Investigation of Foodborne *Salmonella* spp. in Bangladesh and Development of Real-Time PCR Based Identification Method

Ph.D. Thesis

DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF DHAKA
DHAKA-1000
APRIL 2022

SUBMITTED BY
REGISTRATION NO. 139
SESSION: 2013-14

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Dedicated to....

My beloved family, who cherished my life with their blessings

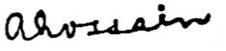
Quotation.....

"Research is to see what everybody else has seen, and to think what nobody else has thought."

-Albert Szent-Gyorgi -

Certification

It is hereby certified that student bearing Reg. No. 139, Session 2013-2014 has carried out the research work entitled "**Investigation of Foodborne *Salmonella* spp. in Bangladesh and Development of Real-Time PCR Based Identification Method**" for the fulfillment of her PhD Degree from University of Dhaka, Bangladesh, under my academic supervision in the Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka.



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Author

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ABSTRACT

Background: Avian non-typhoidal salmonellosis is a major concern for to the development of poultry sector in Bangladesh. The conventional microbiological tests of *Salmonella* are time consuming, laborious and costly. Further confirmatory and rapid methods for detection of *Salmonella* spp. and its distribution are very crucial.

Hypothesis: *Salmonella* spp., the etiological agent of salmonellosis is poultry derived zoonotic pathogen. Epidemiological studies based on the molecular genetics to identify clonal and strain distribution among particular locality or within the country are invaluable to track down the routes of transmission of *Salmonella* spp. and their distribution.

Method: A total of 307 poultry samples were collected from fourteen poultry farms, live bird markets, hotel and household kitchens in the supply line from producers to consumers. Among these, 154 farm samples were found to have *Salmonella* spp. using selective culture and PCR amplification of *invA* gene. The isolates were further genotyped through Randomly Amplified Polymorphic DNA (RAPD), Amplified Ribosomal DNA Restriction Analysis (ARDRA), and Multi Locus Sequence Typing (MLST) methods. Antimicrobial profiles and genotypic variations were compared to address Multi Drug Resistance (MDR) among circulating genotypes. In addition to develop an economical and rapid method, all the 307 samples were analyzed using *invA* gene targeted SYBR green-qPCR to quantify the *Salmonella* spp. bacterial load and their characterization. Purified 284 amplicons of *invA* were cloned in the TOPO TA vector. *Salmonella* gDNA was used for the development of a standard template for SYBER green qPCR. The standard curve showed good linearity (R2 _ 0.97) and efficiency (99%). The bacterial load among farm samples were identified using the standard reference C_t value and the C_t values of the test samples.

Results: Out of 687 isolates collected from farm samples, 200 (29.11%) were confirmed as *Salmonella* spp.. These 200 isolates were differentiated into 18 RAPD genotypes while MLST of these 18 groups assigned the isolates into 3 sequence types (STs) - ST198, ST11, and ST214. The prevalent MLST type, ST198 (50.5%) was represented as *Salmonella enterica* Kentucky, followed by ST214 (33%) representing *S. enterica* Litchfield and ST11 (16.5%) for *S. enterica* serovar Enteritidis. The present study revealed that farm-originated *Salmonella* spp. were multidrug-resistant, including the high level of resistance against doxycycline (96.49%) followed by ampicillin (88.30%), oxytetracycline (88.30%), and ciprofloxacin (66.08%).

We also developed a novel SYBR green-qPCR quantification method that detected the highest load of *Salmonella* spp. in the poultry dropping samples up to 1.3×10^7/ml followed by 6.8×10^6/ml in the cloacal swab, 3.8×10^5/ml count for poultry feed and 2.7×10^4/ml for poultry farm water samples respectively.

Among the live bird market samples, water was analyzed and found to be highly contaminated with *Salmonella* (78%) through SYBR green-qPCR detection. Other market samples including cage of chicken (55%), processing board (60%), knives (40%) as well as transport van of chicken (from farm to bazar) (60%) were also found highly contaminated. Besides, a high percentage of *Salmonella* contamination among raw chicken (55%) and raw food (30%) processing areas of hotel kitchen indicates the possible means of transmission throughout the routes.

Conclusion: The present investigation can be summarized as- (i) the collected *Salmonella* serovars from poultry farms are zoonotic in nature, indicating that poultry could be a major source of non-typhoidal zoonotic salmonellosis; (ii) the dominant MLST type, *Salmonella* ST198 with multidrug resistance traits in different farms confirm the probability of intra-farm transmission; (iii) the risk of *Salmonella* contamination is considerably high in different supply chain points; and (iv) SYBR Green Real-Time PCR can be a reliable and rapid method for *Salmonella* spp. detection.

Figures

Tables

ABBREVIATIONS

NTS- Non-typhoidal *Salmonella*

MDR- Multi-drug resistant

WHO- World Health Organization

MLST- Multi-Locus Sequence Typing

MPN- Most Probable Number

XLD- Xylose Lysine Deoxycholate Agar

PCR- Polymerase Chain Reaction

qPCR- Quantitative Polymerase Chain Reaction

dsDNA- Double Stranded Deoxyribonucleic Acid

dNTP- Deoxyribonucleotide triphosphate

FAO- Food and Agriculture Organization

BPW- Buffered Peptone Water

FDA- Food and Drug Administration

TSI- Triple Sugar Iron

KIA- Kligler Iron Agar

BLAST– Basic Local Alignment Search Tool

MEGA – Molecular Evolutionary Genetics Analysis

NCBI – National Centre for Biotechnology Information

ARDRA- Amplified Ribosomal DNA Restriction Analysis

RAPD- Randomly Amplified Polymorphic DNA

EDTA- Ethylenediamine Tetraacetic Acid

TE Buffer- Tris-EDTA Buffer

UPGMA- Unweighted Pair Group Method with Arithmetic Mean

ST- Sequence Type

CDC- Centers for Disease Control and Prevention

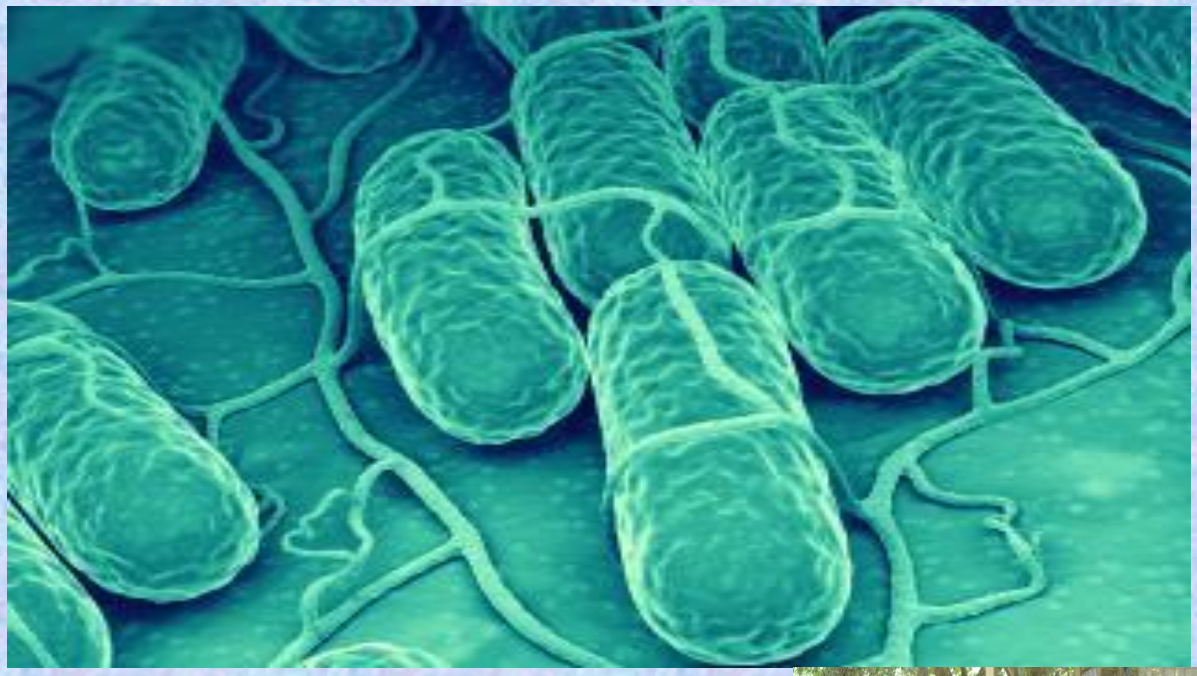
ECDC- European Centre for Disease Prevention and Control

SYBR Green- Synergy Brands, Inc.

SYBR Green RT-PCR- SYBR Green Real Time PCR

C_T Value- Cycle Threshold Value

T_m Value- Melting Temperature Value



Chapter 01: Introduction and Literature Review

1. Introduction and Literature Review

Globalized world with extensive travel and merchandising among countries accelerates the global transmission and spread of food borne pathogens. This highlights the 'control of infectious diseases and food safety management' crucial for all countries.

Salmonella is one of the major foodborne enteropathogenic bacteria universally. Infections caused by this pathogen are a serious concern for economic and healthy living. This bacterium has the ability to infect both household and primitive animal species. The acquaintance between humans and wild animals is one of the important factors imparting to human infections with this pathogen. Almost 93.8 million human infections, with 155,000 deaths caused by *Salmonella* are estimated per annum universally (Majowicz *et al.*, 2010). On account of misdiagnosis and inadequate discloser, the actual numbers of gastrointestinal illnesses are probably significantly higher than available reports (Voetsch *et al.*, 2004). *Salmonella* control programs based on the sources and origin need to be established in developing and developed countries considering the worldwide interest of lowering this particular infection in sake of medical and economic concern. Rapid, intuitive and economical characterization and diagnostic protocols are required for identification, monitoring and control of *Salmonella* in raw food, poultry, livestock and other transmission routes. The subsequent human exposure can be controlled through accurate and rapid detection of *Salmonella* from various sources and the characterization of their subgroups. The global food production and food safety regulations consider the conventional microbiological testing methods as an integral part of the salmonellosis. Use of genotypic based methods for potential pathogenic *Salmonella* characterization is considered as an important tool to encounter the prospect of the food industry regulations (Hoorfar *et al.*, 2000). Alternative protocols in food industry legislation need to be validated and standardized regularly for proper food control and foodborne outbreaks investigations.

For developing countries like Bangladesh, infections caused by *Salmonella* are one of the major obstacles in animal and public health sectors development. Cost effective, rapid and proper validated protocols along with updated dataset based on genotypic and antibiotic resistance profiling variations may help in combating the associated losses.

The present study aims to provide a validated protocol for the qualitative and quantitative detection of *Salmonella* in farm and market-based poultry samples. This study also aimed to

present a minimum dataset of the circulating *Salmonella* and their antibiotic resistance patterns in sampling regions of Bangladesh. The genotyping variability of the pathogens addressed with updated protocols, like, MLST, RAPD, ARDRA, reflects the potential hazard of this pathogen for human in relation to the food chain production. Database with wider sampling regions based on genotypes and antibiotic resistance profiling may contribute to track down the transmission routs of multi-drug resistant (MDR) pathogens, thus help to develop a proper control program along with effective treatments. Rapid, validated protocols for detection and quantification of pathogens are prerequisite to minimize the adverse effects and transmission of outbreaks among localities.

1.1 *Salmonella*- a group of Gram-negative bacteria

Salmonella is a genus of rod-shaped, gram-negative, non-spore forming, oxidase negative, predominantly motile (peritrichous) bacteria belonging to the family *'Enterobacteriaceae'*. *Salmonella* are approximately 2.0 to 5.0 µm in length and 0.7 to 1.5 µm wide (Giannella and Ralph, 1996). The bacterium can ferment glucose usually with gas production. This bacterium can use glucose as the sole source of carbon and ammonium ion as a nitrogen source (prototrophic), thus may grow in a minimal media. Phenotypically most of the serovars are identified by urea hydrolysis, non-lactose fermentation, the absence of tryptophan deaminase, decarboxylate lysine and ornithine, the production of hydrogen sulphide (H_2S), and growth on Simmons citrate agar (*Salmonella* in Domestic Animals - Google Books).

In 1886, Daniel Elmer Salmon and Theobald Smith discovered the genus *Salmonella*, first known as *Salmonella choleraesuis*. Theobald Smith discovered the genus from swine fever (hog cholera) sample and named the genus after Daniel E. Salmon, who was his supervisor at the U.S. Department of Agriculture (USDA) (Salmonella in Domestic Animals - Google Books).

Salmonella spp. are widely spread universally and causing illnesses in human beings and animals. *Salmonella enterica* and *Salmonella bongori* are the two species so far for this specific genus (Michel *et al*., 2003). *S. enterica* is divided into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*), each of which has several serovars or serotypes (Table 1.1). Thus, a serotype can be presented in the following way: *Salmonella enterica* subspecies enterica serotype Kentucky, which may be simplified as *Salmonella* Kentucky. More than 2,500 serotypes are known today and almost 1,500 of them are belonging to subspecies enterica (Porwollik *et al*., 2004).

Table 1.1: *Salmonella* species, subspecies and their usual habitats

***Salmonella* species and subspecies**	**Number of serotypes within subspecies**	**Usual habitat**
S. enterica subsp. *enterica* (I)	1454	Warm-blooded animals
S. enterica subsp. *salamae* (II)	489	Cold-blooded animals and the environment[a]
S. enterica subsp. *arizonae* (IIIa)	94	Cold-blooded animals and the environment
S. enterica subsp. *diarizonae* (IIIb)	324	Cold-blooded animals and the environment
S. enterica subsp. *houtenae* (IV)	70	Cold-blooded animals and the environment
S. enterica subsp. *indica* (VI)	12	Cold-blooded animals and the environment
S. bongori (V)	20	Cold-blooded animals and the environment
Total	**2463**	

[a] Isolates of all species and subspecies recorded in humans.

1.2 *Salmonella*- zoonotic pathogen

A large number of warm and cold-blooded animals across the world harbor *Salmonella* without showing any disease. Besides, it is also the most important diarrheaogenic bacterial pathogens in man among many countries. This bacterium may transmit from animals to humans trough consuming contaminated foods of animal origin, thus salmonellosis are classified as zoonotic disease (Eng *et al.*, 2015).

All species of animals including humans are commonly infected with most of the 2,400 serovars of *Salmonella* sp. *Salmonella* cases in humans normally follow one of two courses, depending on the clinical symptoms and infectious doses:

I. The typhoid resembling disease is mainly caused by the serovars *S. Typhi, S. paratyphi A, B* and *C*. The infectious dose is low (10^2 - 10^3cfu/ml) for this type of infections. The pathogens are ingested orally and can be transmitted via blood. Before main course of clinical symptoms appear, the pathogen have a short incubation period (a few days up to 3 weeks) in human. After that the symptoms emerged, like, diarrhea, high temperature and possible damage to the intestines, liver, heart, gallbladder, and liver. These pathogens are transmissible from man to man.

II. The second type of salmonellosis are characterized by enteritic infections, termed as Non typhoidal Salmonellosis (enteritis = intestinal inflammation). For this course of infection, the infectious dose can vary from human to human depending on the health condition. The minimum infection dose is considerably higher (10^6cfu/ml). The incubation period is shorter (1-3, possibly 5 days). The infection may pose no symptoms at all. Inflammation of the intestinal mucosa may lead to diarrhea. In animals infection with these pathogens frequently occurs without any clinical symptoms.

1.3 Non typhoidal *Salmonella* and salmonellosis

Non-typhoidal *Salmonella* (NTS) infections most commonly result in self-limiting diarrheal illness with limited cases of mortality. Besides diarrheal diseases, non-typhoidal *Salmonella* infections also can invade in sterile sites of human body, causing bacteremia, meningitis, and other focal infections. The cases may invasive or non-invasive infections. Generally, the invasive non-typhoidal *Salmonella* diseases are not typically associated with diarrhea. Still the infection may present as non-specific febrile illnesses that are clinically indistinguishable from other febrile illnesses. This invasive infections cause higher cases of fatality than non-invasive infections (Crump *et al.*, 2015). Elderly people, malnourished infants, and individuals with HIV, sickle-cell disease, and acute malaria are at particular risk (Graham *et al.*, 2000; Vugia *et al.*, 2004; Feasey *et al.*, 2012; Park *et al.*, 2016; Keddy *et al.*, 2017).

Most of the human salmonellosis cases are reported as foodborne, still there are also infections occur through direct and indirect contacts with reservoir animals in homes, veterinary clinics, zoological gardens, farm environments or other public, professional or private settings each year.

Though clinically positive affected animals are mostly accountable for viral transmission, both affected and reservoir animals may shed *Salmonella* for long periods of time (Hoelzer *et al.*, 2011). The indirect transmission through contaminated food and water and environmental issues also often creates complications in control efforts. Certain human subpopulations may pose higher risk of infection due to biological or behavioral risk factors, because the risk of infection varies by animal species, age group, health status, and husbandry practice. Among the several species, some may infect wider range of host species, like *Salmonella* Enteritidis, whereas, serotypes such as *Salmonella* Dublin are adapted only to certain individual host species (McDonough *et al.*, 1999). The implementation of proper management strategies and proper hygiene practices can efficiently mitigate the risks associated with animal contacts.

1.4 Sources of non-typhoidal *Salmonella*

As being the most common human pathogen and zoonosis in nature, there are lots of food sources for non-typhoidal *Salmonella* transmission into human (Figure:1.1). The largest and most common source of NTS transmission is the poultry population, in particular chicken and turkey. These animals are frequently colonized with *Salmonella* without visible symptoms. They play a vital role in horizontal and vertical transmission of *Salmonella* at primary production level (Barrow *et al.*, 2012; Cosby *et al.*, 2015). The presence of *Salmonella* in healthy animals can be considered as the main risk factor for transmitting the bacteria in table eggs and poultry feed to human (Hugas and Beloeil, 2014).

One of the important sources of human salmonellosis is the red meat itself and the food products prepared from red-meat. One of the sub species, *Salmonella* Dublin, has been reported from red meat (Neto *et al.*, 2010). This species commonly causes infections in cows, and can be fetal, especially for calves. The Dublin species is unexpectedly dangerous for its extremely high resistance against the antibiotics. In addition, there is no vaccine available for this particular species. The infection from *Salmonella* Dublin is growing throughout the world.

Dairy products also implicate in food-borne salmonellosis in human. The milk and milk products from animal origin are generally give into pasteurization, which kills *Salmonella* serovars. Therefore, the consumption of raw or inadequately pasteurized milk and contamination after pasteurization often causes milk-borne salmonellosis in patients.

Reports are available on outbreaks of food borne salmonellosis caused by unpasteurized orange juice, prepared salads, tomatoes etc. (Little and Gillespie, 2007; Jain *et al.*, 2009). The vegetable

contamination mostly caused by cross-contamination, infected food handler or inappropriate storage management.

Pet animals including amphibians, cats, birds, dogs, guinea pigs, fish, horses, mice, snakes, lizards, and turtle; are commonly infected with *Salmonella* serovars. The infected pet animals are also an important reservoir of transmission of *Salmonella* from animal to human (Bruins *et al.*, 2006; Bertrand *et al.*, 2008).

In some cases, salmonellosis may be associated with unusual sources. The contaminated ingredients, improperly cleaned or disinfected equipment in the food industry may also cross-contaminate the users, as well as, infected employees in kitchen may also cause transmission of *Salmonella* among humans.

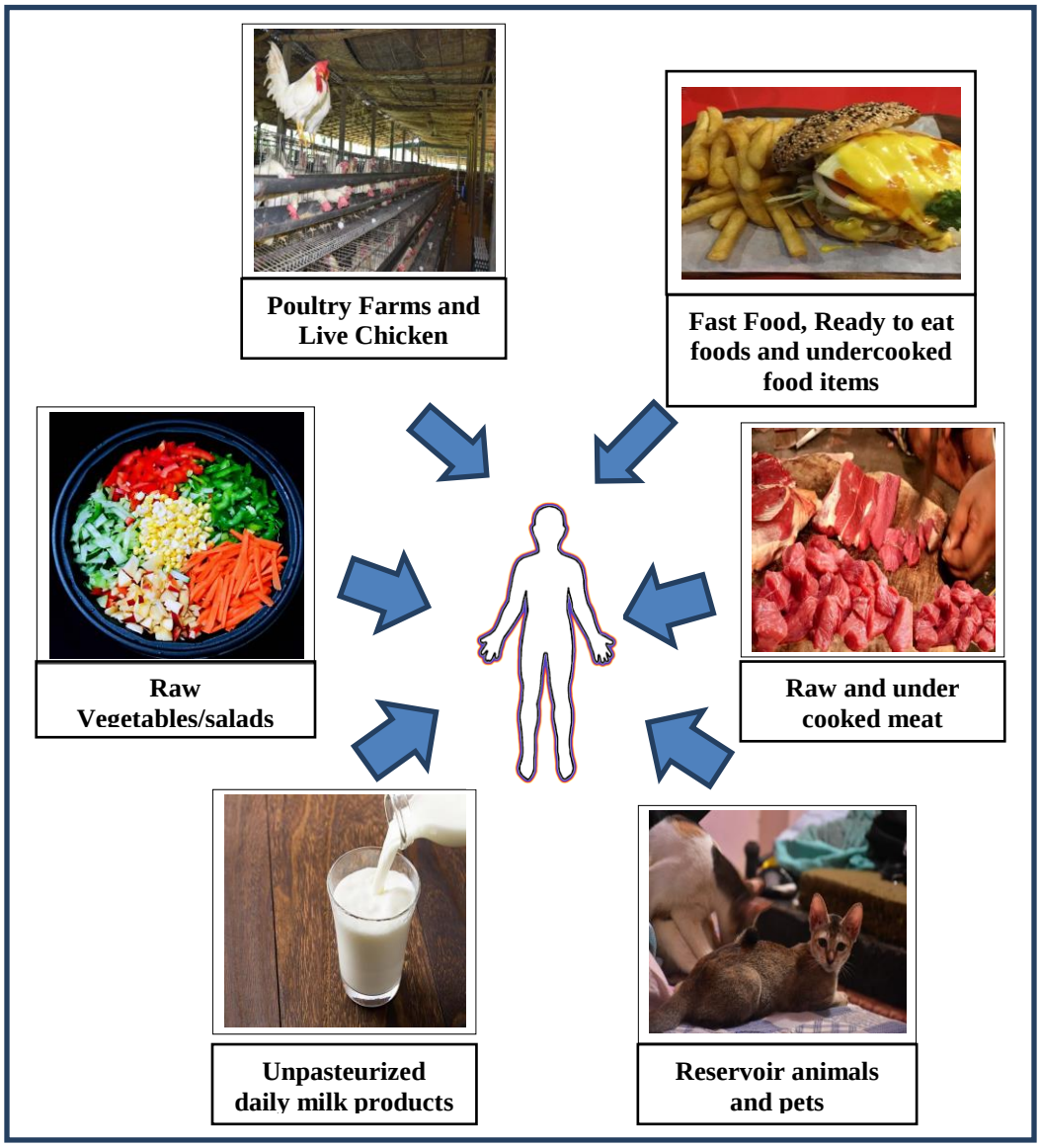


Figure 1.1: Sources of *Salmonella* transmission to human. A number of sources belong to poultry, meat, beef, vegetables, milk; ready to eat foods may contribute to the transmission process of *Salmonella* if these are mishandled or improperly cooked and stored.

1.5 Non-typhoidal salmonellosis in Bangladesh

The NTS are matter of concern because of its approximately 94 million human cases, with 150000 deaths annually in human throughout the world (Majowicz *et al.*, 2010). Both adult and children in, NTS is a common cause of bacteremia. Especially the areas of higher HIV and malaria prevalence are mostly affected with this infection (Feasey *et al.*, 2012). Comparing to

sub-Saharan Africa, the invasive NTS are not so common phenomenon in Asian countries. The low incidence of disease limits data regarding the clinical symptoms, associated risk factors, emerging resistance patterns, and outcomes for NTS bacteremia in South Asia.

The retrospective data from the 'International Centre for Diarrhoeal Disease Research, Bangladesh' (icddr'b) Hospital, Dhaka reports approximately 120,000 incidence of diarrhea cases annually (Shahunja *et al.*, 2015). It has been reported that patients with invasive NTS to the hospital can be characterized with high rates of malnutrition (Feasey *et al.*, 2010). There are also evidences of associated clinical signs, like, kidney injury, alarming WBC count, sepsis, septic shock, associated with NTS infections among hospitalized patients.

1.6 Poultry associated salmonellosis- a threat to poultry industry in Bangladesh

1.6.1 Poultry as a major source of protein

The Poultry sub-sector is an important avenue in fostering agricultural growth. This sector plays a vital role to reduce the malnutrition in people of Bangladesh (Silva and Ranking, 2013). It is an integral part of farming system in Bangladesh and has created direct or indirect employment opportunities including support services for about 6 million people fostering the entire economy (Ahmed *et al.*, 2016). The whole poultry industry itself is the center of a number of other industries relating to the inputs and outputs of poultry along with a number of service providing organizations. Poultry industry contributes 1 per cent to the country's GDP. At least 60 lakh people of Bangladesh are involved in this sector.

Poultry meat alone contributes 37% of the total meat production and 22-27% of the total animal protein supply in Bangladesh (Ahmed *et al.*, 2016). Nowadays the production of chicken meat, egg and live chicken are beyond the national demand, which indicates that poultry industry has the potentials to export to India, Pakistan, Nepal, Malaysia, Indonesia and other countries (Rahman *et al.*, 2017).

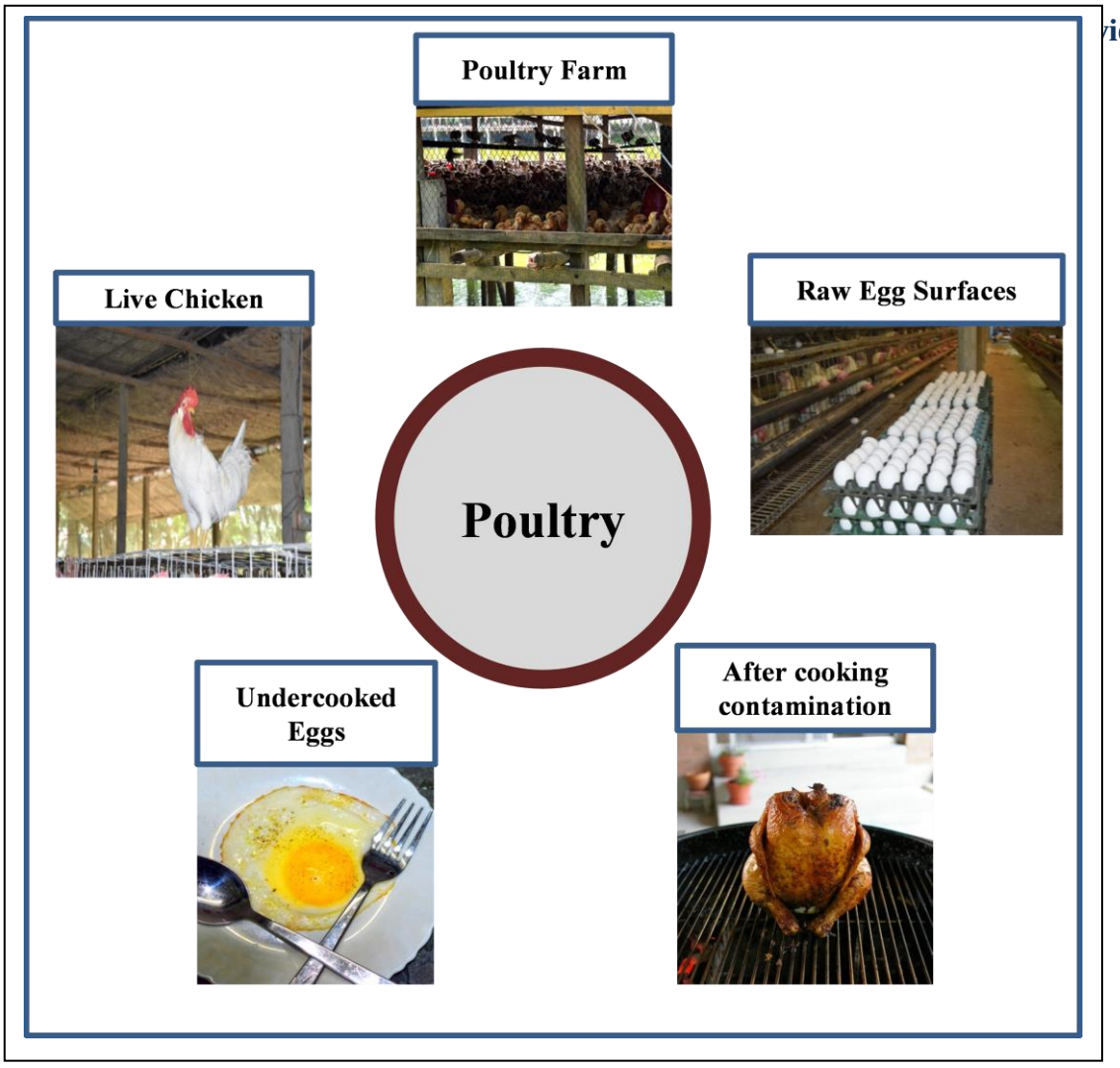


Figure 1.2: Potential sources for *Salmonella* transmission from poultry production and food processing. Raw chicken itself, egg surface and under cooked poultry food items may be contaminated with *Salmonella* and thus transmit to Human.

There are some vital challenges in the poultry sector of Bangladesh, including, the financial supports and access, endemic and seasonal diseases with higher mortality, competition with foreign farm houses. Among several bacterial and viral diseases, *Salmonella* infections are one of the major obstacles in poultry farming sector in country (Islam *et al.*, 2003; Haider *et al.*, 2012).

1.6.2 Salmonellosis in poultry

In poultry sector of Bangladesh, salmonellosis is a common concern like other developing countries. In our country the major source of protein can be revealed from chickens and eggs produced from layer farms throughout the country (Barua *et al.*, 2012). Several bacterial infections, including *Salmonella* contamination are the critical restrictions in development of poultry farming in the country (Barua *et al.*, 2014).

Zoonotic motile serovars of *Salmonella enterica* causes contamination in meat and egg products easily and originate a transmission route to human. These sources of transmission have a larger negative impact on public health globally. Bangladesh is not out of this impact, rather mostly ignored in this impactful losses, the country is facing different obstacles into this sector (Islam *et al.*, 2003; Haider *et al.*, 2012).

Salmonella serotypes MDR phenotypes are a threat to the poultry of Bangladesh (Sultana *et al.*, 2014). The prevalence of MDR *Salmonella* at farm industries is increasing day by day in Bangladesh whereas small-scale commercial farms are predominant (Barua *et al.*, 2012).

According to literature, salmonellosis is the most prevalent disease in different poultry farms of Gazipur district of Bangladesh (Hoque *et al.*, 2019). Previous studies reported 52.29% bacterial diseases among layers, including 38.56% salmonellosis. In case of broiler, 21.30% salmonellosis in 28.99% overall bacterial diseases are reported (Kabir, 2010; Al-Ferdous *et al.*, 2013).

In Bangladesh, prevalence of *Salmonella* spp. is also significantly higher in egg shell compared to egg contents and might be associated with human illnesses during consumption of contaminated poultry eggs (Hoque *et al.*, 2019). Poultry eggs from different retail markets of Savar was found contaminated by *Salmonella spp*. with 86% prevalence (Mahmud *et al.*, 2015).

A number of poultry-based products are responsible for *Salmonella* transmission from poultry to human (Figure 1.2). The primary transmission occurs through raw egg handling. But the direct and indirect contact and handling the chickens, cleaning the carcasses, droppings without proper hygienic maintenance may also cause cross contamination of pathogens. Other environmental factors such as air, unclean facilities, and vectors, such as insects, and rodents are also responsible for *Salmonella* contamination in poultry farms. The prevalence of salmonellosis in breeder flocks and specially layer flocks is increasing in Bangladesh (Islam *et al.*, 2003; Sikder *et al.*, 2005). These contaminated flocks eventually spread the pathogen gradually among farm,

transport, poultry bazar, bazar place, kitchens and then transmit to human. This sector is a larger reservoir of this pathogen as well as threat to public health of country.

1.7 Antibiotic resistance in *Salmonella*: a matter of concern for public health

Antimicrobial resistance in bacterial pathogens is a worldwide challenge associated with high morbidity and mortality (Akova, 2016). Multidrug resistant patterns in bacteria have resulted in difficult-to-treat or even untreatable infections with conventional antimicrobials. Dramatic increases in emerging resistance occur due to liberal and unnecessary use of broad-spectrum antibiotics. Along with the poor infection control practices cause the frequent dissemination of resistant pathogens to the other patients and the environment.

The improper use of antimicrobial agents in clinical, industrial or laboratory sector creates selection pressures that favor the survival of antibiotic-resistant pathogens. According to the infectious-disease report that was released by the World Health Organization in 2000, such organisms have become increasingly prevalent worldwide (WHO, 2001). The resistance to antimicrobial erects a burden with increased morbidity, mortality, and financial losses associated with disease. The routine practice of giving antimicrobial agents to domestic livestock as a means of preventing and treating diseases, as well as promoting growth, is an important factor in the emergence of antibiotic-resistant bacteria that are subsequently transferred to humans through the food chain (Tollefson *et al.*, 1997; Witte, 1998).

Almost all the countries, especially in developed countries, usage of antimicrobial drugs in food-producing animals, either therapeutically or prophylactically, or for growth promotion are common incident. The improper usage of antimicrobial drugs prompts the emergence of resistance in non-typhoidal *Salmonella*. Most infections with antimicrobial-resistant *Salmonella* are acquired by eating contaminated foods of animal origin (Scallan *et al.*, 2011). Of particular concern in such organisms is the development of resistance to key antimicrobials such as the fluoroquinolones (Kumar *et al.*, 2019) and extended-spectrum β-lactamases (Vahaboglu *et al.*, 2001).

Antibiotics such as ciprofloxacin, azithromycin, and ceftriaxone are commonly used to treat patients with severe *Salmonella* infections. The arising resistance causing less susceptibility of non-typhoidal *Salmonella* is increasing day by day, thus limiting the treatment options.

Table 1.2: Resistance snapshot of non-typhoidal *Salmonella* according to the data from CDC 2019 threat report based on USA

	Percentage of all non-typhoidal *Salmonella*[a]	Estimated numbers of infections per year
Ceftriaxone resistance	3%	41000
Ciprofloxacin nonsusceptible	7%	89200
Decreased susceptibility to Azythromycin	0.5%	7400
Resistant to ≤1 essential antibiotic[b]	16%	212500
Resistant to ≤3 antibiotics[b]	2%	20800

[a]Average (2015-2017) in USA (CDC, 2019)

[b] Represents the following: ciprofloxacin nonsusceptible, decreased susceptibility to azithromycin, resistance to ceftriaxone, ampicillin, or trimethoprim-sulfamethoxadole.

The resistance mechanisms are not unique for all serotypes of NTS organisms. Sometimes these are specifically significant for the specific strain. The genomic research analysis from late 90's to 2000s revealed several clones of MDR *Salmonella* universally. For instance, in *Salmonella enterica* serotype Typhimurium, the resistance to common antibiotics, such as, ampicillin, chloramphenicol, tetracycline etc. are carried by mobile genetic elements and thus are horizontally transmissible among the strains.

Table 1.3: Resistance to fluoroquinolones in NTS (WHO)

Data sources based on at least 30 tested isolates	Overall reported range of resistant proportion (%)	Reported range of resistant proportion (%) in blood isolates (no. of reports)
Africa (n=17 countries data)	0–35 0–30	0–30 (n=4)
United States of America (n=14 countries data)	0–96 0	
Eastern Mediterranean (n=8 countries data)	2–49 0–46	6 (n=1)
Europe (n=30 countries)	2–3 13	
South-East Asia (n=3 countries)	0.2–4 1.4	
Western Pacific (n= 11countries)	0–14 0–0.3	

FWD-Net, Foodborne and Waterborne Diseases and Zoonoses Network. (WHO, 2014)

a. ciprofloxacin, norfloxacin.

Despite the severe invasive infections, NTS causing infections are generally common and usually self-limiting. Multidrug-resistance in several serotypes of *Salmonella enterica* has been associated with higher risk of invasive infections. The MDR pathogens also cause higher frequency and duration of hospitalization with prolonged illness, and increased risk of death as compared to infections caused by susceptible strains (Osazuwa *et al.*, 2011).

In Bangladesh, higher prevalence of poultry associated clinical MDR *Salmonella* have been reported in several studies (Ferdous *et al.*, 2013, 2019; Mannan *et al.*, 2014; Munna *et al.*, 2015; Rahman *et al.*, 2018). These studies also revealed the occurrence of resistant *Salmonella* among diverse food and animal sources other than poultry sector. Though there are a number of reports on prevalence and antimicrobial resistance patterns, the sources and transmission routes of *Salmonella* in developing countries, are poorly understood due to the lack of coordinated national epidemiological surveillance systems (Aferstein, 2003).

