measured with a Qubit fluorometer (Life Technologies, Carlsbad, CA), standardised to 0.2 ng/µl, and the samples were stored at 4°C before library preparation.

Whole genome sequencing of *Salmonella* isolates was performed by the Public Health Agency of Canada (PHAC) Laboratory and Food and Drug Administration (FDA): Center for Food Safety and Applied Nutrition genomics laboratory (FDA-CFSAN) and Center for Veterinary Medicine (FDA-CVM), Maryland, USA. The WGS was generated on an Illumina MiSeq using 2 x 250 and 2 x 300 bp paired-end chemistry (Illumina Inc., San Diego, CA, USA) according to manufacturer's instructions, at 50-150X coverage. According to the manufacturer's instructions, the libraries were constructed using 100 ng of genomic DNA using the Illumina DNA Prep (M) Tagmentation kit (Illumina Inc., San Diego, CA, USA) and the Nextera XT kit (Illumina Inc., San Diego, CA, USA).

7.3.3 Genomic data analysis and in silico determination of genetic elements

Quality control including adapter removal of the raw data was done using BBDuk (v.37.90; https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbduk-guide/). SPAdes v.3.12.0 [216] was used to create a de novo assembly of each isolate. Only contigs larger than 500 bp were retained for further analysis. Serovar prediction was done using command-line version of SISTR [208] (Version: sistr_cmd v.1.1.1).

Gene finding in each isolate was done with Prodigal v.2.6.3 [520] (parameters -c -n). VFDB [521] was used to assign virulence factors. This was done with the predicted genes (amino acid format) from Prodigal using NCBI-blast-2.9.0+. Results were filtered for the top hit with 100% identity and 100% alignment length.

CARD [522] was used to assign antimicrobial resistance. This was done with the predicted genes (amino acid format) from Prodigal using NCBI-blast-2.9.0+. Results were filtered for the top hit with 100% identity and 100% alignment length.

7.3.4 Phenotypic methods used for comparison with WGS

The results from conventional serotyping methods described previously [300, 328, 519] were compared to the genomic data. Antimicrobial resistance determined by

the disk diffusion method [327, 328, 519] described previously was also used. Data generated from these two methods were compared to the genomic data.

7.3.5 Statistical analyses

R version 4.0.2 was used for Chi-square analysis and data visualisation.

7.3.6 Data deposition

The draft genome sequence of all *S.* enterica strains have been deposited at GenBank under the accession listed in Appendix 7.1—Metadata of 146 *Salmonella* isolates detected along the broiler production chain in Trinidad and Tobago.

7.4 Results

7.4.1 Serotyping results

Overall, the 146 isolates of *Salmonella* subjected to conventional serotyping methods were classified into 23 serovars and 3 unspecific groups (unknown serotype). In silico analysis of the WGS data generated from these 146 isolates using the SISTR software identified 23 different serovars where Kentucky 20.5% (30/146), Javiana 19.2% (28/146), Infantis 13.7% (20/146), and Albany 8.9% (13/146) were the predominant serovars. There was a 76.0% (111/146) agreement in the test results of both methods. Isolates classified as *S.* Albany (n = 2), Gaminara (n = 2), Oranienburg (n = 1), and Soerenga (n = 1) by SISTR were all classified as *S.* Infantis (n = 6) using the traditional method. Three *S.*Warragul isolates detected using the conventional method were classified as *S.* Caracas on SISTR analysis. The distribution of serovars of *Salmonella* isolates from various sources is shown in Table 7.1.

Table 7.1. The distribution of serovars of *Salmonella* isolates from various sources based on in silico analysis.

	No. of Strains of *Salmonella* Detected from the Following:				
Serovars	**Hatchery**	**Farm**	**Processing Plant**	**Pluck Shop** [a]	**Supermarket** [a]
Aberdeen	0	0	1	1	0
Alachua	0	0	1	0	0
Albany	0	4	8	1	0
Anatomy	0	0	5	0	0
Caracas	0	0	0	3	0
Chester	0	0	0	0	2
Enteritidis	0	0	9	0	0
Fresno	1	0	0	0	0
Gaminara	0	3	0	0	0
Infantis	0	11	9	0	0
Javiana	0	0	10	17	1
Kentucky	8	0	7	12	3
Liverpool	0	0	1	0	0
Manhattan	0	0	0	7	0
Mbandaka	0	0	1	0	0
Molade	0	0	0	0	1
Montevideo	0	0	0	2	1
Oranienburg	0	1	0	0	0
Schwarzengrund	0	0	7	1	0
Senftenberg	1	0	0	2	1
Soerenga	0	1	0	0	0
Virchow	0	0	1	0	0
Weltevreden	0	0	1	0	0
Sub-total	10	20	61	46	9

[a] Retail outlets comprised pluck shops and supermarkets.

7.4.2 Antimicrobial resistance profiles

A total of 71 ARO accessions (Antibiotic Resistance Ontology, as defined by CARD) were detected among 22 isolates. Genes associated with aminoglycoside resistance, i.e., *aac(3)-IV* (plasmid-encoded), *aac(60)-Iaa* (chromosomal-encoded), *aac(60)Iy* (chromosomalencoded), *aph(30)-Ia* (plasmid-encoded), and *aph(4)-Ia* (plasmid-encoded) (Table 7.2) were found at frequencies ranging from 1.4% to 7.5%. All our *S.* Manhattan and *S.* Aberdeen strains containing the often silent, chromosomal-encoded *aac(60)-Iaa* and *aac(60)Iy* genes, exhibited phenotypic aminoglycoside resistance. Ten (6.8%) of 146 isolates contained the $bla_{CTX\text{-}M\text{-}65}$ gene, which confers cephalosporin resistance. This gene was identified

in *S.* Infantis isolates only. Genes *qacEDelta1* and *sul1*, responsible for antiseptic and sulfonamide resistance, were each detected at a frequency of 8.2% (12/146). *mcr-9*, the mobilized and plasmid-mediated colistin resistance gene, was found in only one isolate. Table 7.3 shows the distribution of AROs among *Salmonella* isolates from various sources. Isolates from broiler farms accounted for 83.1% (59/71) of AROs where the predominance of *aac(3)-IV* (9.9%; 7/71), *aph(4)-Ia* (9.9%; 7/71), *qacEdelta1* (9.9%; 7/71), *sul1* (9.9%; 7/71), and $bla_{CTX\text{-}M\text{-}65}$ (9.9%; 7/71) among cloacal swab isolates (62.7%; 37/59) was evident. *Salmonella* isolated from the water supply at farms (18.6%; 11/59) were found to contain 66.7% (6/9) of the AROs found in this study except for *mcr-9.1*, *aac(60)-Iaa* and *aac(60)-Iy*.

Overall, 6.8% (10/146) MDR (resistance to 3 or more classes of antimicrobial agents, according to CARD classification) isolates were detected, of which 100% were recovered at broiler farms and belonged to serovar Infantis.

Table 7.2. Antimicrobial class and genes detected in 146 *Salmonella* isolates used in this study.

Antimicrobial Class and Genes Detected [a]							
Pattern	**Aminoglycoside**	**Disinfectant**	**Cephalosporin**	**Peptide**	**Sulphonamide**	**Number of Isolates (%)**	**Serovar (n, %)**
Pattern 1	*aph(4)-Ia* *aac(3)-IV*	*qacEDelta1*	*bla*$_{CTX\text{-}M\text{-}65}$	-	*sul1*	6 (4.2)	Infantis (6, 100.0)
Pattern 2	*aph(3')-Ia* *aph(4)-Ia* *aac(3)-IV*	*qacEDelta1*	-	-	*sul1*	1 (0.7)	Infantis (1, 100.0)
Pattern 3	*aph(3')Ia* *aph(4)-Ia* *aac(3)-IV*	*qacEDelta1*	*bla*$_{CTX\text{-}M\text{-}65}$	-	*sul1*	4 (2.8)	Infantis (4, 100.0)
Pattern 4	-	*qacEDelta1*	-	-	*sul1*	1 (0.7)	Senftenberg (1, 100.0)
Pattern 5	*aac(6')-Iaa*	-	-	-	-	7 (4.9)	Manhattan (7, 100.0)
Pattern 6	*aac(6')-Iy*	-	-	-	-	2 (1.4)	Aberdeen (2, 100.0)
Pattern 7	-	-	-	*mcr-9.1*	-	1 (0.7)	Senftenberg (1, 100.0)
Total	20 (14.9)	12 (9.0)	10 (7.5)	1 (0.7)	12 (9.0)	22 (16.1)	

[a] Of a total of 146 isolates subjected to CARD analyses, AMR genes were detected in 22 isolates shown, 121 isolates possessing the core gene *golS* (regulator of a multidrug efflux pump) were not included in the table and three isolates were negative for resistance genes (Liverpool, Mbandaka, and Oranienburg).

Table 7.3. Frequency of ARO accessions detected in this study.

			Distribution of AROs among the Various Sampling Levels			
ARO Name [a]	**No. of AROs**	**Overall Frequency (%) [b]**	**Hatchery**	**Farm**	**Processing Plant**	**Retail Outlet**
aac(3)-IV	11	7.5	0	11	0	0
aac(6')-Iaa	7	4.8	0	0	0	7
aac(6')-Iy	2	1.4	0	0	1	1
aph(3')-Ia	5	3.4	0	5	0	0
aph(4)-Ia	11	7.5	0	11	0	0
$bla_{CTX\text{-}M\text{-}65}$	10	6.8	0	10	0	0
mcr-9.1	1	0.7	1	0	0	0
qacEDelta1	12	8.2	0	11	0	1
sul1	12	8.2	0	11	0	1
Total	71		1	59	1	10

[a] Antibiotic resistant ontology name in accordance with the Comprehensive Antibiotic Resistance Database (CARD) software.
[b] A total of 71 ARO counts were detected in 146 isolates.

7.4.3 Virulence profile

Overall, for the *Salmonella* strains from the four sources (hatcheries, farms, processing plants, and retail outlets), 4463 different virulence factors belonging to five virulence classes were identified. Genes classified as secretion systems and fimbrial adherence determinant classes accounted for the predominant virulence classes of 69.3% (3091/4463) and 29.2% (1302/4463) counts, respectively. Magnesium uptake, stress adaptation, and toxin classes accounted for less than 1.3% (56/4463) counts, respectively. *Salmonella* isolates (n = 10) recovered from the hatcheries contained virulence factors belonging to secretion systems (4.2%, 187/4463) and fimbrial adherence determinants (2.0%, 91/4463), whereas farm isolates (n = 20) were found to contain fimbrial adherence determinants, 4.4% (198/4463), and secretion system, 10.1% (451/4463). Processing plant *Salmonella* isolates (n = 61) contained predominantly factors in the secretion systems, fimbrial adherence determinants, and toxins, accounting for 30.0% (1341/4463), 12.4% (553/4463), and 0.6% (26/4463) counts, respectively. Retail outlet isolates (n = 55) contained fimbrial adherence determinants, 10.3% (460/4463), secretion system, 24.9% (1112/4463), and toxin-related factors, 0.6% (27/4463). The differences in the detection of virulence factors among the sources were statistically significant ($p < 0.001$).

Serovars Kentucky, Javiana, and Infantis contained higher numbers of virulence factors (all related to secretion systems), accounting for 13.0% (578/4463), 12.1% (540/4463), and 12.1% (517/4463), respectively, of the virulence factors (Appendix 7.2). Therefore, it is pertinent to mention that they were the predominant serovars detected in this study.

S. Infantis isolates contained factors associated with secretion systems (TTSS-1 translocated effectors, TTSS-SPI-1-, and TTSS-SPI-2-encoded genes), 12.1% (540/4463), and factors associated with fimbrial adherence determinants (*bcfA, D, F, csg A, B, C, E, F, G,* and *lpfB, E*), 4.9% (220/4463). For the isolates of *S.* Javiana, 11.6% (517/4463), 4.4% (196/4463), and 0.6% (28/4463) were positive for factors

associated with secretion systems, fimbrial adherence determinants *(bcfA, csgA, C, D, F, G*, and *fimF*), and toxins (*cdtB*), respectively. Only secretion system and fimbrial adherence determinant factors were detected among Kentucky isolates, accounting for 13.0% (578/4463) and 5.9% (263/4463), respectively. *S.* Schwarzengrund, Senftenberg, and Caracas contained predominantly factors associated with secretion systems at frequencies ranging from 1.3% to 2.8%. Seven serovars (Caracas, Chester, Enteritidis, Gaminara, Javiana, Montevideo, and Schwarzengrund) contained virulence factors related to toxins, where the *cdtB* was detected in all except serovar Enteritidis, where the *spvB* gene was detected.

7.4.4 Comparison of frequency of detection of resistance and virulence factors in Salmonella strains

Comparisons between the possession of virulence factors (VFDB accessions) and AMR genes (ARO accessions) across serovars were performed and detected 10 sources and years (farm-to-fork, hatcheries, processing plants, retail outlets, 'pluck shops', supermarkets, 2016, 2017, 2018, and 2019). Statistically significant positive correlations in *Salmonella* serovars isolated from farms, retail outlets, and 'pluck shops', as well as those isolated in 2016 and 2019 were detected, respectively ($p < 0.05$) (Figure 7.1). Negative and non-significant positive correlations are not displayed.

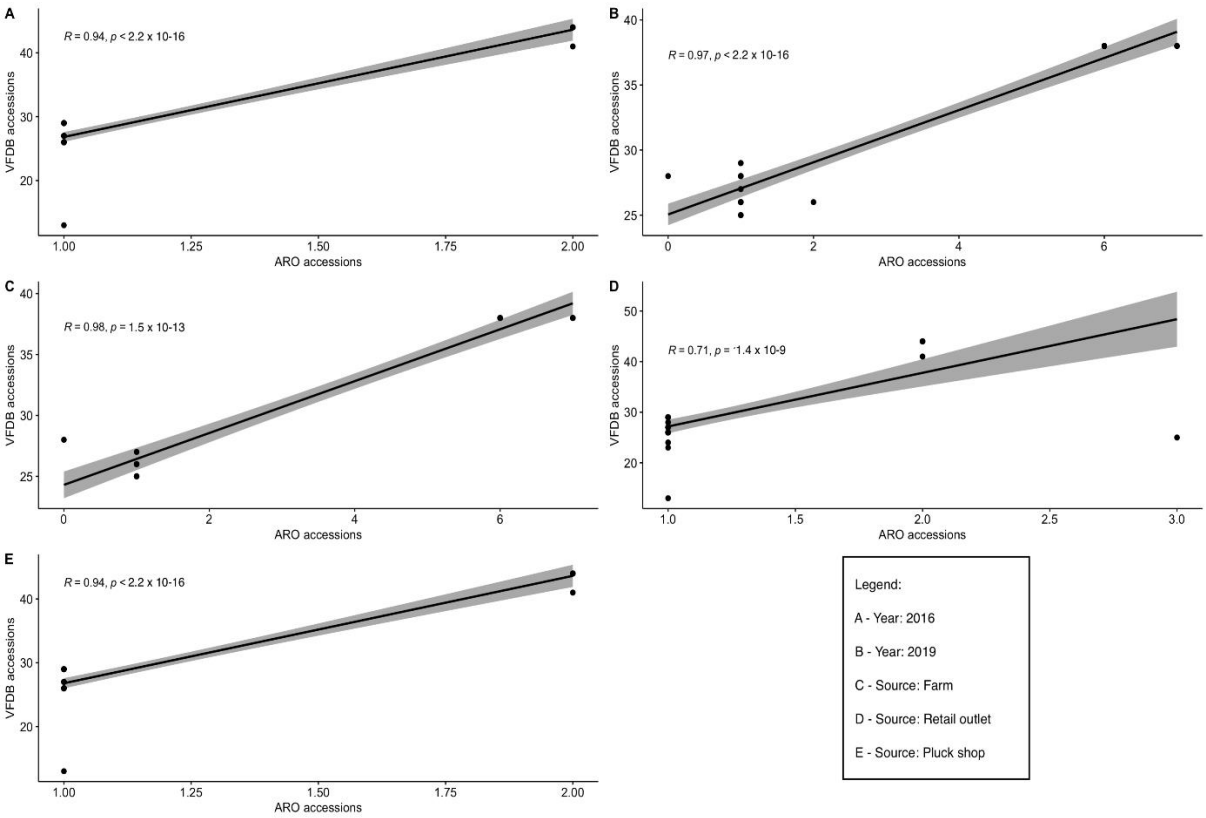

Figure 7.1. VFDB accessions (virulence) versus ARO accessions (AMR)

7.4.5 Detection of ESBL resistance genes and virulence genes in isolates of S. Infantis

A comparison of the phenotypic and genotypic resistance patterns in *S.* Infantis isolates is displayed in Table 7.4. The *bla$_{CTX\text{-}M\text{-}65}$* gene was only detected among the *S.* Infantis isolates. Of the 10 isolates of serovar Infantis positive for *bla$_{CTX\text{-}M\text{-}65}$* gene, phenotypically (using the disk diffusion method), two were resistant to two classes of antimicrobial agents, and six were MDR. However, genotypically, all 10 Infantis isolates exhibited MDR. Furthermore, the resistance gene *qacEDelta1* responsible for antiseptic resistance was found in all the 10 serovar Infantis isolates. Additionally, virulence factors associated with fimbrial adherence determinants and the secretion system were detected in all the 10 isolates of serovar Infantis.

Table 7.4. Detection of ESBL resistance genes and virulence genes in *S.* Infantis

BioSample	Isolate No.[a]	Phenotypic AMR Using the Disk Diffusion Method [b,c,d]							Genotypic Characteristics [e]	
		P	TE	CE	AM	PH	S	F	Other Resistance Genes Detected [f]	Virulence Factors
SAMN25867756	F 17	S	R	R	R	S	R	S	*qacEDelta1* *aph(4)-Ia* *aac(3)-IV* *sul1*	*agf/csg* *bcf* *lpf* TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors
SAMN25867757	F 22	S	R	R	R	S	R	S	*qacEDelta1* *aph(4)-Ia* *aac(3)-IV* *sul1*	*agf/csg* *bcf* *lpf* TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors
SAMN14677229	F 11	S	R	R	R	S	R	S	*qacEDelta1* *aph(4)-Ia* *aac(3)-IV* *sul1*	*agf/csg* *bcf* *lpf* TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors
SAMN14677211	F 32	S	R	R	R	S	R	S	*aph(3')-Ia* *qacEDelta1* *aph(4)-Ia* *aac(3)-IV* *sul1*	*agf/csg* *bcf* *lpf* TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors
SAMN14677232	F 36	S	R	R	R	S	R	S	*aph(3')-Ia* *qacEDelta1* *aph(4)-Ia* *aac(3)-IV* *sul1*	*agf/csg* *bcf* *lpf* TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors
SAMN14677210	F 2	S	S	S	S	S	S	S	*qacEDelta1*	*agf/csg*

									aph(4)-Ia	*bcf*
									aac(3)-IV	*lpf*
									sul1	TTSS (SPI-1 encode)
										TTSS (SPI-2 encode)
										TTSS-1 translocated effectors
SAMN14677203	F 4	S	R	R	R	S	S	R	*qacEDelta1*	*agf/csg*
									aph(4)-Ia	*bcf*
									aac(3)-IV	*lpf*
									sul1	TTSS (SPI-1 encode)
										TTSS (SPI-2 encode)
										TTSS-1 translocated effectors
SAMN14677209	UWI-F30	S	S	S	S	S	S	S	*aph(3')-Ia*	*agf/csg*
									qacEDelta1	*bcf*
									aph(4)-Ia	*lpf*
									aac(3)-IV	TTSS (SPI-1 encode)
									sul1	TTSS (SPI-2 encode)
										TTSS-1 translocated effectors
SAMN14677207	UWI-F9	S	R	S	R	S	S	S	*qacEDelta1*	*agf/csg*
									aph(4)-Ia	*bcf*
									aac(3)-IV	*lpf*
									sul1	TTSS (SPI-1 encode)
										TTSS (SPI-2 encode)
										TTSS-1 translocated effectors
SAMN14677208	UWI-F31	S	R	S	R	S	S	S	*aph(3')-Ia*	*agf/csg*
									qacEDelta1	*bcf*
									aph(4)-Ia	*lpf*
									aac(3)-IV	TTSS (SPI-1 encode)
									sul1	TTSS (SPI-2 encode)
										TTSS-1 translocated effectors

[a] All 10 isolates were obtained from broiler farms comprising 7 (70%) cloacal swabs, 2 (20%) water supply (UWI-F30 and UWI-F9), and 1 drag swab of litter (UWI-F31) from where *blaCTX-M-65*, the only *ESβL*-resistance gene was detected. [b] P, penam (amoxicillin–clavulanic acid, 30 µg); TE, tetracycline (doxycycline, 30 µg); CE, cephalosporin (ceftriaxone, 30 µg); AM, aminoglycoside (gentamicin, 10 µg, and kanamycin, 30 µg); PH, phenicol (chloramphenicol, 30 µg); S, sulphonamide (sulfamethoxazole–trimethoprim, 23.75 and 1.25 µg); F, fluoroquinolone (ciprofloxacin, 5 µg). [c] A total of 146 (151 with controls) isolates of *Salmonella* were tested for AMR by the disk diffusion method, resistance genes, and virulence genes by WGS where 6.6% (10/151) were positive for *ESβL* resistance genes ($bla_{CTX\text{-}M\text{-}65}$). [d] S:

Susceptible and R: Resistance. [e] Antimicrobial resistance and virulence analyses were performed using CARD and VFDB. [f] All isolates belonged to serovar Infantis and contained the *golS* gene, not shown.

7.5 Discussion

This is the first documented WGS study conducted in the poultry (broilers and layers) industry along the broiler production chain in Trinidad and Tobago, and the Caribbean region at large. Whole-genome sequencing analysis has been used to investigate genetic characteristics and phylogenies among *Salmonella* strains isolated from different origins, such as humans, food, animals, and the environment [523-526]. The current study was comprised of isolates from four cross-sectional studies conducted at the level of retail outlets (2016-2017) [300], broiler processing plants (2018) [328], broiler farms and broiler hatcheries (2019) [519]. Although several limitations exist with the use of cross-sectional instead of longitudinal studies, this approach provides valuable information on the status of *Salmonella* shedding and contamination at the four levels (hatcheries, farms, processing plants, and retail outlets) of the broiler production chain in the country. Furthermore, the information obtained will lead to a better understanding of the epidemiology of *Salmonella* and the associated public health implications. Finally, this approach will also facilitate the implementation of an effective surveillance system across the poultry production system in the country.

Using the SISTR pipeline, a 76% agreement was detected with the traditional conventional serotyping method, which utilizes the White–Kauffman–Le Minor (WKL) scheme, which is based on immunological reactions to somatic (O) and flagellar (H) antigens [527]. However, it has been documented that conventional serotyping is time-consuming, labor-intensive, costly, and some isolates do not express serotype antigens due to a single nucleotide change in the genome [528, 529]. On the other hand, SISTR pipeline has been validated and a 94.6% overall serovar prediction accuracy was reported when 4,291 genomes were analysed [208]. In silico serotyping, pipelines like SISTR provide us with an understanding of the antigenic genes that are carried by an isolate and not necessarily what is expressed by that isolate, an advantage over traditional serotyping methods. In a study that compared three in silico pipelines, SISTR, SeqSero and MLST, to

traditional serotyping techniques using a set of 813 verified clinical and laboratory isolates, 94.8%, 88.2%, and 88.3% accuracy, respectively was reported [530]. Of significance in our study was the incorrect serotype classification by the conventional method of potential public health important serotypes such as *S*. Albany, Senftenberg, Infantis, and Caracas. Variations in *Salmonella* serovars in poultry have been reported in different countries and are said to be a function of geographic location [504]. In Egypt [531], serovars Enteritidis and Typhimurium were isolated from broiler chickens at retail outlets; in Japan [350], serovars Infantis, Manhattan, Schwarzengrund from caecal samples in broilers; and in China [532], serovars Pullorum, Gallinarum, Enteritidis and Typhimurium were the predominant serotypes. Unlike our study, *S*. Kentucky, Javiana, Infantis and Albany were the predominant serovars isolated along the broiler production chain.

Gene $bla_{CTX\text{-}M\text{-}65}$ was detected in 77% (31/40) of the *S*. Infantis strains isolated at Colombian processing plants [533], a finding comparable to the 50% (10/20) detected in the current study. Worldwide, the rapid development of resistance to extended-spectrum cephalosporins, predominantly associated with the production of β-lactamase-producing bacteria (EsβL) in different *Salmonella* serovars, have been reported. In agreement with our study, EsβL resistance genes have been detected in *Salmonella* strains isolated from animal products in several countries including Korea (food animals and humans), 1.6% [534], Mexico (humans and animals), 6.6% [535], and Brazil (broiler chickens), where 27.8% [536] of isolates were positive for the $bla_{CTX\text{-}M}$ gene. The detection of 6.8% MDR strains among serovar Infantis isolates possessing the $bla_{CTX\text{-}M\text{-}65}$ gene is of public health significance due to the reported cross transmission of EsβL-producing bacterial strains from poultry farms to other livestock farms and humans with potential for wide-spread population infections [537, 538]. A cause for concern is the detection of 10 MDR serovar Infantis strains, each possessing the $bla_{CTX\text{-}M\text{-}65}$ gene, in addition to 38 virulence homologs according to VFDB. In 2014, the detection of a MDR emergent *Salmonella* Infantis (ESI) strain, often containing the $bla_{CTX\text{-}M\text{-}65}$ gene,

was first reported in Israel, and subsequently detected in Italy, Japan and Russia [539-542]. However, retrospective sequencing tracked the origins of this clone to South America [543, 544]. This ESI strain was documented to carry a large plasmid ESI (pESI) with several antimicrobial resistance, metal and virulence genes. This clone was detected in retail meats in Tennessee, USA, in 2014, but by 2019 had spread throughout the USA to comprise 29% *Salmonella* isolated from retail chickens and 7% from retail turkey [545]. This clone also accounted for nearly 10% of all human Infantis cases by 2017 in the United States and was highly related to chicken sources [546]. The most frequently described ESβL genotype in Colombia between 1997-2018 was *CTX-M*, which was detected primarily in *S*. Typhimurium (40%; 65/164) and *S*. Infantis (29%; 48/164). Detection of bla_{CTX} genes has been reported in Latin American countries, such as Brazil and Argentina [547]. The assumption was that cephalosporin resistance development was due to the injection of ceftiofur into fertile eggs at hatcheries to prevent *E. coli*-induced omphalitis in day-old chicks [548]. This assumption was supported by a Canadian study that revealed a strong correlation between this practice and the increase in ceftiofur-resistant strains of *S.* Heidelberg [549]. This practice was not evident at hatcheries in our study, nor was *bla* genes detected among hatchery isolates.

A quick look into the NCBI Pathogen detection browser (https://www.ncbi.nlm.nih.gov/pathogens) allows us to determine that the eight strains of *S.* Infantis (ST32) detected in this study were highly related to the MDR emergent *S.* Infantis strains carrying $bla_{CTX\text{-}M\text{-}65}$ (https://www.ncbi.nlm.nih.gov/pathogens/tree#*Salmonella*/PDG000000002.2405/PDS000089910.160?term=CFSAN103822,%20CFSAN103806,%20CFSAN103805,%20CFSAN103797,%20CFSAN103801,%20CFSAN103796,%20CFSAN103798,%20CFSAN103802, accessed on 2 March 2022), reported in previous studies [539-546]. This highlights the usefulness of WGS approaches for AMR surveillance in a country or region, in this case, Trinidad and Tobago, considering the significant public health and clinical implications resulting from the presence of this large plasmid ESI. The plasmid detected in our *S.* Infantis carrying the *CTX-M-65* gene (Accession: CP066336.1) contained 312,952 bp,

differing from the plasmids reported in USA [550] and Italy [541] which ranged from 316,160- 323,122bp. These eight strains exhibited two resistance profiles: *aac(3)-IVa, aadA1, aph(3')-Ia, aph(4)-Ia, bla$_{CTX-M-65}$, dfrA14, gyrA_D87Y, mdsA, mdsB, sul1, tet(A)* (4 strains) and *aac(3)-IVa, aadA1, aph(4)-Ia, bla$_{CTX-M-65}$, dfrA14, gyrA_D87Y, mdsA, mdsB, sul1, tet(A)* (4 strains), according to the NCBI database (AMRFinderPlus). Our findings were similar to the *aph(4)-Ia, aac(3)-IVa, aph(3')-Ic, bla$_{CTX-M-65}$, fosA3, floR, dfrA14, gyrA_D87Y, sul1, tetA, aadA1* pattern detected in the USA [550] and *aph(4)-Ia, aac(3)-IVa, aph(3')-Ic, bla$_{CTX-M-65}$, fosA3, floR, dfrA14, sul1, tetA, aadA1* detected clinically in Italy [541], where both studies used ResFinder.

