CHAPTER-1

INTRODUCTION

Among the bacterial diseases, Salmonellosis is one of the major problems in poultry industry causes high morbidity, mortality and production loss (Haider et al., 2009). Salmonella infection also remains public health concern worldwide with the hazard of transmitting food poisoning and gastroenteritis in human (Khan et al., 2007). Several environmental factors including air, dirty litter, feed, water and vectors, such as insects, humans, and rodents are also responsible for spreading Salmonella infection in poultry farms (Jones et al., 1991; Hoover et al., 1997; Amick-Morris, 1998). Antimicrobials are generally applied to treat diseases as well as growth promoter in poultry, exposing a large number of birds to frequently subtherapeutic concentrations (White et al., 2001), and leading to the development of antimicrobial resistant *Salmonella* sp that might subsequently be transferred to humans through the food chain. Recently, the emergence of antibiotic-resistant *Salmonella* sp has also led to the ineffective treatment of salmonellosis by several antibiotics e.g. ampicillin (Padungtod and Kaneene, 2006). Diverse virulence factors found in Salmonella species are required for host infection and disease transmission. Salmonella sp. that are multidrug resistant (MDR) may be transmitted from poultry to people at any point in the food chain due to their zoonotic nature. In recent years, the development of MDR among foodborne pathogens, such as *Salmonella* spp., have been associated with an increase in human mortality, and longtime hospitalization due to therapy failure (Mahmud et al., 2011). Therefore, utilizing cloacal swabs, we looked at certain resistance genes, including those that are in charge of multidrug resistance in broiler farms. Additionally, research into the pathological lesions in commercial poultry is vital for veterinary professionals to use for postmortem diagnosis and treatment.

This study will emphasize how important it is for governmental organizations, poultry researchers, and producers to identify strategies to lessen the effects of antibiotic usage in chicken, paying particular attention to active surveillance and the discovery of antibiotic substitutes.

So, our objectives were:

1. *Salmonella* sp. was identified, isolated, and characterized from broiler cloacal swab samples.
2. AMR profiling of Salmonella isolates.
3. Determination of antimicrobial resistant gene(s) in each isolate.
4. Study of the risk factors for salmonella infection.
5. Investigation of histopathological changes in Salmonella affected samples.

CHAPTER-2

REVIEW OF LITERATURE

2.1. An overview of Salmonella

2.1.1. Classification and nomenclature

Salmonella, a member of the family Enterobacteriaceae (Dunkley et al., 2009), is a Gram-negative, non-spore forming bacillus. The genus has only two genetically distinct species: *S. enterica* and *S. bongori* (Grimont and Weill, 2007). On the basis of biochemical reactions (Malorny et al., 2011) and susceptibility to lysis by bacteriophage Felix O1, 6 subspecies of *S. enterica* has been determined. These subspecies are designated by taxonomic names, and occasionally the serotypes are designated by Roman numbers.

Table 2.1 Taxonomic names of subspecies

Taxonomic names of subspecies	Roman Numeral
Salmonella enterica subsp. *enterica*	I
Salmonella enterica subsp. *salamae*	II
Salmonella enterica subsp. *arizonae*	IIIa
Salmonella enterica subsp. *diarizonae*	IIIb
Salmonella enterica subsp. *houtenae*	IV
Salmonella enterica subsp. *indica*	VI

2.1.2. Morphology

Salmonella organisms are facultative anaerobic gram-negative rods within the family Enterobacteriaceae (Yan et al., 2003). Generally, all members but *S. Pullorum* and *S. Gallinarum* of this genus are motile by peritrichous flagella though motility in *S. Pullorum* can be induced under special medium conditions (Desmidt et al., 1997). On agar media, typical Salmonella colonies are 2-4 mm in diameter, rounded with smooth edges, slightly elevated, and shiny. Colonies are pink or red and are surrounded by pink to red medium on Brilliant Green Agar (BGA). Colonies on Xylose-lysine-deoxycholate (XLD) agar are red with black cores. Colonies on Salmonella-Shigella

(SS) agar are normally colorless with black cores. On Rambach agar, Salmonella produce red colonies (Quinn et al., 2011).

2.1.3. Growth requirements

Salmonella grow optimally at 35^{o}C to 37^{o}C, catalyze a variety of carbohydrates into acid and gas, use citrate as the sole carbon source, produce H_2S and decarboxylate lysine and ornithine to cadaverine and putrescine, respectively (Whaley et al., 2000). Historically Salmonella catabolized glucose and lysine, but failed to metabolize lactose, sucrose and urea, however due to widespread exchange of genetic elements between compatible bacterial strains in environment, atypical Salmonella biotypes that cannot decarboxylate lysine or that readily use lactose (Falcao et al., 1975; Kohbata et al., 1983), sucrose (Johnson et al., 1976; Reid et al., 1993) and urea, have been isolated. They are chemo-organ trophic organisms having both a respiratory and a fermentative type of metabolism (Savalia, 2010).

2.1.4. Antigenic structure

Salmonella strain pathogenicity is determined by the flagellar, polysaccharide, and capsular antigens that are expressed; as a result, variation of these antigens has served as the basis for Salmonella serotyping. Till now, 57 O antigens and 117 H antigens have been identified and more than 2500 serotypes have been described (Mortimer et al., 2004). Antigen factors that are shared by some H antigens. These antigens are found in five complexes: the E, G, L, Z4, and I complex. There are various stages of Salmonella H antigen expression. Most serotypes are diphasic, i.e., they express two flagellar antigens, and a minor part are monophasic, i.e., express one flagellar antigen (Sonne Hansen et al., 2005).