1.8 Genotypic methods for surveillance- a step to control

Global surveillance of *Salmonella* infections and outbreaks are conducted and facilitated by WHO in regular basis since 2000. The advanced protocols and epidemiological methodologies enhance the capability to epidemiologists to address the outbreaks and conduct regular surveillance of specific clonal variants of pathogens in more scientific and acceptable manner. Furthermore, bacterial typing techniques are now more widely used to measure genetic relatedness among emerging pathogenic strains, clones or clusters of specific bacterial species.

In the beginning of the bacterial typing era, typing systems were based solely on phenotypic methods such as serotyping (Grimont and Weill, 2007), phage typing (Sechter and Gerichter, 1968; Petrow *et al.*, 1974; Ward *et al.*, 1987) and antibiogram typing (Figure 1.3). For a long period of time, the epidemiological studies are based on different phenotypic tests, like serotyping, for NTS characterization. Recently, several DNA fingerprinting and array techniques have been developed for upgrading the characterization protocols based on molecular and genomic organizations (McQuiston *et al.*, 2004; Fitzgerald *et al.*, 2007).

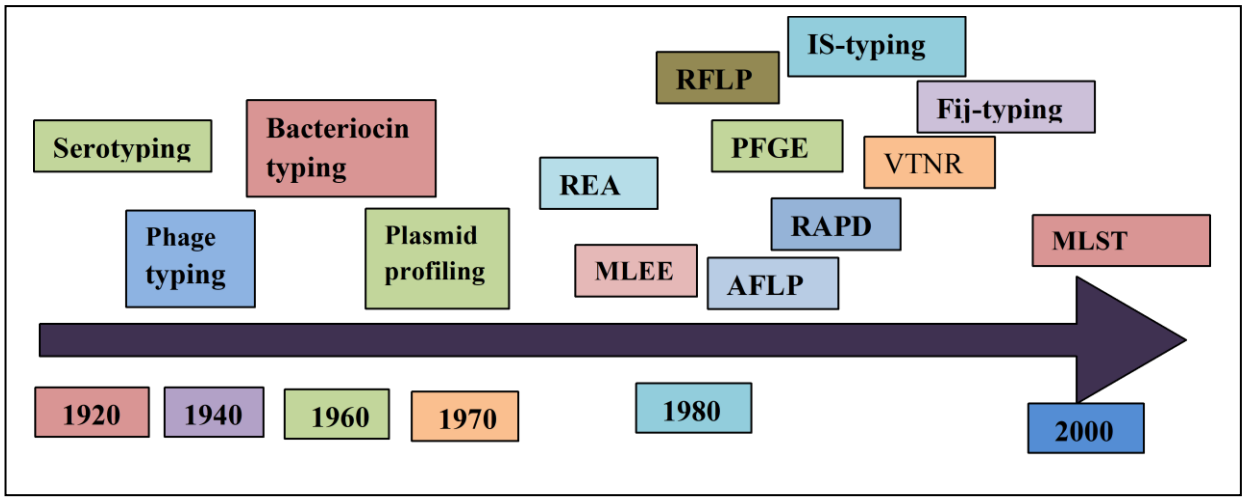


Figure 1.3: **Evolution of molecular typing methods for evolutionary analysis of bacterial pathogens.**

The basis of phylogenetic study is to perceive the variations in distantly related isolates of same genus of organisms. These variations accumulate relatively slowly and impacts on global epidemiology in long term. Generally, the housekeeping genes are considered to be objective in evolutionary reconstructions, and are also scientifically well researched and documented. These features attract the housekeeping genes for genotypic variation analysis. In the past, multilocus

enzyme electrophoresis assessed the allelic variation of the genes in a strain by determination of electromorphs (allozymes) of an enzyme (Selander *et al.*, 1986). Recently new concept of MLST has been developed by simply using DNA sequencing protocols, which is not only rapid, but also cost effective. In MLST method, enzymatic electrical mobilities are not consider, rather the nucleotide sequences of housekeeping genes are analyzed and compared with universal open database (Maiden *et al.*, 1998). There is also database for *Salmonella enterica* is available, among which the nucleotide sequences of seven housekeeping genes are compared to determine the relatedness among similar species in serovar level (Achtman *et al.*, 2012). The changes in DNA level are revealed in MLST method thus can recognize the phylogenetic lineages in individual serovars. The prophylactic serovars, which are originated from more than one common ancestor, can be identified using MLST.

1.9 Methods for *Salmonella* detection and enumeration- integral part of control program

Though *Salmonella* is a widely recorded food borne pathogen, numerous typing methodologies have been developed to trace salmonellosis outbreaks to the contamination source and to explore the epidemiology of *Salmonella* infections. In conventional detection methods, generally physiological and biochemical markers of that organism have been used for detection and characterization (Williams, 1981). Cultural methods are based on nutrient acquisition, biochemical characteristics, and metabolic products unique to *Salmonella* spp. (Ricke *et al.*, 1998).

1.9.1 Traditional cultural methods for detection and isolation

An internationally accepted procedure is established for the detection and isolation of *Salmonella* in standard document ISO 6579:2002/A1:2007 (Microbiology of food and animal feeding stuffs-horizontal method for the detection of Salmonella spp. Amendment 1: Annex D: Detection of Salmonella spp. in animal faeces and in environmental samples from the primary production stage., 2007). This method consists of four stages: (i) pre-enrichment of the sample in non-selective buffered peptone water (BPW) for 18 h at 37°C ± 1°C, (ii) enrichment in two different selective liquid media, Tetrathionate Broth Base for 24 h at 37°C ± 1°C and Rappaport-Vassiliadis Broth (RSV) for 24 h at 41.5°C ±1°C, (iii) inoculation and identification on XLD agar plate after 24 h incubation at 37°C ± 1°C and use another selective agar medium plate of free choice, (iv) identification with confirmatory approaches using biochemical and serological tests. It requires 4-6 working days for confirmatory identification.

After identification, enumeration of bacteria is laborious and time consuming. The conventional bacteriological methods (MPN) test or cell count on agar plate is used for bacterial enumeration. Rapid, user friendly, less human handling, cheap enumeration methods could provide quantitative data for proper control measure analysis. On the other hand, for a production system, early diagnosis with quantification could provide proper information about source and transmission of contamination (Malorny *et al.*, 2008). In clinical diagnosis to trace the source, only highly sensitive and specific enumeration method could detect the minimal number of bacteria in primary production or ready-to-eat food items.

1.9.2 Conventional PCR method for *Salmonella* detection

Polymerase Chain Reaction (PCR) has been demonstrated by Kary Mullis in the mid-1980s. Since then, this procedure has been considered as an efficient diagnostic tool for clinical and food microbiology. A number of scientific protocols have been published for establishment of PCR as a successful and reproducible technique (Hoorfar *et al.*, 2000; Malorny *et al.*, 2003).

Today for detection of *Salmonella* the universal target gene, *invA* is used for PCR method (Rahn *et al.*, 1992). This gene locates within the highly conserved pathogenicity island 1 of *Salmonella*. Initially *oriC* gene was targeted for *Salmonella* identification in agarose-gel electrophoresis based PCR assay (Widjojoatmodjo *et al.*, 1991). After validation of the PCR protocol for *Salmonella*, *invA* gene is widely used and considered as universal with highest selectivity (Malorny *et al.*, 2003). There are also other primers published for *Salmonella* detection, but their detection limit, accuracy varies, thus acceptability also (Bej *et al.*, 1994; Cohen *et al.*, 1996; Makino *et al.*, 1999; Ziemer and Steadham, 2003).

1.9.3 Real-time PCR methods

In 1990s, the PCR technologies were introduced to fluorescent ds-DNA binding dyes or DNA probes. Real-time PCR uses some basic components as traditional PCR like dsDNA, primers, dNTPs, PCR buffer, Taq polymerase etc. As with traditional PCR, reactions of real-time PCR are cycled in a temperature block. However, in real-time PCR some form of fluorescent dye is added to the PCR mix.

- ✓ **Fluorescence detection systems**

All the Real-time fluorescence detection technologies are on the fluorescent signal that is proportional to the amount of PCR products produced in each PCR cycle. The choice of fluorescence system is determined by the method and requirement of the protocol. The three main fluorescence detection systems are:

(i) DNA-binding agents (e.g., SYBR® Green and SYBR® Green ER™ technologies)

(ii) Fluorescent primers (e.g., LUX™ Fluorogenic Primers and Amplifluor™ qPCR primers)

(iii) Fluorescent probes (e.g., TaqMan® probes, Scorpions, Molecular Beacons)

- ✓ **DNA-binding dyes**

Intercalating dyes have the feature to emit fluoresce while bound to dsDNA. This feature is used in Green I and SYBR® Green ER™ technologies. SYBR® also use this type of detection mechanism. SYBR® is a cyanine dye, can be used to stain nucleic acid in dsDNA detection protocols. This binding dye can be used to quantify amplicon amount during the PCR reaction through fluorescence emission. The signal significantly increases when bound to double-stranded DNA (dsDNA). The intensity of the fluorescent signal depends on the amount of dsDNA that is present. The intensity of the signal is proportional to the DNA concentration and is presented continuously on real-time PCR instruments. As the dye indiscriminately binds all dsDNA in reaction mixture, it may lack specificity in some extent. But the specificity can be assessed using melting curve analysis.

The basic mode of action of SYBR Green PCR has been diagrammatically presented in Figure 1.4.

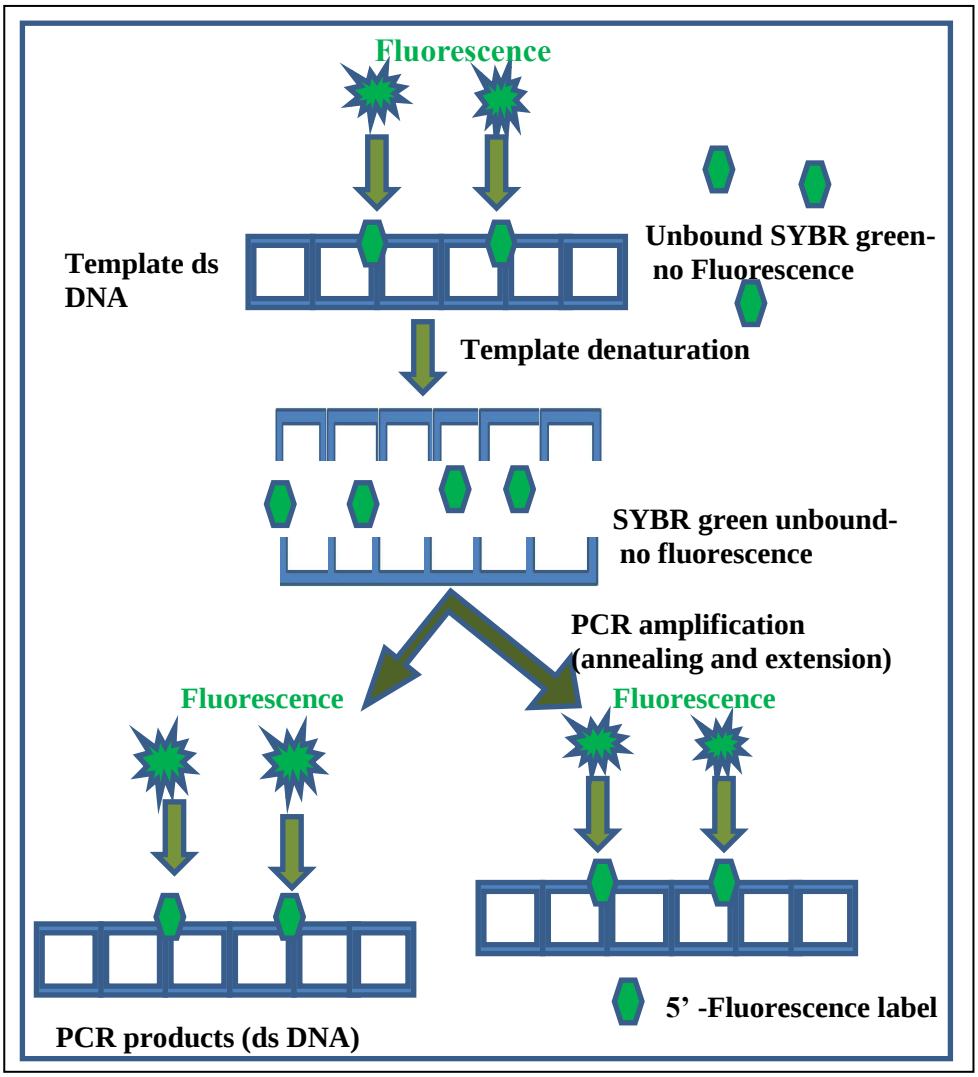


Figure 1.4: Mode of action of SYBR green dye [modified from (Smith and Osborn, 2009)].

1.9.3.1 PCR sample preparation

For PCR analysis of raw samples, a pre enrichment of the target pathogen is needed to get an acceptable concentration of cells in the analytical (Löfström *et al.*, 2004; Malorny *et al.*, 2009). For this pre-enrichment step, an extra time period had to be added with the total protocol, but it provides some essential benefits, like help to differentiate the viable from non-viable cells and also dilutes the inhibitors. This step also helps to repair the stressed or injured cells in raw samples.

The next step of enrichment is to isolate the target bacteria from the sample using different culture based selective methods. The genomic DNA of the target organism also has to be purified from the raw sample as well. There are a number of biochemical purification methods which are designed to concentrate the target DNA from the inhibitors in raw samples. Many commercial kits for DNA extraction are available for different sample types and conditions (Elizaquível and Aznar, 2008). In some methods of bacterial DNA extraction, a chelating resin, e.g. 6% (w/v) Chelex 100 suspension is used in simpler manner (Malorny *et al.*, 2003; Vázquez-Novelle *et al.*, 2005).

A number of physical non-destructive purification methods are used for purification of *Salmonella* based on the principle of bacterial cell density properties. The methods utilize buoyant density centrifugation, aqueous two-phase systems and floatation (Löfström *et al.*, 2004; Wolffs *et al.*, 2006). Floatation can separate biological particles and microorganisms that differ in buoyant density in between cells and media, which allows the cells to float. In other methods, different substances are directly added to the reaction tube to neutralize PCR inhibitors for enhancing the efficiency, bovine serum albumin, Triton X-100, Tween 20 are some examples of such substances used for inhibitor neutralization (Wilson, 1997; Waleed and Peter, 2000; Hedman *et al.*, 2013).

1.9.3.2 Real-time PCR and quantification

The enumeration method of real-time PCR is based on the exponential increase of the initial amount of DNA during the reaction period rather than the end point signal (Mackay, 2004). There are several advantages of real-time quantitative PCR over conventional PCR, such as, detection limit, speed, cost and high throughput of quantitative data on target organism in various matrices (Guy *et al.*, 2006; Wolffs *et al.*, 2006).

1.9.3.3 Validation of real-time PCR

Real-time PCR in combination with enrichment can be used for national epidemiological surveillance and monitoring. The method is increasingly applied to identify *Salmonella* in potentially contaminated food samples as well as raw samples of animal and human origin. The methods based on real-time PCR need to be approved by recognized certification bodies such as the Association of Official Analytical Chemists (AOAC) in the USA, the European Validation and Certification Organization (MicroVal) in Europe.

The method has to be validated for certification with proper protocols comprises two steps: comparing study of alternative method and the reference method in expert laboratory (in-house validation), and an inter-laboratory study against reference method carried out indifferent laboratories. Generally the detection limits, selectivity, potentiality of the method are determined by in-house validations authorities (Hoorfar *et al.*, 2004; Qvist, 2011).

1.10 Detection of *Salmonella* in each point of poultry production system- possible way of prevention

According to several studies, the prevalence of various *Salmonella* serotypes among live birds ranges from 6% to 30% (Liljebjelke *et al.*, 2005; Srinivasan *et al.*, 2014), while the incidence of *Salmonella* in poultry and poultry products ranges from 1% to 65.5% (Fearnley *et al.*, 2011; Hyeon *et al.*, 2011; Yang *et al.*, 2011). The infected live birds may harbor the pathogens asymptomatically, while others may disseminate via lateral transmission, mainly through feces, feathers, litter etc. (Wakenell, 2016).

In poultry sector, the processing periods of live chickens can cross-contaminate the production system with the existing bacteria or viruses. Each stage of poultry processing from farm to bazar to kitchen is a potential point for cross contamination of *Salmonella* to environment. In farm areas, the contamination may occur during handling the live chicken, cleaning the hatches, or directly through the egg surfaces. In bazar, the sanitation practices are beyond expectation, thus the condition is more antagonized comparing to farm areas. The transporting vehicle, cages in transports or bazar are also major sources of contamination. The processing instruments, knives, cutting boards in bazar also act as reservoir of potential pathogenic organisms harbored by live chickens. In Bangladesh, several studies addressed the higher prevalence of *Salmonella* in poultry sector (Mahmud *et al.*, 2011; Barua *et al.*, 2012; Karim *et al.*, 2017), but these studies focus on the occurrence of *Salmonella* rather than studying the source and dissemination mode of *Salmonella*. There are lack of etiological studies covering each transmission point is not available from the country. In chicken, *Salmonella* may exist in the feathers, feet, intestines; so the poor sanitation and cleaning measures can easily contaminate the various sites of poultry farms and bazar areas. The high moisturized environment in bazar of our country mostly stimulates the colonization of such bacteria in to processing area and helps the transmission up to kitchen.

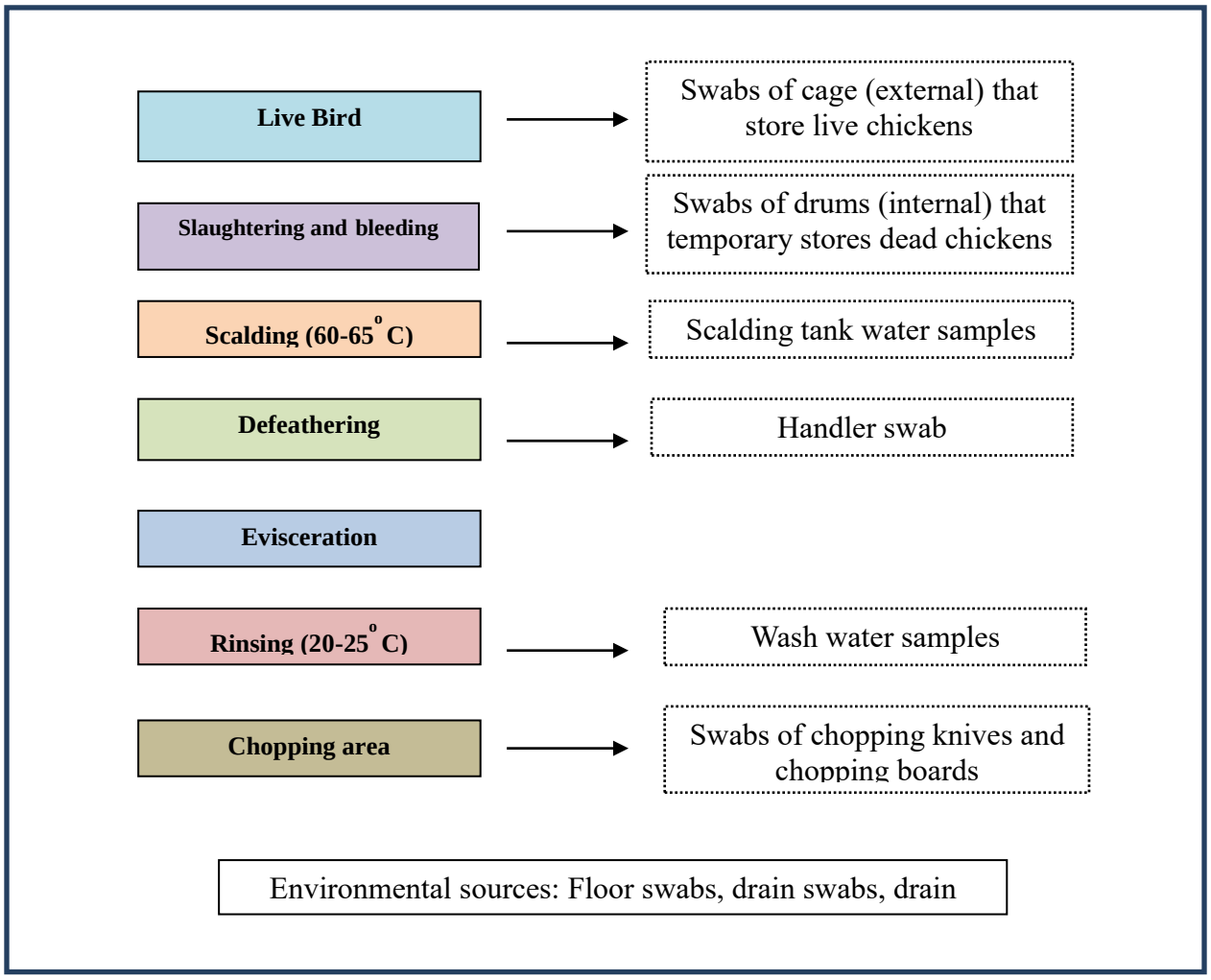


Figure 1.5: Possible market sources for *Salmonella* transmission and the sampling points for analysis.

The improperly cleaned surfaces promote biological soil build-up, and, in the presence of water, contribute to the development of bacterial biofilms which may contain pathogenic microorganisms (Chmielewski and Frank, 2003). *Salmonella* can easily attach and form biofilms on surfaces found in food processing plants, including plastic, cement, and stainless steel (Chmielewski and Frank, 2003). Poultry processing in bazar areas in Bangladesh involve constant rinsing steps. Wet environment encountered in poultry processing plants is ideal for biofilm formation. Studies have shown that *Salmonella* prevalent in poultry processing environment can be isolated from poultry processing equipment, especially in the slaughter and evisceration areas (Helke *et al.*, 1993; Helke and Wong, 1994; Joseph *et al.*, 2001).

As the poultry farm and bazar can contribute as a source of *Salmonella* transmission, there is huge possibility to transmit these bacteria from these areas direct to hotel kitchen and household kitchen. Identification of prevalent *Salmonella* spp. among the kitchen area also required to trace the route. In kitchen, the raw meat and vegetable preserving areas, chopping boards, knives may cause cross contamination among raw foods and salad items. Proper hygienic practice may minimize the chance of cross contamination. So, a countrywide survey of *Salmonella* throughout the routes from poultry farm to kitchen with proper, affordable and quick methods will help to getting steps regarding prevention and control management of poultry associated *Salmonella* in Bangladesh.

Objectives of this study:

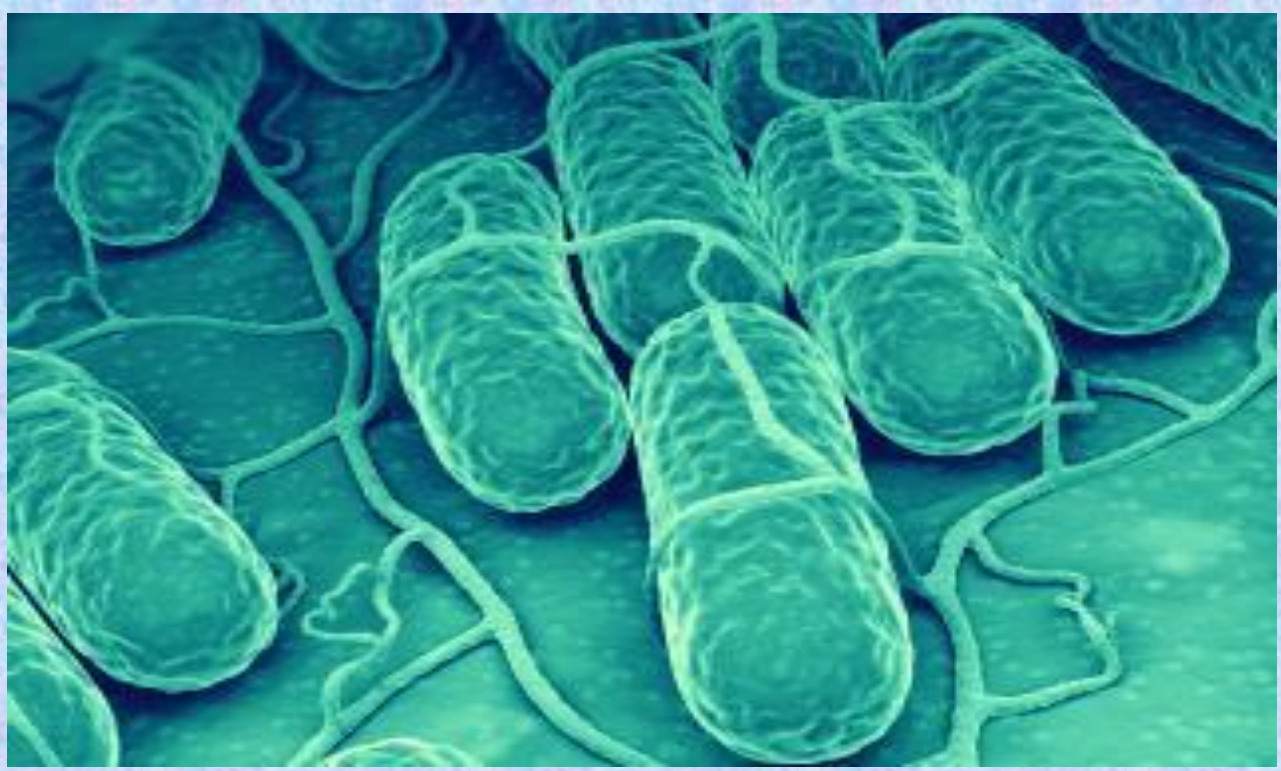
Non typhoidal Salmonellosis is a common phenomenon in Bangladesh. For introduction and establishment of a control panel to prevent NTS salmonellosis as well as food borne illnesses, it is necessary to create a database about the pathogens. Upgraded genotypic variants, their evolution, associations, zoonotic potentials, correlation with antibiotic resistance, correlation among global isolates and transmission rout identification are prerequisite for creating a database and establishing any preventive measures. To track down the prevalence and rapid transmission of *Salmonella*, a rapid, simple and affordable method is required.

This study intends to achieve this by developing a method for quantification using real-time PCR, and developing a validation protocol.

The first part of this study war aimed to gather information about the genetic variations among poultry farm samples. The second part was targeted to establish a simple and robust real-time PCR method using SYBR Green that would be suitable for routine analysis of *Salmonella* spp. and finally, the finding of the study was accumulated to sum up in a link of transmission of farm *Salmonella* into household kitchen through the transmission route.

The study has four sub-objectives:

1. Assessment of *Salmonella* burden in selected poultry farms;
2. Isolation and characterization of *Salmonella* spp. and their antibiotic resistance profile analysis;
3. Molecular characterization and distribution analysis of the isolated *Salmonella* spp. using different typing approaches, such as, ARDRA, RAPD, MLST; and
4. Validation of SYBR green Real-Time qPCR method and identification the transmission route of *Salmonella* from producers to end users.



Chapter 02: Materials and Methods

2. Materials and Methods

2.1 Farm survey

A survey was done to collect primary data by pre-formatted questionnaire (Annex-1) which was designed for all the farmers from 14 layer poultry farms located in five different districts of Dhaka division. These regional places were selected due to the higher number of layer poultry farms and farmers. The questionnaire was based on the regular hygienic practices applied in the poultry farm houses. A veterinarian was present during sample collection and queries on disease and treatment was collected through him (Table 2.2).

Table 2.1: The farms studied during present work.

Farm No	Poultry Farm Name	District	Type of Chicken	Flock size
F1	Kawsar Poultry Farm	Narayanganj	Layer	1000
F2	Khajapolli Poultry Farm		Layer	2500
F3	Mayer Doa Poultry Farm		Layer	7000
F4	Pandhoa Poultry Farm	Savar	Layer	2000
F5	Savar Poultry Farm		Layer	5000
F6	Shiraj Poultry Farm	Gazipur	Layer	2000
F7	Kapasia Poultry Farm		Layer	2500
F8	Alam Poultry Farm		Layer	2000
F9	Sujon Poultry Farm	Manikganj	Layer	3000
F10	Balaka Poultry Farm		Layer	3000
F11	Ma Poutry Farm		Layer	2000
F12	Nadira Poultry Farm	Gopalganj	Layer	2000
F13	Ali poultry Farm		Layer	2700
F14	Reza Poultry Farm		Layer	1200

Fourteen different poultry farms were selected for this study. Though all the farms contain same type of chicken but vary in amount. All the collected data based on questionnaire were tabulated using excel sheets. The pie chart, bar chart and tables were prepared for the easy interpretation. The farmers were divided into five groups based on their location/districts.

Table 2.2: The prevalent diseases and antibiotics used in poultry farms of Bangladesh.

Area	Farms	Prevalent Bacterial Diseases	Mostly Used Antibiotics
Narayanganj	F1, F2, F3	Fowl cholera, collibacillosis, Necrotic enteritis, Infectious coryza, Salmonellosis	Amoxicillin, Ciprofloxacin, Enrofloxacin, Tetracycline, Pleuromutilins, Macrolide, Aminoglycosides
Savar	F4, F5,F6	Fowl cholera, collibacillosis, Necrotic enteritis, Infectious coryza, Salmonellosis	Amoxicillin, Ciprofloxacin, Enrofloxacin, Tetracycline, Pleuromutilins, Macrolide, Aminoglycosides
Gazipur	F7, F8,F9	Fowl cholera, collibacillosis, Necrotic enteritis, Infectious coryza, Salmonellosis	Amoxicillin, Ciprofloxacin, Enrofloxacin, Tetracycline, Pleuromutilin, Macrolide, Aminoglycosides
Manikganj	F10, F11,F12	Fowl cholera, collibacillosis, Necrotic enteritis, Infectious coryza, Salmonellosis	Amoxicillin, Ciprofloxacin, Tetracycline, Aminoglycosides
Gopalganj	F13, F14,F15	Fowl cholera, collibacillosis, Necrotic enteritis, Infectious coryza, Salmonellosis	Ciprofloxacin, Enrofloxacin, Tetracycline, Aminoglycosides

2.2 Isolation and identification of non-typhoidal *Salmonella* in poultry samples

Poultry meat alone contributes 36 per cent of total meat production in Bangladesh (BBS, 2013).The presence of urban consumers is a precondition for the development of commercial poultry production. Almost 72.9% of the total commercial chicken production in Bangladesh is located in the divisions of the country's two largest cities of Chittagong and Dhaka (Source: Bangladesh Bureau of Statistics, June 2016 and FAO 2010). As Dhaka Division is the largest

source of poultry business and marketing, 5 districts of this division were selected through analyzing the number of poultry farms, business and distance between the places (Table 2.1).

2.2.1. Sampling time

All poultry samples were collected between 20th October, 2015 to 5th May, 2017. The specific sampling times are listed in Table 2.3.

Table 2.3: Timeline of the poultry sample collection from five different districts of Dhaka Division, Bangladesh

Districts	Number of Farms	Number of samples	Date of Sampling
Narayanganj	3 (F1, F2, F3)	23	20th May, 2015
Savar	2 (F4, F5)	20	1st May, 2016
Gazipur	3 (F6,F7, F8)	33	3rd July, 2016
Manikganj	3 (F9,F10, F11)	48	6th September, 2016
Gopalganj	3 (F12,F13, F14)	30	5th May, 2017
Total	14	154	

2.2.2. Sampling Area

In Bangladesh, the highest numbers of poultry farms are located in Dhaka Division (http://www.dls.gov.bd/2014). For this study, poultry samples were collected from five districts of Dhaka Division, Bangladesh. Savar (23° 51' 30.0024" N and 90° 16' 0.0120" E), Narayanganj (23° 37' 21.5076" N and 90° 29' 59.2584" E.), Gazipur (23° 59' 59.7876" N and 90° 25' 12.9828" E), Manikganj (24° 78' 09'' N, 91° 87'72''E) and Gopalganj (26° 28' 12.00" N,84° 25' 48.00" E) (Figure 2.1) are five higher poultry farm containing districts among Dhaka Division. Fourteen different poultry farms were selected for collecting farm samples among these areas.

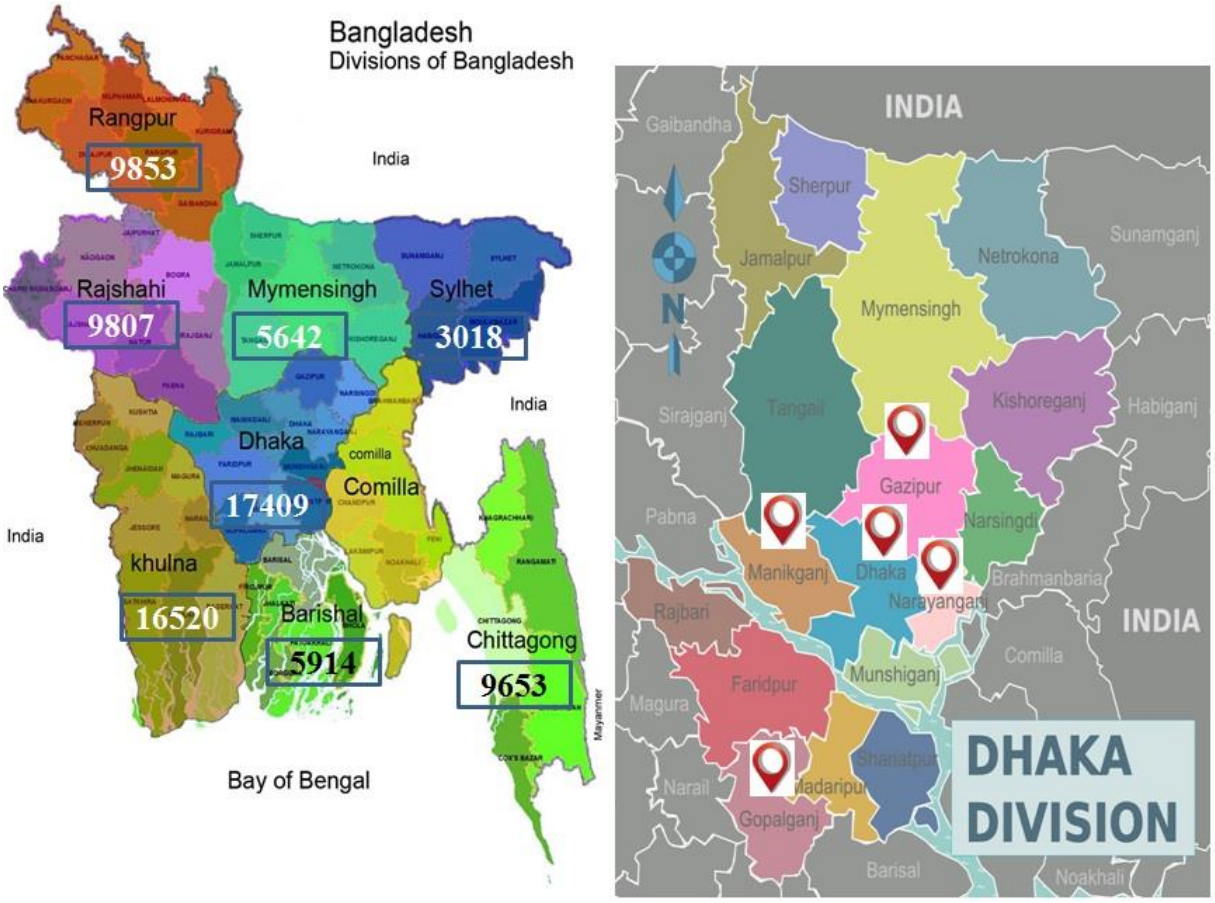
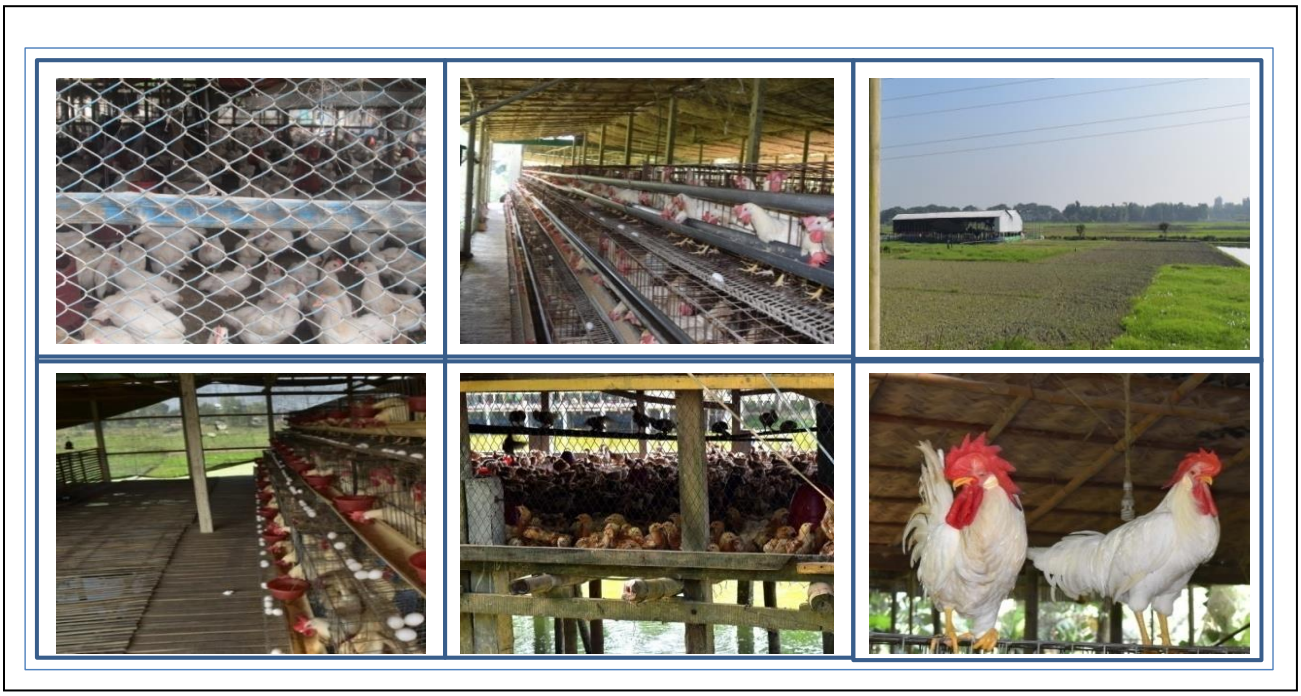


Figure 2.1: (a) The Number of Poultry Farms in Different Divisions of Bangladesh; (Reference: Central Disease Investigation Laboratory, Bangladesh; http://www.dls.gov.bd/2014) **(b) Selected Sampling Areas among Dhaka Division.**

2.2.3. Sample collection:

The selected farms were physically visited once to collect samples (Figure 2.2). Different poultry farm samples including droppings, cloacal swab, poultry feed, poultry water, egg-shell swab, and handlers swab were collected for further microbiological analysis. All samples were collected within appropriate biosafety manner (Figure 2.2). The collectors always had face mask and gloves during sampling. All the culture media, cotton swab were autoclaved and transported in proper temperature.