It must be highlighted that EsβL-producing *K. pneumoniae* was detected in 78.8% (41/52) of clinical isolates originating from a tertiary care hospital in Trinidad and Tobago, where the *bla$_{SHV}$* and *bla$_{CTX-M}$* genes were predominantly detected [551]. It is of interest that all the MDR Infantis strains isolated in the current study originated from broiler farms. This is because there is a potential for *Salmonella* strains positive for *bla$_{CTX}$* gene, AMR, and associated virulence genes, to enter the human food chain through the processing plants and chicken products at the retail outlets. This is supported by reports that documented close association of MDR Infantis strains recovered from the broiler population to animal production environments, eventually spread into the food chain and potentially humans [552, 553].

As with this study, aminoglycoside resistance genes and *sul1* genes were also detected in *Salmonella* Infantis strains isolated in a recent study conducted at three Colombian broiler processing plants [533]. Sulphonamide resistance conferred by *sul* genes [554] were reported in Canadian swine and chicken *Salmonella* isolates [555] and at a broiler processing plant in China [556], however, in our study only the *sul1* gene was detected in all our *S*. Infantis strains and the only Senftenberg strain asssessed. Arkali et al. [557] detected *sul1* gene among 58% of *Salmonella* isolated from chickens in Eastern Turkey. The detection of one mobilized colistin resistance, *mcr-9.1* gene [558] in an isolate of serovar Senftenberg was not a significant finding. This gene is not associated with colistin resistance in

Salmonella or *E. coli* in the United States [559]. However, detecting this novel *mcr-9* homolog is crucial as it can confer phenotypic resistance to colistin and warrants close monitoring [513].

The *qacEdelta1* gene, known to confer resistance to antiseptics, was also detected in *Salmonella* from retail foods of animal origin [517]. It must be considered that the presence of antimicrobial resistance genes can represent the phenotypic resistance of antimicrobial agents, and thus diminish their effectiveness when used on farms or processing plants. However, it is important to mention that there are several mechanisms of antimicrobial resistance in bacteria. It is not always associated with a specific gene responsible to resistance. This supports our findings where resistance genes were found in two Infantis strains but phenotypically, they were all sensitive. Cross-resistance to antimicrobial agents can occur with resistance within group members of chemical-related compounds, and/ or with a similar mechanism of action [560, 561]. The correlation of genotypic and phenotypic resistance was variable in our study, contrary to the findings of other studies where harmonic correlation was evident [556, 562]. The lack of correlation between phenotypic and genotypic resistance profiles may occur due to low sensitivity and specificity of disk method, inoculum concentration, laboratory capacity and individual skill. Misalignments between phenotypic and genotypic resistance patterns have been reported by others [563, 564].

In the current study, only 6.8% (10/146) of the isolates, based on genotypic characterisation, exhibited multidrug resistance, at variance with the 96.6% reported in *Salmonella* isolated from chickens sampled at chicken farms in South Africa [565] and the 27.3% reported for *Salmonella* strains isolated from broilers in Egypt [337]. Therefore, our low frequency of detecting MDR is of therapeutic significance at the broiler farm level in the country.

SPI-1 and *SPI-2* genes enable invasion of eukaryotic cells, induction of macrophage cytotoxicity, invasion of phagocytes and survival inside phagocytic cells [566-569]. The inactivation of the TTSS-1 translocated effector gene *sipB* in *S.* Dublin has been associated with reduced fluid secretion and inflammation [570]. This is of

public health significance because of the 73 genes detected in the current study, 49.3% and 35.6% were detected in *Salmonella* strains isolated from processing plants and retail outlets, respectively, highlighting the risk posed to consumers should they be infected with a serovar positive for the gene. In the current study, serovars Aberdeen, Anatum, Enteritidis, Infantis, Javiana, Manhattan, Virchow, and Weltevreden were all positive for the *sipB* gene.

Fimbrial adherence factors that aid intestinal adhesion such as long polar fimbriae (*lpfA*) and aggregative fimbriae (*agfA/csgA*) are highly conserved in *Salmonella* and have been implicated in biofilm formation and adhesion to surfaces and epithelial cells that is an important stage prior to biofilm formation, respectively [571, 572]. This is of importance in the current study because 99.3% of the isolates were positive for the *csgA* gene, therefore having the potential for biofilm formation and persistence in the environment. The high incidence of *csgA* in our study is comparable to the findings in different serovars, as reported by others [573, 574].

Typhoid toxin/*cdtB* cytolethal distending toxin B, previously thought to be a unique virulence factor in *S*. Typhi was recently characterised in at least 40 non-typhoidal *Salmonella* serovars [575], as evident in our study. The detection of virulence genes *invA, csgA, lpfA, sopE* and *spvC* in our *S.* Enteritidis strains agrees with the findings of studies conducted on chickens sold at Bangladeshi retail outlets [576], in food and humans in Brazil [577] and in humans and animals in Iran [578].

The positive correlations in the detection of AMR and virulence genes in the *Salmonella* serovars isolated from farms, retail outlets, and 'pluck shops' are indicative of close similarities in the occurrence of AMR and virulence genes in different serovars and isolates in the study area or source-dependent AMR/virulence profiles. The presence of virulence genes and the occurrence of AMR *Salmonella* isolates can potentially accelerate the pathogenicity of microbes [579]. It has also been reported that the emergence of resistant *Salmonella enterica* solely depends on genetic and pathogenicity mechanisms that may enhance survivability by preserving their drug resistance genes [580]. However, the correlation between AMR and virulence has been shown to vary in studies

conducted by others. The acquisition of AMR by *Salmonella* isolates decreases [581, 582], increases [583, 584], or does not change [585, 586] their potential virulence according to those authors.

7.6 Conclusions

This study highlighted the antimicrobial resistance and virulence genes associated with *Salmonella* serovars isolated along the broiler production chain in Trinidad and Tobago. The detection of the $bla_{CTX\text{-}M\text{-}65}$ gene, MDR, and highly virulent *S.* Infantis isolates based on their genotypes, is cause for concern given their international emergence and implications for human health. The positive correlation of resistance and virulence genes detected at broiler farms, processing plants, and retail outlets (‘pluck shops’) is significant since the latter two stages of the broiler continuum can directly impact consumers of contaminated, improperly handled, or cooked chicken.

The availability of these genomes will help future source tracking during epidemiological investigations associated with *Salmonella* foodborne outbreaks in the region and worldwide. Therefore, the abundance of data from several sources in the country will benefit the scientific community at large.

Connecting statement to the next chapter

Based on the findings on the frequency of detecting resistance genes and virulence factors presented in this chapter (Chapter 7), the genetic relatedness of the serovars of *Salmonella* recovered from different types of samples collected from the four levels (hatcheries, farms, processing plants and retail outlets) of the broiler industry in the country, was determined using their phylogenies and bioinformatic analysis in Chapter 8.

CHAPTER 8: MOLECULAR CHARACTERISATION OF *SALMONELLA* ISOLATED FROM THE BROILER INDUSTRY IN TRINIDAD USING A 'FARM TO FORK' APPROACH: A COMPARISON OF THE PHYLOGENIES OF *SALMONELLA* SEROVARS

The cross-sectional study aspect of this chapter is published in Poultry Science

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8.1 Abstract

This study was conducted to determine the phylogenies of *Salmonella* strains isolated from cross-sectional studies conducted at the hatcheries, broiler farms, processing plants, and retail outlets (broiler production chain) in Trinidad and Tobago over four years (2016-2019). Whole genome sequencing (WGS) was used to characterise *Salmonella* isolates. Core genome phylogenies of eight serovars of public health significance were analysed for similarities in origin and relatedness. In addition, *Salmonella* strains isolated from human salmonellosis cases in Trinidad were analysed for their relatedness to the isolates detected along the broiler production chain. The common source of these isolates of diverse serovars within farms, within processing plants, between processing plants and retail outlets, and among farm-processing plant-retail outlet continuum was well-supported (bootstrap value >70%) by the core genome phylogenies for the respective serovars. Also, genome analyses revealed clustering of *Salmonella* serovars of regional (intra-Caribbean) and international (extra-Caribbean) origin. Similarly, strains of *S.* Enteritidis and *S.* Infantis isolated from human clinical salmonellosis in 2019 from Trinidad and Tobago clustered with our processing plant isolates recovered in 2018. Another study was conducted by sampling a batch of chickens from production at a broiler farm through processing at a plant, to retailing at a supermarket, within a period of two months. Resistance genes *aac-(3)IV, aph(4)-Ia, bla*$_{\text{CTX-M-65}}$ and *qacEdelta1* were detected in 84.6% (11/13) of the isolates. Based on core genome phylogenies, the *S.* Infantis strains were grouped together and well-supported by bootstrap values, within and across the three levels of sampling. This study, which used a batch sampling method and conducted within two months demonstrated a direct measure of the dynamics of transmission of *Salmonella* during a farm to fork approach. This is first phylogenetic analysis of *Salmonella* isolates using WGS from the broiler industry in the Caribbean region. The food safety risk posed to consumers of inadequately cooked or handling *Salmonella*-positive chickens, emphasises the potential for transmission of *Salmonella* in the farm-processing plant-retail outlet chain. The study also demonstrates the importance of detection

of the *bla*$_{CTX\text{-}M65}$ gene in all the multi-drug resistant (MDR) *S.* Infantis strains, consistent with other emergent Infantis strains detected worldwide.

8.2 Introduction

The recent technological advancements and declining costs of next-generation sequencing tools such as whole-genome sequencing (WGS) have increased the efficiency and resolution of detection, characterisation, and surveillance of infectious pathogens of public health. The vast potential of WGS in surveillance of infectious diseases [587, 588] has been demonstrated in many studies [589-591]. A more effective and rational approach for prevention of microbial threats is essential at the global level due to increases in food trade, population density and movement, and consumption habits which have increased the risk of contracting foodborne diseases and their potential global spread. To harness the full potential of WGS, the global microbial identifier (GMI) initiative was developed as a shared global database of genomes in order to diagnose infectious diseases in humans and animals, identify microorganisms in food and environment, as well as track and trace microbial agents globally [592]. Sharing of sequencing data within the global scientific community supports the mission of public health institutions and the One Health concept. Furthermore, this approach facilitates early recognition and investigation of international outbreaks in addition to providing invaluable information to clinicians, veterinarians, environmental scientist as well as policymakers, regulators and industry [593, 594].

The risk of salmonellosis exists along the farm-to-fork continuum posing a threat to public health [595]. Therefore, utilising molecular analysis of *Salmonella* strains to investigate their distribution, transmission, and characteristics is vital. Serotyping using traditional techniques is the predominant practice to determine serovars and the relatedness of *Salmonella* strains from foods, animals, environment and disease outbreaks in the Caribbean [30, 596-600]. PFGE has been

used previously to investigate the diversity of *Salmonella* strains isolated in the poultry industry [601] and in human gastroenteritis in the region [602]. However, published studies on the use of WGS of *Salmonella* isolated from the broiler industry in the country and the Caribbean region at large are non-existent. The high molecular resolution offered by WGS compared with PFGE allows for more accurate identification and characterisation of bacterial strains [603]. Surveillance systems that integrate public heath, animal health and food production are optimal to detect, investigate and solve infection commonly transmitted through food.

Therefore, the primary objective of this study was to investigate the genetic relatedness of *Salmonella* serovars recovered from the broiler industry in Trinidad and Tobago using WGS to determine their phylogenies. The investigation comprised both cross-sectional and longitudinal components.

8.3 Materials and methods

8.3.1 Sample selection and preparation

The sample set and sample preparations used in the previous chapter (Chapter 7) were subjected to in silico phylogenetic analysis. The following is a summary of the number of isolates included from earlier studies: hatcheries (n= 10), farms (n= 20), processing plant (n= 52) and retail outlets (n= 55). Five additional human clinical isolates of *Salmonella* belonging to serovars *S.* Enteritidis and *S.* Infantis, obtained from the Caribbean Public Health Agency (CARPHA), Trinidad and Tobago were included in our panel of isolates subjected to WG*S.* These human strains were included to compare *Salmonella* strains detected along the broiler production chain to human salmonellosis, since to date there has been no association between the two. The selected isolates represent *Salmonella* strains recovered from various samples, the serovars, and different enrichment (selective and non-selective) broths and agar used to isolate the pathogen.

8.3.1.1 Determination of phylogeny

Maximum likelihood phylogenies of all serovars (i.e. serovars comprising 4 or more isolates) from this study, in addition to reference genomes (completely, closed genomes) for each serovar, downloaded from NCBI, were calculated using core genome alignment. Reference genomes used for each serovar was as follows: Albany_strain_R17.2117 (Accession: NZ_CP063330), Anatum_strain_ATCC_BAA_1592 (Accession: CP007531), Enteritidis_strain_P125109 (Accession: NC_011294), Infantis_strain_119944 (Accession: NZ_CP047881), Javiana_strain_CFSAN001992 (Accession: NC_020307), Kentucky_strain_CVM_30177(Accession: NZ_CP051346), Manhattan_strain_SA20084699 (Accession: NZ_CP022497), Schwarzengrund_strain_CVM_30168 (Accession: NZ_CP051350), and Senftenberg_strain_CVM_34514 (Accession: NZ_CP051329). Other publicly available *Salmonella* strains were randomly selected, downloaded and calculated using core genome alignment using the following criteria for each serovar: detected along the broiler production chain, originating from the Caribbean, South America, North America, Europe and/or Asia as well as the availability of the raw sequences on the NCBI database. It was important to include other publicly available strains in this study due to the worldwide trade of poultry and moreso because this is the first phylogenetic study focusing on *Salmonella* detected from the broiler industry in the region. As such, any comparison with international/regional strains was deemed valuable. MLST ST was also generated (https://github.com/tseemann/mlst).

Core Genome Phylogeny was inferred by creating a core genome alignment with Roary [604] and using the resulting alignment in IQ-TREE v.2.1.2 [605] to produce a tree file and 1,000 bootstrap pseudo-replications. The tree file was visualized with the R package ggtree [606]. Bootstrap values ≥ 70% were considered well-supported/reliable and were indicated by a black dot on the node in the respective figures.

8.3.2 Batch sampling study

8.3.2.1 Study design

The study's design was to conduct three cross-sectional studies at a selected broiler farm, processing plant, and supermarket outlet using a batch sampling approach. This entailed following broiler chicks reared on a broiler farm, following the same batch of chickens to slaughter at a processing plant, and finally sampling chilled carcasses from the same processing plant at a supermarket. The samples were collected longitudinally, over a period of 2 months.

8.3.2.2 Selection of broiler farm to use in the study

Initially, samples were collected at 4 broiler farms, comprising 2 each from two processors in the country. On each of the four farms, cloacal swabs were randomly collected from chicks aged 10 to 25 days and at day 35 from a selected pen to determine their *Salmonella* status.

8.3.2.3 Type and number of samples collected

Figure 8.1 shows the types and number of samples collected at the three levels of the study. Overall, a total of 32, 40, and 10 samples were collected from the broiler farm, processing plant, and supermarket, respectively.

8.3.2.4 Collection of samples

The procedure for collecting cloacal swabs (broiler farm and pre-slaughter at processing plant), necks, pre-packaged whole chicken and pre-packaged chicken parts (processing plants), and chilled chickens (supermarket) was earlier described (Chapters 3, 4 and 5).

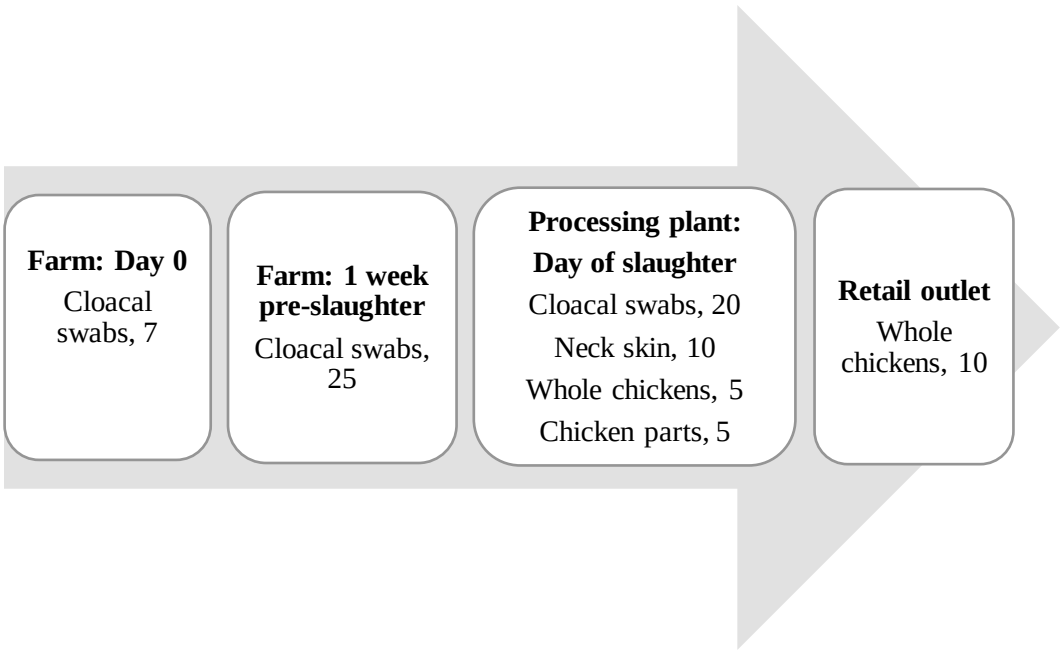

Figure 8.1. Schematics showing the number of samples collected from the positive farm, at the processing plant and retail outlet.

8.3.2.5 Processing of samples, isolation and identification of Salmonella

The methods used for the processing, isolation and phenotypic identification of *Salmonella* from farms, processing plants and retail outlets were described in earlier chapters (Chapter 3,4 and 5).

8.3.2.6 Determination of antimicrobial resistance

Forty-nine isolates of *Salmonella* representative of the various sources and enrichment media/plating were subjected to disc diffusion antimicrobial susceptibility to 8 antimicrobial agents as earlier described (Chapter 3).

8.3.2.7 Molecular characterisation

Thirteen, non-duplicate isolates of *Salmonella*, representative of the various sources were subjected to WGS. The methods and software used were earlier described in the previous chapters (Chapter 7).

8.3.2.8 Statistical analyses

Chi-square analyses were conducted using the Statistical Package for Social Sciences, SPSS (version 27, IBM Corp., Somers, NY) to determine statistically significant associations in the frequency of isolation of *Salmonella* spp. The level of significance was set at an alpha level of 0.05.

8.4 Results

8.4.1 Core genome phylogenies of serovars detected over a period of four years

8.4.1.1 S. Albany

Strain SAMN16678613, isolated in 2016 from a chicken carcass sold at a retail outlet in the current study, was grouped with all the other isolates collected in 2018 and 2019 from processing plants and farms in Trinidad, respectively (Figure 8.2, Table 8.1), supported by a bootstrap value of 100%. SAMN14677224 isolated from broiler farm C (Processor A; cloacal swab) in 2019 was grouped with SAMN14677213 (boot swab taken at farm F operated by Processor B in 2019), SAMN25867743 (neck skin collected at processing plant B in 2018), and SAMN25867755 (cloacal swab collected at a farm A operated by Processor B in 2019) and was also supported by a bootstrap value of 100%. SAMN16678613, detected in 2016 from a retail outlet clustered to the previously mentioned isolates, highlighting the linkages of *S.* Albany isolated at different sampling levels in this study (retail outlet, various farms, and processing plants). Publicly available isolates, SAMN16986415 and SAMN16986826, detected at retail outlets in China

in 2014 and 2016, respectively, were clustered to all the Albany isolates from various types of samples and origin isolated between 2016 and 2019 in the current study. Two strains included in this tree were isolated in Taiwan from a retail outlet (SAMN16987445) and human feces (reference strain; Albany_strain_R17.2117) from 2016 and 2017, respectively not clustered together nor to any of the isolates.

8.4.1.2 S. Anatum

All the *S.* Anatum isolates recovered at processing plants in 2018 from chilled whole/chicken parts, and neck skin samples (Table 8.2) were clustered together and well-supported by a bootstrap value of 100% (Figure 8.3). All five isolates (SAMN25867742, SAMN16678587, SAMN14404266, SAMN14404264, and SAMN14404265) were detected on the same sampling day and at the same processing plant B. Two publicly available isolates, SAMN03275929 (USA processing plant in 2006) and SAMN09788964 (Taiwan, farm in 2007), were clustered with isolate SAMN17974661 (USA processing plant in 2019), and all the processing plant isolates detected in 2018 with supporting bootstrap values. Reference strain Anatum_strain_ATCC_BAA_1592 (raw tomato, 2012, USA) and publicly sourced isolate SAMN09914823 (Taiwan processing plant isolate, 2018) exhibited no clustering to one another as well as to the isolates in the current study.

Table 8.1 Sources of serovar Albany isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country isolated
(n=17)								
Albany_strain_R17.2117	292	Reference	Reference	Human faeces	2017	Reference	Albany	Taiwan
SAMN25867743	292	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Albany	Trinidad
SAMN25867755	292	Cross sectional	Farm	Cloacal swab	2019	Farm	Albany	Trinidad
SAMN14677213	292	Cross sectional	Farm	Boot swab	2019	Farm	Albany	Trinidad
SAMN14677224	292	Cross sectional	Farm	Cloacal swab	2019	Farm	Albany	Trinidad
SAMN14677212	292	Cross sectional	Farm	Cloacal swab	2019	Farm	Albany	Trinidad
SAMN25867754	292	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Albany	Trinidad
SAMN14404261	292	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Albany	Trinidad
SAMN14404276	292	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Albany	Trinidad
SAMN14404277	292	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Albany	Trinidad
SAMN16678591	292	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Albany	Trinidad
SAMN25867744	292	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Albany	Trinidad
SAMN16678589	292	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Albany	Trinidad
SAMN16678613	292	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Albany	Trinidad
SAMN16986415	292	PublicData	Supermarket	Chicken carcass	2014	Retail Outlet	Albany	China
SAMN16986826	292	PublicData	Pluck Shop	Chicken carcass	2016	Retail Outlet	Albany	China
SAMN16987445	292	PublicData	Supermarket	Chicken carcass	2016	Retail Outlet	Albany	Taiwan

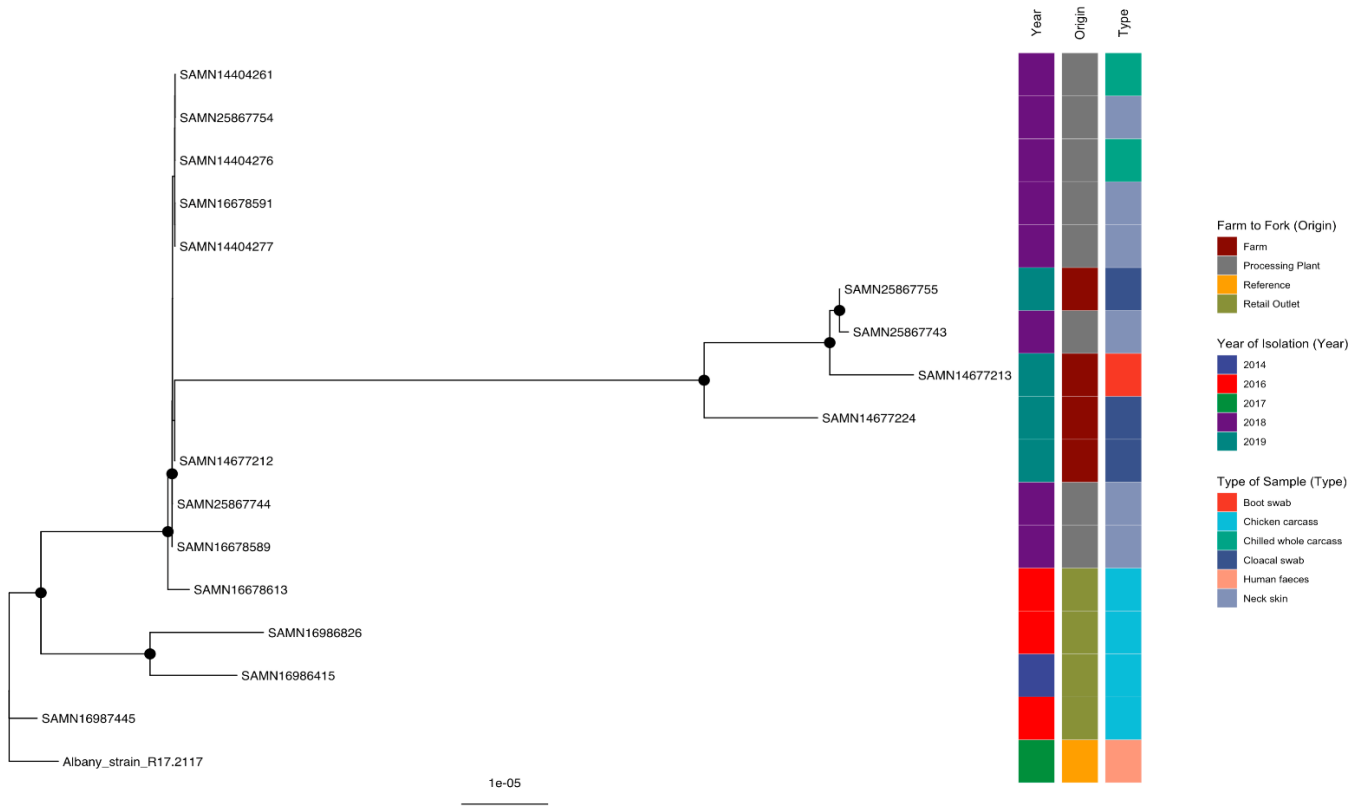

Figure 8.2. Phylogenetic analysis of *S.* Albany. Shown is a maximum-likelihood phylogeny of *S.* Albany calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

Table 8.2 Sources of serovar Anatum isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country isolated
(n=10)								
Anatum_strain_ATCC_BAA_1592	64	Reference	Reference	Tomato-whole-raw	2012	Reference	Anatum	USA
SAMN25867742	64	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Anatum	Trinidad
SAMN16678587	64	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Anatum	Trinidad
SAMN14404266	64	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Anatum	Trinidad
SAMN14404264	64	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Anatum	Trinidad
SAMN14404265	64	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Anatum	Trinidad
SAMN03275929	64	PublicData	Processing Plant	Chilled chicken parts	2006	Processing Plant	Anatum	USA
SAMN17974661	64	PublicData	Farm	Cloacal swab	2019	Processing Plant	Anatum	USA
SAMN09788964	64	PublicData	Farm	Cloacal swab	2007	Farm	Anatum	Taiwan
SAMN09914823	64	PublicData	Processing Plant	Chilled whole carcass	2018	Processing Plant	Anatum	Taiwan

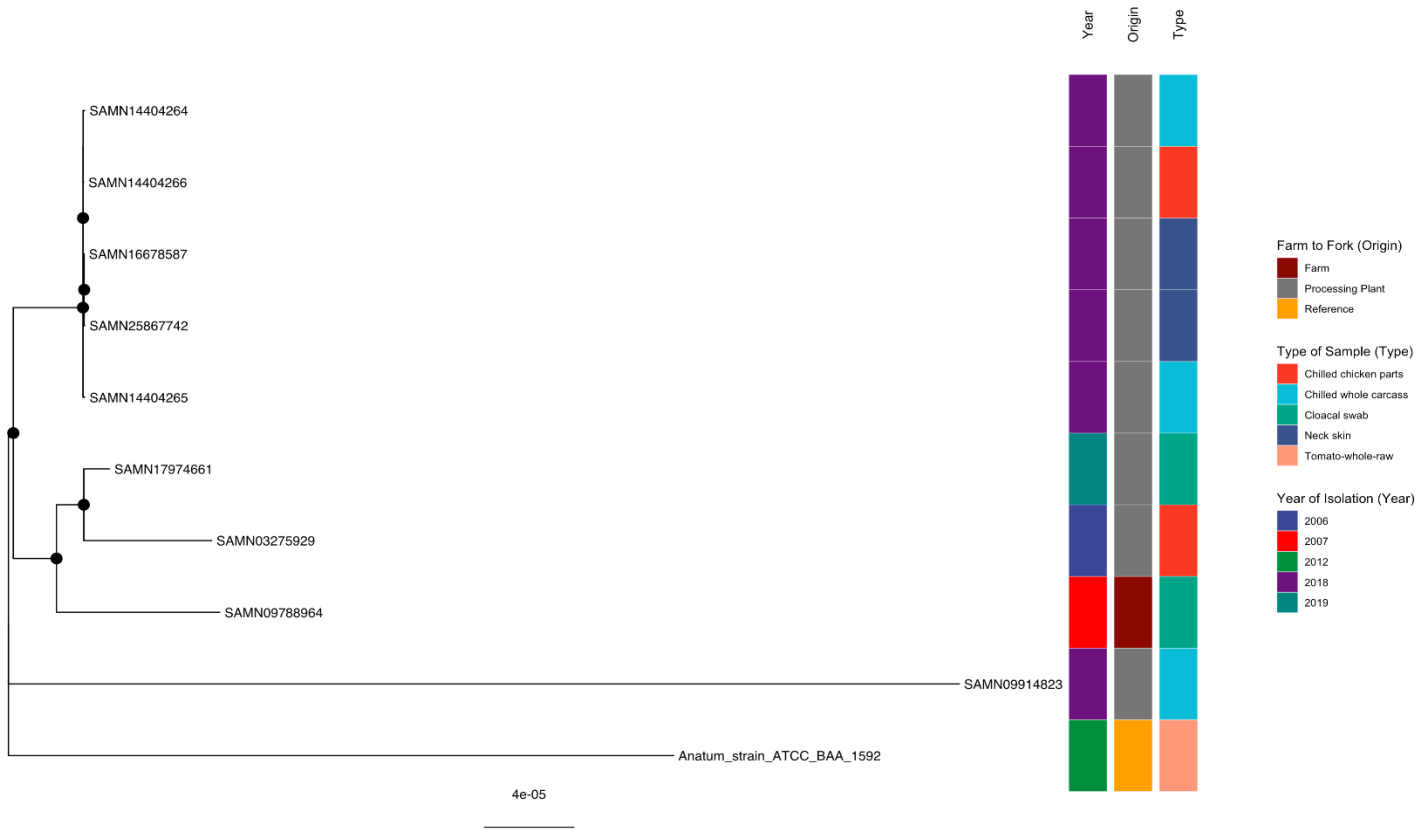

Figure 8.3. Phylogenetic analysis of *S.* Anatum. Shown is a maximum-likelihood phylogeny of *S.* Anatum calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.4.1.3 S. Enteritidis

The four human clinical isolates of *Salmonella* from Trinidad and Tobago isolated in 2019 exhibited clustering to one another, as well as to all processing plant isolates recovered in 2018 (Table 8.3) from the chilled whole carcass, chilled chicken parts, and neck skin in the current study, well-supported by bootstrap values. Within processing plant isolates, two clustered strains were detected during the same sampling visit to Plant A (SAMN25867749 and SAMN14404255) and Plant C (SAMN25867750 and SAMN14404256) (Figure 8.4). Also, four strains were detected at Plant D on two separate sampling days: strains SAMN25867752 and SAMN14404259 on sampling day 1 and strains SAMN14404260 and SAMN14404258 on day 2.