2.1.5. Biochemical properties

Salmonella that causes typhoid do not hydrolyze urea or gelatin or generate indole. Most paratyphoid Salmonella, can be readily distinguished from the avian host-adapted serotypes, *S. Pullorum and S. Gallinarum* on the basis of the inability of *S. Pullorum* strains to ferment mucate or dulcitol and the inability of *S. Gallinarum* strains to decarboxylate ornithine or produce gas from glucose fermentation (Gast et al., 2011).

2.1.6. Transmission of Salmonella

There are several ways that salmonella might enter flocks of poultry. Feeds containing contaminated animal proteins, vegetable proteins, or cereals, or contaminated by vermin or wildlife, are potential sources of Salmonella in both chickens and turkeys (Rose et al., 1999; Davies and Wales, 2010; Danguy des Déserts et al., 2011), can be infected by very low levels of paratyphoid Salmonella in feed (Li et al., 2007). Salmonella contamination was found in 28% of finished food samples from commercial broiler farms (Alali et al., 2010). Meal or mash feeds are more often contaminated with salmonellae than pelleted feeds (Rose et al., 1999; Danguy des Déserts et al., 2011). Salmonella can survive for 2 years in inoculated feeds (Davies and Wray, 1996). The wide range of hosts results in numerous reservoirs of Salmonella infection for poultry.

2.1.7. The Factors Affecting Salmonella Colonization in Chickens

The following factors are known to influence Salmonella colonization: 1) chicken age, 2) physiological and environmental stressors (such as lack of food or water, abrupt temperature changes, etc.), 3) Salmonella's ability to survive through the gastric barrier, 4) the health and disease status of the chicken, 5) the use of antibiotics and/or coccidiostats, 6) diet, and 7) the genetic make-up of the chicks. Bacterial colonization and invasion are influenced by parameters specific to Salmonella and the effects of environmental stimuli (avian gastrointestinal tract) on gene expression (Dunkley et al., 2008). One of the most important factors is the age of the birds. Newly hatched chicks are most susceptible to Salmonella colonization because they lack mature gut microflora or feed in the alimentary tract (Snoeyenbos et al., 1978). While very low doses of Salmonella, as low as 10 cells, can readily infect 1-day-old chicks, the susceptibility of chicks to infection with Salmonella tends to decrease with age (Milner and Shaffer, 1952). Cox et al., (1990) found that 38% of intracloacally inoculated 1-day-old chicks could be colonized with as few as 2 Salmonella cells. The production of internally contaminated eggs by a hen who was not excreting *Salmonella Enteritidis* suggests that prolonged persistence in internal organs can occur at a low frequency, despite the fact that *Salmonella Enteritidis* was typically cleared from internal organs within 8 wks. post inoculation, according to the research. (Beal et al., 2005) determined that age and genetics affect the ability of chickens to resist Salmonella colonization. Competitive exclusion is one method used to assist prevent Salmonella colonization in

chicks, especially those without developed intestinal microbiota (CE). First reported by Nurmi and Rantala (Nurmi and Rantala, 1973), competitive exclusion (CE) as a treatment involves the oral administration of intestinal microflora from healthy, salmonellae-free adult chickens to newly hatched chicks. This CE intestinal microflora can either be defined (known bacterial strains) or undefined (a complex of unidentified bacterial strains from an adult chicken's intestinal tract), and it is utilized to speed up the maturation of the chick's gut. Salmonella colonization resistance is subsequently increased by both defined and undefined CE cultures. The concept behind the use of probiotics is similar to that of competitive exclusion with the distinction that probiotics are intended to enhance the functions of the existing microflora (Wagner, 2006; Wagner et al., 2009). A second factor that can affect colonization is the ability of Salmonella to survive the passage through the pH of the gastrointestinal tract. Natural infection occurs mainly through the oral route and, in poultry, Salmonella encounters the acidic (pH ~4.5 to 5) environment of the crop (Farner, 1942). Lactobacillus strains present in the crop assist in maintaining the low pH associated with the crop environment, but upon feed withdrawal, a decrease in the lactobacilli population causes the crop pH to increase to approximately pH 6.0 to 6.3 (Humphrey et al., 1993; Durant et al., 1999), providing a more suitable environment for survival of Salmonella. Salmonella needs to endure transit via the gizzard and proventriculus, both of which are acidic conditions. The pH of the proventricular content's changes to an acidic range (pH 2.0 to 4.0) around the 20th day of egg incubation. This is a sign that the proventricular glands are secreting significant amounts of hydrochloric acid, with the secretions actually starting between the 11th and 13th day of egg incubation in response to the embryo ingesting albumin. In an in vitro study, (Cox et al., 1972) reported a decreased survival rate for *Salmonella* spp. at pH 4.4 which corresponds to the proventriculus, with limited survival at pH 2.6 which is encountered in the gizzard. Finally, the pH of the small intestine (6.2) and large intestine (6.3) are closer to neutral and therefore more suited for Salmonella survival and proliferation in 3-week-old chickens (Jayne-Williams and Fuller, 1971). As with lactobacilli colonization, antimicrobial or anticoccidial feed additives may also influence Salmonella colonization by altering or reducing normal intestinal microflora (Cox et al., 2003). A chicken's susceptibility to Salmonella colonization can be increased by changes in the protective gut microbiota, regardless of what causes the change. A third factor associated with colonization includes both the dose and strain of