A.



B.

Figure 2.2: Different types of samples were collected (cloacal swab, droppings, egg swab, handler swab, feeding water) (A.), from fourteen different poultry farms located in five different districts (B.).

Each poultry sample was collected directly in buffered peptone water (BPW; CM0009; Oxoid Ltd., England), and was placed separately in a sterile plastic bag, and finally transferred to the laboratory (Microbial Genetics and Bioinformatics Laboratory, Department of Microbiology, University of Dhaka)at ambient temperature. After arrival at the laboratory, the samples were stored at 5^{o} C until further processing and examination.

2.2.4 Isolation and identification of poultry *Salmonella*

2.2.4.1 Pre enrichment of the samples

Food and Drug Administration (FDA) guideline (BAM, 2016) was followed for the isolation and characterization of *Salmonella* species. The collected samples in BPW were incubated at 37^{o} C for 18 hours. After that 0.1ml of this culture was inoculated into Rappaport Vassiliadis (RV) broth (02-379; ScharlauChemie, EU), and Tetrathionate (TT) broth (MM032; HiMedia Lab. Netherlands).

All the inoculated RV and TT broth tubes were then incubated with proper condition. Inoculated RV medium were incubated for 24 ± 2 h at 42 ± 0.2°C (circulating, thermostatically-controlled, water bath). TT broth culture was incubated for 24 ± 2 h at 43 ± 0.2°C (circulating, thermostatically-controlled, water bath).

2.2.4.2 Culture based and biochemical identification of *Salmonella* spp.

After enrichment with RV and TT media, 0.01 ml fresh culture from each tube was streaked onto Xylose Lysine Deoxycholate (XLD) Agar (CM0469; Oxoid, England), Salmonella-Shigella (SS) Agar (CM0099; Oxoid, England), and MacConkey Agar (CM0115; Oxoid, England) media. The inoculated media were incubated overnight at 37^{o} C. The specific colonies were then tested with Gram staining followed by biochemical tests. The biochemical tests were performed based on the guideline of the Bergey's Manual of Determinate Bacteriology (Buchanan, 1974). The performed biochemical tests included: Urease Test, Oxidase Test, Catalase Test, Triple Sugar Iron (TSI), Kligler Iron Agar (KIA) test. The principles of these biochemical tests have been discussed in following:

Urease Test: This test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease. Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Rapid urease-positive organisms turn the entire medium pink within 24 hours. This test can be used as part of the identification of several genera and species of Enterobacteriaceae including *Salmonella*, *Klebsiella*, and *Proteus*. Suspected isolates producing negative result in this test can be biochemically confirmed as *Salmonella* (Brink, 2010).

Oxidase Test: The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. If present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless. *Salmonella* isolates are expected to be oxidase negative (Shields and Cathcart, 2010).

Catalase Test: The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. As a facultative anaerobe, *Salmonella* generally produce positive result for catalase (Reiner, 2010).

Triple Sugar Iron (TSI) Test: The objective of this test is to identify the ability of an organism to ferment glucose, lactose, and sucrose, and their ability to produce hydrogen sulfide. An agar slant of a special medium with multiple sugars constituting a pH-sensitive dye (phenol red), 1% lactose, 1% sucrose, 0.1% glucose, as well as sodium thiosulfate and ferrous sulfate or ferrous ammonium sulfate is used for carrying out the test (Skillern and Overman, 1983). All of these ingredients when mixed together and allowed solidification at an angle result in a agar test tube at a slanted angle. The slanted shape of this medium provides an array of surfaces that are either exposed to oxygen-containing air in varying degrees (an aerobic environment) or not exposed to air (an anaerobic environment) under which fermentation patterns of organisms are determined. The expected results for *Salmonella* contain; red slant, yellow butt, and production of both gas and H_2S. This result can be interpreted that *Salmonella* ferments only glucose with gas and H_2S production. This test is often used to differentiate enteric bacteria including *Salmonella* and *Shigella*.

Kligler's Iron Agar (KIA) Test: Kligler's Iron Agar (KIA) is used for the detection of carbohydrate fermentation. KIA often used for the presumptive identification of *Salmonella, Shigella* and other members of the Enterobacteriaceae family. *Salmonella* isolates supposed to produce alkaline slant/acid butt along with gas production. The result indicates that *Salmonella* isolates can ferment glucose but not lactose (Skillern and Overman, 1983).

2.2.5 Extraction of genomic DNA

The distinguished suspected *Salmonella* isolates were subjected to DNA extraction following the boiling of the cells at 100^{o}C and then immediately transferred into ice for 10 minutes. The

process was then followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was used for amplification by PCR with *Salmonella* specific primers (Nandi *et al.*, 2013). Concentration and purity of the extracted DNA was determined by using NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).

2.2.6 Screening of virulence genes by gene specific PCR

The genomic DNA of the respective isolates were used for PCR amplification with *Salmonella* specific primers S139 and S141 (Rahn *et al.*, 1992) targeting the *invA* gene of *Salmonella*- 5´-GTGAAATTATCGCCACGT TCGGGCAA- 3´and 5´ TCATCG CACCGT CAAAGGAACC -3´ respectively (Table 2.4). PCR reaction condition was 95ºC for 10 minutes (initial denaturation) followed by 95ºC for 15 seconds (cycle denaturation), 58ºC for 30 seconds (annealing) and 72ºC for 30 seconds (extension). Final Extension was set at 72ºC for 5 minutes. The amplified PCR products were subsequently visualized by agarose gel-electrophoresis using 1.5% agarose gel.

2.2.7 16S rRNA gene PCR and sequencing

Salmonella isolates from each sampling area were selected further for 16S rRNA gene amplification using primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTT ACGACTT-3’ followed by sequencing of approximately 1465 bp amplicon (Table 2.4). The sequences were aligned with reference sequences and a neighbor-joining analysis was used to construct a phylogenetic tree by using MEGA7 (Kumar *et al.*, 2016).

Table 2.4: All the primers used in this study for identification and genotypic classification of *Salmonella* isolates

PCR Target	Primer Sequence	Annealing Temperature (^{0}C)	Product size (bp)	Reference
invA	5'GTGAAATTATCGCCACGTTCGGGCAA 3' 5' TCA TCG CAC CGT CAA AGG AAC C 3'	**64**	**284bp**	(Rahn *et al.*, 1992)
16S rRNA	27F- 5'AGAGTT TGATCMTGGCTCAG 3' 1492R-5'TACGGYTACCTTGTTACGACTT 3'	**55**	**1465bp**	(Marchesi *et al.*, 1998)
1283	5′-GCGATCCCCA-3′	**40**		(Chansiriporn chai *et al.*, 2000)
MLST Primers				
thrA(aspartokinase+homoserine dehydrogenase)	5' GTCACGGTGATCGATCCGGT 3' 5' CACGATATTGATATTAGCCCG 3'	**55**	**852**	https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-senterica.html
purE (phosphoribosylaminoimi dazolecarboxylas)	5' ATGTCTTCCCGCAATAATCC 3' 5' TCATAGCGTCCCCCGCGGATC 3'	**55**	**510**	
sucA (alpha ketoglutarate dehydrogenase)	5′-AGCACCGAAGAGAAACGCTG-3′ 5′-GGTTGTTGATAACGATACGTAG-3′	**55**	**643**	
hisD (histidinol dehydrogenase)	5′-GAAACGTTCCATTCCGCGCAGAC-3′ 5′-CTGAACGGTCATCCGTTTCTG-3′	**55**	**894**	
aroC (chorismate synthase)	5' CCTGGCACCTCGCGCTATAC 3' 5' CCACACACGGATCGTGGCG 3'	**55**	**826**	
hemD (uroporphyrinogen III cosynthase)	5' ATGAGTATTCTGATCACCCG 3' 5' ATCAGCGACCTTAATATCTTGCCA 3'	**55**	**666**	
dnaN(DNA polymerase III beta subunit)	5′-ATGAAATTTACCGTTGAACGTGA -3′ 5′-AATTTCTCATTCGAGAGGATTGC-3′	**55**	**833**	
Sequencing Primers for MLST				
aroC	5′-GGC GTGACGACCGGCAC-3′ 5′-AGCGCCATATGCGCCAC-3′	**50**	**-**	https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-senterica.html
dnaN	5′-CCGATTCTCGGTAACCTGCT-3′ 5′-ACGCGACGGTAATCCGGG-3′	**50**	**-**	
hemD	5′-GCCTGGAGTTTTCCACTG -3′ 5′-GACCAATAGCCGACAGCGTAG -3′	**50**	**-**	
hisD	5′-GTCGGTCTGTATATTCCCGG -3′ 5′-GGTAATCGCATCCACCAAATC -3′	**50**	**-**	
purE	5′- ACAGGAGTTTTAAGACGCATG-3′ 5′-GCAAACTTGCTTCATAGCG-3′	**50**	**-**	
sucA	5′- CCGAAGAGAAACGCTGGATC-3′ 5′-GGTTGTTGATAACGATACGTAC-3′	**50**	**-**	
thrA	5′-ATCCCGGCCGATCACATGAT -3′ 5′- ACCGCCAGCGGCTCCAGCA-3′	**50**	**-**	

2.3 Genotypic diversity analysis of *Salmonella* isolates

2.3.1 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

For all *invA* positive 200 isolates, 10µl of the amplified 16S rRNA gene (Table 2.4) with approximately 1400 bp product size were digested with 5U of restriction enzyme *Alu*I (Promega, USA) to reveal their ARDRA profiles (Vaneechoutte *et al.*, 1995). The reaction mixture contained 2 µL 10X buffer, 0.2 µL Acetylated bovine serum albumin (10µg/µL), 5U enzyme, 16.3 µL water and ~ 1000 ng PCR product.

The digests were resolved on a 1.5% agarose gel, stained with ethidium bromide (5µg/ml) and bands were observed using a GelDoc (protein sample, USA). Two different-sized DNA markers, 1Kb and 100 bp (Bioneer, South Korea) were used to analyze different restriction fragments.

2.3.2 Randomly Amplified Polymorphic DNA (RAPD)

RAPD was done using primer 1283 for its ability to further discriminate between species of *Salmonella* isolates (Table 2.4) (Chansiripornchai *et al.*, 2000). PCR was carried out in 20 µl reaction volume. The volume was made up of, 10 µl of master mix 2X (Go Taq Colorless Master Mix), 100 pmol of primer, 2 µl of template DNA in each tube. The PCR conditions for 1283 primer included an initial denaturation of 94^{o} for 5min, followed by 30 cycles of denaturation 94^{o} for 1 min, primer annealing at 56^{o} for 1 min, extension at 72^{o} for 2 min and a final delay at 72^{o} for 5min. The PCR products were resolved on a 1.5% agarose gel, stained with ethidium bromide (5 µg/ml) and bands observed using a Geldoc (protein sample, USA). The gel images were further analyzed and phylogenetically clustered using the software PyElph 1.4 (Pavel and Vasile, 2012). From RAPD groups, representative isolates were selected for sequencing with 16S rRNA gene and phylogenetic tree was constructed based on Neighbor-joining method using MEGA5 software.

Isolates from the 18 RAPD groups were serotyped according to White-Kauffmann-Le Minor Scheme. The isolates were serologically confirmed based on slide agglutination test using commercial antisera (S and A Reagents Lab, Thailand). Strains of *Salmonella* spp. were classified into serovars on the basis of extensively diversity of the lipo-polysaccharide (O) antigens and the flagellar protein (H) antigens (Hajna and Damon, 1950).

H (flagellar) antigen may occur in either or both of two forms, phase 1 and phase 2. There are over 1800 known serovars which current classification considers being separate species. The organisms tend to change from one phase to the other.

O (somatic) antigens occur on the surface of the outer membrane and are determined by specific sugar sequences on the cell surface.

PBS (pH 7.38) was used as a control to check for the autoagglutination of the individual antiserum.

2.3.3 Multi-Locus Sequence Typing (MLST)

2.3.3.1 PCR reactions of seven housekeeping genes

Eighteen selected isolates from 18 RAPD groups representing three serovars (*S. enterica* Kentucky, *S. enterica* Enteritidis, *S. enterica* Litchfield) were subjected to MLST analysis (Figure 2.3). Genomic DNA of the isolates was extracted following the boiling DNA method (Hossain et al., 2018). Seven house-keeping genes (*thrA*, *purE*, *sucA*, *hisD*, *aroC*, *hemD*, and *dnaN*) of *Salmonella* were amplified by PCR using the primers published on the MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica) (Table 2.4) (Achtman *et al.*, 2012). PCR conditions consisted of an initial denaturation step of 94^{o} C for 2 min, followed by 30 cycles of 94^{o} C for 1 min, 55^{o} C for 1 min and 72^{o} C for 1 min with a final step of 72^{o} C for 5 min. All PCR products were purified using the PCR purification kit (QIAGEN Inc., USA), and quantified using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop^{TM} 2000/2000c Spectrophotometers, Canada). Purified PCR products were subsequently sequenced using the primers specific for sequence analysis (http://mlst.ucc.ie/mlst/dbs/Senterica). The sequences of seven housekeeping genes were compared, and aligned with the available MLST online database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). The sequences were further submitted to the online *Salmonella* MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica), and assigned to a sequence type for each isolate examined (Table 2.4).

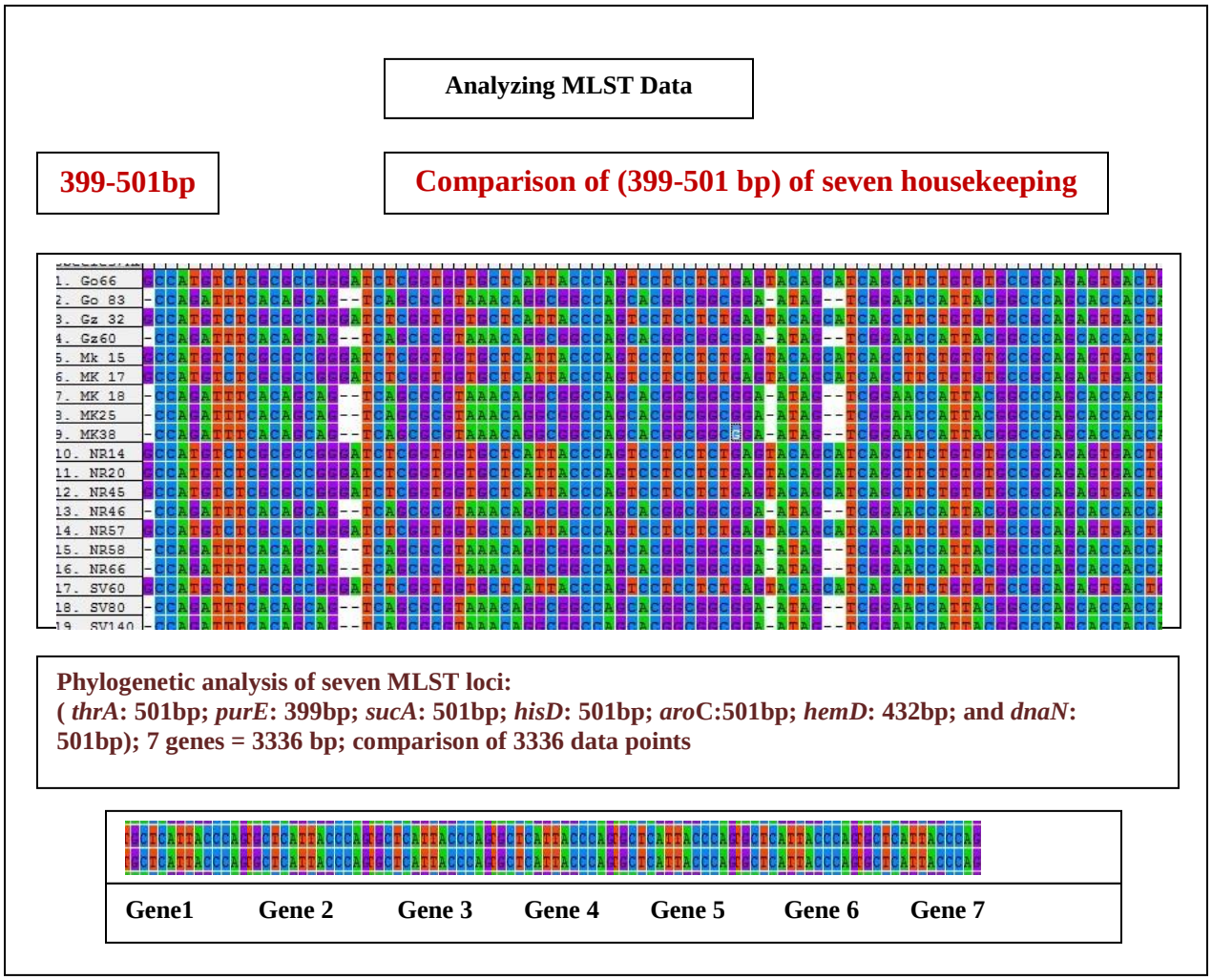


Figure 2.3: Analysis of Multi-Locus Sequence Typing data. Comparison of total 3336 data points of seven housekeeping genes for analyzing the MLST types among isolates of *Salmonella* sp.

2.3.3.2 Phylogenetic reconstruction of MLST data and clustering analysis

The SeqMan software was used for MLST sequence analysis. All the sequences were edited with this software from the Lasergene software package (DNASTAR, USA). A minimal spanning tree was generated from the concatenated sequences of each target isolate. The seven housekeeping genes were concatenated in the order *aroC - dnaN - hemD - hisD - purE - sucA - thrA.* All the reliable STs that belonged to *S. enterica* on the website (http://mlst.warwick.ac.uk) were used for this tree construction using MEGA software 7.0 (Kumar *et al.*, 2016). Phylogenetic Neighbor-joining tree was inferred for concatenated sequences to determine the variable sites in seven loci.

The percentage of bootstrap value of the replicates in the tree was estimated from 1000 replicates.

2.3.4 Molecular evolutionary analysis

The polymorphism analysis of seven housekeeping genes including the mutation rates, the number of alleles, the nucleotide diversity, the number of nonsynonymous and synonymous mutations, was carried out using DnaSPv5.10.00 software (Librado, 2009).

Rates of nonsynonymous (*dN*) and synonymous (*dS*) mutations were computed by using mutation-fraction method (Nei *et al.*, 1986). The number of nonsynonymous or synonymous changes per nonsynonymous or synonymous site, respectively, defines the nonsynonymous mutation rate (*dN*) or synonymous mutation rate (*dS*) in a gene.

Furthermore, the eBURST approach to multi-locus analysis, developed for multi-locus sequence typing (MLST), has been analyzed using eBURSTv3 software (Turner *et al.*, 2007). The split network of STs and individual loci was generated by using neighbor-net method using SplitTree4 (Huson and Bryant, 2006). Separate split network was analyzed for all seven housekeeping genes of *Salmonella* isolates representing all 18 RAPD profiles. This network analysis dissects the dissimilarities, such as, evolutionary distances with more accuracy.

In order to compare the discriminatory power of these three methods, an index of discrimination based on Simpson's index of diversity was used (Hunter and Gaston, 1988). D value ranges from 0.00 to 1. The higher the D values, the more discriminatory the method is (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php).

2.4 Antibiotic resistance analysis of the isolated poultry based *Salmonella*

All 200 *Salmonella* isolates were investigated for their antibiotic resistance pattern using 15 antimicrobials belonging to 11 different antibiotic classes including Penicillins (ampicillin, AMP-10 µg); Tetracyclines (doxycycline, DO-30 µg; tetracycline, TE-30µg; oxytetracycline, OT-30 µg); Nitrofurans- (nitrofurantoin, F-300 µg); Lipopeptides (polymyxin B, PB-30 µg); Monobactams (aztreonam, AZM-30 µg); Quinolones (subclass fluoroquinolone ciprofloxacin, CIP-10 µg; subclass quinolone, nalidixic acid, NA-30µg); Beta Lactams-(subclass Cephalosporins, cefoxitin, FOX-30 µg and subclass Cephems, cephalexin CEX- 30 µg), Penems-(imipenem, IPM-10 µg); Aminoglycosides (gentamycin, GN-10 µg); Phenicols (chloramphenicol, C-30 µg); Macrolides (azythromycin, ATM-15 µg). *In vitro* antibiotic sensitivity test of the isolated *Salmonella* was performed using the standard commercial discs

(Oxoid, USA) through the disc diffusion method on Mueller-Hinton agar. The overall procedure for this sensitivity test is described below:

- ✓ **Inoculum Preparation**

According to the standard guideline described by the Clinical and Laboratory Standards Institute (CLSI) 0.5 McFarland turbidity standard was prepared. The standard inoculums were prepared for each isolate as following described method:

a. The preserved isolates were inoculated on Nutrient agar plates and incubated for overnight at 37ºC. At least 2-3 well isolated colonies were selected from Nutrient Agar plate and transferred into Tripticase Soy Broth (TSB) using sterile loop. Each tube of TSB containing 5ml media were incubated at 37º C after inoculation.

b. The broth cultures were incubated at 37º C to achieve the 0.5 McFarland standard (usually 2-6 hours).

c. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to the point of the 0.5 McFarland standards.

- ✓ **Inoculation of test plates**

Mueller- Hinton plates (Appendix I) were inoculated with the working culture according to the following process:

a. Within 15 minutes of adjusting the turbidity of test culture, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several time and pressed firmly on the inside wall of the respected culture tube above the culture to remove the excess culture from the swab.

b. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times rotating the plate approximately 600 each time to ensure an even distribution of inoculums. As a final step the rim of the agar was swabbed. The procedure was done under laminar air flow to avoid contamination.

c. The lid was left ajar for 3-5 minutes but no more than 15 minutes, to allow for any access surface moisture to be absorbed before applying the drug impregnated disks.

- ✓ **Application of antibiotic disks to inoculated agar plates**

Sterile antimicrobial disks were dispensed onto the surface of the inoculated agar plate using sterile forceps. Each disk was pressed down individually to ensure complete contact with the agar surface. The disk placed in the agar surface was not closer than 24 mm from center to

center. A total of 7 disks were placed on one 150 mm plate. The plates were inverted and placed in an incubator set to 35oC within 15 minutes after the disks were applied.

- ✓ **Reading plates and results interpretation**

After 16-18 hours of incubation, each plate was examined. The resulting zone of inhibition was uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition were measured, including the diameter of the disk. Zones are measured to the nearest whole millimeter.

The results were interpreted according to the guideline of Clinical and Laboratory Standard Institutes (CLSI), 2016 (Table 2.5). These results were also further analyzed for correlation with the MLST types, and the locations from where the isolates originated.

Table 2.5: Antibiotic classes and the resistance zone used in the study (CLSI guideline 2019)

Antibiotic Class	Antibiotic Sub class	Agents included; Generic Names	Short form	Resistance Zone sizes for *Salmonella*
Aminoglycosides		Gentamycin	GN(10µg)	≤12
Carbapenems		Imipenem	IPM(10µg)	≤13
Cephalosporins	First Generations	Cephalexin	CEX(30µg)	≤14
Cephalosporins	First Generations	Cefoxitin	FOX(30µg)	≤14
Macrolides		Azythromycin	ATM(15µg)	≤13
Monobactams		Aztreonam	AZM(30µg)	≤17
Nitrofurans		Nitrofurantoin	F(300µg)	≤14
Penicillins	Aminopenicillin	Ampicillin	AMP(10µg)	≤13
Lipopeptides		Polymyxin B	PB(300U)	≤11
Quinolones	Fluoroquinolone	Ciprofloxacin	CIP (5µg)	≤15
	Quinolone	Nalidixic Acid	NA(30µg)	≤13
Tetracyclines	Tetracycline	Tetracycline	TE(30µg)	≤11
	Doxycycline	Doxycycline	DO (30µg)	≤10
	Oxycycline	Oxycycline	OT(30µg)	≤10
Phenicols		Chloramphenicol	C (30µg)	≤12

2.5 Enumeration of *Salmonella* from poultry farm samples using real-time PCR based rapid identification method

Poultry farm is one of the major repositories of zoonotic *Salmonella*. Regular monitoring with proper quantification of this pathogen in poultry industry is not being practiced in our country. Lack of the availability of rapid, cost effective quantification method is the main obstacle for this monitoring program. In this study, SYBR green real-time based PCR was used to quantify the actual load of *Salmonella* in selected poultry farm samples**.**

2.5.1 Sample collection

Representative poultry farm samples were selected from the previously collected samples originated in Dhaka Division (Table: 2.6). Each type of sample was selected to assess the burden of *Salmonella* among different poultry farm samples including, droppings, cloacal swab, poultry feed, handler swab.

Table 2.6: Farm samples selected for quantitative analysis to assess the burden of *Salmonella.*

Farm Sample Type	Number of Samples	Sampled Farms
Droppings	15	F1,F4,F7,F8,F10,F13,F14
Cloacal Swab	15	F2,F4,F6,F7,F10,F11,F13,F14
Handler Swab	15	F2,F3,F4,F6,F8,F10,F12,F14
Poultry Feed	15	F1,F4,F6,F9,F12
Water	15	F1,F3,F4,F7,F9,F10,F12,F13

2.5.2 DNA extraction

Total DNA was extracted from the selected poultry farm samples using manual DNA extraction method as found mostly efficient in previous study in laboratory. In this method, about 1.0ml of each sample was suspended in 467µl TE buffer by repeated pipetting. About 30µl of 10% SDS and 3µl of 20 mg/ml Proteinase-K was mixed with suspended sample and incubated for 1 hour at 37ºC. An equal volume of phenol-chloroform was added and mixed by inverting the tube until the phases are completely mixed. The tube was centrifuged for 10 minutes at 14000 rpm. The upper aqueous phase was transferred to a new eppendorf tube and an equal volume of phenol-chloroform was added into the tube and again mixed well and centrifuged for 10 minutes at 14000 rpm. The upper aqueous phase was transferred to a new tube. Sodium acetate (3 M) (one

tenth volume of the aqueous phase) was added. Iso-propanol (0.6 volume of aqueous phase) was added and mixed gently until the DNA precipitates. The tube was centrifuged to pellet the DNA for 10 minutes at 14000 rpm. About 50µl of 70% alcohol was used to wash the DNA and centrifugation was carried out for 10 minutes at 14000rpm. The tube was kept on heat block and heated for 25 minutes at 60 ºC to evaporate ethanol completely. After all the ethanol evaporated, extracted DNA was eluted with nuclease free water. DNA concentration was measured by NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., USA) (Sambrook, 2001).

2.5.3 Primer selection

Primers targeting *invA* region were used in this study for the development of specific real-time PCR method for quantification of *Salmonella* sp. using real-time PCR assay (Rahn *et al.*, 1992). We selected this primer pair on the hypothesis that invasin is single copy gene and this primer pair generates a specific 284 bp amplified product. The detail information about the primer and amplicon has listed in table 2.4. The reproducibility and the specificity of the primer pair was tested using bacterial isolates collected from laboratory repository and also from clinical, environmental and poultry isolates.

2.5.4 Standard for real-time PCR

Salmonella sp. Enteritidis IFO 3313 strain (obtained from the Microbial Genetics and Bioinformatics Laboratory, University of Dhaka), was amplified using *invA* primer. Recombinant plasmid was constructed using the amplified 284bp PCR product as insert. The reaction was carried out in a ligase independent manner, using a linearized plasmid vector, pCR™4-TOPO® supplied in the kit (TOPO® TA Cloning® Kit, Invitrogen, USA). Competent cells were prepared in laboratory.

Chemical transformation of the chemically competent *Escherichia coli* DH5α was done with recombinant plasmid constructed by cloning reaction. The day after transformation (usually 18 hours after plating transformation reaction on LB agar containing kanamycin) the colonies that appeared on the plates were analyzed for transformants. Original clones were preserved for long term use. About 0.85 ml of saturated culture of positive clones in LB broth containing 100µg/ml Kanamycin was mixed with 0.15ml sterile molecular biology grade glycerol (Promega, USA) and transferred into Cryovial. Vials were stored at -80º C.

Plasmid DNA was extracted from the saturated overnight culture using PureYield™ Plasmid Miniprep System (Promega, USA). Conventional PCR using *invA* primer was carried out to

reconfirm that the extracted plasmid is the recombinant plasmid which was transformed into *Escherichia coli* DH5α. After electrophoresis in low melting agarose gel (UltraPure^{TM}L.M.P. Agarose, Spain), the gel was visualized in UV and gel containing recombinant plasmid of approximately 4240 bp was cut and taken into an eppendorf tube and weighed to measure. The gel was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA; Appendix II). Further confirmation of gel purified plasmid was performed by setting a conventional PCR with *invA* primer using the same protocol.

The concentration of recombinant plasmid was measured using a NanoDrop^{TM} spectrophotometer (Thermo Fisher Scientific Inc., USA).

The following calculation was used to determine the required amount of recombinant plasmid DNA for standard dilution preparation.

Mass of the recombinant plasmid DNA can be calculated by using the formula,

$m = [n][(1/6.023\times10^{23})]\ [660]$gram

Here,

n= Size of recombinant plasmid DNA (bp)

m= mass of plasmid DNA

Avogadro's number= 6.023×10^{23} molecule/mole

Average molecular weight of double stranded DNA is 660 g/mole.

The simplified expression is, $m = [n]\ [1.096\times10^{-21}]$ g

In this experiment, Size of the vector was 3956 bp and size of the insert was 284 bp.

So, size of the total recombinant plasmid DNA was 4240 bp

So, the mass of single recombinant plasmid is,

$m= 4240 \times [1.096\times10^{-21}]$ g

$= 4.647\times10^{-18}$ g

$= 4.647\times 10^{-3}$fg (1 fg = 10^{-15} g)

The *invA* gene is a target that exists as a single copy gene per plasmid vector. Therefore, 4.65×10^{-3}fg of recombinant plasmid DNA contains one copy of the *inv*A gene.

Therefore, copy number of interest × mass of recombinant plasmid DNA = mass of recombinant plasmid DNA needed. The mass of recombinant plasmid DNA needed was divided by the volume to be pipetted into each reaction to get the final concentration (Table 2.7).

Table 2.7 Dilutions series of recombinant plasmid DNA (2.5 µl of template DNA per PCR reaction)

Copy number of *invA* gene	× mass of recombinant plasmid DNA (fg)	Mass of recombinant plasmid DNA needed	Amount of DNA to be pipette	Final concentration (ng/µl)
10^{10}	4.647×10^{-3}	4.647×10^{7}fg i.e. 46.47 ng	2.5 µl	18.588
10^{9}	4.647×10^{-3}	4.647×10^{6}fg i.e. 4.647 ng	2.5 µl	1.8588
10^{8}	4.647×10^{-3}	4.647×10^{5}fg i.e. 0.4647 ng	2.5 µl	0.18588
10^{7}	4.647×10^{-3}	4.647×10^{4}fg i.e. 0.04647 ng	2.5 µl	0.018588
10^{6}	4.647×10^{-3}	4.647×10^{3}fg i.e. 0.004647 ng	2.5 µl	0.0018588
10^{5}	4.647×10^{-3}	4.647×10^{2}fg i.e. 0.0004647 ng	2.5 µl	0.00018588
10^{4}	4.647×10^{-3}	4.647×10 fg i.e. 0.00004647 ng	2.5 µl	0.000018588
10^{3}	4.647×10^{-3}	4.647fg i.e. 0.000004647 ng	2.5 µl	0.0000018588
10^{2}	4.647×10^{-3}	4.647×10^{-1}fg i.e. 0.0000004647 ng	2.5 µl	0.00000018588
10	4.647×10^{-3}	4.647×10^{-2}fg i.e. 0.00000004647 ng	2.5 µl	0.000000018588

A serial dilution of the recombinant plasmid DNA was prepared using the formula $C_1V_1 = C_2V_2$and calculations were performed to generate a series of standards from 10^{10} to 10 concentration. Figure 2.4 is showing preparation of dilution for genomic DNA based standard and Table 2.8 is showing dilution series of genomic DNA.

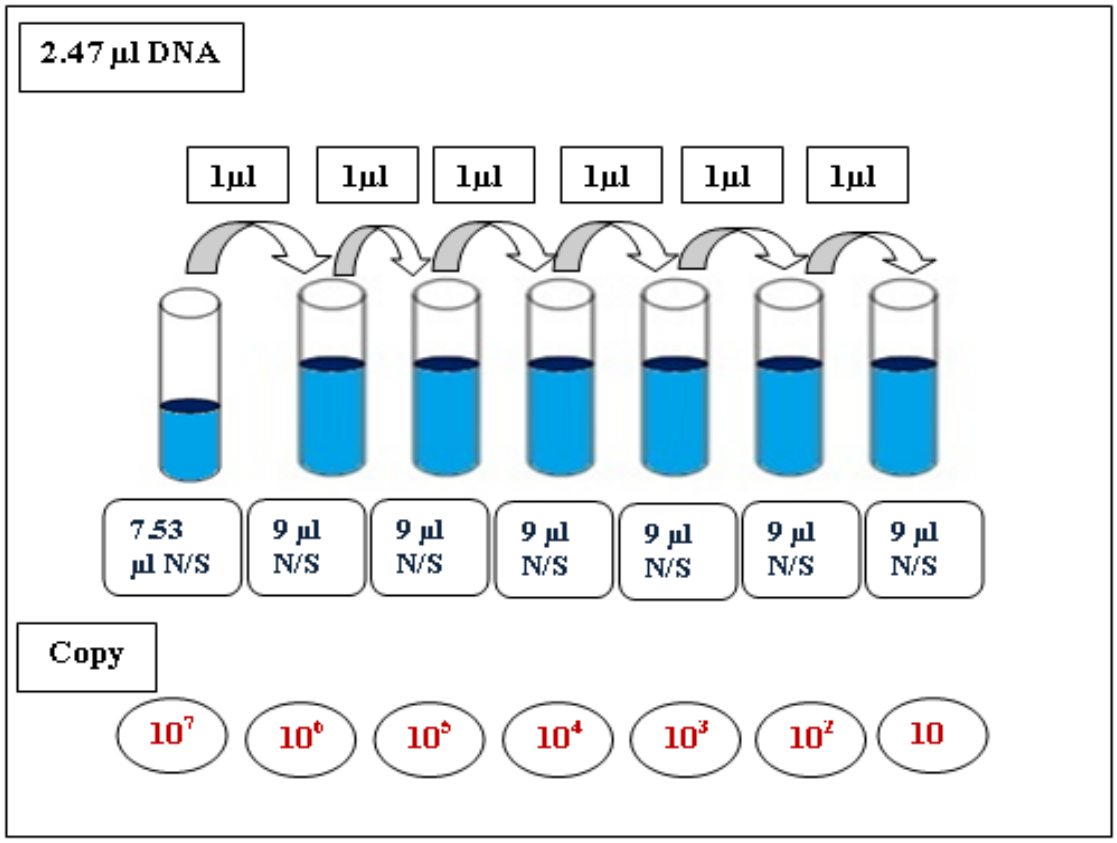


Figure 2.4: Dilution of genomic DNA for standard curve preparation

Table 2.8: Final concept of dilutions for standard preparation of SYBR green Real-Time PCR

Dilution no	Source of gDNA for dilution	Initial conc. (ng/µl)	Volume of gDNA (µl)	Volume of diluent (µl)	Final conc. (ng/µl)	Final Volume (µl)	Resulting copy of invA gene/ 2.5µl
1	Stock	83.1	2.47	7.53	20.54	10	10^7
2	Dilution 1	20.54	1.0	9.0	2.054	10	10^6
3	Dilution 2	2.054	1.0	9.0	0.2054	10	10^5
4	Dilution 3	0.2054	1.0	9.0	0.02054	10	10^4
5	Dilution 4	0.02054	1.0	9.0	0.002054	10	10^3
6	Dilution 5	0.002054	1.0	9.0	0.0002054	10	100
7	Dilution 6	0.0002054	1.0	9.0	0.00002054	10	10

2.5.5 Quantitative real-time PCR assay

After standard dilution preparation, reaction mix was prepared. During reaction mixture preparation SYBR green master mix (SYBR Green, Applied Biosystems, USA) (2.0×) was used whose final concentration was found to be (1×). A reaction mix was prepared (except template DNA) for each 25µl reaction to a tube at room temperature. All solutions were gently vortexed and briefly centrifuged after thawing. Primer used in the assay was the same as the primer described in Table 2.4. The master mix was mixed thoroughly and dispensed in appropriate volumes into well of Micro Amp™ Optical 8-tube Strip, containing 25µl of reaction mixture with template DNA. Each strip was sealed properly with Micro Amp™ Optical 8-Cap Strip. Template DNA was added to a volume of 2.5µl/reaction to the individual PCR tubes containing the master mix. The concentration of extracted DNA was measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Concentration was measured as ng/µl. The ratio of the reading was between at 260 nm and 280 nm (OD 260 /OD 280). This OD 260/280 ratio provides an estimate of the purity of the nucleic acid (DNA) which is should preferably have a value of 1.8. After this, calculations (section 2.5.4) were performed. The reactions were mixed by gentle centrifugation without creating bubbles after addition of template. Assay mix was kept protected from light, in the freezer, until use.