Table 8.3. Sources of serovar Enteritidis isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country isolated
(n-17)								
Enteritidis_strain_P125109	11	Reference	Reference	Human faeces	1988	Reference	Enteritidis	UK
SAMN25867749	11	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Enteritidis	Trinidad
SAMN14404255	11	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Enteritidis	Trinidad
SAMN25867750	11	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Enteritidis	Trinidad
SAMN25867752	11	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Enteritidis	Trinidad
SAMN14404257	11	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Enteritidis	Trinidad
SAMN14404260	11	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Enteritidis	Trinidad
SAMN14404258	11	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Enteritidis	Trinidad
SAMN14404259	11	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Enteritidis	Trinidad
SAMN14404256	11	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Enteritidis	Trinidad
SAMN14404292	11	Control	Human clinical*	Human faeces	2019	Control	Enteritidis	Trinidad
SAMN14404291	11	Control	Human clinical*	Human faeces	2019	Control	Enteritidis	Trinidad
SAMN25867775	11	Control	Human clinical*	Human faeces	2019	Control	Enteritidis	Trinidad
SAMN14404290	11	Control	Human clinical*	Human faeces	2019	Control	Enteritidis	Trinidad
SAMN08951173	11	PublicData	Processing Plant	Chilled whole carcass	2016	Processing Plant	Enteritidis	Brazil
SAMN10103914	NA	PublicData	Processing Plant	Chilled chicken parts	2015	Processing Plant	Enteritidis	China
SAMN03743889	11	PublicData	Processing Plant	Chilled whole carcass	2006	Processing Plant	Enteritidis	Argentina

*Isolates from human gastroenteritis in Trinidad provided by the Caribbean Public Health Agency (CARPHA)
NA: ST could not be assigned with confidence or an allele was missing

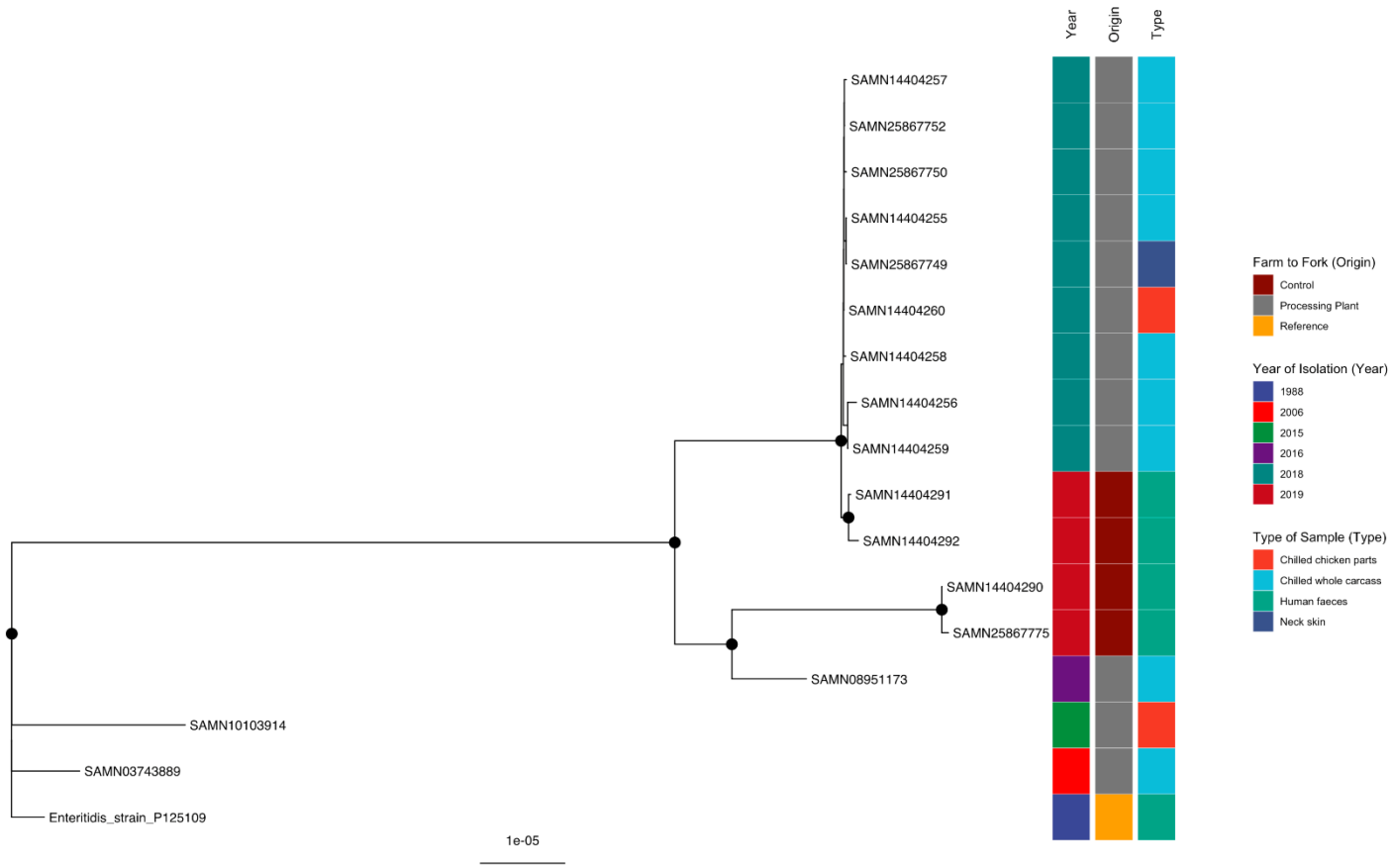

Figure 8.4. Phylogenetic analysis of *S.* Enteritidis. Shown is a maximum-likelihood phylogeny of *S.* Enteritidis calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.4.1.4 S. Infantis

Two distinct clusters were observed. In the first, SAMN08951144, isolated from a Brazilian processing plant in 2016 (Table 8.4), showed well-supported clustering with isolates from neck skin (SAMN16678590 and SAMN16678588) and chilled chicken parts (SAMN16678592 and SAMN16678593) isolates, all from processing plant B (same sample day) in the current study, as well as the human clinical isolate of *Salmonella* recovered (SAMN14404289) in 2019 in the present study (Figure 8.5). SAMN14404263 isolated from chilled whole carcass exhibited well-supported clustering with the other chilled whole carcass (SAMN16678604 and SAMN14404262), chilled chicken parts (SAMN16678605), and post-evisceration carcass (SAMN16678603), all isolated at Plant A on the same sampling day. The latter 4 isolates were clustered together with a bootstrap value of 97 %. Our farm isolates were clustered with three isolates detected at retail outlets in Barbados and an isolate detected at a processing plant in the USA (SAMN18407134).

In the second cluster, well-supported clustering existed among the farm isolates of *Salmonella*, which originated from cloacal swabs, water supply, and drag swabs of litter in our study. Clustering between the drag swab isolate (SAMN14677208, detected at Farm U, operated by Processor B) and cloacal swab samples (SAMN14677211 and SAMN14677232 detected at Farm C, operated by Processor A&C) was observed. There was also clustering with the water supply sample (SAMN14677207, Farm J, Processor B) and two cloacal swab samples (SAMN25867756 and SAMN25867757, Farm K, Processor B), lineages supported by a bootstrap value of 88%. Additionally, clustering was observed among the cloacal swab isolates detected at two farms operated by Processor B, Farm K (SAMN25867756, SAMN14677229, and SAMN25867757) and Farm A (SAMN14677203 and SAMN14677210).

Table 8.4. Sources of serovar Infantis isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country of isolation
(n=30)								
Infantis_strain_119944	32	Reference	Reference	Human faeces	2008	Reference	Infantis	Israel
SAMN25867756	32	Cross sectional	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad
SAMN25867757	32	Cross sectional	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad
SAMN14677203	32	Cross sectional	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad
SAMN14677207	32	Cross sectional	Farm	Water supply	2019	Farm	Infantis	Trinidad
SAMN14677210	32	Cross sectional	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad
SAMN14677229	32	Cross sectional	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad
SAMN14677211	32	Cross sectional	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad
SAMN14677232	32	Cross sectional	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad
SAMN14677208	32	Cross sectional	Farm	Drag swab litter	2019	Farm	Infantis	Trinidad
SAMN14677209	32	Cross sectional	Farm	Water supply	2019	Farm	Infantis	Trinidad
SAMN14677226	32	Cross sectional	Farm	Feed	2019	Farm	Infantis	Trinidad
SAMN18407134	32	PublicData	Processing Plant	Chilled whole carcass	2021	Processing Plant	Infantis	USA
SAMN15964920	32	PublicData	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Barbados
SAMN15964921	32	PublicData	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Barbados
SAMN15964932	32	PublicData	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Barbados
SAMEA3538994	32	PublicData	Processing Plant	Chilled whole carcass	2007	Processing Plant	Infantis	Italy
SAMEA3539001	32	PublicData	Processing Plant	Chilled whole carcass	2014	Processing Plant	Infantis	Italy
SAMN08951144	32	PublicData	Processing Plant	Chilled whole carcass	2016	Processing Plant	Infantis	Brazil
SAMN14404263	32	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Infantis	Trinidad
SAMN14404262	32	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Infantis	Trinidad

SAMN16678605	32	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Infantis	Trinidad
SAMN16678604	32	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Infantis	Trinidad
SAMN16678603	32	Cross sectional	Processing Plant	Post-evisceration carcass	2018	Processing Plant	Infantis	Trinidad
SAMN14404289	32	Control	Human clinical*	Human faeces	2019	Control	Infantis	Trinidad
SAMN16678593	32	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Infantis	Trinidad
SAMN16678588	32	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Infantis	Trinidad
SAMN16678592	32	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Infantis	Trinidad
SAMN16678590	32	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Infantis	Trinidad
SAMN16986393	32	PublicData	Pluck Shop	Chicken carcass	2014	Retail Outlet	Infantis	China

*Isolated from a human gastroenteritis case and provided by the Caribbean Public Health Agency (CARPHA)

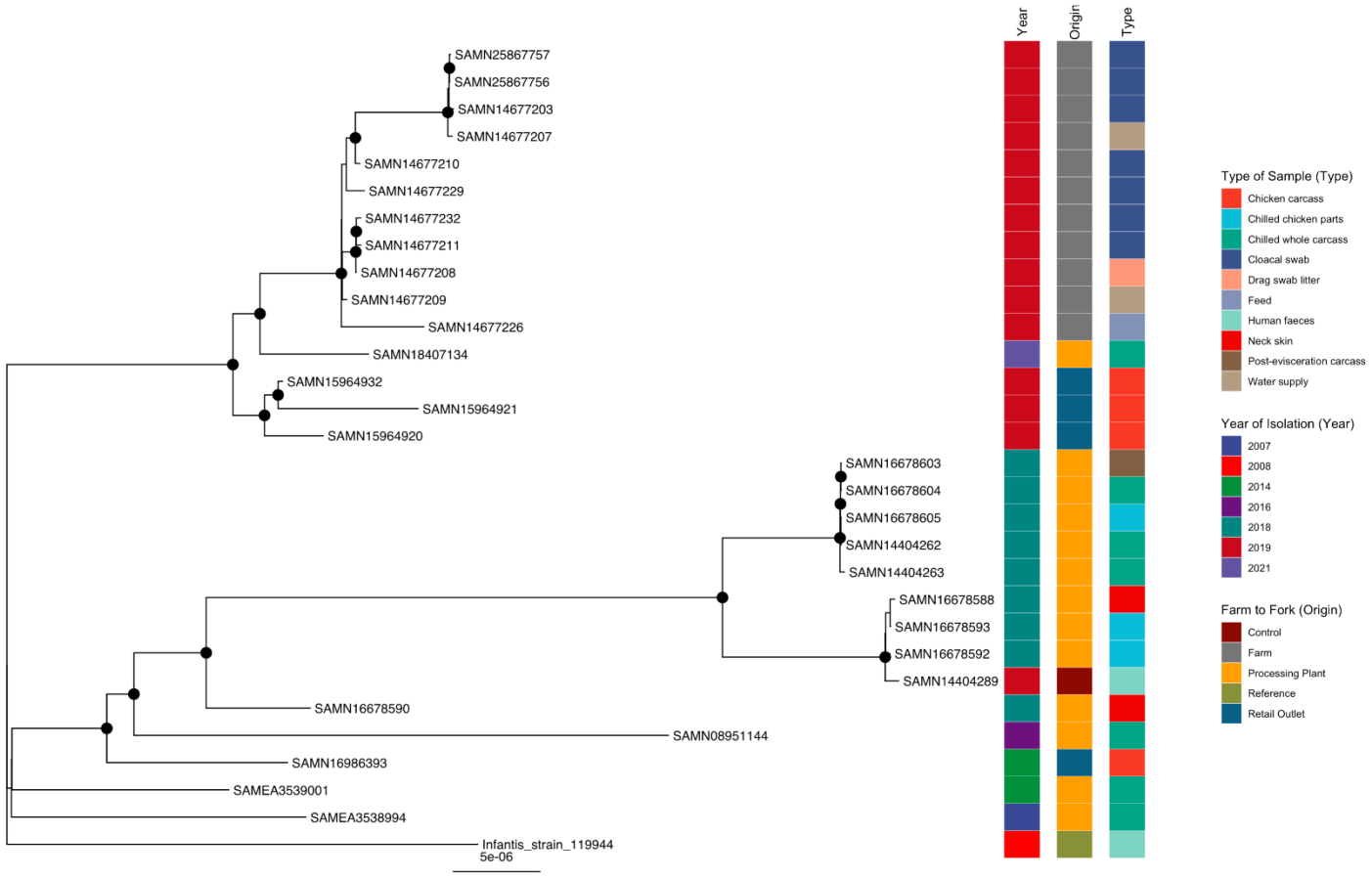

Figure 8.5. Phylogenetic analysis of *S.* Infantis. Shown is a maximum-likelihood phylogeny of *S.* Infantis calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.4.1.5 S. Javiana

Two main clusters supported by bootstrap values of 77% and 97%, respectively, were observed in the Javiana phylogenetic tree (Figure 8.6). Of significance was the well-supported clustering of SAMN16986692, a publicly available isolate (Table 8.5) detected in retail outlets in China in 2016 with retail outlet isolates, SAMN16678614, SAMN14404241, SAMN16678615, SAMN16678616, and SAMN25867712 all detected at one cottage poultry processor in St. George Central. In addition, this subcluster of retail outlet isolates showed reliable clustering with SAMN07682510, isolated from a processing plant in 2016 in a previous study conducted in Trinidad and Tobago, and most (8/10; 80%) of our 2018 processing plant isolates (Plants A and D). Further analyses of our processing plant isolates showed reliable clustering amongst the Javiana isolates detected on all (6/6) neck samples detected at Plant A on the same sample day.

In the second main cluster, isolates SAMN09771033 and SAMN14911214 recovered from processing plants in 2017 and 2019 (both from the USA) exhibited reliable clustering with SAMN11252889 isolated at a Peruvian processing plant in 2017. There was evidence of well-supported clustering of this Peruvian isolate with all the 2016 isolates from retail outlets in the current study. A 2016 retail outlet isolate, SAMN14404242 (detected in the county of St. George East), and a 2018 processing plant isolate, SAMN14404246 (Plant B), were grouped, supported by a bootstrap value of 100%. Isolates SAMN16678596 (Plant B) and SAMN14404244 (cottage processor in the county of Nariva/Mayaro) were also grouped and supported by a bootstrap value of 100%.

Reference strain Javiana_strain_CFSAN001992 isolated from human feces in the USA (2012) exhibited no clustering to any of the isolates included in the tree.

Table 8.5. Sources of serovar Javiana isolates

BioSample	ST	Study type	Source	Type of sample	Year of solation	FarmtoFork	SISTR Serovar	Country of isolation
(n=36)								
Javiana_strain_CFSAN001992	24	Reference	Reference	Human faeces	2012	Reference	Javiana	USA
SAMN25867712	24	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN14404241	24	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN16678614	24	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN16678615	24	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN16678616	24	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN25867747	24	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Javiana	Trinidad
SAMN25867748	24	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Javiana	Trinidad
SAMN14404248	24	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Javiana	Trinidad
SAMN14404247	24	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Javiana	Trinidad
SAMN16678602	24	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Javiana	Trinidad
SAMN16678601	24	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Javiana	Trinidad
SAMN16678600	24	Cross sectional	Processing Plant	Post-evisceration carcass	2018	Processing Plant	Javiana	Trinidad
SAMN25867753	24	Cross sectional	Processing Plant	Post-evisceration carcass	2018	Processing Plant	Javiana	Trinidad
SAMN07682510	24	PublicData	Processing Plant	Cloacal swab	2016	Processing Plant	Javiana	Trinidad
SAMN16986692	24	PublicData	Supermarket	Chicken carcass	2016	Retail Outlet	Javiana	China
SAMN25867718	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN25867720	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN16678621	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN16678620	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad

SAMN25867721	24	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN14404244	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN16678596	1674	Cross sectional	Processing Plant	Post-evisceration carcass	2018	Processing Plant	Javiana	Trinidad
SAMN14404246	1674	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Javiana	Trinidad
SAMN14404242	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN25867729	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN25867730	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN25867731	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN16678624	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN14404243	1674	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN14404245	1674	Cross sectional	Supermarket	Chicken carcass	2016	Retail Outlet	Javiana	Trinidad
SAMN11252889	NA	PublicData	Processing Plant	Chilled chicken parts	2017	Processing Plant	Javiana	Peru
SAMN02846870	1674	PublicData	Fresh coriander	Food	2010	Food	Javiana	Trinidad
SAMN04397451	1674	PublicData	Unknown	Food	1999	Food	Javiana	Trinidad
SAMN09771033	24	PublicData	Processing Plant	Chilled chicken parts	2017	Processing Plant	Javiana	USA
SAMN14911214	24	PublicData	Processing Plant	Lung	2019	Processing Plant	Javiana	USA

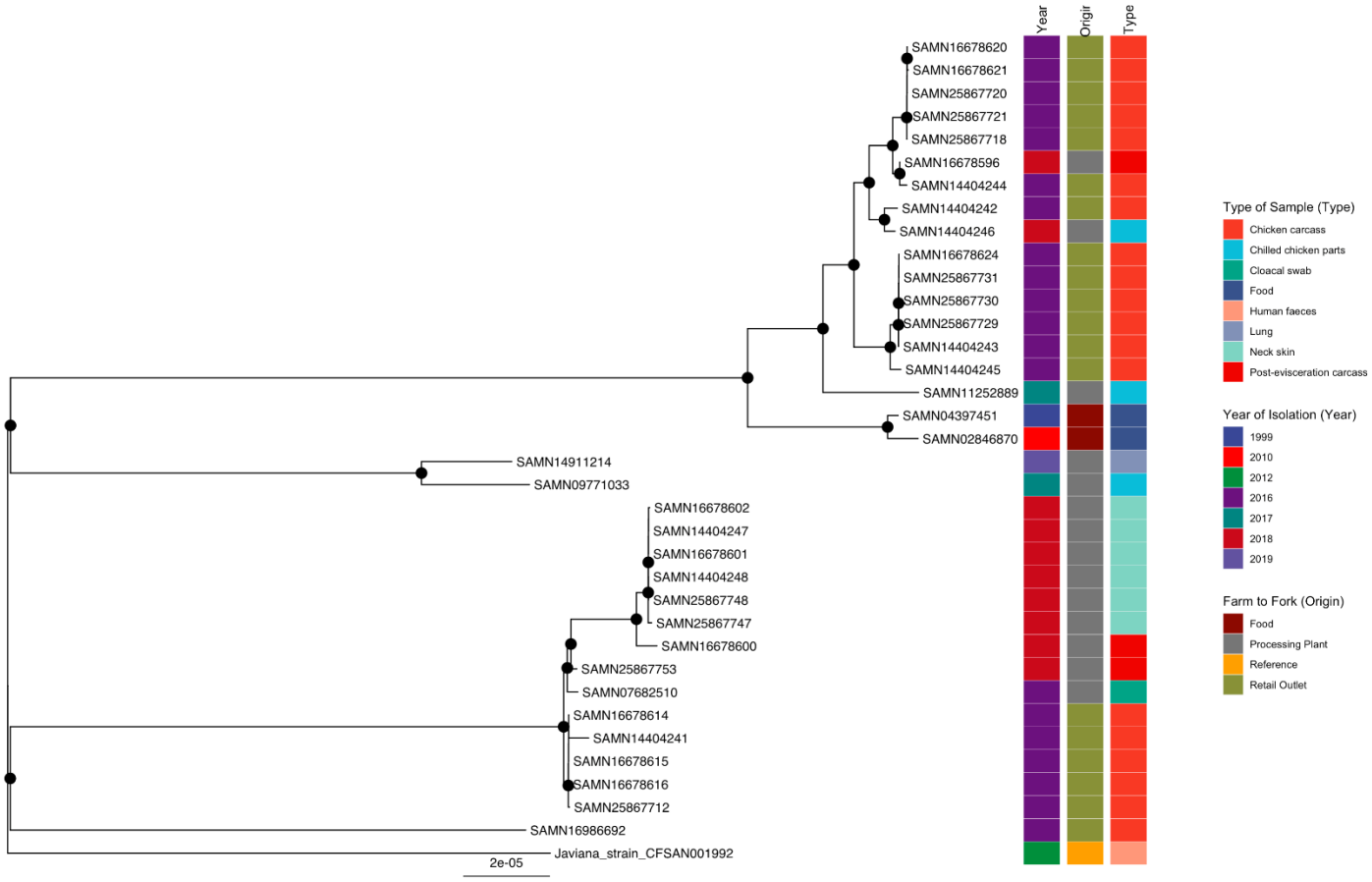

Figure 8.6. Phylogenetic analysis of *S.* Javiana. Shown is a maximum-likelihood phylogeny of *S.* Javiana calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.4.1.6 S. Kentucky

Among the 38 *S.* Kentucky isolates compared, clustering was observed amongst all the processing plant isolates, supported by bootstrap values of 100%. SAMN14404281, isolated at a retail outlet in 2016 in this study, exhibited clustering supported by a bootstrap value of 100% with other retail outlet isolates (SAMN16678617, SAMN16678618, SAMN25867716, SAMN25867715, and SAMN25867713), all detected at the same cottage poultry processor in the county of Victoria (Figure 8.7, Table 8.6). Well-supported clustering was also observed with a 2016 retail outlet isolate from Barbados (SAMN07682504), the retail outlet isolates from 2017 (SAMN25867738 and SAMN14404252, chickens sold at different supermarkets), and a hatchery isolate from 2019 (SAMN25867761, operated by Processor A). An isolate recovered from a litter drag swab sample from a farm in the USA in 2017, SAMN13322254, exhibited well-supported clustering with 100% (5/5) of the hatchery serovar Kentucky isolates (SAMN25867762- stillborn chick, SAMN25867767- hatcher environmental swab, SAMN25867763- eggs in the incubator, SAMN14677227- eggs in hatcher and SAMN16678573- hatcher environmental swab) detected at the hatchery operated by Processor B in 2019. International strains, SAMN16812935 and SAMN16987098, detected in a Brazilian processing plant and Chinese retail outlet, respectively, showed well-supported clustering with the previously mentioned hatchery isolates in the current study, in addition to SAMN13322254 (USA, drag swab litter, 2017). Two retail outlet isolates, SAMN25867732, and SAMN25867733 were detected in county Victoria (same cottage processor). The other two Kentucky strains detected at hatcheries were clustered together and well-supported (operated by Processor A) but exhibited no clustering to other members of the tree.