Salmonella to which the chickens are exposed (Milner and Shaffer, 1952; Sadler et al., 1969), including the ability of the strain to attach, colonize, and invade the various intestinal tissues (Daoust et al., 1991). Higher levels (104 to 105 cfu) of Salmonella are more likely to colonize chickens, and some Salmonella serotypes can colonize the avian intestinal tract more efficiently at lower levels than others (Barrow et al., 1988). However, Salmonella must first attach themselves to the host epithelial cells to initiate the processes of colonization and invasion (Finiay and Falkow, 1989; Khan et al., 2003). Attachment is mediated by cell surface proteins known as adhesins, with the *Salmonella enterica* serovars possessing several fimbrial and nonfimbrial adhesins that are capable of binding to intestinal epithelial cells (Korhonen, 2007). The Salmonella Pathogenicity Island (SPI) 1 (discrete genetic units) contributes to colonization of the chicken with Salmonella, while SPI2, in the absence of SPI1, inhibits colonization (Dieye et al., 2009). Salmonella invasion is mediated by genes located on SPI1 (Bohez et al., 2006). Several studies have shown that mutations in these SPI1-specific genes can affect the intestinal colonization of young chicks (Porter and Curtiss III, 1997; Turner et al., 1998; Morgan et al., 2004). Rabsch et al., (2000), Callaway et al., (2008) and Foley et al., (2011) all analyzed epidemiological data collected through surveillance studies from the last half of the 20th century in the United States and Europe to explain the reduction of host specific Salmonella, specifically *Salmonella Gallinarum* and *Salmonella Pullorum*, in poultry production. These 3 studies support the theory that the increase in the prevalence of *Salmonella Enteritidis* and other nonhost-specific Salmonella serotypes in poultry and poultry products might be the result of the reduction and/or elimination of the host-specific Salmonella serovar Gallinarum which includes the 2 biovars, Gallinarum and Pullorum. Rabsch et al., (2000) proposed that the increase in prevalence of *Salmonella Enteritidis* was a result of the industry's actions which resulted in the reduction in the prevalence of *Salmonella Gallinarum* and *Salmonella Pullorum*. Since *Salmonella Gallinarum* has no animal reservoirs other than domestic and aquatic fowl, the eradication left a niche which was filled by nonhost specific Salmonella serovars; Heidelberg, Typhimurium, and Enteritidis in particular (Foley et al., 2011). Thomson et al., (2008) sequenced the genomes of *Salmonella Enteritidis* PT4 isolate P125109, a host-promiscuous serovar, and *Salmonella Gallinarum* isolate 287/91, a chicken-restricted serovar. Genomic comparisons between these 2 genomes indicate that *Salmonella Gallinarum* 287/91 is highly related

to and likely a direct descendent of *Salmonella Enteritidis*, which has undergone extensive degradation through deletion and pseudogene formation, which might explain the increase in Salmonella Enteritidis colonization of chickens following the reduction and/or elimination of *Salmonella Gallinarum* in the poultry industry (Thomson et al., 2008). Other studies looking at the competition between Salmonella serotypes in the gut of broiler chicks are almost nonexistent. (Nógrády et al., 2003)examined the growth suppression of *Salmonella Hadar*, in vitro under strict anaerobiosis and in vivo in the intestine of 1-day-old chicks. Four strains were selected for evaluation of their ability to suppress the growth of *Salmonella Enteritidis*, Typhimurium, (Nógrády et al., 2003) were able to show that precolonization of the chicken with *Salmonella Hadar* prevented the super-infection with any of the 4 mentioned serotypes. Ngwai et al., (2006) looked at the in vitro growth suppression of antibiotic-resistant *Salmonella Typhimurium* DT-104 by non-DT104 strains. When the antibiotic-resistant DT104 strain was given sparingly to 24-hour cultures of the non-DT104 strains, the non-DT104 strains were able to stop the DT104 strain from spreading. The consequence is that one Salmonella serotype may be able to stop another Salmonella serotype from colonizing.

2.2. Salmonella infections in layer chicken

Salmonella serotypes from sources of poultry vary in their regional and temporal distribution, despite the fact that many of them are consistently present at high incidence. Only a small subset of the known Salmonella serotypes linked to Para typhoid have ever been found in chicken—roughly 10%—and only a small subset of these is common. Among clinical and environmental isolates submitted in the United States in 2009, the most frequently identified Para typhoid serotypes were *S. Enteritidis, S. Kentucky, S. Heidelberg, S. Senftenberg, S. Sofia* (Mellor *et al*., 2010) and *S. Mbandaka* in chickens and *S. Senftenberg, S. Hadar, S. Worthington, S. Muenster*, and *S. Saintpaul* in turkeys. Salmonella reservoirs in poultry and humans share substantial epidemiological connections, which can sometimes be shown in comparable serotype distributions. The unique epidemiological association of *S. Enteritidis* with disease transmission via contaminated eggs (CDC, 2011) has made the prevalence of this serotype a topic of special interest. *S. Enteritidis* is often the most common serotype found in surveys of egg-producing chickens in many nations (Rousi et al., 2010), and has been reported as the most common serotype present in even when other serotypes are predominant in associated laying flocks (Otomo et al., 2007).

2.3. Salmonella infections in broiler chicken

The transmission of salmonellae among broiler chickens has been demonstrated in studies conducted worldwide (Byrd et al., 1998; Liljebjelke et al., 2005; Thomas et al., 2009; De Vylder et al., 2011; Roll et al., 2011) found that after colonizing a minimum of 5 chicks per treatment pen with as few as 102 cfu/chick of Salmonella, *Salmonella Typhimurium* approximately 57% of the remaining birds became colonized with log10 2.2 cfu *S. Typhimurium* per gram of cecal contents by d 17 of grow-out. This population of salmonellae in the ceca increased when the seeder chicks were orally gavaged with larger concentrations of *Salmonella Typhimurium* (Liljebjelke et al., 2005) also recovered *Salmonella Typhimurium* from litter samples at d 17, which indicates the potential for horizontal transmission of salmonellae from seeder chicks to contact chicks through the litter (Liljebjelke et al., 2005) recovered *Salmonella enterica* from 2 integrated poultry systems over 7 consecutive flocks isolating 15 different serotypes. *Salmonella Typhimurium* and Enteritidis isolates, respectively, from poultry carcasses shared the same PFGE pattern as those isolated from the rearing environment and from

rodents caught in the same house implicating horizontal transmission as one means of spread of these Salmonella serotypes (Liljebjelke et al., 2005). However, indistinguishable PFGE types of *Salmonella Typhimurium*, *Enteritidis*, and *Heidelberg* were isolated from carcasses, the broiler chicken environment and chick-box liners which also implicate the hatchery as a source for these persistent serotypes on this farm (Liljebjelke et al., 2005).