For preparation of standard curve, calculations were performed according to the section 2.5.4 and standard dilutions were prepared from 10^{11} to 10 copy plasmid DNA using the purified PCR product (*invA*). After standard dilution preparation, reaction mixture was prepared.

Each reaction was present in duplicate having copy number from 10^{7} to 10 copy DNA and two set of negative control was included in the assay.7500 software, ver. 2.0.6 (Applied Biosystems, USA) was used for data analysis.

Reaction condition in real-time PCR assay was 95ºC for 10 minutes (initial denaturation) followed by 95ºC for 15 seconds (cycle denaturation), 58ºC for 30 seconds (annealing) and 72ºC for 30 seconds (extension).Final Extension was set at 72ºC for 5 minutes.

Melt curve analysis was also included in the assay where there was a range of temperature upshift and downshift ranging from 95ºC for 15 seconds, 60ºC 1 minute, 95ºC for 30 seconds and 60ºC for 15 seconds. During template selection the extracted DNA from each farm sample was used as template and added into reaction mixture.

2.5.6 Enumeration of farm samples

Poultry farm samples were selected for enumeration to count the *Salmonella* load using the established SYBR green real-time PCR protocol. Different types of farm samples including dropping, cloacal swab, feed, water and handler swab were selected for the analysis. All these samples were tested using cultural and molecular analysis. Both culture positive and culture negative samples were selected to assess the sensitivity of the methods.

2.6 Detection of *Salmonella* burden in samples from each point of poultry production from farm to kitchen

To determine the risk factors and lead to methods for prevention and/or reduction of pathogenic bacteria colonizing poultry, information about entry, transmission, and overall prevalence of pathogen in the production chain is needed. Aimed to this point at the last part of the thesis samples from different points of poultry from farm to kitchen were collected.

2.6.1 Sample collection

The poultry farms of selected five sampling regions were surveyed about their poultry business areas. Information found about the transports, market places to which the farmers sell their poultry chickens. Poultry market places have been selected based on the business of the farmers of poultry farms from where farm samples were collected. Samples were collected from the poultry markets of Savar Bazar, Mirpur 6 Kacha Bazar and Karwan Bazar (Figure: 2.5). Total 153 samples from transport and market area were collected from different rout points (Figure 2.5; Figure 2.6). The types and number of sample has been summarized in Table 2.9.

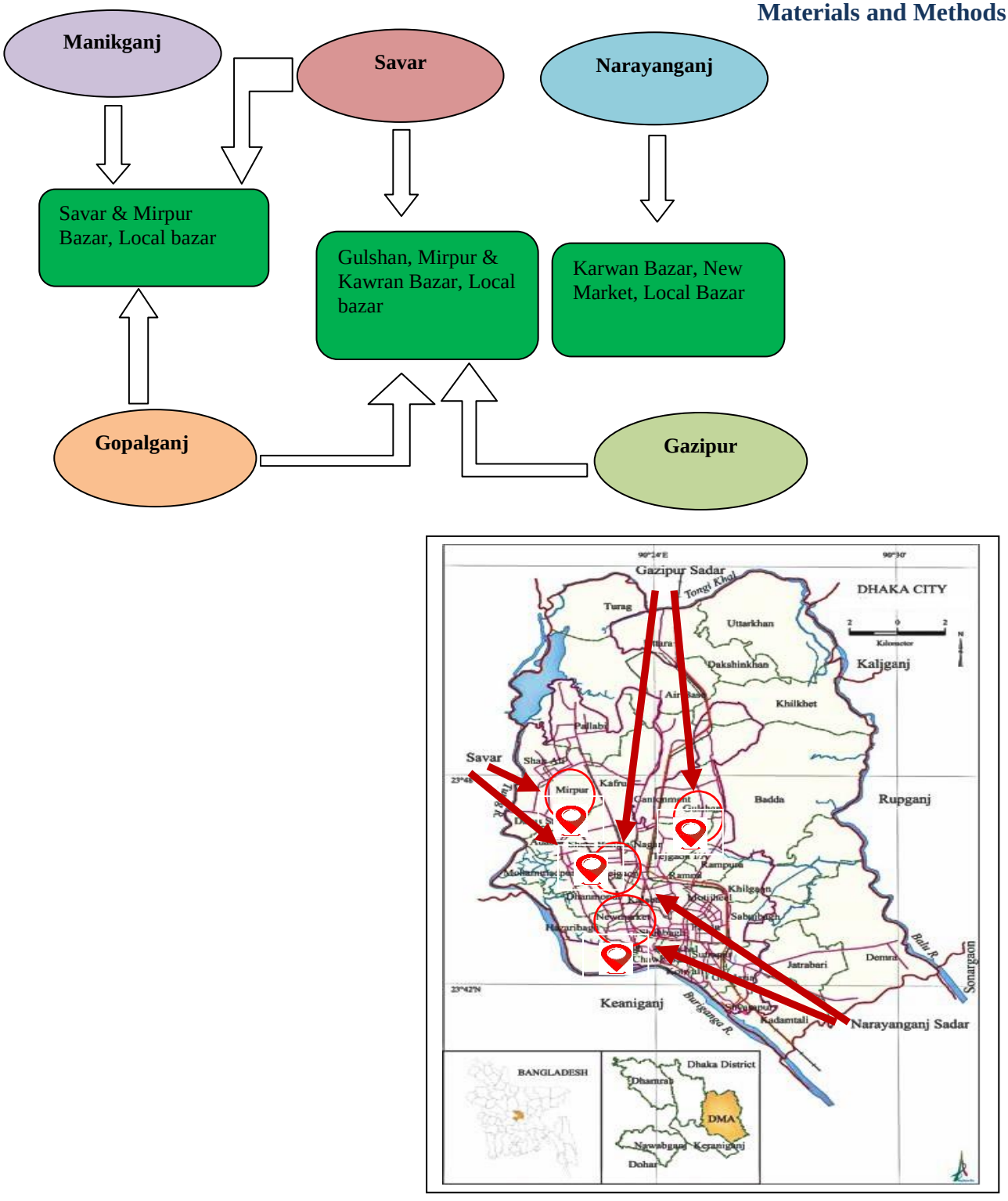


Figure 2.5: The bazar sampling areas in relation with sampling farms. The three poultry bazar were selected based on the sampled poultry farm businesses, in which areas they transport their chicken.

2.6.2 Extraction of genomic DNA

Total DNA was extracted using manual DNA extraction method described in section 2.5.2 from the selected poultry farm samples.

Table2.9: Types and total number of poultry samples collected from three different bazars located in Dhaka city

Poultry Market Area	Sample Types	Amount
Mipur 6 Kach Bazar	Transport	8
	Chicken Cage	10
	Cutting board	8
	Cutting knife	8
	Washing Water	7
Karwan Bazar	Transport	8
	Chicken Cage	10
	Cutting board	10
	Cutting knife	8
	Washing Water	8
Savar Bazar	Transport	8
	Chicken Cage	10
	Cutting board	9
	Cutting knife	8
	Washing Water	8
Home	Cutting board, knife	10
Hotel	Processing area, cutting knife	15
Total Samples		**153**

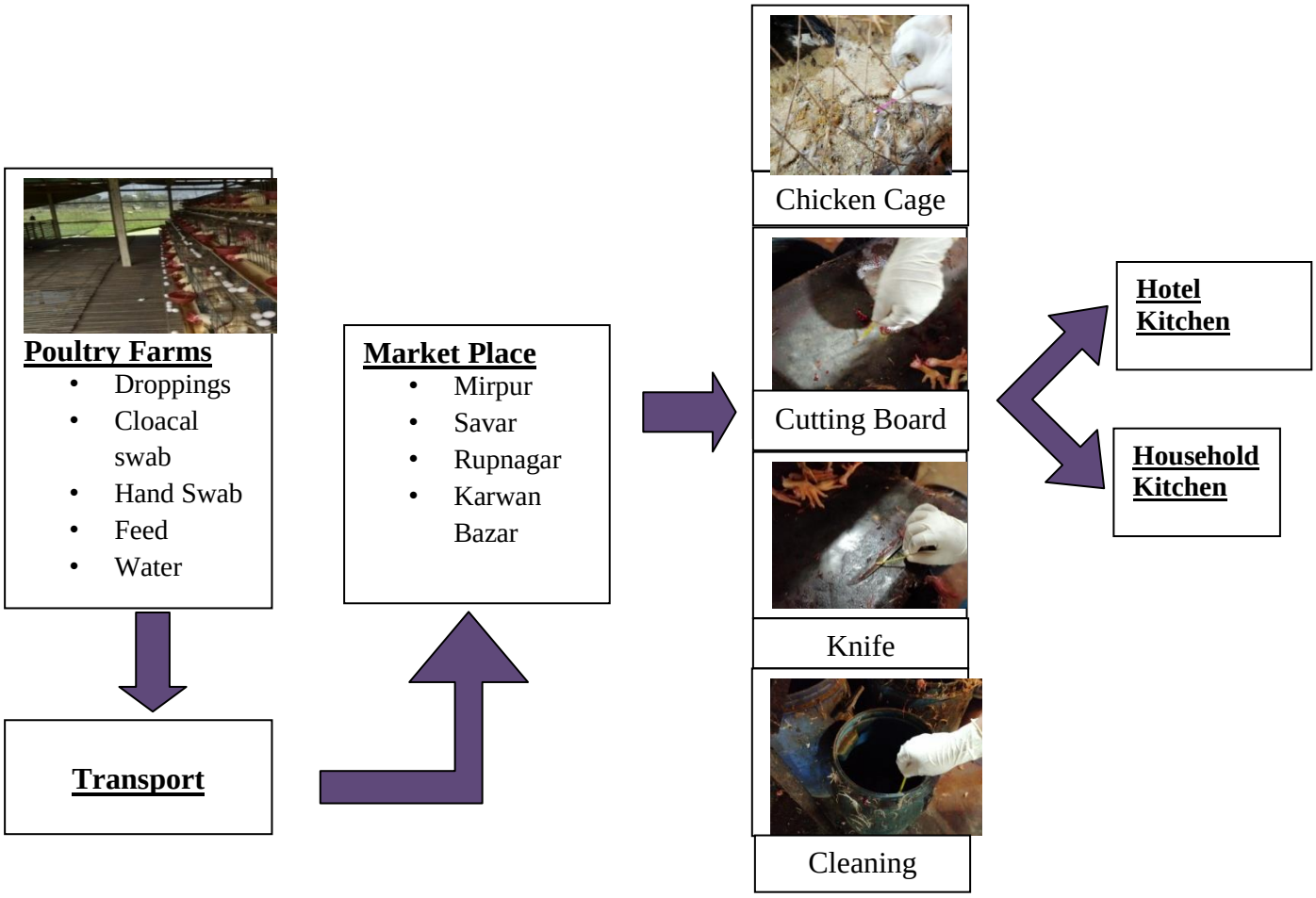


Figure 2.6: Samples collected from the poultry based areas for transmission analysis of non-typhoidal *Salmonella*.

2.6.3 Detection of *Salmonella* in each point of transmission

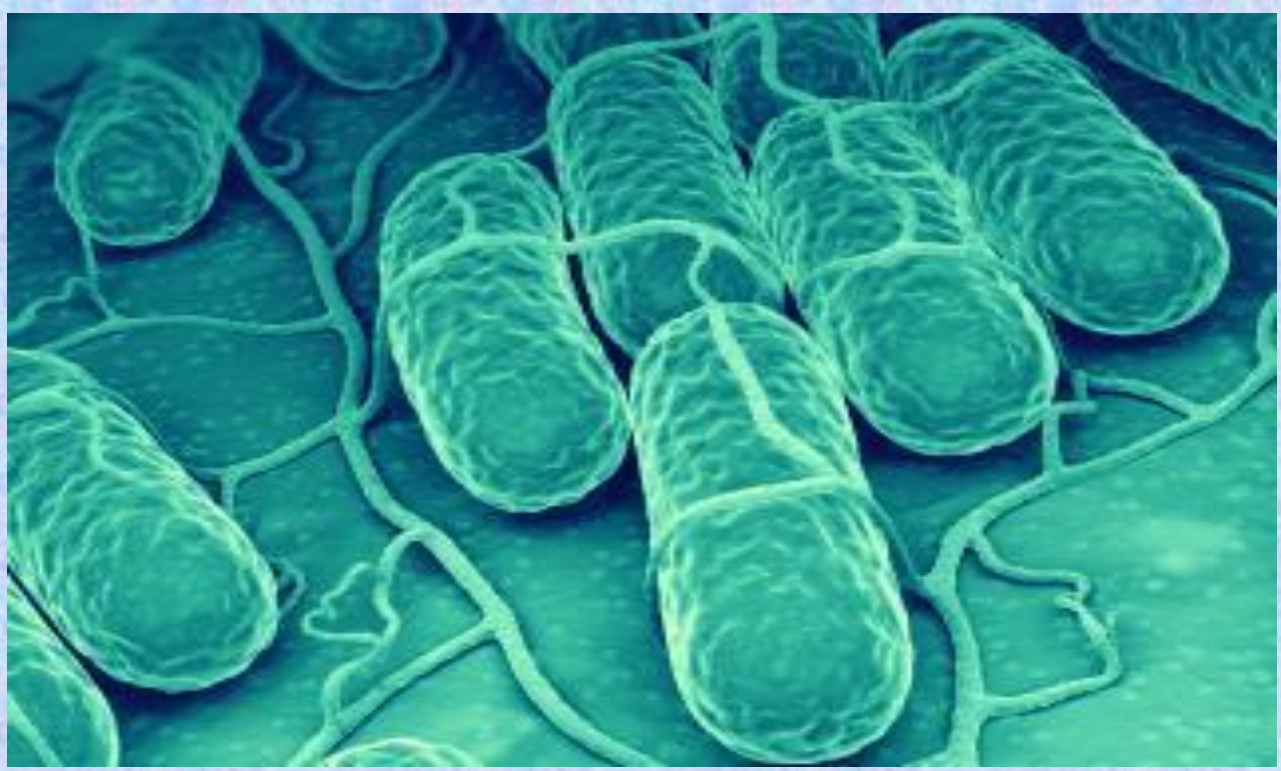
The optimized and validated real-time PCR method which was done previously has been used for detection of *Salmonella* in the samples collected from different transmission rout points. Quantification did not perform in this section. Reaction mixture was prepared and assay was performed following the same protocol described in section 2.5.5. Positive Standard template was prepared using cloned plasmid DNA which had been described in section 2.5.4. The entire extracted DNA from transport and bazar samples were used as template and mixed with the reaction mixtures.

Reaction condition consisted initial denaturation at 95ºC for 10 minutes followed by cycle denaturation at 95ºC for 15 seconds, annealing at 58ºC for 30 seconds and extension at 72ºC for

30 seconds. Final extension was 72ºC for 5 minutes.

The reaction of real-time PCR was carried out using Real-time PCR system 7500, (Applied Biosystems, USA) and data analyzed with the 7500 software, ver. 2.0.6 (Applied Biosystems, Foster City, CA, USA)**.**

Salmonella load in all types of poultry associated samples were analyzed to trace the rout of transmission from poultry farm to kitchen.



Chapter 03: Results

3. Results

3.1 Survey on non-typhoidal *Salmonella* in poultry farms

According to the Central Disease Investigation Laboratory, Bangladesh, the largest numbers of poultry farms are located in the Dhaka Division. In total, 154 poultry samples were collected from five different districts in the Dhaka Division for this study. Although no infection was observed at the time of sampling, all farms (100%; 14/14) were found positive as a *Salmonella* reservoir following microbiological analysis. Our survey report revealed that *Salmonella* infection is a common infection in farmed chickens during the summer and rainy season (May to September).

Table 3.1: Background information about poultry farms and farmers

District	Gender		Education level of farmers				Flock size				Production cycle/year			Main income source	
	Male	Female	No	Primary	Secondary	Graduation	1000-1999	2000-3000	4000-5000	>5000	3	4	5	Yes	No
Narayanganj	8	2	2	5	3	0	4	3	0	3	6	4	0	7	3
Savar	6	4	0	4	4	2	0	5	5	0	0	3	7	8	2
Gazipur	7	3	3	2	3	2	0	10	0	0	2	3	5	6	4
Manikganj	8	2	4	4	2	0	0	10	0	0	4	2	4	8	2
Gopalganj	7	3	3	4	1	2	3	7	0	0	0	3	7	9	1
Total	36	14	12	19	13	6	7	35	5	3	12	15	23	38	12
Percentage (%)	72	28	24	38	26	12	14	70	10	6	24	30	46	76	24

A total of fifty (N=50) farmers were interviewed in the areas of Narayanganj, Savar, Gazipur, Manikganj and Gopalganj, where all locations had 10 respondents. All data were retained in the questionnaire and subsequently analyzed. The survey found that a higher percentage of male farmers (72%) engaged in poultry farming than female farmers (28%).The majority of farmers had primary education (38%), while few graduates (12%) have also become involved in a family

poultry business in the past few years. The majority (46%) of the poultry farms had 5 production cycles per year (Table: 3.1).

Baseline knowledge of farm hygienic practices was collected and compiled in Table 3.2. More than half (55%) of farmers use disinfectant sprays on their farms (Figure 3.1).

Table3.2: Basic knowledge of poultry hygienic protocols in farmers

Hygienic practices	Narayanganj		Savar		Gazipur		Manikganj		Gopalganj		Total	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Having foot bath disinfectant	5	5	7	3	4	6	0	10	0	10	16	34
Use of water disinfectant	8	2	10	0	8	2	3	7	4	6	33	17
Experience of Pest	6	4	8	2	8	2	10	0	10	0	42	8
Facing Layer disease problems	4	6	2	8	3	7	4	6	5	5	18	32
Antibiotics use for the treatment	10	0	10	0	10	0	7	3	8	2	45	5
Cleaning and disinfection of poultry house	8	2	10	0	7	3	6	4	5	5	36	14
Awareness about disease transmission	6	4	8	2	7	3	4	6	4	6	31	19
Washing hands after handling the chickens	7	3	7	3	5	5	4	6	5	5	28	22
Awareness of withdrawing feeds before slaughter	6	4	7	3	6	4	3	7	3	7	25	25

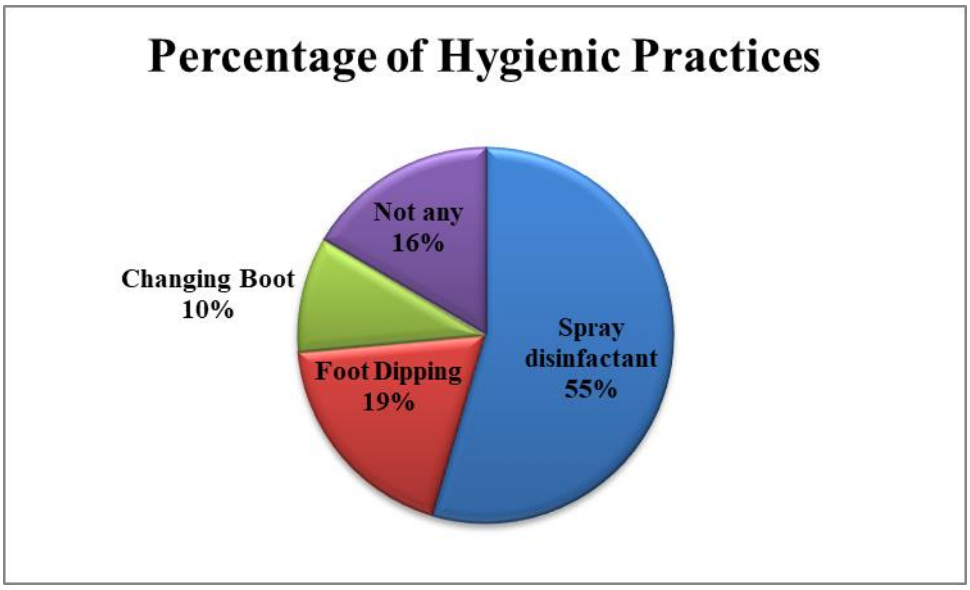


Figure 3.1: Hygienic practices applied in poultry farms.

Few farmers use more than one method of disinfection, while very few farmers from particular areas (Manikganj and Gopalganj) are not aware of disinfection practices (16%).The mortality rate for young flocks was calculated for approximately 5% to 10% of all poultry farms. According to the assumption of the poultry farmers, a relatively higher percentage of mortality was caused by bacterial infections.

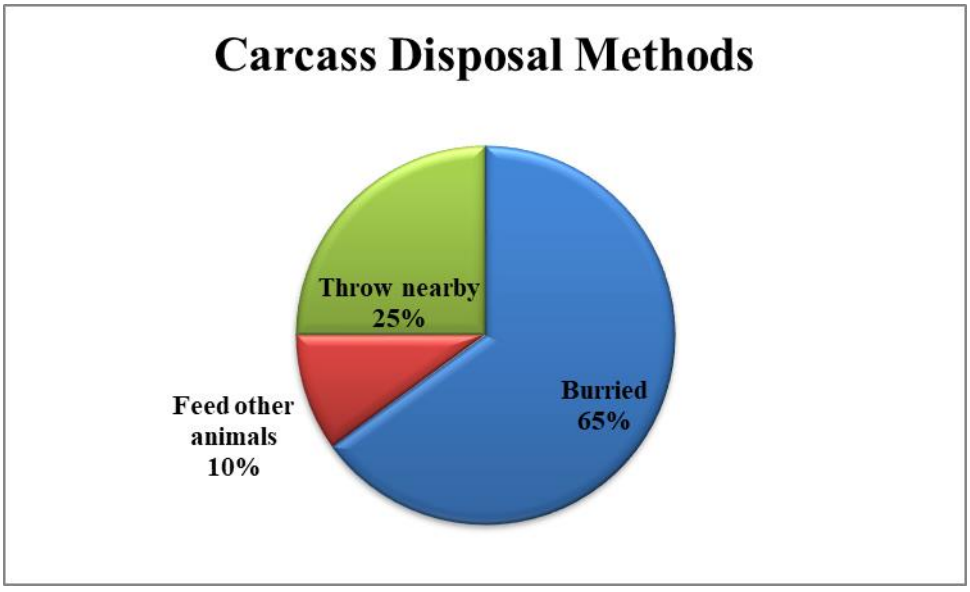


Figure 3.2: Carcass disposal methods followed by poultry farmers.

Farmers were also asked about their most common method of carcass disposal. The majority of the farmers (65%) bury the carcass near their poultry farmhouse followed by throwing a nearby (25%) and feed other pet animals (10%) (Figure 3.2).

3.2 Prevalence of non-typhoidal *Salmonella* spp. in poultry samples

A number of scientific publications have documented the high prevalence of non-typhoidal *Salmonella* spp. in the poultry industry globally. Eventually, this study was designed to evaluate the genetic variation of non-typhoidal *Salmonella* spp.of poultry origin; and to establish a rapid, sensitive identification and quantification protocol for this organism.

3.2.1 Isolation and identification of *Salmonella*

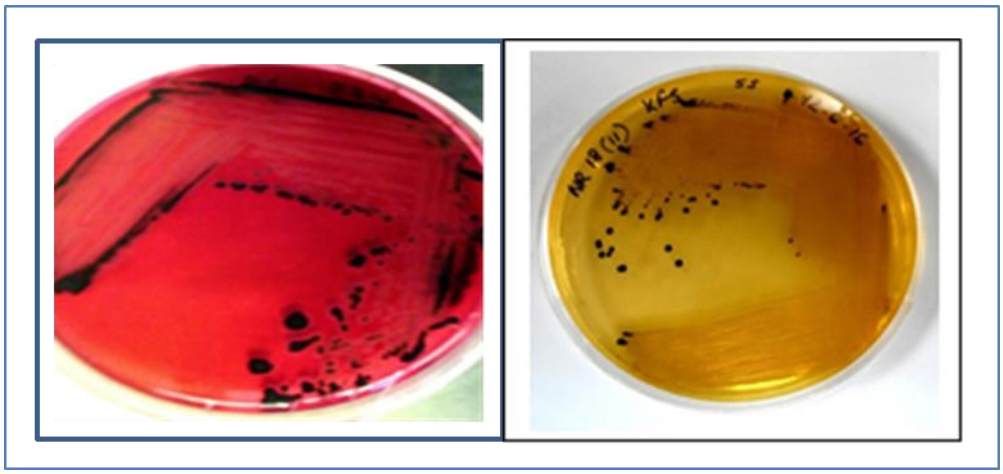
Salmonella spp. was detected and identified from 70% of the poultry samples (108/154) collected from five different districts in the Dhaka Division (Table 3.3).

According to the morphology and culture-based characteristics, 687 isolates from selective media plates with specific colony characteristics (black centered colonies on XLD, SS agar plates and colorless colonies on MacConkey agar) were selected for further analysis as presumptive *Salmonella* spp. (Figure 3.3).

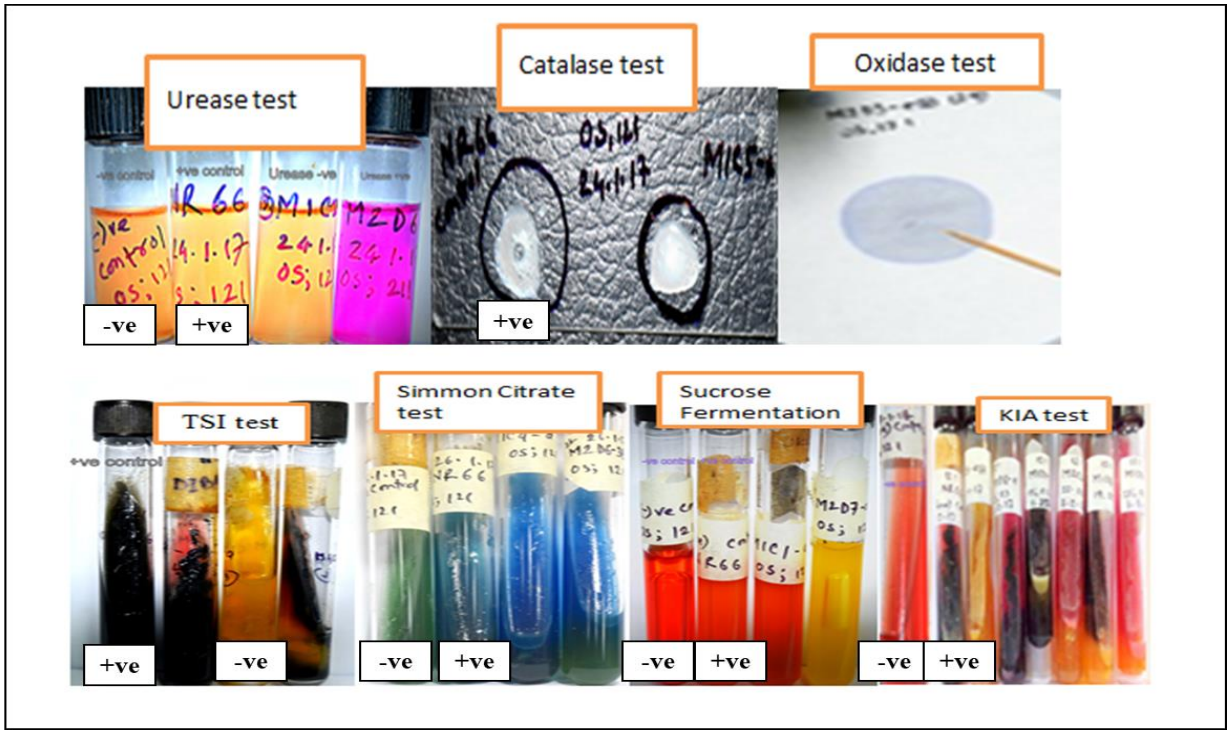
Table 3.3: **Precise information about the sampling farms, sampling time, and isolation of *Salmonella* from poultry origin in Dhaka Division, Bangladesh;** ***F- poultry farm**

Area	Date of Sampling	Number of Farms	Total Sample	Number of positive samples	Total Isolates	Total Number of *Salmonella*
Narayanganj	20th October, 2015	3 (*F1, F2, F3)	23	18 (78%)	95	30 (15%)
Savar	1st March, 2016	2 (F4, F5)	20	8 (4%)	102	17 (8.5%)
Gazipur	3rd October, 2016	3 (F6, F7, F8)	33	26 (78%)	162	48 (24%)
Manikganj	6th December, 2016	3 (F9, F10, F11)	48	31 (64%)	180	54 (27%)
Gopalganj	5th May, 2017	3 (F12, F13, F14)	30	25 (83%)	148	51 (25.5%)
Total			154	108	687	200 (29%)

All suspected isolates were tested for the biochemical characterization. Almost 58.22% (400/687) of the total isolates did not produce urease, 56.8% (390/687) were positive for H_2S production in TSI test, 62.6% isolates (430/687) and 65.5% isolates (450/687) were found negative for indole and oxidase test results, respectively (Figure: 3.3). Based on biochemical results, 380 poultry isolates were selected for molecular confirmation of *Salmonella* spp.



A i. B



ii.

Figure 3.3: Cultural and biochemical identification of the isolated *Salmonella* strains.

i. Selective media plates; A. XLD Agar- Black colonies, B. SS Agar- Black colonies. ii. Biochemical Tests; A. Urease test; B. Catalase test; C. Oxidase test; D.TSI test. Control isolate was included with the test batch of organisms, labeled as '+ve'. A negative control media tube without inoculation was also incubated with culture media, labeled as '-ve'.

3.2.2 Molecular confirmation of *Salmonella* isolates

These 380 selected isolates were further analyzed for molecular confirmation using gene specific *invA* primer targeted Polymerase Chain Reaction (PCR) method. The characteristic 284 bp amplicon was retrieved from a total of 200 isolates, thus, confirmed as *Salmonella* spp. (Figure 3.4).

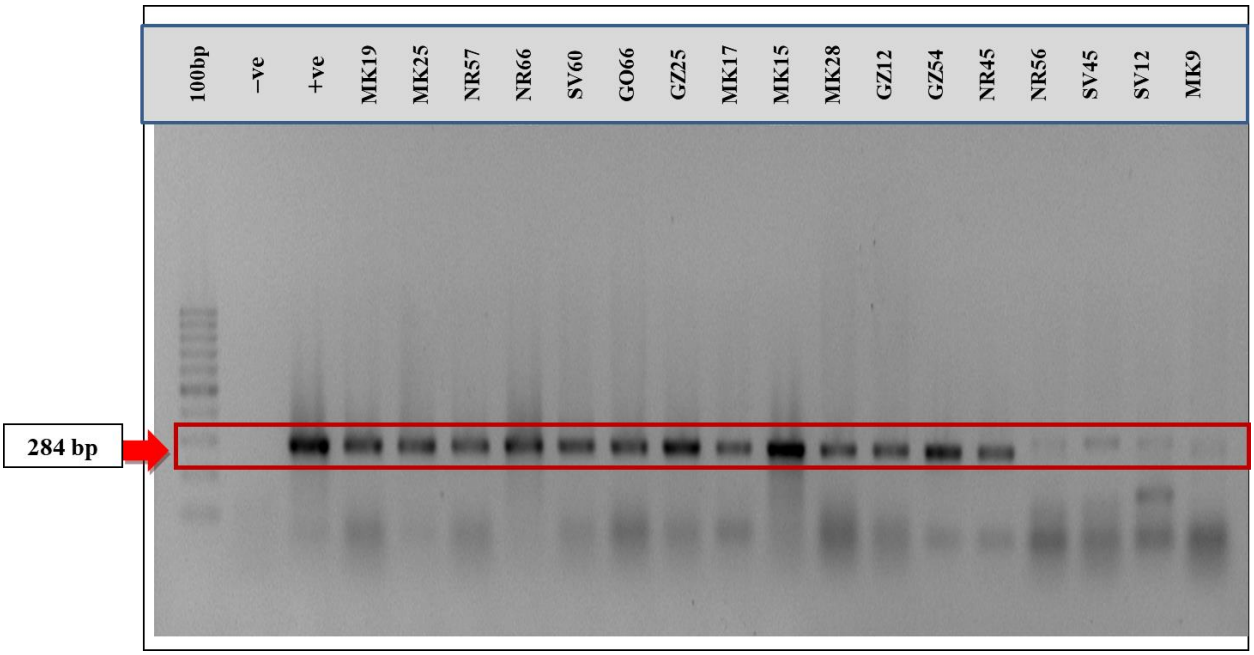


Figure 3.4: *Salmonella* specific *invA* gene PCR result showing characteristic 284 bp amplicon. The 100bp ladder was used for the characterization of bands amplified by *invA* gene in sample.

The highest number of *Salmonella* isolates were retrieved from poultry farms located in Manikganj (54/200; 27%) followed by Gopalganj (51/200; 25.5%), Gazipur (48/200; 24%), Narayanganj (30/200; 15%), and Savar (17/200; 8.5%). Maximum *Salmonella* isolates were identified from dropping samples (47%), followed by cloacal swab (43%), poultry feed (5%) and feeding water (6%) (Figure 3.5).

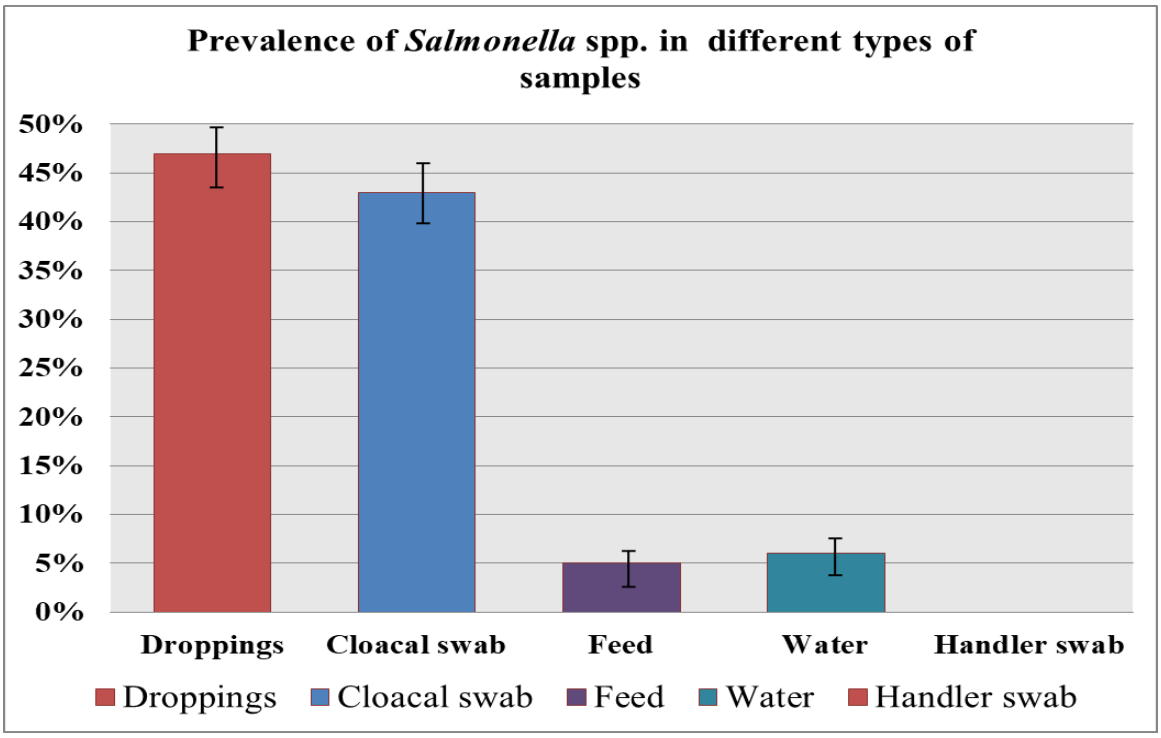


Figure 3.5: Prevalence of *Salmonella* spp. in different types of samples collected from different poultry farms. The highest percentage of *Salmonella* was identified from dropping samples, whereas, handler swabs were negative for the *Salmonella* spp.