Table 8.6. Sources of serovar Kentucky isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country of isolation
(n=38)								
Kentucky_strain_CVM_30177	152	Reference	Reference	Chicken carcass	2003	Reference	Kentucky	USA
SAMN25867713	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN25867715	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN25867716	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN16678618	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN16678617	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN14404281	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN14404251	152	Cross sectional	Supermarket	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN07682514	152	PublicData	Supermarket	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN25867746	152	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Kentucky	Trinidad
SAMN16678599	152	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Kentucky	Trinidad
SAMN14404254	152	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Kentucky	Trinidad
SAMN14404253	152	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Kentucky	Trinidad
SAMN16678598	152	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Kentucky	Trinidad
SAMN07504769	2132	PublicData	Processing Plant	Cloacal swab	2016	Processing Plant	Kentucky	Barbados
SAMN16678619	NA	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN07514696	2132	PublicData	Supermarket	Chicken carcass	2016	Retail Outlet	Kentucky	Barbados
SAMN14404250	2132	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN14404249	2132	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN16678622	2132	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN25867738	152	Cross sectional	Supermarket	Chicken carcass	2017	Retail Outlet	Kentucky	Trinidad
SAMN14404252	152	Cross sectional	Supermarket	Chicken carcass	2017	Retail Outlet	Kentucky	Trinidad

SAMN25867761	152	Cross sectional	Hatchery	Hatcher fluff	2019	Hatchery	Kentucky	Trinidad
SAMN07682504	152	PublicData	Supermarket	Chicken carcass	2016	Retail Outlet	Kentucky	Barbados
SAMN25867732	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN25867733	152	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Kentucky	Trinidad
SAMN25867762	152	Cross sectional	Hatchery	Still born chick	2019	Hatchery	Kentucky	Trinidad
SAMN25867763	152	Cross sectional	Hatchery	Eggs in incubator	2019	Hatchery	Kentucky	Trinidad
SAMN14677227	152	Cross sectional	Hatchery	Eggs in hatcher	2019	Hatchery	Kentucky	Trinidad
SAMN16678573	152	Cross sectional	Hatchery	Hatcher environmental swab	2019	Hatchery	Kentucky	Trinidad
SAMN25867767	152	Cross sectional	Hatchery	Hatcher environmental swab	2019	Hatchery	Kentucky	Trinidad
SAMN13322254	152	PublicData	Farm	Drag swab litter	2017	Farm	Kentucky	USA
SAMN16812935	198	PublicData	Processing Plant	Chilled whole carcass	2015	Processing Plant	Kentucky	Brazil
SAMN16987098	314	PublicData	Supermarket	Chicken carcass	2017	Retail Outlet	Kentucky	China
SAMN25867751	152	Cross sectional	Processing Plant	Chilled chicken parts	2018	Processing Plant	Kentucky	Trinidad
SAMN16678609	152	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Kentucky	Trinidad
SAMN25867764	152	Cross sectional	Hatchery	Stillborn chick	2019	Hatchery	Kentucky	Trinidad
SAMN16678575	152	Cross sectional	Hatchery	Stillborn chick	2019	Hatchery	Kentucky	Trinidad

NA: ST could not be assigned with confidence or an allele was missing

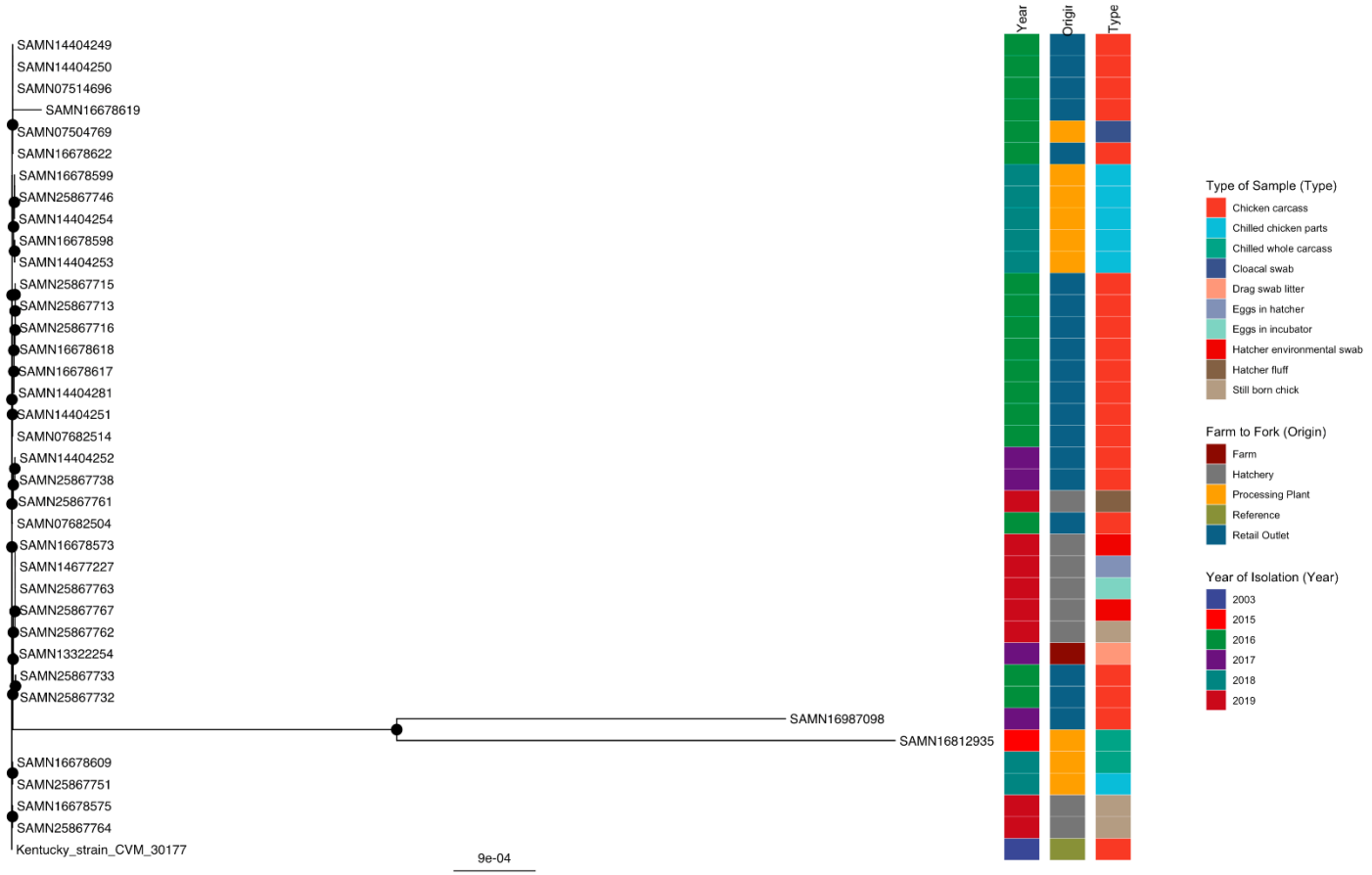

Figure 8.7. Phylogenetic analysis of *S.* Kentucky. Shown is a maximum-likelihood phylogeny of *S.* Kentucky calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.4.1.7 S. Manhattan

Three international strains (all isolated in the USA) of *Salmonella* isolated from processing plants (SAMN10261776, 2018 and SAMN17188031, 2020) and a farm (SAMN10718822, 2018) were clustered with all seven retail outlet isolates recovered in 2016, well-supported by a bootstrap value of 100% (Figure 8.8). In addition, intra-cluster analyses of the seven retail outlet strains in this study exhibited well-supported clustering where all strains were detected from the same pluck shop (Table 8.7).

8.4.1.8 S. Schwarzengrund

Strain SAMN14404267, isolated from a retail outlet in this study (2016), was clustered with a USA reference strain (2003) as well as two other Brazilian strains from a farm (2016) and processing plant (2015) (Figure 8.9, Table 8.8) and supported by a bootstrap value of 100%. All the Schwarzengrund strains isolated from neck skins and chilled whole carcasses that originated from Plants B and D were clustered together and well-supported except for SAMN25867745 (neck skin, Plant D), which was unclustered with the other strains in this tree.

Table 8.7. Sources of serovar Manhattan isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country of isolation
(n=12)								
Manhattan_strain_SA20084699	18	Reference	Reference	Unknown	Unknown	Reference	Manhattan	Unknown
SAMN25867722	18	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Manhattan	Trinidad
SAMN25867725	18	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Manhattan	Trinidad
SAMN25867726	18	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Manhattan	Trinidad
SAMN25867724	18	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Manhattan	Trinidad
SAMN25867723	18	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Manhattan	Trinidad
SAMN14404275	18	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Manhattan	Trinidad
SAMN14404274	18	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Manhattan	Trinidad
SAMN10261776	18	PublicData	Processing Plant	Chilled whole carcass	2018	Processing Plant	Manhattan	USA
SAMN10718822	18	PublicData	Farm	Drag swab litter	2018	Farm	Manhattan	USA
SAMN17188031	18	PublicData	Processing Plant	Minced chicken	2020	Processing Plant	Manhattan	USA
SAMN10458815	18	PublicData	Processing Plant	Chilled whole carcass	2018	Processing Plant	Manhattan	USA

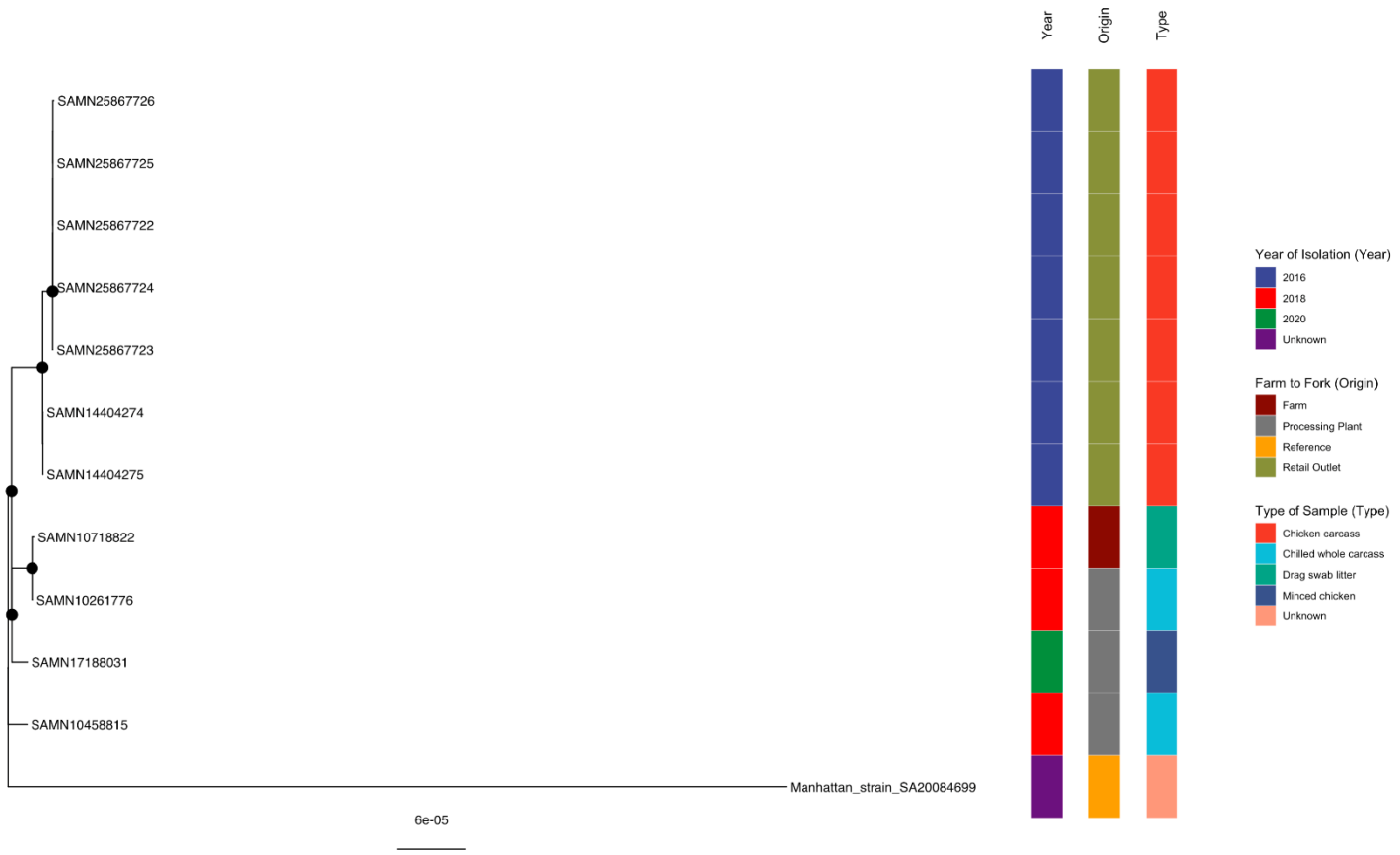

Figure 8.8. Phylogenetic analysis of *S.* Manhattan. Shown is a maximum-likelihood phylogeny of *S.* Manhattan calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.4.1.9 S. Senftenberg

The only Senftenberg serovar (SAMN14677201) isolated at hatcheries in our study was clustered with four international strains (Senftenberg_strain_CVM_34514, SAMN03462287, SAMN13682938, and SAMN14360776) (Figure 8.10, Table 8.9). This tree is included and supported by a bootstrap value of 100%. Of significance is the clustering of SAMN14677201 (stillborn chick, 2019) in this study with SAMN13682938, a cloacal swab sample detected at a farm in the USA in 2019, and SAMN14360776, isolated from minced chicken at a processing plant in the USA (2020), well-supported branches. In addition, one of the retail outlet strains, SAMN14404283 in the current study, showed well-supported clustering with SAMN08951190, a processing plant strain isolated in Brazil in 2016. The other retail outlet strains in this study were unclustered to one another and other members of this tree.

Table 8.8. Sources of serovar Schwarzengrund isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country of isolation
(n=11)								
SAMN25867745	96	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Schwarzengrund	Trinidad
SAMN08874436	96	PublicData	Farm	Drag swab litter	2016	Farm	Schwarzengrund	Brazil
SAMN17170969	96	PublicData	Processing Plant	Chilled chicken parts	2015	Processing Plant	Schwarzengrund	Brazil
SAMN14404267	96	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Schwarzengrund	Trinidad
Schwarzengrund_strain_CVM_30168	96	Reference	Reference	Turkey	2003	Reference	Schwarzengrund	USA
SAMN14404269	96	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Schwarzengrund	Trinidad
SAMN16678610	96	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Schwarzengrund	Trinidad
SAMN14404285	96	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Schwarzengrund	Trinidad
SAMN14404268	96	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Schwarzengrund	Trinidad
SAMN16678597	96	Cross sectional	Processing Plant	Chilled whole carcass	2018	Processing Plant	Schwarzengrund	Trinidad
SAMN16678594	96	Cross sectional	Processing Plant	Neck skin	2018	Processing Plant	Schwarzengrund	Trinidad

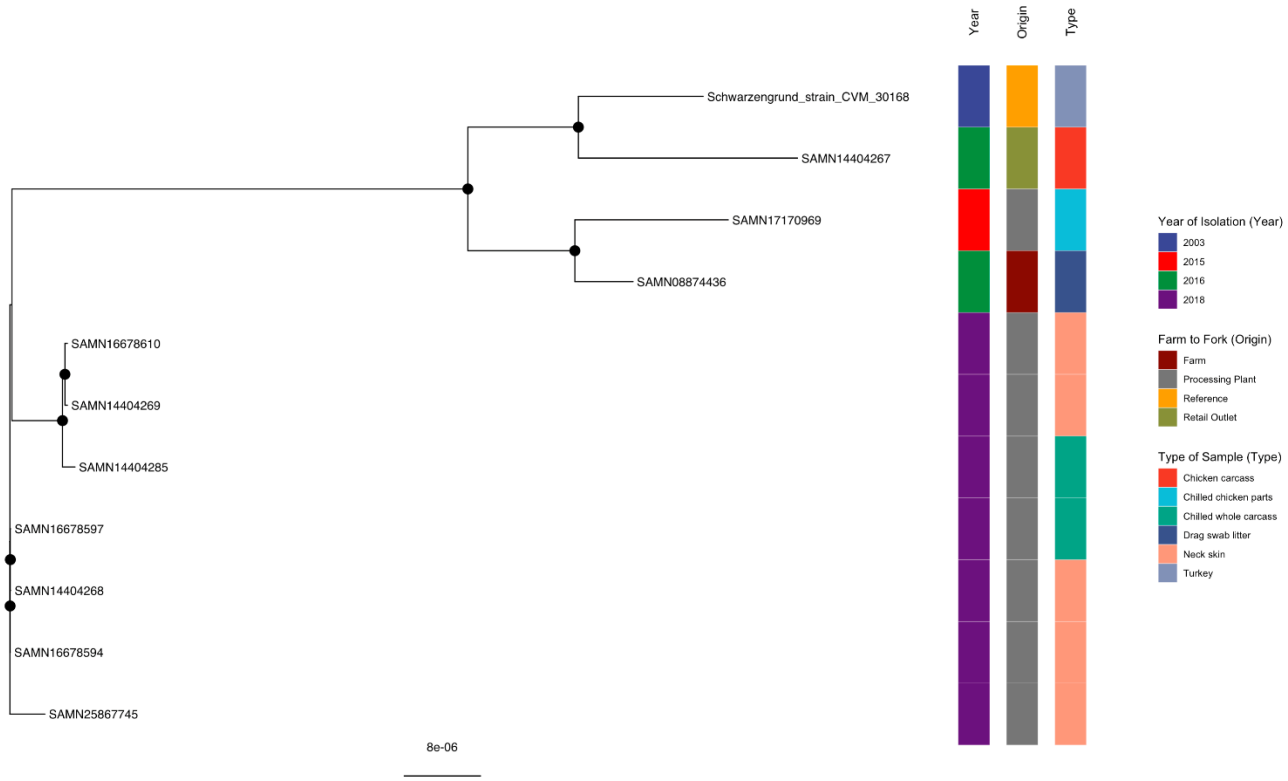

Figure 8.9. Phylogenetic analysis of *S.* Schwarzengrund. Shown is a maximum-likelihood phylogeny of *S.* Schwarzengrund calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

Table 8.9. Sources of serovar Senftenberg isolates

BioSample	ST	Study type	Source	Type of sample	Year of isolation	FarmtoFork	SISTR Serovar	Country of isolation
(n=10)								
SAMN25867719	14	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Senftenberg	Trinidad
SAMN03462287	14	PublicData	Processing Plant	Chilled chicken parts	2014	Processing Plant	Senftenberg	USA
SAMN13682938	14	PublicData	Farm	Cloacal swab	2019	Farm	Senftenberg	USA
SAMN14360776	14	PublicData	Processing Plant	Minced chicken	2020	Processing Plant	Senftenberg	USA
SAMN14677201	14	Cross sectional	Hatchery	Still born chick	2019	Hatchery	Senftenberg	Trinidad
Senftenberg_strain_CVM_34514	14	Reference	Reference	Turkey	2004	Reference	Senftenberg	USA
SAMN04255325	14	PublicData	Processing Plant	Chilled chicken parts	2009	Processing Plant	Senftenberg	USA
SAMN08951190	14	PublicData	Processing Plant	Chilled whole carcass	2016	Processing Plant	Senftenberg	Brazil
SAMN14404283	14	Cross sectional	Supermarket	Chicken carcass	2017	Retail Outlet	Senftenberg	Trinidad
SAMN14404280	14	Cross sectional	Pluck Shop	Chicken carcass	2016	Retail Outlet	Senftenberg	Trinidad

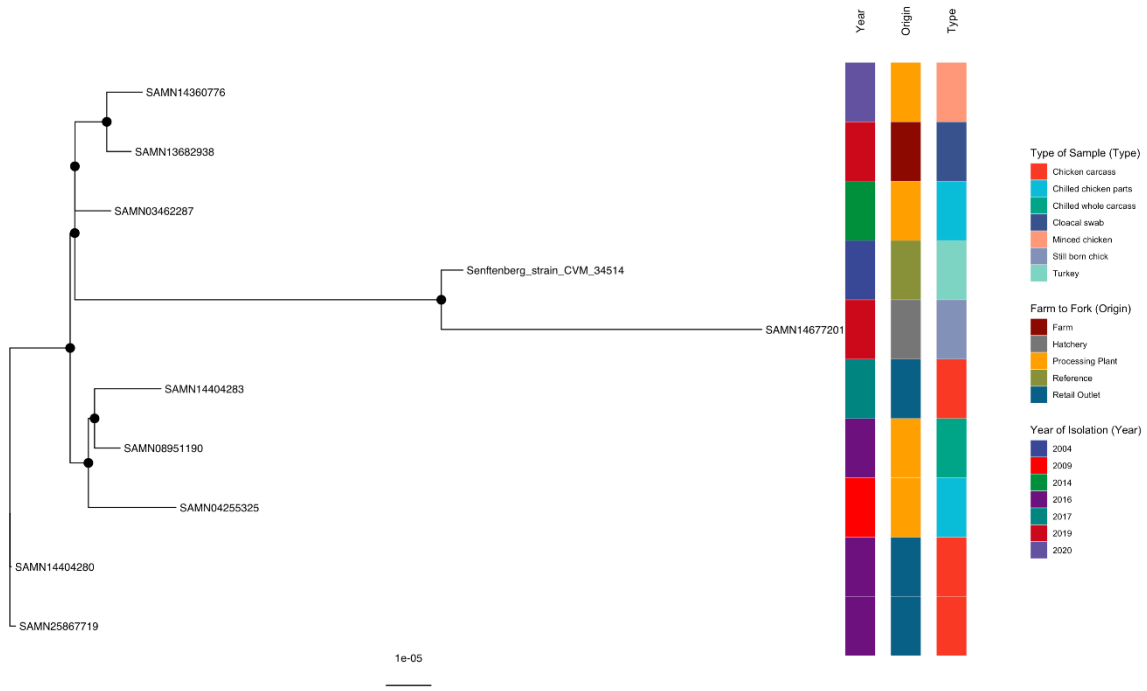

Figure 8.10. Phylogenetic analysis of *S.* Senftenberg. Shown is a maximum-likelihood phylogeny of *S.* Senftenberg calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.4.2 Batch sampling study results

8.4.2.1 Frequency of isolation of Salmonella and serovars isolated from the batch sampling study

Only one of the four farms sampled yielded *Salmonella* -positive chickens and was therefore chosen for the study. Four (57.1%) of 7 and 12 (48.0%) of 25 cloacal swab samples collected from the poultry farm were positive for *Salmonella* on Day 0 (10 days old) and 35 days old (1-week pre-slaughter), respectively (Table 8.10). Overall, the frequency of isolation of *Salmonella* was 17.5% (7/40) and 40.0% (4/10) for samples collected from the processing plant and supermarket, respectively. The differences in the frequency of isolation of *Salmonella* from the farm, processing plant, and supermarket were statistically significant (p=0.03). *S.* Infantis was isolated across all three sampling levels, whereas *S.* Kentucky was isolated only at broiler processing and *S.* Typhimurium at the retail outlet.

8.4.2.2 Frequency of antimicrobial resistance of Salmonella isolated during the batch sampling study

All 49 (100%) isolates of *Salmonella* from farms, processing plant, and retail outlet were resistant to one or more antimicrobial agents (Table 8.11). All isolates were susceptible to chloramphenicol and ciprofloxacin. The frequency of resistance was statistically significantly different for ceftriaxone (CRO, p= 0.003), gentamicin (CN, p=0.002), and sulphamethoxazole-trimethoprim (SXT, p=0.02) among the sampling levels. All (100%) of the 34 farm isolates of *Salmonella* were resistant to doxycycline (DO), ceftriaxone (CRO), gentamicin (CN), and kanamycin (K). All (100.0%) isolates that originated from the processing plant and retail outlet were resistant to doxycycline (DO).

Table 8.10. Frequency of isolation of *Salmonella* from samples in the batch sampling study

Study	**Type of sample**	**Time of sample collection**	**No. samples collected**	**No. (%) positive for *Salmonella***	**Serotypes (No., %)[a]**
Farm	Cloacal swab	Day 0 (10 days old)	7	4 (57.1)	Infantis (4, 100.0)
Farm	Cloacal swab	1 week pre-slaughter (35 days old)	25	12 (48.0)[a]	Infantis (11, 91.7)
	Sub-Total		32	16 (50.0)	
Processing plant	Pre-slaughter cloacal swabs	Day of slaughter (39 days old)	20	0 (0.0)	NA[b]
	Neck skins		10	5 (50.0)[a]	Infantis (3, 60.0)
					Kentucky (1, 20.0)
	Pre-packaged whole birds		5	1 (20.0)	Infantis (1, 100.0)
	Pre-packaged chicken parts		5	1 (20.0)	Infantis (1, 100.0)
	Sub-Total		40	7 (17.5)	
Retail outlet	Chilled chickens		10	4 (40.0)	Infantis (3, 75.0)
					Typhimurium (1, 25.0)
	Total		82	27 (32.9)	
p value				0.030	

[a] Conventional serotyping method conducted on 25 *Salmonella* isolates due to the high cost of conventional serotyping.
[b] NA: Not available

Table 8.11. Phenotypic frequency of resistance of *Salmonella* isolated during the batch sampling study

			No. (%) isolates resistant[b]:					
Study	**No. of isolated tested**	**No. (%) isolates resistant[a]**	**AMC**	**DO**	**CRO**	**CN**	**K**	**SXT**
Farm	34	34 (100.0)	3 (8.8)	34 (100.0)	34 (100.0)	34 (100.0)	34 (100.0)	33 (97.1)
Processing plant	10	10 (100.0)	0 (0.0)	10 (100.0)	8 (80.0)	9 (90.0)	9 (90.0)	8 (80.0)
Retail outlet	5	5 (100.0)	0 (0.0)	5 (100.0)	3 (60.0)	3 (60.0)	4 (80.0)	3 (60.0)
Total	49	49 (100.0)	3 (6.1)	49 (100.0)	45 (91.8)	46 (93.9)	47 (95.9)	44 (89.8)
p value			0.494	NA[c]	0.003	0.002	0.061	0.02

[a] Resistance to one or more agents tested

[b] AMC:Amoxycillin-clavulanic acid, DO: Doxycycline; CRO: Ceftriaxone; CN: Gentamicin; K: Kanamycin; C: Chloramphenicol; SXT: Sulphamethoxazole- trimethoprim and CIP: Ciprofloxacin

[c] All the 49 isolates of *Salmonella* were susceptible to chloramphenicol and ciprofloxacin

8.4.2.3 Detection of antimicrobial genes in the batch sampling study

Resistance genes *aac(3)IV*, *aph(4)Ia*, *bla*$_{CTX\text{-}M65,}$ and *qacEdelta1* were detected in 84.6% (11/13) of the isolates. All isolates except *S.* Kentucky (n=1) contained resistance genes. The genotypic resistance profiles of the serovars detected throughout this batch study are shown in Table 8.12. Pattern A (Farm, Processing plant, and Retail outlet) observed in serovar Infantis isolates was identical (*aac(3)-IV-aph(3')-Ia-aph(4)-Ia-qacEdelta1-bla* $_{CTX\text{-}M65}$*-sul1*). Pattern B (Farm and Processing plant) was identical (*aac(3)-IV-aph(4)-Ia-qacEdelta1-bla* $_{CTX\text{-}M65}$*-sul1*).

8.4.2.4 Core genome phylogenies of serovars from batch sampling study

8.4.2.4.1 S. Infantis

Two distinct clusters were observed in the tree displayed in Figure 8.11. Well-supported clustering was observed among our farm, processing plants, and retail outlet serovar Infantis isolates. Within this cluster, SAMN16678586 and SAMN16678584 were found on the same branch, supported by a bootstrap value of 100%. A significant finding was the reliable clustering of all our isolates from the three sampling levels to SAMN18407134, isolated at a USA processing plant in 2021, and three Infantis isolates detected at retail outlets in Barbados in 2019 (SAMN15964920, SAMN15964921, and SAMN15964932) (Table 8.13). The human clinical isolate was branched together and well supported by a bootstrap value of 100% with a Brazilian processing plant isolate detected in 2016 and a retail outlet sample isolated in China in 2014, but no clustering was observed on any of our isolates. This human strain was included to compare *Salmonella* strains detected along the broiler production chain to human salmonellosis since to date, there has been no association between the two. No clustering was observed among the isolates and the reference strain (Infantis_strain_119944).

Table 8.12. Antimicrobial class and genes detected in the batch sampling study

		Antimicrobial class and genes detected:					
Study	**Pattern**	**Aminoglycoside**	**Antiseptics[a]**	**Cephalosporin**	**Sulphonamide**	**Number of isolates (%)[b]**	**Serovar (n, %)**
Farm	A	*aac(3)-IV*	*qacEdelta1*	$bla_{CTX\text{-}M\text{-}65}$	*sul1*	5	Infantis (5, 100.0)
		aph(3')-Ia					
		aph(4)-Ia					
	B	*aac(3)-IV*	*qacEdelta1*	$bla_{CTX\text{-}M\text{-}65}$	*sul1*	1	Infantis (1, 100.0)
		aph(4)-Ia					
Processing plant	A	*aac(3)-IV*	*qacEdelta1*	$bla_{CTX\text{-}M\text{-}65}$	*sul1*	1	Infantis (1, 100.0)
		aph(3')-Ia					
		aph(4)-Ia					
	B	*aac(3)-IV*	*qacEdelta1*	$bla_{CTX\text{-}M\text{-}65}$	*sul1*	1	Infantis (1, 100.0)
		aph(4)-Ia					
Retail outlet	A	*aac(3)-IV*	*qacEdelta1*	$bla_{CTX\text{-}M\text{-}65}$	*sul1*	3	Infantis (3, 100.0)
		aph(3')-Ia					
		aph(4)-Ia					
	C[c]	*aac(6')-Iaa*	-	-	*sul2*	1	Typhimurium (1, 100.0)

[a] *qacEdelta1* is a resistance gene conferring resistance to quaternary ammonium compound (QAC) antiseptics, conferring resistance to the following drug classes: disinfecting agents, intercalating dyes and acridine dye

[b] All isolates except *S.* Kentucky contained resistance genes (n=1)

[c] Resistance genes *mdsA*, *mdsB*, and *sdiA* were also detected

8.4.2.4.2 S. Kentucky

SAMN1667858, the only serovar Kentucky isolate recovered from a processing plant, showed no clustering with any publicly available isolates, including the reference strain.

8.4.2.4.3 S. Typhimurium

The only isolate of *S.* Typhimurium detected in our study, SAMN16678585, showed reliable clustering with two Jamaican processing plant isolates (SAMN07504229 and SAMN07504964) detected in 2016, in addition to the reference strain detected in 2001 in the USA (Typhimurium_strain_LT2).