2.4. The Overall Prevalence of Salmonella in poultry

Poultry appears to be a general reservoir of Salmonella (Hoque et al., 2019). An elevated amount of salmonella contamination is hazardous to both human health and chicken production. Since the egg surface may have been contaminated with Salmonella via excrement during lay in an unsanitary environment from tainted fowl, the average Salmonella content is 18.09% (Mahbub et al., 2011; Mahmud et al., 2013; Paul et al., 2017; Hossain et al., 2019). In this review, we found that 17.19%, 28.57% and 30% of salmonellae were present in water, transport swab and air samples from poultry farm environments in Bangladesh (Jahan et al., 2013; Md et al., 2017; Hossain et al., 2020; Mridha et al., 2020). This study also observed that average 26.30% of the cloacal swab samples, 42% of the visceral organ samples and 60% of intestinal fluid samples were infected with Salmonella (Akond et al., 2012; Paul et al., 2017; Hoque et al., 2019; Alam et al., 2020; Sarker et al., 2021; Uddin et al., 2021). Salmonella infection in poultry farm samples has been documented from many regions of the world, with rates of 17%, 35%, 36%, 39% and 53% in the United States, Spain, Korea, Brazil and Vietnam, respectively (Forshell and Wierup, 2006; Lu et al., 2011). Salmonella was found in 23.44% of poultry handlers, indicating a possible breakdown in personal hygiene during bird handling and shipment of chicken products (Akond et al., 2012; Paul et al., 2017; Hossain et al., 2020; Mridha et al., 2020) . Poultry droplets and litters in various chicken farms in Bangladesh were found to contain an average of 26% and 25.71% Salmonella, respectively (Parvej et al., 2016; Alam et al., 2020; Hossain et al., 2020). Commercial poultry feed should be free from Salmonella but average 18.75% Salmonella was found within poultry feeds in different farms due to accidental contamination with feces or litter (Md et al., 2017; Alam et al., 2020; Hossain et al., 2020). A significant source of bacterial contamination of chicken feeds among the sources of animal protein and regular components of poultry feed was locally processed

fish by-products. Salmonella has also been found in feed and feeding materials of poultry and animals as a natural microflora (Fallon and Whittlestone, 1969).

2.5.1 The Antimicrobial Resistance Pattern of Salmonella Infection

Similarly it was determined that through oral and intracloacal inoculation, the number of cells required for a colonizing dose50 was 100 times fewer than that of 3-day-old chicks that had been fed (Gast and Holt, 1998) challenged 1-day-old chicks to evaluate the persistence of *Salmonella Enteritidis* through maturity (24 wks. age) and demonstrated that although Salmonella Enteritidis was usually cleared from internal organs within 8 wk. post inoculation, the production of internally contaminated eggs by a hen that was not shedding *Salmonella Enteritidis* in her feces suggest that extended persistence in internal organs can occur at a low frequency (Beal et al., 2005) determined that age and genetics affect the ability of chickens to resist Salmonella colonization. Competitive exclusion is one method used to assist prevent Salmonella colonization in chicks, especially those without developed intestinal microbiota (CE). First reported by (Nurmi and Rantala, 1973), CE as a treatment involves the oral administration of intestinal microflora from healthy, salmonellae-free adult chickens to newly hatched chicks. This CE intestinal microflora can either be defined (known bacterial strains) or undefined (a complex of unknown bacterial strains from an adult chicken's intestinal tract), and it is used to speed up the maturation of the chick's gut. Following Salmonella colonization, both defined and undefined CE cultures boost resistance. The concept behind the use of probiotics is similar to that of competitive exclusion with the distinction that probiotics are intended to enhance the functions of the existing microflora (Wagner, 2006; Wagner et al., 2009). The capacity of Salmonella to survive passage through the pH of the gastrointestinal system is a second element that may have an impact on colonization. Natural infection occurs mainly through the oral route and, in poultry, Salmonella encounters the acidic (pH ~4.5 to 5) environment of the crop (Farner, 1942). Lactobacillus strains present in the crop assist in maintaining the low pH associated with the crop environment, but upon feed withdrawal, a decrease in the lactobacilli population causes the crop pH to increase to approximately pH 6.0 to 6.3 (Farner, 1942; Humphrey et al., 1993), providing a more suitable environment for survival of Salmonella. Salmonella needs to endure transit via the gizzard and proventriculus, both of which are acidic conditions. The pH of the proventricular contents becomes acidic (pH 2.0 to 4.0) about the 20th d of egg