3.2.3 Molecular identification of *Salmonella* based on 16S rRNA gene sequencing

Representative *Salmonella* isolates from each sampling area were selected for ribosomal gene expression (16S rRNA gene). All the sequences were edited, aligned to generate a phylogenetic tree along with reference sequences. The phylogenetic tree revealed three different clusters (cluster A, B and C). The A B and C clusters possessed 11, 8 and 6 strains of *Salmonella*, respectively (Figure 3.4). The isolates flocked in cluster A had 98-100% similarity with *S. enterica* Kentucky (CP026327) whereas in cluster B the isolates found closely related with *S. enterica* Litchfield (NBRY01000034), and in cluster C with *S. enterica* Enteritidis (ATCC13076) (Figure 3.6).

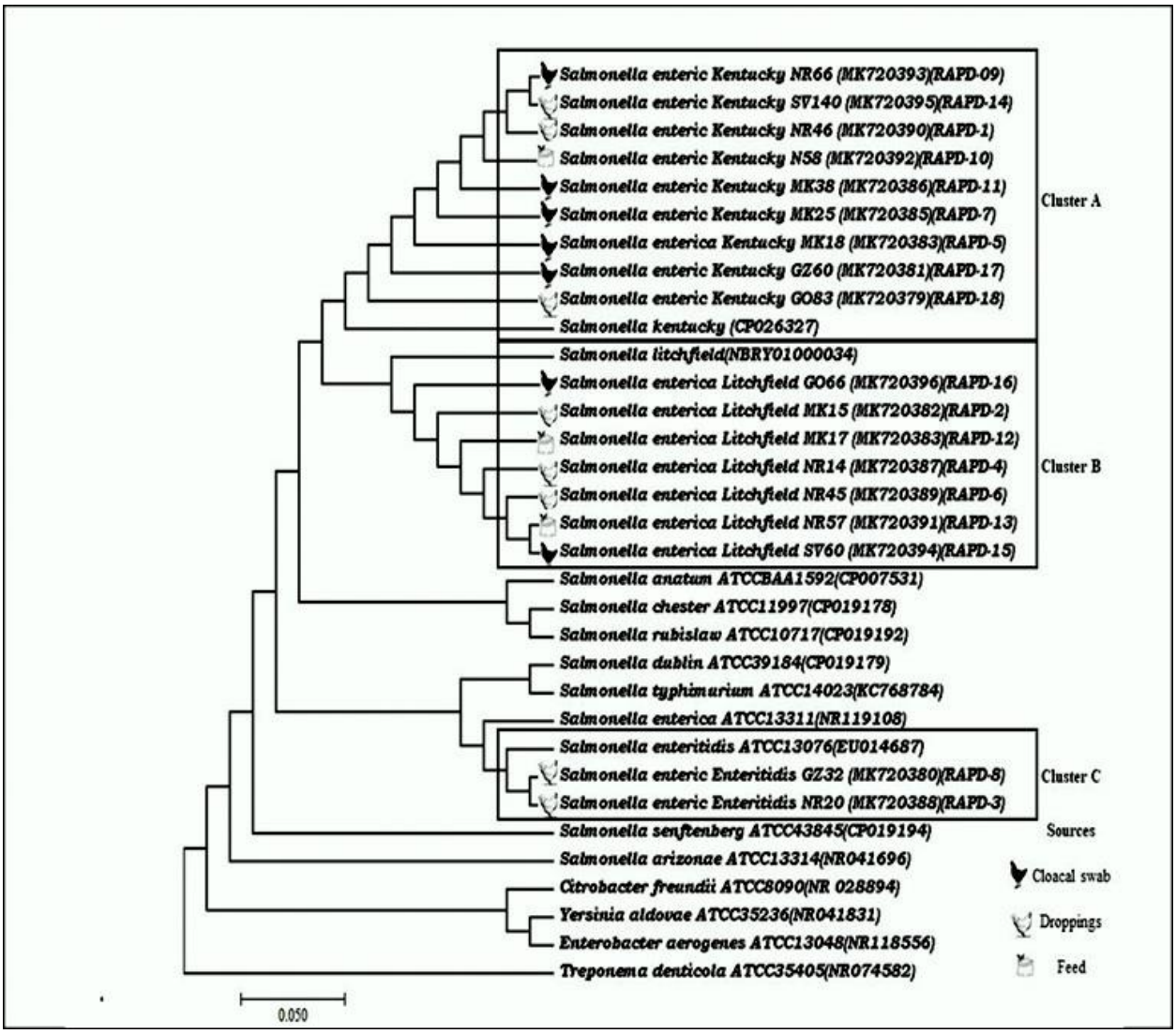


Figure 3.6: Phylogenetic tree predicted by the neighbor-joining method using 16S rRNA gene sequences. The evolutionary distances were computed using the Kimura 2-parameter model method and are in the units of the number of base substitutions per site. The bootstrap considered 1000 replicates. The scale bar represents the expected number of substitutions averaged over all the analyzed sites. The optimal tree with the sum of branch length = 0 0.46891257 is shown here.

The 16S rRNA gene sequences for *S. enterica* Kentucky, *S. enterica* Litchfield and *S. Enterica* Enteritidis have been submitted to the GenBank database with the accession numbers MK720379 to MK720396.

Table 3.4: Detail information of poultry originated *Salmonella* isolates with genotypic diversities

Area	Farm Name	ID of Selected Samples	Sample Type	Representative Isolates	ARDRA group	RAPD group	16S rRNA gene
Narayanganj	Kawsar Poultry Farm (F1)	NR_DR1 NR_DR2	Droppings	7,**14**, 19, **20**	I, II, V	1,3,4,8, 12,13	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		NR_CS1	Cloacal swab	12,25			
		NR_F1	Feed	10,**57**			
	Khajapolli Poultry Farm (F2)	NR_DR5	Droppings	39**,45**,46,50	I,III,IV	1,2,4,6, 8, 10	*S. enterica* Kentucky *S enterica* Enteritidis
		NR_CS3 NR_CS4	Cloacal swab	26,28,34,35,38, 44,**46**,48,54			
		NR_F3	Feed	43,55			
	Maer Doa Poultry Farm (F3)	NR_DR7	Droppings	**58,**60	I,II,V,VI	1,6,9,10 , 12,13	*S. enterica* Enteritidis *S.enterica* Litchfield
		NR_CS7	Cloacal swab	56,59,**66,**67			
		NR_W3	Water	62			
Savar	Pandhoa Poultry Farm (F4)	SV_DR6 SV_DR7	Droppings	14,48,65,**140,** 122	I,II,IX	1,4,10,11, 12,14,15	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		SV_CS8	Cloacal swab	37,45,70,72,80, 107,108			
		SV_W4	Water	47			
	Savar Poultry Farm (F5)	SV_DR8	Droppings	12	III,V,X	1,3,15	*S. enterica* Enteritidis
		SV_CS9	Cloacal swab	15,16,**60**			
Gazipur	Shiraj Poultry Farm (F6)	GZ_DR10 GZ_DR11	Droppings	7,9,10,15,17,**32,** 39,40	I,II,IV	7,8,17,18	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		GZ_CS10 GZ_CS11	Cloacal swab	28,62,92			
		GZ_W5	Water	35			
	Kapasia Poultry Farm (F7)	GZ_DR14 GZ_DR15	Droppings	42,44,47,51,58, 61, 59, 95	V,II,IV	1,7,8,12 , 13, 17,18	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		GZ_CS13 GZ_CS14	Cloacal swab	43,34, 64,65, 66, 38,76			
		GZ_W6	Water	70,71,72,90,94			
	Alam Poultry Farm (F8)	GZ_DR16	Droppings	2,37,41, 48, 85, 91,93	I,VI	3,5,8,12 ,13, 15	*S.enterica* Enteritidis *S. enterica* Litchfield
		GZ_CS15	Cloacal swab	31,45, **60,** 96, 98,99			
		GZ_W7	Water	49,68			
		GZ_F9	Feed	97			

Area	Farm Name	ID of Selected Samples	Sample Type	Representative Isolates	ARDRA group	RAPD group	16S rRNA
Manikganj	Sujon Poultry Farm (F9)	MK_DR17 MK_DR18	Droppings	1,2,**15,**16,22,29, 21,40, 86, 39, 92,115	I, II,III	1,2,7,8, 9	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		MK_CS16 MK_CS17	Cloacal swab	7,21,30,48,83, 85, 90, **25,**35, 24			
		MK_W9	Water	62, 28			
		MK_F10	Feed	36			
	Bolaka Poultry Farm (F10)	MK_DR19 MK_DR20	Droppings	4,14,44,46,80, 81, 87, 88,89, 122, 126,	I,II,IV	3,4,11, 12,15	*S. enterica* Kentucky *S. enterica* Enteritidis
		MK_CS19	Cloacal swab	33,**38,**47,49, 22			*S. enterica* Enteritidis
		MK_F12	Feed	**17**			
	Ma Poultry Farm (F11)	MK_DR21	Droppings	13,27, 31, 65,8,19,9	I,II	1,5,6	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		MK_CS20	Cloacal swab	6,**18,**23			
Gopalganj	Nadira Poultry Farm (F12)	GO_DR23	Droppings	15,21,25,26,30	II,IV	1,3,5,7, 8, 18	*S.enterica* Litchfield
		GO_CS21	Cloacal swab	19,27,28,46,116 , 126,127			
		GO_F12	Feed	125,128			
	Ali poultry Farm (F13)	GO_DR25 GO_DR26	Droppings	63,72,73**,83,** 22,102,103,104, 105,88	V,VI	2,4,5,7, 8,9, 11,13,1 5, 16,18	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		GO_CS22	Cloacal swab	23,32,33,35,37			
		GO_F13	Feed	8			
		GO_W16	Water	62			
	Reza Poultry Farm (F14)	GO_DR28 GO_DR29	Droppings	13,47,49,50,14, 58,48,51, 65,10	I,II,III,VI	1,5,8,9, 13,15,1 6, 17,18	*S. enterica* Kentucky *S. enterica* Enteritidis *S. enterica* Litchfield
		GO_CS23	Cloacal swab	51,52,46,43,55, **66**			
		GO_W18	Water	106			

*Highlighted isolates were selected for MLST analysis.

3.3 Genotypic diversity analysis of the poultry *Salmonella* isolates

3.3.1 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

For all 200 strains of *Salmonella*, 10 µl of the 16S rRNA gene PCR product was digested with 5U of restriction enzyme *aluI* (Fermentas, Lithuania, sequence: AG^CT). The ARDRA profiling for all isolates yielded six different restriction patterns.

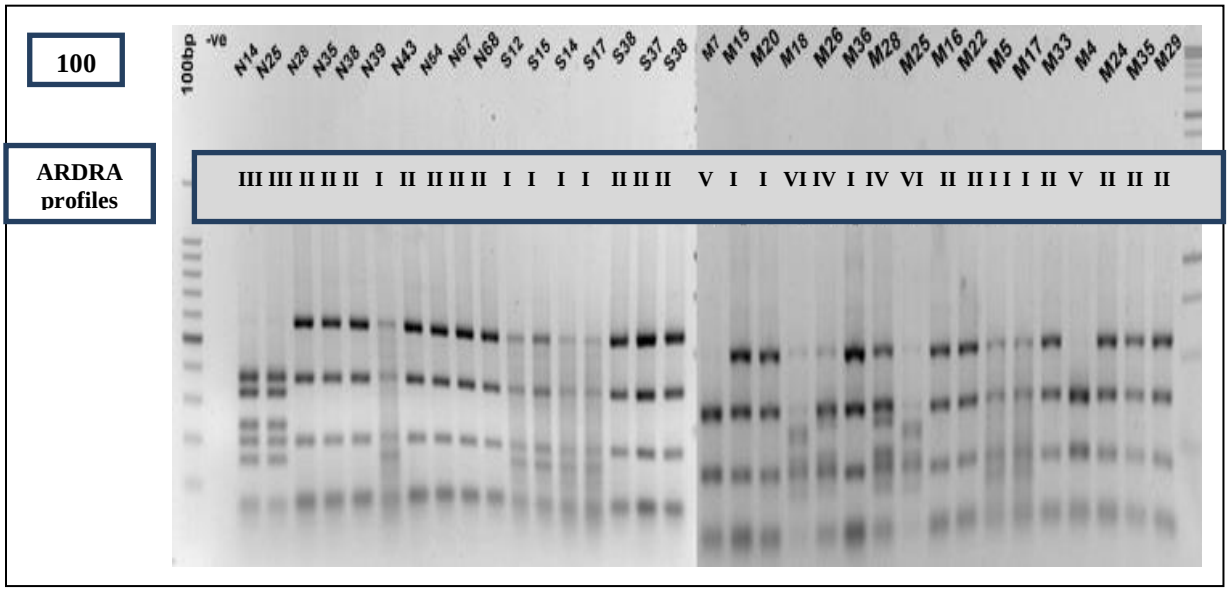


Figure 3.7: The six different ARDRA profiles of the poultry *Salmonella* strains. The restriction enzyme *aluI* was used for digestion. The 100 bp marker was used to align the band patterns.

The six different ARDRA profiles obtained with *aluI* are shown in Figure 3.7. The strains from all sampling areas were almost equally distributed among the profiles. The profiles were designated as I, II, III, IV, V and VI. The restriction pattern II was predominant containing 52 isolates, followed by 31 in profile I, 23 in profile III, 36 in profile IV, 37 in profile V and 21 in profile VI (Table 3.4).

3.3.2 Random Amplification of Polymorphic DNA (RAPD) based diversity

The 200 isolates developed 18 distinct RAPD profiles using specific 1283 primer (Figure 3.8). The profiles were analyzed in duplicate to check the reproducibility of the isolates. Additionally, an UPGMA tree was developed based on RAPD profiles using PyElph 1.4 software (Figure 3.9).

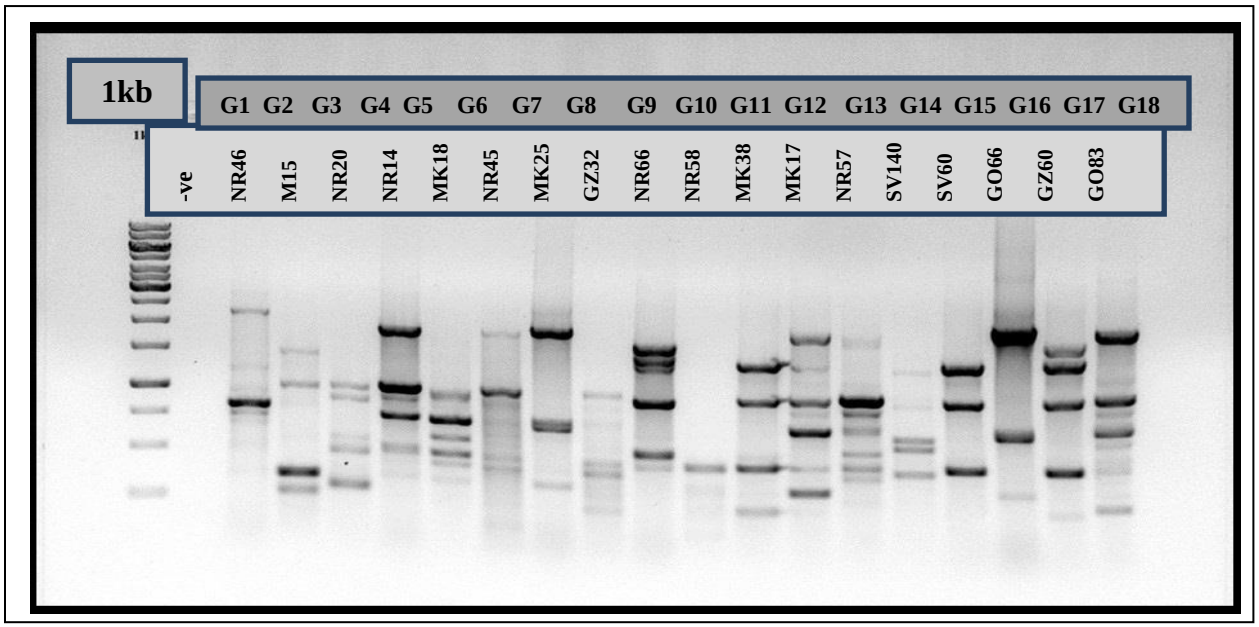


Figure 3.8: Eighteen distinct RAPD groups of poultry *Salmonella* isolates. The first well of the agarose gel contains the 1 kb marker and second one contains the negative control.

The software segregated genotypic variations between isolates based on their agarose gel electrophoresis image. Analysis of PyElph revealed 18 different groups of RAPD patterns among poultry-derived *Salmonella* isolates (Figure 3.9).

The largest cluster (cluster 6), contained the isolates only from Savar area. The isolates from Gazipur grouped together in 1, 4 and 5 clusters. Groups 8, 11, 12 and 18 consisted of only one isolate. The other RAPD groups created by the PyElf software were overlapped with organisms in multiple areas.

The comparative analysis of RAPD profiles and *Salmonella* strains revealed that three different serotypes were split between the 18 RAPD profiles. *S. enterica* serovar Kentucky were distributed among the RAPD profiles -1, 5, 7, 9, 10, 11, 14, 17, and 18; *S. enterica* serovar Litchfield belonged to the profiles 2,4,6,12,13,15,16; and *S. enterica* serovar Enteritidis were

detected in profiles 3 and 8 (Figure 3.6). The phylogenetic tree of the 16S rRNA gene also clustered the same serovar of *Salmonella* isolates into specific distinct groups. RAPD profiles 1, 5, 7, 9, 10, 11, 14, 17, and 18 clustered in clade A, the RAPD profiles 2,4,6,12,13,15,16 clustered in clade B and 3, 8 groups clustered in C clade separately (Figure 3.6).

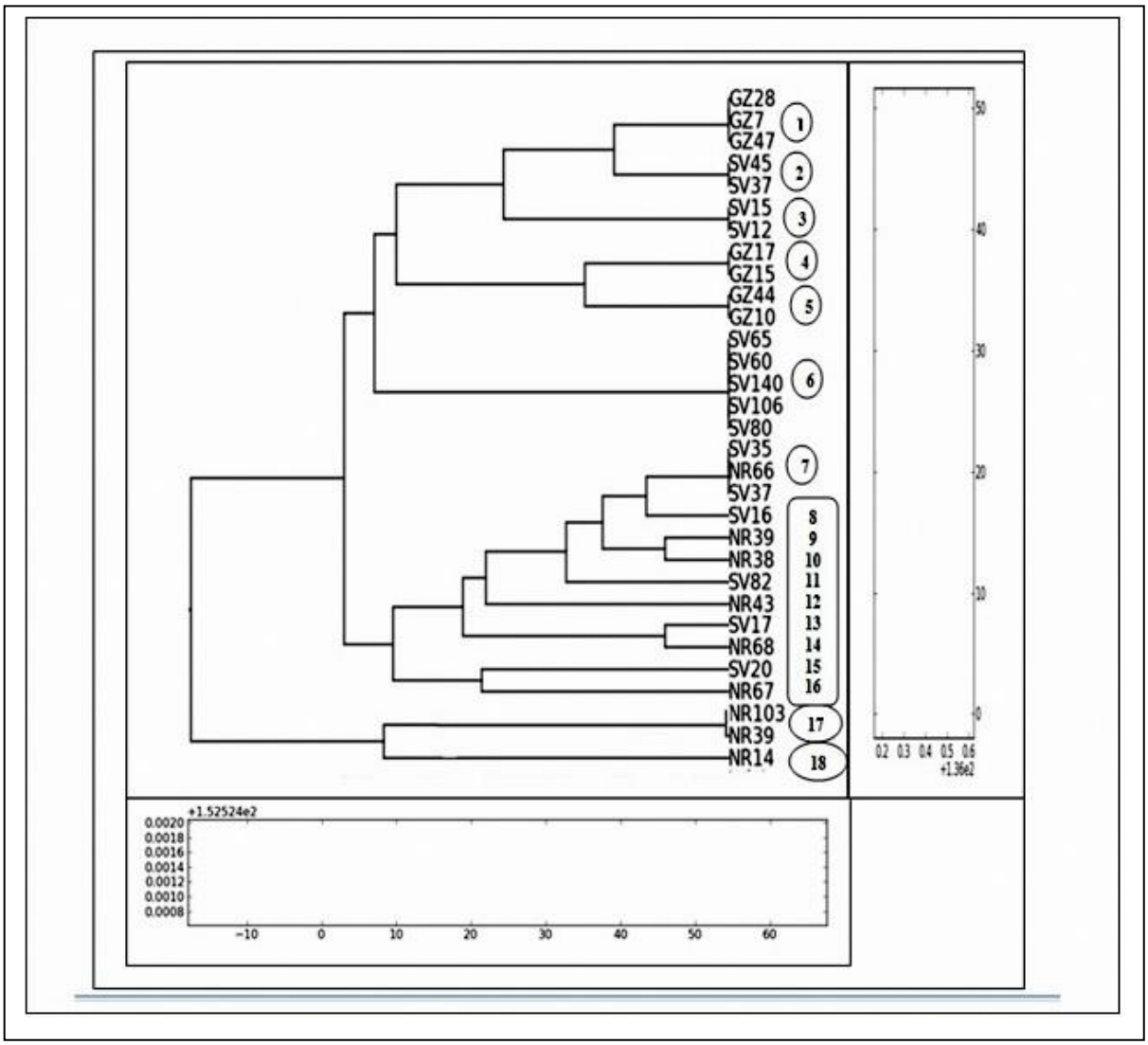


Figure 3.9: UPGMA tree based on RAPD profiles using PyElph 1.4. The band picture after gel electrophoresis of RAPD genotypic method was analyzed using this software. This software generated a tree based on the genotypic variations based on RAPD profiling. The tree also separated the 18 RAPD profiles for the isolates same as the electrophoresis results.

3.3.3 Subtype discrimination of poultry *Salmonella* spp. by Multi-Locus Sequence Typing (MLST)

The MLST sequencing of seven housekeeping genes of isolates representative of each of the 18 genotypical RAPD groups revealed 3 sequence types (STs); ST11, ST198, and ST214.Most of the isolates were assigned to the ST198 (50.5%) followed by ST214 (33%) and ST11 (16.5%) (Table 3.5).The three STs were distributed across the all sampling areas. ST198 was most prevalent in all regions except Narayanganj, whereas ST214 was the most common type of MLST relative to others (Figure3.10).The three STs belonged to three separate serotypes according to serological identification. *Salmonella enterica* ST11 resulted to serogoup Enteritidis; whereas, ST198 to Kentucky; and ST214 to Litchfield.

As all the sequences of seven housekeeping genes were submitted to the online database http://mlst.ucc.ie/mlst/dbs/Senterica, the outcome produced 15 separate allelic types for the genes. The allelic numbers include 2, 3, 5, 6, 11, 12, 14, 15, 19, 21, 64, 67, 72, 76 and 77 (Table 3.5). The most common genotype, ST198, was observed in all five geographic areas (Figure3.10).

The concatenated sequence data was divided into three diverging phylogroups, where *S. enterica* Litchfield, *S. enterica* Enteritidis and *S. enterica* Kentucky phylogroups individually clustered into single sequence complexes (Figure 3.11).

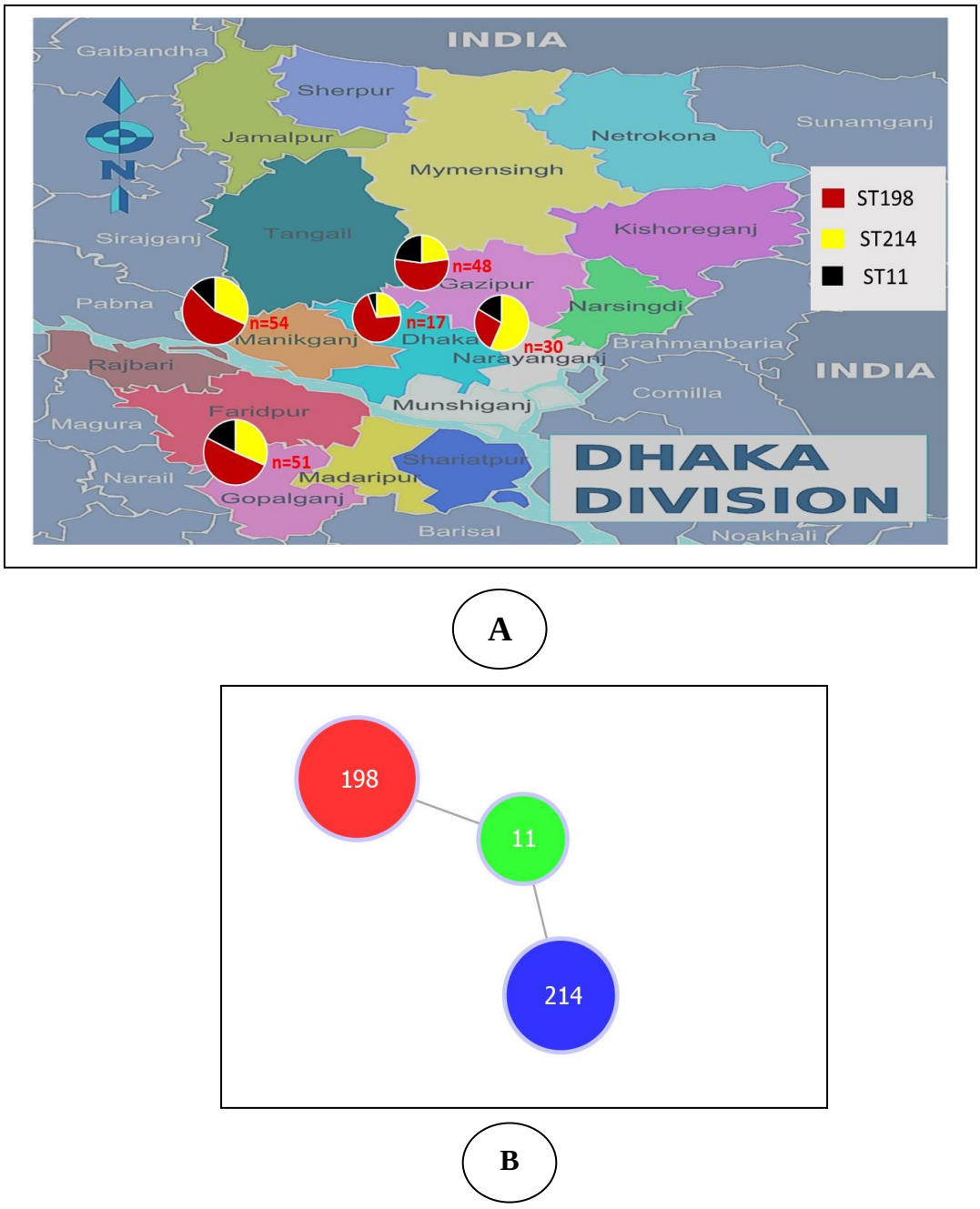


Figure 3.10: A. Distribution of three MLST types (ST11, ST198 and ST214), in all sampling areas of Dhaka Division, Bangladesh. B. Populations structure analysis. eBURST analysis of the 3 STs present in the database. Each circle represents the single ST.

Table 3.5: The allelic distribution in the 3 STs originated from poultry *Salmonella* serovars and their specific antibiotic resistance profiles

No	Sample Id	Source	Area	Serotype	Allele Number							Antibiotic Resistant profiles **
					aroC	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	ST
1	NR46	Droppings	Narayanganj	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-FOX-TE-OT-ATM-CIP-CEX-NA
2	NR58	Feed	Narayanganj	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-PB-AZM-TE-CIP-C-CEX
3	NR66	Cloacal swab	Narayanganj	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-FOX-PB-TE-CIP-ATM-CEX
4	SV140	Cloacal swab	Savar	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-FOX-TE-CIP-OT-ATM-IMP-C
5	GZ60	Cloacal swab	Gazipur	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-PB-AZM-TE-CIP-OT-NA-C
6	GO83	Droppings	Gopalganj	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-FOX-TE-CIP-OT-IPM-C-CEX
7	MK18	Cloacal swab	Manikganj	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-FOX-PB-TE-CIP-OT-ATM-C-NA
8	MK25	Cloacal swab	Manikganj	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-FOX-TE-CIP-OT-ATM-C
9	MK38	Cloacal swab	Manikganj	Kentucky	76	14	3	77	64	64	67	**198**	AMP-DO-F-FOX-TE-CIP-CEX-OT-AZM-ATM-C
10	NR14	Droppings	Narayanganj	Litchfield	14	72	21	12	6	19	15	**214**	AMP-DO-F-FOX-TE-CIP-OT-IPM-CEX
11	NR45	Droppings	Narayanganj	Litchfield	14	72	21	12	6	19	15	**214**	AMP-DO-F-FOX-PB-TE-CIP-GN-OT-C-CEX-NA
12	NR57	Feed	Narayanganj	Litchfield	14	72	21	12	6	19	15	**214**	AMP-DO-F-FOX-PB-TE-C-CIP-NA-CEX
13	SV60	Droppings	Savar	Litchfield	14	72	21	12	6	19	15	**214**	AMP-DO-FOX-TE-CIP-CEX-OT-NA-C
14	MK15	Droppings	Manikganj	Litchfield	14	72	21	12	6	19	15	**214**	AMP-DO-F-FOX-TE-NA-AZM-CIP-IMP-OT
15	MK17	Feed	Manikganj	Litchfield	14	72	21	12	6	19	15	**214**	AMP-DO-F-FOX-TE-AZM-CIP-CEX-OT-C-NA
16	GO66	Cloacal Swab	Gopalganj	Litchfield	14	72	21	12	6	19	15	**214**	AMP-DO-F-FOX-PB-TE-NA-OT-ATM-C-CEX
17	NR20	Droppings	Narayanganj	Enteritidis	5	2	3	7	6	6	11	**11**	AMP-DO-F-PB-TE-CIP-OT-ATM-C
18	GZ32	Droppings	Gazipur	Enteritidis	5	2	3	7	6	6	11	**11**	AMP-DO-F-FOX-PB-AZM-TE-NA-C-IMP-CEX

*NR- Narayanganj, SV-Savar, Gz-Gazipur, MK- Manikganj, GO-Gopalganj

**AMP-Ampicillin ,DO- Doxyxycline, F- Fluoroquinolone, ATM- Aztreonam,, FOX-Cefoxitin , TE- Tetracycline , OT- Oxytetracycilne,CIP- Ciprofloxacin , CEX-Cefalexin, NA-Nalidixic Acid , PB- Polymixin B.

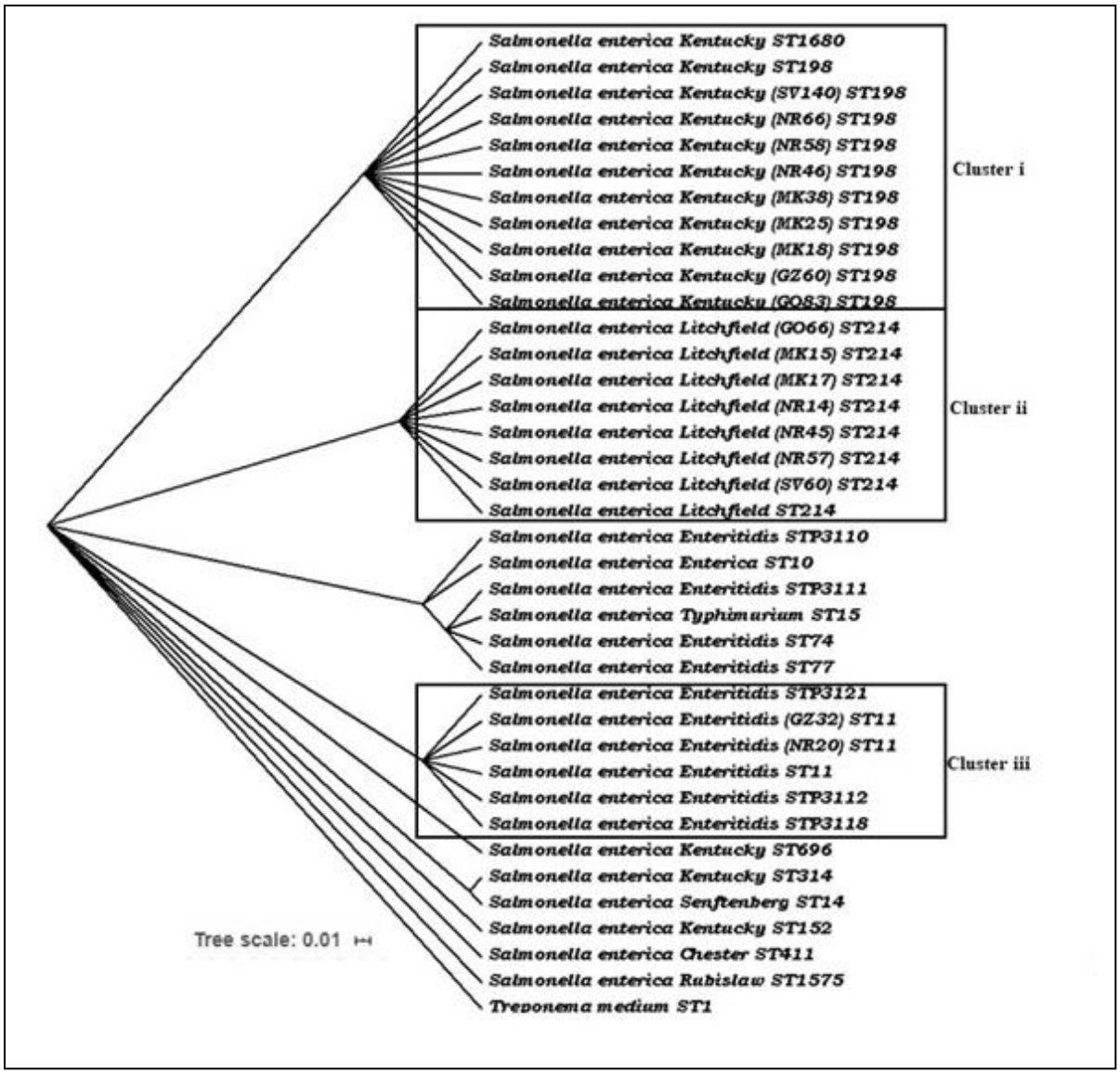


Figure 3.11: Concatenated gene DNA phylogenetic tree for seven housekeeping genes for *Salmonella* spp. Each bacterium is labeled with the isolate name, the ST to which it belongs.

3.3.4 Diversity analysis of poultry *Salmonella* population

Polymorphism in a population is measured by the analysis of nucleotide diversity. The nucleotide diversity of seven housekeeping genes in the poultry isolates varied from 0.00374 for *aroC* to 0.595 for *dnaN* (Table 3.6). There are 11 polymorphic sites identified for the *dnaN* gene sequence (Table 3.6). The average nucleotide diversity was 0.091 for the seven housekeeping genes. The minimum diversity resulted from a limited variation of isolates of three different types of MLST. The numbers of polymorphic sites in *thrA, sucA, aroC, purE, , hisD, hemD,* and *dnaN* were 5,5, 5, 11,14,7and 10, respectively (Table 3.4).

The test of Tajima's D helps to make inferences about population demographics thus supports the hypothesis that ecological adaptation or little geographic expansion occurred within the STs (Table 3.3). This is because the cut off values were significant and are different from zero for all seven loci ($p< 0.05$) except for *hisD*, where the value was ($p<0.01$) (Table 3.4).

eBURST divides an MLST data set of any size into groups of related isolates and clonal complexes. This analytic presentation predicts the founding (ancestral) genotype of each clonal complex, and computes the bootstrap support for the assignment. The eBURST analysis denoted ST11 as the founder genotype among 3 STs in clonal complex. The other two STs (ST198 & ST214) are linked to ST11 as single-locus variants (Figure 3.10).

