Defining the food safety landscape in Cambodia: the ecology of *Salmonella enterica* in informal markets

by

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B.S., Federal University of Santa Maria - Brazil, 2013
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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Abstract

The lack of hygiene and sanitation practices and insufficient infrastructure in Cambodian informal markets may increase the risk of food contamination, specifically raw vegetables, which in turn may increase the chances of contracting a foodborne disease. The aims of this research were i) to quantify the prevalence of *Salmonella enterica* based upon seasonal differences (rainy and dry) between surface types (food contact surface [FCS] and non-food contact surface [NFCS]) and between location of vendors within the market (inside and outside), ii) to characterize *Salmonella enterica* serotypes abundance, and iii) identify and characterize the genotypic antimicrobial resistance profiles of *Salmonella enterica* strains isolated from environmental samples in informal markets in Cambodia. A total of 310 samples were screened for *Salmonella enterica* prevalence following the U.S. Department of Agriculture guidelines and confirmed by PCR. Whole Genome Sequencing was performed and the serotype for each isolate was determined in-silico using SeqSero 1.0 on draft genomes. Antimicrobial resistance (AMR), stress and virulence genotypes were retrieved from the National Center for Biotechnology Information (NCBI) Pathogen Detection engine. *Salmonella enterica* pathogenicity islands (SPIs) were identified with SPIFinder (Center for Genomic Epidemiology). A total of 81 samples were confirmed positive for *Salmonella enterica*. During the dry season, *Salmonella enterica* was more prevalent on FCS compared to NFCS (estimated probability of detection [confidence interval]: 0.41 [0.25,0.59] and 0.17 [0.08, 0.32], respectively; P=0.002), though no differences were apparent during the rainy season. Further, there was no evidence of any differences in *Salmonella enterica* prevalence based on location within the market (P=0.61). Sixteen *Salmonella enterica* serotypes were detected across multiple surfaces, Rissen (n=19); Hvittingfoss (n=13); Corvallis (n=10); Krefeld (n=8); Weltevreden (n=6); Altona (n=6); Mbandaka (n=5); Typhimurium (n=3); Javiana,

Uganda, and Derby (n=2 each); Anatum, Braenderup, Lexington, Virchow, and the potential monophasic variant of *S. enterica* serotype Typhimurium (I 4,[5],12:i:–) (n=1 each). AMR genes were identified in 43 out of 81 strains, including those encoding tetracycline, beta-lactam, sulfonamide, quinolone, aminoglycoside, phenicol, trimethoprim, and fosfomycin resistance. A total of 10 SPIs (SP1, 3-5, 8, 9, 12-14, centisome 63) were detected in 59 genomes. SPI-1, SPI-4 and SPI-9 were present in 13%, 2%, and 5% of the isolates, respectively. The availability of robust and accurate data on the ecology of *Salmonella enterica* in these markets is crucial for active surveillance, implementation of suitable intervention strategies, and prevention of domestic foodborne illness cases. These studies may serve as guiding references to develop further food safety research, education and intervention programs, and even policy drafting. They will support decision making within the Ministry of Health and the Ministry of Agriculture, Forestry, and Fisheries in Cambodia and foster public health protection, as well as support global epidemiological investigations of outbreaks.

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Major Professor
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Table of Contents

List of Figures

List of Tables

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Dedication

I want to dedicate this dissertation to my parents Iris and Inacio Schwan (*in memoriam*), who have always inspired, supported, and encouraged me to pursue my dreams. They are the reason why I was able to finish my doctorate degree. My forever gratitude to them.

Chapter 1 - Literature review

1.1 Overview of food safety in low and middle-income countries

The prevalence of foodborne diseases (FBD) remains a significant cause of morbidity and preventable mortality, specifically in low and middle-income countries (LMICs), despite advancements in technologies to prevent pathogens from entering the food chain (*1*). The burden of FBD for those living below the poverty line (i.e., less than $1.90 USD/day) is further amplified due to limited access to safe food, water, sanitation, health care, and education. Each of these factors contribute to the cycle of poverty, which ultimately perpetuates the cycle of foodborne diseases (*22*).

The level of economic development of a country is directly related to the level of economic burden of FBD. This food safety life cycle is divided into four phases: i) traditional, ii) transitioning, iii) modernizing, and iv) postmodern (*33*). Countries in the transitioning phase like lower-middle-income countries generally experience more severe food safety issues (i.e., higher exposure to food safety hazards) than countries in other phases due to several factors. Those factors include a rapid diet change that is centered in fresh vegetables, salads and animal source proteins; higher consumption of foods outside the home; food safety management systems devoted only to export markets; and limited government support for implementing effective food safety management systems (*33*).

Several risk factors have been identified as the primary contributors to food contamination frequently observed in LMICs, including unsafe water, poor hygiene practices, unsafe handling of food products, inadequate food storage practices, and regulatory compliance (*1*, *76*). The use of contaminated water to clean and process food can potentially lead to cross-contamination. Poor hygiene practices are mostly a result of the lack of knowledge and limited access to hygienic

amenities (i.e., clean bathroom with potable water). Unsafe handling of food products may be related to the lack of awareness on cross-contamination between food products, environmental surfaces, and food handlers. Poor food storage practices are a result of the absence of refrigeration systems, the lack of knowledge on temperature control, and the use of contaminated ice to store food products. Finally, inadequate regulatory compliance stems from lack of law enforcement, untrained personnel to conduct inspections, and corruption within the regulatory system (i.e., bribery of food safety inspectors). These factors exacerbate the food safety burden in LMICs and demonstrate both a lack of personal health protection measures and infrastructure (i.e., access to potable water, adequate sanitation systems, healthcare, education, and safe and nutritious foods) (*72*), and a lack of commitment to food safety as a national priority.

The burden of FBD may be further characterized into three major categories: i) public health, ii) economic growth, and iii) international trade. The effect of FBD on public health is usually measured by the number of people that get sick, its frequency, and severity (*33*). The global burden of morbidity and mortality caused by FBD was uncertain until 2006 when the Foodborne Disease Burden Epidemiology Reference Group (FERG) was created to investigate and report on the global burden of foodborne diseases (*76*). This report provides global estimates of foodborne illnesses and a list of its corresponding pathogens. One of the major findings published in this report was that the burden of foodborne diseases is at a similar level of importance as the three major infectious diseases (HIV/AIDS, malaria, and tuberculosis) (*76*).

Globally, a total of 31 hazards (e.g., bacteria, viruses, parasites, toxins, and chemicals) were identified, causing nearly 600 million foodborne illnesses, 420,000 deaths, and consequently resulting in the loss of 33 million healthy life years (*76*). An unequal share of the burden was observed in children under five years of age, who represented 40% of the population affected by

FBD (125,000 deaths) (*76*), yet accounted only for 9% of the global population (*33*). Thus, besides carrying the greatest burden, premature deaths of children resulted in more years of life lost (YLL) when compared to adult premature deaths.

The burden of FBD was classified into regions, as illustrated by figure 1.1. All countries were grouped into a total of six regions: Africa (AFR E & D), America (AMR A, B & D), Eastern Mediterranean (EMR B & D), Europe (EUR A, B & C), Southeast Asia (SEAR B & D) and the Western Pacific (WPR A & B) (76). Child and adult mortality rates were estimated and used to further classify countries into sub-regions, which were represented by different letters; stratum A: very low child and adult mortality, stratum B: low child mortality and very low adult mortality, stratum C: low child mortality and high adult mortality, stratum D: high child and adult mortality, and stratum E: high child mortality and very high adult mortality (76).

The highest foodborne disease burden was seen in subregions in Africa, South-East Asia, and Eastern Mediterranean (*76*). Sub-Saharan Africa, South and Southeast Asia represent 41% of the global population and yet account for more than 50% of all foodborne illnesses (*33*). Therefore, food safety measures present high potential for improving livelihoods and life expectancy, especially in regions where LMIC are predominant.

One of the primary agents responsible for causing foodborne diarrheal diseases was non-typhoidal *Salmonella.* It accounted for approximately 54% of deaths (230,000 people) and 19% (6.43 million) of Disability Adjusted Life Years (DALYs) (*76*) of the total global burden. Thus, food safety research and intervention strategies targeting this pathogen are largely needed and have great potential for positively improving the livelihood of LMIC communities.

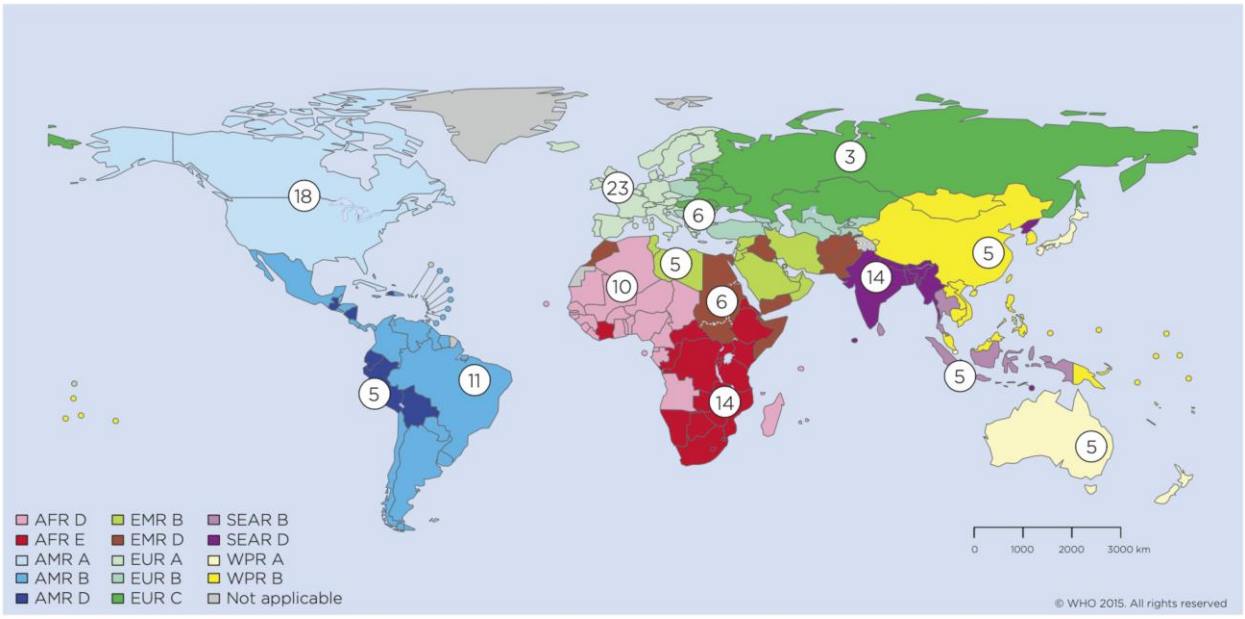

Figure 1.1. Geographical distribution of sub-regions according to the foodborne disease burden estimates (*76*).

The second major category affected by FBD burden is economic growth. The combined estimates of the DALYs for 2010 (from the burden of FBD report) (76) and the gross national income per capita estimates for 2016 (from the World Bank's World Development Indicators Database) (33), indicates that approximately US$ 95.2 billion per year in total productivity was lost due to FBD in LMICs (*33*). The greatest economic burden was observed in LMICs within the Asia and Sub-Saharan Africa regions, with a loss of US$ 63.1 billion and US$ 16.7 billion, respectively. Besides productivity losses, an estimated US$15 billion per year was spent on foodborne disease treatments (*33*). The combined economic burden of public health costs and productivity losses associated with unsafe food was estimated at approximately US$110 billion every year in LMICs (*33*).

The third major category affected by FBD burden is international trade. The exporting ability of a country is directly affected by its capacity to meet export food safety standards (*73*). LMICs commonly fail to meet international standards and regulations due to a fragmented system

that is often deficient in food safety policies and limited on foodborne pathogen data. In fact, studies have shown that poor monitoring and surveillance systems are recognized as the main gaps in LMIC, preventing the improvement and implementation of better food safety systems (*25*, *26*). As a result, targeted food safety interventions to prevent food contamination cannot be developed, and international food safety requirements are not met, thus limiting economic export growth (*38*).

Food products designated to feed export markets that do not meet international food safety standards end up being recycled into the already fragile domestic market. This differential level of care between export and domestic markets increases the risk of unsafe foods reaching local populations, extending the burden beyond the economic level and diminishing development outcomes. The combined effect of restricted exportation and recycled supply of unsafe foods domestically increases the likelihood of FBD incidence, morbidity and mortality, further halting economic growth.

1.2 Overview of food safety in Cambodia

Cambodia has a population of 16.2 million people, of which 76% lives in rural areas, 45% experiences food insecurity (*23*), and 13.5% lives under the poverty line (*67*). Food insecurity and economic distress, along with lacking infrastructure and policies, are two main drivers of diarrheal-related diseases originating from multiple contaminated sources including water and food. Every year, 10,000 children under five years of age die due to diarrheal diseases (*70*). About 29% of diarrheal-related diseases worldwide are caused by contaminated food (*28*). Between 2014 and 2019, Cambodia's Food Safety Bureau and the Department of Drug and Food (DDF) reported 134 foodborne outbreaks, resulting in 5,825 illnesses, 5,598 hospitalizations, and 81 deaths (*48*). The causes of these outbreaks were attributed to poor hygiene and sanitation practices, storage and temperature control, cross-contamination, and the use of unsafe water and raw materials (*48*).

Additionally, due to low-socioeconomic status, lack of availability of hospitals and diagnostic clinics in rural areas (where most of the population is located), the population rarely seeks medical attention, resulting in higher numbers of underreported foodborne disease cases (*17*, *48*). An indication of underreported cases was observed by the high percentage of illnesses resulting into hospitalizations (96%) as described above (48).

Other aggravating factors that contribute to the burden of FBD in Cambodia are the lack of established and enforced food safety policies. Since 1993, a joint effort by several Cambodian ministries (e.g., Ministry of Health, Ministry of Agriculture Forestry and Fisheries, Ministry of Commerce, and Ministry of Good Manufacturing Practice) resulted in 452 food safety standard drafts. To date, only 12 have been officially published (*62*). This lack of food safety regulations was recognized again in 2015 when the ministry of commerce in partnership with the U.N.'s Food and Agriculture Organization (FAO) drafted the first national Food Safety Law, which has remained under revision for the past five years (*49*). Furthermore, the limited food safety standards that currently exist are insufficiently and inadequately enforced, indicating that food safety challenges may persist in the Cambodian food chain. The main barriers to the success of food safety efforts in Cambodia are the lack of: i) trained food safety inspectors; ii) appropriate infrastructure, hygiene and sanitation practices by food facilities; and iii) surveillance system to monitor foodborne pathogens and outbreaks in the country (*48*, *49*). Food safety is the basic building block towards a healthier society. Unless the food safety agenda is prioritized and regulations are properly enforced, food safety issues will remain an imminent public health concern.

1.2.1 Informal market systems and environmental contamination

Informal markets are common in Cambodia and they play an important role in the country's economy, culture, and lifestyle. Typical informal markets are usually found in open-air environments, with individual vendors selling a variety of food products, often in the absence of basic food safety infrastructure, practices, and/or oversight (Figure 1.2) (*26*). Frequently, vendors sell a variety of food products (i.e., fresh vegetables, fruits, seafood, and animal products; live or processed poultry, beef, goat, and pork), and household items side-by-side in the same stand.

Due to the high vegetable demand in Cambodia, approximately 70% of the vegetables sold at these markets are imported from neighboring countries such as Vietnam and Thailand (63). The vegetable value-chain is composed by several stakeholders including; farmers, collectors, distributors, vendors and consumers. The vegetables that are not imported are grown by local farmers. Oftentimes the vegetables grown locally can take two main routes i) vegetables are sold to collectors, or ii) vegetables are sold to distributors. The collectors can act as middlemen between farmers, distributors and vendors. The collectors purchase vegetables from both local farmers, as well as from neighboring countries. Finally, the distributors may purchase the vegetables directly from farmers and/or collectors, and sell them in large quantities to the vendors, which are located at the informal markets (63).

At the informal markets, most of the food items lack proper refrigeration and are directly exposed to the environment (*15*, *55*). Vendors are often allocated into inside and outside locations, where the inside location is commonly characterized by the presence of concrete walls, metal or wood fencing sustaining a roof, whereas outside location is characterized by open air tents (Figure 1.3).

The lack of hygiene and sanitation practices, food safety regulations, and infrastructure present a risk of contamination to the food products sold at these markets. Further, the absence of potable water, unsanitary handling, storage, and preparation of foods may contribute to the cross-contamination process involving infectious agents such as *Salmonella enterica* (henceforth referred to as *Salmonella*) (*39, 57, 58, 64*). In fact, existing literature shows that the highest FBD burden in LMICs is attributed to biological hazards, where the main source of contamination comes from the consumption of fresh, perishable foods sold in informal markets (*26*).

Figure 1.2. Typical informal market in Cambodia. Picture credit: Dr. Jessie Vipham.

Figure 1.3. Typical informal market environment setting with vendors displayed within outside and inside areas of the market. Picture credit: Dr. Jessie Vipham.

Overall, informal markets lack appropriate sanitation practices and damage to the physical infrastructure (i.e., cracks in the walls and on the floors is a common sight). These characteristics can serve as potential reservoirs for bacterial pathogens, such as *Salmonella* (*5*, *59*). This source of environmental contamination is commonly observed among informal markets in Cambodia (*41*, *51*, *55*).

Environmental contamination can further originate from food contact surfaces (FCS) and non-food contact surfaces (NFCS). FCS are characterized as any surface that is directly in contact with food items (e.g., baskets, mats), whereas NFCS are defined as any surface that is not directly in contact with food, but in the proximate vicinity of food items (e.g., floor, wall). Nidaullah and colleagues (*55*) investigated the prevalence of *Salmonella* on FCS and NFCS in informal poultry markets in Malaysia, where they found a prevalence of 100% and 89%, respectively (*55*).

Other studies on meat samples have identified *Salmonella* in informal markets in Cambodia, with contamination varying from 42.8% to 88.2% in poultry, and 18.9% to 71.1% in pork (*41*, *69*). Other studies have reported *Salmonella* in various food samples, environmental sites, and food surfaces, suggesting potential cross-contamination between raw meat products, food contact surfaces, and the market environment (*15*), indicating that the market environment may be consistently contaminated with this pathogen. However, a cross-contamination hypothesis needs to be further evaluated since there is a gap in the literature on prevalence and serotype characterization of *Salmonella*-contaminated vegetable foods.

Salmonella has the potential to cause disease; however, specific serotypes have been proven to be more virulent than others (*34*). The most common serotypes identified in meat foods sold in informal markets were *S.* Agona (*69*), *S.* Rissen (*69*), *S.* Anatum, *S.* Typhimurium, and *S.*

Corvallis (*41*). Knowledge about specific serotypes can also be used to track foodborne illnesses occurrence with the contamination source.

Contamination by *S.* Corvallis has been identified in food environments, as well as in clinical samples from patients in Southeast Asia (*27*, *47*, *82*). A report published by the Institute of Public Health in Japan described a case of travel-related bacteremia caused by *S.* Corvallis in an immunocompetent adult that had traveled to Vietnam and Cambodia prior to the appearance of symptoms (*53*). This case indicates that *S.* Corvallis has recurrently established clinical infections in Southeast Asia and could be the common cause of both reported food contamination and human infections.

The availability of quality data on the prevalence of *Salmonella* and other bacterial contaminants in these markets is crucial for active surveillance, implementation of suitable intervention strategies, and prevention of future foodborne illness cases. Quality data is characterized by precise detection methods and serotype-specific characterization. Such data can be generated by the implementation of Whole Genome Sequencing (WGS). This technology has been used to surveil and monitor foodborne pathogens and outbreaks in developed countries such as the United States, Australia and the United Kingdom (*11*, *13*, *65*).

In these countries, the use of WGS has improved the accuracy of results (*4*) and reduced the turnaround time by 50% (*13*). Additionally, WGS has made sample processing a simpler task, minimizing laboratory errors and employee exposure to pathogens. The benefits of the use of WGS further include i) the creation of a robust national surveillance system, ii) the identification of emerging pathogens, iii) the identification of virulence factors and antibiotic resistance patterns, and iv) the generation of data to support clinical treatment options (*13*). Therefore, the use of

WGS technology should be considered as an investment to public health, not only in developed countries, but in all nations around the globe.

1.3 The use of next generation sequencing technologies and their benefits to public health

Next-generation sequencing techniques, such as pulsed-field gel electrophoresis (PFGE), Multiple Locus Variable Number Tandem Repeat Analysis (MLVA), and WGS can be used to understand the ecology of foodborne pathogens due to high throughput results. This not only allows for comprehensive analysis of the genetic content of community microbiota, but also enables the discovery of their interactions with the environment (*6*). The ability to fully sequence a microbiome allows researchers to explore and better understand the taxonomic diversity of various microorganisms present in a certain microbiome, including those microorganisms that are non-culturable (*7*). These approaches provide a complete understanding of complex interactions that happen between the microbiome and its surrounding environment. Examples of such interactions were described by a study conducted by Tan et al. (*66*), which evaluated the diversity of a complex microbiota and the built environment of apple processing plants and their association with the prevalence of *Listeria monocytogenes*. Researchers found that lower microbiota diversity may result in less competition for nutrients among microorganisms and the production of fewer inhibitory metabolites (e.g., bacteriocin-like compounds). These factors could potentially contribute to the growth and persistence of *Listeria monocytogenes* in the apple processing environment. Additionally, the high abundance of *Pseudomonas* in the environment may shelter *Listeria monocytogenes* through biofilm formation, conferring protection against sanitizers (*66*).

Another study revealed that *Wausteria paucula* and *Enterobacter asburiae* interacted differently with *Escherichia coli* O157:H7 on lettuce leaves and roots. *E. asburiae* decreased *E.*

coli O157:H7 survival while *W. paucula* enhanced *E. coli* O157:H7 survival. According to research findings, *E. asburiae* utilizes the same sources of carbon and nitrogen as *E. coli* O157:H7, which adversely affects *E. coli* O157:H7 survival. Conversely, *W. paucula* only utilizes two substrates metabolized by *E. coli* O157:H7, and thus leaves more nutrients available for *E. coli* O157:H7 to grow (*16*). These determinations were made possible by next-generation sequencing techniques, specifically WGS.

Whole genome sequencing is revolutionizing the way scientists view and conceptualize microbiology (*50*). With WGS, it is possible to determine the complete DNA sequence of a bacterial isolate. The technology allows for fast sequencing and data sharing. For many years, the Centers for Disease Control and Prevention (CDC) relied on the gold standard (i.e., Pulsed-field Gel Electrophoresis [PFGE]) to identify and solve outbreaks. However, recently, CDC replaced PFGE for WGS (*11*) to better track FBD throughout the United States. WGS provides the whole genome of a bacterium instead of only partial genome information provided by PFGE and MLVA. In WGS, microbial DNA is extracted and purified before PCR amplification. Numerous steps of library preparation are done before the sheared DNA is loaded into the sequencer. The combination of nucleotides that compose each DNA fragment is read through the sequencer, and an output (i.e., DNA reads) is generated. All of the DNA fragments are then assembled into one complete genome reading (*11*). Different software (e.g., GalaxyTrakr) and bioinformatic tools can be used to perform the assembly. Finally, the sequence is then compared to known references, and conclusions are made based on how identical sequences are to the references in a database (e.g., NCBI database) (*50*).

Further, WGS has given health agencies a greater power of precision and speed of strain characterization to differentiate genomic potential between organisms. Through WGS, the ability

to detect, resolve and even prevent foodborne outbreaks has improved tremendously (*7*). An example of the use of WGS to solve an outbreak was described in 2017. The outbreak investigation involved *E. coli* O157:H7 and leafy greens in fifteen U.S. states. The CDC and FDA shared efforts to elucidate the outbreak that caused 210 infections and five deaths (*24*). The use of WGS in this outbreak revealed the benefits of this technology. Authorities were able to prove that isolates from infected people were genetically closely related, meaning that ill people in this outbreak were more likely to have a common source of infection. At the same time that the U.S. outbreak was active, the Canadian government reported an outbreak with romaine lettuce. The rapid strain identification and information sharing process between Canadian and U.S. health authorities revealed that U.S. states and Canadian provinces shared the same DNA sequence of *E. coli* O157:H7. The CDC and FDA were able to trace back the source of the outbreak to romaine lettuce from specific growing regions in the U.S. (*12*). This demonstrated that WGS was able to aid in solving multi-national foodborne outbreaks by allowing accurate comparison of genetic strains between various outbreaks.

Additionally, with the use of WGS, authorities were able to compare the outbreak strain to strains collected from innumerous environmental sources. Interestingly, the same outbreak strain of *E. coli* O157:H7 was confirmed in water samples collected from a canal in the growing region, suggesting a common source of contamination. Further, data generated by WGS was also used to identify antibiotic resistance genes from isolates collected from human specimens affected by the outbreak. In this case, these findings did not change the treatment course since antibiotics are not recommended for patients with *E. coli* O157 infections. On the other hand, results of antibiotic resistant strains of *Salmonella* Typhi may be important in selecting adequate treatment options for severe typhoid fever (*12, 24*). Overall, WGS significantly helped investigations by accurately

identifying other routes of outbreak contamination, as well as guiding physicians in selecting the best course of treatment for infected patients.

The use of WGS has proven to be a rapid and precise tool in the identification of pathogens, foodborne outbreaks, and human infections. A study by Hoffmann et al. (2015) used a novel combination of WGS and geographic metadata to trace the origins of *S.* Bareilly, which was previously linked to a wide-spread scraped tuna outbreak in the United States. Bacterial isolates from patients, food, feed, and environmental sources were processed using WGS. Pathogen genomes were compared, and single-nucleotide polymorphism (SNP; extracted from WGS data) matrixes were built. SNPs represent a specific genetic variation and are commonly used to differentiate the genetic relatedness and evolutionary origin in a bacterial population (*19*). For this reason, SNP data is extremely valuable in the investigation process of epidemiological outbreaks. Hoffmann et al. (2015) were able to conclude that *S.* Bareilly strains directly linked to the outbreak originated in a facility in India. These conclusions were possible through the integration of genomic analysis (WGS) and geographic mapping, revealing the complex transmission network involved in an outbreak (*31*).

Next-generation sequencing methods, specifically WGS, can be used to understand the ecology of foodborne pathogens and provide insights on the interactions between complex microbial communities and environments. In fact, WGS could be extremely valuable in the investigation process of cross-contamination between food products, food surfaces, and environmental sites in informal markets in Cambodia. The use of WGS would i) advance source-attribution studies, ii) enable the development of proper interventions in the market environment, and iii) enlighten the presence and dissemination of drug resistance among pathogens. Ultimately, the benefits of WGS would create positive public health outcomes for the population of Cambodia.

1.4 Overview of antimicrobial resistance in Southeast Asia

Antimicrobial resistance (AMR), and more specifically, antibiotic resistance (ABR) is commonly observed in bacteria. The resistance can be developed and rapidly spread by the misuse of antibiotics in humans and animals (Figure 1.4) (*46*). The term antimicrobial resistance describes any drug resistance presented by parasites, viruses, bacteria and fungi, while antibiotic resistance is specifically used for any drug resistance presented by bacteria only (*75*). Antimicrobial resistance has become a serious threat to global public health. The indiscriminate use of antibiotics in food production (i.e., food animals) contributes to the development and spread of ABR among different pathogens (*45*). The spread of ABR bacteria threatens the ability to effectively treat bacterial infections, potentially leading to an extended illness period, disability, or death (*78*).

The lack of success in introducing new chemical classes of antibiotics for the past 30 years, raises a global concern about the increasing ABR spread and the limited options of effective antibiotics for treating infections (*74*). The failure to introduce new antibiotics combined with the extensive overuse of existing antibiotics are the reasons why the WHO has declared AMR as a high global priority (*78*). In order to address this issue, in 2015, the WHO developed a global action plan – One Health – that includes five main strategies: 1) improve awareness of antimicrobial resistance, 2) strengthen surveillance and research programs, 3) reduce the incidence of infections, 4) optimize the use of antimicrobial drugs, and 5) ensure sustainable investment in countering antimicrobial resistance (*77*).

The WHO Antimicrobial Resistance Global Report and Surveillance has highlighted high rates of antibiotic resistance in bacteria that are commonly associated with urinary tract infections and pneumonia in all WHO regions. Resistance to fluroquinolones was observed among non-typhoidal *Salmonella* and *Shigella* species (*75*). Fluroquinolones are particularly important as they

are often used to treat salmonellosis in humans and are classified as critically important for human health (*75*). Additionally, the report emphasizes the significant gaps in monitoring and surveillance, particularly from regions with major public health issues (e.g., Southeast Asia and African regions).