incubation and is indicative of the considerable secretion of hydrochloric acid by the proventricular glands with the actual onset of secretions beginning between d 11 and 13 of egg incubation in response to the ingestion of albumin by the embryo (Clench and Mathias, 1995). In an in vitro study, (Cox et al., 1972) reported a decreased survival rate for *Salmonella* spp. at pH 4.4 which corresponds to the proventriculus, with limited survival at pH 2.6 which is encountered in the gizzard. Finally, the pH of the small intestine (6.2) and large intestine (6.3) are closer to neutral and therefore more suited for Salmonella survival and proliferation in 3-week-old chickens (Jayne-Williams and Fuller, 1971). As with lactobacilli colonization, antimicrobial or anticoccidial feed additives may also influence Salmonella colonization by altering or reducing normal intestinal microflora (Cox et al., 2003). A chicken can become more vulnerable to Salmonella colonization due to changes in the protective gut microbiota, regardless of what causes the change. A third factor associated with colonization includes both the dose and strain of Salmonella to which the chickens are exposed (Milner and Shaffer, 1952; Sadler et al., 1969), including the ability of the strain to attach, colonize, and invade the various intestinal tissues (Daoust et al., 1991). Higher levels (104 to 105 cfu) of Salmonella are more likely to colonize chickens, and some Salmonella serotypes can colonize the avian intestinal tract more efficiently at lower levels than others (Barrow et al., 1988). However, Salmonella must first attach themselves to the host epithelial cells to initiate the processes of colonization and invasion (Finiay and Falkow, 1989; Khan et al., 2003). Attachment is mediated by cell surface proteins known as adhesins, with the *Salmonella enterica* serovars possessing several fimbrial and nonfimbrial adhesins that are capable of binding to intestinal epithelial cells (Korhonen, 2007). The Salmonella Pathogenicity Island (SPI) 1 (discrete genetic units) contributes to colonization of the chicken with Salmonella, while SPI2, in the absence of SPI1, inhibits colonization (Dieye et al., 2009). Salmonella invasion is mediated by genes located on SPI1 (Bohez et al., 2006). Several studies have shown that mutations in these SPI1-specific genes can affect the intestinal colonization of young chicks (Porter and Curtiss III, 1997; Turner et al., 1998; Morgan et al., 2004). Rabsch et al., (2000); Callaway et al., (2000) and Foley et al., (2011) all analyzed epidemiological data collected through surveillance studies from the last half of the 20th century in the United States and Europe to explain the reduction of host specific Salmonella, specifically *Salmonella Gallinarum* and *Salmonella Pullorum*, in poultry production. These 3

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(Nógrády et al., 2003) examined the growth suppression of *Salmonella Hadar*, in vitro under strict anaerobiosis and in vivo in the intestine of 1-day-old chicks. Four strains were selected for evaluation of their ability to suppress the growth of *Salmonella Enteritidis*, *Typhimurium*, Virchow, and Saintpaul. Nogrady et al., (2003) were able to show that precolonization of the chicken with *Salmonella Hadar* prevented the super-infection with any of the 4 mentioned serotypes. Ngwai et al., (2006) looked at the in vitro growth suppression of antibiotic-resistant *Salmonella Typhimurium* DT-104 by non-DT104 strains. When the antibiotic-resistant DT104 strain was given sparingly to 24-hour cultures of the non-DT104 strains, the non-DT104 strains were able to stop the DT104 strain from spreading. The consequence is that one Salmonella serotype may be able to stop another Salmonella serotype from colonizing.

2.5.2. The Resistance to Penicillin

Penicillins are antimicrobials that are classified as β-lactams (Hoque et al., 2019). Ampicillin, amoxicillin, oxacillin and cloxacillin are broadly utilized semi-synthetic antimicrobials within the penicillin class. Akond et al., (2012) reported 100% penicillin-resistant and 88% ampicillin-resistant Salmonella in hand wash, intestinal fluid, cloacal swab, egg surface and soil samples from a layer farm in Dhaka. Sarkar et al., (2021) reported similar results ten years later, both in broilers and layers in Rajshahi. Ampicillin was the first broad-spectrum antibiotic of the penicillin group. Ahmed et al., (2008) reported that 87.50% of Salmonella exhibited resistance to ampicillin and amoxicillin in egg surface samples from laying hens at different markets in Dhaka city. Additionally, Mahmud et al., (2013) and Talukder et al., (2021) observed 100% resistance to ampicillin and amoxicillin in Chittagong and Mymensingh. Furthermore, Salmonella was found to be 40% to 92.86% resistant to ampicillin and amoxicillin in broilers and layers (Pui et al., 2011; Rahman et al., 2018; Hossain et al., 2019; Haque et al., 2021). Jahan et al., (2013) detected 100% ampicillin- and amoxicillin-resistant Salmonella from dressed broilers, water and device surface samples, whereas Alam et al., (2020) and Hossain et al., (2020) reported 66.67% to 82.85% ampicillin-resistant Salmonella in cloacal, fecal, litter, feed, water, air and hand washing samples collected from different broiler farms in Mymensingh. Previously, Mir et al., (2015) showed that 100% Salmonella were resistant to penicillin and oxacillin, and Sharma et al., (2019) found 95.71% ampicillin-resistant Salmonella from poultry samples in India. There are several genes that have been associated to penicillin resistance. A study in Bangladesh has recently confirmed the presence of the β-lactam-resistant *blaTEM* gene in 73.30%, 63.60% and 50% of S. Typhimurium isolates from broilers, sonali and indigenous chickens, respectively (Siddiky et al., 2021). Parvin et al., (2020) also detected the *blaTEM-1*-resistant gene of Salmonella from chicken in Bangladesh. Previously, Alam et al., (2020) detected the *blaTEM-1* (82.85%) gene in Salmonella from broiler samples in Bangladesh. Likely, in Egypt, Sabry et al., (2020) reported the β-lactam-resistant *blaTEM* gene from healthy and diseased chickens. Earlier, Wajid et al., (2019) detected the *blaTEM-1* (72.70%) gene in Salmonella from the layers in Pakistan. In addition, Giuriatti et al., (2017) detected the *blaTEM-1* (83.33%) gene from chickens in Brazil. Similarly, the *blaTEM-1*-resistant gene of Salmonella from poultry was detected in Brazil and China by Souza et al., (2020) and Wang et al., (2017),

respectively. Therefore, prolonged usage of these antibiotics may be related to Salmonella exhibiting increased resistance patterns to the penicillin family of antibiotics in chicken.