Table 8.13. Sources of serovar Infantis isolates (batch sampling study)

Sample Identification	ST	Study type	Source	Type of sample	Year of isolation	Sampling level	SISTR serovar	Country source of isolate
(n=21)								
Infantis_strain_119944	32	Reference	Reference	Human faeces	2008	Reference	Infantis	Israel
SAMN25867770	32	Longitudinal	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad and Tobago
SAMN16678579	32	Longitudinal	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad and Tobago
SAMN25867771	32	Longitudinal	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad and Tobago
SAMN16678582	32	Longitudinal	Processing Plant	Chilled whole carcass	2019	Processing Plant	Infantis	Trinidad and Tobago
SAMN16678577	32	Longitudinal	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad and Tobago
SAMN16678578	32	Longitudinal	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad and Tobago
SAMN16678576	32	Longitudinal	Farm	Cloacal swab	2019	Farm	Infantis	Trinidad and Tobago
SAMN25867773	32	Longitudinal	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Trinidad and Tobago
SAMN16678583	32	Longitudinal	Processing Plant	Chilled chicken parts	2019	Processing Plant	Infantis	Trinidad and Tobago
SAMN16678584	32	Longitudinal	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Trinidad and Tobago
SAMN16678586	32	Longitudinal	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Trinidad and Tobago
SAMN18407134	32	Public Data	Processing Plant	Chilled whole carcass	2021	Processing Plant	Infantis	USA
SAMN15964920	32	Public Data	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Barbados
SAMN15964921	32	Public Data	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Barbados
SAMN15964932	32	Public Data	Supermarket	Chicken carcass	2019	Retail Outlet	Infantis	Barbados
SAMEA3538994	32	Public Data	Processing Plant	Chilled whole carcass	2007	Processing Plant	Infantis	Italy
SAMEA3539001	32	Public Data	Processing Plant	Chilled whole carcass	2014	Processing Plant	Infantis	Italy
SAMN08951144	32	Public Data	Processing Plant	Chilled whole carcass	2016	Processing Plant	Infantis	Brazil
SAMN14404289	32	Control	Human clinical	Human faeces	2019	Control	Infantis	Trinidad and Tobago
SAMN16986393	32	Public Data	Pluck Shop	Chicken carcass	2014	Retail Outlet	Infantis	China

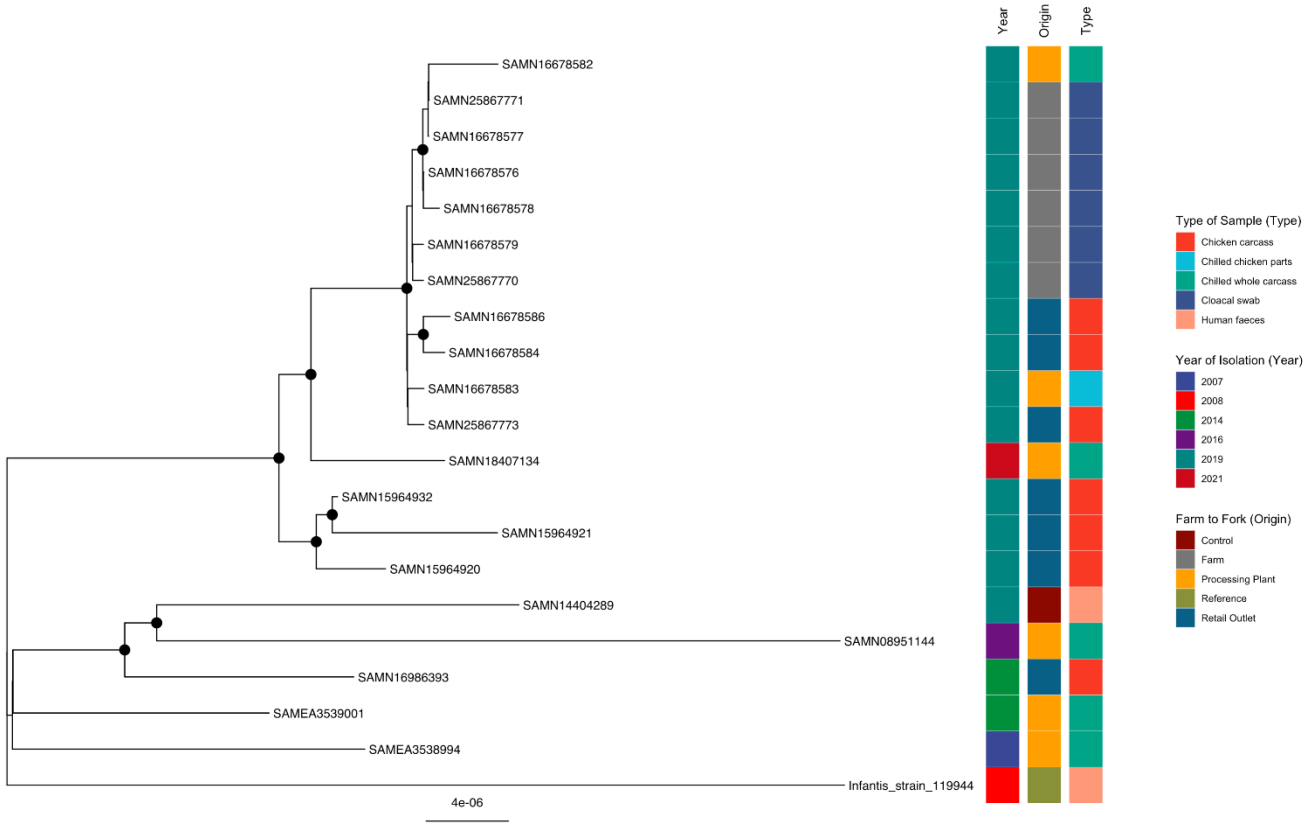

Figure 8.11. Phylogenetic analysis of *S.* Infantis (batch sampling study). Shown is a maximum-likelihood phylogeny of *S.* Infantis calculated using a core genome alignment with IQ-TREE v.2.1.2 and 1,000 bootstraps specified. A black dot on the node indicates bootstrap values >70%.

8.5 Discussion

This is the first documented phylogenetic study conducted using WGS in the poultry industry (broiler production, processing, and retailing) in Trinidad and Tobago and the Caribbean region at large. Whole-genome sequencing analysis has been used to investigate genetic characteristics and phylogenies among *Salmonella* isolated from different origins, such as humans, food, animals and environmental samples elsewhere [523, 525, 526, 607]. This discussion analysed the four-year phylogenetic analyses followed by the batch sampling study.

WGS analyses revealed well-supported clustering of isolates recovered within farms as detected with *S.* Infantis, within processing plants (Anatum, Infantis and Schwarzengrund), between processing plants and retail outlets (Javiana and Kentucky), along the farm-processing plant-retail outlet chain (Albany). Additionally, Intra-Caribbean (Kentucky; Barbados) and international associations (USA, Brazil, China and Peru) clustering of *Salmonella* serovars were demonstrated in the current study. These findings are significant as they highlight the well-supported clustering of *Salmonella* serovars within and between different levels of the broiler production chain in the country, as well as regional and international sources, respectively, therefore having public health significance because the serovars studied have been implicated in human salmonellosis.

All fertile broiler hatching eggs were imported from two companies in the USA. Whilst all imported fertile hatching eggs were negative for *Salmonella* [519], *S.* Kentucky strains were detected at the two hatcheries sampled (operated by Processor A and B), therefore it was important to determine the relatedness of these strains. It is significant that the Kentucky strains detected at the respective hatcheries were not clustered together, a finding that could be attributed to different sources of the imported fertile hatching eggs. At variance to the findings in the current study, Kim et al. [330] reported that *S.* Enteritidis strains isolated from different hatcheries in Korea exhibited the same PFGE types, indicating that cross-transmission among hatcheries possibly due to the sharing of the same source of eggs, a practice common at local hatcheries in this study. However, a serotype

Kentucky strain detected at the hatchery operated by Processor A showed well-supported clustering with retail outlet strains detected in the current study, as well as to a retail outlet strain recovered in Barbados. It is known that day old chicks in Barbados are exported to other Caribbean countries [608] thereby potentially disseminating this strain. Another important finding was the well-supported clustering exhibited among most of the Kentucky strains detected at the hatchery operated by Processor B to a drag swab sample in the USA. Based on this, it can be hypothesized that this strain was imported into the country through fertile broiler hatching eggs. This is supported by the findings of a recent study that implicated *Salmonella* dissemination [329]. *S.* Kentucky is a well-known broiler derived serovar having limited public health significance, therefore its detection and relatedness is important to the poultry industry, moreso, with the increasing development of AMR.

At the farm level in this study, well-supported clustering among *S.* Albany and *S.* Infantis strains (mostly cloacal swab) detected at different farms operated by the same and different processors was observed. Whilst all broiler farms in the country received chicks from processors to which they are attached, it is unclear why the clustering of strains across farms was observed since the farms were located in different counties. A possibility may be the spread of the serovar across poultry farms by pests, such as pigeons, which were a common pest found on most farms, and have been implicated in *Salmonella* dissemination [240].

Within processing plants, reliable clustering was observed among *S.* Anatum (Plant B) and Infantis (Plant A) strains detected on the same sampling day at the respective plant. Whereas *S.* Schwarzengrund strains detected on different sampling days at different plants (Plant B and D) exhibited well-supported clustering among each other. This finding was expected as it is a common practice at processing plants to receive birds that would have originated from farms operated by different processors due to chicken shortages at some of the smaller processors. Mendonca et al. [536], reported the persistence of 7 strains of serovar Infantis in environmental swabs taken at broiler farms over a period of 5 months and attributed this to biofilm

formation. They also isolated homologous *Salmonella* strains from environmental swabs of the farm and the meat matrices at the processing plant which were suggestive of negligence in biosafety protocols in the production line which resulted in contaminated carcasses and meat cuts. Additionally, the authors detected another cluster of strains which included various samples from within the processing plant, which was indicative of persistence of the strain within the plants and subsequent contamination of chickens during processing. The findings in the study conducted in Brazil [536], corroborates with the current study since serovars Anatum, Infantis and Schwarzengrund were detected in chilled whole, chilled chicken parts and neck skin samples, at various time periods during the sampling day. These findings are indicative of ineffective control strategies or biofilm formation at the processing plants. A study conducted by Hyeon et al. [609] on *S.* Enteritidis strains in South Korea reported the persistence of genetically related *Salmonella* strains in carcasses at processing plants and in the environment of slaughterhouses (evisceration room, chilling rooms) which were suggestive of resident strains. These findings agree with our detection of genetic relatedness of *Salmonella* isolates within processing plants. The detection of well-supported clustering among isolates of serovars Infantis recovered from broiler farms (cloacal swabs, drinking water and drag swabs of litter) to isolates of the same serovar subsequently recovered from the processing plant where the broilers were slaughtered, agrees with a published report [536].

Strains of Javiana and Kentucky detected at retail outlets across the country, exhibited reliable clustering. Serovar Javiana strains detected at retail outlets (pluck shops) showed reliable clustering to processing plant strains (Plant B) which could be attributed to the fact the chickens sold at pluck shops could have originated from the same farm (with resident strains). It is common practice that chickens from farms are either processed at processing plants or sold to the ‘live market’ where they are slaughtered at pluck shops. Therefore, it was possible that chickens sold at pluck shops and those processed at Plant B originated from the same farm, with resident strains. Strains of Kentucky detected at the same pluck shop as well as at different supermarkets exhibited reliable clustering. Similar to the findings at pluck

shops, chickens sold at supermarkets were processed at processing plants, therefore, it was possible that chickens sold at different supermarkets originated from the same farm or processing plant with resident Kentucky strains. It should be highlighted that retail outlet and processing plant sample collection was conducted in 2016-2017 and 2018, respectively, therefore the presence of resident strains is a plausible cause.

Dissemination to downstream stages of the broiler production chain, persistence and difficulty of elimination from environment has been attributed to biofilm-formation ability [610, 611]. Several phylogenetic studies conducted on *S.* Kentucky, Typhimurium and Schwarzengrund strains concluded that animals are reservoirs of *Salmonella* spp. and a source of human and environmental contamination [612, 613]. The detection of strains of international relevance in this study agrees with the statement that global dissemination of *Salmonella* spp. is facilitated by travelers, imports and exports of livestock and livestock products [614]. Internationally related *S.* Infantis strains detected in the Galapagos Island [615], showed close relatedness to strains in the United States, Peru and Ecuador [550, 616]. Similarly, in the current study well-supported clustering was observed between strains of serovar Infantis and those recovered in the USA, Brazil, China and Peru. Even though the years of detection varied it may be attributable to their persistence at hatcheries (through imported fertile hatching eggs), farms and processing plants due to biofilm formation and inadequate sanitation protocols at the different localities. Of major significance in this study was the clustering of serovar Kentucky strains recovered from various sources within a hatchery to a farm isolate detected in the USA, considering that all fertile hatching broiler eggs imported into Trinidad and Tobago, originates from the USA. Even though 450 imported fertile hatching eggs tested were all *Salmonella* negative, the detection of Kentucky strains from various sources within hatcheries has the potential to proliferate and disseminate rapidly at hatcheries, to farms, processing plants and retail outlets, given the conditions maintained in incubators and hatchers [536]. The regional clustering of *S.* Kentucky strains between Trinidad and Tobago and

Barbados could be explained by the practice of exportation/importation of poultry livestock in the Caribbean.

Of food safety and clinical significance was the fact that two serovars (Infantis and Enteritis) isolated locally from human gastroenteritis cases during the period of our cross-sectional studies exhibited well-supported clustering with our isolates from the broiler industry (neck skins, chilled whole carcasses, chilled chicken parts). The possibility therefore exists of human exposure through consumption of improperly cooked chicken or chicken products which originated from the processing plants in the country.

To overcome the limitation of time, the batch sampling study followed a batch of chickens from a *Salmonella*-positive flock during the grow-out stages, through processing at a plant and then to a retail outlet. Within a 2-month period of sampling, *Salmonella* was detected in 50% (16/32), 17.5% (7/40) and 40.0% (4/10) of samples originating from one broiler farm, processing plant and supermarket, respectively.

At the processing plant, none (0.0%) of the 20 cloacal swabs were *Salmonella*-positive which was an unexpected finding. This finding may be explained, in part, by the limited number (20) of samples collected which may not be representative of the 25,500 birds in the pen from where the sampled chickens originated. Introduction of feed additives such as probiotics [617, 618], prebiotics, [619, 620] and organic acids [621, 622] in preparation for slaughter might have contributed to our cloacal samples being negative since supplementation with organic acids is a common protocol used by broiler farms in the country, having shown premise in *Salmonella* control [23, 621, 623].

It is important to have detected that serotype Infantis was isolated from the three (3) sampling levels whereas Kentucky and Typhimurium were only isolated at processing plant and retail outlet, respectively. Phylogenetic analyses of the Infantis strains revealed reliable clustering among our farm, processing plant, and retail outlet isolates. This highlighted the transmission throughout the entire production, processing, and retail chain and inadequate sanitisation and disinfection protocols

employed at the processing plants. It can be speculated that presence of serovars Kentucky and Typhimurium at retail outlets only, was as a result of being undetected at the farm or was as a result of cross-contamination at the processing plant since chickens originating from other farms were processed before the tagged positive flock.

The detection of *S*. Infantis agrees with the findings on the serovars detected in the cross-sectional study conducted on 27 broiler farms (Chapter 3) [519], where serovar Infantis was the most frequently isolated, and it accounted for 62.5% of all serovars detected. Crabb et al. [624] similarly detected *S*. Infantis at all levels of their longitudinal study. *S*. Typhimurium, although detected at low frequency in this study, has been the predominant cause of broiler-associated human salmonellosis, therefore this finding has public health significance [625-627].

The overall frequency of resistance exhibited by the *Salmonella* serovars were high (89.9% to 100.0%) to DO, CRO, CN, K and SXT, and the findings were like what were detected in the isolates of *Salmonella* recovered from a cross-sectional study earlier conducted at hatcheries and broiler farms [519]. These findings can be attributed in part by the exposure of broilers on the farms to antimicrobial agents used for prophylaxis, therapy, and growth promotion since the use of these agents is regulated but not routinely enforced [519].

A significant finding of our phenotypic resistance testing was the detection of a 91.8% (41/48) overall prevalence of resistance to the extended spectrum cephalosporin, ceftriaxone. This phenotypic finding correlated with our genotypic detection of $bla_{CTX\text{-}M65}$ in all the isolates of Infantis serovar tested. Additionally, all the Infantis strains in the batch study exhibited MDR resistance to aminoglycosides, cephalosporins, sulfonamides, as well as disinfecting agents, genotypically. Of these 11 MDR Infantis strains, 54.5% (6/11), 18.2% (2/11) and 27.3% (3/11) were detected at farm, processing plant and retail outlet levels, respectively. Among the 20 Infantis strains detected in the previously published cross-sectional molecular study [628] (Chapter 7), 50.0% (10/20) exhibited MDR, contained the $bla_{CTX\text{-}M\text{-}65}$ gene, and were detected at broiler farms. A comparison of the findings of this batch

sampling study to the cross-sectional molecular study, revealed two circulating Infantis strains, one MDR and the other a sensitive strain based on molecular characterisation. This is evident in the phylogenies in the respective studies where reliable clustering was exhibited between the human clinical isolate and processing plant strains (antimicrobial sensitive), however, in the batch study there was no clustering between the clinical isolate and any (MDR) strain detected in this batch sampling study. The MDR Infantis strains (ST32) detected in this batch sampling study and cross-sectional studies (Chapter 7) [628] contained a large plasmid ESI (pESI) as evidenced by the NCBI Pathogen detection browser (https://www.ncbi.nlm.nih.gov/pathogens) where all eleven (11) strains in the batch study were highly related to the MDR emergent *S.* Infantis strains carrying the $bla_{CTX\text{-}M\text{-}65}$ gene (https://www.ncbi.nlm.nih.gov/pathogens/tree/#*Salmonella*/PDG000000002.2411/PDS000089910.165?accessions=PDT000880734.1, accessed March 18, 2022). This large plasmid contains several antimicrobial resistance, metal and virulence genes found in the emergent *S.* Infantis strain reported in several countries, including South America [543, 544] and the United States [545]. This clone was responsible for nearly 10% of all human Infantis cases by 2017 in the USA and was highly related to chicken sources [546].

Resistance to disinfecting agents is of significance as disinfectants are used throughout the broiler production chain in the country and elsewhere to limit cross-contamination. The detection of 81.8% (9/11) of Infantis strains exhibiting the genotypic resistant pattern *aac(3)-IV-aph(3')la- aph(4)la-*$bla_{CTX\text{-}M65}$*-sul1*, is similar to the genes detected in Pennsylvania, USA from chicken breast meat sampled at retail outlets [629]. In the current study, AMR patterns exhibited by serovar Infantis isolates were like what was reported for the same serovar recovered from German broiler farms between 2014-2020, where resistance to aminoglycoside, sulfonamide, tetracycline and quaternary ammonium compounds (*qacEdelta1*) were detected [630]. Comparing the resistance genes detected in the earlier cross-sectional study (Chapter 7) [628] and this batch sampling study, Pattern A and Pattern B were detected in both studies where 36.4% (4/11) and 81.8% (9/11) (Pattern A), and 54.5% (6/11) and 18.2% (2/11) (Pattern B) of MDR strains were

reported, respectively. The *bla*$_{CTX\text{-}M65}$ gene, detected in our study, was the most predominant extended spectrum β-lactamase gene detected in Ecuadorian broiler production chain where *S.* Infantis was also the predominant serovar detected (94%; 66/70) [631].

Failure in biosecurity measures that enable the dissemination of *Salmonella* within an integrated system as documented in our study have been by others elsewhere [632, 633]. The risk of cross-contamination of carcasses of broilers that originated from *Salmonella*-free flocks after processing of birds from *Salmonella*-positive flocks also exists [634].

8.6 Conclusions

Phylogenetic analyses proved useful in comparing the genetic sequences among strains of *Salmonella* to investigate their homology and their relatedness using the demographic and epidemiological data generated from the cross-sectional studies conducted at three levels (production, processing and retailing) of the broiler industry in the country. Well supported clustering among isolates recovered within hatcheries, farms and processing plants, between processing plants and retail outlets and farm-processing plant-retail outlets, as well as inter-Caribbean (Kentucky; Barbados) and international associations (USA, Brazil, China and Peru) were highlighted in this study. It can be deduced that the human clinical *S.* Enteritidis and Infantis isolates originated from chickens based on the homology of the respective strains.

Despite the limitations (sample size, number of farms sampled and logistical challenges to conduct classical longitudinal study), the batch sampling approach used in this study demonstrated the transmission dynamics of *Salmonella* strains in a farm to fork context. This was evident in the antimicrobial resistance patterns and phylogenies exhibited by serovar Infantis detected at different sampling levels. The findings of this study proved the dissemination of serovar Infantis from the farm, through processing plant to retail outlets, posing a threat to consumers of

contaminated broiler meat in the country. However, to further demonstrate the validity and usefulness of this approach, it is imperative to conduct a larger study, using a higher number of farms and samples in the country.

Chapter 9. GENERAL DISCUSSION, RECOMMENDATIONS AND CONCLUSIONS

9.1 General discussion

Salmonellosis is an important zoonotic foodborne disease contributing to economic burden in both LMIC and high-income countries [635]. In Trinidad and Tobago, like the rest of the world, there is an underreporting of salmonellosis and other foodborne disease-causing organisms due to inadequacy of diagnostic tests done at hospitals, if the disease process is progressive enough to require a hospital visit. This relaxed approach when it comes to salmonellosis is due to the lack of public information on the disease status and absence of active and passive surveillance of zoonoses, such as salmonellosis, in the country in both animal and human populations. High income countries employ up-to-date surveillance programs of their poultry production system, enforced food safety legislations regarding limits of *Salmonella* contamination at processing plants and at broiler farms, enforced controlled usage of antimicrobial agents as growth promoters or prophylactically [636, 637]. Additionally, the public awareness of the sources of potential *Salmonella* infections, how to limit cross contamination via proper handling of raw meat/meat products and real time outbreak tracking/source attribution [638] highlights the significance of *Salmonella* to the broiler industry and public health.

Given the role broiler meat plays in the spread of *Salmonella* to humans and the high broiler meat consumption rate in Trinidad and Tobago, it was important to survey the entire broiler production chain to better understand the sources, risk factors, characteristics, and relatedness of *Salmonella* spp. detected. *Salmonella* has previously been associated with consumption of raw eggs in homemade eggnog, ice cream, cake batter and egg-containing beverages in the country, where 65% of the patients required hospitalisation [599]. However, to date there has not been an established relationship between detection of *Salmonella* in the broiler food chain and human salmonellosis in Trinidad and Tobago. Furthermore, there are few

published information on the characteristics of broiler-associated *Salmonella* such as their possession of resistance genes and virulence factors among these strains which may have zoonotic and therapeutic implications.

The overall aim of the investigation was to use the farm to fork approach to assess the risk of *Salmonella* dissemination originating from production (imported fertile eggs, hatcheries and broiler farms), to processing at broiler processing plants, and finally, to retailing at 'pluck shops'/cottage processors and supermarkets, where *Salmonella* can directly enter the food chain through improper handling of raw chickens.

Overall, the various serovars with public health significance detected from the broiler production chain, more so those detected at retail outlets highlight the potential risk posed to consumers of poultry when improperly handled. Serovars implicated in foodborne illness in the Caribbean region [348] also detected throughout this study included *S.* Infantis, Kentucky, Weltevrden, Montevideo, Javiana, Typhimurium and Enteritidis. However, the other serotypes that were subjected to phylogenetic analysis are known to have global public health significance. Traditional serotyping proved inadequate to in silico analysis based on the agreement rate of 76% (111/146) between both methods. This limitation of conventional serotyping is well documented to be cause false-positive reactions because of weak nonspecific agglutination or autoagglutination and false-negative test results due to the loss of antigen expression [128, 152]. Additionally, serovars Caracas, Senftenberg, Liverpool, Gaminara, Oranienburg, Soerenga and Fresno were detected using SISTR but by serotyping with conventional method different or inconclusive results were obtained. Inappropriate serotyping results can have public health significance given the history of certain serotypes causing disease in humans therefore requiring further characterisation.

Regarding the frequency of phenotypic antimicrobial resistance among the various studies, resistance to one or more agents was reported in 100% of the retail outlet (MDR- 100%), 90.5% processing plant (MDR- 12.3%), 87.5% farm (MDR- 85.7%) and 44% hatchery isolates (MDR- 4.0%). This is concerning since the drugs used in the AMR studies were also used in the poultry industry and it is well known that bacteria can transmit AMR genes to other more pathogenic bacteria [639], therefore this should not be taken lightly from a public health perspective. Of public health significance is the finding of high ceftiofur resistant (phenotypic) *Salmonella* strains isolated at farms (75%) and retail outlets (100%). Ceftiofur is commonly administered in-ovo to broilers [640] or subcutaneously to day-old broiler chicks [641] together with Marek's vaccine in certain commercial hatcheries prophylactically. Reports have demonstrated the link between off-label antibiotic use of antimicrobials in animals and the increase in AMR, supporting the hypothesis that fluctuations in ceftiofur resistance was most likely due to a common exposure (or reduction of exposure) to ceftiofur in chicken hatcheries as opposed to being secondary to natural spread and disappearance of a ceftiofur-resistant clone unrelated to ceftiofur use [642-644]. The use of ceftiofur at hatcheries in this current study was not disclosed and should be further investigated given the importance of third generation cephalosporins to human health. Genotypically, the AMR genes detected did not reflect the phenotypic findings and this could have been to the 100% setting used for the AMR genes with CARD. 100% was used to ensure reliable, reproducible results without any unnecessary speculation since the strains used originated from the most profitable agricultural sector in Trinidad and Tobago. Therefore, it is recommended that another database such as ResFinder be used to compliment the initial findings with slightly lowered settings.

WGS was useful in detecting the presence of the $bla_{CTX\text{-}M\text{-}65}$ gene in strains detected in 2019 at the farm, processing plant and retail outlet. This finding holds importance to public health and poultry industry because there are currently emergent *S.* Infantis strains being implicated in food-producing animals and human outbreaks

being linked to poultry sources [540, 645] which were found to be highly related to strains detected in this study. This is concerning and warrants continuous surveillance of not only the broiler industry, but all sources of foodborne disease transmission given its public health significance. It was comforting to know however, that the clinical strain of *S.* Infantis detected in Trinidad did not cluster or share the MDR pattern exhibited by this emerging Infantis strain detected in the farm isolates as shown in the batch study phylogenetic tree based on core genome alignment. However, there is potential for this MDR Infantis strain to cause human salmonellosis in Trinidad and Tobago. The use of WGS to characterise these Infantis strains using AMR genes and phylogeny highlighted the circulation of two subgroups of Infantis strains. The ability to use WGS based phylogeny of *Salmonella* for example, knowing the evolutionary rate of 1.01 nucleotide substitution per year [646] is a useful tool. However, in source attribution studies, several factors must be taken into consideration such as single nucleotide polymorphism (SNP) <21, bootstrapping >89%, monophyletic tree topology as well as epidemiological and traceback evidence [526]. A closely related reference strain is also necessary for SNP analysis. SNP-based phylogeny may be useful for future epidemiological studies where source attribution is needed and highlights the many uses of WGS instead of traditional methods of characterisation. Source attribution was not the aim of this study hence the use of the highly conserved core genome alignment for phylogeny based on the objective of comparing strains in this study to each other and those detected globally. Implications of international trade as a source of *Salmonella* dissemination [329] led to the inclusion of reference strains that were publicly available to be compared to strains detected in this study.

It was of food safety, public health and epidemiological significance to have detected reliable clustering (having a common recent ancestor/ showing relatedness) of the serovars of *Salmonella* isolated from human clinical cases and processing plants. This is therefore the first genetic documentation of the association of *Salmonella* of poultry origin (poultry meat) with human

salmonellosis, possibly through improperly handled raw chicken, in the country. The recovery of isolates of serovar Infantis and Enteritidis in 2018 which were genetically related to isolates of the same serovars recovered from human cases of salmonellosis in 2019 may be due to the reported persistent *Salmonella* serovars due poor sanitary practices [536]. This is attributed to the failure of existing practices to limit cross-contamination by *Salmonella* serovars in the poultry industry in the country. The risk of exposing humans to antimicrobial resistant *Salmonella* exists and is further complicated by the spread of AMR genes via horizontal transfer of plasmids to other microorganisms of public health importance [639]. Although control measures can reduce the bacterial load and distribution at processing plants, they unable to eliminate them [647]. Therefore, proper raw meat handling by food preparers and consumers play a more important role in food safety than government or broiler producers [648]. This finding warrants continued surveillance of possible sources of FBD causing organisms since this was an incidental retrospective finding.

The sequencing data generated in this study was uploaded onto the National Center for Biotechnology Information (NCBI) database. This study focused on AMR and virulence gene detection and phylogenetic inference, however plasmid and integron detection should be analysed in a future study, retrospectively. WGS and sharing of sequencing data is vital to epidemiological studies since real- time comparisons are made to sequences uploaded. Therefore, studies across the region and Trinidad should transition towards the use of WGS as the gold standard method of characterisation for *Salmonella* and other FBD pathogens where possible, to grow the database in order to properly conduct epidemiological studies of foodborne outbreaks. The deposition of data, which is publicly available also allows for retrospective studies, important when monitoring for implications of regulatory changes. With the rapid progression and international use of WGS technology for foodborne surveillance, it is recommended that the relevant health authorities

responsible for public health in Trinidad and Tobago and the region employ WGS to adequately characterise *Salmonella* and other foodborne pathogens.

Risk factors associated with *Salmonella* isolation in processing plants included use of a pre-chiller (OR 2.3, 95% CI: 1.45-3.74), addition of chlorine to chiller (OR 3.2, 95% CI: 1.22-8.30). Similarly, at cottage processors the use of chilled water bath to cool carcasses was the only risk factor significantly (p=0.044) associated with isolation of *Salmonella* spp. Based on these findings, improper use of chiller water was evident and should be further investigated to improve the effectiveness of the chiller where possible. Ideally, a counter current flow, adequate freshwater input with a chlorine concentration of 50 ppm, water temperature below 40 °F/ 4 °C, low organic matter content, and control of the chlorine level to ensure a free available chlorine concentration of 1 to 5 ppm with an adjusted pH ranging from 5 to 6.5 (achieved by using citric acid, sodium acid sulfate or carbon dioxide) as well as adequate contact time are all parameters essential to reducing *Salmonella* in immersion chillers [291]. These parameters might be difficult to achieve with the smaller scaled cottage processors as opposed to processing plants however the effectiveness of chlorine concentration, water temperature, pH and organic matter concentration and contact time should be scaled and investigated for use at cottage processors.