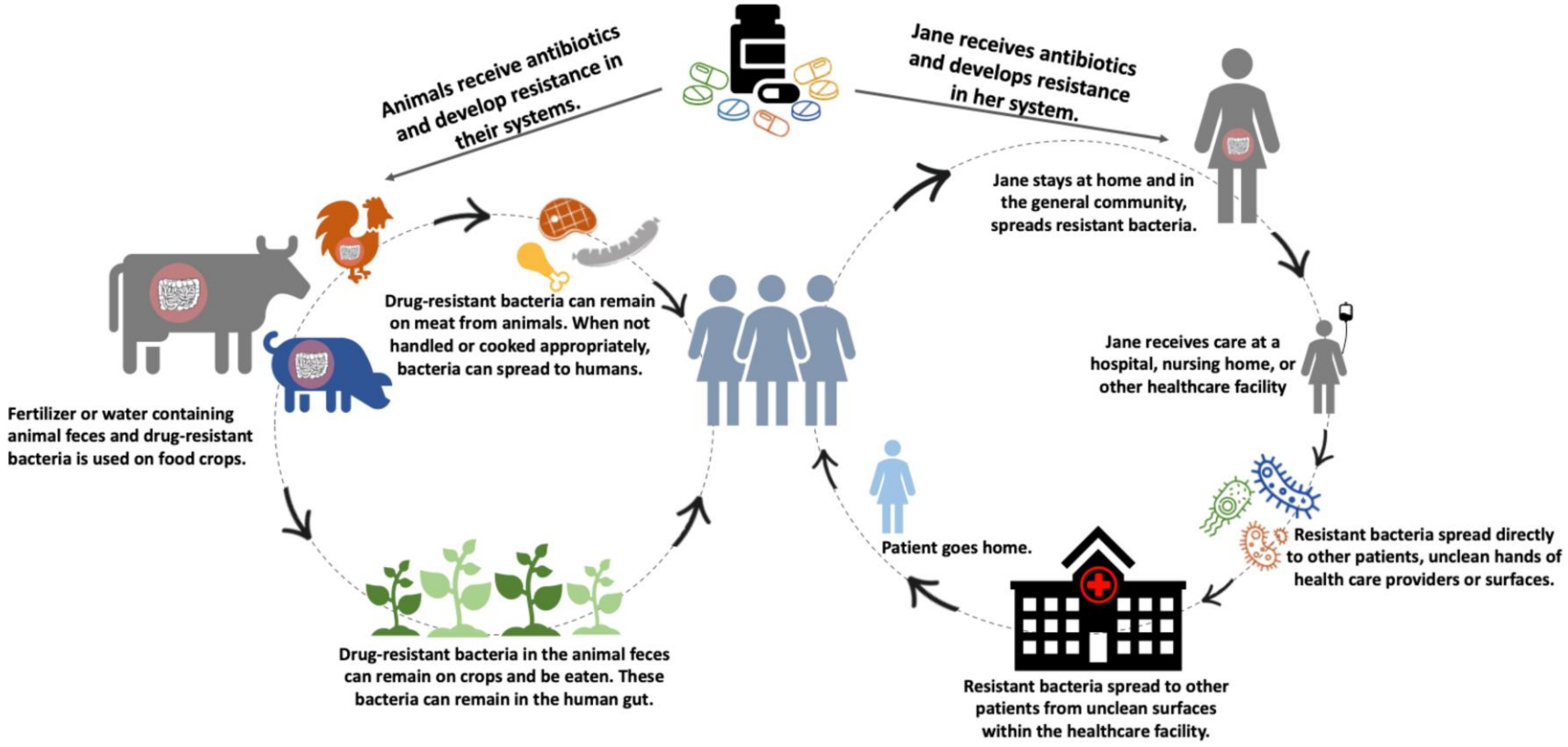

Figure 1.4. Spread of antibiotic-resistant bacteria. Adapted from (*46*).

The economic burden of AMR has been estimated in the United States and the findings are alarming. The U.S. health system alone has an average cost of US $27.5 billion dollars per year, followed by approximately eight million days in the hospital (*75*). Developed countries, such as the U.S., have established a strong AMR monitoring and surveillance system, and therefore the economic burden of AMR can be estimated. On the contrary, LMIC lack such systems and have limited ability to detect and track emerging AMR pathogens.

In Southeast Asia, the burden of AMR is less understood and epidemiological data is limited. For example, for non-typhoidal *Salmonella*, the resistance to fluroquinolones ranged from 0.2 to 4% (*75*). This low resistance observed in Southeast Asia may be attributed to the limited amount of data and to divergent opinions about *Salmonella* serotypes that are included in the statistics (*75*). Higher rates of resistance (5-10%) among *Salmonella* serotypes have been reported in animals, informal markets, and on slaughterhouse equipment in Southeast Asia (*41*, *51*, *61*). The increased use of unregulated antibiotics in food animals is known to accelerate the antibiotic resistance selection process among bacteria (*8*). This practice contributes to the spread of ABR to humans through contaminated food products (*77*). In fact, the inappropriate, unregulated, and excessive use of antibiotics in agriculture and livestock production systems has been previously reported in Cambodia (*9*).

Broad spectrum beta-lactam resistance of non-typhoidal *Salmonella* strains has been reported in several countries in Southeast Asia (*42*, *43*). More specifically, multidrug resistant (MDR) *S.* Krefeld was involved in a large outbreak in Thailand. The MDR strains presented resistance to five classes of antibiotics (i.e., aminoglycoside, sulfonamide, tetracycline, β-lactam, and phenicol) (35). In another study, 100% of *S.* Krefeld isolates collected from human patients

presented resistance to five different antibiotics (i.e., amoxicillin, chloramphenicol, tetracycline, streptomycin and sulfamethoxazole) (*3*).

Multidrug resistance was also reported among *S.* Derby isolates in Cambodia (*51*), China (80), and Thailand (*61*, *69*). Historically, *S.* Derby is mainly recovered from pork, potentially indicating that the uncontrolled use of antibiotics in the pig production chain plays an important role in the antimicrobial resistance selection pressure (*80*). Further, approximately 30% of poultry and pork samples collected from informal markets and retail shops in Cambodia revealed contamination with MDR *Salmonella* strains (*41*, *51*). Similarly, Nadimpalli and colleagues (2019) identified MDR *S.* Rissen from pork, chicken and fish samples collected from informal markets in Cambodia. The isolates revealed antibiotic resistance profiles to six classes (e.g., aminoglycosides, phenicols, quinolone, tetracyclines, sulfonamides, and trimethoprims) (*51*).

Another study by Lay et al. (2011) collected poultry samples from retail markets in Cambodia. *S.* Anatum isolates presented high rates (23.1-53.8%) of resistance to quinolone, sulfonamide, and tetracycline (*41*). Trongjit et al. (2017) reported resistance profiles to aminoglycoside and trimethoprim among *S.* Anatum isolates collected from pig samples at the Thailand-Cambodia border (*69*). Additionally, studies from Cambodia and Thailand have shown that *S.* Corvallis isolated from various sample types (i.e., informal markets, food products and patients) had similar AMR profiles (51). The resistance profiles observed over multiple studies in Southeast Asia demonstrate that several *Salmonella* serotypes (i.e., *S.* Krefeld, *S.* Derby, *S.* Rissen, *S.* Corvallis, and *S.* Anatum) have developed MDR. The presence of genes that are commonly associated with resistance to third generation cephalosporins is a growing problem that reduces bacteremia treatment options and jeopardizes public health. Moreover, the limited antibiotic

resources in low and middle-income countries (e.g., Cambodia) pose a big challenge to treat infections when pathogens are resistant to several classes of antibiotics.

Surveillance networks are limited or non-existent in Southeast Asia (*81*). The lack of laboratory infrastructure, trained personnel, and standardized and consistent data collection reveals the immense gap faced by Southeast Asian countries in determining the burden of AMR. Therefore, impacting the ability of stakeholders to implement interventions to reduce AMR spread. Reports from Cambodia revealed that physicians frequently prescribe inappropriate antibiotics due to the lack of microbiological confirmation or a defined diagnostic (*56*). To address this problem, the Association of Southeast Asian Nations (ASEAN) has included AMR as a priority in their five-year work program (2016–2020) (*79*). Member states have developed guidelines and strategies to control AMR spread, while the health ministry of each ASEAN member is responsible for endorsing and monitoring the progress of AMR control (*79*).

The presence of ABR bacteria in the food chain and in food-producing animals is a complex global challenge. Integrated surveillance systems and data sharing are major elements in combating antibiotic resistance in foodborne bacteria and its impact on animal and human health. An integrated surveillance system would enable data comparison from food-producing animals, food products and humans. This type of system may assist in understanding ABR development among pathogens, humans and animals, guiding the implementation of policies to reduce the misuse of antibiotics in humans, agriculture and animal food systems.

1.5 Antimicrobial resistance and its risks to public health

The use of antibiotics has transformed modern medicine by reducing the risks of infection during surgeries, transplants and chemotherapy. Further, advantages include the reduction in mortality and morbidity from infectious diseases (*79*). The extensive expansion of antibiotic use

in healthcare systems is evident. After accounting for the global population growth between 2000 and 2015, the use of antibiotics has increased by 35%, ranging from 22 billion daily doses in 2000 to 35 billion doses in 2015 (*40*). Additionally, antibiotics are commonly used in food-producing animals to prevent and treat infections, while also used as growth promoters. Unfortunately, the abusive and widespread use of antibiotics has accelerated the selection pressure of genes that encode ABR mechanisms, conferring resistance to different antibiotics (*79*). Evidence shows that antimicrobial drugs commonly used as treatment or prophylaxis in animals contribute to the development and spread of ABR among foodborne pathogens, in particular, non-typhoidal *Salmonella*, pathogenic *E. coli*, and *Campylobacter* spp. (*44*).

Antimicrobial resistance is considered a serious threat to public health (*75*). It decreases the effectiveness of drug therapy and contributes to the severity of disease, increasing hospitalizations, cost of treatment, and incidence of infections (*44*). In fact, every year in the United States approximately 2.8 million people are infected with ABR bacteria, resulting in more than 35,000 deaths (*10*). Antibiotic resistant bacteria have been reported all around the world; however, their prevalence varies among regions, countries and continents. Many factors affect ABR, including diversity and levels of antibiotic consumption, access to healthcare, adequate sanitation, and appropriate regulations (*79*). Additionally, the increase in travel and international food trade are important contributors in the spread of MDR pathogens around the globe (*71*, *83*). In fact, reports have shown that extended spectrum beta-lactamase (ESBL) producing Enterobacteriaceae (e.g., *Salmonella* and *Escherichia coli*) have increased the proportion of ABR pathogens causing traveler's diarrhea (*18*, *20*, *30*, *68*).

Southeast Asia is considered to have the highest risk of emergence and spread of ABR bacteria (*14*, *37*). In this region, antibiotic resistance is often seen as an invisible threat since the

risks of acquiring ABR bacteria are not readily recognized by individuals. Moreover, the spread of ABR bacteria is worsened by environmental factors (i.e., such as poor sanitation and waste management and high levels of air pollution). For instance, respiratory illnesses attributed to these environmental factors are commonly mistaken as bacterial infections and are treated with antibiotics (*2*, *40*). For this reason, the perceived benefit of recovering from an illness in Southeast Asian countries are often the result of antibiotic misuse. Further, the use of antibiotics without the prescription of a physician is a common practice reported in several countries in Southeast Asia, aggravating the selection pressure of ABR strains (*54*). These practices highlight the lack of awareness and poor law enforcement for antibiotic stewardship in LMICs (*54*).

The use of cephalosporins and fluroquinolones in food animals has been linked to beta-lactam and quinolone resistance in non-typhoidal *Salmonella*, respectively (*21*, *60*). Since animals are important carriers of antibiotic resistant *Salmonella* (*45*), fecal shedding becomes a significant source of contamination in meat and poultry products, as well as in vegetables through fecal contamination of the environment (*44*). More importantly, studies have demonstrated that antibiotic resistance in more virulent *Salmonella* serotypes (e.g., Newport, Enteritidis, Heidelberg, Typhimurium, and I 4,[5],12:i:) has been associated with increased risk of infection and complications in humans (*29*, *52*).

Surveillance system networks are critical in combating the spread of ABR and improving public health outcomes. This can be exemplified by the strong surveillance system adopted by the CDC. The 2019 Antibiotic Resistance Threats report revealed that nearly twice as many annual deaths from ABR infections were observed compared to the previous report in 2013 – when data sources were not available. Since then, continued vigilance through the surveillance system revealed positive outcomes, where deaths by ABR infections were reduced by 18% overall and

30% in hospitals (*10*). This example demonstrates the importance of having a surveillance system to monitor and predict ABR emergence and spread. Ultimately, the presence of appropriate surveillance systems will enable countries to i) create a reliable database, ii) detect emerging and spreading ABR pathogens, iii) collect information on the burden of ABR, iv) support healthcare with appropriate treatment guidelines, v) estimate the costs associated with ABR, vi) inform priorities to develop a research agenda, vii) guide stakeholders in policy development, and viii) create national and global strategies to combat and prevent ABR.

A majority of the current literature reports the burden of foodborne disease and its associated pathogens over large heterogeneous regions (e.g., Southeast Asian, Western Pacific, African region, etc.). These regionally-summarized data are inherently inaccurate for any given country and either underestimate or overestimate a response at the country level. In the case of Cambodia, the reality is greatly underestimated when the few studies that have been locally reported are compared to regional estimates. One reason for the discrepancy is that regional estimates commonly do not account for country-specific factors. For example, Cambodia and China are grouped in the same region, even though these two countries have large differences in socio-economic levels and infrastructure capabilities. Extrapolation from regional estimates may lead to an inaccurate depiction of the public health status of the country. For this reason, in-country studies should be conducted in order to better understand the burden of FBD in LMICs, specifically Cambodia.

Literature on food safety topics in Cambodia reveals immense gaps and limitations on the availability of data, especially from informal markets. It intensifies the need for region-specific research studies focusing on pathogen identification, detection, and monitoring. More specifically, in-country studies investigating the ecology (prevalence, abundance, antibiotic resistance,

virulence factors, and genomic potential) of *Salmonella* in informal markets are necessary. These data will be essential for the development of effective surveillance and implementation of suitable intervention strategies at the domestic level. Lastly, the use of next-generation technologies (e.g., whole genome sequencing) will be valuable in understanding antibiotic resistance profiles, as well as the dynamics of *Salmonella* diversity in informal markets in Cambodia, supporting in-country and global epidemiological investigations of outbreaks.

1.6 References

1. Akhtar, S., M. R. Sarker, and A. Hossain. 2014. Microbiological food safety: a dilemma of developing societies. *Crit. Rev. Microbiol.* 40:348–359.

2. Alirol, E., L. Getaz, B. Stoll, F. Chappuis, and L. Loutan. 2011. Urbanisation and infectious diseases in a globalised world. *Lancet Infect. Dis.* 11:131–141.

3. Angkititrakul, S., C. Chomvarin, T. Chaita, K. Kanistanon, and S. Waethewutajarn. 2005. Epidemiology of antimicrobial resistance in *Salmonella* isolated from pork, chicken meat and humans in Thailand. *Southeast Asian J Trop Med Public Health* 36:6.

4. Ashton, P. M., S. Nair, T. M. Peters, J. A. Bale, D. G. Powell, A. Painset, R. Tewolde, U. Schaefer, C. Jenkins, T. J. Dallman, E. M. de Pinna, K. A. Grant. *Salmonella* Whole Genome Sequencing Implementation Group. 2016. Identification of *Salmonella* for public health surveillance using whole genome sequencing. *PeerJ* 4:e1752.

5. Barker, J., and S. F. Bloomfield. 2000. Survival of *Salmonella* in bathrooms and toilets in domestic homes following salmonellosis. *J. Appl. Microbiol.* 89:137–144.

6. Boughner, L. A., and P. Singh. 2016. Microbial Ecology: Where are we now? *Postdoc J. J. Postdr. Res. Postdr. Aff.* 4:3–17.

7. Brown, E., U. Dessai, S. McGarry, and P. Gerner-Smidt. 2019. Use of whole-genome sequencing for food safety and public health in the United States. *Foodborne Pathog. Dis.* 16:441–450.

8. Caniça, M., V. Manageiro, H. Abriouel, J. Moran-Gilad, and C. M. A. P. Franz. 2019. Antibiotic resistance in foodborne bacteria. *Trends Food Sci. Technol.* 84:41–44.

9. Caron, Y., R. Chheang, N. Puthea, M. Soda, S. Boyer, A. Tarantola, and A. Kerléguer. 2018. Beta-lactam resistance among Enterobacteriaceae in Cambodia: The four-year itch. *Int. J. Infect. Dis.* 66:74–79.

10. Centers for Disease Control and Prevention. 2019. Antibiotic resistance threats in the United States, 2019. Centers for Disease Control and Prevention (U.S.). Available at https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html. Accessed 19 February 2020.

11. Centers for Disease Control and Prevention. 2019. Foodborne Diseases Active Surveillance Network (FoodNet). Available at https://www.cdc.gov/foodnet/index.html. Accessed 19 February 2020.

12. Centers for Disease Control and Prevention. 2018. Multistate outbreak of *E. coli* O157:H7 Infections linked to romaine lettuce. Available at https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html. Accessed 11 September 2019.

13. Chattaway, M. A., T. J. Dallman, L. Larkin, S. Nair, J. McCormick, A. Mikhail, H. Hartman, G. Godbole, D. Powell, M. Day, R. Smith, and K. Grant. 2019. The

transformation of reference microbiology methods and surveillance for *Salmonella* with the use of whole genome sequencing in England and Wales. *Front. Public Health* 7:317.

14. Chereau, F., L. Opatowski, M. Tourdjman, and S. Vong. 2017. Risk assessment for antibiotic resistance in South East Asia. *BMJ* j3393.

15. Chuah, L.-O., A.-K. Shamila Syuhada, I. Mohamad Suhaimi, T. Farah Hanim, and G. Rusul. 2018. Genetic relatedness, antimicrobial resistance and biofilm formation of *Salmonella* isolated from naturally contaminated poultry and their processing environment in northern Malaysia. *Food Res. Int.* 105:743–751.

16. Critzer, F. J., and M. P. Doyle. 2010. Microbial ecology of foodborne pathogens associated with produce. *Curr. Opin. Biotechnol.* 21:125–130.

17. Culas, R. J., and K. Tek. 2016. Food security in Cambodia: trends and policy objectives. *Int. J. Dev. Issues* 15:306–327.

18. Doi, Y., A. Iovleva, and R. A. Bonomo. 2017. The ecology of extended-spectrum β-lactamases (ESBLs) in the developed world. *J. Travel Med.* 24:S44–S51.

19. Dong, H.-J., S. Cho, D. Boxrud, S. Rankin, F. Downe, J. Lovchik, J. Gibson, M. Erdman, and A. M. Saeed. 2017. Single-nucleotide polymorphism typing analysis for molecular subtyping of *Salmonella* Tennessee isolates associated with the 2007 nationwide peanut butter outbreak in the United States. *Gut Pathog.* 9:25.

20. DuPont, H. L., and R. Steffen. 2017. Use of antimicrobial agents for treatment and prevention of travellers' diarrhoea in the face of enhanced risk of transient fecal carriage

of multi-drug resistant enterobacteriaceae: setting the stage for consensus recommendations. *J. Travel Med.* 24:S57–S62.

21. Eguale, T., J. Birungi, D. Asrat, M. N. Njahira, J. Njuguna, W. A. Gebreyes, J. S. Gunn, A. Djikeng, and E. Engidawork. 2017. Genetic markers associated with resistance to beta-lactam and quinolone antimicrobials in non-typhoidal *Salmonella* isolates from humans and animals in central Ethiopia. *Antimicrob. Resist. Infect. Control* 6:13.

22. Faour-Klingbeil, D., and E. C. D. Todd. 2018. A review on the rising prevalence of international standards: threats or opportunities for the agri-food produce sector in developing countries, with a focus on examples from the Mena region. *Foods* 7.

23. Food and Agriculture Organization of the United Nations. 2019. World food and agriculture - statistical pocketbook 2019. Food & Agriculture Org.

24. Food and Drug Administration. 2018. FDA ends investigation of E. coli O157:H7 outbreak likely linked to leafy greens. *FDA*. Available at http://www.fda.gov/food/outbreaks-foodborne-illness/fda-ends-investigation-e-coli-o157h7-outbreak-likely-linked-leafy-greens. Accessed 11 September 2019.

25. Grace, D. 2017. Food safety in developing countries: research gaps and opportunities. Nairobi, Kenya: International Livestock Research Institute.

26. Grace, D. 2015. Food safety in low and middle income countries. *Int. J. Environ. Res. Public. Health* 12:10490–10507.

27. Hadziabdic, S., M. Borowiak, A. Bloch, B. Malorny, I. Szabo, B. Guerra, A. Kaesbohrer, and J. Fischer. 2018. Complete genome sequence of an avian native NDM-1-producing *Salmonella enterica* subsp. *enterica* serovar corvallis strain. *Genome Announc.* 6:e00593-18, /ga/6/26/e00593-18.atom.

28. Havelaar, A. H., M. D. Kirk, P. R. Torgerson, H. J. Gibb, T. Hald, R. J. Lake, N. Praet, D. C. Bellinger, N. R. de Silva, N. Gargouri, N. Speybroeck, A. Cawthorne, C. Mathers, C. Stein, F. J. Angulo, B. Devleesschauwer, and on behalf of World Health Organization Foodborne Disease Burden Epidemiology Reference Group. 2015. World health organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLOS Med.* 12:e1001923.

29. Helms, M., J. Simonsen, and K. Mølbak. 2004. Quinolone resistance is associated with increased risk of invasive illness or death during infection with *Salmonella* serotype Typhimurium. *J. Infect. Dis.* 190:1652–1654.

30. Hitch, G., and N. Fleming. 2018. Antibiotic resistance in travellers' diarrhoeal disease, an external perspective. *J. Travel Med.* 25:S27–S37.

31. Hoffmann, M., Y. Luo, S. R. Monday, N. Gonzalez-Escalona, A. R. Ottesen, T. Muruvanda, C. Wang, G. Kastanis, C. Keys, D. Janies, I. F. Senturk, U. V. Catalyurek, H. Wang, T. S. Hammack, W. J. Wolfgang, D. Schoonmaker-Bopp, A. Chu, R. Myers, J. Haendiges, P. S. Evans, J. Meng, E. A. Strain, M. W. Allard, and E. W. Brown. 2016. Tracing origins of the *Salmonella* Bareilly strain causing a food-borne outbreak in the United States. *J. Infect. Dis.* 213:502–508.

33. Jaffee, S., S. Henson, L. Unnevehr, D. Grace, and E. Cassou. 2019. The safe food imperative: accelerating progress in low- and middle-income countries. World Bank Group, Washington, DC.

34. Jajere, S. M. 2019. A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Vet. World* 12:504–521.

35. Jayanetra, P., M. Vorachit, A. Pilantanapak, W. Panbangred, A. Bangtragulnonth, and D. Chiewsilp. 1990. *Salmonella* krefeld in Thailand: II. Molecular biology of drug resistance. *Southeast Asian J. Trop. Med. Public Health* 21:361–366.

36. Jayanetra, P., M. Vorachit, A. Pilantanapak, W. Panbangred, A. Bangtragulnonth, and R. Pan-urai. 1990. *Salmonella* krefeld in Thailand: I. Epidemiology, infection and drug resistance. *Southeast Asian J. Trop. Med. Public Health* 21:354–360.

37. Kang, C.-I., and J.-H. Song. 2013. Antimicrobial resistance in Asia: current epidemiology and clinical implications. *Infect. Chemother*. 45:22.

38. Keiichiro, H., T. Otsuki, and J. S. Wilson. 2015. Food safety standards and international trade: the impact on developing countries' export performance, p. 151–166. *In* A. Hammoudi, C. Grazia, Y. Surry, and J.-B. Traversac (eds.), Food Safety, Market Organization, Trade and Development. Springer International Publishing, Cham.

39. Keithellakpam, L. 2018. Study on hygienic practices of street food vendors in Allahabad city and determination of critical control points for safe street foods. doi:https:10.13140/RG.2.2.22360.14080

40. Klein, E. Y., T. P. Van Boeckel, E. M. Martinez, S. Pant, S. Gandra, S. A. Levin, H. Goossens, and R. Laxminarayan. 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci.* 115:E3463–E3470.

41. Lay, K. S., Y. Vuthy, P. Song, K. Phol, and J. L. Sarthou. 2011. Prevalence, numbers and antimicrobial susceptibilities of *Salmonella* serovars and *Campylobacter* spp. in retail Poultry in Phnom Penh, Cambodia. *J. Vet. Med. Sci.* 73:325–329.

42. Lee, H.-Y., L.-H. Su, M.-H. Tsai, S.-W. Kim, H.-H. Chang, S.-I. Jung, K.-H. Park, J. Perera, C. Carlos, B. H. Tan, G. Kumarasinghe, T. So, A. Chongthaleong, P.-R. Hsueh, J.-W. Liu, J.-H. Song, and C.-H. Chiu. 2009. High rate of reduced susceptibility to ciprofloxacin and ceftriaxone among nontyphoid *Salmonella* clinical isolates in Asia. *Antimicrob. Agents Chemother*. 53:2696–2699.

43. Li, W.-C., F.-Y. Huang, C.-P. Liu, L.-C. Weng, N.-Y. Wang, N.-C. Chiu, and C.-S. Chiang. 2005. Ceftriaxone resistance of nontyphoidal *Salmonella enterica* isolates in Northern Taiwan attributable to production of CTX-M-14 and CMY-2 -lactamases. *J. Clin. Microbiol.* 43:3237–3243.

44. Mackenzie, J. S., and M. Jeggo. 2019. One health and zoonoses.*Trop. Med Infect Dis.* https://doi.org/10.3390/books978-3-03921-296-5

45. Marshall, B. M., and S. B. Levy. 2011. Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24:718–733.

46. Matthews, K. R., K. E. Kniel, and T. J. Montville. 2017. Food microbiology: an introduction. Fourth edition. ASM Press, Washington, DC.

47. McCutchen, E. L., M. Galac, C. Kapsak, S. H. Hinrichs, P. C. Iwen, and B. Abdalhamid. 2019. Genome sequence of a *Salmonella enterica* subsp. *enterica* serovar Corvallis strain isolated from human blood. *Microbiol. Resour. Announc.* 8:e01025-19, /mra/8/44/MRA.01025-19.atom.

48. Mekong Institute. 2019. MI, MoH Support FORT Team Responding to Foodborne Disease Outbreak. Available at https://www.mekonginstitute.org/news-activities/detail/2019/12/04/mi-moh-support-fort/. Accessed 24 April 2020.

49. Ministry of Agriculture Forestry and Fisheries. 2004. Country paper on food safety overview in Cambodia. Available at https://www.mekonginstitute.org/news-activities/detail/2019/12/04/mi-moh-support-fort/. Accessed 3 March 2020.

50. Montville, T. J., and K. R. Matthews. 2008. Food microbiology: an introduction. 2nd ed. ASM Press, Washington, DC.

51. Nadimpalli, M., L. Fabre, V. Yith, N. Sem, M. Gouali, E. Delarocque-Astagneau, N. Sreng, S. Le Hello, the BIRDY study group, B. T. Raheliarivao, F. Randrianirina, P. Herindrainy, Z. Z. Andrianirina, F. M. J. Rakotoarimanana, B. Garin, J.-M. Collard, A. de Lauzanne, L. Borand, P. Piola, A. Kerléguer, T. Chon, S. Touch, A. Tarantola, S. Goyet, S. Lach, V. Ngo, M. Vray, M. Diatta, J. Faye, A. Ndiaye, V. Richard, A. Seck, R. Bercion, A. G. Sow, J. B. Diouf, P. S. Dieye, B. Sy, B. Ndao, D. Guillemot, B. Huynh, M. Seguy, L. Watier, A. A. Youssouf, and M. Padget. 2019. CTX-M-55-type ESBL-producing *Salmonella enterica* are emerging among retail meats in Phnom Penh, Cambodia. *J. Antimicrob. Chemother.* 74:342–348.

52. Nair, D., K. Venkitanarayanan, and A. Kollanoor Johny. 2018. Antibiotic-resistant *Salmonella* in the food supply and the potential role of antibiotic alternatives for control. *Foods* 7:167.

53. Nakakubo, S., K. Nagaoka, M. Suzuki, S. Konno, Y. Shibue, T. Ikeda, and M. Nishimura. 2019. A case of primary bacteraemia caused by *Salmonella enterica* serovar Corvallis in an immunocompetent adult after travel to Southeast Asia. *Access Microbiol.* 1.

54. Nepal, G., and S. Bhatta. 2018. Self-medication with antibiotics in WHO Southeast Asian region: A systematic review. *Cureus*.

55. Nidaullah, H., N. Abirami, A. K. Shamila-Syuhada, L.-O. Chuah, H. Nurul, T. P. Tan, F. W. Zainal Abidin, and G. Rusul. 2017. Prevalence of *Salmonella* in poultry processing environments in wet markets in Penang and Perlis, Malaysia. *Vet. World* 10:286–292.

56. Om, C., F. Daily, E. Vlieghe, J. C. McLaughlin, and M.-L. McLaws. 2016. "If it's a broad spectrum, it can shoot better": inappropriate antibiotic prescribing in Cambodia. *Antimicrob. Resist. Infect. Control* 5:58.

57. Rane, S. 2011. Street vended food in developing world: hazard analyses. *Indian J. Microbiol.* 51:100–106.

58. Shafini, A. B., R. Son, N. A. Mahyudin, Y. Rukayadi, and T. C. T. Zainazor. 2017. Prevalence of *Salmonella* spp. in chicken and beef from retail outlets in Malaysia. *Int. Food Res. J. Selangor* 24:437–449.

59. Sinclair, R. G., and C. P. Gerba. 2011. Microbial contamination in kitchens and bathrooms of rural Cambodian village households: microbes in Cambodian households. *Lett. Appl. Microbiol.* 52:144–149.

60. Singh, O. V. 2016. Foodborne pathogens and antibiotic resistance. John Wiley & Sons, Hoboken, New Jersey. Library of Congress ISBN: 978-1-119-13917-1.

61. Sirichote, P., A. Bangtrakulnonth, K. Tianmanee, A. Unahalekhaka, A. Oulai, P. Chittaphithakchai, W. Kheowrod, and R. S. Hendriksen. 2010. Serotypes and antimicrobial resistance of *Salmonella enterica* in central Thailand, 2001-2006. *Southeast Asian J. Trop. Med. Public Health* 41:1405–1415.

62. Smith, G. 2018. Update on the draft Food Safety Law in Cambodia. Available at https://apps.fas.usda.gov/newgainapi/api/report/downloadreportbyfilename?filename=Update%20on%20the%20draft%20Food%20Safety%20Law_Ho%20Chi%20Minh%20City_Cambodia_1-4-2018. Accessed 9 April 2020.

63. Sokhen, C., D. Kanika, and Moustier, P. 2004. Vegetable market flows and chains in Phnom Penh.

64. Sousa, C. P. de. 2008. The impact of food manufacturing practices on food borne diseases. *Braz. Arch. Biol. Technol.* 51:615–623.