The nucleotide diversity of coding genes is both non-synonymous (amino acid replacement) and synonymous (structurally silent) in nature. The number of non-synonymous or synonymous changes per non-synonymous or synonymous site respectively, defines the non-synonymous mutation rate (*dN*) or synonymous mutation rate (*dS*) in a gene. Calculating the dN/dS can reveal the polymorphisms segregating within a population. In this study, the dN/dS ratio was < 1 for all housekeeping genes, implies either weak negative or strong positive selection within the population (Table 3.6).

Table 3.6: Nucleotide and allelic diversity of the seven housekeeping genes including the average G+C, polymorphic diversity, *dN/dS*, and Tajima's D

Locus	Length	Avg G+C	Number of polymorphic site	Average nucleotide difference, K	Nucleotide diversity per site, π	*dN*	*dS*	Average *dN/dS*	Tajima's D
aroC	501	0.589	5	1.87135	0.00374	0	0.015	0	-0.958
dnaN	501	0.595	11	0.595	0.595	0	.047	0	-2.778
hemD	432	0.558	5	2.45614	0.00569	0.002	0.018	.111	-2.229
hisD	501	0.622	14	5.33333	0.01065	0.001	0.041	0.024	-0.010
purE	399	0.609	5	2.63158	0.00660	0	0.002	0	-2.610
sucA	501	0.585	7	2.66667	0.00532	0	0.021	0	-1.106
thrA	501	0.579	10	4.10526	0.00819	0	0.035	0	-1.542

Split decomposition analysis suggested that, in general, recombination had a marked influence on the divergence of STs within all three phylogroups. Multi-parallelogram formations indicated recombination events (Figure 3.12). The split graphs of *aroC*, *hemD*, *purE*, *sucA* and *dnaN* shows divided into two clusters. And *thrA* and *hisD* form three clusters with tree-like structures (Figure 3.12). The result suggests that all the genes were clonal and there was no recombination among those studied genes.

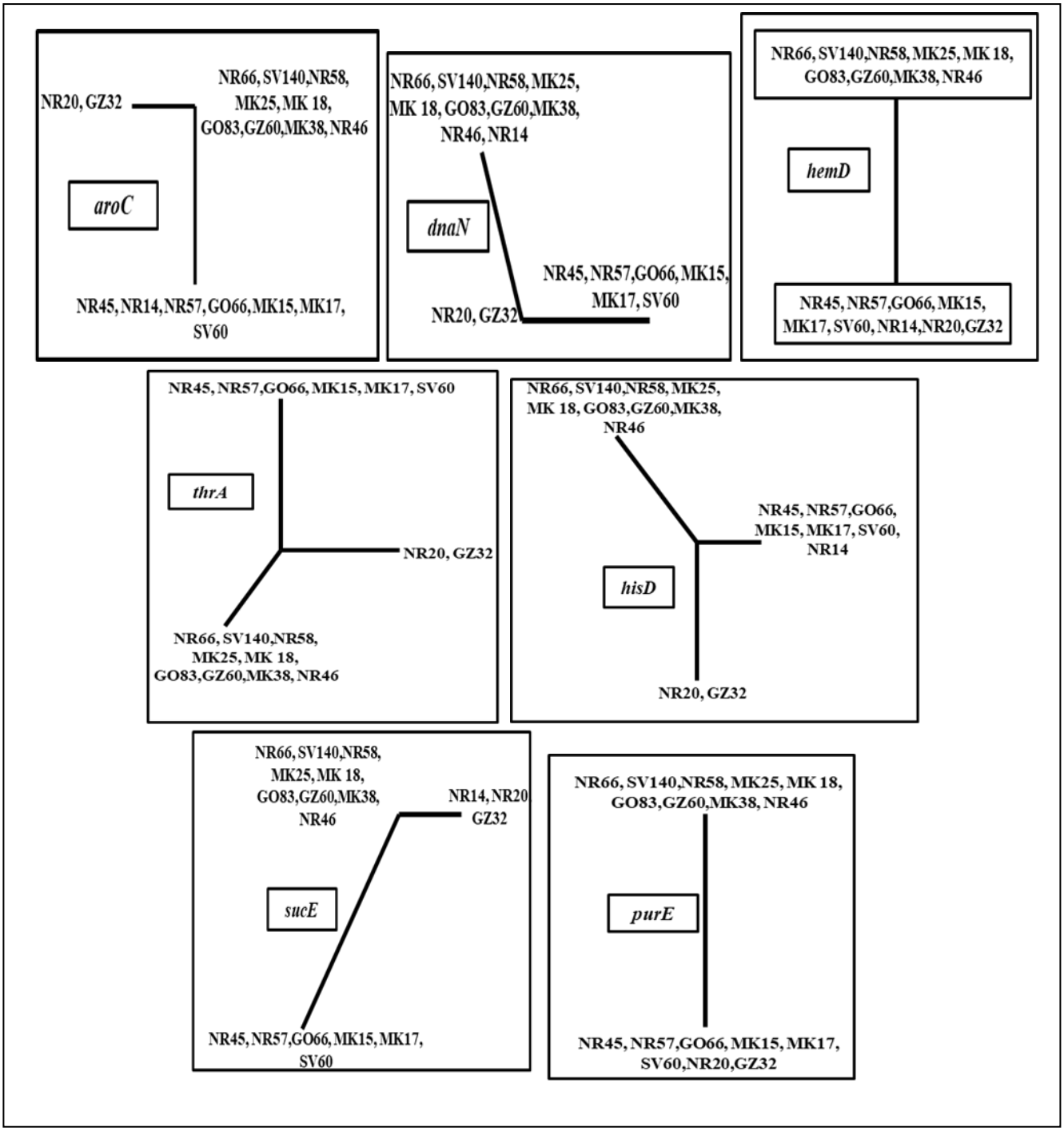
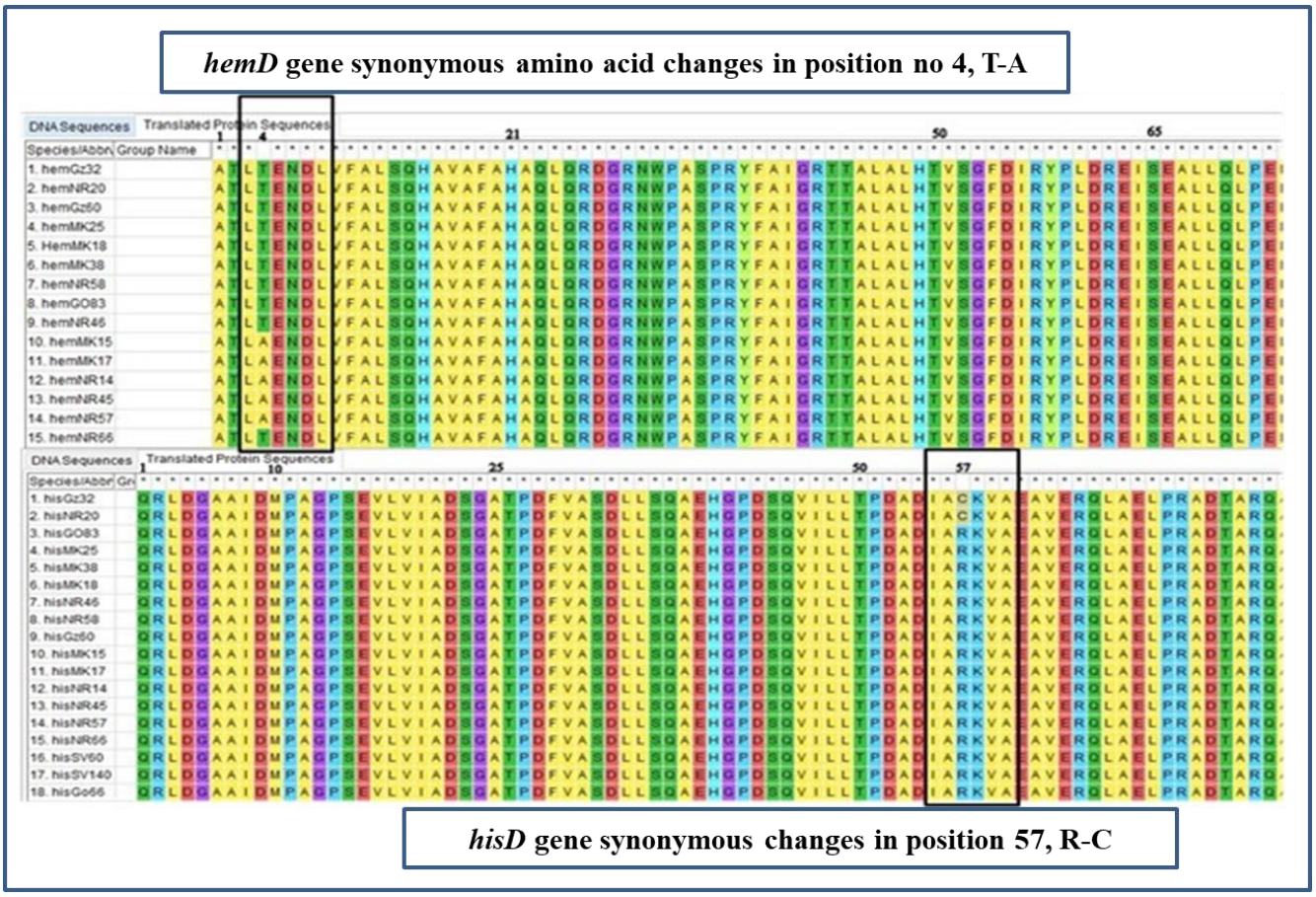


Figure 3.12: Split network analysis of the 18 isolates from each RAPD group revealed different structures in the split graphs for seven loci. The numbering in the figure refers to allele types: a. *aroC*; b.*dnaN*; c. *hemD*; d. *hisD*; e. *thrA*; f. *sucE* & g. *purE*.

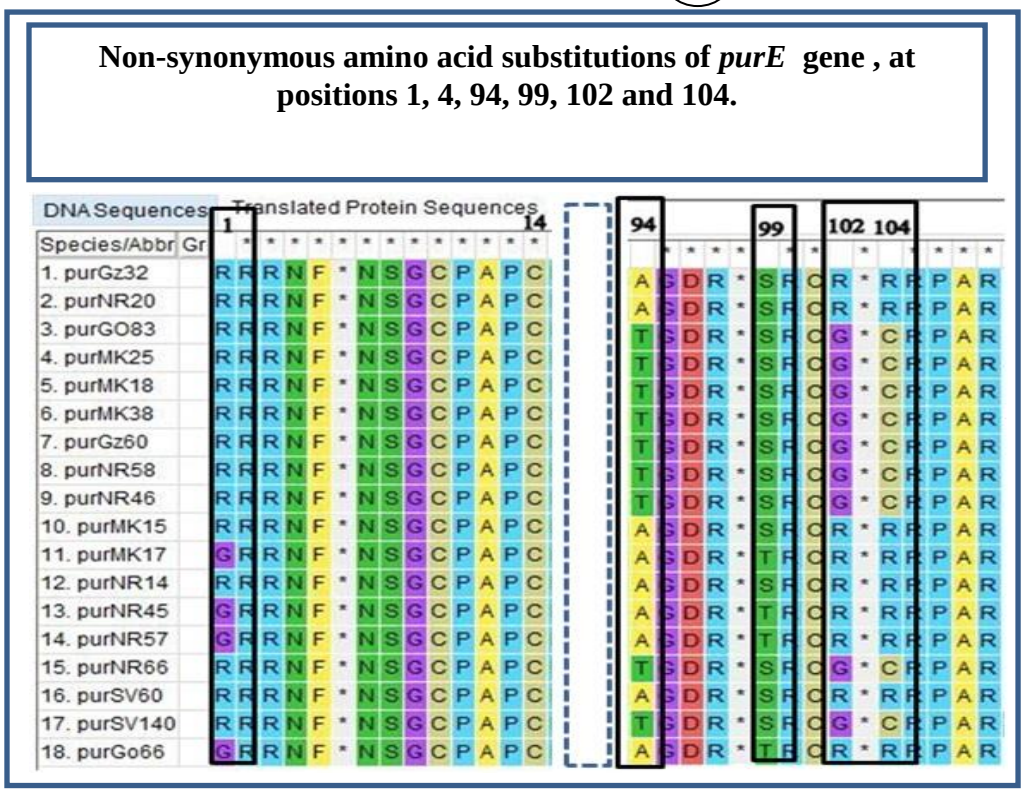
In the mutational analysis of the hotspot regions of *Salmonella* spp., housekeeping genes showed various non-synonymous and synonymous amino acid variations. All of the seven housekeeping genes had multiple non-synonymous changes (G-R, A-T, R-C, S-T), while *hisD* and *hemD* showed synonymous substitutions. The hot spot regions of *hemD* displayed one change (T-A) at position number 4, while *hisD* showed one amino acid substitution (R-C) at position 57 (Figure 3.13).

Furthermore, Simpsons index of diversity indicated that the discriminatory power of MLST (D=0.67) was close to that serotyping method (D=0.63) than RAPD (D=0.92).

The sequences for *Salmonella* specific seven housekeeping genes like *aroC, dnaN, hemD, sucA, thrA, purE and hisD* of the isolates had also been submitted to the GenBank database with the accession numbers MK732157 to MK732282 respectively.



A



B

Figure 3.13: Synonymous and non-synonymous amino acid substitutions of the protein sequences of housekeeping genes in *Salmonella* spp. A. Synonymous substitutions in *hemD* and *hisD* genes **B.** non-synonymous substitutions in *purE*

3.4 Antibiotic resistance profiles of poultry *Salmonella* isolates

The 200 isolated non-typhoidal *Salmonella* from poultry were subjected to antibiotic resistance profiling. (3.14). All isolates were resistant to one or more antibiotics; therefore, the percentage of resistance was 100%. Nearly 72% (144/200) of *Salmonella* were resistant to five or more classes of antibiotics, which may therefore be considered multi-drug resistant (MDR) (CDC, 2019).

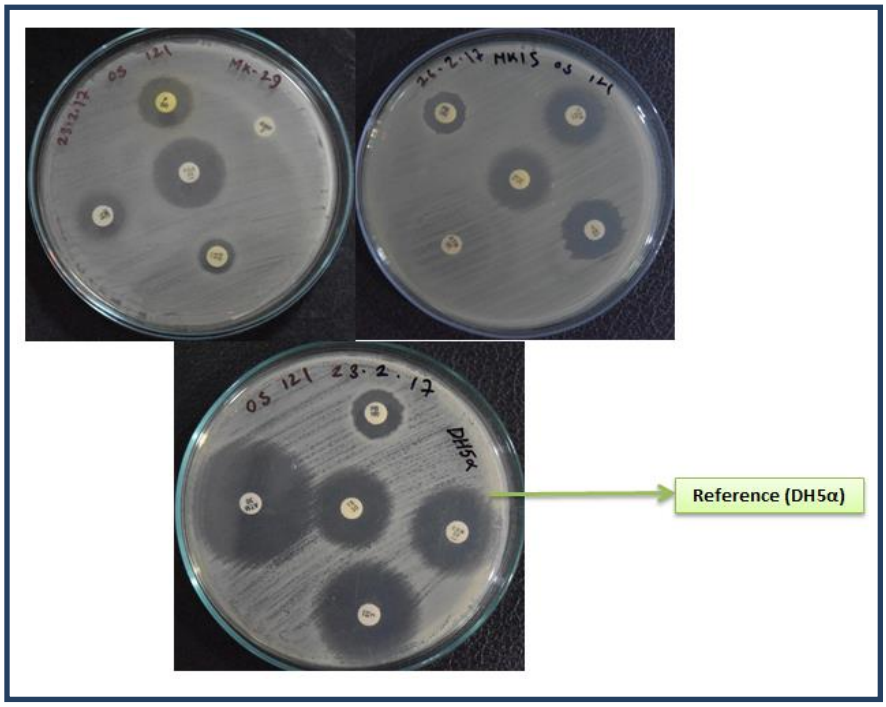


Figure 3.14: Disk diffusion test for antibiotic resistance profiling. Representative culture plates along with the reference DH5α isolate as control organism.

Fifteen groups of antibiotics were selected for disc diffusion testing to establish antibiotic profiles of *Salmonella* isolates. Doxycycline (96.49%), ampicillin (88.30%), oxytetracycline (88.30%) and ciprofloxacin (66.08%) exhibited the highest percentage of resistance.

The resistance profile based on the sampling areas indicated that Narayanganj isolates were highly resistant to doxycycline (100%) followed by tetracycline (96%), ampicillin (84%) and ciprofloxacin (76%). Savar isolates also demonstrated high resistance to ciprofloxacin (100%), oxytetracycline (100%) followed by ampicillin and tetracycline (93%). Gazipur isolates exhibited similar resistance to tetracycline, oxytetracycline and doxycycline (95% each). Isolates from Manikganj were found highly resistant to oxytetracycline (88%) and ampicillin (83%), and

Gopalganj isolates were highly resistant to ampicillin (100%) followed by doxycycline (88%), oxytetracycline and tetracycline (76%) (Figure 3.15).

Despite the fact that, there were no significant differences in antibiotic resistance patterns for *Salmonella* within different sampling areas, but the prevalence of MDR *Salmonella* was lower in the Manikganj and Gazipur compared to other parts of the sampling regions.

3.4.1 Antibiotic resistance patterns in MLST variants

Depending on the types of MLST and the antibiotic resistance profile, this study revealed a higher percentage of resistance against certain antibiotic groups. *S. enterica* ST198 isolates displayed high resistance to Doxycycline (100%), Fluoroquinolone (100%) and Tetracycline (100%). Alternatively, ST214 isolates exhibited 100% resistance to the antibiotic ciprofloxacin and 98% resistance to tetracycline. . ST11 isolates were 100% resistant to both Tetracycline and Oxytetracycline (Figure 3.16)

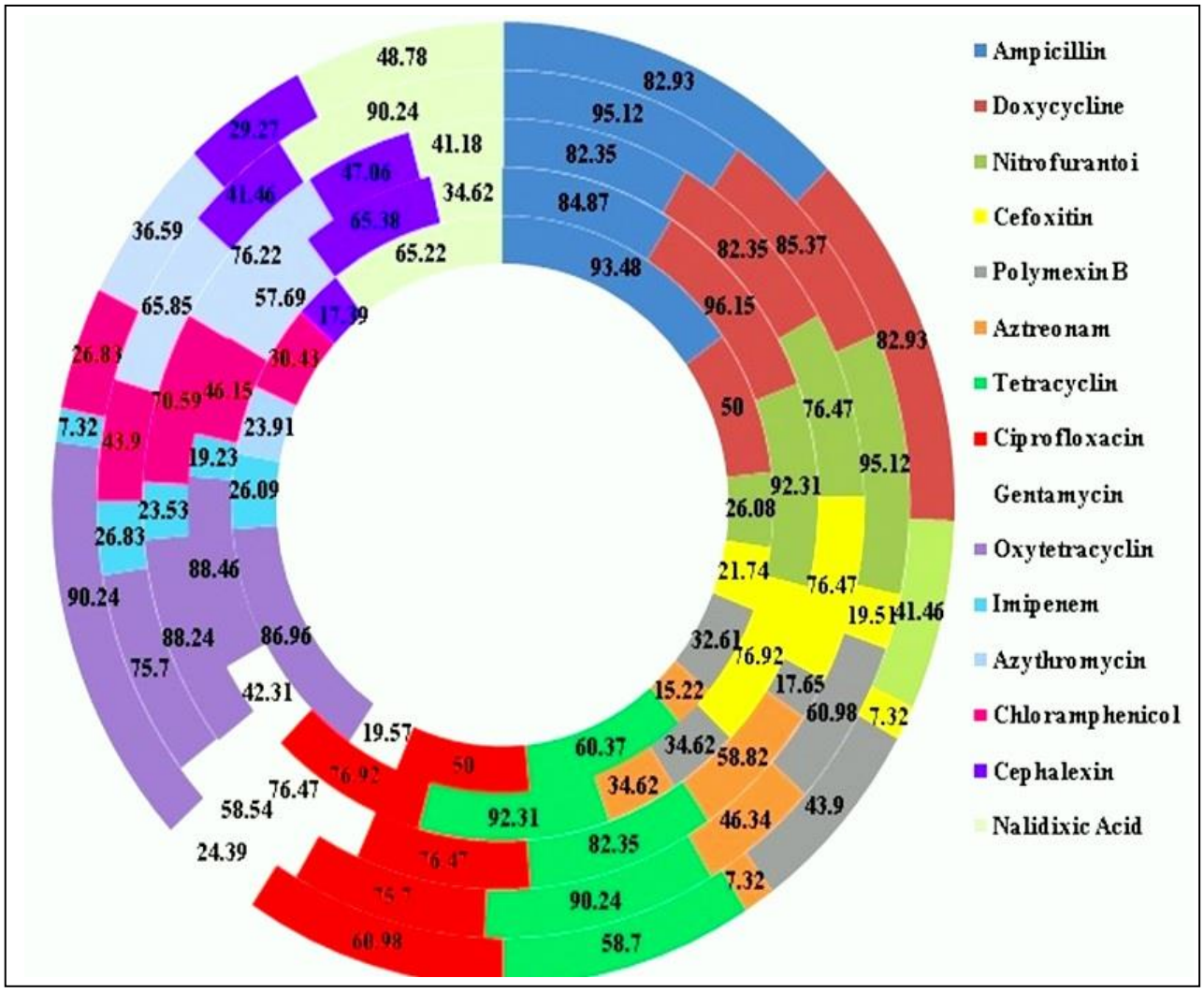
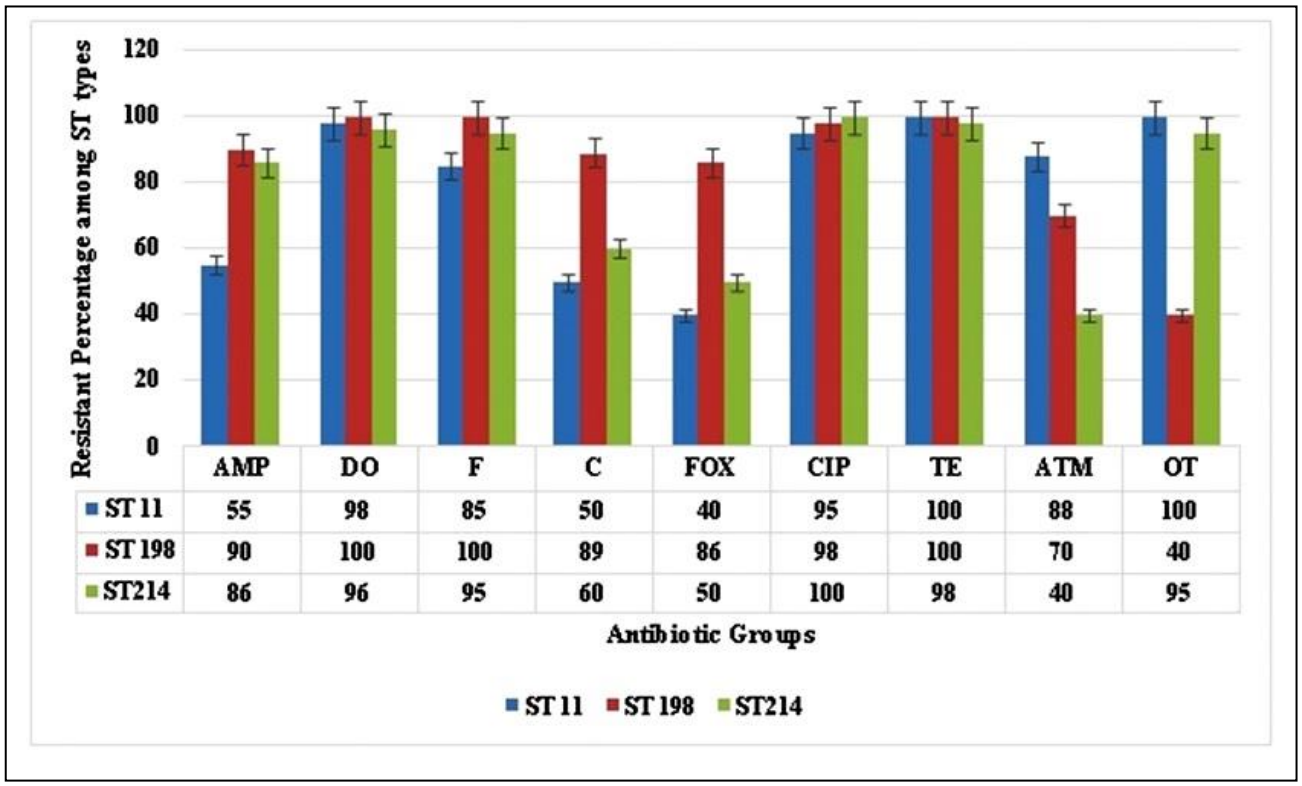


Figure 3.15: Antibiotic resistance pattern (%) of isolated *Salmonella* based on sampling locations. Antibiotic resistance pattern (%) chart of isolated microorganism against different antibiotics on area basis (From inner side 1st circle: Manikganj; 2nd Circle: Narayanganj; 3rd circle: Savar; 4th circle: Gazipur and 5th circle: Gopalganj).



*AMP- Ampicillin, DO- Doxycycline, F- Fluoroquinolone, C- Chloramphenicol, FOX- Cefoxitin, CIP- Ciprofloxacin, TE- Tetracycline, ATM- Azythromycin, OT- Oxytetracycline.

Figure 3.16: Antibiotic resistance patterns of all the three MLST types of *Salmonella* from poultry origin.

Antibiotic resistance patterns in comparison to MLST types revealed a higher percentage of MDRs for all three STs. The resistance profiles for each STs can be stated as like- AMP-DO-F-C-FOX-CIP-TE-CEX-NA for ST198, AMP-DO-F-FOX-TE-CIP-OT-CEX and AMP-DO-F-PB-CIP-C-NA for ST11 and ST214, respectively. AMP-DO-F-CIP-C the typical resistance pattern for *Salmonella* spp. was found in nearly all tested isolates (Figure 3.17). Though the isolates of ST11 and ST214 were separately specifically showed resistance to OT and PB antibiotics, respectively; none of such specific resistance was observed for ST198 to particular group of antibiotic (Figure 3.17).

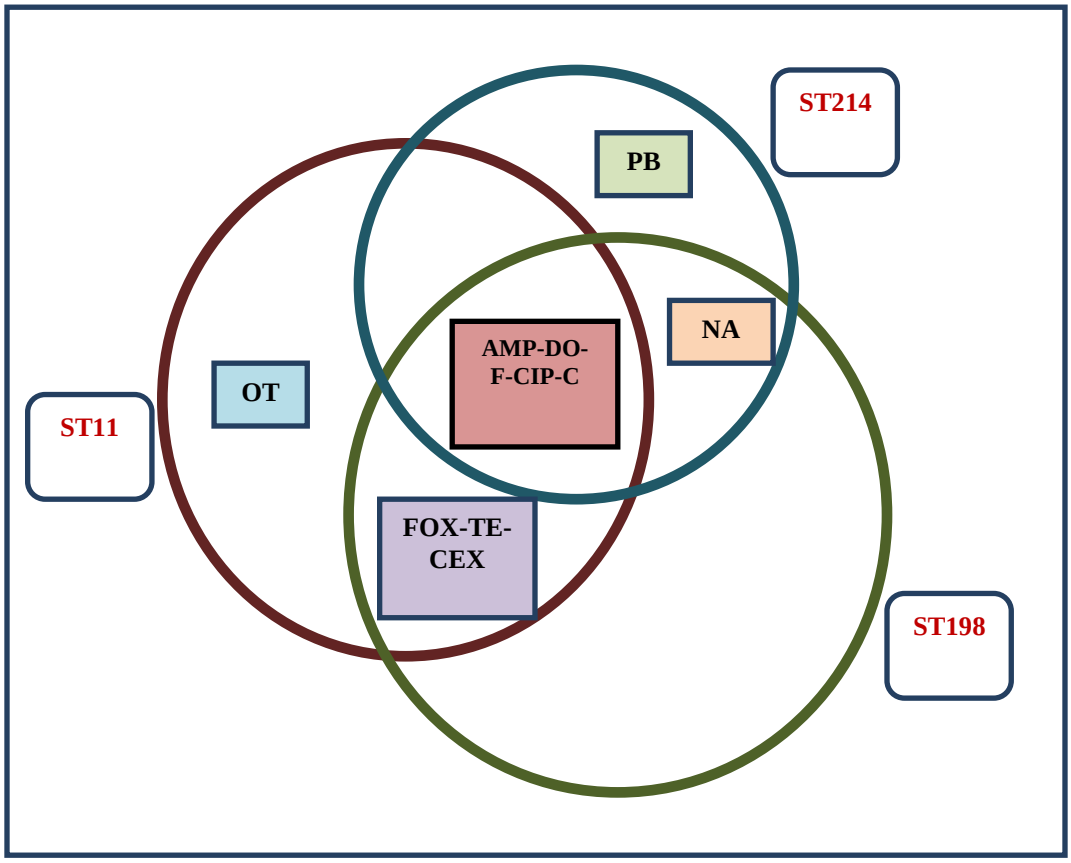


Figure 3.17: Comparative analysis of resistance patterns of MDR *Salmonella* isolates from all three STs (ST198, ST11, ST214). All three STs showed resistance to AMP-DO-F-CIP-C, these antibiotics nearly equally. The antibiotics, OT and PB found to be resistant to the specific ST, ST11 and ST214, respectively. ST198 did not result such kind of specific resistance to any antibiotic. AMP- Ampicillin, DO- Doxycycline, F-Fluoroquinolone, CIP- Ciprofloxacin,C-Chloramphenicol, FOX-Cefoxitin, TE- Tetracycline, CEX-Cefoxitin, OT-Oxytetracycline, PB-Polymyxin B, NA-Nalidixic Acid.

According to the definition of Centers for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC), bacterial isolates resistant to ≥3 class antimicrobials are considered as MDR (Magiorakos *et al.*, 2012). In this study, approximately 72% (144) of the selected *Salmonella* isolates showed resistance to 5 or more than 5 antibiotics so can be referred to as MDR. An increased proportion of MDR isolates were observed in all STs (Table 3.4).

3.5 Real-time PCR based detection and quantification of non-typhoidal *Salmonella* circulating in poultry sector of Dhaka Division, Bangladesh

Non-typhoid *Salmonella* is a public health and economical threat to poultry in Bangladesh. In order to control this threat, a cost-effective molecular method for the rapid detection and quantification of *Salmonella* in raw poultry samples is essential.

3.5.1 Standard curve construction for SYBR green real-time PCR

The microbial Genetics and Bioinformatics Laboratory, University of Dhaka, has established a SYBR green real-time PCR protocol for detection and quantification of *Salmonella* in raw poultry samples. The PCR method was 100% inclusive and detected less than 10 copies of *Salmonella* DNA per reaction.

The lowest detection limit was 10 copies with a mean C_t value of 29.729 (Table 3.7). The quantity of lowest amount of DNA was 0.00000003942, measured by the real-time PCR (Table 3.7). The C_t mean of standard dilution series of *invA* gene in this study were compared with previous studies where C_t values were 18.35 to 35.63 for *Salmonella* in accordance with 10^6 to 10 genome concentrations respectively (Calvó *et al.*, 2008).

Table 3.7: Standard dilution series of *invA* gene, quantity of DNA, C_t mean and melting temperature (Tm) of recombinant plasmid DNA based standard curve

Standard dilution of *invA* gene	Quantity of DNA (ng)	C_t mean± s.d.	Tm
10^7	0.03942	14.900 ± 0.029	82.66
10^6	0.003941	18.302 ± 0.267	82.84
10^5	0.0003942	21.411 ± 0.255	82.80
10^4	0.00003944	24.815 ± 0.049	83.02
10^3	0.000003942	27.835 ± 0.04	83.22
100	0.0000003940	30.537 ± 0.182	83.21
10	0.00000003942	29.729± 0.023	83.39

The standard curve constructed using a standard based on recombinant plasmid DNA showed good linearity. R^2 value was found to be 0.97 and it was statistically significant (3.16). All data

analyses were conducted using 7500 software, ver. 2.0.6 (Applied Biosystems, Foster City, CA, USA). The slope of a standard curve is mathematically correlated to PCR efficiency according to the equation $E=10^{-1/slope}-1$, where E is the PCR efficiency (Cikos and Koppel, 2009). A100% efficiency corresponds to a slope value of −3.32. Slop of this real-time PCR was -3.31, thus the test efficiency was 99.01% (Figure 3.18).

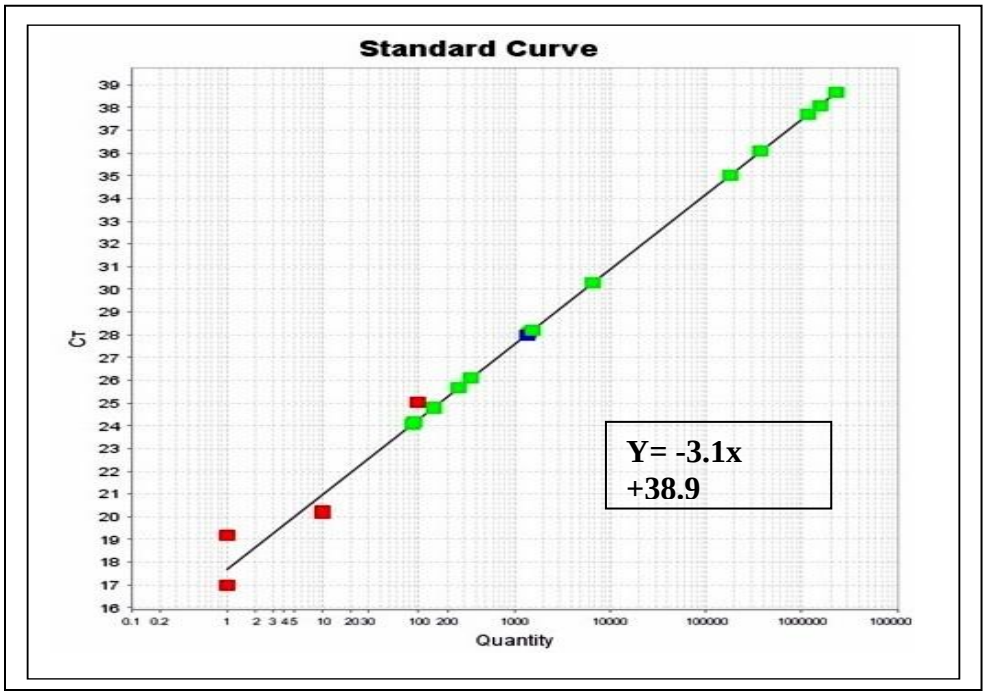


Figure 3.18: Recombinant plasmid DNA based standard curve for quantitative analysis of poultry farm samples. Here the slope is -3.1, Y intercept is 38.9, correlation coefficient R^2 value is 0.94 and efficiency is 99.1%. Along x axis quantity of DNA is present and along y axis C_T value is represented.

3.5.2 Quantification of *Salmonella* DNA in poultry samples

A significant number of poultry farm samples have already been found contaminated with *Salmonella* in this study using cultural and molecular analyses. To localize the specific transmission points/routes of *Salmonella* in the poultry sector, appropriate quantification and identification with a more sensitive molecular protocol is a prerequisite.

In this study, the established and validated SYBR green real-time PCR was used for absolute detection and quantification of *Salmonella* DNA loads in raw samples of poultry origin. Each type of farm sample (droppings, cloacal swab, feeding water, poultry feed, handler swab) taken from 14 different poultry farms in five sampling regions was subjected for quantification. In total, 25 farm samples were selected for quantification using real-time PCR, of which both positive and culturally negative samples were included. The genomic DNA for each sample was selected to a volume of 2.5µl/RT-PCR reaction. The initial DNA concentration was measured after calculations (2.5.5) and then measured using a NanoDrop^{TM} spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

In the SYBR green real-time PCR assay, approximately 84% (21/25) of poultry farm samples were detected positive for *Salmonella* contamination. On an average, the largest amount of contaminated DNA was quantified from dropping samples of poultry farms, followed by cloacal swab samples. The dropping samples were highly contaminated with *Salmonella* DNA up to 13.5×10^{8} /per ml. Although the poultry handler swab samples were negative in microbiological culture techniques, three of the five samples were positive in the real-time PCR results (Figure 3.19). The C_t mean and T_m value for each DNA sample are listed in Table 3.8.