None of the imported fertile hatching eggs were *Salmonella* positive, however the hatcheries were found to contain *Salmonella*. A limitation of this fertile hatching egg study was that the number of imported fertile hatching samples collected was relatively small therefore it was not possible to make inferences. Fertile hatching broiler eggs from *Salmonella*-infected breeder flocks have been reported to serve as a source of the pathogen for hatched day-old chicks, the contamination of hatchery environments and the exposure of broiler flocks on farms to the *Salmonella* [330]. Since all broiler fertile eggs are imported into Trinidad, it was not possible to sample the breeder flock. Sanitation, biosecurity and control

measures should be prioritised downstream, as these criteria are within the jurisdiction of the relevant local authority.

Similarly, only 2 of the 4 functional hatcheries agreed to participate in the study and efforts made to increase the number of hatcheries to be sampled in the study were unsuccessful. Therefore, it was difficult to assess risk factors, moreso due to the limited number of positive samples detected. Similarly at processing plants, in order to make inferences regarding the operational and management practices of the plants, increasing the number of sampling visits is suggested, as this was a limitation the study.

A common limitation among all the cross-sectional studies conducted was the high cost of conventional serotyping, therefore only representative isolates were serotyped. Additionally, the regional laboratory, the Public Health Laboratory, Ministry of Health, Barbados, where the isolates of *Salmonella* were serotyped was unable to type some isolates beyond the Group level due to the unavailability a complete panel of antisera required for serotyping. This limitation resulted in obtaining incomplete serotyping results, a well-known drawback of this method of serotyping [152].

Overall, the data provided by this thesis highlight the public health significance of the resistant *Salmonella* serovars recovered at the three levels of poultry production, processing and retailing in the country. The highest risk is posed to humans exposed to AMR and/or MDR *Salmonella* through handling and consumption of improperly cooked chicken products. The isolation of resistant *Salmonella* from chickens may negatively impact on the successful treatment of salmonellosis and other bacterial infections in humans in the country.

9.2 Recommendations and Conclusions

The high MDR reported in *Salmonella* detected at broiler farms and retail outlets, and in particular, the resistance to ceftiofur, suggests that there is need for monitoring of unauthorized use of ceftiofur [649] and enforcement by those in authority given its public health significance. Alternative control measures should replace the use, misuse and over-use of antimicrobial agents since these generate selection pressure favoring the multiplication of antimicrobial-resistant organisms [650]. Effective biosecurity and hygiene practices such as disinfection practices, pest controls, animal, person and vehicle movements, feed and water sanitation should complement other control measures. The following have shown to not only improve animal health but can also protect birds from *Salmonella* infections: acidic compounds or organic acids [275], prebiotics [276], competitive exclusion products [277], probiotics [278], phytobiotics [279], potential feed additives such as flavophospholipol [283] and bacteriophages [27, 284, 285]. The feasibility of these methods should be assessed by the poultry industry and relevant authorities.

It is recommended that risk assessment studies be conducted by the relevant broiler processing plants to review and update the current control measures based on the findings of this study. In particular, there is need for further research to assess the role immersion chillers might be playing in cross-contamination of *Salmonella* . Currently, there is no routine surveillance of the poultry industry where samples are collected from various stages of the broiler production chain by the regulatory food safety authority. Therefore, it is suggested that the use of the neck skin maceration method is a practical, low invasive method that can be used for routine processing plant and cottage processor surveillance for internal quality control (QC) and by the relevant authorities for food safety assurance.

At cottage processors, standardised poultry processing guidelines should be enforced by the regulatory authority in the country. Implementation of this

recommendation in the country has the potential to reduce broiler meat-borne salmonellosis in humans given the 20.5% prevalence of *Salmonella* and the associated risk posed to customers at these establishments. This initiative is currently being undertaken in Trinidad and Tobago. Further research and scaling of the recommendations given to processing plant regarding immersion chillers needs to be done due to the much smaller throughput and the finding that *Salmonella* was detected more frequently in establishments that cooled carcasses in an iced water bath.

Given on the possibilities offered by WGS in this study, it is recommended to the regulatory authority in Trinidad that it implements an in-house WGS laboratory as part of its national food safety surveillance program. Secondly, public awareness campaigns regarding food safety highlighting ways to avoid cross-contamination, keeping foods at safe temperatures, and avoiding food from unsafe sources as risk factors associated with acquiring foodborne diseases should be implemented. Increasing the public's engagement in safe food-handling behaviours by using education programs has been useful [651]. However, ease of reporting FDB at the respective health facilities is also important as this can potentially deter the public from reporting their illness [652] since only a small proportion of people with FBD seek medical attention and only a fraction of these cases clinical samples were collected by health workers [54]. Thirdly, based on previous risk assessment studies, data points and trends, implementing routine testing as part of the national public health inspection service, throughout the country utilising WGS, focusing on AMR in foodborne zoonotic and animal pathogens. For example, in the EU, the standardisation of targeted bacteria, samplings procedures, antimicrobials to be used, and their respective cut-off values have been implemented through the Commission Decision 2007/516/EC and Decision 2013/652/EU and were guided by important reports published by EFSA [653-656]. Closer to our shores, in the United States, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) is responsible for food safety and is supplemented by

NARMS (National Antimicrobial Resistance Monitoring System, responsible for the surveillance system tracking antimicrobial resistance in foodborne pathogens from different sources. Guidelines from international agencies such as EFSA, FSIS and NARMS should be scaled and enforced to suite the requirements of Trinidad and Tobago based on data and further research which should guide our legislation.

The role of wildlife and environmental contamination in the indirect transmission of AMR to production animals and humans is often underestimated [657]. A One-Health approach to tackle AMR should be established. Routine sampling should not be limited to where outbreak strains or individual clinical strains originated but as part of routine mandatory testing of food-producing establishments, food-producing animal farms, abattoirs, retail outlets, soil and waterways. This will aid in the deposition of sequencing data necessary for epidemiological studies in Trinidad. Private sector may deem it an unnecessary additional cost that will negatively affect their profitability. Therefore, it is suggested that as part of the health authority's certification process, establishments would be subjected to routine testing to deem these establishments 'food safe' to ensure compliance. Data generated from this initiative will be invaluable since it will also be useful in subsequent regulatory interventions, policy-making and performance tracking.

This is the first study to associate human salmonellosis with *Salmonella* isolated in chickens in Trinidad and Tobago. It is suggested that future epidemiological studies be conducted using a similar approach for other zoonotic FBD causing pathogens using WGS in Trinidad and Tobago. Future studies that aim to overcome the limitations of this study are also recommended.

The findings of this extensive study should not be misrepresented regarding the poultry industry since the industry plays a role in reducing the presence of *Salmonella* in the food chain, however, food preparers and consumers also play a

role in food safety. A study conducted by Oscar [648] demonstrated that highly contaminated carcasses (>100 *Salmonella*/carcass) exiting the processing plant did not necessarily pose a greater risk of salmonellosis when compared to carcasses that had low contamination rates (<10 *Salmonella*/carcass). He also found a greater risk of salmonellosis from carcasses with low levels of contamination when they were temperature abused, undercooked and consumed by someone from the high-risk population. Therefore, the role of consumers and food preparers in food safety cannot be ignored.

In conclusion, this study was able to confirm the association of human salmonellosis and *Salmonella* detected in broilers, detection of known serovars implicated in human salmonellosis, high phenotypic MDR exhibited at farms and retail outlets and the detection of the emergent *S.* Infantis strain harbouring the $bla_{\text{CTX-M-65}}$ gene. Of immense public health significance was the detection of these MDR strains harbouring the $bla_{\text{CTX-M-65}}$ gene, that can pose a direct threat to public health but more importantly, the possession of antimicrobial resistance genes in mobile genetic elements can potentially be a source of transmission to other pathogenic bacteria within a given host or in the environment [658, 659]. These findings warrant continuous monitoring and surveillance of the food production chain, environment and animals in an attempt to maintain food safety and human health by reducing AMR. This research also highlights the need for public awareness of the risks associated with the handling of raw chickens to reduce the potential for cross-contamination, salmonellosis in humans and further spread of AMR genes.

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APPENDICES

Appendix 1. Approval of research project by UWI St. Augustine Campus, Post-graduate Research Committee

THE UNIVERSITY OF THE WEST INDIES
ST. AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
OFFICE OF THE CAMPUS REGISTRAR
SCHOOL FOR GRADUATE STUDIES & RESEARCH
TELEPHONE: (1-868) 645-3232 ext. 83797 FAX: (1-868) 645 7327 E-mail: sarah.kalloo@sta.uwi.edu

Our Reference: PF December 08, 2015

Prof. A.A. Adesiyun,
School of Veterinary Medicine,
Faculty of Medical Sciences,
EWMSC., Champs Fleurs.

Dear Prof. Adesiyun,

CRP.3.NOV15.14 - Prevalence and characteristics of Salmonella spp. isolated during broiler production, processing and sale in Trinidad and Tobago using the 'farm to fork' approach

Further to our letter to you dated November 11, 2015, the Chair, Campus Research and Publication Fund Committee received notice of ethics approval and the revised budget and agreed approve a grant in the sum of TT$73,376.98 as a contribution towards consumables for Year 1 of the above mentioned project from Medical Sciences Funds.

The Committee, in its meeting of November 9, 2015 advised that a new grant application be submitted for Year 2 of the project along with an interim report detailing the progress of the project.

Kindly note the following

43. Under the rules of the Fund, the Bursar makes payment of grants for Conference fees for buying equipment and research material and for meeting research expenses only on presentation of vouchers certifying expenditure.
44. In the hiring of Research Assistants, priority must be given to graduate students who are registered for higher degrees at the University of the West Indies or senior undergraduates of the University of the West Indies who may be interested in pursuing a higher degree on graduation.
45. Once you have received funding from this Committee, you are asked to acknowledge the Campus Research and Publication Fund Committee in your articles, books etc.
46. When funds are awarded for a grant, the funds should be **utilized within one (1) year** from the date of this letter. Should you not have completed the project by this date, you are required to submit an **interim report** (see link for report form in #6 below) to the Office of Graduate Studies & Research (OGS&R).
47. On completion of the project/visit/allocation for which the grant was provided, you must submit a **full/final report** to the **Office of Graduate Studies & Research (OGS&R).** Please visit the website given below for the reporting form: (http://sta.uwi.edu/postgrad/documents/ReportingFormatforStaffGrants.doc).
48. Your report must include any **evidence of output** e.g. abstract of conference presentation and a copy of the acceptance email from the conference organizers, publication of papers in journals, an acceptance email from the publishers of the journal article or the publication of a book chapter etc. Adequate evidence of output from a previous grant must be submitted before funding for new projects could be approved. A final report must be submitted which would signal the completion of the project inclusive of any evidence of output emanating from the project. Failure to report fully may result in the non-consideration of future applications for research funding.
49. **Please note that should the funds not be used within one (1) year from the date of this letter, it will be returned to the Bursary's accounts. These funds are not returned to the Campus Research and Publication Fund Committee for redistribution. You are urged to request an extension in time (with a specific completion date) via email to complete the project in a timely manner.**

Kindly contact the Projects Section of the Bursary at extensions 83389 or 82146 for information pertaining to access of the funds.

Please be guided accordingly.

Yours sincerely,
Deborah Charles-Smythe (Mrs.)
Senior Assistant Registrar,
For Campus Registrar

cc: Head, School of Veterinary Medicine
Dean, Faculty of Medical Sciences
Dr. Kenneth Charles

/tf

Appendix 2. Exemption of study from Faculty of Medical Sciences, Campus Ethics Committee

THE UNIVERSITY OF THE WEST INDIES
ST AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
FACULTY OF MEDICAL SCIENCES
CAMPUS ETHICS COMMITTEE
Telephone: (868) 645-3232 Ext. 5021 Fax: (868) 663-9836 email: campusethics@sta.uwi.edu

December 7, 2015

Prof. Abiodun Adesiyun
School of Veterinary Medicine
Faculty of Medical Sciences

Dear Prof. Adesiyun

Prevalence and characteristics of *Salmonella* spp. isolated during broiler production, processing and sale in Trinidad and Tobago using the 'farm to fork' approach

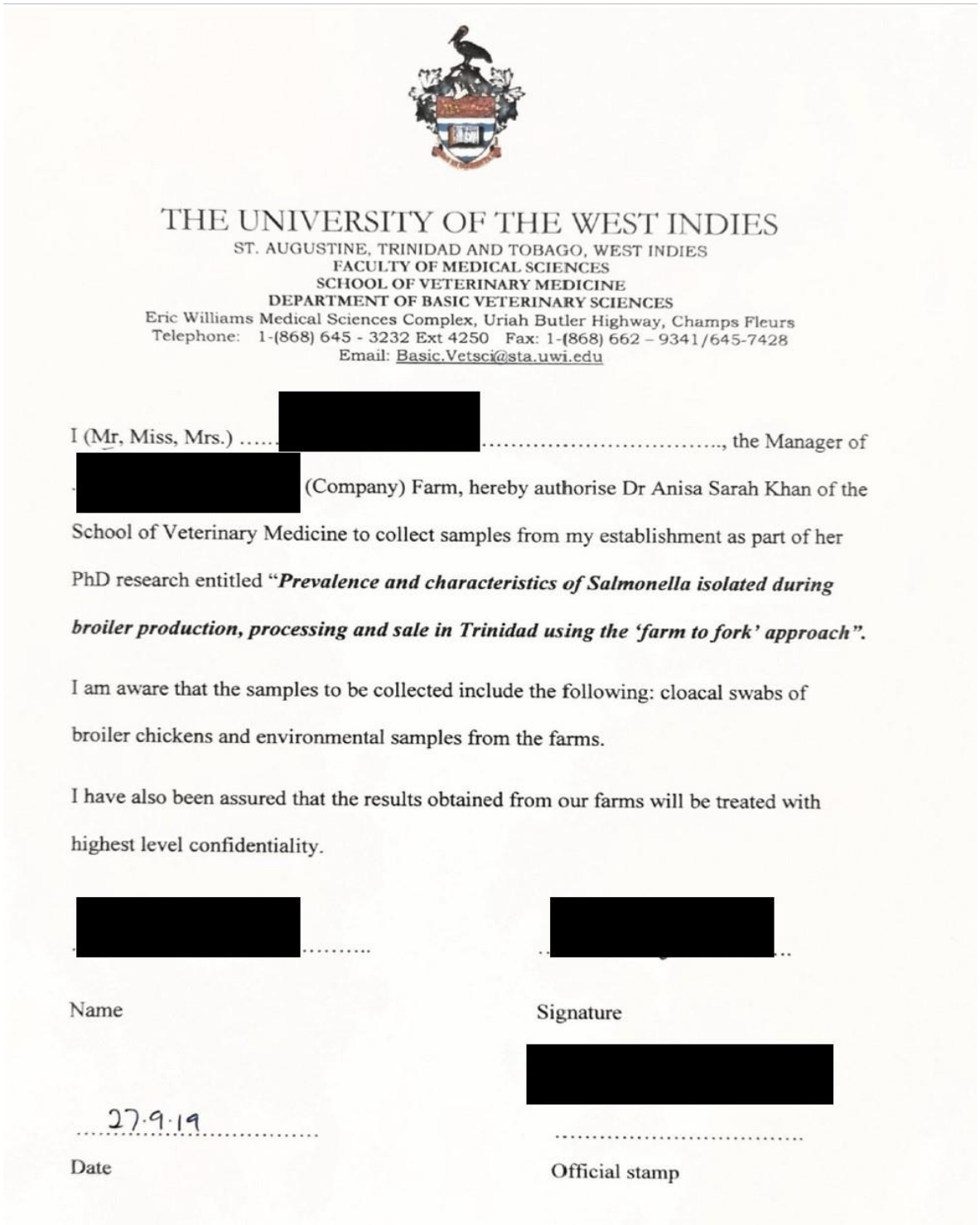
This letter is to confirm that your application for research on the topic captioned above is exempted from ethical review, by Campus Ethics Committee.

Yours sincerely

Shivananda Nayak (Prof.)
Chairman, Campus Ethics Committee
Faculty of Medical Science

erf

Appendix 3.A. Consent form completed by hatchery and farm managers



THE UNIVERSITY OF THE WEST INDIES

ST. AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
FACULTY OF MEDICAL SCIENCES
SCHOOL OF VETERINARY MEDICINE
DEPARTMENT OF BASIC VETERINARY SCIENCES
Eric Williams Medical Sciences Complex, Uriah Butler Highway, Champs Fleurs
Telephone: 1-(868) 645 - 3232 Ext 4250 Fax: 1-(868) 662 – 9341/645-7428
Email: Basic.Vetsci@sta.uwi.edu

I (Mr, Miss, Mrs.) ..... [redacted] ....., the Manager of [redacted] (Company) Farm, hereby authorise Dr Anisa Sarah Khan of the School of Veterinary Medicine to collect samples from my establishment as part of her PhD research entitled "***Prevalence and characteristics of Salmonella isolated during broiler production, processing and sale in Trinidad using the 'farm to fork' approach".***

I am aware that the samples to be collected include the following: cloacal swabs of broiler chickens and environmental samples from the farms.

I have also been assured that the results obtained from our farms will be treated with highest level confidentiality.

[redacted] ..... [redacted] ...

Name Signature

[redacted]

27·9·19 ....

Date Official stamp

THE UNIVERSITY OF THE WEST INDIES
ST. AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
FACULTY OF MEDICAL SCIENCES
SCHOOL OF VETERINARY MEDICINE
DEPARTMENT OF BASIC VETERINARY SCIENCES
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I am aware that the samples to be collected include the following: broken eggs shells, still born chicks, hatcher fluff and swabs of the interior of incubators and hatchers.

I have also been assured that the results obtained from our hatchery will be treated with highest level confidentiality.

[redacted]
Name

[redacted]
Signature

27.9.19
Date

Official stamp

THE UNIVERSITY OF THE WEST INDIES
ST. AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
FACULTY OF MEDICAL SCIENCES
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I am aware that the samples to be collected include the following: cloacal swabs of broiler chickens and environmental samples from the farms.

I have also been assured that the results obtained from our farms will be treated with highest level confidentiality.

[redacted]..

Name

[redacted]

Signature

30/9/2019

Date

[redacted]

THE UNIVERSITY OF THE WEST INDIES
ST. AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
FACULTY OF MEDICAL SCIENCES
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I have also been assured that the results obtained from our hatchery will be treated with highest level confidentiality.

[redacted] [redacted]

Name Signature

30/9/19

Date

[redacted]

Official stamp

Appendix 3.1 Questionnaire- Hatcheries

Name of Hatchery

Contact Person

Address of Hatchery

Telephone Number Email Address

Section A: Operation information

1. What is the maximum hatching capacity of your facility i.e. total number of eggs that can be hatched?

   _____

2. What is the current hatching capacity?

   Hatchers: _____eggs Incubators: ____ eggs

3. On an average, how many chicks are normally hatched at your facility per hatching day?

   _____

4. How many incubators and hatchers do you have at your facility?

   Incubators: _____ Hatchers: _____

5. On an average, how many dead chicks are normally recovered per hatcher on a hatching day?

   Normal/hatcher: _____ Today/hatcher: _____

6. What is the average number of chicks that were hatched but die in the boxes before being sent to the farms?

   _____

7. What breed/s of chicks are normally hatched?

_____

8. Within the last 12 months, list the countries of origin of the eggs hatched at your facility:

_____

9. Within the last 12 months have you experienced any problems with hatchability at your facility? ☐ Yes ☐ No

If yes, specify:

_____

_____

_____

10. What is the average incubation condition at your facility?

a. Duration (days/weeks):
_____

b. Temperature (degree Celsius):
_____

c. Humidity (%):
_____

d. Frequency of turning the eggs:
_____

11. What type of production system do you have?

☐ Manual ☐ Semi- automated ☐ Fully automated

12. What type of ventilation system (hatchers/incubators) do you have?

☐ Open air/common air space (internal of building)

☐ Closed system (each incubator/hatcher)

☐ Dust trap ☐ Exterior of building ☐ Other:
_____

13. How many air intakes/out puts do you have per incubator and hatcher?

Incubators (__)		Hatchers (__)	
# Air intake	# Air output	# Air intake	# Air output

14. Are incubators and hatchers housed in the same building?

☐ Yes ☐ No

15. Do you export day old chicks from your hatchery to other countries in the Caribbean? ☐ Yes ☐ No

If 'Yes', name the countries:

a. _____

b. _____

c. _____

16. Is there any particular reason why you import eggs from two difference sources?

_____

17. Have you ever changed sources of hatching eggs? ☐ Yes ☐ No

If Yes, why?

_____

_____

Section B: Biosecurity/Sanitation measures

1. Are your eggs fumigated/sanitized before placement into incubators?

   ☐ Yes ☐ No

   If Yes, what agents, concentrations and time of contact are used?

Agent	Concentration	Time of contact with agent
a.		
b.		
c.		

2. Are workers mandated to wear gloves/protective wear when handling and transferring eggs?

   ☐ Yes ☐ No

3. Are workers' movement between different areas/buildings restricted?

   ☐ Yes ☐ No

   If Yes, what are the areas that are restricted?

   a. _____

   b. _____

   c. _____

   d. _____

4. What pest control measures are in place at your facility?

   a. _____

   b. _____

   c. _____

   d. _____

5. What are your cleaning and disinfection (general) protocols? How often is it done?

Agents used:

_____

_____

Frequency and timing of cleaning:

_____

_____

6. What agents are used to sanitize the hatchers and incubators?

a. _____

b. _____

c. _____

d. _____

7. Are routine environmental/egg sampling done at your facility to ensure quality assurance?

☐ Yes ☐ No

If Yes, what tests are done and how often is it done?

Test	Type of sample collected	Frequency
a.		
b.		
c.		
d.		

8. Have you ever isolated *Salmonella* from samples taken at your hatchery within the

last 12 months? ☐ Yes ☐ No

If Yes, which type of samples tested positive?

_____

9. On average, what is the number of eggs with dirty shells that are place in each incubator? _____

10) On average, what is the number of eggs with cracks in the shells that are placed in each incubator? _____

Section C: Vaccination protocol

1. What is the route of vaccine administration given at your hatchery?

   ☐ In Ovo (Day: ___) ☐ IM Injection ☐ SubQ injection

   ☐ Ocular ☐ Nasal ☐ Cloacal ☐ Feather follicle

   ☐ Wing stab ☐ Spray ☐ Oral

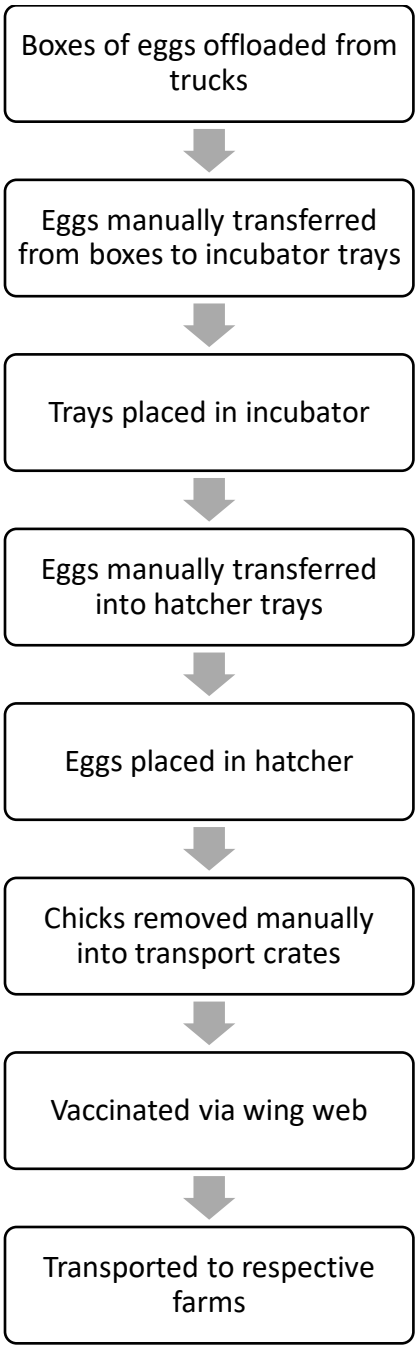
   Other: _____

2. What vaccines are administered via which route?

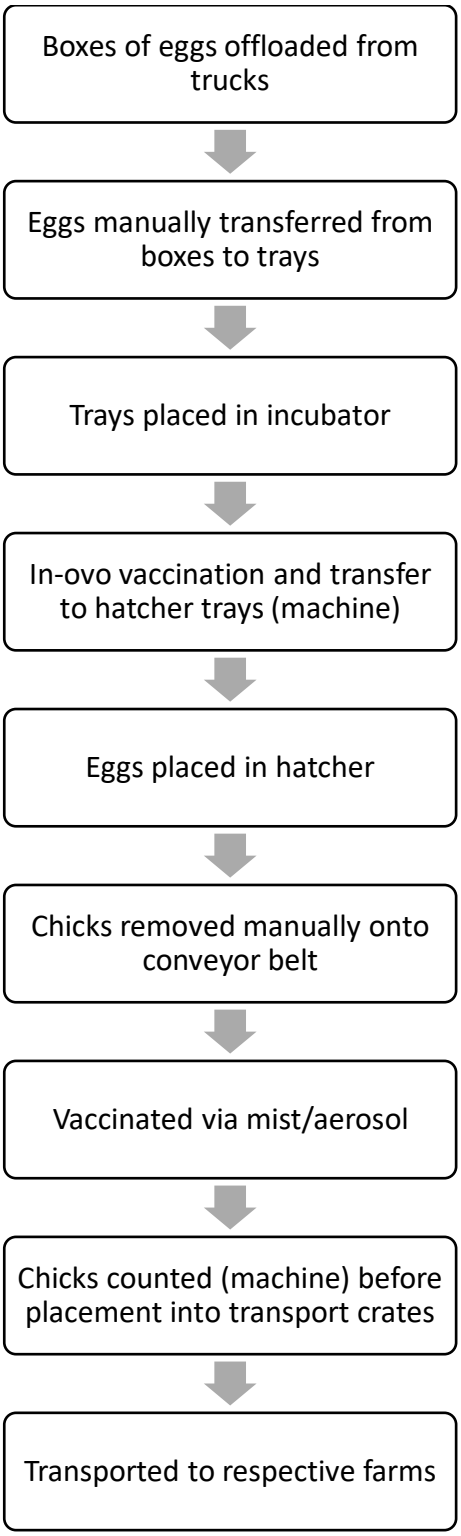
Vaccine/Agent	Route administered	Age
a.		
b.		
c.		
d.		
e.		

END.

Thank you for your time and cooperation.



Appendix 3.2 Flow chart showing protocols of hatchery owned by poultry integrator A/ Hatchery A



Appendix 3.3. Flow chart showing protocols of hatchery owned by poultry integrator B/ Hatchery B

Appendix 3.4 Questionnaire – Farms

Date of administration: _____ Code: _____

Farmer/ Contact Person **Contractor**

Address of Farm/GPS coordinates

Telephone Number

SECTION A: Worker's information

1. **How many workers are employed on your farm? _____**
2. **Length of time working in industry:**

☐ < 1 year……Number of workers: _____

☐ 1-3 years……...Number of workers: _____

☐ 3-5 years……...Number of workers: _____

☐ 5-7 years……...Number of workers: _____

☐ >7 years……...Number of workers: _____

3. **Are specific workers assigned to each pen?**

☐ Yes ☐ No

If Yes, how many workers are assigned to each pen? _____

SECTION B: Farm Management- Pens

1. What type of management system do you have on your farm?

☐ Automatic ☐ Semi-automatic ☐ Manual

2. **Are your pens tunnel ventilated or open sided?**

   ☐ Tunnel ventilation ☐ Open sided

3. **What is the maximum number of broilers your farm can house, i.e., maximum bird capacity?**

   ☐ < 1000 birds ☐ 1001- 5000 birds

   ☐ 5001- 10,000 birds ☐ 10,001- 15,000 birds

   ☐ 15,001- 20,000 birds ☐ 20,001- 25,000 birds

   ☐ > 25,000 birds

4. **How many broilers do you currently have on your farm?**

   ☐ 1001- 5000 birds ☐ 5001- 10,000 birds

   ☐ 10,001- 15,000 birds ☐ 15,001- 20,000 birds

   ☐ 20,001- 25,000 birds ☐ > 25,000 birds

5. **How many actively used pens do you have on this farm?**

   ☐ 1 ☐ 5 ☐ 9

   ☐ 2 ☐ 6 ☐ 10

   ☐ 3 ☐ 7 ☐ > 10: ____

   ☐ 4 ☐ 8

6. **Do you use fans in all of your pens?** ☐ Yes ☐ No

7. **What is/are the average sizes of your pens/cages/barn? (in metres)**

   P1_____

   P2_____

   P3_____

P4_____

8. **In the following table please state the number of birds housed in each pen and their respective ages.**

Pen #	Number of birds in each pen	Age of birds in each pen	Orientation of each pen	# & type of sample taken
☐1				
☐2				
☐3				
☐4				
☐5				
☐6				
☐7				
☐8				
☐9				
☐10				

9. **What type of ventilation system do you use in your pens?**

☐ Fans ☐ Extraction fans ☐ Natural ventilation

☐ Insulation (ceiling) ☐ Others (specify)_____

10. On average, how often do you grow-out birds for slaughter (how often do birds leave your farm to go to slaughter)?

☐ Once a week ☐ Twice a week ☐ Every other week

☐ Once/month ☐ Other:

_____

11. What kind of flooring system do you use in your pens?

☐ Dirt ☐ Concrete ☐ Dirt and Concrete

☐ Other _____

Section C: Farm Management- Husbandry Practices

12. How many hours per day are the birds exposed to light (day/artificial/natural)? What type of lighting is used on your farm? What is the light to dark ratio?