65. Stark, Z., T. Boughtwood, P. Phillips, J. Christodoulou, D. P. Hansen, J. Braithwaite, A. J. Newson, C. L. Gaff, A. H. Sinclair, and K. N. North. 2019. Australian Genomics: A federated model for integrating genomics into healthcare. *Am. J. Hum. Genet.* 105:7–14.

66. Tan, X., T. Chung, Y. Chen, D. Macarisin, L. LaBorde, and J. Kovac. 2019. The occurrence of *Listeria monocytogenes* is associated with built environment microbiota in three tree fruit processing facilities. *Microbiome* 7:115.

67. The World Bank. 2020. The World Bank in Cambodia. Available at https://www.worldbank.org/en/country/cambodia/overview. Accessed 24 March 2020.

68. Tribble, D. R. 2017. Resistant pathogens as causes of traveller's diarrhea globally and impact(s) on treatment failure and recommendations. *J. Travel Med.* 24:S6–S12.

69. Trongjit, S., S. Angkititrakul, R. E. Tuttle, J. Poungseree, P. Padungtod, and R. Chuanchuen. 2017. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand-Cambodia border provinces: AMR in *Salmonella enterica*. *Microbiol. Immunol.* 61:23–33.

70. United Nations Children's Fund. 2018. Diarrhoea as a cause of death. Available at https://data.unicef.org/topic/child-health/diarrhoeal-disease/. Accessed 17 September 2019.

71. Vila, J. 2015. Multidrug-resistant bacteria without borders: role of international trips in the spread of multidrug-resistant bacteria. *J. Travel Med.* 22:289–291.

72. Vipham, J. L., B. D. Chaves, and V. Trinetta. 2018. Mind the gaps: how can food safety gaps be addressed in developing nations? *Anim. Front.* 8:16–25.

73. Wilson, J., and T. Otsuki. 2001. Global trade and food safety: winners and losers in a fragmented system. The World Bank, Washington DC,USA.

74. Wilson, M., R. McNab, and B. Henderson. 2002. Bacterial disease mechanisms: an introduction to cellular microbiology. Cambridge University Press, Cambridge ; New York, NY.

75. World Health Organization. 2014. Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva, Switzerland.

76. World Health Organization. 2015. WHO estimates of the global burden of foodborne diseases. World Health Organization, Geneva, Switzerland.

77. World Health Organization. 2018. Antibiotic resistance. Available at https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance. Accessed 8 June 2020.

78. World Health Organization. 2019. Antimicrobial resistance. Geneva, Switzerland. Available at https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance. Accessed 24 April 2020.

79. Yam, E. L. Y., L. Y. Hsu, E. P.-H. Yap, T. W. Yeo, V. Lee, J. Schlundt, M. O. Lwin, D. Limmathurotsakul, M. Jit, P. Dedon, P. Turner, and A. Wilder-Smith. 2019. Antimicrobial resistance in the Asia Pacific region: a meeting report. *Antimicrob. Resist. Infect. Control* 8:202.

80. Yang, X., Q. Wu, J. Zhang, J. Huang, L. Chen, S. Wu, H. Zeng, J. Wang, M. Chen, H. Wu, Q. Gu, and X. Wei. 2019. Prevalence, bacterial load, and antimicrobial resistance of *Salmonella* serovars isolated from retail meat and meat products in China. *Front. Microbiol.* 10:2121.

81. Zellweger, R. M., J. Carrique-Mas, D. Limmathurotsakul, N. P. J. Day, G. E. Thwaites, S. Baker, on behalf of the Southeast Asia Antimicrobial Resistance Network, Members of the Southeast Asia Antimicrobial Resistance Network, E. Ashley, K. de Balogh, K. Baird, B. Basnyat, C. Benigno, L. Bodhidatta, N. Chantratita, B. Cooper, D. Dance, M. Dhorda, R. van Doorn, G. Dougan, N. T. Hoa, M. Ip, T. Lawley, C. Lim, T. K. Lin, C. Ling, Y. Lubell, A. Mather, F. Marks, V. R. Mohan, P. Newton, D. Paris, N. Thomson, P. Turner, O. Serichantalergs, F. Smithuis, V. Wuthiekanun, N. White, and H. Li Yang. 2017. A current perspective on antimicrobial resistance in Southeast Asia. *J. Antimicrob. Chemother.* 72:2963–2972.

82. Zhang, L., Y. Fu, Z. Xiong, Y. Ma, Y. Wei, X. Qu, H. Zhang, J. Zhang, and M. Liao. 2018. Highly prevalent multidrug-resistant *Salmonella* from chicken and pork meat at retail markets in Guangdong, China. *Front. Microbiol.* 9:2104.

83. Zhou, Y. P., A. Wilder-Smith, and L. Hsu. 2014. The role of international travel in the spread of methicillin-resistant *Staphylococcus aureus. J. Travel Med.* 21:272–281.

Chapter 2 - Prevalence of *Salmonella enterica* isolated from food contact and non-food contact surfaces in Cambodian informal markets

2.1 Abstract

The lack of hygiene and sanitation practices and insufficient infrastructure in Cambodian informal markets may increase the risk of food contamination, specifically raw vegetables, which in turn may increase the chances of contracting a foodborne disease. The aims of this study were i) to quantify the prevalence of *Salmonella enterica* based upon seasonal differences (rainy and dry) between surface types (food contact surface [FCS] and non-food contact surface [NFCS]) and between location of vendors within the market (inside and outside), and ii) to characterize *Salmonella enterica* serotypes abundance in informal markets in Cambodia. A total of 310 samples were screened to determine *Salmonella enterica* prevalence following the U.S. Department of Agriculture guidelines and confirmed by PCR. Whole Genome Sequencing was performed and the serotype for each isolate was determined in-silico using SeqSero 1.0 on draft genomes. A total of 81 samples were confirmed positive for *Salmonella enterica*. During the dry season, *Salmonella enterica* was more prevalent on FCS compared to NFCS (estimated probability of detection [confidence interval]: 0.41 [0.25,0.59] and 0.17 [0.08, 0.32], respectively; P=0.002), though no differences were apparent during the rainy season. Further, there was no evidence of any differences in *Salmonella enterica* prevalence based on location within the market (P=0.61). Sixteen *Salmonella enterica* serotypes were detected across multiple surfaces. The most common serotypes were Rissen (n=19), Hvittingfoss (n=13), Corvallis (n=10), Krefeld (n=8), Weltevreden (n=6), and Altona (n=6). The availability of accurate data on the prevalence of *Salmonella enterica*

in informal markets is crucial for the development of effective surveillance and implementation of suitable intervention strategies at the domestic level, thus preventing future foodborne illness.

2.2 Introduction

Foodborne diseases have been present since begging of the human race. However, only in the last century has the discipline of food safety been recognized as an essential pillar of public health. Foodborne diseases differ in their prevalence, degree of severity, and impacts on health outcomes at community, regional, and country levels (*32*). According to the World Health Organization, the health burden of foodborne diseases is comparable to that of the three major infectious diseases, namely HIV/AIDS, malaria, and tuberculosis (*47*). Despite their global nature, food safety issues pose more severe challenges on human, community, and country prosperity of low and middle-income countries (LMICs) as compared to high-income countries (HICs) (*2*, *14*, *15*).

Biological hazards are responsible for 70% of the global burden of foodborne diseases (*47*). Despite technological advancements to prevent foodborne hazards from entering food supplies, the prevalence of foodborne illnesses remains a significant cause of morbidity and preventable mortality, mainly in LMICs (*2*). In particular, the main gaps recognized in LMICs are inadequate monitoring and surveillance programs, which jointly hinder the implementation of food safety interventions (*15*, *42*).

It can be further argued that the absence of reliable data on foodborne diseases (e.g., surveillance, prevalence, incidence, mortality rates, productivity loss, and costs of medical care) likely prevents the development of effective national food safety programs to meet domestic and international food safety requirements. Oftentimes, food safety programs in LMICs are fragmented into two main scenarios: (i) international export markets, where food safety concerns are

prioritized and highly monitored, and (ii) domestic markets, where food safety issues are commonly omitted. This fragmentation leads to domestic markets featuring food products that, quite simply, fall short of international food safety standards. Generally, domestic market systems lack appropriate measures to control for low levels of contamination, let alone consolidated food products diverted from the export market with confirmed contamination results. This differential treatment on the prioritization of food safety only increases the risk of unsafe foods reaching local populations, adding to an undue burden of diminishing development outcomes (*15*).

The domestic market sector in LMICs often consists of large informal markets, commonly in open-air environments, with individual vendors selling various food products, often in the absence of any basic food safety infrastructure or oversight. Frequently, vendors can be observed selling fresh vegetables, fruits, seafood, animal products (live or processed poultry, beef, goat, and pork), and household items side-by-side in the same stand. Furthermore, most of these food items are sold at ambient temperature and are directly exposed to the environment (*9*, *27*).

The market environment plays an essential role in the cross-contamination process between food products and food contact surfaces, and vice-versa. Studies conducted on informal markets in Cambodia, Thailand, Vietnam, Malaysia, and the Philippines have revealed a high prevalence of foodborne pathogens on fresh vegetables, poultry, and pork products, particularly *Salmonella enterica* (henceforth referred to as *Salmonella*) and pathogenic *Escherichia coli* (*3*, *4*, *7*, *23*, *27*, *39*, *43*). However, few studies have reported the prevalence of foodborne pathogens on environmental surfaces (*1*, *27*, *31*); this is critical to explaining mechanisms of cross-contamination of food products. Further, the literature shows regional gaps on baseline research studies evaluating the presence of *Salmonella* in informal markets, for example in Cambodia.

The aims of this study were i) to quantify the prevalence of *Salmonella enterica* based upon seasonal differences (rainy and dry) between surface types (food contact surface [FCS] and non-food contact surface [NFCS]) and between location of vendors within the market (inside and outside) and ii) to characterize *Salmonella* serotypes abundance in informal markets in Cambodia.

2.3 Material and methods

2.3.1 Sample collection

Two markets in the province of Battambang, Cambodia were selected for this study. These two markets were selected because their vendors sold vegetables that were sourced from local farmers in the region. Each market was sampled during the rainy (June 2018) and dry (January 2019) seasons, as identified by Culas & Tek (*12*). Each market was further segmented into inside and outside locations. The inside location was characterized by the presence of concrete walls and metal or wood fencing sustaining a roof, whereas the outside location was characterized by open air tents. Within each market-season-location, all vendors selling vegetables were sampled (varying from 6 to 10 vendors depending on the market-season-location stratum), for a total of 64 vendors sampled throughout the entire study. For each vendor, samples were collected on FCS (n=3) and on NFCS (n=3), with FCS defined as any surface that was directly in contact with food items (e.g., baskets, mats), whereas NFCS was defined as a surface not directly in contact with food but in the proximate vicinity of food items (e.g., floor, wall). A variety of FCS and NFCS surfaces were sampled and classified accordingly. The samples included baskets, floors, lotus flower leaves, mats, metal stands, platforms, scales, and wood tables. All FCS samples were in direct contact with vegetables at the time of sampling. A grand total of n=384 samples were collected. However, 74 samples were lost to follow up due to a shortage of laboratory supplies. Therefore, a total of 310 samples were used for the analysis (FCS [n=148] and NFCS [n=162]).

Remote weather data loggers (HOBO MX110 Temp/RH logger) were set up in a research station in the Battambang province to measure annual rainfall volume, temperature, and humidity (Table 2.1). Samples were collected according to the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) Directive 10,300.1, Rev. 1, Section VII, A, 12 (*40*). Students from the Royal University of Agriculture were trained in sample collection techniques and assisted during sample collection days. A Sani-stick sponge (LABPLAS, Quebec, Canada) hydrated with 10 mL of buffered peptone water (BPW; Becton Dickinson, Franklin Lakes, NJ-USA) was used to swab 100 cm^2 of an environmental surface area. Only the sponge portion of the Sani-Stick sponge was placed back into the original sterile bag. Bags were labeled and kept on ice directly after sample collection. Between different samples, 70% alcohol was used to decontaminate sample collector's hands. Samples' temperature was monitored during transport to the laboratory at the Royal University of Agriculture in Phnom Penh which occurred within 24 hours, and samples were immediately processed.

2.3.2 Isolation and identification of *Salmonella*

Samples were analyzed for the presence of *Salmonella* by means of USDA-FSIS Microbiology Laboratory Guidebook 4.09 methods (*41*). Fifty mL of BPW was aseptically added to each sample bag, and samples were hand-massaged for one minute. Sponge bags were incubated at 35 ± 2 °C for 20-24 h. An aliquot of 0.5 ± 0.05 mL of the sample was transferred into 10 mL Tetrathionate (TT) broth (HiMedia, West Chester, PA-USA) and 0.1 ± 0.02 mL into 10 mL Rappaport-Vassiliadis (RV) broth (HiMedia, West Chester, PA-USA). Selective enrichments were incubated at 42 ± 0.5 °C for 22-24 h. A 10 µL loopful of the selective enrichment medium was streaked onto a 100 x 15 mm plate of Xylose Lysine Tergitol-4 (XLT-4) agar and Brilliant Green Sulpha agar (BGS; HiMedia, West Chester, PA-USA). Plates were incubated at 35 ± 2 °C for 18-

24 h. After incubation, three yellow colonies with dark centers from XLT-4 agar, and three pink and opaque colonies with a smooth appearance surrounded by a red color from BGS agar were tested for agglutination using a *Salmonella* latex agglutination kit (Oxoid, Hampshire, UK). Colonies that tested positive in the agglutination test were recorded as presumptive positives for *Salmonella.*

2.3.3 *Salmonella* isolates collection

For each sample, one presumptive positive colony of *Salmonella* was streaked onto a 60 x 15 mm plate of Tryptic Soy Agar (TSA; Hardy Diagnostics, Santa Maria, CA-USA), and incubated at 37 ± 2.0 °C for 24 ± 2.0 h. Isolated colonies were transferred into 9 mL Tryptic soy broth (TSB; Hardy Diagnostics, Santa Maria, CA-USA) tubes and further incubated at 37 ± 2.0 °C for 24 ± 2.0 h. The TSB inoculated broth was then used to create a lawn of *Salmonella* growth onto TSA using a sterilized cotton swab. TSA plates were incubated at 37 ± 2.0 °C for 24 ± 2.0 h. The lawn was harvested and transferred to individual cryobeads (Key Scientific Products INC, Stamford, TX-USA), following manufacturer's protocol. Cryobeads were stored at -80 ± 2.0 °C at the Royal University of Agriculture, and replicates were transported to Kansas State University for isolate confirmation.

2.3.4 PCR confirmation of *Salmonella* isolates

Confirmation of isolates was completed at Kansas State University. Isolates were activated by using a cryobead that was streaked for growth onto a TSA plate. Plates were incubated at 37 ± 2.0 °C for 24 ± 2 h. Isolated colonies were transferred to 9 mL TSB tubes and incubated at 37 ± 2.0 °C for 24 ± 2 h. Samples were prepared using the MicroSEQ *Salmonella* detection Kit (with PrepSEQ rapid spin sample preparation), per manufacturer's protocol (Thermofisher Scientific, Waltham, MA-USA) and confirmed using the Applied Biosystems 7500 Fast Real-Time PCR.

A total of 106 isolates were confirmed positive for *Salmonella* by using the applied biosystems 7500 fast real-time PCR. However, results from whole genome sequencing (WGS) identified 25 isolates as being non-*Salmonella*. The applied biosystems 7500 fast real-time PCR presented a false-positive rate of 23.5%. Due to this issue, all the isolates were re-tested using a multiplex PCR assay targeting specific *Salmonella* genes (i.e., *pagC* and *invA*) as previously described by Bai et. al., (2018) (5). The results from the multiplex real-time PCR matched the results from the WGS, indicating that the multiplex real-time PCR should be always used as a confirmatory step in research settings.

2.3.5 DNA preparation

Genomic DNA from each strain was obtained using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), following manufacturer instructions. DNA concentration was determined using a Qubit 4.0 fluorometer (Invitrogen). DNA samples were sent to the Food and Drug Administration Center for Food Safety and Applied Nutrition (FDA-CFSAN) for Whole Genome Sequencing (WGS). The resultant DNA extract was stored at -20°C until WGS analysis.

2.3.6 Whole genome sequencing and *Salmonella* serotypes prediction

Libraries were prepared from genomic DNA with the Nextera XT DNA Library Preparation Kit, and WGS was carried out on either the MiSeq or NextSeq sequencer, using a MiSeq Reagent Kit V2 (500-cycles) or a NextSeq 500/550 High-Output Kit V2 (300-cycles), respectively (Illumina). Isolates were run in two different analyzers due to analytical laboratory scheduling availability. *De novo* assemblies were obtained with Shovill 0.9 (https://github.com/tseemann/shovill). The serotype of each isolate was determined *in-silico* using SeqSero 1.0 on draft genomes (http://denglab.info/SeqSero).

2.3.7 Statistical analysis

For the prevalence analysis of *Salmonella,* a generalized linear mixed model was fitted to a binary response indicative of confirmed *Salmonell*a detection on a given sample. The logit link function was used to connect the Bernoulli probability of detection with a linear predictor that included the fixed effects of season (dry or rainy), market location (inside or outside), surface type (FCS or NFCS), and all two- and three-way interactions. Random effects considered in the linear predictor included i) market as an overarching blocking structure, ii) the cluster defined by a market in a given season and location (to identify the experimental unit for these factors), iii) the vendor nested within market in a given season and location (to identify the blocking factor for surface type), and iv) the set of samples collected for a combination of vendor and surface type (to recognize repeated sampling; i.e., subsampling, of each surface type). Variance components described above as i) and iv) converged to zero, and thus the corresponding random effects were removed from the linear predictor; degrees of freedom were adjusted to recognize the appropriate amount of replication for each factor.

Overdispersion (i.e., checks for greater variability than what is expected based upon the statistical model that is chosen) was evaluated using the maximum-likelihood based fit statistic Pearson Chi-Square/DF. No evidence for overdispersion was apparent in the final model used for inference. The final statistical model used for inference was fitted using a pseudolikelihood. The model was fitted using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute, Cary, NC) implemented using Newton-Raphson with ridging as the optimization technique. Relevant pairwise comparisons were conducted using Tukey-Kramer or Bonferroni adjustments, as appropriate to the level of inference, to avoid inflation of Type I error rate due to multiple comparisons.

2.4 Results

2.4.1 *Salmonella* prevalence

A total of 81 samples were confirmed positive for *Salmonella* out of the 310 samples analyzed. Moreover, *Salmonella* was detected for a majority of the vendors sampled (i.e., 42 out of 64) and in both informal markets in the province of Battambang, Cambodia.

2.4.2 Explanatory factors

There was no evidence for any three-way interaction between season, location, and surface type on the probability of *Salmonella* detection (P=0.93). However, season-specific differences in *Salmonella* detection were apparent between FCS and NFCS. Specifically, during the dry season, *Salmonella* was more prevalent on FCS compared to NFCS (estimated probability of detection [confidence interval]: 0.41 [0.25,0.59] and 0.17 [0.08, 0.32], respectively; P=0.002) regardless of vendor location within the market (Figure 2.1). By contrast, the rainy season showed no significant differences between surface types in *Salmonella* prevalence (P=0.57), though the estimated probability of detection was low in both cases (Figure 2.1).

Regarding market location, there was no evidence for any differences in *Salmonella* detection between inside and outside vendors (P=0.61), even after adjusting for season and surface type.

2.4.3 Frequency of *Salmonella* serotypes

A total of 16 *Salmonella* serotypes were identified from the 81 positive samples obtained from both markets. Table 2.2 characterizes the frequency distribution of *Salmonella* serotypes in each season. Overall, the most common serotypes were *Salmonella* Rissen (19), *Salmonella* Hvittingfoss (13), *Salmonella* Corvallis (10), *Salmonella* Krefeld (8), *Salmonella* Weltevreden (6), *Salmonella* Altona (6), and *Salmonella* Mbandaka (5). Other serotypes such as *Salmonella*

Typhimurium, *Salmonella* Javiana, *Salmonella* Uganda, *Salmonella* Derby, *Salmonella* Anatum, *Salmonella* Braenderup, *Salmonella* Lexington, *Salmonella* Virchow, and *Salmonella* I 4,[5],12:i:- registered few (≤3) isolates.

The most frequently isolated serotype in this study was *Salmonella* Rissen which was isolated from diverse sample types (i.e., basket, floor, lotus flower leaf, mat, metal stand, platform, scale, and wood table), including FCS (n=13) and NFCS (n=6; Table 2.2). Figure 2.2 illustrates the empirical frequency distribution of detected serotypes from a variety of surfaces. Sample types with the most *Salmonella* diversity were the floor, basket, mat, scale, and lotus flower leaf with 13, 11, 5, 5, and 4 different serotypes, respectively.

2.5 Discussion

2.5.1 *Salmonella* prevalence

Overall, *Salmonella* was detected in a considerable number of samples, indicating conditions of unsanitary food handling and poor market sanitation practices; these are known to be contributing factors to cross-contamination (*22*, *33*, *35*, *37*). Moreover, *Salmonella* was pervasive across markets, vendors, and multiple locations within each informal market, suggesting that cross-contamination is likely occurring. Indeed, practices and behaviors that increase the likelihood of cross-contamination between foods and the environment were anecdotally observed in these informal markets. For example, vendors selling raw meats, fruits and vegetables adjacent to each other and in the same physical environment was a common sight. These practices in combination with poor infrastructure (i.e., lack of sanitary design) are likely to increase the probability of cross-contamination.

Interestingly, *Salmonella* was more prevalent on FCS compared to NFCS in the dry season. Although this study did not investigate seasonal determinants (e.g., humidity, temperature, etc.) of

Salmonella in detail, a few local factors may contribute to a higher prevalence in FCS during the dry season. In Cambodia, vegetables are commonly irrigated with unsafe water sources during the dry season, which may contribute to vegetable contamination and thus detection of *Salmonella* on FCS. In fact, reports from Southeast Asia indicate that surface water used for irrigation can be highly contaminated with foodborne pathogens (*44*). This is consistent with reports from other LMIC locations, such as Nigeria, where (*29*) studies have observed greater *Salmonella* contamination of irrigation surface water during the dry season compared to rainy season. If contaminated water is used for vegetable irrigation, this may increase the probability of pathogen transfer to vegetables. These findings may indicate a synergistic seasonal effect in terms of irrigation water, as i) limited precipitation during the dry season calls for increased use of irrigation practices, and ii) irrigation water shows greater contamination levels during the dry season. In the current study, vegetables contaminated at the field level may become a contamination source at the market level, when put in contact with different surface types, explaining the higher prevalence of *Salmonella* in FCS when compared to NFCS during the dry season.

The weather patterns in Cambodia are largely influenced by rainfall, with distinct rainy (May to October) and dry seasons (November to April) (*12*). While the control of *Salmonella* on food contact surfaces and cross-contamination from vegetables and meats is critical, the contributing effect of season on *Salmonella* prevalence is poorly understood. Several studies evaluating the prevalence of *Salmonella* have been conducted in informal markets in Southeast Asia; however, the seasonality effect was not considered in such studies (*1*, *27*, *34*, *38*, *43*).

The combined role of the season and surface type on *Salmonella* detection observed in this study illustrates the complexity of the market microbiological environment and its dynamic factors. Higher humidity environments observed during the rainy season are known to favor

bacterial survival and enhance cross-contamination (*6*), since water can act as a vehicle for pathogen transmission (*17*). Hence, we speculate that high humidity and heavy rain events during the rainy season promoted cross-contamination of *Salmonella* between FCS and NFCS, potentially explaining the lack of evidence for differences between surface types in *Salmonella* prevalence.

2.5.2 *Salmonella* serotypes abundance

This research revealed substantial *Salmonella* contamination of both informal markets in Cambodia; however, it is crucial to consider the serotype diversity and their importance to public health. Our results show that many serotypes found in these informal markets are concerning to public health. Indeed, seven (*S.* Rissen, *S.* Hvittingfoss, *S.* Corvallis, *S.* Krefeld, *S.* Weltevreden, *S.* Altona, and *S.* Mbandaka) of the ten serotypes most frequently found in this study are listed in the top ten most frequently isolated serotypes from human patients in Southeast Asia (*20*). To the best of our knowledge, no published data exist characterizing *Salmonella* serotypes from human specimens in Cambodia. However, this study reiterated six of the ten serotypes most commonly isolated from patients in Thailand, specifically *S.* Weltevreden, *S.* Anatum, *S.* Rissen, *S.* I 4,[5],12:i:-, *S.* Typhimurium, and *S.* Corvallis (*19*, *36*). The similarity of *Salmonella* serotypes identified in human specimens in the region with those isolated in this study suggests that informal markets could be a potential contamination source for *Salmonella*.

The most prevalent serotypes found in this study were *S.* Rissen, *S.* Hvittingfoss, and *S.* Corvallis. Previous studies reported *Salmonella* in poultry and pork from informal markets in Phnom Penh, Cambodia with prevalence of *Salmonella* contamination varying from 42.8% to 88.2% in poultry, and 18.9% to 71.1% in pork (*23*, *39*). The most common *Salmonella* serotypes found in poultry samples were *S.* Anatum, *S.* Typhimurium, *S.* Corvallis (*23*), *S.* Agona (*39*); and

in pork samples was *S.* Rissen (*39*). These serotypes were also observed in this data, indicating that their prevalence in informal markets in Cambodia may be endemic.

In this study, *S.* Hvittingfoss was the second most prevalent serotype isolated mainly from FCS. This serotype has rarely been identified in Southeast Asian countries (*20*, *23*, *25*, *27*, *39*). Our data suggest that *S.* Hvittingfoss may be an emerging serotype in these informal markets. *S.* Hvittingfoss infections have been reported in China, Australia, and Nigeria. Sources of contamination were attributed to reptiles (i.e., turtles and geckos), fruits, and vegetables (*28*, *30*, *45*). While not sampled in this study, it is important to note that turtles are frequently sold in informal markets in Cambodia. This study revealed that *S.* Hvittingfoss is highly prevalent in the informal market setting, posing a significant source of contamination since it was mainly isolated from FCS.

In addition, *S.* Corvallis was the third most prevalent serotype isolated from both FCS and NFCS. Nidaullah et al. (27) identified *S.* Corvallis to be a highly prevalent serotype among poultry and environmental samples collected from informal markets in Malaysia. *S.* Corvallis has raised concerns within public health authorities due to reports of highly antimicrobial resistant strains (*16*, *24*, *46*). A report published by the Institute of Public Health in Japan described a case of travel-related bacteremia caused by *S.* Corvallis in an immunocompetent adult that had traveled to Vietnam and Cambodia prior to the appearance of symptoms (*26*). Previous studies have identified *S.* Corvallis contamination in food environments, as well as in clinical samples from patients in Southeast Asia.

The distribution of *Salmonella* serotypes varies depending on the season, geographical location, surface type and the economic level of development of a country (*10*, *11*, *18*, *20*, *27*). Additionally, the increase in international multilateral trade and commerce of food and agricultural

products may contribute to the shift of specific *Salmonella* serotypes and an increase in its population diversity (*13*, *21*). This study demonstrated a great diversity of *Salmonella* serotypes, the identification of emerging serotypes (i.e., *S.* Hvittingfoss), and their distribution across surface type and seasons. Additionally, these findings demonstrate the complexity of intertwined contributing factors to *Salmonella* prevalence and its imminent risk to public health in Cambodia.

To the best of our knowledge, this is the first study to investigate the prevalence and abundance of *Salmonella* in environmental samples from informal markets in Cambodia. Many interrelated factors, including inadequate infrastructure, environmental sanitation, personal hygiene practices, and a lack of food safety measures, may contribute to the development of foodborne illness. Thus, the availability of accurate data on the prevalence of *Salmonella* in these markets is crucial for active surveillance, implementation of suitable intervention strategies, and prevention of future foodborne illness cases.

The limitations of the current study were i) the sampling of only two markets representative of the rural area of Cambodia; ii) the sampling of only one sample point per season (June and January, representing the rainy and dry season, respectively); and iii) the short timeframe of the study, which was conducted in less than one year only. Future studies should sample a greater diversity of markets at various time points, following individual vendors throughout the seasons to better understand the dynamics of *Salmonella* prevalence, diversity, and persistence at a finer spatial-temporal scale. Future studies conducting confirmatory steps for *Salmonella enterica* using the Applied Biosystems 7500 Fast Real-Time PCR should consider an additional method of confirmation (i.e., WGS or multiplex-real time PCR) due to the false-positive rate of 23.5% identified in this study. Alternatively, the use of a multiplex real-time PCR, as described in this study (5), is highly recommended when additional analysis (i.e., WGS) are not conducted.

Based on these findings, we strongly recommend the development and implementation of proper intervention strategies that prioritize the surface type (i.e., food contact surface), particularly during a problematic season of the year (i.e., dry) to address constraints of the environment and maximize *Salmonella* reduction more efficiently. Sanitation programs should focus on strategies to clean the market environment, in which FCS should be prioritized because i) they presented the highest prevalence of *Salmonella* serotypes during the dry season, and ii) they are in direct contact with food.