Table 3.8: C_t values and copy numbers of different poultry farm samples

Sample Type	Replicate	C_t mean ± s.d.	Quantity mean ± s.d. (BCE/mL)	T_m	Culture Results
Droppings	1	24.10±0.149184	1.1×10^7 ±13.33	85.25146	Positive
	2	29.64 ±0.619511	10.8×10^6±18.4	76.90059	Positive
	3	32.54 ±0.149184	10.4×10^5±20.5	85.84795	Positive
	4	28.34 ±0.12312	10.8×10^6± 13.42	82.18713	Positive
	5	25.21±0.619511	1.3×10^7± 18.2	77.29825	Positive
Cloacal swab	1	27.68 ±0.619511	6.8×10^6 ± 55.4	86.04678	Positive
	2	29.78 ±1.538787	7×10^5±34.6	77.69591	Positive
	3	24.89 ±1.538787	5×10^7 ±45.3	70.33918	Positive
	4	28.38±0.731251	3.5×10^5±18.3	86.24561	Positive
	5	38.52±0.731251	3.8×10^2± 28.9	86.24561	Positive
Handler Swab	1	34.07±0.054969	2.5×10^2± 63.2	78.09357	Positive
	2	38.07 ±1.553107	6.8×10^2 ± 44.6	65.56725	Positive
	3	34.19 ±0.049565	1.4×10^2 ±16.2	76.90059	Positive
	4	C_T Undetermined	C_T Undetermined	86.24561	Negative
	5	C_T Undetermined	C_T Undetermined	61.9883	Negative
Feed	1	29.55±0.917459	3.8×10^5 ± 25.3	84.05848	Positive
	2	30.55 ±0.917459	2.5×10^5 ± 55.2	86.24561	Positive
	3	35.05 ±0.049565	2.7×10^4± 42.2	78.09357	Positive
	4	32.05 ± 0.054969	3.5×10^4 ± 34.4	78.09357	Positive
	5	C_T Undetermined	C_T Undetermined	86.24561	Negative
Water	1	37.20 ±1.553107	8.5×10^2 ±12.2	65.56725	Positive
	2	35.45±0.917459	7.8×10^2± 15.5	86.24561	Positive
	3	30.37±0.386354	1.02×10^3±20.5	65.56725	Positive
	4	35.20±0.386354	2.0×10^2 ±16.5	78.09357	Positive
	5	C_T Undetermined	C_T Undetermined	65.56725	Negative

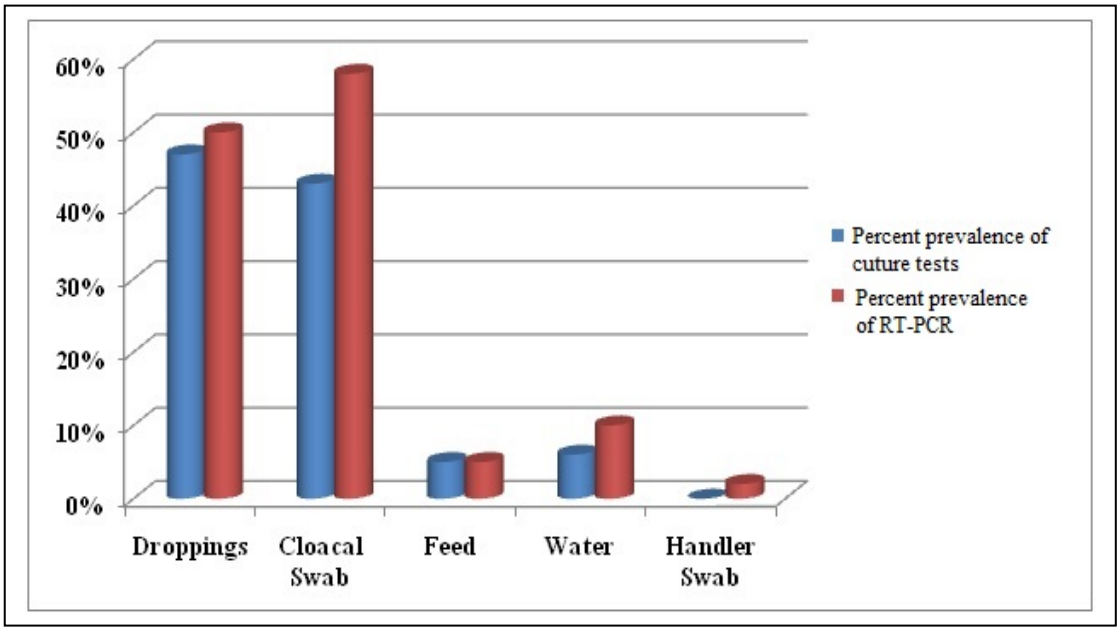


Figure 3.19: Comparative analysis of cultural positive and real-time PCR positive samples. Same farm samples were tested for *Salmonella* contamination using both cultural-molecular combined protocol and real-time PCR based protocol.

A significant difference was detected between the results of microbiological culture and SYBR Green PCR based detection methods. A number of false negative poultry samples were found to be positive in the real-time PCR approach. . The incidence of false negative result was higher for cloacal swab samples (15%) (Figure 3.19).All three out of five negative Handler Swab samples tested positive for real-time PCR. The amount of DNA was comparatively lower for the culture negative samples identified in the real-time PCR method (Table 3.8). All DNA counts identified for *Salmonella* contamination in different poultry samples were plotted graphically for comprehension (Figure 3.20).

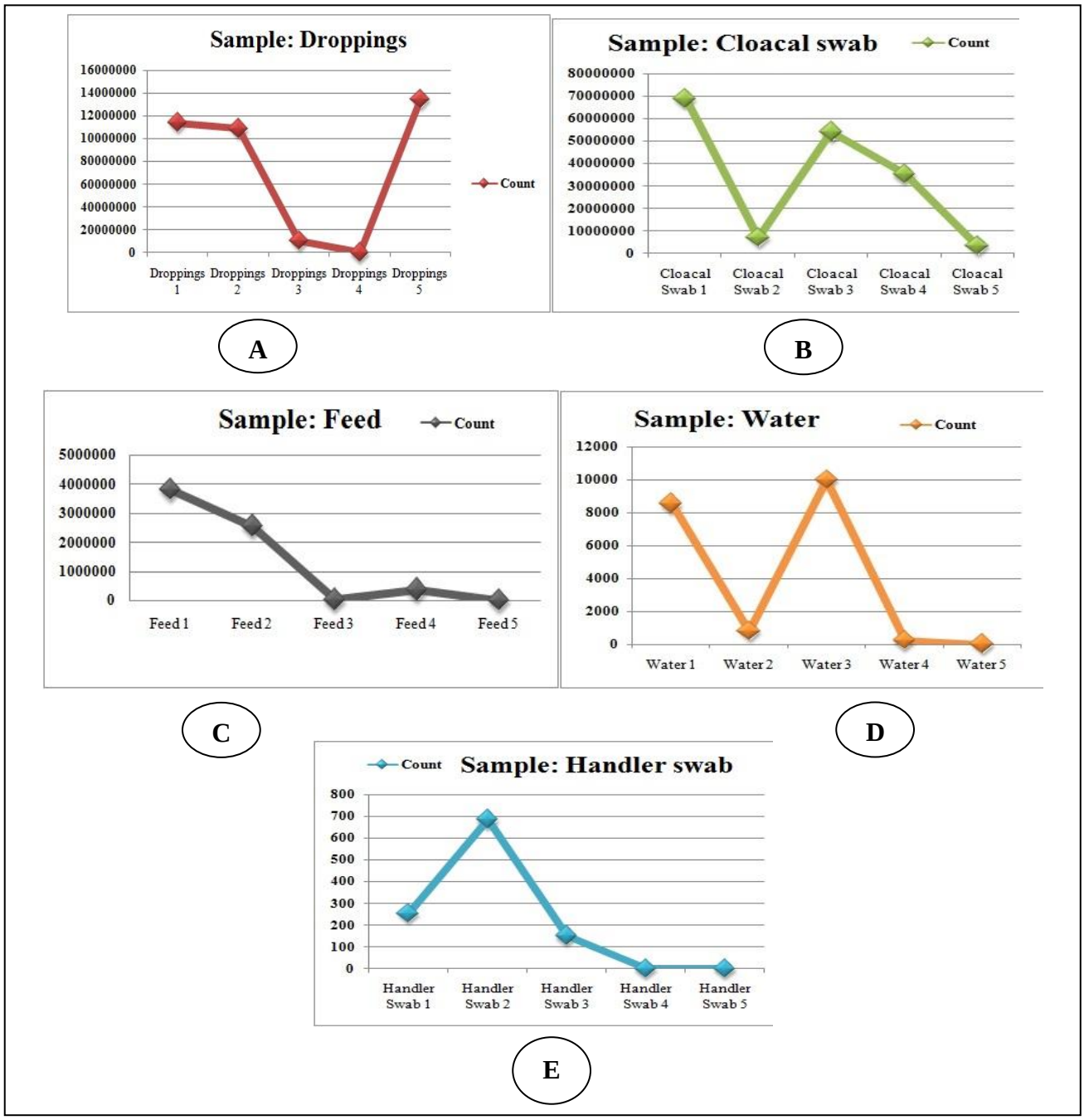


Figure 3.20: Real-time PCR counts for *Salmonella* isolates in different types of poultry farm samples.

3.5.3 Identification of *Salmonella* from farm samples at different sampling regions.

All the samples were equally selected for SYBR green real-time PCR from all five sampling regions. The number of positive samples was compared in between the locations. The poultry samples from Gopalganj area showed the highest *Salmonella* count, followed by Manikganj, Gazipur and Narayanganj. The samples from Savar were least contaminated with *Salmonella* (Figure 3.21).

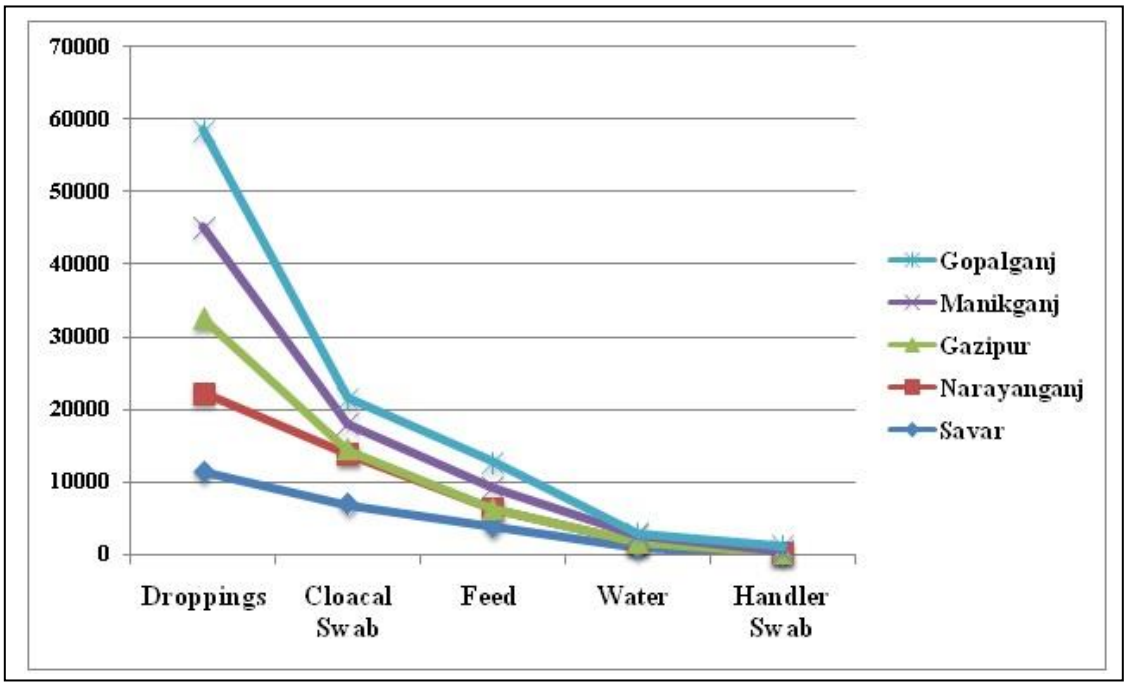


Figure 3.21: Farm quality analysis based on real-time PCR results and comparison among different sampling areas.

3.6 Detection of *Salmonella* in each point of poultry production system using real-time PCR:

Real-time PCR method was carried out for identification of *Salmonella* from each point of poultry farm production system to kitchen. In total, 153 samples were collected from poultry bazar, transport, hotel kitchen and home kitchen for detection analysis (Figure 3.22). All the points were found positive for different range of *Salmonella* contamination.

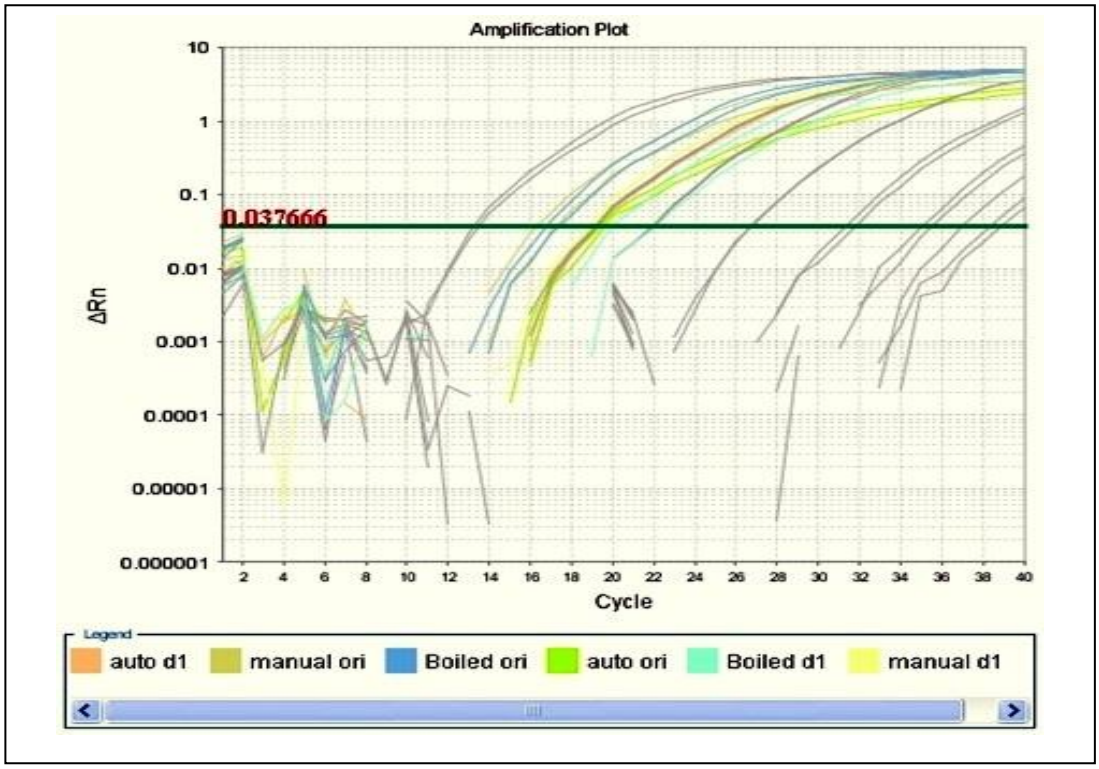
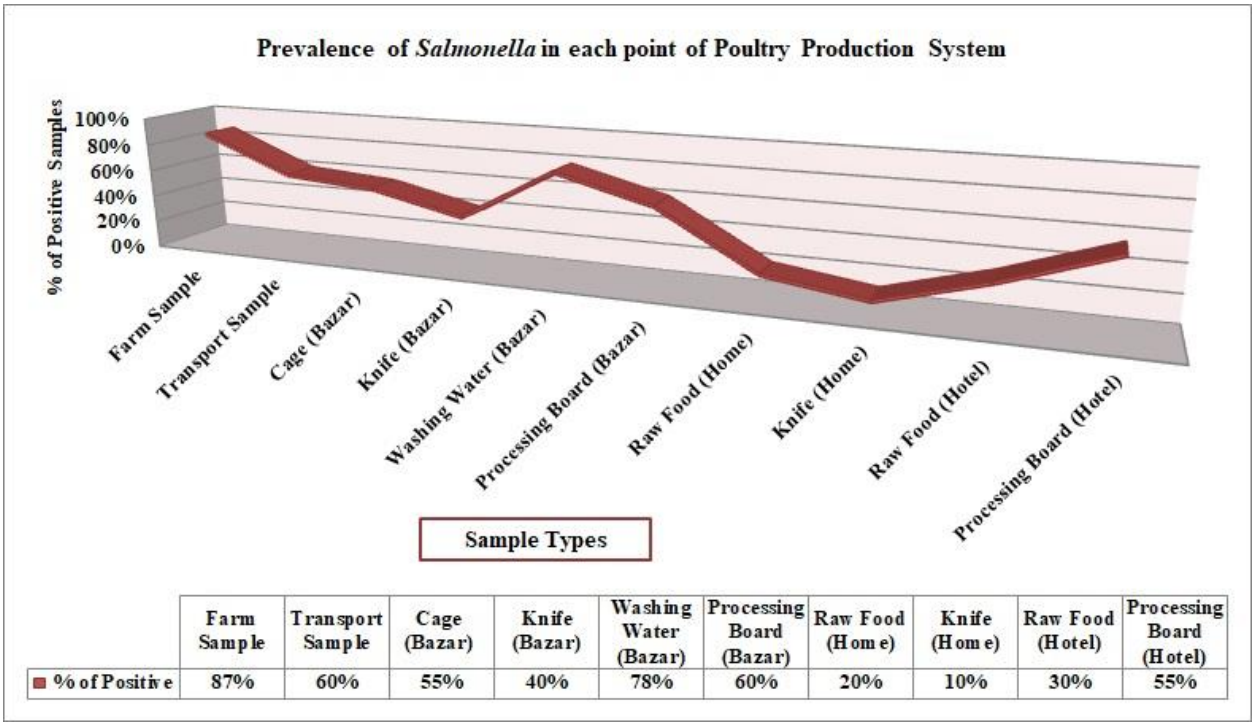


Figure 3.22: The amplification plot of poultry samples collected from different points of poultry production system.

The extracted total DNA from the poultry originated samples collected from transmission rout points were analyzed using SYBR Green real-time PCR based method for *Salmonella* detection. Since enumeration was not the target, the standard curve was not prepared for this analysis. Negative control was used with each reaction batch to authenticate the protocol and reaction conditions.

A higher percentage (87%) of poultry farm samples tested positive for *Salmonella* through the real-time PCR method. A relatively higher proportion of poultry transport (60%) and bazaar samples were also found to be contaminated with the organism. The water sample from the slaughter area (78%), processing board (60%) and chicken cages (55%) at poultry bazars was heavily contaminated with *Salmonella* isolates. All these transportation and bazar points seem to be directly linked to the mass population, which can transmit the pathogen to the cooking areas. As a result, the real-time PCR detected an unpleasant percentage of *Salmonella* in the hotel kitchen (55%) and therefore lower, but not negative for home kitchenware (10%) (Figure 3.23).



	Farm Sample	Transport Sample	Cage (Bazar)	Knife (Bazar)	Washing Water (Bazar)	Processing Board (Bazar)	Raw Food (Home)	Knife (Home)	Raw Food (Hotel)	Processing Board (Hotel)
% of Positive	87%	60%	55%	40%	78%	60%	20%	10%	30%	55%

Figure 3.23: Prevalence of *Salmonella* in each point of poultry production system from farm to kitchen.

Different poultry bazar samples including, transport swab, poultry cage swab, swab from poultry slaughtering knife and processing board were collected for detection analysis. Real-time PCR based identification method used for the analysis.

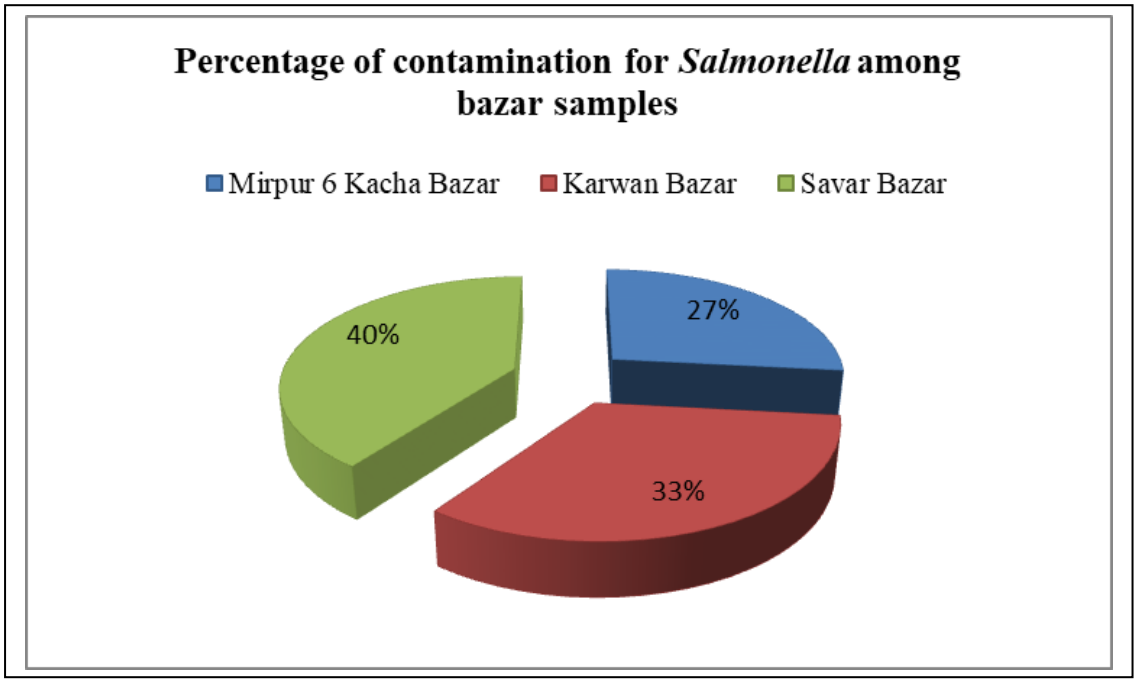
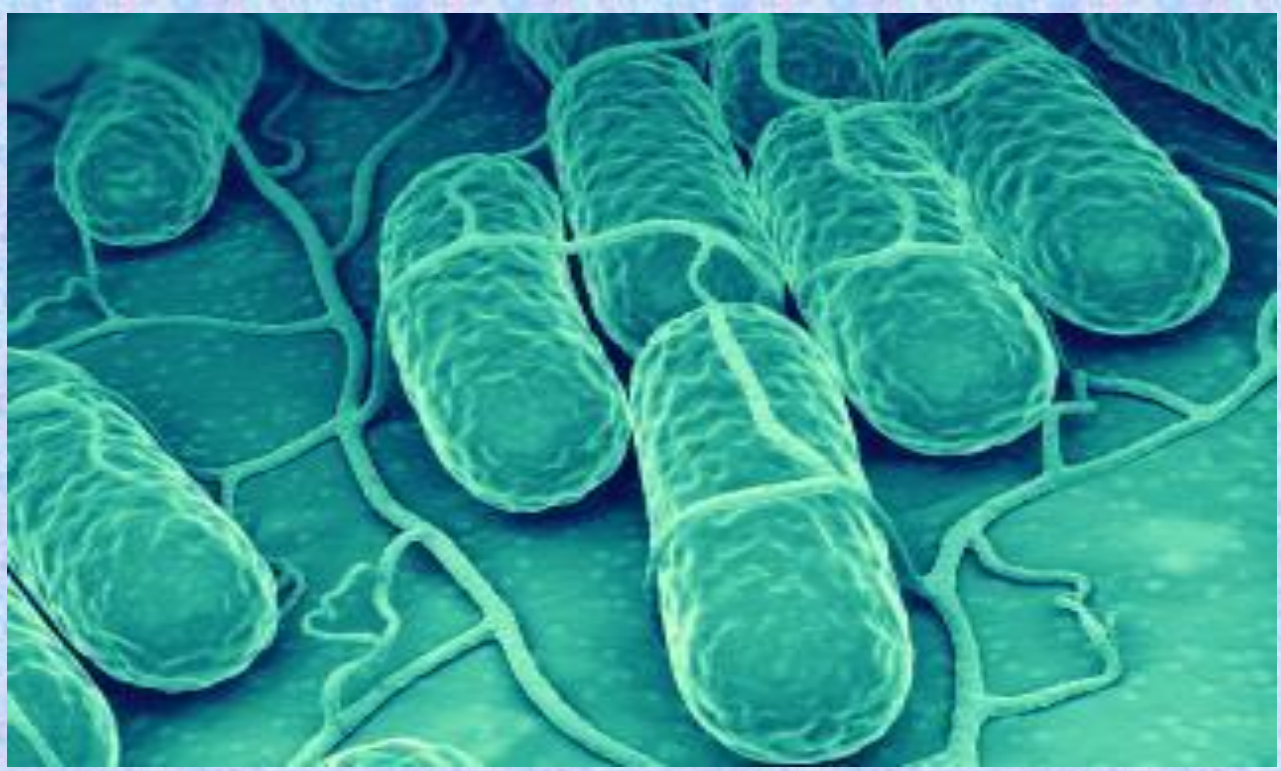


Figure 3.24: Comparative *Salmonella* contamination analysis between bazar areas.

The poultry samples were collected from three well established poultry bazar areas in Dhaka city, Mirpur 6 kacha bazar, Karwan bazar, and Savar bazar. Collected samples from all possible source points were tested for *Salmonella* contamination using real-time PCR based identification method.

A comparative analysis among the three bazar areas resulted that, Savar bazar contained the highest percentage (40%) of *Salmonella* contamination compared to Mirpur 6 (33%) and Karwan bazar (27%) (Figure3.24). No significant differences were found in the management of handling, storage, preparation and sale of chicken in these bazar areas. The practice of maintaining hygiene conditions has been ignored by all retailers and consumers. This may be the primary reason for these high percentages of *Salmonella* contamination in poultry samples at each point of transmission.



Chapter 04: Discussion

4. Discussion

The multi-drug resistant (MDR) *Salmonella* have been considered as a superbug for the public health sector worldwide. World Health Organization (WHO) and Food and Agriculture Organization (FAO) have declared these organisms as the most common zoonotic pathogens. The association of poultry with *Salmonella enterica* serovar Enteritidis and non-Enteriditis mediated foodborne outbreaks in human and animals are a matter of concern throughout the world. The present study found comparatively higher percentage (70%) of non-typhoidal *Salmonella* in the poultry sector in Bangladesh as a whole, and the *Salmonella* contaminations move from farm to kitchen. Furthermore, a broader range of epidemiological understanding of the clonal distribution and MDR properties of these pathogens and their rapid detection and quantification method are focused in the current dissertation to minimize the threat of these zoonotic pathogens.

4.1 Hygiene practice reduces *Salmonella* prevalence but increases antibiotics resistance

Non-typhoidal *Salmonella* infections in farm birds lead to higher rates of loss in the poultry sector each year and the presence of the pathogen in the food supply chain creates barriers in poultry businesses. Moreover, the zoonotic *Salmonella* can also contaminate food and human environment and becomes the mean source of food born salmonellosis in human.

A structured questionnaire was designed and followed up to gather information on the farm management process, as well as local knowledge on hygiene practices and uses of antibiotics. Although the percentage of hygienic knowledge and practices was higher among farmers, the knowledge about antibiotic usage was not satisfactory. As the misuse of antibiotics is a major driver of resistance, integrated strategies are required to improve the on-farm antimicrobial administration and awareness in farmers (Kramer *et al.*, 2017). In this study, exploratory factor analysis (EFA) on the questionnaire items focused on farmer's view, knowledge and usage of antibiotics identified few factors, such as referent beliefs, awareness, self-administration of antibiotics, and educational background. In this analysis, the poultry farmers scored highest for hygienic practices and this score was higher in Savar, Gazipur and Narayanganj poultry farms rather than others. Lowest score was documented for 'awareness' and 'educations' in several farms, specifically in Manikganj and Gopalganj (Table 3.2). These differences also point to the growing urbanization and therefore educational preferences in farmers of Savar, Gazipur and Narayanganj areas. 'Non-prudent misusage of antibiotics' was observed in all the sampling

regions. This observation correlates with previous studies from Bangladesh, where reported 39.1% of farmers possess knowledge of antibiotic usage, while only 20% of farmers consult with veterinarians when they are unable to control the infection and mortality in their poultry farms (Ferdous *et al.*, 2019; Masud *et al.*, 2020). 'Knowledge and education' scores were significantly and inversely related to antimicrobial misuses (P=0.0004), which correspondences with other studies (Kramer *et al.*, 2017). Referent belief and basic knowledge are also significantly associated with proper antimicrobial usage (Caudell *et al.*, 2020). Although the farmers on all selected sampling regions have regular contact with veterinarians in periodical basis, further behavioral interventions in remote farmers, such as educational campaign and increased support from national livestock offices may help to mitigate the unawareness, and thus combat antibiotic misusage and resistance nationally.

Among the sample types, droppings were mostly contaminated with *Salmonella* (47%), followed by cloacal swabs (43%). In an earlier study, the prevalence rate was 46.02% and 40.63% respectively for cloacal swabs and carcasses from Gazipur and Tangail poultry farms (Mridha *et al.*, 2020). The prevalence rate of *Salmonella* in poultry farm samples in other Asian countries found lower than that of Bangladesh from several studies, for instance, the reported percentage was 6.1% for India and 17% for Malaysia in cloacal swab samples from poultry farms (Samanta *et al.*, 2014; Mohammed *et al.*, 2019). Moreover, this study found, lower hygienic poultry farms in Manikganj and Gopalganj had the highest prevalence of *Salmonella* contaminations (27% and 25.5%, respectively).

Among the *Salmonella* isolates, 72% were found resistant to five or more of the antibiotic classes and included under MDR group according to the Centers for Disease Control and Prevention (CDC, 2019). The resistant patters of the isolates revealed that Tetracycline group (doxycycline-91.5%) found the most resistant a common choice of drug for bacterial infection including *Salmonella.* The antibiotic resistance patterns of the *Salmonella* isolates correlates with previous findings from Bangladesh, where high resistances were reported for ampicillin, tetracycline, ciprofloxacin, gentamycin, nitrofurantoin (Aditya A, 2015; Parvej *et al.*, 2016). Similar resistance pattern was observed in sampling areas but the percentage of resistance for specific drug was different. Isolates from all of the sampling regions were found resistant to all of the antibiotic groups. A number of *Salmonella* isolates resistant to nine or more antibiotics were isolated more frequently in the Savar and Gazipur sampling areas (Table 3.5). The relative

abundances of *Salmonella* varies inversely with the hygiene practices observed in poultry farms of Savar, Gazipur and Narayanganj, where more MDR were observed than in other regions (Section 3.4).

The occurrence of non-typhoidal *Salmonella* in the poultry sector in Bangladesh has been documented in a number of scientific studies (Barua *et al.*, 2014; Hassan *et al.*, 2016; Hoque *et al.*, 2019). The higher prevalence and increased percentage of antibiotic resistant *Salmonella* in poultry and poultry-produces are a great threat to public health.

4.2 MLST typing improves precisely characterization of *Salmonella* diversities in poultry

The increased prevalence of *Salmonella* in the poultry sector requires adequate surveillance and more accurate and efficient research methods to control. Generally, molecular typing methods are deliberately used to solve epidemiological surveillance at both the local and international levels. In laboratory programs, the choice of surveillance methods may vary in accordance with the pertinent and episodic use of the results. Molecular typing methods based on hypervariable loci can be used for local and short-term monitoring programs, whereas analysis of conserved regions give an ancestral data analysis platform for a longer time period (Sankar *et al.*, 2013). Multi-locus sequence typing (MLST) method is used for such evolutionary analysis to trace the ancestral lineages in a large number of bacterial populations (Urwin and Maiden, 2003; Torpdahl *et al.*, 2005). Analysis of housekeeping genes along with decentralized public domain of databases has made the MLST method as one of the most adoptable evolutionary and epidemiological tools.

In current study, seven housekeeping genes based MLST method revealed 3 different STs (ST11, ST198, and ST214) from the 18 *Salmonella* isolates representative of different RAPD genotypes and distinct sampling regions. All three STs adhered to specific serotypic varients; ST11- *S. enterica* serovar Enteritidis; ST198- serovar Kentucky and ST214- serovar Litchfield. The most prevalent ST198 has previously been reported from human and poultry samples in Bangladesh (Barua *et al.*, 2014). A wider range of sample types (animals, food items, dairy farms, poultry, and human) has been documented to be contaminated with *Salmonella* ST198 from different countries (Hello *et al.*, 2011; Barua *et al.*, 2012; Howe *et al.*, 2017). The second most prevalent MLST type, ST214 has not been reported before in Bangladesh. A recent study from Shanghai mentioned the presence of *S. enterica* Litchfield (ST214) in poultry farm samples in China (En Ni *et al.*, 2017). The MLST type, *S. enterica* Enteritidis, ST 11 (16.5%), has been reported as a

common ST from poultry samples in Chittagong, Bangladesh (Barua *et al.*, 2014). ST11 has also been categorized as a unique and widely distributed ST circulating a period of decades in Japan, Brazil, and Iran (Noda *et al.*, 2011; Ghaderi *et al.*, 2015; Acurcio *et al.*, 2020). This type of ST can be transmitted not only by foods of animal origin, but also by vegetables, fruits and other plant products (Petridou *et al.*, 2016). It is frequently associated with human infection as well (Fandiño and Verjan-García, 2019).

The resulted common STs among different farms indicate the ancestral lineages over five districts of the country. All three STs are commonly distributed among all five sampling regions in this study whereas ST198 was predominant in all four sampling regions except Narayanganj. In Narayanganj, ST214 was predominant, which is the second prevalent sequence type in other regions (Figure 3.10). Although the circulating MLST types identified in this study have been reported from other Asian countries like, China and Malaysia; studies of neighboring countries such as India, Nepal and Pakistan have not yet been documented (Yang *et al.*, 2019; Zakaria *et al.*, 2020). A wider range of studies in South Asian countries could reveal the current circulating MLSTs in this region and the risk of cross-border transmission of this pathogen.

In this study, almost all the isolates of the three MLST types were MDR. Specifically, all the ST198 isolates were resistant to ampicillin, tetracycline, doxycycline, ciprofloxacin and fluroquinolone antibiotics. Ciprofloxacin-resistant ST198 *Salmonella* Kentucky has already been reported in African and Middle Eastern countries of poultry origin (Hello *et al.*, 2011). ST11 and ST214 were also resistant to doxycycline, ampicillin, and ciprofloxacin. Studies in Africa and Kenya have found that *Salmonella* enteritidis ST11, a MDR bacterium, is the most widespread *Salmonella* ST in poultry samples (Kariuki and Dougan, 2014; Kariuki and Onsare, 2015). Studies have also identified this pathogen as the most common cause of invasive diseases in the community (Akullian *et al.*, 2018).

The current study is one of the initial steps of MLST based surveillance for non-typhoidal *Salmonella* in Bangladesh. The new ST from this study indicates that there could be more STs circulating in this region. It may be a surveillance model which can provide more definitive information on the sources, repository and transmission of resistance genes in non-typhoidal *Salmonella*. A complete dataset based on molecular analysis of non-variable regions in MDR *Salmonella* could be a major and inevitable part for control measures.

4.3. SYBR green real-time PCR: a choice of low-cost method for rapid quantification of *Salmonella*

Zoonotic pathogenic *Salmonella* becomes a major contamination of poultry and poultry-produces. The routine detection of *Salmonella* in foods and raw food items is an important part of public health programs. Therefore, routine monitoring management requires a rapid, less expensive, more sensitive and easier to handle protocol for *Salmonella* detection. In this study, a method of SYBR green RT-PCR was established in the laboratory for raw and ready to eat products targeting *invA* gene for *Salmonella* with an efficiency 99.01% and the correlation between C_t value and copy number of *invA* was well enough (R2=0.972). In this study, the average C_t value detected in real-time PCR was 14.900 ± 0.029 to 30.537±0.182 in the range of 10^7 to 10 gene copy. These results can be correlated with previous studies where C_t values were 18.35 to 35.63 for *Salmonella* in accordance with 10^6 to 10 genome concentrations respectively (Calvó *et al.*, 2008).

The *Salmonella* detection rate by conventional bacteriology versus SYBR green RT-PCR was 20% to 25% among poultry samples. The result can be compared with the study in Turkey having 1.77% deviation from bacteriological analysis to SYBR green-based RT-PCR (Eyigor *et al.*, 2005).

PCR is one of the key molecular-based methods using microbial detection and characterization in recent years. The real time PCR described in this study showed high selectivity and accuracy. Quantitative real time PCR have the ability of enumeration of bacteria in high specific manner, thus have a major advantages over the traditional microbiological methods. The time limit in real-time PCR is surprisingly less than other methods with higher selectivity and specificity. The overall protocol have the ability to generate a larger data in a shorter period of time, thus makes the method useful for epidemiological studies. The personnel workload is lower and consequently the cost of analysis is less. These positive features help to select this molecular method as a method of choice for regular monitoring and epidemiological studies.