2.5.3. The Resistance to Cephalosporins

Cephalosporins are also applied to poultry in Bangladesh. Salmonella was shown to be somewhat resistant to the first-generation cephalosporins, cephalexin. For example, in Dhaka city, Salmonella isolated from egg surface, hand wash, cloacal swab, intestinal fluid and soil samples were found about 50% to 65% resistant to cephalexin (Mahbub et al., 2011; Akond et al., 2012). Similarly, Akond et al., (2012) in Dhaka and Chaudhary et al., (2019) in Chittagong observed 50.00% to 96.44% resistance of Salmonella to ceftriaxone and cefixime since they are used as the third generation of cephalosporins. Dutil et al., (2010) and Jeon et al., (2019) recorded ceftiofur-resistant Salmonella from poultry meat in Canada and Korea, respectively. The use of ceftiofur (a third-generation cephalosporin) in farm animals has severe public health concerns since it leads to resistance to extended-spectrum cephalosporins such as ceftriaxone and cephamycin (Dunne et al., 2000). These results highlight the need for an improved monitoring system and policies for the responsible use of antimicrobial drugs in Bangladesh's poultry industry.

2.5.4. The Resistance to Carbapenems

Antibiotics of the carbapenem class include ertapenem, imipenem, and meropenem. Imipenem has a broad spectrum of effects on both aerobic and anaerobic bacteria. Parvin et al., (2020) reported 48.60% resistance to imipenem in Salmonella isolates from chicken frozen meat. Tawyabur et al., (2020) also observed 40.74% resistance of meropenem in healthy and diseased turkeys. These findings demonstrate that we must be concerned since antibiotics from carbapenem group are frequently used as “last-line agents” to cure diseases caused by MDR Gram-negative bacteria (Zhanel et al., 2007; Nordmann et al., 2011; Patel and Bonomo, 2013). Earlier, Wajid et al., (2019) also reported resistance of S. Typhimurium for imipenem (79.40%), doripenem (61.70%), and meropenem (54.50%) in poultry in Pakistan. Carbapenems are typically regarded as last-resort antimicrobials for the treatment of hospitalized patients with various bacterial infections. Since these antimicrobials are not permitted for use in the poultry sector, it is unclear how this kind of resistance has spread to chickens. It is crucial to

establish quality control and confirmation methods for the poultry processing and production industry since higher incidences of carbapenem resistance in chicken are quite concerning.

2.5.5. The Resistance to Fluroquinolones

There is no end to the uses of the class of antibiotics known as fluoroquinolones. Fluoroquinolone antimicrobials like ciprofloxacin are frequently used to treat a variety of infections in people, poultry, and other animals. As a result, Salmonella isolated from broilers, layers and turkeys showed periodical increase in resistance to ciprofloxacin, ranging from 20% to 100% (Akond et al., 2012; Mahmud et al., 2013; Aditya, 2015; Chaudhary et al., 2019; Tawyabur et al., 2020; Siddiky et al., 2021; Uddin et al., 2021) in different districts of Bangladesh in between 2012 to 2021. The scenario is similar in neighboring countries. (Hassan et al., 2014) revealed 87.50% resistance of Salmonella to pefloxacin in layer chickens, whereas Parvin et al., (2020) reported 70.30% resistance in the broilers. Sharma et al., (2019) observed 82.86% resistance of Salmonella to ciprofloxacin in chickens in India. Similarly, in Pakistan, 92.60% of S. Typhimurium and 100% of S. Enteritidis were resistant to pefloxacin in poultry birds (Wajid et al., 2019). Furthermore, 60% of S. Typhimurium and 65.85% S. Enteritidis showed resistance in layers in Chittagong, Gazipur, Narsingdi, Tangail, and Brahmanbaria (Mahmud et al., 2013; Uddin et al., 2021). Nalidixic acid (NA) is the first of the synthetic quinolone antibiotics. Various degrees of resistance found against NA have been reported in Salmonella in Bangladesh. About 20% to 100% resistance found in Salmonella to NA secluded from poultry and environmental samples at a different region of Bangladesh (Porter and Curtiss III, 1997; Ahmed et al., 2008; Akond et al., 2012; Jahan et al., 2013; Karim et al., 2020; Parvin et al., 2020; Sarker et al., 2021). Early, Nikolic et al., (2017) observed 95.50% resistance of Salmonella to NA in broiler isolates in Serbia. These findings emphasize the need for the implementation of surveillance systems that focus on food cleanliness, employ antimicrobials in chicken production, and regularly assess the quality of retail meat products.

2.5.6. The Resistance to Aminoglycosides

Antimicrobial aminoglycosides prevent bacteria from producing proteins. One of the most common aminoglycoside antibiotics used in human medicine is streptomycin. Salmonella resistance to streptomycin has been documented in chicken in Bangladesh, ranging from 38% to 100% (Jahan et al., 2013; Al-Salauddin et al., 2015; Md, 2018; Alam et al., 2020; Siddiky et al., 2021). Similarly, Souza et al., (2020) reported 98.30% resistance in Salmonella to streptomycin from poultry in Brazil. Bangladesh has long utilized gentamicin, a broad-spectrum aminoglycoside antibiotic, to treat both Gram-negative and Gram-positive bacteria in chicken. Extremely recently, Siddiky et al., (2021) reported 86.70% resistance in Salmonella to gentamicin in the broilers, sonali, and indigenous chickens in Bangladesh. Previously, Wajid et al., (2019) observed 64.70% resistance to gentamicin from S. Typhimurium isolates in poultry in Pakistan. Earlier, Hasan et al., (2014) and Paul et al., (2017) also observed significant amount of resistance to other aminoglycosides in Salmonella such as kanamycin in the layers. Alam et al., (2020) reported the aminoglycoside-resistant gene aadA1 (77.10%) in Salmonella isolates from cloacal swabs and a litter of broilers in Mymensingh. Siddiky et al., (2021) observed the strA/B (33.33%) resistance gene in S. Typhimurium isolates from broilers ceca at wet markets in Dhaka. Earlier, Wajid et al., (2019) reported aminoglycosides aadA1 (35.20%), strA (20.50%) and strB (41.10%) resistance genes, respectively, in S. Typhimurium from poultry in Pakistan.