2.6 Acknowledgments

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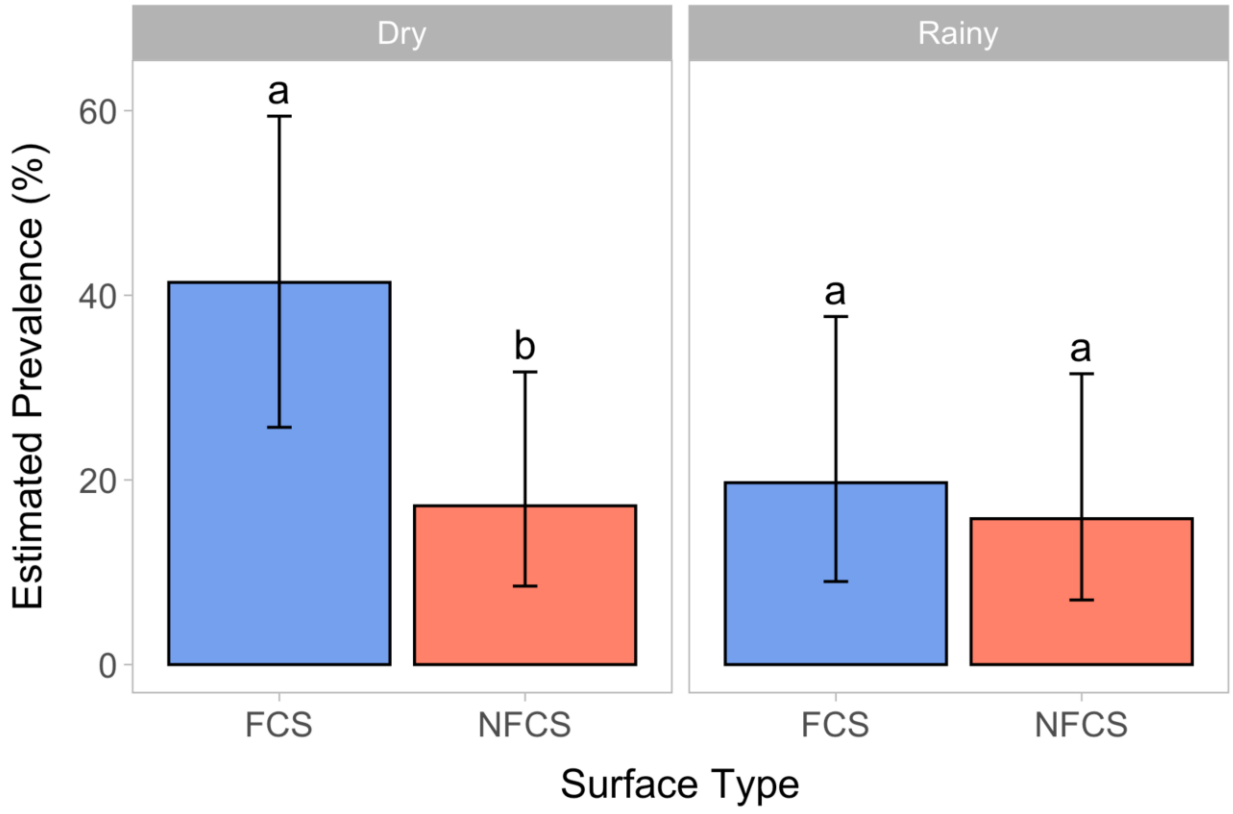

Figure 2.1. Estimated prevalence of *Salmonella enterica* (and corresponding 95% confidence intervals) on food contact surfaces (FCS) and non-food-contact surfaces (NFCS) during dry and rainy seasons. (a,b) Letters indicate significant differences between surface types within each season at alpha = 0.05.

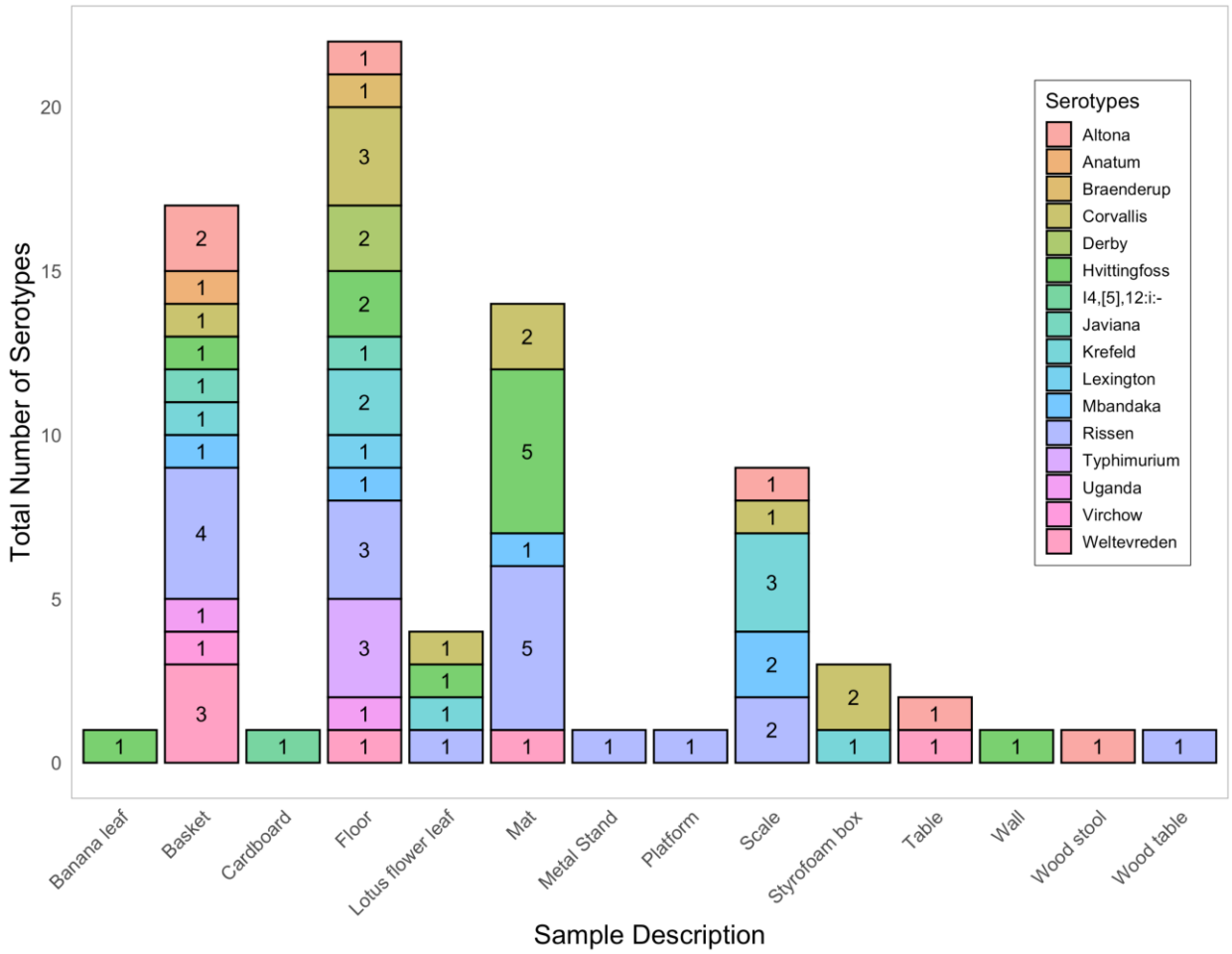

Figure 2.2. Empirical frequency distribution of the 16 serotypes of *Salmonella enterica* detected in various sample types.

Table 2.1. Monthly cumulative rainfall, mean temperature and mean humidity in Battambang, Cambodia during rainy and dry seasons, as recorded using remote weather data logger (HOBO MX110 Temp/RH logger).

Season	Rainfall (mm)	Temperature (°C)	Humidity (%)
June 2018 (Rainy)	109.3	28.4	81.1
January 2019 (Dry)	0.00	25.6	74.8

Table 2.2. Frequency table of *Salmonella enterica* serotypes detected on food contact surface (FCS) and non-food contact surface (NFCS) during dry and rainy seasons in informal markets in Battambang, Cambodia.

	Dry Season		Rainy Season	
Serotype	FCS	NFCS	FCS	NFCS
Altona	0	0	3	3
Anatum	1	0	0	0
Braenderup	0	1	0	0
Corvallis	6	4	0	0
Derby	0	0	0	2
Hvittingfoss	9	3	1	0
Javiana	0	1	1	0
Krefeld	5	2	1	0
Lexington	0	1	0	0
Mbandaka	4	1	0	0
I 4,[5],12:i:-	0	0	1	0
Rissen	10	3	3	3
Typhimurium	1	0	0	2
Uganda	0	0	1	1
Virchow	1	0	0	0
Weltevreden	4	2	0	0

2.7 References

1. Abatcha, M. G., M. E. Effarizah, and G. Rusul. 2018. Prevalence, antimicrobial resistance, resistance genes and class 1 integrons of *Salmonella* serovars in leafy vegetables, chicken carcasses and related processing environments in Malaysian fresh food markets. *Food Control* 91:170–180.

2. Akhtar, S., M. R. Sarker, and A. Hossain. 2014. Microbiological food safety: a dilemma of developing societies. *Crit. Rev. Microbiol.* 40:348–359.

3. Ananchaipattana, C., Y. Hosotani, S. Kawasaki, S. Pongsawat, B. M. Latiful, S. Isobe, and Y. Inatsu. 2012. Prevalence of foodborne pathogens in retailed foods in Thailand. *Foodborne Pathog. Dis.* 9:835–840.

4. Awang Salleh, N., N. A. Salleh, G. Rusul, Z. Hassan, A. Reezal, S. H. Isa, M. Nishibuchi, and S. Radu. 2003. Incidence of *Salmonella* spp. in raw vegetables in Selangor, Malaysia. *Food Control* 14:475–479.

5. Bai, J., V. Trinetta, X. Shi, L. W. Noll, G. Magossi, W. Zheng, E. P. Porter, N. Cernicchiaro, D. G. Renter, and T. G. Nagaraja. 2018. A multiplex real-time PCR assay, based on invA and pagC genes, for the detection and quantification of *Salmonella enterica* from cattle lymph nodes. *Journal of Microbiological Methods* 148:110–116.

6. Carrasco, E., A. Morales-Rueda, and R. M. García-Gimeno. 2012. Cross-contamination and recontamination by *Salmonella* in foods: A review. *Food Res. Int.* 45:545–556.

7. Chau, H. L. Q., H. T. Thong, N. V. Chao, P. H. S. Hung, V. V. Hai, L. V. An, A. Fujieda, T. Ueru, and M. Akamatsu. 2014. Microbial and parasitic contamination on fresh vegetables sold in traditional markets in Hue City, Vietnam. *J. Food Nutr. Res.* 2:959–964.

8. Chhay, N., S. Seng, T. Tanaka, A. Yamauchi, E. C. Cedicol, K. Kawakita, and S. Chiba. 2017. Rice productivity improvement in Cambodia through the application of technical recommendation in a farmer field school. *Int. J. Agric. Sustain.* 15:54–69.

9. Chuah, L. O., A. K. Shamila Syuhada, I. Mohamad Suhaimi, T. Farah Hanim, and G. Rusul. 2018. Genetic relatedness, antimicrobial resistance and biofilm formation of

Salmonella isolated from naturally contaminated poultry and their processing environment in northern Malaysia. *Food Res. Int.* 105:743–751.

10. Clark, G., A. F. Kaufmann, E. J. Gangarosa, and M. A. Thompson. 1973. Epidemiology of an international outbreak of *Salmonella* Agona. *The Lancet* 302:490–493.

11. Crump, J. A., and M. D. Kirk. 2015. Estimating the burden of febrile illnesses. *PLoS Negl. Trop. Dis.* 9:e0004040.

12. Culas, R. J., and K. Tek. 2016. Food security in Cambodia: trends and policy objectives. *Int. J. of Dev. Issues.* 15:306–327.

13. D'Aoust, J.-Y. 1994. *Salmonella* and the international food trade. *Int. J. Food Microbiol.* 24:11–31.

14. Grace, D. 2017. Food Safety in developing countries: research gaps and opportunities. Nairobi, Kenya: international livestock research institute. Available at: https://www.agrilinks.org/sites/default/files/resource/files/whitepaperfoodsafetyftf-branding_06052017_formatted.pdf. Accessed 10 September 2018.

15. Grace, D. 2015. Food safety in low and middle income countries. *Int. J. Environ. Res. Public Health* 12:10490–10507.

16. Hadziabdic, S., M. Borowiak, A. Bloch, B. Malorny, I. Szabo, B. Guerra, A. Kaesbohrer, and J. Fischer. 2018. Complete genome sequence of an avian native NDM-1-producing *Salmonella enterica* subsp. *enterica* serovar corvallis strain. *Genome Announc.* 6:e00593-18, /ga/6/26/e00593-18.atom.

17. Havelaar, A. H., M. D. Kirk, P. R. Torgerson, H. J. Gibb, T. Hald, R. J. Lake, N. Praet, D. C. Bellinger, N. R. de Silva, N. Gargouri, N. Speybroeck, A. Cawthorne, C. Mathers, C. Stein, F. J. Angulo, B. Devleesschauwer, and on behalf of World Health Organization Foodborne Disease Burden Epidemiology Reference Group. 2015. World health organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Med.* 12:e1001923.

18. Hendriksen, R. S., A. Bangtrakulnonth, C. Pulsrikarn, S. Pornreongwong, H. Hasman, S. W. Song, and F. M. Aarestrup. 2008. Antimicrobial resistance and molecular epidemiology of *Salmonella* Rissen from animals, food products, and patients in Thailand and Denmark. *Foodborne Pathog. Dis.* 5:605–619.

19. Hendriksen, R. S., A. Bangtrakulnonth, C. Pulsrikarn, S. Pornruangwong, G. Noppornphan, H. D. Emborg, and F. M. Aarestrup. 2009. Risk factors and epidemiology of the ten most common *Salmonella* serovars from patients in Thailand:2002-2007. *Foodborne Pathog. Dis.* 6:1009–1019

20. Hendriksen, R. S., A. R. Vieira, S. Karlsmose, D. M. A. Lo Fo Wong, A. B. Jensen, H. C. Wegener, and F. M. Aarestrup. 2011. Global monitoring of *Salmonella* serovar distribution from the world health organization global foodborne infections network country data bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathog. Dis.* 8:887–900.

21. Herikstad, H., Y. Motarjemi, and R. V. Tauxe. 2002. *Salmonella* surveillance: a global survey of public health serotyping. *Epidemiol. Infect.* 129:1–8.

22. Keithellakpam, L. 2018. Study on hygienic practices of street food vendors in Allahabad city and determination of critical control points for safe street foods. Available at: http://rgdoi.net/10.13140/RG.2.2.22360.14080. Accessed 6 February 2020.

23. Lay, K. S., Y. Vuthy, P. Song, K. Phol, and J. L. Sarthou. 2011. Prevalence, numbers and antimicrobial susceptibilities of *Salmonella* serovars and *Campylobacter* spp. in retail poultry in Phnom Penh, Cambodia. *J. Vet. Med. Sci.* 73:325–329.

24. McCutchen, E. L., M. Galac, C. Kapsak, S. H. Hinrichs, P. C. Iwen, and B. Abdalhamid. 2019. Genome sequence of a *Salmonella enterica* subsp. *enterica* serovar Corvallis strain isolated from human blood. *Microbiol. Resour. Announc.* 8:e01025-19, /mra/8/44/MRA.01025-19.atom.

25. Nadimpalli, M., L. Fabre, V. Yith, N. Sem, M. Gouali, E. Delarocque-Astagneau, N. Sreng, S. Le Hello, the Birdy study group, B. T. Raheliarivao, F. Randrianirina, P. Herindrainy, Z. Z. Andrianirina, F. M. J. Rakotoarimanana, B. Garin, J. M. Collard, A. de Lauzanne, L. Borand, P. Piola, A. Kerléguer, T. Chon, S. Touch, A. Tarantola, S. Goyet, S. Lach, V. Ngo, M. Vray, M. Diatta, J. Faye, A. Ndiaye, V. Richard, A. Seck, R. Bercion, A. G. Sow, J. B. Diouf, P. S. Dieye, B. Sy, B. Ndao, D. Guillemot, B. Huynh, M. Seguy, L. Watier, A. A. Youssouf, and M. Padget. 2019. CTX-M-55-type ESBL-producing *Salmonella enterica* are emerging among retail meats in Phnom Penh, Cambodia. *J. Antimicrob. Chemother.* 74:342–348.

26. Nakakubo, S., K. Nagaoka, M. Suzuki, S. Konno, Y. Shibue, T. Ikeda, and M. Nishimura. 2019. A case of primary bacteraemia caused by *Salmonella enterica* serovar Corvallis in an immunocompetent adult after travel to Southeast Asia. *Access Microbiol*.

27. Nidaullah, H., N. Abirami, A. K. Shamila-Syuhada, L.-O. Chuah, H. Nurul, T. P. Tan, F. W. Zainal Abidin, and G. Rusul. 2017. Prevalence of *Salmonella* in poultry processing environments in wet markets in Penang and Perlis, Malaysia. *Vet. World* 10:286–292.

28. Oboegbulem, S. I., and A. U. Iseghohimhen. 1985. Wall geckos (Geckonidae) as reservoirs of *Salmonellae* in Nigeria: problems for epidemiology and public health. *Int. J. Zoonoses* 12:228–232.

29. Okafo, C. N., V. J. Umoh, and M. Galadima. 2003. Occurrence of pathogens on vegetables harvested from soils irrigated with contaminated streams. *Sci. Total Environ.* 311:49–56.

30. Oxenford, C. J., A. P. Black, R. J. Bell, S. A. Munnoch, M. J. Irwin, R. N. Hanson, R. L. Owen, and Outbreak Investigation Team. 2005. Investigation of a multi-state outbreak of *Salmonella* Hvittingfoss using a web-based case reporting form. *Commun. Dis. Intell. Q. Rep.* 29:379–381.

31. Padungtod, P., and J. B. Kaneene. 2006. *Salmonella* in food animals and humans in northern Thailand. *Int. J. Food Microbiol.* 108:346–354.

32. Quinlan, J. J. 2013. Foodborne illness incidence rates and food safety risks for populations of low socioeconomic status and minority race/ethnicity: a review of the literature. *Int. J. Environ. Res. Public Health* 10:3634–3652.

33. Rane, S. 2011. Street vended food in developing world: hazard analyses. *Indian J. Microbiol.* 51:100–106.

34. Rusul, G., J. Khair, S. Radu, C. T. Cheah, and R. Md. Yassin. 1996. Prevalence of *Salmonella* in broilers at retail outlets, processing plants and farms in Malaysia. *Int. J. Food Microbiol.* 33:183–194.

35. Shafini, A. B., R. Son, N. A. Mahyudin, Y. Rukayadi, and T. C. T. Zainazor. 2017. Prevalence of *Salmonella* spp. in chicken and beef from retail outlets in Malaysia. *Int. Food Res. J.* 24:437–449.

36. Sirichote, P., A. Bangtrakulnonth, K. Tianmanee, A. Unahalekhaka, A. Oulai, P. Chittaphithakchai, W. Kheowrod, and R. S. Hendriksen. 2010. Serotypes and antimicrobial resistance of *Salmonella enterica* in central Thailand, 2001-2006. *Southeast Asian J. Trop. Med. Public Health* 41:1405–1415.

37. Sousa, C. P. de. 2008. The impact of food manufacturing practices on food borne diseases. *Braz. Arch. Biol. Technol.* 51:615–623.

38. Sripaurya, B., R. Ngasaman, S. Benjakul, and K. Vongkamjan. 2019. Virulence genes and antibiotic resistance of *Salmonella* recovered from a wet market in Thailand. *J. Food Saf.* 39:e12601.

39. Trongjit, S., S. Angkititrakul, R. E. Tuttle, J. Poungseree, P. Padungtod, and R. Chuanchuen. 2017. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand-Cambodia border provinces: AMR in *Salmonella enterica*. *Microbiol. Immunol.* 61:23–33.

40. U.S. Department of Agriculture, Food Safety and Inspection Service. 2013. Directive 10300.1 Revision 1 - Intensified verification testing (IVT) protocol for sampling of

product, food contact surfaces and environmental surfaces for *Listeria monocytogenes* (Lm) or *Salmonella* spp. U.S. Department of Agriculture, Washington, DC.

41. U.S. Department of Agriculture, Food Safety and Inspection Service. 2018. Isolation and identification of *Salmonella* from meat, poultry, pasteurized egg, and siluriformes (fish) products and carcass and environmental sponges. U.S. Department of Agriculture, Washington, DC.

42. Vipham, J. L., B. D. Chaves, and V. Trinetta. 2018. Mind the gaps: how can food safety gaps be addressed in developing nations? *Anim. Front.* 8:16–25.

43. Vital, P. G., K. G. B. Dimasuay, K. W. Widmer, and W. L. Rivera. 2014. Microbiological quality of fresh produce from open air markets and supermarkets in the Philippines. *Sci. World J.*

44. Widmer, K., N. T. Van Ha, S. Vinitnantharat, S. Sthiannopkao, S. Wangsaatmaja, M. A. N. Prasetiati, N. C. Thanh, K. Thepnoo, A. D. Sutadian, H. T. T. Thao, D. Fapyane, V. San, P. Vital, and H. G. Hur. 2013. Prevalence of *Escherichia coli* in surface waters of Southeast Asian cities. *World J. Microbiol. Biotechnol.* 29:2115–2124.

45. Zhang, J., D. Kuang, F. Wang, J. Meng, H. Jin, X. Yang, M. Liao, J. D. Klena, S. Wu, Y. Zhang, and X. Xu. 2016. Turtles as a possible reservoir of nontyphoidal *Salmonella* in Shanghai, China. *Foodborne Pathog. Dis.* 13:428–433.

46. Zhang, L., Y. Fu, Z. Xiong, Y. Ma, Y. Wei, X. Qu, H. Zhang, J. Zhang, and M. Liao. 2018. Highly prevalent multidrug-resistant *Salmonella* from chicken and pork meat at retail markets in Guangdong, China. *Front. Microbiol.* 9:2104.

47. World Health Organization. 2015. WHO estimates of the global burden of foodborne diseases. World Health Organization, Geneva, Switzerland.

Chapter 3 - Draft genome sequences of 81 *Salmonella enterica* strains from informal markets in Cambodia

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3.1 Abstract

Salmonella enterica is an important global pathogen due to its contribution to human morbidity and death. The presence of *S. enterica* in Southeast Asian informal markets is amplified by cross-contamination between market surfaces and food products. Here, we describe the draft genome sequences of 81 *Salmonella enterica* isolates from informal markets in Cambodia.

3.2 Introduction

Non-typhoidal *Salmonella enterica* (*S. enterica*) causes the most cases of diarrheal disease worldwide and is a pathogen of global importance, particularly in low-income countries (LICs) (1). Major data gaps in LICs stem from inadequate national monitoring and surveillance programs. Whole genome sequencing (WGS) can provide accurate information on foodborne pathogens (2). The availability of molecular data is crucial for improving food safety outcomes in LICs and understanding foodborne outbreaks and transmission pathways.

3.3 Material and methods

Herein we describe the draft-genome sequences of 81 *S. enterica* isolates collected from two Cambodian informal markets (i.e., those escaping food safety oversight) (3). Sixty-four vendors classified by market location (inside versus outside) were sampled during the rainy (June 2018) and dry (January 2019) seasons. For each vendor, samples from three food contact surfaces (FCSs) and three non-food contact surfaces (NFCSs) were collected, totaling 384 samples. Screening and isolation of *S. enterica* strains were conducted according to the U.S. Department of Agriculture Food Safety and Inspection Service guidelines (3). Samples were enriched with buffered peptone water, followed by selective enrichments of tetrathionate bile broth and Rappaport-Vassiliadis broth (HiMedia). Selective enrichments were streaked onto xylose-lysine-Tergitol 4 and brilliant green sulfa agar (HiMedia). Typical colonies were tested using the *Salmonella* latex agglutination kit (Oxoid, Basingstoke, UK), and presumptive isolates were confirmed by PCR (4). Genomic DNA was obtained using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), following manufacturer instructions. Libraries were prepared from genomic DNA with the Nextera XT DNA library preparation kit, and WGS was conducted on either a MiSeq or NextSeq sequencer, using a MiSeq reagent kit v2 (500-cycles) or a NextSeq 500/550 high-output kit v2 (300-cycles), respectively (Illumina, San Diego, CA). Minimum sequence quality was represented by average coverage of >30× and Q scores for reads 1 and 2 of >30 (5). Absence of contamination was affirmed with Kraken (6). Default parameters were used for all software unless otherwise specified. *De novo* assemblies were generated with SKESA v. 2.2 either by NCBI Pathogen Detection or in-house using GalaxyTrakr with standard settings (7, 8). Antimicrobial resistance (AMR), stress and virulence genotypes were available at NCBI Pathogen Detection (9). *Salmonella* pathogenicity islands (SPIs) were identified with SPIFinder

(https://cge.cbs.dtu.dk/services/) (Center for Genomic Epidemiology). Draft genomes were annotated using the NCBI's Prokaryotic Genome Annotation Pipeline (PGAP) (10). The serotype of each isolate was determined *in-silico* using SeqSero v1.0 on draft genomes (11).

3.4 Results

Draft genomes ranged between 4,609,585 and 5,198,930 bp in size, with an average GC content of 52.4%. The number of contigs for each isolate ranged from 23 to 468. A total of 16 *S. enterica* serotypes were identified, including Rissen (n = 19); Hvittingfoss (n = 13); Corvallis (n = 10); Krefeld (n = 8); Weltevreden (n = 6); Altona (n = 6); Mbandaka (n = 5); Typhimurium (n = 3); Javiana, Uganda, and Derby (n = 2 each); Anatum, Braenderup, Lexington, Virchow, and the potential monophasic variant of *S. enterica* serotype Typhimurium (I 4,[5],12:i:–) (n = 1 each). AMR genes, including those encoding for tetracycline, β-lactam, sulfonamide, quinolone, aminoglycoside, phenicol, trimethoprim, and fosfomycin resistance, were identified in 43 of 81 strains. A total of 10 SPIs were detected in 59 genomes (Table 3.1) (12, 13).

3.5 Conclusions

The draft genome sequences described will be valuable for understanding the dynamics of *S. enterica* diversity and persistence in informal markets in Cambodia, as well as supporting global epidemiological investigations of outbreaks.

3.6 Data availability

This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession numbers presented in Table 3.1. The versions described in this paper are the first versions.

3.7 Acknowledgments

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Table 3.1. Metadata for the 81 *Salmonella enterica* strains isolated from informal markets in Cambodia.