The developed and established SYBR Green RT-PCR method in 'Microbial Genetics and Bioinformatics Laboratory' used recombinant plasmid DNA (cloned with *invA* amplicon) based standard curve, as it found more efficient having 99.04% efficiency. The estimated Tm value for this modified method was 83.5^0C. Although the Tm value was higher than previous studies, this

temperature found consistently specific for the amplicon obtained (De Medici *et al.*, 1998). Phenol chloroform DNA extraction method was selected as method of choice for its cost effectiveness. The method was validated using intentionally spiking laboratory protocols. Thereafter, crude raw samples were analyzed and quantified for *Salmonella* contamination using the validated SYBR Green RT-PCR. In raw samples, the resulted efficiency was 99.01% and R^2 value was 0.97%. The protocol requires 4 to 6 hours to quantify the actual number of target gene in raw samples, whereas the traditional ones approved by FDA, need up to 10 days to get results (Wallace *et al.*, 2011). Furthermore, the method does not need pre-enrichment steps, thus limiting the time required. Considering the cost-effectiveness and rapid result interpretation, the established SYBR Green RT-PCR method can be used for bulk community study in low-income countries.

4.4 Route of transmission of *Salmonella* from producers to consumers

Using the validated SYBR Green RT- PCR, a wider range of poultry samples from farms, local bazar, hotel kitchen and household kitchen; have been analyzed to understand the route of *Salmonella* transmissions from producers to consumers. It is noteworthy that zoonotic Salmonellosis patients have been increasing considerably over the years in Bangladesh with increased dependency of farmed poultry meets and eggs. Furthermore, simultaneous increased MDR properties in circulating *Salmonella* properties have been documented (Mahmud *et al.*, 2011) The results presented in this dissertation suggests the possible transmission rout of poultry associated non- typhoidal *Salmonella* in Bangladesh from producers to consumers as depicted in Figure 4.1.

From the poultry farm house to poultry bazar all the points can be an important vehicle route for the transmission of *Salmonella*. This may be attributed to increased poultry production and the stress induced during poultry transportation, while in bazar areas, the high prevalence of *Salmonella* can be attributed to poor hygiene and sanitation practices.

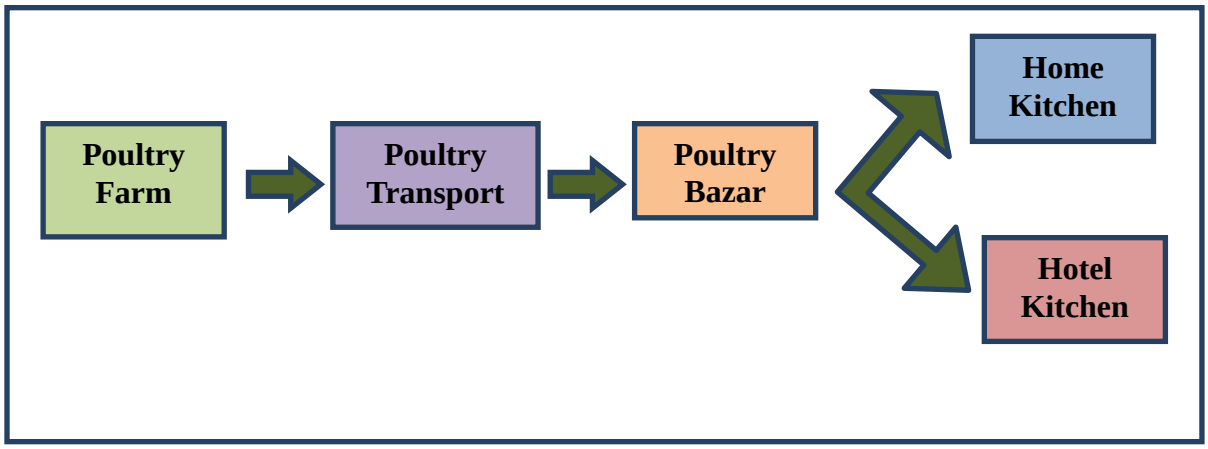
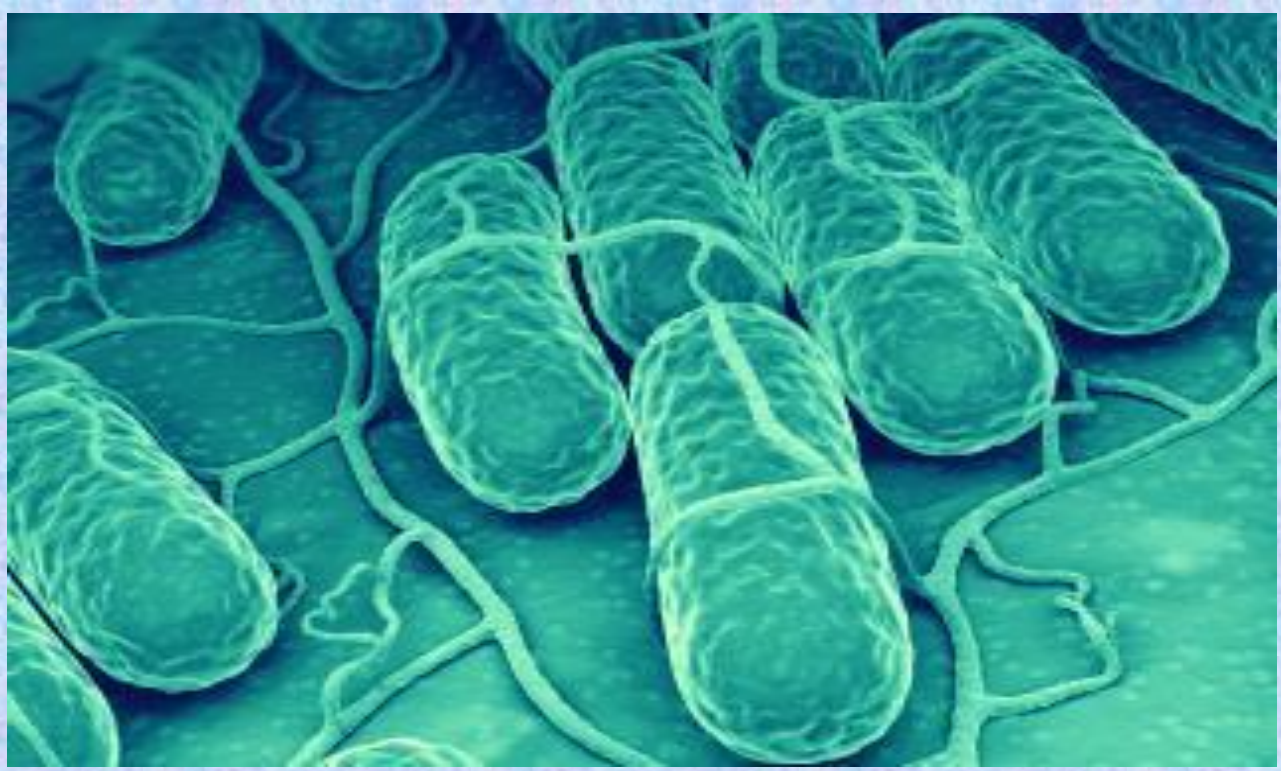


Figure 4.1: Possible rout of poultry *Salmonella* transmission from farm to kitchen.

In this study, all poultry cage samples from transport and bazar were positive for *Salmonella* contamination. The impact of unclean and recycled transport crates for chicken transportation has been well documented (Slader *et al.*, 2002). Studies in Malaysia have demonstrated that poultry processing areas are a significant source of cross-contamination of *Salmonella* between live birds and humans (Nidaullah *et al.*, 2017). The presence of *Salmonella* on processing board (60%) and knife (40%) in bazaar and kitchen samples suggest this pathogen have colonized on these contact surfaces possibly through formation of biofilms and thus able to survive even after cleaning as there were no standard cleaning or sanitization protocol observed in bazaar areas. Generally, the cleaning is carried out by hosing of loose soils such as dirt, blood, feather and cloth residues.

The presence of *Salmonella* in the kitchen is also alarming and should be of concern. The hotel samples found more prevalent (55%) than household samples (20%). The percentage of contamination of household utensils (10%) correlates with a cross sectional study conducted in UK for kitchen dishcloths (10%) (Parry *et al.*, 2005). But there is no previous report for specific household contamination in Bangladesh. Hopefully, the findings of this study will extend the concern in transmission of *Salmonella* and enlighten the control steps to minimize the cross contaminations.



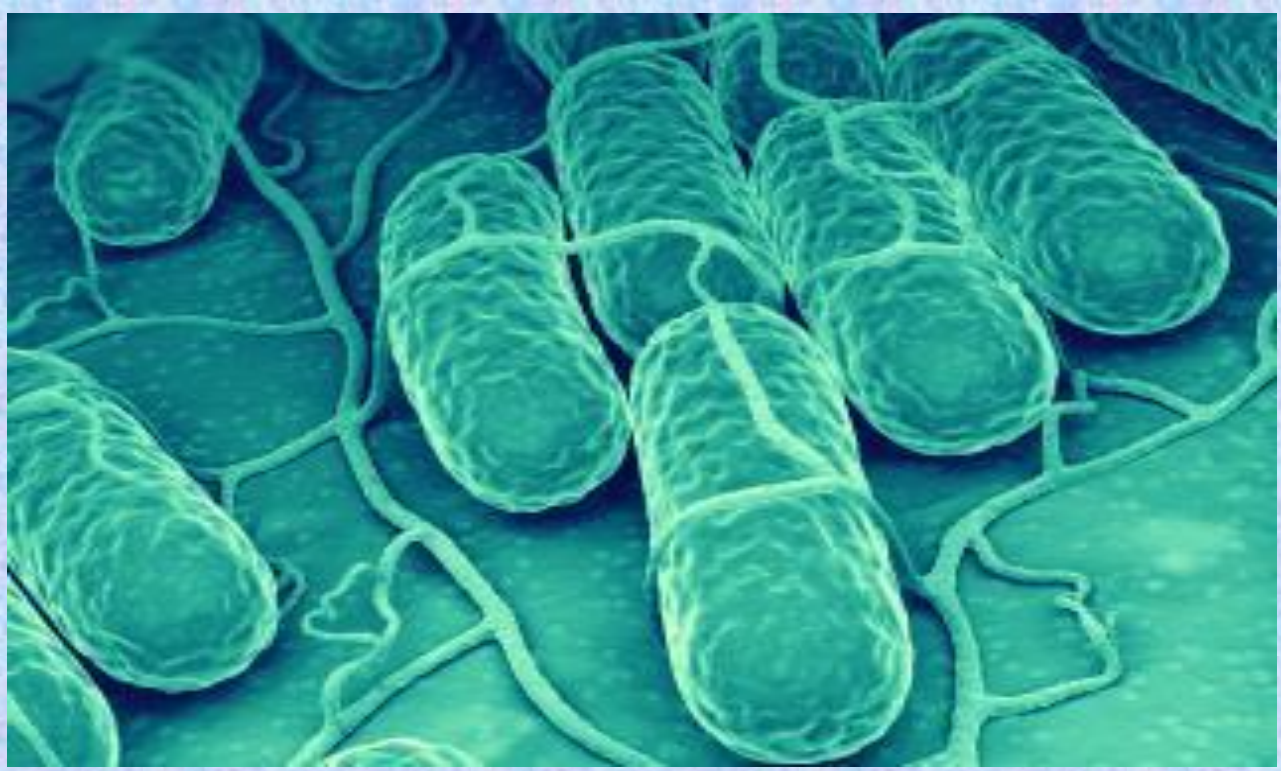
Chapter 05: Conclusion

5. Conclusion

Traditional routine diagnosis of *Salmonella* generally involves culture base identification up to species level tandem with serological methods. Further discrimination is achieved through antimicrobial resistance as well as phage typing for surveillance and epidemiological studies. Moreover, within the past two decades, molecular typing has significantly improved and is used to increase our understanding how pathogens transmit from farm animals to human. This thesis contributes to gain a better knowledge about the spread of *Salmonella* in human community; firstly, by evaluating the presence of non-typhoidal *Salmonella* and their genotypic variants, secondly, by establishing a rapid and sensitive detection and quantification method for raw poultry and food samples. The potential hazard for humans of certain genotypes was estimated by combining antibiotic resistance profiling, MLST typing and epidemiological data analysis.

The current study addressed 3 different MLST types including the novel one, *S. enterica* Litchfield ST214 from Bangladesh. A higher percentage of MDR pathogens have been identified as resistant to frequently prescribed antibiotics like, Penicillin, Ciprofloxacin, Tetracycline. Relatively higher prevalence of *Salmonella* in live chickens and its processing environment of local markets including kitchen areas indicate that poultry is undoubtedly a major potential source of human salmonellosis. Updated, rapid and efficient molecular method, like SYBR Green real time PCR could be the best choice for proper monitoring of *Salmonella* in both raw and processed foods. The continuation of monitoring research with wider area and more samples could reveal the circulating all MLST genotypes including their MDR properties and transmission frequencies.

The policy makers of livestock industry need to implement proper guidelines over the hygiene and sanitation practices in poultry farms, bazar, and hotel places. Periodical research with monitoring will help to minimize and eventually terminate the persistence and transmission of zoonotic *Salmonella* in poultry industry.



Chapter 06: References

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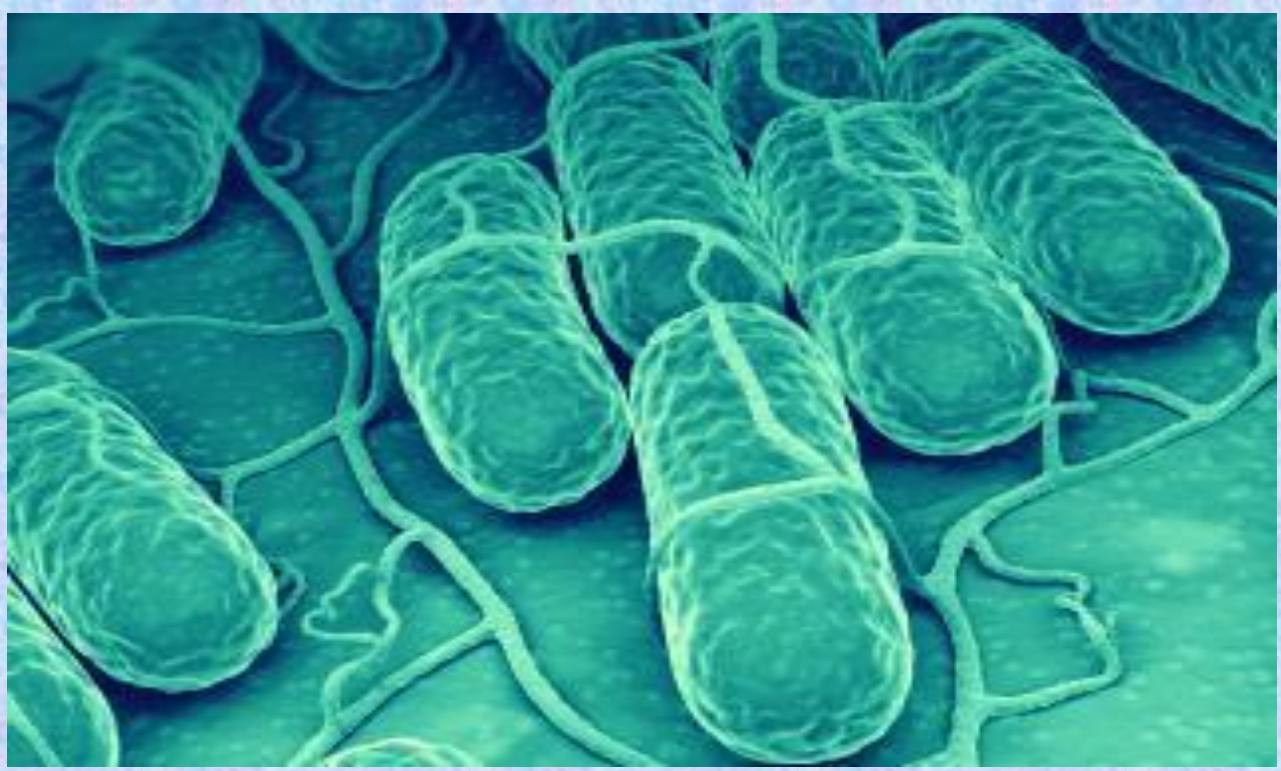

Appendices

Appendix-I

Unless otherwise mentioned, all media were sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this thesis have been given below:

Nutrient Agar (OXOID)

Ingredients	Amount (g/L)
Peptone	5.0
Sodium Chloride	5.0
Beef extract	3.0
Agar	15.0
pH	7.0

Luria Bertani Broth (ROTH)

Ingredients	Amount (g/L)
Trypton	10
Yeast Extract	5
NaCl	10
pH	7.0

Rappaport-Vassiliadis (RV) Enrichment Broth

Typical Formula (Classical)	gm/litre
Soya peptone	5.0
Sodium chloride	8.0
Potassium dihydrogen phosphate	1.6
Magnesium chloride $6H_2O$	40.0
Malachite green	0.04
pH 5.2 ± 0.2 @ 25°C	

Tetrathionate broth

Typical Formula	gm/litre
Casein peptone	2.5
Meat peptone	2.5
Bile salts	1.0
Calcium carbonate	10.0
Sodium thiosulphate	30.0

X.L.D Agar

Typical Formula	**gm/litre**
Yeast extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium desoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5
pH 7.4 ± 0.2 @ 25°C	

MacConkey Agar

Typical Formula	**gm/litre**
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH 7.4 ± 0.2	

Tryptic Soy Agar (OXOID)

Ingredients	Amount (g/L)
Pancreatic Digest of Casein	17.0
Papaic Digest of Soya Bean	3.0
Sodium Chloride	5.0
Dipotassium Hydrogen Phosphate	2.5
Glucose Monohydrate	2.5
Bacteriological Agar	1.5
pH	7.3

Tryptic Soy Broth (OXOID)

Ingredients	Amount (g/L)
Pancreatic Digest of Casein	17.0
Papaic Digest of Soya Bean	3.0
Sodium Chloride	5.0
Dipotassium Hydrogen Phosphate	2.5
Glucose Monohydrate	2.5
pH	7.3

Salmonella Shigella Agar

Typical Formula	gm/litre
`Lab-Lemco' powder	5.0
Peptone	5.0
Lactose	10.0
Bile salts	5.5
Sodium citrate	10.0
Sodium thiosulphate	8.5
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	12.0
pH	7.3

Appendix II

Solutions and Reagents used

Preparations of the stock solutions used in this work are given below: (all the working solutions used in this work were prepared from the stock solutions).

5 M NaCl

29.22 g of NaCl was dissolved in distilled water to a final volume of 100 ml. The solution was autoclaved and stored at room temperature.

1 M KCL

7.444 g of KCl was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M $MgCl_2$

20.33 g of $MgCl_2$ was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M $MgSO_4$

24.648 g of $MgSO_4$ was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

1 M glucose

19.817 g of Glucose was dissolved in deionized water to a final volume of 100 ml. The solution is sterilized by filter sterilization (0.22 µm filter).

0.5 M EDTA

186.1 g of $Na_2EDTA.2H_2O$ and 20.0 g of NaOH pellets were added and dissolved by stirring to 800 ml distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

3 M sodium acetate

40.81 g of $Na_2 (CH_3COOH).H_2O$ was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water and the solution was sterilized by autoclaving. It was stored at 4°C.

TAE buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was taken and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml distilled water and stored at room temperature.

Ethidium bromide solution

10 µl of ethidium bromide was dissolved in 100 ml TAE buffer to make a final concentration of 20 mg/ml and stored at 4°C in the dark.

Gel loading buffer

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04
Distilled water	Up to 1 L

Wizard® SV Gel and PCR Clean-Up System. Catalog No. A9282

Reagents	Purpose
Membrane Binding Solution	Help in binding of PCR product
SV Minicolumn	For Binding of PCR product
Collection Tube	For collection of flow throw
Membrane Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the purified DNA from the GD column
SYBR Green master mix	

Wizard®Plus SV Minipreps DNA Purification System. Catalog No.A1460 (For Plasmid DNA)

Reagents	Purpose
Cell Resuspension Solution	For the resuspension of pelleted cells
Cell Lysis Solution	For lysis of cells
Alkaline Protease Solution	For the degradation of cellular proteins
Neutralization Solution	For Nutralizaton of AlkilineProease
Spin Column	For Binding of the plasmid DNA molecules
Collection Tube	For collection of flothrow
Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the plasmid DNA from the GD column

Maxwell® 16 Total DNA Purification Kit

Reagents	
Maxwell® 16 RNA Cartridges	Maxwell® 16 RNA Cartridges
DNA Dilution Buffer	DNA Dilution Buffer
Nuclease-Free Water	Nuclease-Free Water
Clearing Columns	Clearing Columns
Plungers	Plungers

GoTaq® Hot Start Colorless Master Mix

GoTaq® Hot Start Colorless Master Mix (2×)
GoTaq® Hot Start Polymerase
dNTPs (400µM each)
2× Colorless GoTaq® Reaction Buffer (pH 8.5)
$MgCl_2$(4 mM)

Appendix III

Representative NCBI Sequences from each serotypes

***Salmonella enterica* subsp. enterica serovar Kentucky strain NR66 16S ribosomal RNA gene, partial sequence**

>MK720393.1 Salmonella enterica subsp. enterica serovar Kentucky strain NR66 16S ribosomal RNA gene, partial sequence

GTTCGTAACAAGGTAACCAGGAACCAGGGGTTAGTGCTGGGACGGGTGAGTAATGT
CTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCA
TAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCC
AGATGGGATTAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCT
GGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACCACGGTCCAGACTCCTACG
GGAGGCAGCCAGTGGGGAATATTGCCACAATGGGGGCAAGCCTTGATGCAAGCCAT
GCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGG
TGTTGTTGGTTAATAACCGCCAGCAATTGGACGTTACCCCGCAGAAGGAAGCACCG
GCTAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGT
AGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA
AGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGCC
CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGC
CGCAAGGTTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACTTCCACAGAACTT
TCCAGAGATGGAATTGGTGTCCTTCGGGAAACTGTGAGACAGGTGCTGCATGGCTGT
CGTCCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAT
CCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTG
GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACAC
GTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCAT
AAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG
CTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGC

***Salmonella enterica* subsp. enterica serovar Kentucky strain SV140 16S ribosomal RNA gene, partial sequence**

>MK720395.1 Salmonella enterica subsp. enterica serovar Kentucky strain SV140 16S ribosomal RNA gene, partial sequence

GTTCGTGACAGGTAACGAGGCACCAGGTGACTAGTGGCGGACGGGTGAGTAATGTC
TGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCAT
AACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCA
GATGGGATTAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTG
GTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCG
TGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGT
GGTTAATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGT
GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGG

AACTGCATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGGT
GTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT
GGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTTGAGGCGTGG
CTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA
ACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGCAACGCGAAGAACCTTACCTGGTTCTTGACATCCACAGAACTTTCCAGAGATGGA
ATGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTG
TGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGG
TTAGGTCCGGGAACTCAAAGGAAGACTGCCAGTGATAAACTGGAGGAAGGTGG
GGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATG
GCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCG
TAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGT
GGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 16S ribosomal RNA gene**

>MK720396.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 16S ribosomal RNA gene, partial sequence
GCCTGATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCGCA
AGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATT
AGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTTAGCTGGTTCTGAGG
AGGATGACCAGCCACACTGGAACTGGAGACACGGTCCCAGACTTCCTACGGGGAGG
GCAGCCAGTGGGGAACTATTGCACAATGGGCGGCAAGCCTGATGCAGCCATGCCG
GCGTGTATGAAGAAAGGCCTTCCGGGTTGTAAAAGTACTTTTCAGCGGGGGAGGAA
AGGTGTTGTTGGTTAATAACCGCCAGCAATTGGACGTTACCCCGCAGAAGGAAGCA
CCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGG
AATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCC
GGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGG
GTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTG
CCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACG
GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGGACATCCACAGAA
CTTTCCAGAGATGGACTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATC
CTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGG
AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACAC
ACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTC
ATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAAT
CGCTAG

***Salmonella enterica* subsp. enterica serovar Litchfield strain NR14 16S ribosomal RNA gene, partial sequence**

>MK720387.1 Salmonella enterica subsp. enterica serovar Litchfield strain NR14 16S ribosomal RNA gene, partial sequence

AGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT
GATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGAC
CAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTT
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAG
GCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGC
AGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCG
GTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG
CGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGA
AACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTG
ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAA
CGCGTTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAG
AACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGG
GAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAAC
TCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTC
ATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGA
AGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAG
TCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCAC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG

***Salmonella enterica* subsp. enterica serovar Enteritidis strain GZ32 16S ribosomal RNA gene, partial sequence**

>MK720380.1 Salmonella enterica subsp. enterica serovar Enteritidis strain GZ32 16S ribosomal RNA gene, partial sequence

GAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAA
CTGCCTGATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCG
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGA
TTAGCTTGTTGGTGAGGTAACGGCTCCACCAAGGCGACGATCCCTAGCTGGTCTGGA
GAGGATGACCAGCCACACTGGAACTGRGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTAT
GAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTA
ATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
CACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTG
CATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC
GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA

AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCG
GAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA
CGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGACTGGTG
CCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTAGGC
CGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACA
AAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGA
TTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAA
TGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
GGGTTGCAAAAGAAGTAGGTAG

***Salmonella enterica* subsp. enterica serovar Enteritidis strain NR20 16S ribosomal RNA gene, partial sequence**

>MK720388.1 Salmonella enterica subsp. enterica serovar Enteritidis strain NR20 16S ribosomal RNA gene, partial sequence
GCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG
ATGGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAACGTCGCAAGACC
AAAGAGGGGGACCTTCGGGCCTCTTGCCATCGAGTGCCCAGATGGGATTACTTGTTG
GTGAGGTAACGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAG
CCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTC
GGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAA
TTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTC
TGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACT
GGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
TAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGC
TCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCTACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCG
TTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCTGGTCTTGACATCCACAGAAGAATCCAGAGATGGATTTGTGCCTTCGGGA
ACTGTGAGACAGGTGCTGCATGGTCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACT
CAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA
TCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAA
GCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGT
CTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGC
AAAAGAAGTAGGTAGCTTAACC

NCBI Sequences of all seven housekeeping genes of GO66, MLST type ST214

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 AroC (aroC) gene, partial cds**

>MK732174.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 AroC (aroC) gene, partial cds
GTTTTTCGTCCGGGACACGCGGATTACACCTATGAGCAGAAATACGGCCTGCGCGAT
TACCGTGGCGGTGGACGTTCTTCCGCGCGTGAAACCGCGATGCGCGTAGCGGCAGG
GGCGATTGCCAAGAAATACCTGGCGGAAAAGTTCGGCATCGAAATCCGCGGCTGCC
TGACCCAGATGGGCGACATTCCGCTGGAGATTAAAGACTGGCGTCAGGTTGAGCTT
AATCCGTTCTTTTGTCCCGATGCGGACAAACTTGACGCGCTGGACGAACTGATGC
GCGCGCTGAAAAAAGAGGGTGACTCCATCGGCGCGAAAGTGACGGTGATGGCGAGC
GGCGTGCCGGCAGGGCTTGGCGAACCGGTATTTGACCGACTGGATGCGGACATCGC
CCATGCGCTGATGAGCATTAATGCGGTGAAAGGCGTGGAGATCGGCGAAGGATTTA
ACGTGGTGGCGCTGCGCGGCAGCCAGAATCGCGATGAAATCACGGCGCAGGGT

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 DnaN (dnaN) gene, partial cds**

>MK732192.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 DnaN (dnaN) gene, partial cds
ATGGAGATGGTCGCGCGCGTTACGCTTTCTCAGCCGCATGAGCCGGGTGCTACTACC
GTGCCGGCGCGGAAATTCTTTGATATCTGCCGCGGCCTGCCGGAGGGCGCGGAGATT
GCCGTTCAGTTGGAAGGCGATCGGATGCTGGTGCGTTCTGGCCGTAGCCGCTTCTCG
CTGTCCACGCTTCCTGCCGCCGATTTCCCGAATCTTGACGACTGGCAAAGCGAAGTT
GAATTTACGCTGCCGCAGGCCACGATGAAGCGCCTGATTGAAGCGACCCAGTTTTCG
ATGGCTCATCAGGATGTGCGCTATTACTTAAACGGTATGCTGTTTGAAACGGAAGGT
AGCGAACTGCGCACTGTCGCGACCGACGGCCACCGTCTGGCGGTGTGCTCAATGCC
GCTGGAAGCGTCTTTACCCAGCCACTCGGTGATTGTGCCGCGTAAAGGCGTGATTGA
ACTGATGCGTATGCTCGACGGCGGTGAAAACCCGCTGCGCGTGCAG

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 HemD (hemD) gene, partial cds**

>MK732210.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 HemD (hemD) gene, partial cds
GCGACGCTCGCGGAAAACGATCTGGTTTTTGCCCTTTCACAGCACGCTGTCGCCTTT
GCTCACGCCCAGCTCCAGCGGGATGGTCGAAACTGGCCTGCGTCGCCGCGCTATTTC
GCGATTGGCCGCACCACGGCGCTCGCCCTTCATACCGTTAGCGGGTTCGATATTCGT
TATCCATTGGATCGGGAAATCAGCGAAGCCTTGCTACAATTACCTGAATTACAAAAT
ATTGCGGGCAAACGCGCGCTGATTTTGCGTGGCAATGGCGGCCGCGAACTGCTGGG
CGAAACCCTGACAGCTCGCGGAGCCGAAGTCAGTTTTTGTGAATGTTATCAACGATG
TGCGAAACATTACGATGGCGCGGAAGAAGCGATGCGCTGGCATACTCGCGGCGTAA
CAACGCTTGTTGTTACCAGCGGCGAGATGTTGCAA

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 SucE (sucE) gene, partial cds**

>MK732228.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 SucE (sucE) gene, partial cds
AAACGCTTCCTGAACGAACTGACCGCCGCTGAAGGGCTGGAACGTTATCTGGGCGC
CAAATTCCCGGGTGCGAAACGTTTCTCGCTTGAGGGGGGAGATGCGCTGATACCCAT
GCTGAAAGAGATGGTTCGCCATGCGGGTAACAGCGGCACTCGCGAAGTGGTGCTGG
GGATGGCGCACCGCGGTCGCCTGAACGTGCTGATCAACGTACTGGGTAAAAAACCG
CAGGATCTGTTCGACGAGTTTGCCGGTAAACATAAAGAACATCTGGGTACCGGCGA
CGTGAAGTATCACATGGGCTTCTCGTCAGATATCGAAACCGAAGGCGGTCTGGTTCA
CCTGGCGCTGGCGTTTAACCCATCGCACCTGGAAATTGTGAGCCCGGTGGTGATGGG
CTCCGTGCGTGCCCGTCTGGACCGACTGGACGAACCGAGCAGCAACAAAGTGTTGC
CGATCACTATTCACGGCGACGCCGCGGTGACCGGCCAGGGCGTGGTTCAG

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 ThrA (thrA) gene, partial cds**

>MK732246.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 ThrA (thrA) gene, partial cds
GTGCTGGGGCGTAATGGCTCTGACTACTCTGCCGCCGTGCTGGCCGCCTGTTTACGC
GCTGACTGCTGTGAAATCTGGACTGACGTCGATGGCGTGTATACCTGTGACCCGCGC
CAGGTGCCGGACGCCAGGCTGCTGAAATCGATGTCCTACCAGGAAGCGATGGAACT
CTCTTACTTCGGCGCCAAAGTTCTTCACCCTCGCACCATTACGCCCATCGCCCAGTTC
CAGATCCCCTGTCTGATTAAAAATACCGGTAATCCGCAGGCGCCAGGAACGCTGAT
CGGCGCGTCCAGCGACGATGATAATCTGCCGGTCAAAGGGATCTCTAACCTTAACA
ACATGGCGATGTTTAGCGTCTCCGGCCCTGGAATGAAAGGGATGATTGGGATGGCG
GCGCGTGTTTTCGCCGCCATGTCTCGCGCCGGGATCTCGGTGGTGCTCATTACCCAG
TCCTCCTCTGAGTACAGCATCAGCTTCTGTGTGCCGCAGAGTGACTGC

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 PurE (purE) gene, partial cds**

>MK732264.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 PurE (purE) gene, partial cds
AGCGACTGGGCTACCATGCAATTCGCCGCCGAAATTTTTGAAATTCTGGATGTCCCG
CACCATGTAGAAGTGGTTTCCGCCCATCGCACCCCCGATAAACTGTTCAGCTTCGCC
GAAACGGCGGAAGAGAACGGATATCAAGTGATTATTGCCGGCGCGGGCGGCGCGGC
GCACCTGCCGGGAATGATTGCGGCAAAAACGCTGGTCCCGGTACTCGGCGTGCCGG
TACAAAGCGCTGCGCTAAGCGGCGTGGATAGCCTCTACTCCATTGTGCAGATGCCGC
GCGGCATTCCGGTGGGTACGCTGGCGATCGGTAAAGCCGGTGCCGCTAACGCCGCC
CTGCTCGCCGCGCAGATTCTGGCGCAACACGACGCGGAACTGCATCAGCGCATTGC
CGAC

***Salmonella enterica* subsp. enterica serovar Litchfield strain GO66 HisD (hisD) gene, partial cds**

>MK732282.1 Salmonella enterica subsp. enterica serovar Litchfield strain GO66 HisD (hisD) gene, partial cds
ATTGCGGGATGTCAGAACGTGGTTCTGTGCTCGCCGCCGCCCATCGCTGATGAAATC
CTCTATGCGGCGCAACTGTGTGGCGTGCAGGAAATCTTTAACGTCGGCGGCGCGCAG

GCGATTGCCGCTCTGGCCTTCGGCAGCGAGTCCGTACCGAAAGTGGATAAAATTTTT
GGTCCCGGCAACGCCTTTGTAACCGAAGCCAAGCGTCAGGTCAGCCAGCGCCTCGA
CGGCGCGGCTATCGATATGCCAGCCGGGCCGTCTGAAGTACTGGTGATCGCCGACA
GCGGCGCAACACCGGATTTCGTCGCGTCTGACCTGCTCTCCCAGGCTGAGCACGGTC
CGGATTCGCAGGTGATCCTGCTGACGCCTGATGCTGACATTGCCCGCAAGGTGGCGG
AGGCGGTAGAACGTCAACTGGCGGAACTGCCGCGCGCGGACACCGCCCGGCAGGCC
CTGAGCGCCAGTCGTCTGATTGTGACCAAAGATTTAGCGCAGTGCGTC

Appendix IV

The important instrument and apparatus used through the study are listed below:

Instruments	Origin
ABI Prism 3130 Genetic Analyzer	Applied Biosystem, USA
AlphaImager HP System Versatile Gel Imaging	Cell Bioscience, USA
Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Microcentrifuge (temperature controlled)	Sigma, USA
Class II Microbiological Safety Cabinet	Nuaire, USA
Electric balance, Scout, SC4010	Shimadzu, Japan
Freezer (-30°C)	Liebherr, Germany
Horizontal Gel Elctrophoresis Apparatus Hl-SET	CBS Scientific, UK
Incubator	Japan
Microcentrifuge	Mikro20, Germany
Microcentrifuge tube	Eppendorf, Germany
Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
NanoDrop 2000	Thermo Scientific, USA
Thermal Cycler	Biometra , Germany; Veriti 96 well Thermal Cycler, USA; ProFlex PCR System, USA
pH meter, Model no: MP220	Eppendorf, Germany
Power pack	Toledo, Germany
Refrigerator (4°C)	Vest frost
Room temperature horizontal shaker	Gerhardt, Germany
Sterilizer, Model no: NDS-600D	Japan
Water bath, Model:SUM	England
-80º C Freezer	Nuaire, USA
Maxwell^{R} 16 Instrument	Promega, USA
Real-time PCR system 7500	Applied Biosystems, Foster City, CA, USA

Appendix V

Questionnaire used during sample collection

Questionnaire for Poultry Sampling

Code No. Sample Type: Date:

1. Name:
2. Name of the District:
3. Name of the Village:
4. Poultry house?
5. Number of Chickens:
6. Other Poultry:
7. Other Animals:
8. Type of Chickens
   Cockerel (male, before puberty) / Cock (male, after puberty) / Pullet (female, before laying) / Layer (female, laying) / Roaster (Hybrid, 6-8 weeks old, used for fleshy meat, male or female)
9. Types of food:

10. What kind of problem do you face with the management of the poultry?
    Disease / feeding / theft / others
11. Mortality: (last 3 months)
    i. Number
    ii. Types of chickens
12. Vaccination:
13. Medication: (Antibiotics used-)
14. Markets:
15. What happens with the dead bird-
    Through away / Burry / Burn / Give someone else to eat / Eat / Nothing / Don't know
16. Education
17. Production Cycle per year
18. Other income source
19. Hygienic awareness: foot bath disinfectant, water disinfectant
20. Awareness about disease transmission