2.5.7. The Resistance to Macrolides

Macrolides are bacteriostatic, which means that instead of killing bacteria, they limit or restrain their growth (Giguère et al., 2013). Azithromycin is an azalide, a sort of macrolide antibiotic. Salmonella in Bangladeshi poultry has been found to have varying degrees of azithromycin resistance, ranging from 18.18% to 81.25% (Jahan et al., 2013; Sultana et al., 2014; Md et al., 2017; Md, 2018; Rahman et al., 2018; Karim et al., 2020; Mridha et al., 2020; Haque et al., 2021). Last year, Tirziu et al., (2020) also reported 88.20% resistance in Salmonella to azithromycin was isolated from store raw poultry in Romania. In Bangladesh, erythromycin is frequently used to treat a variety of chicken ailments. About 62.50% to 100.00% resistance found in Salmonella to erythromycin in layer samples (Mahbub et al., 2011; Akond et al., 2012; Mahmud et al., 2013; Paul et al., 2017), while 64.28% to 100.00% resistance observed (Al-Abadi and Al-Mayah,

2011; Jahan et al., 2013; Aditya, 2015; Md et al., 2017; Md, 2018; Rahman et al., 2018; Mridha et al., 2020) where as in case of broiler samples. Cardoso et al., (2006) in Brazil and (Sharma et al., 2019) in India also reported 100% resistance of avian Salmonella to erythromycin. Salmonella's increased sensitivity to macrolides is not unusual given the prevalence of Enterobacteriaceae members who are susceptible to them.

2.5.8. The Resistance to Lincosamides

A number of bacterial illnesses can be treated with the antibiotic clindamycin. It belongs to the lincosamides family and operates by preventing bacteria from producing protein (Hossain et al., 2021). Sultan et al., (2014) reported 84% resistance in Salmonella to clindamycin in poultry in Bangladesh. Similarly, Yildirim et al., (2011) in Turkey and Mir et al., (2015) in India detected 97% and 100% resistance in Salmonella isolated from poultry as resistance to clindamycin, respectively. Therefore, it is essential to have stringent supervision over the use of antimicrobials, particularly in the poultry sector. Examining the non-judicial use of antibiotics requires appropriate rational and transparent health controls.

2.5.9. The Resistance to Tetracyclines

Tetracycline is one of the antibiotics that is frequently used in veterinary medicine. Salmonella to tetracycline in chicken in Bangladesh has shown varying degrees of tetracycline resistance. Several studies had reported about 65% to100% resistance in Salmonella to tetracycline and oxytetracycline in layers and broilers in Bangladesh (Jahan et al., 2013; Mahmud et al., 2013; Hassan et al., 2014; Al-Salauddin et al., 2015; Md et al., 2017; Paul et al., 2017; Rahman et al., 2018; Alam et al., 2020; Karim et al., 2020; Mridha et al., 2020; Parvin et al., 2020; Tawyabur et al., 2020; Siddiky et al., 2021; Talukder et al., 2021). Recently, Alam et al., (2020) and Tawyabur et al., (2020) detected tetracycline-resistant phenotype and the tetracycline-resistant gene *tetA* in Salmonella in poultry in Bangladesh. More specifically, recently (Siddiky et al., 2021) identified tetracycline *tetA* gene 80%, 90.90% and 100% S. Typhimurium isolates of broilers, sonali, and indigenous chickens' ceca, respectively, in Bangladesh. Earlier, Sharif et al., (2009) observed 100% resistance to tetracycline and also detected the *tetA*-resistant gene in Salmonella in India. The antibiotic doxycycline belongs to the broad-spectrum tetracycline class and is frequently prescribed to treat many illnesses in both humans and animals. A significant number of isolates resistant to doxycycline (50.00%

to 79.31%) has also been reported in Salmonella in poultry in Bangladesh (Mahbub et al., 2011; Sultana et al., 2014; Rahman et al., 2018; Haque et al., 2021). Formerly, Waghamare et al., (2018) also observed 100% resistance in Salmonella to doxycycline in India. Salmonella from the environment of poultry has a higher probability of tetracycline resistance detection, which poses a risk to both animals and people. Salmonella's capacity for resistance may allow it to enter the food chain, posing a serious risk to human life. AMR reconnaissance techniques should be used to limit the emergence of bacterial resistance in chicken farms in Bangladesh and other countries.

2.5.10. The Resistance to Phenicols

A broad-spectrum antibiotic called chloramphenicol is not currently in use because it is illegal due to its adverse effects on the host. However, it has long been used to treat numeric types of bacterial maladies in both individuals and animals (Hossain et al., 2021). Studies carried out throughout 2012 to 2021 have reported variable degree (20% to 58%) resistance in Salmonella to chloramphenicol in layer birds in Bangladesh (Mahbub et al., 2011; Paul et al., 2017; Hossain et al., 2019; Hossain et al., 2020; Siddiky et al., 2021). In broilers, about 94.28% to 100% resistance was reported in Salmonella to chloramphenicol (Jahan et al., 2013; Alam et al., 2020). Alam et al., (2020) also detected chloramphenicol resistance floR (94.28%) gene from Salmonella isolates of broilers in Bangladesh. Previously, El-Sharkawy et al., (2017) reported 100% resistance to chloramphenicol in S. Typhimurium isolated from chicken in Egypt. These authors also detected the chloramphenicol-resistant gene floR (79.30%) from these isolates (El-Sharkawy et al., 2017). Salmonella zoonotic type and potential results to penetrate the food web make the discovery of the chloramphenicol-resistant floR gene of Salmonella in broiler carrying intl1 of significant general health hazards.