Sample no.[a]	NCBI BioSample no.	SRA accession no.	GenBank accession no.	No. of contigs	Total length (bp)	N_{50} (bp)	GC content (%)	Coverage (×)	Avg quality scores (read 1, read 2)	No. of reads	Serotype	SPI(s)	AMR gene(s)
Isolates collected in June 2018													
94565 (Inside)	SAMN12663190	SRR10050324	JABGCS000000000	69	4,873,866	219,252	52.4	70	36, 34	1,418,030	*S.* Typhimurium	1, 3, 4, 5, 13, 14, C63PI	None
94567 (Inside)	SAMN12668245	SRR10051127	AAKBCQ000000000	57	4,955,643	172,452	52.5	44	36, 33	897,000	*S.* I 4,[5],12:i:-	1, 3, 5, 13, 14, C63PI	aph(3")-Ib, aph(6)-Id, blaTEM-1, sul2, tet(B)
94568 (Inside)	SAMN12668243	SRR10051124	AAKBCK000000000	55	5,198,930	373,560	51.9	55	36, 34	1,108,382	*S.* Derby	1, 3, C63PI	aac(3)-IId, blaCTX-M-14, floR, fosA7, qnrS1, sul2, tet(A), tet(M)
94569 (Inside)	SAMN12668230	SRR10051109	AAKBBW000000000	26	4,701,855	499,670	52.5	70	36, 34	1,421,354	*S.* Hvittingfoss	1, 3, 4, 5, 13, 14, C63PI	None
94571 (Inside)	SAMN12662740	SRR10049763	AAKAXV000000000	69	5,184,225	226,413	52.2	50	31, 33	995,476	*S.* Derby	3, C63PI	aac(3)-IId, blaCTX-M-14, floR, fosA7, qnrS1, sul2, tet(A), tet(M)
94572 (Inside)	SAMN12663184	SRR10050161	AAKBBL000000000	32	4,680,319	331,356	52.6	55	32, 33	1,111,732	*S.* Altona	9, C63PI	None
94573 (Inside)	SAMN12663133	SRR10050104	AAKBAJ000000000	29	4,680,915	513,284	52.7	62	32, 33	1,250,594	*S.* Altona	1, 3, C63PI	None
94574 (Outside)	SAMN12667774	SRR10051051	AAKBBS000000000	47	4,925,460	283,403	52.5	100	32, 34	2,006,780	*S.* Rissen	C63PI	aph(3")-Ib, aph(6)-Id, blaTEM-1, tet(A)
94575 (Outside)	SAMN12663163	SRR10050134	AAKBAN000000000	27	4,677,013	410,252	52.5	48	32, 33	969,076	*S.* Javiana	13, 14, C63PI	None
94577 (Outside)	SAMN12667725	SRR10050996	AAKBBO000000000	29	4,679,187	328,974	52.7	41	31, 32	824,578	*S.* Altona	C63PI	None
94584 (Outside)	SAMN13293250	SRR10452822	AAPREJ000000000	47	4,889,561	256,881	52.2	52	33*, 32*	1,702,736	*S.* Typhimurium	5, 13, 14, C63PI	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, qacEdelta1, qacL, sul1, sul3, tet(A)
94585 (Outside)	SAMN13293256	SRR10452813	AAQIHK000000000	55	4,916,559	278,635	52.3	73	33*, 32*	2,348,336	*S.* Rissen	C63PI	aph(3")-Ib, aph(6)-Id, blaTEM-1, tet(A)
94586 (Outside)	SAMN13293254	SRR10452812	AAPDIY000000000	33	4,735,977	237,700	52.5	43	33*, 32*	1,410,512	*S.* Uganda	13, 14	None
94587 (Outside)	SAMN13293452	SRR10454469	AAPDKA000000000	47	5,006,945	303,350	52.4	68	33*, 32*	2,229,632	*S.* Rissen	C63PI	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, qacEdelta1, qacL, sul1, sul3, tet(A)
94588 (Outside)	SAMN13293455	SRR10454468	AAQJIL000000000	23	4,738,724	454,824	52.7	98	33*, 32*	3,189,200	*S.* Uganda	13, 14, C63PI	None

94590 (Outside)	SAMN13293118	SRR10452790	AAPCHN000000000	59	4,918,011	213,300	52.6	94	33*, 32*	3,046,128	*S.* Rissen	C63PI	aph(3")-Ib, aph(6)-Id, blaTEM-1, tet(A)
94591 (Inside)	SAMN13293114	SRR10452791	AAQOJH000000000	49	4,918,530	223,933	52.5	93	33*, 32*	3,015,232	*S.* Rissen	none	aph(3")-Ib, aph(6)-Id, blaTEM-1, tet(A)
94592 (Inside)	SAMN13293095	SRR10452788	AAPCJA000000000	29	4,679,294	397,032	52.8	102	33*, 32*	3,329,360	*S.* Altona	C63PI	None
94593 (Inside)	SAMN13293094	SRR10452789	AAQNRR000000000	67	4,960,359	271,790	52.6	99	33*, 32*	3,194,896	*S.* Krefeld	1, 3, C63PI	aph(3")-Ib, aph(6)-Id, blaTEM-1, sul2, tet (A), tet(B)
94595 (Outside)	SAMN13293187	SRR10452793	AAPCIY000000000	201	4,824,904	172,092	51.8	48	33*, 32*	1,598,464	*S.* Altona	none	None
94597 (Outside)	SAMN13293518	SRR10454708	AAPMUB000000000	68	4,687,319	151,326	52	41	33*, 32*	1,373,328	*S.* Altona	none	None
94599 (Outside)	SAMN13293252	SRR10452811	AAPCIC000000000	468	5,167,830	84,530	51.2	30	33*, 32*	998,032	*S.* Rissen	none	aph(3")-Ib, aph(6)-Id, blaTEM-1, tet(A)
Isolates collected in January 2019													
96602 (Inside)	SAMN13293236	SRR10452800	AAPUPR000000000	53	4,846,101	252,467	52.3	70	33*, 32*	2,278,400	*S.* Anatum	3, 13, 14, C63PI	aadA1, blaLAP-2, blaTEM-1, cmlA1, qacL, qnrS1, sul3, tet(A)
96603 (Inside)	SAMN13321513	SRR10483448	AAPLYZ000000000	72	4,997,549	222,935	52.4	74	33*, 32*	2,417,008	*S.* Rissen	none	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96604 (Inside)	SAMN13321512	SRR10483450	AAPMWO000000000	56	4,914,011	238,068	52.3	56	33*, 32*	1,833,712	*S.* Rissen	none	aph(3")-Ib,aph(6)-Id, blaTEM-1,tet(A)
96606 (Inside)	SAMN13321510	SRR10483455	AAOZWR000000000	84	4,609,585	110,513	52.4	30	33*, 32*	1,011,424	*S.* Javiana	13	None
96608 (Inside)	SAMN13321506	SRR10483451	AAPBBZ000000000	125	4,983,354	99,781	52.5	35	33*, 32*	1,265,040	*S.* Rissen	none	aadA1, aadA2, blaTEM, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96609 (Inside)	SAMN13321503	SRR10483453	AAPCYP000000000	102	4,993,067	148,483	52.3	37	33*, 32*	1,255,248	*S.* Rissen	none	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96610 (Inside)	SAMN13322387	SRR10484095	AAQHJG000000000	104	4,979,073	139,864	52.5	36	33*, 32*	1,211,136	*S.* Rissen	none	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96611 (Inside)	SAMN13321502	SRR10483452	AAQKJJ000000000	94	4,994,667	133,140	52.5	51	33*, 32*	1,698,016	*S.* Rissen	none	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96612 (Outside)	SAMN13321624	SRR10483525	AAOYHR000000000	91	4,994,881	170,117	52.5	48	33*, 32*	1,603,888	*S.* Rissen	none	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96613 (Outside)	SAMN13321528	SRR10483478	AAPUSA000000000	86	4,994,170	186,856	52.4	45	33*, 32*	1,488,848	*S.* Rissen	none	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)

96614 (Outside)	SAMN13322384	SRR10484096	AAOZZM000000000	50	4,937,735	231,881	52.4	41	33*, 32*	1,361,888	*S.* Mbandaka	none	tet(A)
96615 (Outside)	SAMN13322378	SRR10484097	AAPMWP000000000	97	4,994,949	130,669	52.3	36	33*, 32*	1,196,224	*S.* Rissen	none	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96616 (Outside)	SAMN13322423	SRR10484100	AAOVVC000000000	91	4,755,022	101,724	52.5	31	33*, 32*	1,037,840	*S.* Corvallis	none	qnrS1
96617 (Outside)	SAMN13322444	SRR10484327	AAPRFQ000000000	46	4,763,876	292,857	52.5	51	33*, 32*	1,696,144	*S.* Corvallis	none	qnrS1
96618 (Outside)	SAMN13322169	SRR10483546	AAPBYN000000000	41	4,763,794	260,239	52.5	59	33*, 32*	1,959,120	*S.* Corvallis	3	qnrS1
96619 (Outside)	SAMN13321508	SRR10483454	AAQKQE000000000	36	4,772,637	292,860	52.6	81	33*, 32*	2,662,560	*S.* Corvallis	3, C63PI	qnrS1
96620 (Outside)	SAMN13321532	SRR10483477	AAOXFD000000000	97	5,023,926	107,850	52.7	83	33*, 32*	2,727,728	*S.* Weltevreden	13, 14, C63PI	None
96622 (Outside)	SAMN13321595	SRR10483508	AAOXHT000000000	127	4,992,049	86,615	52.4	32	33*, 32*	1,060,272	*S.* Weltevreden	none	None
96623 (Outside)	SAMN13321591	SRR10483504	AAOWPT000000000	54	4,884,751	162,476	52.3	44	33*, 32*	1,426,064	*S.* Hvittingfoss	13, 14	None
96624 (Outside)	SAMN13321587	SRR10483507	AAQMOL000000000	111	5,011,432	104,441	52.6	51	33*, 32*	1,674,544	*S.* Weltevreden	13, 14	None
96625 (Outside)	SAMN13321619	SRR10483514	AAOYAQ000000000	103	5,015,404	104,982	52.6	52	33*, 32*	1,694,784	*S.* Weltevreden	13, 14	None
96626 (Outside)	SAMN13321607	SRR10483502	AAPAQG000000000	121	4,988,881	99,968	52.3	32	33*, 32*	1,070,256	*S.* Weltevreden	none	None
96627 (Outside)	SAMN13321604	SRR10483509	AAPUXJ000000000	46	4,886,976	225,268	52.3	41	33*, 32*	1,347,840	*S.* Hvittingfoss	13, 14	None
96628 (Outside)	SAMN13321600	SRR10483506	AAOXYW000000000	117	4,997,196	94,034	52.4	38	33*, 32*	1,269,280	*S.* Weltevreden	13, 14	None
96629 (Outside)	SAMN13321598	SRR10483510	AAOXFL000000000	40	4,763,596	256,627	52.3	38	33*, 32*	1,262,464	*S.* Corvallis	none	qnrS1
96630 (Outside)	SAMN13322150	SRR10483543	AAPRFR000000000	64	4,885,698	149,476	52.7	50	33*, 32*	1,625,728	*S.* Hvittingfoss	13	None
96631 (Outside)	SAMN13321593	SRR10483511	AAQIBY000000000	69	4,880,849	136,339	52.5	33	33*, 32*	1,087,728	*S.* Hvittingfoss	13, 14, C63PI	None
96632 (Outside)	SAMN13321589	SRR10483512	AAQFWT000000000	76	4,882,637	112,271	52.6	39	33*, 32*	1,274,576	*S.* Hvittingfoss	13, 14	None
96633 (Outside)	SAMN13322151	SRR10483544	AAPBIM000000000	48	4,888,754	163,819	52.5	41	33*, 32*	1,339,280	*S.* Hvittingfoss	13, 14	None
96634 (Outside)	SAMN13293471	SRR10454525	AAPDHS000000000	45	4,886,448	297,538	52.5	46	33*, 32*	1,568,144	*S.* Hvittingfoss	3, 13, 14	None
96635 (Outside)	SAMN13293474	SRR10454524	AAPCTJ000000000	47	4,888,472	163,860	52.6	65	33*, 32*	2,130,128	*S.* Hvittingfoss	5, 9, 13, 14, C63PI	None
96636	SAMN13293495	SRR10454571	AAOVNP000000000	38	4,941,926	231,887	52.4	61	33*, 32*	1,975,648	*S.* Mbandaka	none	tet(A)

(Outside)												
96637 (Outside)	SAMN13293478	SRR10454554	AAOZSX000000000	34	4,986,876	336,928	52.1	10233*, 32*	3,260,192	*S.* Mbandaka	C63PI	tet(A)
96638 (Inside)	SAMN13293513	SRR10454709	AAPURY000000000	39	4,941,739	234,898	52.3	79 33*, 32*	2,583,600	*S.* Mbandaka	none	tet(A)
96639 (Inside)	SAMN13293559	SRR10454722	AAPREC000000000	36	4,890,402	263,956	52.3	60 33*, 32*	1,972,576	*S.* Hvittingfoss	5, 12, 13, 14, C63PI	None
96640 (Inside)	SAMN13293548	SRR10454720	AAPUPU000000000	37	4,891,877	225,246	52.4	69 33*, 32*	2,235,248	*S.* Hvittingfoss	5, 13, 14, C63PI	None
96641 (Inside)	SAMN13293552	SRR10454721	AAPBFP000000000	35	4,892,034	237,120	52.2	88 33*, 32*	2,925,152	*S.* Hvittingfoss	5, 12, 13, 14, C63PI	None
96642 (Inside)	SAMN13293550	SRR10454719	AAQJIM000000000	46	4,756,930	279,551	52.4	84 33*, 32*	2,787,552	*S.* Virchow	12, 13, 14	None
96643 (Inside)	SAMN13293560	SRR10454725	AAPBTO000000000	38	4,983,844	199,234	52.5	13933*, 32*	4,528,976	*S.* Mbandaka	C63PI	tet(A)
96644 (Inside)	SAMN13293554	SRR10454718	AAQGLD000000000	39	4,891,900	236,887	52.6	11633*, 32*	3,775,040	*S.* Hvittingfoss	5, 13, 14, C63PI	None
96645 (Inside)	SAMN13293468	SRR10454522	AAOWKQ000000000	50	4,957,603	240,177	52.1	14133*, 32*	4,515,936	*S.* Corvallis	C63PI	None
96646 (Inside)	SAMN13293464	SRR10454477	AAQFPZ000000000	41	4,959,060	266,854	52.3	12933*, 32*	4,143,408	*S.* Corvallis	1, 3, 8, C63PI	None
96647 (Inside)	SAMN13293481	SRR10454526	AAQMBA000000000	52	4,963,011	260,889	52.1	15233*, 32*	4,875,888	S. Lexington	C63PI	None
96648 (Inside)	SAMN13293179	SRR10452792	AAQIFP000000000	50	4,884,485	278,698	52.3	14233*, 32*	4,544,784	*S.* Typhimurium	1, 5, 9, 13, 14, C63PI	None
96649 (Inside)	SAMN13293237	SRR10452801	AAQIIF000000000	55	4,886,407	271,030	52.3	19333*, 32*	6,212,320	S. Rissen	none	None
96650 (Inside)	SAMN13293340	SRR10453836	AAQLUZ000000000	59	4,925,157	233,820	52.2	21933*, 32*	7,025,728	*S.* Krefeld	13, 14, C63PI	aph(3")-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1, sul2, tet(B)
96651 (Inside)	SAMN13293437	SRR10453845	AAOVOL000000000	57	4,914,721	233,820	52.2	18633*, 32*	5,968,960	*S.* Krefeld	1, 3, 8, C63PI	aph(3")-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1, sul2, tet(B)
96652 (Inside)	SAMN13293509	SRR10454712	AAQLVG000000000	59	4,926,743	233,820	52.2	15633*, 32*	4,990,512	*S.* Krefeld	1, 3, 8, C63PI	aph(3")-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1, sul2, tet(B)
96653 (Outside)	SAMN13293549	SRR10454716	AAOYDR000000000	57	4,920,767	277,388	52.2	84 33*, 32*	2,692,272	*S.* Krefeld	3, C63PI	aph(3")-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1, sul2, tet(B)
96654 (Outside)	SAMN13293562	SRR10454724	AAQJMM000000000	62	4,927,585	233,696	52.3	18233*, 32*	5,832,112	*S.* Krefeld	C63PI	aph(3")-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1, sul2, tet(B)
96655 (Outside)	SAMN13293551	SRR10454714	AAQGIL000000000	59	4,924,859	276,634	52.2	12233*, 32*	3,896,496	*S.* Krefeld	3, 8, C63PI	aph(3")-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1, sul2, tet(B)
96656 (Outside)	SAMN13293561	SRR10454723	AAPMVH000000000	50	4,961,397	365,146	52.3	18133*, 32*	5,840,736	*S.* Krefeld	1, 3, 8, C63PI	None
96657 (Outside)	SAMN13293555	SRR10454717	AAQKVT000000000	37	4,971,264	382,215	52.2	12533*, 32*	4,059,616	*S.* Corvallis	3, 8, C63PI	aph(3")-Ib, aph(6)-Id, qnrS1, sul2, tet(A)

96658 (Outside)	SAMN13293462	SRR10454521	AAOVOG000000000	35	4,970,109	464,760	52.1	12333*, 32*	3,959,344	*S.* Corvallis	3, 8, C63PI	aph(3")-Ib, aph(6)-Id, qnrS1, sul2, tet(A)
96659 (Outside)	SAMN13293326	SRR10452834	AAQLZF000000000	39	4,737,400	337,520	52.4	12633*, 32*	4,068,208	*S.* Braenderup	13, 14, C63PI	None
96660 (Outside)	SAMN13293349	SRR10452835	AAPDJJ000000000	79	5,000,081	222,935	52.3	14533*, 32*	4,664,640	*S.* Rissen	8, C63PI	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96661 (Outside)	SAMN13293344	SRR10453838	AAQIFK000000000	80	5,004,186	222,935	52.1	14833*, 32*	4,735,072	*S.* Rissen	8, 9, C63PI	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96662 (Outside)	SAMN13293463	SRR10454475	AAQINH000000000	79	5,002,383	222,935	52.3	16933*, 32*	5,406,656	*S.* Rissen	8, C63PI	aadA1, aadA2, blaTEM-1, cmlA1, dfrA12, floR, qacL, qnrS1, sul2, sul3, tet(A), tet(M)
96663 (Outside)	SAMN13293461	SRR10454463	AAPLYY000000000	32	4,969,793	502,099	52	12833*, 32*	4,091,808	*S.* Corvallis	C63PI	aph(3")-Ib, aph(6)-Id, qnrS1, sul2, tet(A)

[a] Samples collected from food contact surfaces (FCSs) are underlined, while the rest were collected from non-food contact surfaces (NFCSs); the location of the vendor is indicated as inside or outside. All isolates belong to BioProject number PRJNA628951.
[b] NextSeq run with an average of four lanes.

3.8 References

1. World Health Organization. WHO estimates of the global burden of foodborne diseases. 2015. World Health Organization, Geneva, Switzerland. https://www.who.int/foodsafety/publications/foodborne_disease/fergreport/en/.

2. Harris SR, Okoro CK. 2014. Whole-genome sequencing for rapid and accurate identification of bacterial transmission pathways, p. 123–152. In Methods in Microbiology. Elsevier. https://doi.org/10.1016/bs.mim.2014.07.003.

3. U.S. Department of Agriculture, Food Safety and Inspection Service. 2018. Isolation and identification of *Salmonella* from meat, poultry, pasteurized egg, and siluriformes (fish) products and carcass and environmental sponges, 4.09. In Laboratory guidebook. US Department of Agriculture Food Safety and Inspection Service, Washington, DC.

4. Bai J, Trinetta V, Shi X, Noll LW, Magossi G, Zheng W, Porter EP, Cernicchiaro N, Renter DG, Nagaraja TG. 2018. A multiplex real-time PCR assay, based on invA and pagC genes, for the detection and quantification of *Salmonella enterica* from cattle lymph nodes. Journal of Microbiological Methods 148:110–116. https://doi.org/10.1016/j.mimet.2018.03.019.

5. Timme RE, Sanchez Leon M, Allard MW. 2019. Utilizing the public GenomeTrakr database for foodborne pathogen traceback. In: Bridier A. (eds) Foodborne Bacterial Pathogens. Methods in Molecular Biology, vol 1918. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-9000-9_17.

6. Wood DE, Salzberg SL: Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biology 2014, 15:R46.

7. Souvorov A, Agarwala R, Lipman DJ. 2018. SKESA: strategic k-mer extension for scrupulous assemblies. Genome Biol 19:153. https://doi.org/10.1186/s13059-018-1540-z.

8. Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Grüning BA, Guerler A, Hillman-Jackson J, Hiltemann S, Jalili V, Rasche H, Soranzo N, Goecks J, Taylor J, Nekrutenko A, Blankenberg D. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Research 46:W537–W544. https://doi.org/10.1093/nar/gky379.

9. Feldgarden M, Brover V, Haft DH, Prasad AB, Slotta DJ, Tolstoy I, Tyson GH, Zhao S, Hsu C-H, McDermott PF, Tadesse DA, Morales C, Simmons M, Tillman G, Wasilenko J, Folster JP, Klimke W. 2019. Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. Antimicrob Agents Chemother 63:e00483-19, /aac/63/11/AAC.00483-19.atom. https://doi.org/10.1128/AAC.00483-19.

10. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M, Ostell J. 2016. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res 44:6614–6624. https://doi.org/10.1093/nar/gkw569.

11. Zhang S, Yin Y, Jones MB, Zhang Z, Deatherage Kaiser BL, Dinsmore BA, Fitzgerald C, Fields PI, Deng X. 2015. *Salmonella* serotype determination utilizing high-throughput

genome sequencing data. J Clin Microbiol 53:1685–1692. https://doi.org/10.1128/JCM.00323-15.

12. Amavisit P, Lightfoot D, Browning GF, Markham PF. 2003. Variation between pathogenic serovars within *Salmonella* pathogenicity islands. Journal of Bacteriology 185:3624–3635. https://doi.org/10.1128/JB.185.12.3624-3635.2003.

13. Chen L, Xiong Z, Sun L, Yang J, Jin Q. 2012. VFDB 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. Nucleic Acids Research 40:D641–D645. https://doi.org/10.1093/nar/gkr989.

Chapter 4 - Genotypic and phenotypic characterization of antimicrobial resistance profiles in non-typhoidal *Salmonella* strains isolated from Cambodian informal markets.

4.1 Abstract

Non-typhoidal *Salmonella enterica* (*Salmonella*) is a pathogen of global importance, particularly in low and middle-income countries (LMICs). The presence of antimicrobial-resistant (AMR) strains in market environments poses a serious health threat to consumers. The aims of this study were to identify and characterize the genotypic and phenotypic antimicrobial resistance profiles from 81 *Salmonella* strains isolated from environmental samples from informal markets in Cambodia in 2018-2019. Antimicrobial resistance (AMR), stress, and virulence genotypes were retrieved from the NCBI Pathogen Detection engine. *Salmonella* pathogenicity islands (SPIs) were identified with SPIFinder (Center for Genomic Epidemiology - https://cge.cbs.dtu.dk/services/). Antimicrobial susceptibility profiles were determined by Sensititre automated antimicrobial susceptibility system with the National Antimicrobial Resistance Monitoring System (NARMS) gram-negative CMV3AGNF plate. A total of 23 unique resistance genes were detected. AMR genes were identified in 43 out of 81 isolates, including those encoding tetracycline, beta-lactam, sulfonamide, quinolone, aminoglycoside, phenicol, trimethoprim, and fosfomycin resistance. A total of 10 SPIs (SPI-1, 3-5, 8, 9, 12-14, and centisome 63 [C63PI]) were detected in 59 isolates. C63PI, an iron transport system in SPI-1, was observed in 56% of the isolates (n=46). SPI-1, SPI-4 and SPI-9 were present in 13%, 2%, and 5% of the isolates, respectively. This study will contribute to understanding the AMR, stress, and virulence genes present in *Salmonella* from

informal markets in Cambodia, as well as support domestic epidemiological investigations of multidrug resistance (MDR) profiles.

4.2 Introduction

Informal markets are common in Southeast Asian countries. In Cambodia, these markets play an important role in the country's economy, culture, and lifestyle. In fact, a variety of food products (e.g., fresh vegetables, fruits, seafood, and animal products) are sold through these markets, which often lack basic food safety infrastructure or oversight (*33*). As a consequence, cross-contamination between surfaces and food products is recurrent in these markets. Studies have shown a high prevalence of foodborne pathogens, specifically non-typhoidal *Salmonella enterica* (henceforth referred to as *Salmonella*) in food products and surfaces in the informal market settings in Cambodia (*20*, *29*, *42*).

Salmonella is the number one cause of diarrheal disease worldwide. This pathogen has been linked to many foodborne outbreaks in several countries (*7*, *10*, *18*). In the United States, approximately 94% of *Salmonella* infections are attributed to food (*14*). Moreover, *Salmonella* is considered a pathogen of global importance, particularly in low-income countries such as Cambodia (*47*). In Cambodia, however, surveillance data on foodborne disease is limited. Recently, the Mekong Institute (an intergovernmental organization founded by Southeast Asian countries) revealed that thousands of people suffer from unsafe food in Cambodia (*26*). Between 2014-2019, Cambodia's Food Safety Bureau and the Department of Drug and Food (DDF) reported 134 foodborne outbreaks, resulting in 5,825 illnesses, 5,598 hospitalizations, and 81 deaths (*26*). Although informative, these estimates are likely underreported as indicated by the high percentage of illnesses that resulted in hospitalizations (96%), with the actual number of cases likely being much greater.

The lack of regulatory oversight, infrastructure, potable water, and adequate hygiene and sanitation practices have been identified as the leading causes for outbreaks (26) and previously described as a common sight among informal markets in Cambodia (*33*). These factors often promote cross-contamination among food products, food contact surfaces and food handlers, favoring the spread of pathogens. The prevalence of *Salmonella* in the Cambodian informal market system has been previously determined, suggesting that cross-contamination is likely occurring (*37*). The ubiquity of *Salmonella* and its ability to cause human infections demonstrates that research needs to be conducted to better understand its persistence and virulence factors (i.e., antimicrobial resistance) in serotypes present in these markets.

The indiscriminate use of antibiotics in food production (i.e., food animals) has raised immense concern among public health authorities. It contributes to the development and spread of AMR among different pathogens (*23*). The spread of AMR bacteria threatens the ability of public health groups to effectively treat bacterial infections, potentially leading to an extended illness period, disability, or death (*45*). Surveillance and monitoring of AMR bacteria has been identified as a global health priority by the WHO (*45*). Several developed nations (e.g., United States, Switzerland, and Australia) have conducted extensive research in identifying AMR foodborne pathogens (*3*, *17*, *49*). However, limited studies have been conducted to assess the prevalence and genetic markers of AMR foodborne pathogens in Cambodia, where awareness and understanding of AMR is insufficient and needs to be investigated (*48*). The aims of this study were to identify and characterize the genotypic and phenotypic antimicrobial resistance profiles from *Salmonella* strains isolated from environmental samples collected at two informal markets in Cambodia in 2018-2019.

4.3 Material and methods

4.3.1 Bacterial isolates and sample collection

A total of 81 *Salmonella* strains were used in this study. The strains were isolated in 2018 (June) and 2019 (January) from environmental samples collected from two informal markets in Cambodia (*37*). The strains were stored in cryobeads (Key Scientific Products INC, Stamford, TX-USA), following manufacturer protocol and were stored at -80° ± 2.0° C until further analysis. Each isolate was assigned a unique U.S. Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition (CFSAN) identification number as part of the GenomeTrakr network.

4.3.2 DNA preparation

Genomic DNA from each strain was obtained using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany), following manufacturer instructions. DNA concentration was determined using a Qubit 4.0 fluorometer (Invitrogen). DNA samples were sent to the FDA-CFSAN for Whole Genome Sequencing (WGS). The resultant DNA extract was stored at -20°C until WGS analysis.

4.3.3 Whole genome sequencing and serovar prediction

Libraries were prepared from genomic DNA with the Nextera XT DNA Library Preparation Kit, and WGS was carried out on either the MiSeq or NextSeq sequencer, using a MiSeq Reagent Kit V2 (500-cycles) or a NextSeq 500/550 High-Output Kit V2 (300-cycles), respectively (Illumina). De novo assemblies were obtained with Shovill 0.9 (https://github.com/tseemann/shovill). The serotype of each isolate was determined in-silico using SeqSero 1.0 on draft genomes (http://denglab.info/SeqSero).

4.3.4 Detection of antimicrobial resistance, stress and virulence genes

Antimicrobial resistance, stress and virulence genotypes were available on NCBI Pathogen Detection (*6*). *Salmonella* pathogenicity islands (SPIs) were identified with SPIFinder (Center for Genomic Epidemiology - https://cge.cbs.dtu.dk/services/). The reference database used included acquired genes and mutations known to confer resistance to the antimicrobials aminoglycosides, β-lactams, colistin, fluoroquinolones, fosfomycin, macrolides, phenicols, rifampicin, sulfonamides, tetracyclines, and trimethoprim (*52*).

4.3.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility profiles were determined using the Sensititre automated antimicrobial susceptibility system (ThermoFisher Scientific, Waltham, MA) with the National Antimicrobial Resistance Monitoring System (NARMS) gram-negative CMV3AGNF plate (*21*). Fourteen antimicrobials were tested: amoxicillin/clavulanic acid 2:1 ratio (AUG2), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (TIF), ceftriaxone (AXO), ciprofloxacin (CIP), chloramphenicol (CHL), gentamycin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT), representing nine antimicrobial classes defined by the Clinical and Laboratory Standards Institute (CLSI; Clinical and Laboratory Standards Institute 2020). The minimum inhibitory concentration (MIC) for each antimicrobial was interpreted using the CLSI standards and NARMS breakpoints to categorize MIC results as susceptible or resistant (*4*, *9*).

4.3.6 Agreement between genotypic and phenotypic susceptibility

A total of 1,863 genotypic and 1,134 phenotypic data points were generated from the 81 isolates. An isolate was classified as genotypically resistant when presenting at least one gene known to confer resistance to a given antimicrobial agent, and susceptible otherwise. An isolate

was classified as phenotypically resistant to a given antimicrobial agent when presenting an MIC equal to or greater than the resistant threshold based on CLSI standards and NARMS breakpoints, and susceptible otherwise. Intermediate phenotypes were considered as susceptible in this analysis. Overall and antibiotic-specific sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated considering genotypic (predicted) and phenotypic (observed) resistant/susceptible classification using the function *confusionMatrix* from the package caret (*19*) in R (*32*).

4.3.7 Accession number(s)

WGS data of the 81 *Salmonella* isolates were submitted to the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA628951 as previously described by Schwan et al. (*36*).

4.4 Results

Among the 81 total isolates that were characterized, the most common phenotypically observed resistances were to tetracycline (47%; n=38), ampicillin (37%; n=30), streptomycin (20%; n=16), chloramphenicol (17%; n=14), and trimethoprim-sulfamethoxazole (16%; n=13). Low levels of resistance were detected to ceftriaxone (4%; n=3), ceftiofur (4%, n=3), cefoxitin (2%; n=2), and gentamicin (2%; n=2). Among the strains selected, 49% (n=40) presented no resistance to any of the tested antimicrobial compounds.

4.4.1 Comparison between genotypic and phenotype AMR profiles

The overall concordance between phenotypic resistance with the presence of known resistance genes was 78.3%, with genotype agreeing with phenotype for 889 of 1,134 of the phenotypic tests. In total, 121 of the 1,134 antibiotic tests indicated resistance, and associated genes were predicted to cause resistance in all but six instances, where four were related to beta-

lactams, one to aminoglycosides, and one to phenicol. The six instances of mismatch were observed in three serotypes, namely *S.* Altona, *S.* Hvittingfoss, and *S.* Virchow. This resulted in an overall sensitivity of 95% (115/121) (Table 4.1).

Among the 1,013 phenotypically susceptible test results, 239 had resistance genes detected by WGS, but were not identified phenotypically. Among these resistance genes, 116 encoded beta-lactam resistance, 49 encoded aminoglycoside resistance, 44 encoded quinolone resistance, 27 encoded folate pathway inhibitor resistance, and 3 encoded phenicol resistance (Table 4.1). The 239 instances of mismatch were observed in seven serotypes, namely *S.* Rissen (n=18), *S.* Corvallis (n=8), *S.* Krefeld (n=7), *S.* Derby (n=2), *S.* Anatum, *S.* Typhimurium, and *S.* I 4,[5],12:i:- (n=1, each). This resulted in an overall specificity of 76.4% (774/1013) (Table 4.1).

4.4.2 Resistance genes

Antimicrobial resistance genes were identified in 43 out of 81 isolates (53%), including those encoding tetracycline, beta-lactam, sulfonamide, quinolone, aminoglycoside, phenicol, trimethoprim, quaternary ammonium and fosfomycin resistance. A total of 23 unique resistance genes were detected, most commonly *tet*(A) (37%, n=30); $bla_{\text{TEM-1}}$ (35%, n=28); *sul2* (30%, n=24); *qnrS1*(27%, n=22); *aph(6)-Id* and *aph(3'')-Ib* (21%, n=17 each), followed by *sul3*, *qacL*, *cmlA1*, and *aadA1*(17%, n=14 each); *tet*(M), *floR*, *dfrA12*, and *aadA2* (16%, n=13 each); *tet*(B) (10%, n=8). Fewer strains presented *aph(3')-Ia* (7%, n=6); *aac(3)-IId*, $bla_{\text{CTX-M-14}}$, *fosA7*, *qacEΔ1*, and *sul1*(2%, n=2 each); and $bla_{\text{LAP-2}}$, and bla_{TEM} (1%, n=1each) resistance genes. Among all isolates, 38 presented no resistance genes (47%). Genes that are predicted to confer resistance to additional compounds (fosfomycin and disinfectants) were also identified. Seventeen isolates (21%) had genes encoding resistance to at least three classes of antibiotics (Table 4.2).