☐ Daylight: _____h/day

☐ Artificial: _____h/day

☐ Natural light: _____h/day

Ratio (light/dark): ___/____

13. Is <u>litter</u> treated with chemicals before chicks are added to new pens?

☐ Yes ☐ No

If "Yes", what agents are used and how often?

_____

_____

_____

_____

If "No", what is the practice used on the farm before re-stocking pens with chicks?

_____

_____

14. Briefly explain how <u>pens</u> are prepared/disinfected/sanitized before a new batch of chicks are added?

_____

_____

_____

_____

15. How are the pens sanitized within flocks? What agents are used?

☐ Change litter only ☐ Change litter and wash pen only

☐ Change litter and clean with disinfectant or bleach ☐ No response

☐ Other (please explain):

_____

_____

Agents used?

1._____

2. _____

3. _____

4. _____

SECTION D: Farm management- Grow out phase

1. **Who supplies the chicks to you? Please mention all sources of your chicks.**

   a. _____

   b. _____

2. **What is the age of the chickens that you receive?**

   ☐ Day-old ☐ 2 days old

   ☐Other (state age):_____

3. **How are the chicks transported to the farm?**

   _____

4. **Do your chicks receive any vaccines during the grow-out period?**

   ☐ Yes ☐ No

   Which vaccines do they receive (after hatching) and at what age/s?

Name of vaccine	**Agents targeted by vaccine**	**Age given to chicks**
1.		
2.		
3.		
4.		
5.		
6.		
7.		

5. **Are the chicks transferred to a grow out pens or do they stay in one pen for their entire lives?**

   ☐ Transferred to grow out pens ☐ Stay in same pen

   ☐ Transferred at some other point. Please specify:

   _____

   ☐Other_____

6. **What type of feed is used at the different ages of growth? (starter, grower, finisher)**

   Pre Starter: _____

   Starter: _____

   Grower: _____

   Finisher: _____

   Other: _____

7. **How do you prepare broilers to be sent to slaughter (any special feed/minerals/vitamins from chicks up until slaughter age)? At what age are they introduced to this supplement. Please fill out the table.**

Supplement (food/water)	**Age of introduction**
☐Normal finisher ration	
☐Special diet/feed (other than finisher)	
☐Mineral supplement	
☐Vitamin supplement _____	_____

_____ _____ _____	_____ _____ _____
☐Growth enhancer/ promoter	
☐Antibiotics	
☐Other:	
☐Other:	

8. **On average, at what age are your broilers sent for slaughter?**

   ☐ 4 weeks old ☐ 5 weeks old ☐ 6 weeks old

   ☐ Other: _____ days old

9. **On average, what is the goal weight of broilers that you aim to achieve at slaughter? _____** lbs/kg

10. **Is this goal weight regularly achieved? ☐ Yes ☐ No**

    If No, why do you think this was not achieved?

    _____
    _____
    _____
    _____
    _____

11. **What is the estimated percent of broilers that die on the farm between arrival of chicks and being sent for slaughter? (mortality rate)**

    _____

12. Who do you supply broilers to (birds for slaughter)? Insert percentages supplied where applicable.

☐ Arawak ___%

☐ Fine Choice ___%

☐ Nutrimix __%

☐ Pluck shops __%

☐ Both contractors and pluck shops (ratio) __/__

13. Who supplies your feed?

☐ Contractor ☐ Other local supplier

☐ Imported ☐ Mixture of local and imported feeds

14. Where is the feed stored?

☐ Storeroom/shed ☐ Other (state):
_____

☐ Pen

15. What feeding system do you have in place?

☐ Automatic ☐ Manual ☐ Automatic and manual

☐ Other: _____

16. How often are the feed containers cleaned and sanitized? Explain how it is done.

_____

_____

_____

17. Where does your water supply come from?

☐ Pipeborne ☐ Spring

☐ Rain ☐ Boreholes ☐ Pond

☐ Other: _____

18. Do you treat the water yourselves?

☐ Yes ☐ No

If "Yes" what do you use to treat the water and how often do you treat it?

Agent/s:

_____

Frequency of treatments:

_____

19. What drinking water system do you have in place for your broilers?

☐ Automatic ☐ Manual ☐ Automatic and manual

☐ Others: _____

20. Do you add any supplements to the everyday drinking water/feed?

☐ Yes ☐ No

If "Yes" to above, please explain:

Agent used:

_____

Frequency of administration:

_____

Feed/Water:

_____

SECTION E: Biosecurity measures

1. **Are there any pests present on your farm?**

   ☐ Yes ☐ No

   If "Yes", which ones

   ☐ Mice ☐Rats ☐ Birds ☐Others: _____
_____

2. **How do you control your pest population?**

   ☐ Poison ☐ Bait ☐ Others:

   _____

3. **What biosecurity measures do you have in place?**

   ☐ None ☐ Foot dips ☐ Vehicle dips ☐ All in-all out

   ☐ Restricted Access ☐ One-way Traffic ☐ Protective Clothing for workers

   ☐ Others_____

4. **Do you have access to veterinary consultations?**

   ☐ Yes ☐ No

   If Yes, how often does the veterinarian visit your farm?

   ☐ Only when requested ☐ Weekly visits ☐ Every 2 weeks

   ☐ Once a month ☐ Before transported to processing plant

   ☐ Other: _____

5. **Have you ever had any disease outbreaks in the last five years?**

   ☐ Yes ☐ No

   If yes, which diseases and when?

   _____

_____
_____
_____

Salmonellosis?_____
_____
_____

6. **What is the average mortality rate per pen?**

_____
_____
_____

7. **In cases of sudden deaths in a pen/ several pens, what protocol is followed in dealing with such?**

_____
_____
_____
_____

8. **Other Comments**

_____
_____
_____
_____

END.

Thank you for your cooperation.

Appendix 4.A Consent form completed by processing plant managers

THE UNIVERSITY OF THE WEST INDIES
ST. AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
FACULTY OF MEDICAL SCIENCES
SCHOOL OF VETERINARY MEDICINE
DEPARTMENT OF BASIC VETERINARY SCIENCES
Eric Williams Medical Sciences Complex, Uriah Butler Highway, Champs Fleurs
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Email: Basic.Vetsci@sta.uwi.edu

I (Mr, Miss, Mrs.) ..... [redacted] ....., the Manager of [redacted]. (Company) broiler processing plant, hereby authorise Dr Anisa Sarah Khan of the School of Veterinary Medicine to collect samples from my establishment as part of her PhD research entitled "***Prevalence and characteristics of Salmonella isolated during broiler production, processing and sale in Trinidad using the 'farm to fork' approach".***

I am aware that the samples to be collected include the following: cloacal swabs of broiler chickens and chicken carcasses.

I have also been assured that the results obtained from our processing plant will be treated with highest level confidentiality.

[redacted] .....

Name

... [redacted] .....

Signature

27.9.19

Date

.....

Official stamp

THE UNIVERSITY OF THE WEST INDIES
ST. AUGUSTINE, TRINIDAD AND TOBAGO, WEST INDIES
FACULTY OF MEDICAL SCIENCES
SCHOOL OF VETERINARY MEDICINE
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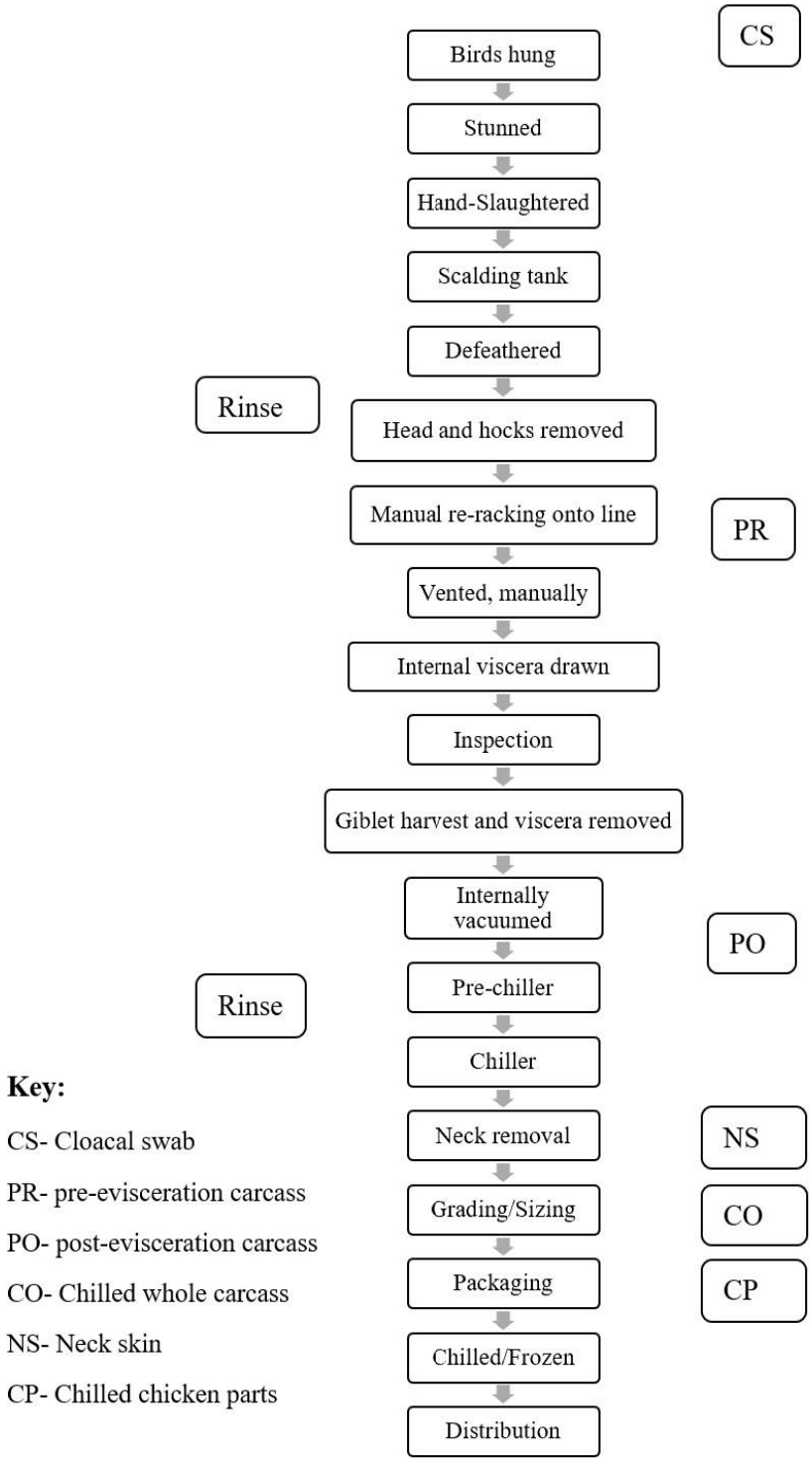
[redacted]..... ..[redacted]

Name Signature

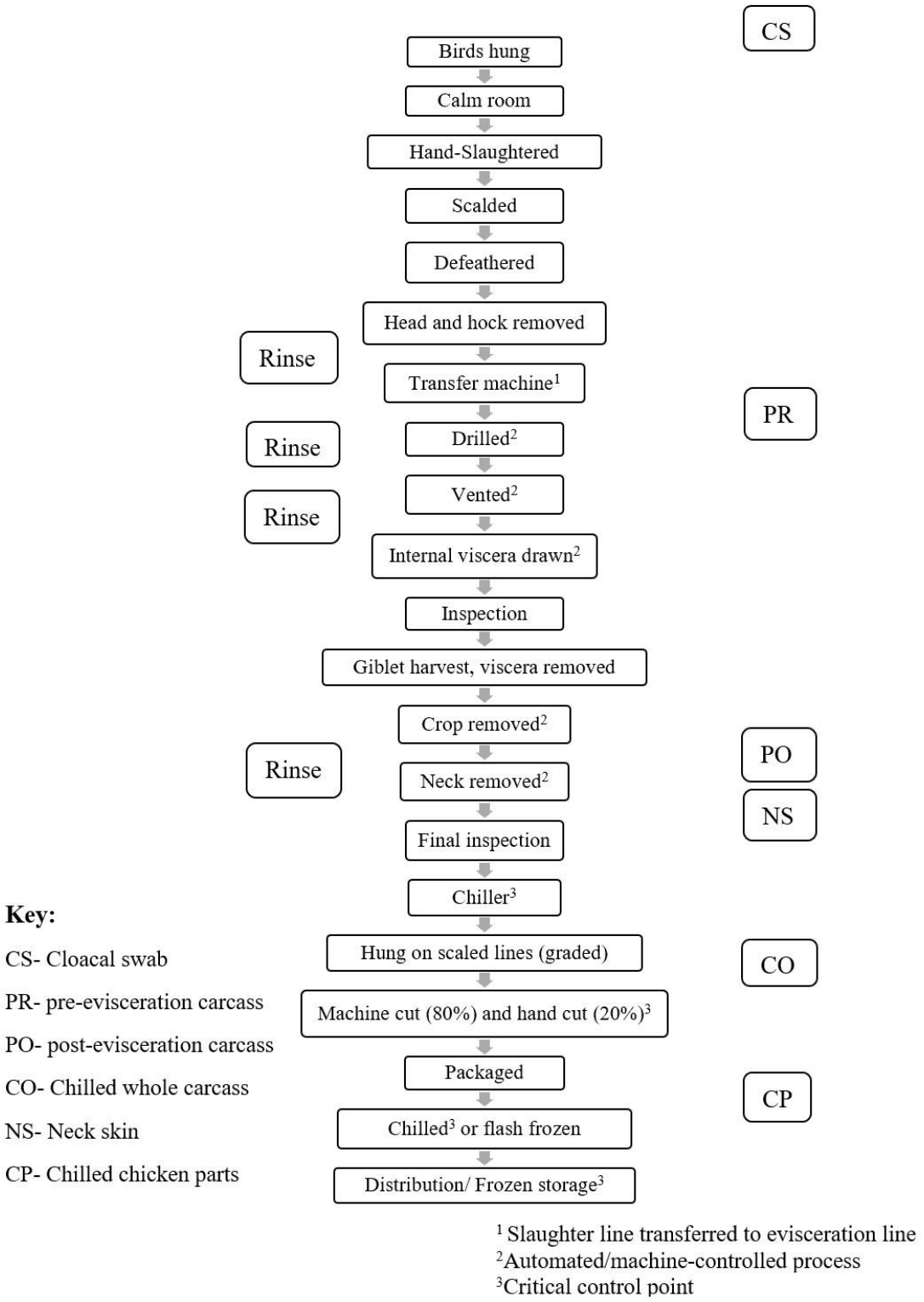
[redacted]

30/09/15.....

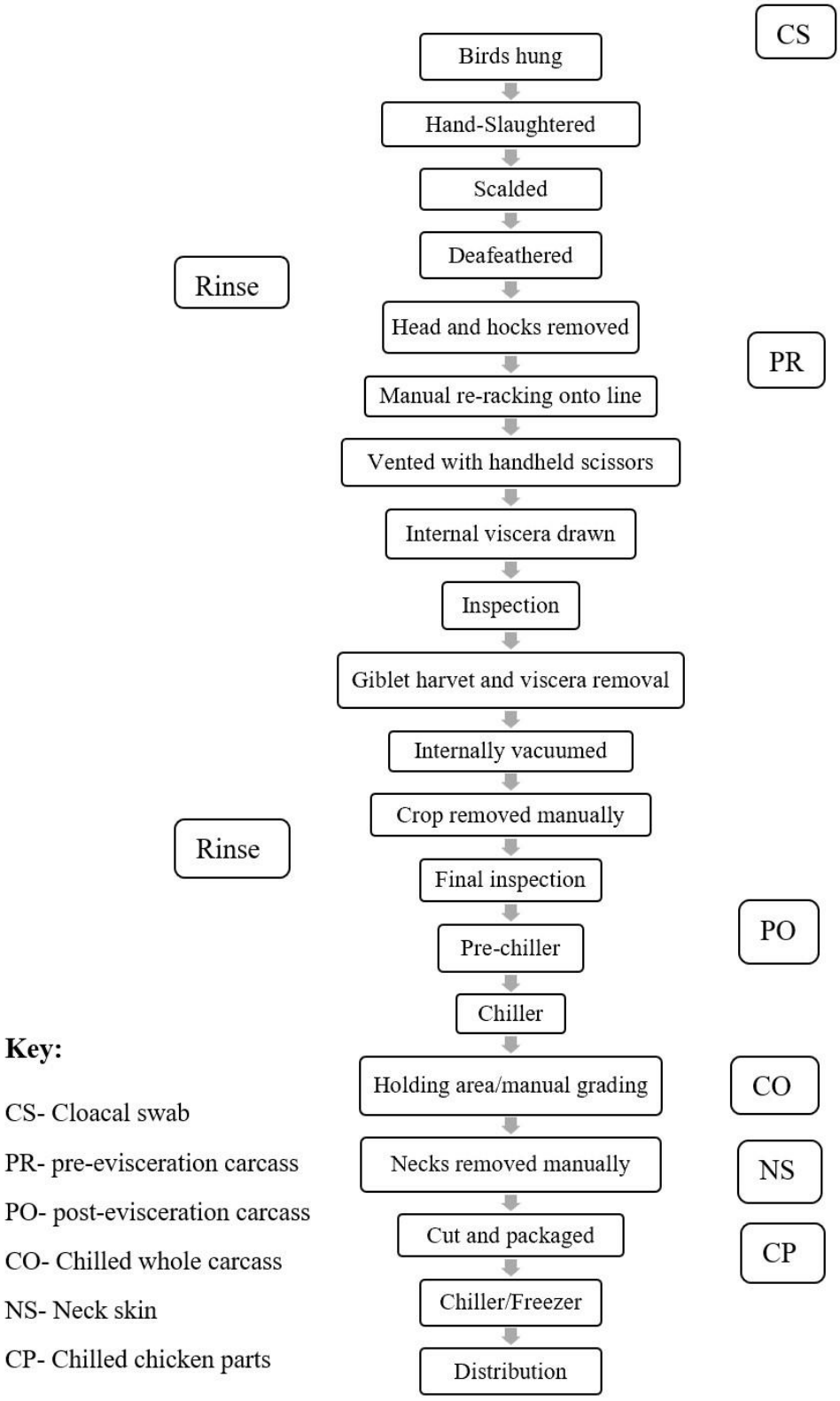
Date Official stamp



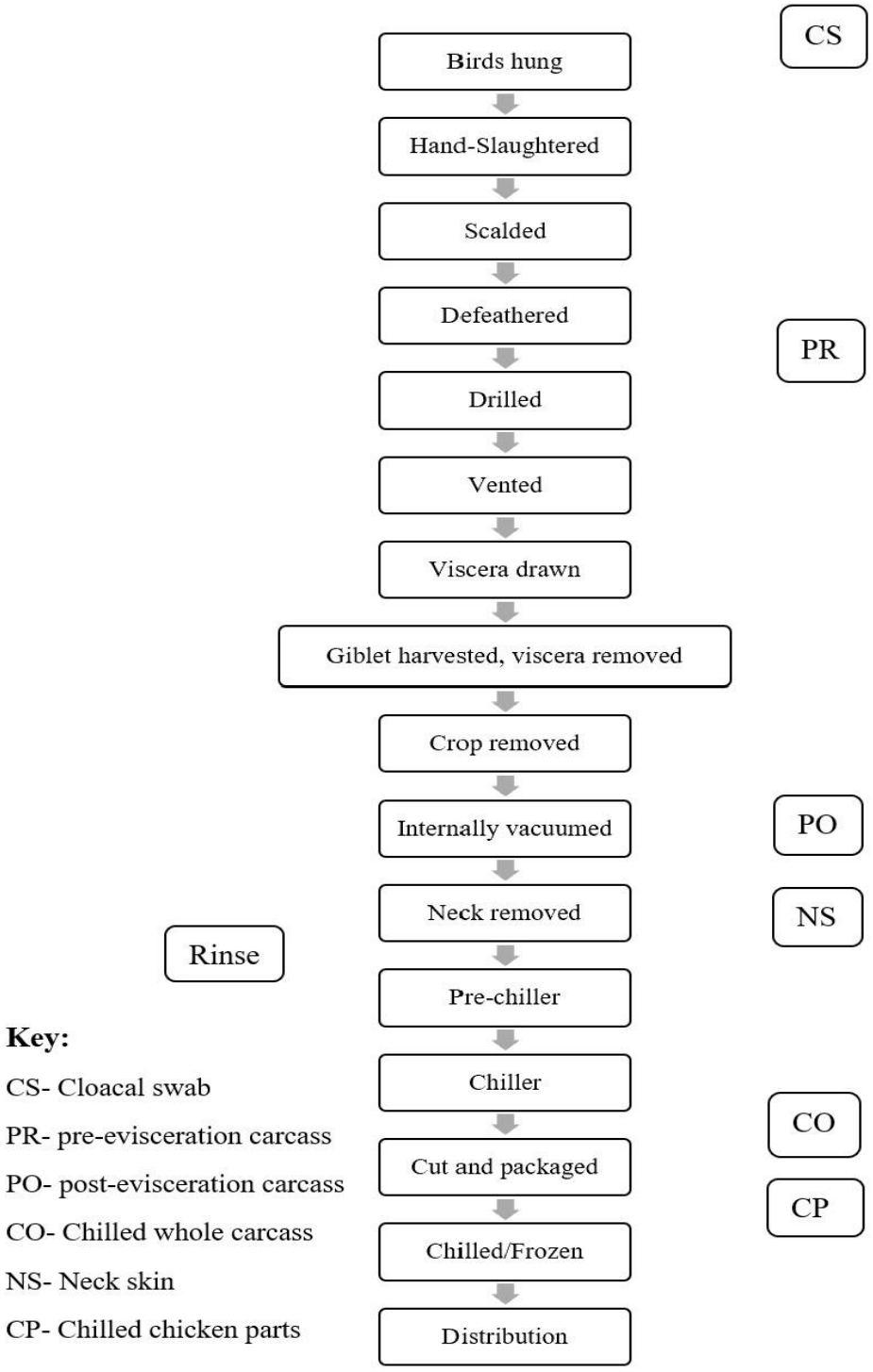
Appendix 4.1 Flow chart showing processing at Plant A



Appendix 4.2 Flow chart showing processing at Plant B



Appendix 4.3 Flow chart showing processing at Plant C



Appendix 4.4 Flow chart showing processing at Plant D

Appendix 4.5 Questionnaire- Processing plant

SECTION A: Plant Information

A1. Date: _____ A2. Code: _____

A3. GPS: _____

_____

A4. Name of Abattoir

A5. Contact Person

A6. Address of Abattoir

A7. Telephone Number A8. Email Address

A9. How many birds are slaughtered at this processing plant for the week?

1.☐500-1000 birds 2.☐1001-5000 birds

3.☐5001-10,000 birds 4.☐ 10,001-15,000 birds

5.☐ 15,001-20,000birds 6.☐20,001-25,000 birds

7.☐25,001-30,000 birds 8.☐ > 30,001 birds

9.☐ Other: _____

A10. a) How many birds are expected to be slaughtered today (day of sampling)

1.☐500-1000 birds 2.☐1001-5000 birds

3.☐5001-10,000 birds 4.☐ 10,001-15,000 birds

5.☐ 15,001-20,000birds 6.☐20,001-25,000 birds

7.☐25,001-30,000 birds 8.☐ > 30,001 birds

A10. b) How many farms are bringing broilers to process today?

_____

A11. How many days/week do you process broilers?

1.☐ 1-2 days/week 2.☐ 3-4 days/week

3.☐ 5 days/week 4.☐ 7 days/week

A12. How long has this plant been in operation?

_____

A13. Do you export the final product? 1☐ Yes 2☐ No

A14. If Yes,

1. Country exported too	2. Product/s exported (whole or parts	3. Frequency of exports/shipment
a.		

b.		
c.		
d.		
e.		
f.		

SECTION B: Operations

B1. List the names of farms supplying broilers to the plant and quantity supplied today (Tick farm where cloacal swabs taken from)

Visit #	Name of Broiler Farm	Location of farm	Quantity supplied today
1)			
2)			
3)			
4)			

B2. How many workers are **directly involved** in the processing operation i.e. handling of the carcass in all shifts?

1.☐ 1-25 2. ☐ 26-50 3. ☐ 51-75 4. ☐ 75-100

5. ☐ 101-125 6. ☐ 126-150 7. ☐ 151-175 8. ☐ 176-200

9. ☐ 201-225 10.☐ 226 or over

B3. How many workers are indirectly (overhead) involved in the processing operation?

1. ☐ 1-25 2. ☐ 26-50 3. ☐ 51-75 4. ☐ 75-100
5. ☐ 101-125 6. ☐ 126-150 7. ☐ 151-175 8. ☐ 176-200
9. ☐ 201-225 10.☐ 226 or over

B4. How long is the waiting period between arrival of birds and slaughter?

1. ☐ 15-30 mins 2. ☐ 30-45 mins 3. ☐ 45-60 mins
4. ☐ > 60 min (1 h)

B5. Are there any measures in place to reduce stress in birds on arrival?

1. ☐ Covered area 2. ☐ High ceiling 3. ☐ Fans
4. ☐ Water spray 5. ☐ Others (mention)_____

B6. What is the average mortality/death rate (%) of broilers on arrival at plant i.e. Of the total number of birds brought to the plant daily, how many arrive dead?

_____

B7. If birds from a farm appear to be diseased, how does this affect its processing?

1. ☐ Proceed as normal 2. ☐ Reject
3. ☐ Process as last batch for the day
4. ☐Other (specify):_____

B8. Please list key steps in the operation.

Location	Temperature	Time spent here	Outflow rate/hr	Agents/chemicals added	Concentrations maintained
Pre-chiller					
Chiller					

SECTION C: Sanitary Practices/Protocols employed

C1. How do you dispose of the faecal material?

_____

_____

_____

C2. How do you dispose of the offal?

_____

_____

_____

_____

_____

C3. How do you dispose of the liquid/effluent waste?

_____

_____

_____

_____

_____

C4. How do you dispose of dead carcasses pre-slaughter?

_____

_____

_____

_____

_____

C5. Is the liquid waste treated before release into public waterways?

1. ☐ Yes 2. ☐ No

C6. If yes, state chemicals/processed used.

1	
2	
3	
4	
5	
6	

C7. How often is quality control (QC)- bacterial contamination analysis performed?

1. ☐ Everyday 2.☐ Every other day 3.☐ 3-4 times a week
4. .☐ Others

(specify)_____

C8. List the types of samples tested for QC?

1. _____
2. _____

3. _____
4. _____
5. _____
6. _____
7. _____

C9. Which bacteria do QC officials focus on?

1. _____
2. _____
3. _____
4. _____
5. _____
6. _____

C10. What manual or method is used for detection of *Salmonella* ?

_____
_____
_____
_____
_____

C11. If *Salmonella* /other zoonotic bacterium found to be present on final product, state protocol.

_____
_____
_____
_____
_____
_____

C12. Are labels of final products tagged using an identification system e.g., Barcode?

1. Yes 2. ☐ No

C13. If Yes, based on what criteria? E.g., Farm/batch/day processed?

_____
_____
_____
_____
_____
_____

C14. How often is general decontamination of processing equipment done?

1.☐ Between batches 2.☐ Every other batch

3. ☐

Other(state):_____

C15. What equipment are included in general decontamination process? If not all equipment coming into contact with carcasses/effluents of processing, state which are excluded.

1.☐All

2.☐Some: a. _____

b. _____

c. _____

d. _____

e. _____

f. _____

C16. What agents are used for general decontamination? State in order of usage

1. _____
2. _____
3. _____
4. _____
5. _____

C17. How often is a thorough decontamination done?