4.4.3 Resistance to β-lactams

A total of 4 genes encoding beta-lactamases were identified, with the most common being bla_{TEM-1} (present in 35% of the isolates), followed by three minor genes (*bla*$_{CTX-M-14}$, *bla*$_{LAP-2}$, and *bla*$_{TEM}$) each present in less than 5% of isolates (n=4). Genotypes predicted phenotypes with 89.4% sensitivity and 68.3% specificity. Results by antibiotic type are shown in Table 4.1.

4.4.4 Resistance to quinolone

Quinolone resistance is commonly observed within multiple mutations of the quinolone resistance-determining regions (QRDR) (i.e., *gyrA*, *gyrB*, *parC*, and *parE*) and/or one or multiple plasmid-mediated quinolone resistance (PMQR) genes (*15*, *30*, *35*, *39*, *51*). QRDR mutations and/or its combinations typically confer resistance to nalidixic acid and ciprofloxacin, respectively (*24*, *30*). Resistance mechanisms that involve a single plasmid-mediated gene typically do not confer resistance to ciprofloxacin or nalidixic acid, except when additional PMQR genes and/or QRDR mutations are present (*16*). In this study, 22 isolates carried only one PMQR gene (i.e., *qnrS1*). Resistance to ciprofloxacin or nalidixic acid was not observed in this study. Genotypic prediction for resistance resulted in a specificity of 72.8%. Results by drug are depicted in Table 4.1.

4.4.5 Resistance to aminoglycosides

Six different aminoglycoside resistance alleles were identified. Aminoglycoside phosphotransferase genes *aph(6)-Id* and *aph(3'')-Ib* (n=17 each), and *aph(3')-Ia* (n=6) were identified. Genes encoding aminoglycoside adenylyltransferases were identified in 27 isolates, most commonly *aadA1* (n=14) and *aadA2* (n=13). Further, two isolates carried an aminoglycoside acetyltransferase *aac(3)* variant (i.e., *aac(3)-IId*) that is commonly associated with resistance to gentamicin (*43*). The two isolates predicted to be resistant to gentamicin were also observed to be

phenotypically resistant to the same drug. Sensitivity and specificity for genotypic-phenotypic correlations were 94.4% and 65.9%, respectively. Results by antibiotic type are depicted in Table 4.1.

4.4.6 Resistance to phenicols

Two genes from the multidrug efflux pumps family were identified in 33% of the isolates: *floR* (n=13) and/or *cmlA1* (n=14). A combination of both *floR* and *cmlA1* genes was observed in 40% of the isolates (n=11). Isolates that had only *floR* (n=2) were phenotypically resistant to chloramphenicol, while the isolates that had only *cmlA1* (n=3) were phenotypically susceptible to the same antibiotic. Isolates that had a combination of both *floR* and *cmlA1* genes were phenotypically resistant to chloramphenicol. Genotypes predicted phenotypes with 92.8% sensitivity and 95.5% specificity.

4.4.7 Resistance to tetracyclines

To date, at least 40 distinct *tet* alleles have been described (*31*), three (i.e., *tet*(A), *tet*(B) and *tet*(M) were identified among the isolates in this study. Tetracycline resistance genes were found in 62% of the isolates (n=51), mostly *tet*(A) (n=30). Further, ribosomal protection protein-producing *tet*(M) (n=13) and efflux pump-encoding *tet*(B) (n=8) were identified less frequently. Sixteen percent of the isolates (n=13) had a combination of two different genes, namely *tet*(A) and *tet*(M). Genotypes predicted phenotypes with 100% sensitivity and 100% specificity.

4.4.8 Resistance to sulfonamides and trimethoprim.

Sulfonamide resistance genes were found in 49% of the isolates (n=40). Three resistance genes (*sul1*, *sul2*, and *sul3*) were identified. Out of the 40 isolates, 24 carried *sul2*, 14 carried *sul3*, and two carried *sul1*. Sixteen percent of the isolates (n=13) had a combination of two distinct *sul* genes. The most common combination was *sul1* and *sul2* and it was observed in 11 isolates.

Sixteen percent of the isolates (n=13) carried the dihydrofolate reductase resistance gene *dfrA12*. Genotypes of the folate synthesis inhibitors predicted phenotypes with a sensitivity of 100% and a specificity of 81.8%.

4.4.9 Resistance to macrolides

Even though no macrolide resistance genes were identified, azithromycin was phenotypically tested among the 81 isolates. All the isolates were phenotypically susceptible to this drug. Genotypic specificity for azithromycin was 100%.

4.4.10 Multidrug resistance

Among the 81 isolates analyzed, 33 (41%) presented genes encoding for multidrug resistance (MDR) to three or more antimicrobial classes (*9*). The most common MDR genes combinations were *aadA1-aadA2-bla*$_{\text{TEM-1}}$*-cmlA1-dfrA12-floR-qacL-qnrS1-sul2-sul3-tet*(A)-*tet*(M) (n=12), and *aph(3'')-Ib-aph(3')-Ia-aph(6)-Id-bla*$_{\text{TEM-1}}$*-sul2-tet*(A)-*tet*(B) (n=11) (Table 4.2). Genotypic MDR was observed in 95% of *S.* Rissen (n=18), 30% of *S.* Corvallis (n=3), 87% of *S.* Krefeld (n=7), 100% of *S.* Derby (n=2), 100% of *S.* Anatum (n=1), 33% *S.* Typhimurium (n=1), and 100% of *S.* I 4,[5],12:i:- isolates (n=1).

Seventeen percent (n=14) displayed resistance to three classes of antibiotics, and 16% (n=13) had resistance to four of the nine classes tested. MDR was observed in 84% of *S.* Rissen (n=16), 87% of *S.* Krefeld (n=7), 100% of *S.* Derby (n=2), 33% *S.* Typhimurium (n=1), and 100% of *S.* I 4,[5],12:i:- (n=1). Phenotypic MDR was not observed among *S.* Corvallis and *S.* Anatum isolates.

4.4.11 *Salmonella* pathogenicity islands

A total of 10 *Salmonella* pathogenicity islands (SPI-1, 3-5, 8, 9, 12-14, and centisome 63 [C63PI]) (*53*) were detected in 59 isolates (Table 4.2). C63PI, an iron transport system in SPI-1,

was observed in 57% of the isolates (n=46). SPI-1, SPI-4 and SPI-9, which encode predicted type I and type III secretion systems (*1*, *41*) were present in 14%, 2%, and 5% of the isolates, respectively. Twenty-seven percent of the isolates (n=22) had no PIs. SPI-1 was found in *S.* Krefeld (n=4), *S.* Typhimurium (n=2), *S.* Derby, *S.* Corvallis, *S.* Hvittingfoss, *S.* I 4,[5],12:i:-, *S.* Altona (n=1 each). SPI-8, known to be involved with resistance to bacteriocins (*41*) was identified in *S.* Krefeld (n=4), *S.* Rissen and *S.* Corvallis (n=3 each).

4.5 Discussion

This study reports on the identification and characterization of the genotypic and phenotypic AMR profiles from *Salmonella* serotypes isolated from environmental samples from informal markets in Cambodia. Overall, a high diversity of resistance genes encoding resistance to several classes of antibiotics was identified.

More than 50% of the *Salmonella* isolates presented resistance to five different classes of antibiotics. Resistance to beta-lactams, tetracyclines, aminoglycosides, phenicol and folate pathway inhibitor antibiotic (e.g., trimethoprim-sulfamethoxazole) were the most common among the isolates. Similarly, previous studies conducted in Cambodia have shown that *Salmonella* isolates were resistant to β-lactams (33%) and tetracyclines (33%) (*27*, *33*). The development and spread of AMR among *Salmonella* serotypes is particularly important when found in retail environments, such as informal markets. Contaminated environments may be the source of cross-contamination between food products and environmental surfaces. When cross-contamination occurs, AMR pathogens become a threat to public health since the effectiveness of antibiotic therapy may be reduced (*41*).

Twenty-seven isolates (33%) were multidrug resistant. Previous studies in Cambodia revealed that MDR *Salmonella* was found in various sample types and ranged from 23% to 52%

(*33*, *42*). The presence of genes that are commonly associated with MDR to third generation cephalosporins (i.e., belong to the beta-lactam class of antibiotics) is a growing problem that reduces bacteremia treatment options and jeopardizes public health. Third generation cephalosporins are particularly important as they are often used to treat *Salmonellosis* in humans and are classified as critically important for human health (*43*). In this study, 4% of the isolates (n=3) demonstrated resistance to third generation cephalosporins (e.g., ceftriaxone and ceftiofur).

Genotypic MDR was predicted in 95% of *S.* Rissen isolates, revealing the presence of genes encoding resistance to seven antibiotic classes. However, phenotypic testing confirmed MDR in only 84% of *S.* Rissen isolates and resistance to five classes of antibiotics, namely beta-lactams, tetracyclines, trimethoprims, phenicols, and aminoglycosides. Similarly, Nadimpalli and colleagues (2019) identified MDR *S.* Rissen from pork, chicken and fish samples collected from informal markets in Cambodia (*29*), revealing that MDR among *S.* Rissen is recurrent in the market environment. This scenario is especially concerning in LMICs (e.g., Cambodia) since antibiotic resources are limited, and treatment options are compromised when strains are resistant to several classes of antibiotics.

Thirty percent of *S.* Corvallis isolates exhibited genes encoding resistance to four classes of antibiotics: aminoglycosides, quinolones, sulfonamides, and tetracyclines. However, phenotypic resistance was only observed to two classes: aminoglycosides and tetracyclines. Previous studies have reported similar AMR profiles in *S.* Corvallis isolated from various sources (i.e., informal markets, food products and patients) from Cambodia and Thailand (*29*). The resistance pattern observed over multiple studies demonstrates that the AMR profile of *S.* Corvallis has remained similar and reoccurring over the past decade in Southeast Asian countries (e.g., Cambodia and Thailand).

Although *S.* Typhimurium presented genes encoding resistance to six classes of antibiotics (i.e., beta-lactam, tetracycline, trimethoprim, aminoglycoside, phenicol, and sulfonamide), phenotypic resistance was observed only to beta-lactam, tetracycline, and trimethoprim. Similar rates of MDR have been reported from poultry and pork samples collected from informal markets and retail shops in Phnom Penh, Cambodia (*20*, *29*). In neighboring Thailand, however, higher rates (over 50%) of MDR isolates have been reported in human specimens, indicating that this strain is highly prevalent in patients (*38*).

S. Derby exhibited MDR in 100% of the isolates, which harbored eight different genes (e.g., *aac(3)-IId*, *bla*$_{\text{CTX-M-14}}$, *floR*, *fosA7*, *qnrS1*, *sul2*, *tet*(A), *tet*(M)) representing seven classes of antibiotics. However, phenotypic resistance was observed only to four antibiotic classes: aminoglycosides, beta-lactam, phenicol, and tetracycline. These results are similar to previous studies from Cambodia (*29*), China (*46*), and Thailand (*38*, *42*), revealing that MDR is commonly found among *S.* Derby strains in Southeast Asia. Historically, *S.* Derby is mainly recovered from pork, potentially indicating that the uncontrolled use of antibiotics in the pig production chain plays an important role in the antimicrobial resistance selection pressure (*50*).

S. I 4,[5],12:i:-, the monophasic variant of *S.* Typhimurium, has increasingly been associated with cases of human disease in several countries around the world (*28*). Sources of contamination were attributed to beef, pork, and chicken products, suggesting that these animal-based food products may serve as critical vectors for human contamination (*28*, *40*). In this study, *S.* I 4,[5],12:i:- was resistant to aminoglycosides, beta-lactams, and tetracycline. Multidrug resistance among *S.* I 4,[5],12:i:- has also been reported from pork samples in Cambodia (*29*), Australia (*2*), Spain (*5*), and Germany (*13*). Human specimens and pork samples have been linked

to the same MDR *S.* I 4,[5],12:i:- in Australia, where the samples were only 10 single nucleotide polymorphisms (SNPs) apart, indicating that pork could be the source of the human infection (*2*).

Although WGS revealed genes encoding resistance to several classes of antibiotics, many of those were not expressed during phenotypic testing, and thus exhibiting phenotypic susceptibility to certain drug classes (i.e., seen in *S.* Rissen, *S.* Corvallis, *S.* Derby, *S.* I 4,[5],12:i-). When resistance genes are plasmid-encoded and phenotypic susceptibility testing is performed retrospectively, these plasmids may be lost during storage and sub-culture. Therefore, the genes that were detected previously by WGS, may not be present when retrospective phenotypic testing is carried out on a different colony (*30*). Interestingly, isolates that had only *floR* genes were phenotypically resistant to chloramphenicol, while the isolates that had only *cmlA1* were phenotypically susceptible to the same antibiotic. This scenario highlights that *cmlA1* may be targeting other antibiotics within the phenicol class other than chloramphenicol. Similar results were found by Neuert et al. that identified isolates genotypically (e.g., *cmlA1*) predicting resistance to chloramphenicol but showed phenotypic susceptibility (30).

Additionally, three isolates (e.g., *S.* Altona, *S.* Hvittingfoss, and *S.* Virchow) were genotypically predicted to be susceptible but exhibited phenotypic resistance to beta-lactams, aminoglycosides, and phenicol. This scenario highlights that emerging antimicrobial resistance mechanisms may be present, and mismatches are likely occurring due to unknown mechanisms not being included in the reference gene database used for genotypic prediction. Similar results have been observed previous studies with nontyphoidal *Salmonella enterica* (*24*, *30*).

A total of 10 SPIs (SPI-1, 3-5, 8, 9, 12-14, and centisome 63 [C63PI]) were detected in 73% of isolates. Pathogenicity islands are especially important due to their capability of carrying genes encoding virulence factors that contribute to the adhesion, invasion, and infection process

(*22*). To the best of our knowledge, this is the first study to explore the presence and abundance of SPIs in environmental samples from informal markets in Cambodia. SPI-1 was identified in 14% of the isolates. SPI-1, located at centisome 63 encoding predicted type III secretion system (T3SS), is a fundamental complex of genetic elements necessary during the initial stages of infection (*11*, *12*). The T3SS machinery, which is an extremely important part of pathogenesis, facilitates the invasion by its unique needle apparatus that is utilized to deliver effector proteins into the host cell cytoplasm and create proinflammatory responses (*8*, *25*). Interestingly, *S.* Krefeld, *S.* Derby and *S.* I 4,[5],12:i:- (n=1 each) presented a combination of both SPI-1 and MDR, indicating that such strains are extremely important to public health due to their ability to successfully establish an infection and to resist several classes of antibiotics. The combination of an important invasion system (i.e., T3SS [SPI-1]) and the resistance profile to several different antimicrobial classes, demonstrates the importance of controlling pathogen contamination in the food chain.

4.6 Conclusions

Overall, this study indicates that AMR *Salmonella* serotypes are prevalent in informal market environments in Cambodia. Resistance to antibiotic classes commonly used to treat *Salmonella* infections in humans were present in a small percentage of the isolates. The findings of this research revealed the presence of important pathogenicity islands related to bacteria's successful adhesion, invasion, and host cell infection. The combined effect of specific SPIs with the diversity and distribution of AMR phenotypes highlights the need for improvement in food safety practices within informal markets and the increase of antibiotic awareness in agriculture and livestock production systems.

While this study elucidated the diversity of predicted resistance genes and phenotypic resistance profiles of *Salmonella* from environmental samples, future studies are needed to identify the dissemination of AMR profiles among the food production chain. Future source-attribution studies should be conducted to investigate the dissemination of resistance among agriculture, animal food systems, and the market environment.

4.7 Acknowledgments

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Table 4.1. Genotype and phenotype comparison of *Salmonella* isolates from informal markets in Cambodia, 2018-2019[a]

	No. of test results							
	Phenotype: resistant		Phenotype: susceptible					
Antibiotic	Genotype: resistant	Genotype: susceptible	Genotype: resistant	Genotype: susceptible	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Aminoglycosides								
GEN	2	0	31	48	100	60.7	6.06	100
SRT	15	1	18	47	93.7	72.3	45.4	97.9
Beta-lactam/beta-lactam inhibitor								
AUG2	0	0	30	51	DBZ[b]	62.9	DBZ	DBZ
Cephems								
FOX	0	2	30	49	0	62.0	0	96.0
AXO	2	1	28	50	66.6	64.1	6.67	98.0
XNL	2	1	28	50	66.6	64.1	6.67	98.0
Penicillin								
AMP	30	0	0	51	100	100	100	100
Folate pathway inhibitor								
FIS	0	0	27	54	DBZ	66.6	DBZ	DBZ
SXT	13	0	0	68	100	100	100	100
Macrolide								
AZI	0	0	0	81	DBZ	100	DBZ	DBZ
Phenicol								
CHL	13	1	3	64	92.8	95.5	81.2	98.4
Quinolones								
CIP	0	0	22	59	DBZ	72.8	DBZ	DBZ
NAL	0	0	22	59	DBZ	72.8	DBZ	DBZ
Tetracycline								
TET	38	0	0	43	100	100	100	100
Total	115	6	239	774	95.0	76.4	32.4	99.2

[a]Abbreviations: GEN, gentamicin; STR, streptomycin; AUG2, amoxicillin-clavulanic acid; FOX, cefoxitin; AXO, ceftriaxone ; XNL, ceftiofur; AMP, ampicillin; FIS, sulfisoxazole; SXT, trimethoprim-sulfamethoxazole; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; PPV, positive predictive value; NPV, negative predictive value.

[b]Division by zero (DBZ): values were not able to be calculated when phenotypic resistance was not observed due to division by zero.

Table 4.2. Serotypes, pathogenicity island, resistance genotypes, and antimicrobial classes identified in non-typhoidal *Salmonella enterica* isolates collected from Cambodian informal markets.

Serotype (No of isolates)	Pathogenicity island	Genotypic AMR	Antimicrobials (No of classes)
Rissen* (7)	-	aadA1; aadA2; blaTEM-1; cmlA1; dfrA12; floR; qacL; qnrS1; sul2; sul3; tet(A); tet(M)	Aminoglycoside, Phenicol, Quaternary ammonium, Quinolone, Sulfonamide, Tetracycline, Trimethoprim, β-lactam (8)
Rissen* (2)	8; C63PI	aadA1; aadA2; blaTEM-1; cmlA1; dfrA12; floR; qacL; qnrS1; sul2; sul3; tet(A); tet(M)	Aminoglycoside, Phenicol, Quaternary ammonium, Quinolone, Sulfonamide, Tetracycline, Trimethoprim (7)
Rissen* (1)	8; 9; C63PI	aadA1; aadA2; blaTEM-1; cmlA1; dfrA12; floR; qacL; qnrS1;sul2; sul3; tet(A); tet(M)	Aminoglycoside, Phenicol, Quaternary ammonium, Quinolone, Sulfonamide, Tetracycline, Trimethoprim, β-lactam (8)
Rissen* (1)	C63PI	aadA1; aadA2; blaTEM-1; cmlA1; dfrA12; *qacEΔ1*; qacL; sul1; sul3; tet(A)	Aminoglycoside, Phenicol, Quaternary ammonium, Sulfonamide, Tetracycline, Trimethoprim, β-lactam (7)
Rissen* (1)	-	aadA1; aadA2; blaTEM; blaTEM-1; cmlA1; dfrA12; floR; qacL; qnrS1; sul2; sul3; tet(A); tet(M)	Aminoglycoside, Phenicol, Quaternary ammonium, Quinolone, Sulfonamide, Tetracycline, Trimethoprim, β-lactam (8)
Rissen* (3)	C63PI	aph(3")-Ib; aph(6)-Id; blaTEM-1; tet(A)	Aminoglycoside, Tetracycline, β-lactam (3)
Rissen* (3)	-	aph(3")-Ib; aph(6)-Id; blaTEM-1; tet(A)	Aminoglycoside, Tetracycline, β-lactam (3)
Rissen (1)	-	-	-
Hvittingfoss (1)	1; 3; 4; 5; 13; 14; C63PI	-	-
Hvittingfoss (4)	13; 14	-	-
Hvittingfoss (1)	13	-	-

Hvittingfoss (1)	13; 14; C63PI	-	-
Hvittingfoss (1)	3; 13; 14	-	-
Hvittingfoss (1)	5; 9; 13; 14; C63PI	-	-
Hvittingfoss (2)	5; 12; 13; 14; C63PI	-	-
Hvittingfoss (2)	5; 13; 14; C63PI		-
Corvallis* (2)	3; 8; C63PI	aph(3")-Ib; aph(6)-Id; qnrS1; sul2; tet(A)	Aminoglycoside, Quinolone, Sulfonamide, Tetracycline (4)
Corvallis* (1)	C63PI	aph(3")-Ib; aph(6)-Id; qnrS1; sul2; tet(A)	Aminoglycoside, Quinolone, Sulfonamide, Tetracycline (4)
Corvallis (3)	-	qnrS1	Quinolone (1)
Corvallis (1)	3	qnrS1	Quinolone (1)
Corvallis (1)	3; C63PI	qnrS1	Quinolone (1)
Corvallis (1)	C63PI	-	-
Corvallis (1)	1; 3; 8; C63PI	-	-
Krefeld* (1)	13; 14; C63PI	aph(3")-Ib; aph(3')-Ia; aph(6)-Id; blaTEM-1; sul2; tet(B)	Aminoglycoside, Sulfonamide, Tetracycline, β-lactam (4)
Krefeld* (3)	1; 3; 8; C63PI	aph(3")-Ib; aph(3')-Ia; aph(6)-Id; blaTEM-1; sul2; tet(B)	Aminoglycoside, Sulfonamide, Tetracycline, β-lactam (4)
Krefeld* (1)	3; C63PI	aph(3")-Ib; aph(3')-Ia; aph(6)-Id; blaTEM-1; sul2; tet(B)	Aminoglycoside, Sulfonamide, Tetracycline, β-lactam (4)
Krefeld* (1)	C63PI	aph(3")-Ib; aph(3')-Ia; aph(6)-Id; blaTEM-1; sul2; tet(B)	Aminoglycoside, Sulfonamide, Tetracycline, β-lactam (4)
Krefeld* (1)	1; 3; C63PI	aph(3")-Ib; aph(6)-Id; blaTEM-1; sul2; tet (A); tet(B)	Aminoglycoside, Sulfonamide, Tetracycline, β-lactam (4)
Krefeld (1)	1; 3; 8; C63PI	-	-
Weltevreden (1)	13; 14; C63PI	-	-
Weltevreden (2)	-	-	-
Weltevreden (3)	13; 14	-	-

Altona (1)	9; C63PI	-	-
Altona (1)	1; 3; C63PI	-	-
Altona (2)	C63PI	-	-
Altona (2)	-	-	-
Mbandaka (3)	-	tet(A)	Tetracycline (1)
Mbandaka (2)	C63PI	tet(A)	Tetracycline (1)
Typhimurium* (1)	5; 13; 14; C63PI	aadA1; aadA2; blaTEM-1; cmlA1; dfrA12; *qacEΔ1*; qacL; sul1; sul3; tet(A)	Aminoglycoside, Phenicol, Quaternary ammonium, Sulfonamide, Tetracycline, Trimethoprim, β-lactam (7)
Typhimurium (1)	1; 3; 4; 5; 13; 14; C63PI	-	-
Typhimurium (1)	1; 5; 9; 13; 14; C63PI	-	-
Javiana (1)	13; 14; C63PI	-	-
Javiana (1)	13	-	-
Uganda (1)	13; 14	-	-
Uganda (1)	13; 14; C63PI	-	-
Derby* (2)	1; 3; C63PI	aac(3)-IId; blaCTX-M-14; floR; fosA7; qnrS1; sul2; tet(A); tet(M)	Aminoglycoside, Fosfomycin, Phenicol, Quinolone, Sulfonamide, Tetracycline, β-lactam (7)
Anatum* (1)	3; 13; 14; C63PI	aadA1; blaLAP-2; blaTEM-1; cmlA1; qacL; qnrS1; sul3; tet(A)	Aminoglycoside, Phenicol, Quaternary ammonium, Quinolone, Sulfonamide, Tetracycline, β-lactam (7)
Braenderup (1)	13; 14; C63PI	-	-
Lexington (1)	C63PI	-	-
Virchow (1)	12; 13; 14	-	-
I 4,[5],12:i:-* (1)	1; 3; 5; 13; 14; C63PI	aph(3")-Ib; aph(6)-Id; blaTEM-1; sul2; tet(B)	Aminoglycoside, Sulfonamide, Tetracycline, β-lactam (4)

*Genotypically multidrug resistant strain (i.e., resistance to three or more antimicrobial classes) (*9*).

4.8 References

1. Amavisit, P., D. Lightfoot, G. F. Browning, and P. F. Markham. 2003. Variation between pathogenic serovars within *Salmonella* pathogenicity islands. Journal of Bacteriology 185:3624–3635.

2. Arnott, A., Q. Wang, N. Bachmann, R. Sadsad, C. Biswas, C. Sotomayor, P. Howard, R. Rockett, A. Wiklendt, J. R. Iredell, and V. Sintchenko. 2018. Multidrug-resistant *Salmonella* enterica 4,[5],12:i:- sequence type 34, New South Wales, Australia, 2016–2017. Emerg. Infect. Dis. 24:751–753.

3. Barlow, R. S., K. E. Mcmillan, L. L. Duffy, N. Fegan, D. Jordan, and G. E. Mellor. 2015. Prevalence and antimicrobial resistance of *Salmonella* and *Escherichia coli* from Australian cattle populations at slaughter. Journal of Food Protection 78:912–920.

4. Clinical and Laboratory Standards Institute. 2020. Performance standards for antimicrobial susceptibility testing. 30th ed. CLSI supplement M100. Wayne, PA: CLSI.6. Echeita, M. A., A. Aladueña, S. Cruchaga, and M. A. Usera. 1999. Emergence and spread of an atypical *Salmonella enterica* subsp. enterica serotype 4,5,12:i:− strain in Spain. Journal of Clinical Microbiology 37:3425–3425.

5. Echeita, M. A., A. Aladueña, S. Cruchaga, and M. A. Usera. 1999. Emergence and spread of an atypical *Salmonella enterica* subsp. *enterica* serotype 4,5,12:i:− strain in Spain. *Journal of Clinical Microbiology* 37:3425–3425.

6. Feldgarden, M., V. Brover, D. H. Haft, A. B. Prasad, D. J. Slotta, I. Tolstoy, G. H. Tyson, S. Zhao, C.-H. Hsu, P. F. McDermott, D. A. Tadesse, C. Morales, M. Simmons, G. Tillman,

J. Wasilenko, J. P. Folster, and W. Klimke. 2019. Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. Antimicrob Agents Chemother 63:e00483-19, /aac/63/11/AAC.00483-19.atom.

7. Finger, J. A. F. F., W. S. G. V. Baroni, D. F. Maffei, D. H. M. Bastos, and U. M. Pinto. 2019. Overview of foodborne disease outbreaks in Brazil from 2000 to 2018. Foods 8:434.

8. Foley, S. L., and A. M. Lynne. 2008. Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance1. Journal of Animal Science 86:E173–E187.

9. Food and Drug Administration. 2015. National antimicrobial resistance monitoring system – enteric bacteria (NARMS): 2011 Executive Report. Rockville, MD: U.S. Department of Health and Human Services, Food and Drug Administration, 2015.

10. Ford, L., Q. Wang, R. Stafford, K.-A. Ressler, S. Norton, C. Shadbolt, K. Hope, N. Franklin, R. Krsteski, A. Carswell, G. P. Carter, T. Seemann, P. Howard, M. Valcanis, C. F. S. Castillo, J. Bates, K. Glass, D. A. Williamson, V. Sintchenko, B. P. Howden, and M. D. Kirk. 2018. Seven *Salmonella* Typhimurium outbreaks in Australia linked by trace-back and whole genome sequencing. Foodborne Pathogens and Disease 15:285–292.

11. Hansen-Wester, I., and M. Hensel. 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. Microbes and Infection 3:549–559.

12. Hardt, W.-D., H. Urlaub, and J. Galan. 1998. A substrate of the centisome 63 type III protein secretion system of *Salmonella* typhimurium is encoded by a cryptic bacteriophage. Proceedings of the National Academy of Sciences 95:2574–2579.

13. Hauser, E., E. Tietze, R. Helmuth, E. Junker, K. Blank, R. Prager, W. Rabsch, B. Appel, A. Fruth, and B. Malorny. 2010. Pork Contaminated with *Salmonella enterica* serovar 4,[5],12:i:−, an emerging health risk for humans. AEM 76:4601–4610.

14. Hoffmann, S., M. B. Batz, and J. G. Morris. 2012. Annual cost of illness and quality-adjusted life year losses in the united states due to 14 foodborne pathogens. Journal of Food Protection 75:1292–1302.

15. Hopkins, K. L., M. Day, and E. J. Threlfall. 2008. Plasmid-mediated quinolone resistance in *Salmonella enterica*, United Kingdom. Emerg. Infect. Dis. 14:340–342.

16. Jacoby, G. A., J. Strahilevitz, and D. C. Hooper. 2014. Plasmid-mediated quinolone resistance. *Microbiology Spectrum* 2.

17. Jans, C., E. Sarno, L. Collineau, L. Meile, K. D. C. Stärk, and R. Stephan. 2018. Consumer exposure to antimicrobial resistant bacteria from food at Swiss retail level. Front. Microbiol. 9:362.

18. Jiang, M., F. Zhu, C. Yang, Y. Deng, P. S. L. Kwan, Y. Li, Y. Lin, Y. Qiu, X. Shi, H. Chen, Y. Cui, and Q. Hu. 2020. Whole-genome analysis of *Salmonella enterica* serovar enteritidis isolates in outbreak linked to online food delivery, Shenzhen, China, 2018. Emerg. Infect. Dis. 26:789–792.

19. Kuhn, M., Wing, J., Weston, S., Williams, A., Keefer, C., Engelhardt, A., Cooper, T., Mayer, Z., Benesty, M., Lescarbeau, R., Ziem, A., Scrucca, L., Tang, Y., Candan, C., Hunt, T. 2020. Package "Caret" Classification and Regression Training.

20. Lay, K. S., Y. Vuthy, P. Song, K. Phol, and J. L. Sarthou. 2011. Prevalence, numbers and antimicrobial susceptibilities of *Salmonella* Serovars and *Campylobacter* spp. in retail poultry in Phnom Penh, Cambodia. J. Vet. Med. Sci. 73:325–329.

21. Maradiaga, M., A. Echeverry, D. Orellana, M. Miller, and M. Brashears. 2017. Antibiotic resistance of *Salmonella enterica* isolated from whole-muscle beef cuts and beef carcasses in Mexico. Meat and Muscle Biology 1:132–132.

22. Marcus, S. L., J. H. Brumell, C. G. Pfeifer, and B. B. Finlay. 2000. *Salmonella* pathogenicity islands: big virulence in small packages. Microbes and Infection 2:145–156.

23. Marshall, B. M., and S. B. Levy. 2011. Food animals and antimicrobials: impacts on human health. Clinical Microbiology Reviews 24:718–733.

24. McDermott, P. F., G. H. Tyson, C. Kabera, Y. Chen, C. Li, J. P. Folster, S. L. Ayers, C. Lam, H. P. Tate, and S. Zhao. 2016. Whole-genome sequencing for detecting antimicrobial resistance in nontyphoidal *Salmonella*. Antimicrob. Agents Chemother. 60:5515–5520.

25. Meena, M., P. Swapnil, A. Zehra, M. Aamir, M. K. Dubey, C. B. Patel, and R. S. Upadhyay. 2019. Virulence factors and their associated genes in microbes, p. 181–208. In New and Future Developments in Microbial Biotechnology and Bioengineering. Elsevier.

26. Mekong Institute. 2019. MI, MoH Support FORT Team Responding to Foodborne Disease Outbreak. Available at https://www.mekonginstitute.org/news-activities/detail/2019/12/04/mi-moh-support-fort/. Accessed on 24 April 2020.

27. Meng, C. Y., B. L. Smith, L. Bodhidatta, S. A. Richard, K. Vansith, B. Thy, A. Srijan, O. Serichantalergs, and C. J. Mason. 2011. Etiology of diarrhea in young children and patterns of antibiotic resistance in Cambodia. The Pediatric Infectious Disease Journal 30:331–335.

28. Moreno Switt, A. I., Y. Soyer, L. D. Warnick, and M. Wiedmann. 2009. Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i:–. Foodborne Pathogens and Disease 6:407–415.

29. Nadimpalli, M., L. Fabre, V. Yith, N. Sem, M. Gouali, E. Delarocque-Astagneau, N. Sreng, S. Le Hello, the BIRDY study group, B. T. Raheliarivao, F. Randrianirina, P. Herindrainy, Z. Z. Andrianirina, F. M. J. Rakotoarimanana, B. Garin, J.-M. Collard, A. de Lauzanne, L. Borand, P. Piola, A. Kerléguer, T. Chon, S. Touch, A. Tarantola, S. Goyet, S. Lach, V. Ngo, M. Vray, M. Diatta, J. Faye, A. Ndiaye, V. Richard, A. Seck, R. Bercion, A. G. Sow, J. B. Diouf, P. S. Dieye, B. Sy, B. Ndao, D. Guillemot, B. Huynh, M. Seguy, L. Watier, A. A. Youssouf, and M. Padget. 2019. CTX-M-55-type ESBL-producing *Salmonella enterica* are emerging among retail meats in Phnom Penh, Cambodia. Journal of Antimicrobial Chemotherapy 74:342–348.

30. Neuert, S., S. Nair, M. R. Day, M. Doumith, P. M. Ashton, K. C. Mellor, C. Jenkins, K. L. Hopkins, N. Woodford, E. de Pinna, G. Godbole, and T. J. Dallman. 2018. Prediction of phenotypic antimicrobial resistance profiles from whole genome sequences of non-typhoidal *Salmonella enterica*. Front. Microbiol. 9:592.

31. Nguyen, F., A. L. Starosta, S. Arenz, D. Sohmen, A. Dönhöfer, and D. N. Wilson. 2014. Tetracycline antibiotics and resistance mechanisms. Biological Chemistry 395:559–575.

32. R Core Team. 2020. R: A Language and environment for statistical computing. R foundation for statistical computing. Vienna, Austria.

33. Reed, T. A. N., S. Krang, T. Miliya, N. Townell, J. Letchford, S. Bun, B. Sar, K. Osbjer, S. Seng, M. Chou, Y. By, L. Vanchinsuren, V. Nov, D. Chau, T. Phe, A. de Lauzanne, S. Ly, and P. Turner. 2019. Antimicrobial resistance in Cambodia: a review. International Journal of Infectious Diseases 85:98–107.

34. Roesel, K., and D. Grace. 2014. Food safety and informal markets. Routledge. Agriculture, Nutrition & Health Academy Food Safety Working Group.

35. Saenz, Y. 2003. Mutations in gyrA and parC genes in nalidixic acid-resistant *Escherichia coli* strains from food products, humans and animals. Journal of Antimicrobial Chemotherapy 51:1001–1005.

36. Schwan, C. L., V. Trinetta, M. Balkey, P. W. Cook, R. K. Phebus, S. Gragg, J. Kastner, J. L. Vipham, and S. Lomonaco. 2020. Draft genome sequences of 81 *Salmonella enterica* strains from informal markets in Cambodia. *Microbiol Resour Announc* 9:e00773-20, /mra/9/36/MRA.00773-20.atom.

37. Schwan, C. L., K. Desiree, N. M. Bello, L. Bastos, L. Hok, R. K. Phebus, S. Gragg, J. Kastner, and J. Vipham. 2020. Prevalence of *Salmonella enterica* isolated from food contact and non-food contact surfaces in Cambodian informal markets. *Journal of Food Protection*.

38. Sirichote, P., A. Bangtrakulnonth, K. Tianmanee, A. Unahalekhaka, A. Oulai, P. Chittaphithakchai, W. Kheowrod, and R. S. Hendriksen. 2010. Serotypes and antimicrobial resistance of *Salmonella enterica* in central Thailand, 2001-2006. Southeast Asian J. Trop. Med. Public Health 41:1405–1415.

39. Sorlozano, A., J. Gutierrez, A. Jimenez, J. de Dios Luna, and J. L. Martinez. 2007. Contribution of a new mutation in pare to quinolone resistance in extended-spectrum- -lactamase-producing *Escherichia coli* isolates. Journal of Clinical Microbiology 45:2740–2742.

40. Soyer, Y., A. Moreno Switt, M. A. Davis, J. Maurer, P. L. McDonough, D. J. Schoonmaker-Bopp, N. B. Dumas, T. Root, L. D. Warnick, Y. T. Grohn, and M. Wiedmann. 2009. *Salmonella enterica* serotype 4,5,12:i:-, an emerging *Salmonella* serotype that represents multiple distinct clones. Journal of Clinical Microbiology 47:3546–3556.

41. Thanner, S., D. Drissner, and F. Walsh. 2016. Antimicrobial resistance in agriculture. mBio 7:e02227-15, /mbio/7/2/e02227-15.atom.

42. Trongjit, S., S. Angkititrakul, R. E. Tuttle, J. Poungseree, P. Padungtod, and R. Chuanchuen. 2017. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand-Cambodia border provinces: AMR in *Salmonella enterica*. Microbiol Immunol 61:23–33.

43. Tyson, G. H., S. Zhao, C. Li, S. Ayers, J. L. Sabo, C. Lam, R. A. Miller, and P. F. McDermott. 2017. Establishing genotypic cutoff values to measure antimicrobial resistance in *Salmonella*. Antimicrob Agents Chemother 61:e02140-16, e02140-16.

44. Wisner, A., T. Desin, A. White, A. Potter, and W. Kster. 2012. The *Salmonella* pathogenicity island-1 and -2 encoded type III secretion systems, In Y. Kumar (ed.), *Salmonella* - A Diversified Superbug. In Tech.

45. World Health Organization. 2018. Antimicrobial resistance. Geneva, Switzerland. Available at https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance. Accessed on 8 May 2020.

46. World Health Organization. 2017. Critically important antimicrobials for human medicine: ranking of antimicrobial agents for risk management of antimicrobial resistance due to non-human use. Available at https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1. Accessed on 11 March 2020.

47. World Health Organization. 2015. WHO estimates of the global burden of foodborne diseases. World Health Organization, Geneva, Switzerland.

48. Yam, E. L. Y., L. Y. Hsu, E. P.-H. Yap, T. W. Yeo, V. Lee, J. Schlundt, M. O. Lwin, D. Limmathurotsakul, M. Jit, P. Dedon, P. Turner, and A. Wilder-Smith. 2019. Antimicrobial resistance in the Asia Pacific region: a meeting report. Antimicrob Resist Infect Control 8:202.

49. Yang, K., A. Wang, M. Fu, A. Wang, K. Chen, Q. Jia, and Z. Huang. 2020. Investigation of incidents and trends of antimicrobial resistance in foodborne pathogens in eight countries from historical sample data. International Journal of Environmental Research and Public Health 17:472.

50. Yang, X., Q. Wu, J. Zhang, J. Huang, L. Chen, S. Wu, H. Zeng, J. Wang, M. Chen, H. Wu, Q. Gu, and X. Wei. 2019. Prevalence, bacterial load, and antimicrobial resistance of *Salmonella* serovars isolated from retail meat and meat products in China. Front. Microbiol. 10:2121.

51. Yoshida, H., M. Bogaki, M. Nakamura, L. M. Yamanaka, and S. Nakamura. 1991. Quinolone resistance-determining region in the DNA gyrase gyrB gene of *Escherichia coli*. Antimicrobial Agents and Chemotherapy 35:1647–1650.

52. Zankari, E., H. Hasman, S. Cosentino, M. Vestergaard, S. Rasmussen, O. Lund, F. M. Aarestrup, and M. V. Larsen. 2012. Identification of acquired antimicrobial resistance genes. Journal of Antimicrobial Chemotherapy 67:2640–2644.

53. Zhou, D., W.-D. Hardt, and J. E. Galán. 1999. *Salmonella* typhimurium encodes a putative iron transport system within the centisome 63 pathogenicity island. Infect Immun 67:1974–1981.

Chapter 5 - A case report of *Salmonella enterica* serovar Corvallis from environmental isolates from Cambodia and clinical cases in the United Kingdom

5.1 Introduction

Salmonella enterica (henceforth referred to as *Salmonella*) is the primary causative agent of diarrheal diseases worldwide, with the most severe affect seen in low-income countries (LICs; e.g., Southeast Asian countries) (26). *Salmonella* is a ubiquitous bacterium with over 2500 different serotypes reported to date (8). A pathogenic serotype of interest, *Salmonella enterica* serovar Corvallis (*S.* Corvallis) was reported worldwide in humans and food products (7, 11, 15, 22). In fact, *S.* Corvallis is among the top ten most frequently isolated serotypes from human patients in Southeast Asia (7).

Foreign tourists who visit Southeast Asian countries (i.e., Cambodia, Thailand and Vietnam) (14) can potentially acquire diarrheal diseases, such as salmonellosis, by coming into contact with contaminated food and water (9, 20). Interestingly, *S.* Corvallis was identified as a common causative agent for travel-related salmonellosis (i.e., travel to Southeast Asian countries) by numerous foreign public health institutes (3, 10, 15, 19).

Characterization of *Salmonella* serotypes can be accomplished by whole genome sequencing (WGS), which is also a powerful tool to aid in the identification of bacterial strain similarities and source attribution pathways. Here, we report the genetic relatedness of *S.* Corvallis from environmental samples from Cambodia and in clinical cases in the United Kingdom through WGS.

5.2 Case Report

In January of 2016 and April of 2019, the Public Health of England (PHE) reported two cases of salmonellosis caused by *S.* Corvallis. The 2016 case was a 20-year-old native British woman who had traveled to Vietnam prior to the onset of symptoms. The WGS for this sample was uploaded to the National Center for Biotechnology Information (NCBI) platform in March of 2019. The 2019 sample of *S.* Corvallis was from a 60-year-old native British man and WGS data was uploaded to the NCBI platform in June of 2019. Unfortunately, it is unclear if this patient had recently visited Southeast Asia, as the "travel" field in his case file was missing.

In January of 2019, a group of researchers from Kansas State University traveled to Cambodia to investigate the prevalence of *Salmonella* on environmental surfaces in informal markets. *Salmonella* was isolated from environmental samples and shipped to the United States for WGS analysis. In November of 2019, the Center for Food Safety and Applied Nutrition (CFSAN) at the U.S. Food and Drug Administration (FDA) conducted WGS analysis and uploaded those sequences to the NCBI platform.

Through the NCBI platform, it is possible to create phylogenetic trees using isolate sequences that have been uploaded into the NCBI's library (1). Phylogenetic trees are built by comparing differences in single nucleotide polymorphisms (SNPs) among different isolates. The SNPs represent a specific genetic variation and are commonly used to compare the genetic relatedness and evolutionary origin in a bacterial population (2).

A SNP analysis was conducted on the NCBI Pathogen Detection platform to identify strain similarities between the Cambodia environmental isolate genomes and all other genomes available in the library. It was discovered using the SNP analysis that the two clinical cases (e.g., 2016 and 2019) and several of the environmental isolates were identified as *S.* Corvallis and apparently

closely related. A SNP difference of three and two nucleotides between the 2016 clinical isolate (e.g., PDT000475677.1) and five environmental isolates (e.g., PDT000630261.1, PDT000630248.1, PDT000630194.1, PDT000630209.1, and PDT000630173.1), is depicted in Figure 5.1.

A SNP difference of one and zero nucleotides was identified between the 2019 clinical isolate (e.g., PDT000522124.1) and the same environmental isolates (e.g., PDT000630261.1, PDT000630248.1, PDT000630194.1, PDT000630209.1, and PDT000630173.1), depicted in Figure 5.2. Although supplementary information on additional clinical patients was not available, the SNP analysis also revealed 4 clinical cases in the United States and additional 29 clinical cases in the United Kingdom that shared similar genetic content with the environmental isolates from Cambodia. These clinical cases were reported between 2014-2019, as shown in Table 5.1. The average SNP difference for the additional clinical cases was 12 SNPs (range 0 – 20 SNPs), depicted in figure 5.3.

5.3 Discussion

A SNP difference of less than 21 SNPs for *Salmonella* supports a match between two or more genomes (16). In this report, an average SNP difference of 1 (0–3 SNPs) between the 2016 and 2019 clinical isolates and the environmental isolates was observed, indicating a high level of genetic similarity. Interestingly, the additional 33 clinical isolates from two countries (e.g., United States and United Kingdom) were also considered genetically related and ranged from 0 – 10 SNPs different relative to the environmental isolates from Cambodia. These results reveal that this pathogenic strain of *S.* Corvallis was recurrently associated with human disease since 2014.

Human infections caused by *S.* Corvallis has been reported in mostly in Asian countries with relatively few infections reported outside this region (i.e., China, Thailand, Vietnam, and

Malaysia (6, 12, 21, 23). For example, a case of travel-related bacteremia caused by *S.* Corvallis was reported in an immunocompetent adult from Japan that had traveled to Cambodia and Vietnam prior the beginning of symptoms (15). These reports indicate that this serotype may be endemic in geographical areas of Asia.

S. Corvallis has been isolated from food products and environments in Southeast Asia, the United States, Brazil, North Africa and Europe (7, 17, 24). Reports of highly antibiotic-resistant strains of *S.* Corvallis raise concerns within public health authorities around the world (5, 13, 25). Further, multidrug resistant *S.* Corvallis was previously recovered from patients with travel history to Vietnam and Thailand (19).

In Cambodia, studies revealed the presence of *S.* Corvallis in poultry (11), pork (22), and environmental (17) samples from retail and informal markets. The informal markets are characterized by open-air environments that are lack basic food safety infrastructure, hygiene, sanitation or oversight (4). The informal markets are often a touristic attraction for foreigners, who frequently purchase and consume food products at these locations. Reports indicate that the consumption of contaminated food and water has resulted in traveler's diarrhea cases worldwide (9, 18). In fact, the risk of *Salmonella* infection in travelers returning to the United States is highest for travelers returning from Africa, Latin America, and Asia (9).

The environmental isolates presented in this report (e.g., isolated from surfaces in informal markets in Cambodia), share high genetic similarity to the human clinical cases in the United Kingdom. *S.* Corvallis from clinical isolates have been reported in the United States (e.g., in 2014, 2016, and 2017) and in the United Kingdom (e.g., in 2015, 2016, 2017, 2018, and 2019) for many consecutive years, indicating that this serotype has recurrently established infection in humans.

Although *S.* Corvallis has caused bacteremia and diarrheal disease (15, 19), few studies have investigated its source of contamination and routes of transmission.

This report indicates that these isolates of *S.* Corvallis isolated from environmental surfaces of informal markets in Cambodia are concerning to public health due to their genetic similarity to strains that have caused human disease. Since the majority of the Cambodian population acquires their food products from these informal markets, it is important to focus on strategies to control and prevent the contamination of *S.* Corvallis (along with other pathogenic serotypes of *Salmonella*) in these locations. Future work should focus on source attribution and persistence studies identifying common sources of contamination and determining major transmission routes.

Figure 5.1. Phylogenetic tree identifying the SNPs difference between the environmental isolates and the 2016 clinical isolate (e.g., PDT000475677.1).

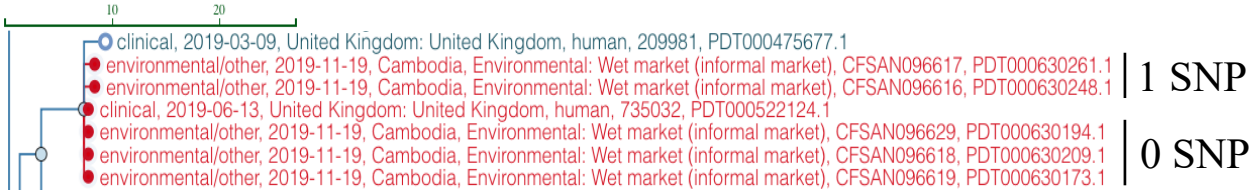

Figure 5.2. Phylogenetic tree identifying the SNPs difference between the environmental isolates and the 2019 clinical isolate (e.g., PDT000522124.1).

Figure 5.3. Phylogenetic tree identifying the SNPs difference between the environmental isolates from Cambodia and clinical cases in the United States and United Kingdom.

Table 5.1. *Salmonella enterica* isolates from Cambodia (e.g., environmental), United Kingdom (e.g., clinical) and the United States (e.g., clinical) within the same SNP cluster (e.g., PDS000056420.14).

Serovar	Isolate	Create date	Location	Isolation type	*Min-same	**Min-diff	BioSample
Year: 2019							
Corvallis	PDT000457118.1	2019-01-31	United Kingdom	clinical	8	15	SAMN10848942
Corvallis	PDT000469598.1	2019-02-27	United Kingdom	clinical	4	16	SAMN11025553
Corvallis	PDT000470700.1	2019-02-28	United Kingdom	clinical	7	14	SAMN11031785
Corvallis	PDT000471288.1	2019-03-02	United Kingdom	clinical	3	10	SAMN11042238
Corvallis	PDT000475677.1	2019-03-09	United Kingdom	clinical	2	2	SAMN11093783
Corvallis	PDT000475844.1	2019-03-10	United Kingdom	clinical	3	8	SAMN11095741
Corvallis	PDT000477511.1	2019-03-15	United Kingdom	clinical	5	16	SAMN11128906
Corvallis	PDT000477513.1	2019-03-15	United Kingdom	clinical	5	12	SAMN11128913
Corvallis	PDT000522124.1	2019-06-12	United Kingdom	clinical	2	0	SAMN12039715
Corvallis	PDT000470764.1	2019-03-01	United Kingdom	clinical	6	17	SAMN11038684
NA	PDT000630173.1	2019-11-19	Cambodia	environmental	0	0	SAMN13321508
NA	PDT000630194.1	2019-11-19	Cambodia	environmental	0	0	SAMN13321598

NA	PDT000630209.1	2019-11-19	Cambodia	environmental	0	0	SAMN13322169
NA	PDT000630248.1	2019-11-19	Cambodia	environmental	1	1	SAMN13322423
NA	PDT000630261.1	2019-11-19	Cambodia	environmental	1	1	SAMN13322444
Corvallis	PDT000639610.1	2019-12-04	United Kingdom	clinical	8	17	SAMN13474428
Year: 2018							
Corvallis	PDT000317841.1	2018-05-22	United Kingdom	clinical	4	11	SAMN09233616
Corvallis	PDT000319348.1	2018-05-25	United Kingdom	clinical	4	10	SAMN09257893
Corvallis	PDT000323021.1	2018-06-03	United Kingdom	clinical	6	13	SAMN09298374
Corvallis	PDT000327612.1	2018-06-10	United Kingdom	clinical	2	11	SAMN09388926
Corvallis	PDT000332213.1	2018-06-19	United Kingdom	clinical	7	14	SAMN09444533
Corvallis	PDT000336426.1	2018-06-26	United Kingdom	clinical	5	15	SAMN09484468
Corvallis	PDT000338111.1	2018-06-28	United Kingdom	clinical	3	11	SAMN09504423
Corvallis	PDT000342475.1	2018-07-06	United Kingdom	clinical	2	11	SAMN09624182
Corvallis	PDT000377617.1	2018-09-15	United Kingdom	clinical	4	16	SAMN10067798
Corvallis	PDT000379076.1	2018-09-17	United Kingdom	clinical	4	13	SAMN10076048
Corvallis	PDT000312919.2	2018-05-10	United Kingdom	clinical	6	17	SAMN09100973
Corvallis	PDT000340695.1	2018-07-03	United Kingdom	clinical	9	16	SAMN09534667
Year: 2017							
NA	PDT000230176.2	2017-08-01	United States	clinical	7	14	SAMN07277150
Corvallis	PDT000214300.2	2017-05-31	United Kingdom	clinical	7	15	SAMN07180127
NA	PDT000215685.2	2017-06-05	United States	clinical	4	11	SAMN07173395
Year: 2016							
Corvallis	PDT000129606.2	2016-05-10	United States	clinical	3	10	SAMN04913844
Chailey	PDT000103370.2	2016-01-26	United Kingdom	clinical	0	7	SAMN04437636
Year: 2015							
Corvallis	PDT000639610.1	2019-12-04	United Kingdom	clinical	8	17	SAMN13474428
Corvallis	PDT000042987.4	2015-02-10	United Kingdom	clinical	9	10	SAMN03168761
Corvallis	PDT000043024.4	2015-02-10	United Kingdom	clinical	4	11	SAMN03168799
Chailey	PDT000058820.2	2015-04-12	United Kingdom	clinical	0	7	SAMN03479345
Chailey	PDT000059500.2	2015-04-13	United Kingdom	clinical	0	7	SAMN03479962
Corvallis	PDT000053912.2	2015-04-10	United Kingdom	clinical	6	17	SAMN03468587
Year: 2014							
Corvallis	PDT000040841.2	2014-10-17	United States	clinical	6	13	SAMN03098691

*Min-same: Minimum SNP distance from this isolate to one of the same isolation type.
**Min-diff: Minimum SNP distance from this isolate to one of a different isolation type.

5.4 References

1. Cherry, J. L. 2017. A practical exact maximum compatibility algorithm for reconstruction of recent evolutionary history. *BMC Bioinformatics* 18:127.

2. Dong, H.-J., S. Cho, D. Boxrud, S. Rankin, F. Downe, J. Lovchik, J. Gibson, M. Erdman, and A. M. Saeed. 2017. Single-nucleotide polymorphism typing analysis for molecular subtyping of *Salmonella* Tennessee isolates associated with the 2007 nationwide peanut butter outbreak in the United States. *Gut Pathog* 9:25.

3. Ekdahl, K., B. de Jong, R. Wollin, and Y. Andersson. 2005. Travel-associated non-typhoidal salmonellosis: geographical and seasonal differences and serotype distribution. *Clinical Microbiology and Infection* 11:138–144.

4. Grace, D. 2015. Food safety in low and middle income countries. *Int J Environ Res Public Health* 12:10490–10507.

5. Hadziabdic, S., M. Borowiak, A. Bloch, B. Malorny, I. Szabo, B. Guerra, A. Kaesbohrer, and J. Fischer. 2018. Complete genome sequence of an avian native NDM-1-producing *Salmonella enterica* subsp. *enterica* serovar Corvallis strain. *Genome Announc* 6:e00593-18, /ga/6/26/e00593-18.atom.

6. Hendriksen, R. S., A. Bangtrakulnonth, C. Pulsrikarn, S. Pornruangwong, G. Noppornphan, H.-D. Emborg, and F. M. Aarestrup. 2009. Risk factors and epidemiology of the ten most common *Salmonella* serovars from patients in Thailand: 2002–2007. *Foodborne Pathogens and Disease* 6:1009–1019.

7. Hendriksen, R. S., A. R. Vieira, S. Karlsmose, D. M. A. Lo Fo Wong, A. B. Jensen, H. C. Wegener, and F. M. Aarestrup. 2011. Global monitoring of *Salmonella* serovar distribution from the World Health Organization global foodborne infections network country data bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathogens and Disease* 8:887–900.

8. Jajere, S. M. 2019. A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Vet World* 12:504–521.

9. Kendall, M. E., S. Crim, K. Fullerton, P. V. Han, A. B. Cronquist, B. Shiferaw, L. A. Ingram, J. Rounds, E. D. Mintz, and B. E. Mahon. 2012. Travel-associated enteric infections diagnosed after return to the United States, foodborne diseases active surveillance network (FoodNet), 2004–2009. *Clinical Infectious Diseases* 54:S480–S487.

10. Koch, K., B. Kristensen, H. M. Holt, S. Ethelberg, K. Mølbak, and H. C. Schønheyder. 2011. International travel and the risk of hospitalization with non-typhoidal *Salmonella* bacteremia. A Danish population-based cohort study, 1999-2008. *BMC Infect Dis* 11:277.

11. Lay, K. S., Y. Vuthy, P. Song, K. Phol, and J. L. Sarthou. 2011. Prevalence, numbers and antimicrobial susceptibilities of *Salmonella* serovars and *Campylobacter* spp. in retail poultry in Phnom Penh, Cambodia. *J. Vet. Med. Sci.* 73:325–329.

12. Ma, Y., X. Xu, Y. Gao, Z. Zhan, C. Xu, X. Qu, Z. Chen, J. Bai, M. Liao, and J. Zhang. 2020. Antimicrobial resistance and molecular characterization of *Salmonella enterica*

serovar Corvallis isolated from human patients and animal source foods in China. *International Journal of Food Microbiology* 335:108859.

13. McCutchen, E. L., M. Galac, C. Kapsak, S. H. Hinrichs, P. C. Iwen, and B. Abdalhamid. 2019. Genome sequence of a *Salmonella enterica* subsp. *enterica* serovar Corvallis strain isolated from human blood. *Microbiol Resour Announc* 8:e01025-19, /mra/8/44/MRA.01025-19.atom.

14. McDowall, S. 2010. International tourist satisfaction and destination loyalty: Bangkok, Thailand. *Asia Pacific Journal of Tourism Research* 15:21–42.

15. Nakakubo, S., K. Nagaoka, M. Suzuki, S. Konno, Y. Shibue, T. Ikeda, and M. Nishimura. 2019. A case of primary bacteraemia caused by *Salmonella enterica* serovar Corvallis in an immunocompetent adult after travel to Southeast Asia. *Access Microbiology* 1.

16. Pightling, A. W., J. B. Pettengill, Y. Luo, J. D. Baugher, H. Rand, and E. Strain. 2018. Interpreting whole-genome sequence analyses of foodborne bacteria for regulatory applications and outbreak investigations. *Front Microbiol* 9.

17. Schwan, C. L., K. Desiree, N. M. Bello, L. Bastos, L. Hok, R. K. Phebus, S. Gragg, J. Kastner, and J. Vipham. 2020. Prevalence of *Salmonella enterica* isolated from food contact and non-food contact surfaces in Cambodian informal markets. *Journal of Food Protection*.

18. Steffen, R., D. R. Hill, and H. L. DuPont. 2015. Traveler's Diarrhea: A Clinical Review. *JAMA* 313:71.

19. Taguchi, M., R. Kawahara, K. Seto, K. Inoue, A. Hayashi, N. Yamagata, K. Kamakura, and E. Kashiwagi. 2009. Plasmid-mediated quinolone resistance in *Salmonella* isolated from patients with overseas travelers' diarrhea in Japan. *Jpn. J. Infect. Dis.* 62:312–314.

20. Taylor, D. N., and P. Echeverria. 1986. Etiology and epidemiology of travelers' diarrhea in Asia. *Clinical Infectious Diseases* 8:S136–S141.

21. Thuy Duong, V., H. Chung The, T. Do Hoang Nhu, H. Thanh Tuyen, J. I. Campbell, P. Van Minh, H. Le Phuc, T. Thi Hong Chau, N. Minh Ngoc, L. Lan Vi, A. E. Mather, and S. Baker. 2020. Genomic serotyping, clinical manifestations, and antimicrobial resistance of non-typhoidal *Salmonella* gastroenteritis in hospitalized children in Ho Chi Minh City, Vietnam. *J Clin Microbiol* JCM.01465-20, jcm;JCM.01465-20v1.

22. Trongjit, S., S. Angkititrakul, R. E. Tuttle, J. Poungseree, P. Padungtod, and R. Chuanchuen. 2017. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand-Cambodia border provinces: AMR in *Salmonella enterica*. *Microbiol Immunol* 61:23–33.

23. Woh, P. Y., K. L. Thong, J. M. Behnke, J. W. Lewis, and S. N. M. Zain. 2017. Characterization of nontyphoidal *Salmonella* isolates from asymptomatic migrant food handlers in Peninsular Malaysia. *Journal of Food Protection* 80:1378–1383.

24. Yamatogi, R. S., H. C. Oliveira, C. H. Camargo, S. A. Fernandes, R. T. Hernandes, J. P. Pinto, V. L. Rall, and J. P. Araújo. 2015. Clonal relatedness and resistance patterns of *Salmonella* Corvallis from poultry carcasses in a Brazilian slaughterhouse. *J Infect Dev Ctries* 9:1161–1165.