1.☐Overnight 2.☐Every other day

3.☐Once a week 4.☐ Twice a week

C18. What equipment are included in the thorough decontamination process? If not, all equipment coming into contact with carcasses/effluents of processing, state which are excluded.

1.☐All

2.☐Some: a. _____

b. _____

c. _____

d. _____

e. _____

f. _____

C19. What agents are used for the thorough decontamination? State in order of usage

1. _____
2. _____

3. _____
4. _____

C20. What is the source of water used during processing? ie. Water coming into contact with carcasses e.g., Spraying of carcasses/use in chilled water bath.

_____
_____
_____
_____

C21. Do you treat your water in-house?

1.☐ Yes 2.☐ No

C22. If yes, what chemicals and concentrations are used?

	Agent	Concentration used
1		
2		
3		
4		
5		

SECTION D: Protocols employed

D1. Are workers colour coded (color of coverall) based on the area that they are working? i.e., Dirty vs clean areas

1.☐ Yes 2.☐ No

D2. If yes, how many areas are selective and which areas?

1	
2	
3	
4	
5	
6	

D3. Attire of workers handling birds/carcasses:

Location	Aprons	Hair net	Gloves			
Pre slaughter						
Initial slaughter/processing						
Final processing						

SECTION E: Products sold to the public

E1. What 'raw' products are made available to consumers? (Whole carcass, parts) Please list.

1	
2	
3	
4	
5	
6	

7	
8	
9	
10	
11	
12	
13	
14	

E2. What further-processing products (cooked) are sold to the public?

1	
2	
3	
4	
5	
6	
7	
8	
9	

E3. Are broilers the only species processed in this plant? If No, state other species processed

1.☐Yes 2.☐No List:

_____

THANK YOU FOR YOUR ASSISTANCE

Appendix 5.1. Questionnaire- Retail outlet (Cottage processors/ 'pluck shops')

DATE:_____ CODE: _____

SECTION A- General information

1) Name of Pluck Shop:

_____

2) Address of Pluck Shop:

_____

3) Contact Person: _____

4) Telephone Number: _____

5) How many year(s) has the Pluck Shop been in operation?

_____

SECTION B- Worker's information

1) Number of workers (not including owner/owners):

☐ Temporary (T) Number: _____

☐ Permanent (P) Number: _____

2) Level of Training of workers:

☐ Ministry of Health Number of workers: _____

☐ Ministry of Agriculture Number of workers: _____

☐ Caribbean Poultry Association Number of workers: _____

☐ Other(specify): _____ Number of workers: _____

3) Length of time working in industry:

☐ < 1 year……Number of workers: _____

☐ 1-3 years……...Number of workers: _____

☐ 3-5 years……...Number of workers: _____

☐ 5-7 years……...Number of workers: _____

☐ >7 years……...Number of workers: _____

Owner experience in the industry: ☐ <1 year ☐ 1-3 years ☐ 3-5 years

☐ 5-7 years ☐ >7 years

SECTION C- Operational Information

1) What is your average sale of broilers:

a) Mon- Fri (Or Weekdays): _____

b) Sat-Sun: _____

2) How many days is the pluck shop open per week?

_____

3) What is the source of your live birds?

☐ Arawak ☐ Malabar Farms ☐ Nutrimix

☐ Master Mix Feed Mill ☐ Warner Grain Mill (WGM)

☐ Self-growing

☐ Other (specify): _____

4) What method is used to rinse carcasses?

☐ Sink ☐ Drum/Tank

☐ Other (Specify): _____

5) If Drum/Tank is used, how often is the rinse water changed?

☐ every 20 carcasses or less processed

☐ every 21- 40 carcasses processed

☐ every 41- 75 carcasses processed

☐ > 100 carcasses processed

☐ once a day

☐ never

6) Are there any quality control measures or practices in effect at the shop? (e.g., How often do you perform general and complete cleaning of equipment/working areas)

☐ Yes ☐ No

If Yes, please describe the following:

General cleaning:

_____

Thorough cleaning:

_____

Do you use a chilled water bath after processing to cool carcasses before packaging?

☐ Yes ☐ No

If Yes, to above, how long do you leave the carcass to chill in the water bath? _____mins

How often are pens where live birds are kept pre-slaughter cleaned?

_____

What material is the flooring of pens made of? ☐Dirt flooring
☐Concrete flooring

Other measures:

_____

_____

_____

7) Do you sell carcasses from the countertop?

☐ Yes ☐ No

If Yes, please state length of time before carcasses stored on counter tops are sold or stored in a chiller: _____mins

8) Please list the complaints you received from your customers.

_____

_____

_____

_____

Thank you for your cooperation!

Appendix 5.2. General sanitary condition report- Cottage processors/'pluck shops'

	Score					
Category	**1 (worst)**	**2**	**3**	**4**	**5(best)**	**NA**
1. Handlers of chickens at outlet						
1.1 Cleanliness of clothes						
Clean clothes with sleeves					**X[a]**	
Clean clothes without sleeve						
Dirty clothes without sleeve						
Dirty clothes with sleeves						
Dirty clothes with dirty sleeves	**X[b]**					
1.2 Wore aprons						
Wore very clean aprons						
Wore clean aprons						
Did not wear aprons						
Wore moderately dirty clothes						
Wore very dirty aprons						
1.3 Had hair covered						
Yes						
No						
2. Cleanliness in cages or areas where live birds are kept						
Relatively clean and not crowded						
Relatively clean and crowded						
Relatively dirty- faeces and crowded						
Relatively filthy and crowded						
Very filthy and very crowded						
3. Sanitation in slaughter area						
Kept very clean- little blood/feathers/faeces						
Kept clean- some blood/feathers/faeces						
Moderately kept clean- blood/feathers/lot of faeces						

Poorly kept- blood/feathers/lot of faeces/few flies						
Very poorly kept- blood/feathers/faeces/ many flies						
4. Sanitation in de-feathering or 'plucking' area						
Kept very clean- little blood/feathers/faeces						
Kept clean- some blood/feathers/faeces						
Moderately kept clean- blood/feathers/lot of faeces						
Poorly kept- blood/ feathers/lot of faeces/few flies						
Very poorly kept- blood/ feathers/faeces/many flies						
5. Sanitation in evisceration area						
Kept very clean- little blood/ feathers/faeces						
Kept clean- some blood/ feathers/faeces						
Moderately kept clean- blood/ feathers/lot of faeces						
Poorly kept- blood/ feathers/lot of faeces/ few flies						
Very poorly kept- blood/ feathers/faeces/many flies						
6. Sanitation in rinsing of carcases						
Use of running water in sink						
Use of 3 rinsing buckets/ clean water						
Use of 2 rinsing buckets/ clean water						
Use of 1 rinsing bucket/ bloody water/feathers						
Do not rinse						
7. Sanitation in packaging and sale areas						
Kept very clean- no blood/ feathers/ faeces or flies						
Kept clean- no blood/feathers or faeces but few flies						

Kept moderately clean- some blood/feathers/faeces and flies						
Poorly kept- blood/ feathers/ lots of faeces with few flies						
Very poorly kept- some blood/ lots of feathers/lots of faeces/many flies						

X^a- Example of scoring of the overall cleanliness of handlers clothing: Clean clothes with sleeves- Score of 5 was recorded.

X^b- Example of scoring of the overall cleanliness of handlers clothing: Dirty clothes with dirty sleeves- Score of 1 (worst) was recorded.

Appendix 7.1 Metadata of 146 *Salmonella* isolates detected along the broiler production chain in Trinidad and Tobago.

BioSample	Sample Code	Study type	Source	Type of sample	Year of isolation	Processor	Farm	County	Class of Supermarket	convenSerotype	SISTR Serovar	SISTR Serovar CGMLST
SAMN14404241	PS13	Cross sectional	Pluck Shop	Chicken carcass	2016			St George Central		Javiana	Javiana	Javiana
SAMN14404242	PS40	Cross sectional	Pluck Shop	Chicken carcass	2016			St George East		Javiana	Javiana	Javiana
SAMN14404243	PS78	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Javiana	Javiana	Javiana
SAMN14404244	PS54	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Javiana	Javiana	Javiana
SAMN14404245	SM52	Cross sectional	Supermarket	Chicken carcass	2016				Chain	Javiana	Javiana	Javiana
SAMN14404246	PP76	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Javiana	Javiana	Javiana
SAMN14404247	PP142	Cross sectional	Processing Plant	Neck skin	2018	A				Javiana	Javiana	Javiana
SAMN14404248	PP143	Cross sectional	Processing Plant	Neck skin	2018	A				Javiana	Javiana	Javiana
SAMN14404249	PS1	Cross sectional	Pluck Shop	Chicken carcass	2016			Caroni		Kentucky	Kentucky	Kentucky
SAMN14404250	PS68	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN14404251	SM34	Cross sectional	Supermarket	Chicken carcass	2016				Chain	Kentucky	Kentucky	Kentucky
SAMN14404252	SM114	Cross sectional	Supermarket	Chicken carcass	2017				Large	Kentucky	Kentucky	Kentucky
SAMN14404253	PP100	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Kentucky	Kentucky	Kentucky
SAMN14404254	PP103	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Kentucky	Kentucky	Kentucky

SAMN14404255	PP151	Cross sectional	Processing Plant	Chilled whole carcass	2018	A				Enteritidis	Enteritidis	Enteritidis
SAMN14404256	PP165	Cross sectional	Processing Plant	Chilled whole carcass	2018	C				Enteritidis	Enteritidis	Enteritidis
SAMN14404257	PP172	Cross sectional	Processing Plant	Chilled whole carcass	2018	D				Enteritidis	Enteritidis	Enteritidis
SAMN14404258	PP196	Cross sectional	Processing Plant	Chilled whole carcass	2018	D				Enteritidis	Enteritidis	Enteritidis
SAMN14404259	PP171	Cross sectional	Processing Plant	Chilled whole carcass	2018	D				Enteritidis	Enteritidis	Enteritidis
SAMN14404260	PP204	Cross sectional	Processing Plant	Chilled chicken parts	2018	D				Enteritidis	Enteritidis	Enteritidis
SAMN14404261	PP48	Cross sectional	Processing Plant	Chilled whole carcass	2018	B				Infantis	Albany	Albany
SAMN14404262	PP144	Cross sectional	Processing Plant	Chilled whole carcass	2018	A				Infantis	Infantis	Infantis
SAMN14404263	PP147	Cross sectional	Processing Plant	Chilled whole carcass	2018	A				Infantis	Infantis	Infantis
SAMN14404264	PP50	Cross sectional	Processing Plant	Chilled whole carcass	2018	B				Anatum	Anatum	Anatum
SAMN14404265	PP66	Cross sectional	Processing Plant	Chilled whole carcass	2018	B				Anatum	Anatum	Anatum
SAMN14404266	PP85	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Anatum	Anatum	Anatum

SAMN14404267	PS17	Cross sectional	Pluck Shop	Chicken carcass	2016			St George Central		Schwarzengrund	Schwarzengrund	Schwarzengrund
SAMN14404268	PP88	Cross sectional	Processing Plant	Neck skin	2018	B				Schwarzengrund	Schwarzengrund	Schwarzengrund
SAMN14404269	PP206	Cross sectional	Processing Plant	Neck skin	2018	D				Schwarzengrund	Schwarzengrund	Schwarzengrund
SAMN14404270	PS2	Cross sectional	Pluck Shop	Chicken carcass	2016			Caroni		Montevideo	Montevideo	Montevideo
SAMN14404272	PS32	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Aberdeen	Aberdeen	Aberdeen
SAMN14404273	PP99	Cross sectional	Processing Plant	Chilled whole carcass	2018	B				Aberdeen	Aberdeen	Aberdeen
SAMN14404274	PS57	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Manhattan	Manhattan	Manhattan
SAMN14404275	PS65	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Manhattan	Manhattan	Manhattan
SAMN14404276	PP62	Cross sectional	Processing Plant	Chilled whole carcass	2018	B				Albany	Albany	Albany
SAMN14404277	PP30	Cross sectional	Processing Plant	Neck skin	2018	B				Albany	Albany	Albany
SAMN14404280	PS42	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Bloomsbury	Senftenberg	Senftenberg
SAMN14404281	PS20	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Molade	Kentucky	Kentucky
SAMN14404282	PS55	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Warragul	Caracas	Caracas
SAMN14404283	SM124	Cross sectional	Supermarket	Chicken carcass	2017				Large	Westhampton	Senftenberg	Senftenberg
SAMN14404284	PP72	Cross sectional	Processing Plant	Chilled whole carcass	2018	B				Mbandaka	Mbandaka	Mbandaka
SAMN14404285	PP198	Cross sectional	Processing Plant	Chilled whole carcass	2018	D				Ayinde	Schwarzengrund	Schwarzengrund

SAMN14404286	SM105	Cross sectional	Supermarket	Chicken carcass	2017				Small	Chester/San diego	Chester	Chester
SAMN14404287	PP113	Cross sectional	Processing Plant	Neck skin	2018	A				Madjorio	Liverpool	Liverpool
SAMN14404288	PP162	Cross sectional	Processing Plant	Pre-evisceration carcass	2018	C				Weltevreden	Weltevreden	Weltevreden
SAMN25867712	PS 18	Cross sectional	Pluck Shop	Chicken carcass	2016			St George Central		Javiana	Javiana	Javiana
SAMN25867713	PS 29	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN25867715	PS 24	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN25867716	PS 31	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN25867718	PS 41	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Javiana	Javiana	Javiana
SAMN25867719	PS 43	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Bloomsbury	Senftenberg	Senftenberg
SAMN25867720	PS 44	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Javiana	Javiana	Javiana
SAMN25867721	PS 51	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Javiana	Javiana	Javiana
SAMN25867722	PS 62	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Manhattan	Manhattan	Manhattan
SAMN25867723	PS 58	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Manhattan	Manhattan	Manhattan
SAMN25867724	PS 59	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Manhattan	Manhattan	Manhattan
SAMN25867725	PS 66	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Manhattan	Manhattan	Manhattan
SAMN25867726	PS 67	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Manhattan	Manhattan	Manhattan
SAMN25867727	PS 75	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Warragul	Caracas	Caracas
SAMN25867728	PS 73	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Warragul	Caracas	Caracas

SAMN25867729	PS 79	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Javiana	Javiana	Javiana
SAMN25867730	PS 88	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Javiana	Javiana	Javiana
SAMN25867731	PS 84	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Javiana	Javiana	Javiana
SAMN25867732	PS 98	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN25867733	PS 97	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN25867736	SM 106	Cross sectional	Supermarket	Chicken carcass	2017				Small	Chester/San diego	Chester	Chester
SAMN25867737	SM 109	Cross sectional	Supermarket	Chicken carcass	2017				Small	Montevideo	Montevideo	Montevideo
SAMN25867738	SM 111	Cross sectional	Supermarket	Chicken carcass	2017				Medium	Kentucky	Kentucky	Kentucky
SAMN25867739	SM 112	Cross sectional	Supermarket	Chicken carcass	2017				Large	Kentucky	Molade	Molade
SAMN25867742	PP 35	Cross sectional	Processing Plant	Neck skin	2018	B				Anatum	Anatum	Anatum
SAMN25867743	PP 47	Cross sectional	Processing Plant	Neck skin	2018	B				Albany	Albany	Albany
SAMN25867744	PP 86	Cross sectional	Processing Plant	Neck skin	2018	B				Albany	Albany	Albany
SAMN25867745	PP 89	Cross sectional	Processing Plant	Neck skin	2018	B				Schwarzen grund	Schwarzen grund	Schwarzen grund
SAMN25867746	PP 105	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Kentucky	Kentucky	Kentucky
SAMN25867747	PP 140	Cross sectional	Processing Plant	Neck skin	2018	A				Javiana	Javiana	Javiana
SAMN25867748	PP 121	Cross sectional	Processing Plant	Neck skin	2018	A				Javiana	Javiana	Javiana
SAMN25867749	PP 150	Cross sectional	Processing Plant	Neck skin	2018	A				Enteritidis	Enteritidis	Enteritidis

SAMN25867750	PP 163	Cross sectional	Processing Plant	Chilled whole carcass	2018	C				Enteritidis	Enteritidis	Enteritidis
SAMN25867751	PP 167	Cross sectional	Processing Plant	Chilled chicken parts	2018	C				Kentucky	Kentucky	Kentucky
SAMN25867752	PP 169	Cross sectional	Processing Plant	Chilled whole carcass	2018	D				Enteritidis	Enteritidis	Enteritidis
SAMN25867753	PP 194	Cross sectional	Processing Plant	Post-evisceration carcass	2018	D				Javiana	Javiana	Javiana
SAMN25867754	PP 33	Cross sectional	Processing Plant	Neck skin	2018	B				Group C2	Albany	Albany
SAMN25867755	F 6	Cross sectional	Farm	Cloacal swab	2019	B	A			Albany	Albany	Albany
SAMN25867756	F 17	Cross sectional	Farm	Cloacal swab	2019	B	K			Infantis	Infantis	Infantis
SAMN25867757	F 22	Cross sectional	Farm	Cloacal swab	2019	B	K			Infantis	Infantis	Infantis
SAMN14677211	F 32	Cross sectional	Farm	Cloacal swab	2019	A&C	C			Infantis	Infantis	Infantis
SAMN25867760	F 28	Cross sectional	Farm	Water-tank	2019	B	M			*Salmonella* sp	Gaminara	Gaminara
SAMN25867761	H 30	Cross sectional	Hatchery	Hatcher fluff	2019	A&C				Kentucky	Kentucky	Kentucky
SAMN25867762	H 10	Cross sectional	Hatchery	Still born chick	2019	B				Kentucky	Kentucky	Kentucky
SAMN25867763	H 19	Cross sectional	Hatchery	Eggs in incubator	2019	B				Kentucky	Kentucky	Kentucky
SAMN25867764	H 26	Cross sectional	Hatchery	Still born chick	2019	A&C				Kentucky	Kentucky	Kentucky
SAMN25867767	H 24	Cross sectional	Hatchery	Hatcher environmental swab	2019	B				Kentucky	Kentucky	Kentucky
SAMN14677232	F 36	Cross sectional	Farm	Cloacal swab	2019	A&C	C			Infantis	Infantis	Infantis

SAMN14677229	UWI-F11	Cross sectional	Farm	Cloacal swab	2019	B	K			Infantis	Infantis	Infantis
SAMN14677212	UWI-F1	Cross sectional	Farm	Cloacal swab	2019	B	A			Albany	Albany	Albany
SAMN14677210	UWI-F2	Cross sectional	Farm	Cloacal swab	2019	B	A			Albany	Infantis	Infantis
SAMN14677203	UWI-F4	Cross sectional	Farm	Cloacal swab	2019	B	A			Albany	Infantis	Infantis
SAMN14677213	UWI-F7	Cross sectional	Farm	Boot swab	2019	B	F			*Salmonella* sp	Albany	Albany
SAMN14677207	UWI-F9	Cross sectional	Farm	Water supply	2019	B	J			*Salmonella* sp	Infantis	Infantis
SAMN14677205	UWI-F19	Cross sectional	Farm	Cloacal swab	2019	B	K			Infantis	Gaminara	Gaminara
SAMN14677202	UWI-F27	Cross sectional	Farm	Boot swab	2019	B	K			Infantis	Oranienburg	Oranienburg
SAMN14677209	UWI-F30	Cross sectional	Farm	Water supply	2019	B	Q			*Salmonella* sp	Infantis	Infantis
SAMN14677208	UWI-F31	Cross sectional	Farm	Drag swab litter	2019	B	U			Othmarschen	Infantis	Infantis
SAMN14677224	UWI-F37	Cross sectional	Farm	Cloacal swab	2019	A&C	C			Infantis	Albany	Albany
SAMN14677226	UWI-F38	Cross sectional	Farm	Feed	2019	A&C	C			Infantis	Infantis	Infantis
SAMN14677228	UWI-F39	Cross sectional	Farm	Drag swab litter	2019	A&C	C			Infantis	Soerenga	Soerenga
SAMN14677206	UWI-F40	Cross sectional	Farm	Boot swab	2019	A&C	D			Infantis	Gaminara	Gaminara
SAMN14677201	UWI-H4	Cross sectional	Hatchery	Still born chick	2019	B				Kentucky	Senftenberg	Senftenberg
SAMN14677225	UWI-H17	Cross sectional	Hatchery	Broken eggshell	2019	B				Westhampton	Fresno	Fresno
SAMN14677227	UWI-H21	Cross sectional	Hatchery	Eggs in hatcher	2019	B				Group D	Kentucky	Kentucky
SAMN16678588	UWI PP 31	Cross sectional	Processing Plant	Neck skin	2018	B				Infantis	Infantis	Infantis

SAMN16678590	UWI PP 41	Cross sectional	Processing Plant	Neck skin	2018	B				Infantis	Infantis	Infantis
SAMN16678603	UWI PP 124	Cross sectional	Processing Plant	Post-evisceration carcass	2018	A				Infantis	Infantis	Infantis
SAMN16678604	UWI PP 145	Cross sectional	Processing Plant	Chilled whole carcass	2018	A				Infantis	Infantis	Infantis
SAMN16678605	UWI PP 149	Cross sectional	Processing Plant	Chilled chicken parts	2018	A				Infantis	Infantis	Infantis
SAMN16678593	UWI PP 79	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Infantis	Infantis	Infantis
SAMN16678575	UWI H 29	Cross sectional	Hatchery	Still born chick	2019	A&C				Kentucky	Kentucky	Kentucky
SAMN16678573	UWI H 22	Cross sectional	Hatchery	Hatcher environmental swab	2019	B				Kentucky	Kentucky	Kentucky
SAMN16678609	UWI PP 166	Cross sectional	Processing Plant	Chilled whole carcass	2018	C				Kentucky	Kentucky	Kentucky
SAMN16678618	UWI PS 22	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN16678599	UWI PP 108	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Kentucky	Kentucky	Kentucky
SAMN16678622	UWI PS 69	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Kentucky	Kentucky	Kentucky
SAMN16678598	UWI PP 102	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Kentucky	Kentucky	Kentucky
SAMN16678617	UWI PS 21	Cross sectional	Pluck Shop	Chicken carcass	2016			Victoria		Kentucky	Kentucky	Kentucky
SAMN16678596	UWI PP 97	Cross sectional	Processing Plant	Post-evisceration carcass	2018	B				Javiana	Javiana	Javiana

SAMN16678600	UWI PP 117	Cross sectional	Processing Plant	Post-evisceration carcass	2018	A				Javiana	Javiana	Javiana
SAMN16678620	UWI PS 45	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Javiana	Javiana	Javiana
SAMN16678601	UWI PP 119	Cross sectional	Processing Plant	Neck skin	2018	A				Javiana	Javiana	Javiana
SAMN16678624	UWI PS 80	Cross sectional	Pluck Shop	Chicken carcass	2016			Nariva/Mayaro		Javiana	Javiana	Javiana
SAMN16678602	UWI PP 122	Cross sectional	Processing Plant	Neck skin	2018	A				Javiana	Javiana	Javiana
SAMN16678616	UWI PS 16	Cross sectional	Pluck Shop	Chicken carcass	2016			St George Central		Javiana	Javiana	Javiana
SAMN16678621	UWI PS 48	Cross sectional	Pluck Shop	Chicken carcass	2016			St Patrick		Javiana	Javiana	Javiana
SAMN16678591	UWI PP 44	Cross sectional	Processing Plant	Neck skin	2018	B				Albany	Albany	Albany
SAMN16678615	UWI PS 14	Cross sectional	Pluck Shop	Chicken carcass	2016			St George Central		Group D	Javiana	Javiana
SAMN16678614	UWI PS 10	Cross sectional	Pluck Shop	Chicken carcass	2016			St George Central		Group D	Javiana	Javiana
SAMN16678594	UWI PP 90	Cross sectional	Processing Plant	Neck skin	2018	B				Schwarzengrund	Schwarzengrund	Schwarzengrund
SAMN16678610	UWI PP 205	Cross sectional	Processing Plant	Neck skin	2018	D				Schwarzengrund	Schwarzengrund	Schwarzengrund
SAMN16678597	UWI PP 98	Cross sectional	Processing Plant	Chilled whole carcass	2018	B				Schwarzengrund	Schwarzengrund	Schwarzengrund
SAMN16678587	UWI PP 29	Cross sectional	Processing Plant	Neck skin	2018	B				Anatum	Anatum	Anatum
SAMN16678619	UWI PS 39	Cross sectional	Pluck Shop	Chicken carcass	2016			St Andrew/St David		Kentucky	Kentucky	Kentucky
SAMN16678589	UWI PP 37	Cross sectional	Processing Plant	Neck skin	2018	B				Group C2	Albany	Albany
SAMN16678613	UWI PS 9	Cross sectional	Pluck Shop	Chicken carcass	2016			St George East		Group C2	Albany	Albany

SAMN16678595	UWI PP 96	Cross sectional	Processing Plant	Post-evisceration carcass	2018	B				Virchow	Virchow	Virchow
SAMN16678611	UWI PS 4	Cross sectional	Pluck Shop	Chicken carcass	2016			Caroni		Aberdeen	Montevideo	Montevideo
SAMN16678606	UWI PP 152	Cross sectional	Processing Plant	Chilled chicken parts	2018	A				Alachua	Alachua	Alachua
SAMN16678592	UWI PP 74	Cross sectional	Processing Plant	Chilled chicken parts	2018	B				Hindmarsh	Infantis	Infantis

Appendix 7.2. Distribution of virulence genes and classes among the various serovars of *Salmonella* Isolated in this study.

	Virulence class and factors detected amongst the various serovars in this study[a]			
Serovar	**Fimbrial adherence determinants**	**Secretion system**	**Toxin**	**No. (%)[b] of isolates**
Aberdeen[c]	*bcf, agf/csg, fim, lpf*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors TTSS-2 translocated effectors	-	2 (1.4)
Alachua	*bcf, agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS effectors translocated via both systems	-	1 (0.7)
Albany	*bcf, agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode)	-	13 (8.9)
Anatum	*bcf, agf/csg, fim*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors TTSS-2 translocated effectors		5 (3.4)
Caracas	*agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS effectors translocated via both systems TTSS-1 translocated effectors	Typhoid toxin	3 (2.1)
Chester	*agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors	Typhoid toxin	2 (1.4)

Enteritidis[d]	*bcf, agf/csg, lpf, pef*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors TTSS-2 translocated effectors	*spvB*	9 (6.2)
Fresno	*bcf, agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors	-	1 (0.7)
Gaminara	*bcf, agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors	Typhoid toxin	3 (2.1)
Infantis	*bcf, agf/csg, lpf*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors	-	20 (13.7)
Javiana	*bcf, agf/csg, fim*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors	Typhoid toxin	28 (19.2)
Kentucky	*bcf, agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode)	-	30 (20.5)
Liverpool	*bcf, agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode)	-	1 (0.7)
Manhattan	*bcf, agf/csg, lpf*	TTSS (SPI-1 encode) TTSS (SPI-2 encode) TTSS-1 translocated effectors TTSS-2 translocated effectors	-	7 (4.8)
Mbandaka	*bcf, agf/csg*	TTSS (SPI-1 encode) TTSS (SPI-2 encode)	-	1 (0.7)
Molade	*bcf, agf/csg*	TTSS (SPI-1 encode)	-	1 (0.7)

		TTSS (SPI-2 encode)		
Montevideo	*bcf, agf/csg*	TTSS (SPI-1 encode)	Typhoid toxin	3 (2.1)
		TTSS (SPI-2 encode)		
Oranienburg	*bcf, agf/csg*	TTSS (SPI-1 encode)	-	1 (0.7)
		TTSS (SPI-2 encode)		
		TTSS-1 translocated effectors		
Schwarzengrund	*bcf, agf/csg*	TTSS (SPI-1 encode)	-	8 (5.5)
		TTSS (SPI-2 encode)		
		TTSS-1 translocated effectors		
Senftenberg	*bcf, agf/csg*	TTSS (SPI-1 encode)	-	4 (2.7)
		TTSS (SPI-2 encode)		
Soerenga	*bcf, agf/csg*	TTSS (SPI-1 encode)	-	1 (0.7)
		TTSS (SPI-2 encode)		
		TTSS-1 translocated effectors		
Virchow	*bcf, agf/csg, fim*	TTSS (SPI-1 encode)	-	1 (0.7)
		TTSS (SPI-2 encode)		
		TTSS-1 translocated effectors		
Weltevreden[d]	*bcf, agf/csg*	TTSS (SPI-1 encode)	-	1 (0.7)
		TTSS (SPI-2 encode)		
		TTSS-1 translocated effectors		
Total				146

[a]Virulence factors and virulence classes in accordance with the Virulence Factor Database (VFDB)

[b]Of a total of 146 isolates of *Salmonella*

[c]Factors associated with magnesium uptake (Mg2+ transport) and stress adaptation (*sodC1*) were also detected

[d] Stress adaptation gene, *sodC1* was also detected