25. Zhang, L., Y. Fu, Z. Xiong, Y. Ma, Y. Wei, X. Qu, H. Zhang, J. Zhang, and M. Liao. 2018. Highly prevalent multidrug-resistant *Salmonella* from chicken and pork meat at retail markets in Guangdong, China. *Front. Microbiol.* 9:2104.

26. World Health Organization. 2015. WHO estimates of the global burden of foodborne diseases. World Health Organization, Geneva, Switzerland.

Chapter 6 - Conclusions

As discussed in Chapter 1, the burden of FBD in Cambodia is less understood and epidemiological data on foodborne pathogens (i.e., *Salmonella enterica*) is limited. Therefore, studies investigating the prevalence, distribution and diversity of *Salmonella* serotypes is crucially important. The studies discussed in this dissertation provide insights into the microbial ecology of *Salmonella* in informal markets in Cambodia.

Chapter 2 determined the prevalence and diversity of *Salmonella* serotypes across multiple surface types. Results from this study revealed that *Salmonella* was detected across several surfaces, mainly from FCS during the dry season. The diversity and distribution of *Salmonella* serotypes varied depending on the season and surface type. A wide range of *Salmonella* serotypes and identification of emerging serotypes (i.e., *S.* Hvittingfoss) demonstrate the complexity of intertwined contributing factors to *Salmonella* prevalence and its imminent risk to public health in Cambodia. Many interrelated factors, including inadequate infrastructure, limited environmental sanitation, poor personal hygiene practices, and a lack of food safety measures, may contribute to cross-contamination and potentially to the development of foodborne illness.

Chapter 3 described the draft-genome sequences of *Salmonella* isolates collected from informal markets. The high diversity and genomic potential identified among *Salmonella* serotypes revealed the presence of harmful and emerging serotypes (i.e., *S.* Typhimurium, *S.* Corvallis, *S.* I 4,[5],12:i:-, and *S.* Hvittingfoss) that may compromise public health due to their potential to cause human infections.

Chapter 4 identified and characterized the genotypic antimicrobial resistance profiles of *Salmonella* strains isolated from environmental samples from informal markets in Cambodia. A high diversity of AMR genes which encode for several major classes of antibiotics were present

in a high percentage of the isolates. The development and spread of AMR among *Salmonella* serotypes is of particular concern when found in food-selling environments, such as the informal markets. Informal markets with a high degree of environmental bacterial contaminants may be the source of cross-contamination between food products and environmental surfaces. When cross-contamination occurs, AMR pathogens become a threat to public health, as the effectiveness of antibiotic therapy may be required to cure a resulting illness is reduced. AMR genes which encode for antibiotic classes commonly used to treat salmonellosis in humans were identified among the *Salmonella* serotypes collected from informal markets. This is critically important since it reduces bacteremia treatment options and jeopardizes public health. Further, important pathogenicity islands related to bacteria's successful adhesion, invasion, and host cell infection were identified. The combination of important invasion systems (i.e., T3SS [SPI-1]) and the resistance profile to several antibiotic classes found in Chapter 4 demonstrates the urgency of controlling pathogen contamination in informal markets and increasing antibiotic resistance awareness in agriculture and livestock production systems.

Chapter 5 described the genetic relatedness between *S.* Corvallis from environmental isolates from informal markets in Cambodia and clinical cases in the United Kingdom. The SNP analysis revealed a high level of genetic similarity between the environmental and clinical isolates (0–3 SNPs). Additionally, clinical isolates in the United Kingdom with similar genetic profiles have been reported since 2015, indicating that this serotype has recurrently established infection in humans. *S.* Corvallis isolates from environmental surfaces in Cambodia are concerning to public health since they share similar genetic content to the strains that have been documented to caused human disease in the United Kingdom.

These studies serve as a baseline for the evaluation of *Salmonella* prevalence, distribution, and diversity. However, gaps are still present, and more studies are needed. Future studies should sample a greater diversity of markets at various time points and follow individual vendors throughout the seasons to better understand the dynamics of *Salmonella* prevalence, diversity, and persistence on a finer spatial-temporal scale. The availability of robust and accurate data on the ecology of *Salmonella* in these markets is crucial for active surveillance, implementation of suitable intervention strategies, and prevention of future foodborne illness cases.

These studies and related strategies may serve as guiding references to develop further food safety research, education and intervention programs, and even policy drafting. They will support decision making within the Ministry of Health and the Ministry of Agriculture, Forestry, and Fisheries in Cambodia and foster public health protection, as well as support global epidemiological investigations of outbreaks.

Appendix A - Raw data used for statistical analysis

Table A.1. Raw data for prevalence and serotype distribution of *Salmonella enterica*

Internal ID	Market	Location	Vendor	Sample Description	Status	Surface	Sample	Season	Serotype
	M1	Inside	1	Mat	Negative	FCS	3	Rainy	
VL-0618-1	M1	Inside	1	Table	Negative	NFCS	1	Rainy	
	M1	Inside	1	Floor	Negative	NFCS	3	Rainy	
	M1	Inside	2	Cardboard	Negative	FCS	3	Rainy	
	M1	Inside	2	Table	Negative	NFCS	1	Rainy	
	M1	Inside	3	Basket	Negative	FCS	1	Rainy	
	M1	Inside	3	Mat	Negative	FCS	3	Rainy	
	M1	Inside	3	Chair	Negative	NFCS	1	Rainy	
VL-0618-3	M1	Inside	3	Floor	Positive	NFCS	2	Rainy	Typhimurium
	M1	Inside	4	Cardboard	Negative	FCS	3	Rainy	
	M1	Inside	4	Chair	Negative	NFCS	1	Rainy	
	M1	Inside	4	Floor	Negative	NFCS	2	Rainy	
	M1	Inside	5	Mat	Negative	FCS	1	Rainy	
VL-0618-5	M1	Inside	5	Cardboard	Positive	FCS	2	Rainy	I 4,[5],12:i:-
	M1	Inside	5	Basket	Negative	FCS	3	Rainy	
VL-0618-6	M1	Inside	5	Floor	Positive	NFCS	1	Rainy	Derby
	M1	Inside	5	Chair	Negative	NFCS	2	Rainy	
VL-0618-7	M1	Inside	6	Mat	Positive	FCS	3	Rainy	Hvittingfoss
	M1	Inside	6	Floor	Negative	NFCS	1	Rainy	
	M1	Inside	6	Wall	Negative	NFCS	3	Rainy	
	M1	Inside	7	Basket	Negative	FCS	2	Rainy	
VL-0618-9	M1	Inside	7	Floor	Positive	NFCS	1	Rainy	Derby

	M1	Inside	7	Wall	Negative	NFCS	2	Rainy	
	M1	Inside	7	Table	Negative	NFCS	3	Rainy	
	M1	Inside	8	Mat	Negative	FCS	3	Rainy	
	M1	Inside	8	Chair	Negative	NFCS	1	Rainy	
	M1	Inside	8	Floor	Negative	NFCS	3	Rainy	
	M1	Inside	9	Mat	Negative	FCS	2	Rainy	
	M1	Inside	9	Scale	Negative	FCS	3	Rainy	
VL-0618-10	M1	Inside	9	Floor	Positive	NFCS	1	Rainy	Altona
	M1	Inside	9	Wall	Negative	NFCS	2	Rainy	
VL-0618-11	M1	Inside	9	Table	Positive	NFCS	3	Rainy	Altona
	M1	Inside	10	Basket	Negative	FCS	1	Rainy	
	M1	Inside	10	Mat	Negative	FCS	3	Rainy	
	M1	Inside	10	Wall	Negative	NFCS	2	Rainy	
VL-0618-12	M1	Outside	11	Basket	Positive	FCS	1	Rainy	Rissen
	M1	Outside	11	Basket	Negative	FCS	3	Rainy	
	M1	Outside	11	Container	Negative	NFCS	3	Rainy	
VL-0618-13	M1	Outside	12	Basket	Positive	FCS	1	Rainy	Javiana
	M1	Outside	12	Basket	Negative	FCS	2	Rainy	
	M1	Outside	12	Mat	Negative	FCS	3	Rainy	
	M1	Outside	12	Floor	Negative	NFCS	1	Rainy	
	M1	Outside	12	Table	Negative	NFCS	2	Rainy	
	M1	Outside	12	Container	Negative	NFCS	3	Rainy	
	M1	Outside	13	Lotus_flower_leaf	Negative	FCS	2	Rainy	
VL-0618-15	M1	Outside	13	Basket	Positive	FCS	3	Rainy	Altona
	M1	Outside	13	Table	Negative	NFCS	1	Rainy	
	M1	Outside	13	Floor	Negative	NFCS	2	Rainy	
	M1	Outside	13	Fabric_Covering	Negative	NFCS	3	Rainy	
	M1	Outside	14	Basket	Negative	FCS	2	Rainy	
	M1	Outside	14	Floor	Negative	NFCS	1	Rainy	

	M1	Outside	14	Table	Negative	NFCS	2	Rainy	
	M1	Outside	15	Mat	Negative	FCS	3	Rainy	
	M1	Outside	15	Floor	Negative	NFCS	1	Rainy	
	M1	Outside	15	Table	Negative	NFCS	2	Rainy	
	M1	Outside	15	Covering	Negative	NFCS	3	Rainy	
	M1	Outside	15	Scale	Negative	FCS	3	Rainy	
	M1	Outside	16	Mat	Negative	FCS	2	Rainy	
VL-0618-22	M1	Outside	16	Floor	Positive	NFCS	1	Rainy	Typhimurium
	M1	Outside	16	Covering	Negative	NFCS	2	Rainy	
	M1	Outside	16	Metal_Stand	Negative	NFCS	3	Rainy	
	M1	Outside	17	Mat	Negative	FCS	1	Rainy	
	M1	Outside	17	Cardboard	Negative	FCS	3	Rainy	
	M1	Outside	17	Wood_table	Negative	NFCS	2	Rainy	
	M1	Outside	17	Covering	Negative	NFCS	3	Rainy	
VL-0618-23	M1	Outside	18	Basket	Positive	FCS	2	Rainy	Rissen
VL-0618-24	M1	Outside	18	Floor	Positive	NFCS	1	Rainy	Uganda
	M1	Outside	18	Covering	Negative	NFCS	2	Rainy	
VL-0618-25	M1	Outside	18	Metal_Stand	Positive	NFCS	3	Rainy	Rissen
VL-0618-26	M1	Outside	19	Basket	Positive	FCS	1	Rainy	Uganda
	M1	Outside	19	Cardboard	Negative	FCS	3	Rainy	
VL-0618-28	M1	Outside	19	Wood_table	Positive	NFCS	2	Rainy	Rissen
	M1	Outside	19	Covering	Negative	NFCS	3	Rainy	
	M1	Outside	20	Basket	Negative	FCS	3	Rainy	
	M1	Outside	20	Table	Negative	NFCS	1	Rainy	
	M1	Outside	20	Covering	Negative	NFCS	3	Rainy	
VL-0618-29	M2	Inside	21	Scale	Positive	FCS	1	Rainy	Rissen
	M2	Inside	21	Table	Negative	NFCS	1	Rainy	
	M2	Inside	21	Wall	Negative	NFCS	2	Rainy	
VL-0618-30	M2	Inside	22	Basket	Positive	FCS	1	Rainy	Altona

	M2	Inside	22	Scale	Negative	FCS	3	Rainy	
	M2	Inside	22	Metal_Stand	Negative	NFCS	1	Rainy	
	M2	Inside	23	Mat	Negative	FCS	1	Rainy	
	M2	Inside	23	Basket	Negative	FCS	3	Rainy	
	M2	Inside	23	Plataform	Negative	NFCS	1	Rainy	
	M2	Inside	23	Wall	Negative	NFCS	2	Rainy	
VL-0618-31	M2	Inside	24	Scale	Positive	FCS	2	Rainy	Krefeld
	M2	Inside	24	Basket	Negative	FCS	3	Rainy	
	M2	Inside	24	Wall	Negative	NFCS	1	Rainy	
	M2	Inside	24	Plataform	Negative	NFCS	2	Rainy	
	M2	Inside	25	Mat	Negative	FCS	1	Rainy	
	M2	Inside	25	Plataform	Negative	NFCS	2	Rainy	
	M2	Inside	25	Wall	Negative	NFCS	3	Rainy	
	M2	Inside	26	Scale	Negative	FCS	1	Rainy	
	M2	Inside	26	Basket	Negative	FCS	2	Rainy	
	M2	Inside	26	Styrofoam_box	Negative	FCS	3	Rainy	
	M2	Inside	26	Wall	Negative	NFCS	2	Rainy	
VL-0618-33	M2	Outside	27	Scale	Positive	FCS	1	Rainy	Altona
	M2	Outside	27	Plastic_box	Negative	FCS	2	Rainy	
VL-0618-35	M2	Outside	27	Stool	Positive	NFCS	2	Rainy	Altona
	M2	Outside	27	Stool	Negative	NFCS	3	Rainy	
	M2	Outside	28	Basket	Negative	FCS	1	Rainy	
	M2	Outside	28	Scale	Negative	FCS	2	Rainy	
	M2	Outside	28	Bucket	Negative	NFCS	2	Rainy	
VL-0618-37	M2	Outside	28	Plataform	Positive	NFCS	3	Rainy	Rissen
	M2	Outside	29	Basket	Negative	FCS	1	Rainy	
	M2	Outside	29	Basket	Negative	FCS	3	Rainy	
	M2	Outside	29	Floor	Negative	NFCS	1	Rainy	
	M2	Outside	29	Plataform	Negative	NFCS	2	Rainy	

M2	Outside	29	Stool	Negative	NFCS	3	Rainy
M2	Outside	30	Basket	Negative	FCS	1	Rainy
M2	Outside	30	Basket	Negative	FCS	2	Rainy
M2	Outside	30	Mat	Negative	FCS	3	Rainy
M2	Outside	30	Stool	Negative	NFCS	3	Rainy
M2	Outside	31	Mat	Negative	FCS	1	Rainy
M2	Outside	31	Scale	Negative	FCS	3	Rainy
M2	Outside	31	Plastic_box	Negative	NFCS	2	Rainy
M2	Outside	31	Stool	Negative	NFCS	3	Rainy
M2	Outside	32	Wall	Negative	NFCS	1	Rainy
M2	Outside	32	Stool	Negative	NFCS	3	Rainy
M1	Inside	1	Basket	Negative	FCS	1	Dry
M1	Inside	1	Basket	Negative	FCS	2	Dry
M1	Inside	1	Mat	Negative	FCS	3	Dry
M1	Inside	1	Floor	Negative	NFCS	1	Dry
M1	Inside	1	Floor	Negative	NFCS	2	Dry
M1	Inside	1	Chair	Negative	NFCS	3	Dry
M1	Inside	2	Mat	Negative	FCS	1	Dry
M1	Inside	2	Mat	Negative	FCS	2	Dry
M1	Inside	2	Basket	Negative	FCS	3	Dry
M1	Inside	2	Floor	Negative	NFCS	1	Dry
M1	Inside	2	Floor	Negative	NFCS	2	Dry
M1	Inside	2	Chair	Negative	NFCS	3	Dry
M1	Inside	3	Basket	Negative	FCS	1	Dry
M1	Inside	3	Mat	Negative	FCS	2	Dry
M1	Inside	3	Mat	Negative	FCS	3	Dry
M1	Inside	3	Wall	Negative	NFCS	1	Dry
M1	Inside	3	Floor	Negative	NFCS	2	Dry
M1	Inside	3	Floor	Negative	NFCS	3	Dry

	M1	Inside	4	Basket	Negative	FCS	1	Dry	
	M1	Inside	4	Basket	Negative	FCS	2	Dry	
	M1	Inside	4	Mat	Negative	FCS	3	Dry	
	M1	Inside	4	Chair	Negative	NFCS	1	Dry	
	M1	Inside	4	Floor	Negative	NFCS	2	Dry	
	M1	Inside	4	Floor	Negative	NFCS	3	Dry	
	M1	Inside	5	Mat	Negative	FCS	1	Dry	
VL-0119-45	M1	Inside	5	Basket	Positive	FCS	2	Dry	Anatum
	M1	Inside	5	Basket	Negative	FCS	3	Dry	
	M1	Inside	5	Chair	Negative	NFCS	1	Dry	
	M1	Inside	5	Wall	Negative	NFCS	2	Dry	
	M1	Inside	5	Floor	Negative	NFCS	3	Dry	
VL-0119-46	M1	Inside	6	Basket	Positive	FCS	1	Dry	Rissen
VL-0119-47	M1	Inside	6	Mat	Positive	FCS	2	Dry	Rissen
	M1	Inside	6	Mat	Negative	FCS	3	Dry	
	M1	Inside	6	Wall	Negative	NFCS	1	Dry	
	M1	Inside	6	Chair	Negative	NFCS	2	Dry	
	M1	Inside	6	Floor	Negative	NFCS	3	Dry	
	M1	Inside	7	Mat	Negative	FCS	1	Dry	
	M1	Inside	7	Basket	Negative	FCS	3	Dry	
	M1	Inside	7	Chair	Negative	NFCS	1	Dry	
	M1	Inside	7	Wall	Negative	NFCS	2	Dry	
VL-0119-49	M1	Inside	7	Floor	Positive	NFCS	3	Dry	Javiana
	M1	Inside	8	Basket	Negative	FCS	1	Dry	
	M1	Inside	8	Basket	Negative	FCS	2	Dry	
	M1	Inside	8	Scale	Negative	NFCS	1	Dry	
	M1	Inside	8	Wall	Negative	NFCS	2	Dry	
	M1	Inside	8	Floor	Negative	NFCS	3	Dry	
VL-0119-51	M1	Inside	9	Mat	Positive	FCS	1	Dry	Rissen

VL-0119-52	M1	Inside	9	Basket	Positive	FCS	2	Dry	Rissen
	M1	Inside	9	Basket	Negative	FCS	3	Dry	
	M1	Inside	9	Scale	Negative	NFCS	1	Dry	
	M1	Inside	9	Wall	Negative	NFCS	2	Dry	
VL-0119-53	M1	Inside	9	Floor	Positive	NFCS	3	Dry	Rissen
	M1	Inside	10	Mat	Negative	FCS	1	Dry	
	M1	Inside	10	Basket	Negative	FCS	2	Dry	
VL-0119-54	M1	Inside	10	Mat	Positive	FCS	3	Dry	Rissen
	M1	Inside	10	Scale	Negative	NFCS	1	Dry	
	M1	Inside	10	Wall	Negative	NFCS	2	Dry	
	M1	Inside	10	Floor	Negative	NFCS	3	Dry	
VL-0119-55	M1	Outside	11	Mat	Positive	FCS	1	Dry	Rissen
	M1	Outside	11	Basket	Negative	FCS	2	Dry	
	M1	Outside	11	Styrofoam_box	Negative	FCS	3	Dry	
	M1	Outside	11	Fabric_Covering	Negative	NFCS	1	Dry	
	M1	Outside	11	Table	Negative	NFCS	2	Dry	
	M1	Outside	11	Floor	Negative	NFCS	3	Dry	
	M1	Outside	12	Mat	Negative	FCS	1	Dry	
VL-0119-56	M1	Outside	12	Mat	Positive	FCS	2	Dry	Rissen
VL-0119-57	M1	Outside	12	Basket	Positive	FCS	3	Dry	Mbandaka
VL-0119-58	M1	Outside	12	Floor	Positive	NFCS	1	Dry	Rissen
	M1	Outside	12	Fabric_Covering	Negative	NFCS	2	Dry	
	M1	Outside	12	Table	Negative	NFCS	3	Dry	
	M1	Outside	13	Basket	Negative	FCS	1	Dry	
VL-0119-59	M1	Outside	13	Mat	Positive	FCS	2	Dry	Corvallis
VL-0119-60	M1	Outside	13	Basket	Positive	FCS	3	Dry	Corvallis
	M1	Outside	13	Fabric_Covering	Negative	NFCS	1	Dry	
VL-0119-61	M1	Outside	13	Scale	Positive	NFCS	2	Dry	Corvallis
VL-0119-62	M1	Outside	13	Floor	Positive	NFCS	3	Dry	Corvallis

	M1	Outside	14	Mat	Negative	FCS	1	Dry	
VL-0119-63	M1	Outside	14	Basket	Positive	FCS	2	Dry	Weltevreden
	M1	Outside	14	Basket	Negative	FCS	3	Dry	
	M1	Outside	14	Floor	Negative	NFCS	1	Dry	
	M1	Outside	14	Scale	Negative	NFCS	2	Dry	
	M1	Outside	14	Fabric_Covering	Negative	NFCS	3	Dry	
VL-0119-65	M1	Outside	15	Basket	Positive	FCS	1	Dry	Weltevreden
VL-0119-66	M1	Outside	15	Mat	Positive	FCS	2	Dry	Hvittingfoss
VL-0119-67	M1	Outside	15	Basket	Positive	FCS	3	Dry	Weltevreden
	M1	Outside	15	Fabric_Covering	Negative	NFCS	1	Dry	
VL-0119-68	M1	Outside	15	Floor	Positive	NFCS	2	Dry	Weltevreden
VL-0119-69	M1	Outside	15	Table	Positive	NFCS	3	Dry	Weltevreden
	M1	Outside	16	Mat	Negative	FCS	1	Dry	
	M1	Outside	16	Basket	Negative	FCS	2	Dry	
VL-0119-70	M1	Outside	16	Mat	Positive	FCS	3	Dry	Hvittingfoss
	M1	Outside	16	Fabric_Covering	Negative	NFCS	1	Dry	
	M1	Outside	16	Table	Negative	NFCS	2	Dry	
	M1	Outside	16	Floor	Negative	NFCS	3	Dry	
VL-0119-71	M1	Outside	17	Mat	Positive	FCS	1	Dry	Weltevreden
	M1	Outside	17	Basket	Negative	FCS	2	Dry	
VL-0119-72	M1	Outside	17	Mat	Positive	FCS	3	Dry	Corvallis
	M1	Outside	17	Fabric_Covering	Negative	NFCS	1	Dry	
VL-0119-73	M1	Outside	17	Floor	Positive	NFCS	2	Dry	Hvittingfoss
VL-0119-74	M1	Outside	17	Table	Positive	NFCS	3	Dry	Hvittingfoss
VL-0119-75	M1	Outside	18	Banana_leaf	Positive	FCS	1	Dry	Hvittingfoss
VL-0119-76	M1	Outside	18	Basket	Positive	FCS	2	Dry	Hvittingfoss
VL-0119-77	M1	Outside	18	Basket	Positive	FCS	3	Dry	Hvittingfoss
	M1	Outside	18	Table	Negative	NFCS	1	Dry	
	M1	Outside	18	Scale	Negative	NFCS	2	Dry	

	M1	Outside	18	Floor	Negative	NFCS	3	Dry	
VL-0119-78	M1	Outside	19	Lotus_flower_leaf	Positive	FCS	1	Dry	Hvittingfoss
VL-0119-79	M1	Outside	19	Mat	Positive	FCS	2	Dry	Mbandaka
	M1	Outside	19	Lotus_flower_leaf	Negative	FCS	3	Dry	
	M1	Outside	19	Fabric_Covering	Negative	NFCS	1	Dry	
	M1	Outside	19	Table	Negative	NFCS	2	Dry	
	M1	Outside	19	Floor	Negative	NFCS	3	Dry	
	M1	Outside	20	Banana_leaf	Negative	FCS	1	Dry	
	M1	Outside	20	Basket	Negative	FCS	2	Dry	
	M1	Outside	20	Mat	Negative	FCS	3	Dry	
VL-0119-80	M1	Outside	20	Floor	Positive	NFCS	1	Dry	Mbandaka
	M1	Outside	20	Table	Negative	NFCS	2	Dry	
	M1	Outside	20	Scale	Negative	NFCS	3	Dry	
	M2	Inside	21	Basket	Negative	FCS	1	Dry	
VL-0119-81	M2	Inside	21	Scale	Positive	FCS	2	Dry	Mbandaka
VL-0119-82	M2	Inside	21	Floor	Positive	FCS	3	Dry	Hvittingfoss
	M2	Inside	21	Wall	Negative	NFCS	1	Dry	
	M2	Inside	21	Floor	Negative	NFCS	2	Dry	
VL-0119-83	M2	Inside	21	Wall	Positive	NFCS	3	Dry	Hvittingfoss
	M2	Inside	22	Basket	Negative	FCS	1	Dry	
VL-0119-84	M2	Inside	22	Mat	Positive	FCS	2	Dry	Hvittingfoss
	M2	Inside	22	Scale	Negative	FCS	3	Dry	
	M2	Inside	22	Floor	Negative	NFCS	1	Dry	
	M2	Inside	22	Wall	Negative	NFCS	2	Dry	
	M2	Inside	22	Floor	Negative	NFCS	3	Dry	
VL-0119-85	M2	Inside	23	Basket	Positive	FCS	1	Dry	Virchow
VL-0119-86	M2	Inside	23	Scale	Positive	FCS	2	Dry	Mbandaka
VL-0119-87	M2	Inside	23	Mat	Positive	FCS	3	Dry	Hvittingfoss
	M2	Inside	23	Wall	Negative	NFCS	1	Dry	

VL-0119-88	M2	Inside	23	Floor	Positive	NFCS	2	Dry	Corvallis
	M2	Inside	23	Floor	Negative	NFCS	3	Dry	
VL-0119-89	M2	Inside	24	Styrofoam_box	Positive	FCS	1	Dry	Corvallis
	M2	Inside	24	Floor	Negative	FCS	2	Dry	
	M2	Inside	24	Scale	Negative	FCS	3	Dry	
VL-0119-90	M2	Inside	24	Floor	Positive	NFCS	1	Dry	Lexington
	M2	Inside	24	Floor	Negative	NFCS	2	Dry	
	M2	Inside	24	Wall	Negative	NFCS	3	Dry	
	M2	Inside	25	Floor	Negative	FCS	1	Dry	
VL-0119-91	M2	Inside	25	Floor	Positive	FCS	2	Dry	Typhimurium
VL-0119-92	M2	Inside	25	Scale	Positive	FCS	3	Dry	Rissen
	M2	Inside	25	Wall	Negative	NFCS	1	Dry	
	M2	Inside	25	Floor	Negative	NFCS	2	Dry	
VL-0119-93	M2	Inside	25	Floor	Positive	NFCS	3	Dry	Krefeld
VL-0119-94	M2	Inside	26	Styrofoam_box	Positive	FCS	1	Dry	Krefeld
	M2	Inside	26	Basket	Negative	FCS	2	Dry	
	M2	Inside	26	Scale	Negative	FCS	3	Dry	
VL-0119-95	M2	Inside	26	Floor	Positive	NFCS	1	Dry	Krefeld
	M2	Inside	26	Wall	Negative	NFCS	2	Dry	
	M2	Inside	26	Floor	Negative	NFCS	3	Dry	
	M2	Outside	27	Lotus_flower_leaf	Negative	FCS	1	Dry	
	M2	Outside	27	Scale	Negative	FCS	2	Dry	
	M2	Outside	27	Basket	Negative	FCS	3	Dry	
	M2	Outside	27	Styrofoam_box	Negative	NFCS	1	Dry	
	M2	Outside	27	Floor	Negative	NFCS	2	Dry	
	M2	Outside	27	Chair	Negative	NFCS	3	Dry	
	M2	Outside	28	Basket	Negative	FCS	1	Dry	
VL-0119-96	M2	Outside	28	Scale	Positive	FCS	2	Dry	Krefeld
VL-0119-97	M2	Outside	28	Lotus_flower_leaf	Positive	FCS	3	Dry	Krefeld

	M2	Outside	28	Floor	Negative	NFCS	1	Dry	
	M2	Outside	28	Chair	Negative	NFCS	2	Dry	
	M2	Outside	28	Fabric_Covering	Negative	NFCS	3	Dry	
	M2	Outside	29	Lotus_flower_leaf	Negative	FCS	1	Dry	
VL-0119-98	M2	Outside	29	Scale	Positive	FCS	2	Dry	Krefeld
VL-0119-99	M2	Outside	29	Basket	Positive	FCS	3	Dry	Krefeld
	M2	Outside	29	Basket	Negative	NFCS	1	Dry	
VL-0119-100	M2	Outside	29	Floor	Positive	NFCS	2	Dry	Corvallis
	M2	Outside	29	Styrofoam_box	Negative	NFCS	3	Dry	
	M2	Outside	30	Banana_leaf	Negative	FCS	1	Dry	
VL-0119-101	M2	Outside	30	Lotus_flower_leaf	Positive	FCS	2	Dry	Corvallis
	M2	Outside	30	Basket	Negative	FCS	3	Dry	
VL-0119-102	M2	Outside	30	Floor	Positive	NFCS	1	Dry	Braenderup
	M2	Outside	30	Basket	Negative	NFCS	2	Dry	
	M2	Outside	30	Floor	Negative	NFCS	3	Dry	
	M2	Outside	31	Basket	Negative	FCS	1	Dry	
VL-0119-103	M2	Outside	31	Mat	Positive	FCS	2	Dry	Rissen
VL-0119-104	M2	Outside	31	Lotus_flower_leaf	Positive	FCS	3	Dry	Rissen
VL-0119-105	M2	Outside	31	Floor	Positive	NFCS	1	Dry	Rissen
	M2	Outside	31	Chair	Negative	NFCS	2	Dry	
	M2	Outside	31	Bucket	Negative	NFCS	3	Dry	
	M2	Outside	32	Lotus_flower_leaf	Negative	FCS	1	Dry	
	M2	Outside	32	Basket	Negative	FCS	2	Dry	
VL-0119-106	M2	Outside	32	Styrofoam_box	Positive	FCS	3	Dry	Corvallis
	M2	Outside	32	Floor	Negative	NFCS	1	Dry	
	M2	Outside	32	Basket	Negative	NFCS	2	Dry	
	M2	Outside	32	Table	Negative	NFCS	3	Dry	