2.5.11. The Resistance to Rifampicin

Rifampicin is used for the treatment of a few sorts of bacterial diseases, counting tuberculosis, Mycobacterium avium complex disease, and Legionnaires' disease (Hossain et al., 2021). It has been utilized experimentally to some extent in livestock and poultry. However, reports are available showing resistance in Salmonella to rifampicin. Akond et al., (2012) reported 60% resistance in Salmonella to rifampicin isolated from the egg surface, cloacal swabs, intestinal fluid, soil and hand washing samples of the layers. Later, Sultana et al., (2014) also observed 88% resistance in

Salmonella to rifampicin isolated from the layers in Bangladesh. Previously Zdragas et al., (2012) reported 33.30% rifampicin resistance in avian Salmonella in Greece and Ramatla et al., (2019) reported 100% rifampicin resistance in avian Salmonella in South Africa. The reported resistance in avian isolates may be caused by horizontal transfer of rifampicin-resistant genes from human isolates to avian species.

2.5.12. The Resistance to Glycopeptides

Vancomycin is a glycopeptide antimicrobial useful to treat skin diseases, circulatory system diseases, endocarditis, bone and joint diseases, and meningitis in humans (Hossain et al., 2021). Although it is not used in poultry, Sultana et al., (2014) reported 78% resistance in Salmonella to vancomycin in the layers in Savar. In India, Singh et al., (2023) recorded 100% resistance in avian Salmonella to vancomycin. Since vancomycin is effective against the Gram-positive cell wall, the presence of vancomycin resistance in Salmonella was not surprising.

2.5.13. The Resistance to Sulphur Drugs

Sulfonamides such as sulfamethoxazole are a widely used group of antimicrobials in poultry Hossain et al., (2021). In Bangladesh, variable degrees of sulfamethoxazole resistance in Salmonella in the layers were recorded (Paul et al., 2017; Haque et al., 2021; Sarker et al., 2021). Rahman et al., (2018) detected 75.86% resistance to sulfamethoxazole and Parvin et al., (2020) also reported 89.20% resistance in Salmonella to sulfamethoxazole in the broilers. In Bangladesh, the percentages of resistance to sulfur medicines detected are close to those reported in other studies in Malaysia, which was 67.50% Chuah et al., (2018) and Tibaijuka et al., (2002) resistance was 60%. Recently, (Siddiky et al., 2021) detected sulfonamide resistance sul1 gene in 36.40%, 66.70% and 80% S. Typhimurium isolated from broiler, sonali and indigenous hens' ceca in Bangladesh, respectively. This gene has also been identified in Salmonella in India (Adesiji et al., 2014). Sulfamethoxazole resistance in chickens could develop as a result of excessive antimicrobial use during manufacturing or environmental dripping. Therefore, in order to ensure food safety and market control, we should be worried about creating and implementing an effective national AMR surveillance strategy.

2.5.14. The Resistance to Polymyxins

A range of MDR bacterial diseases in people are treated as a last resort with the reserve-group antibiotic colistin. Although there are limitations on its use in farmed chicken in Bangladesh, colistin has long been used to treat Gram-negative bacterial infections. A significant level of colistin resistance was observed as ranging from 50% to 92.68% in Salmonella in broilers and layers in Bangladesh (Mahmud et al., 2013; Hassan et al., 2014; Aditya, 2015; Hossain et al., 2020; Uddin et al., 2021). Similarly, (Phiri et al., 2020) also reported 78.70% colistin resistance in Salmonella in Zambia. The main reservoirs for colistin resistance and transmission have been identified as livestock and poultry (Hoelzer et al., 2017). Detection of colistin resistance is extremely concerning for public health. In Bangladesh, Uddin et al., (2021) detected colistin resistance mcr1 gene in Salmonella in poultry. Earlier, Quesada et al. (2016) and Moreno et al., (2019) also identified colistin resistancemcr1 gene in poultry in Spain and Brazil, respectively. Increased colistin resistance is swiftly spreading around the world and is a threat to human health. The plasmid contains genes that are resistant to colistin. The issue could become more problematic if these resistance genes were transferred from resistant to other susceptible strains.

2.3. Antimicrobial resistant gene in Salmonella

Gram-negative bacteria have developed a number of plasmid-mediated -lactamases over the past ten years, which has decreased their resistance to broad-spectrum -lactams. These -lactamases included -lactamases with an extended spectrum (ESBLS) and -lactamases with AmpC. The most common ESBL families encountered are CTX-M, TEM, and SHV, while CMY is the most common AmpC family. The most common genes associated with this resistance in animals are $bla_{\text{CTX-M-1}}$ (the most commonly identified ESBL) and $bla_{\text{CTX-M-14}}$, followed by $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ (Paterson and Bonomo,2005). BlaCMY-2 is the most prevalent gene encoding AmpC-type -lactamases. These genes are most frequently found in the bacteria E. coli and Salmonella that are not typhoidal. ESBL/AmpC transmission is mainly driven by integrons insertion sequences, transposons and plasmids, some of which are homologous in isolates from both food-production animals and humans (EFSA, 2011).

2.4. Diagnosis of Salmonella

2.4.1. Serological diagnosis

Using a variety of agglutination and enzyme immune test (EIA) techniques, specific antibodies to paratyphoid salmonellae can be detected in infected chickens with great sensitivity. Additionally, serology produces conclusive results considerably later than bacteriological culture does after infection. Other serologic testing limitations include subclinical infections which lead to fecal shedding without eliciting detectable antibody responses, immunologic unresponsiveness in very young birds, cross reactions between antibodies to similar PT serotypes (Biswas et al., 2010) and vaccine-induced antibody responses which confound serologic differentiation of vaccinated and infected birds. Agglutination tests have detected both natural and experimental infections of chickens with paratyphoid salmonella (Gast and Beard, 1990). Whole blood and serum are subjected to agglutination assays in plate, tube, and microwell formats.

2.4.2. Molecular diagnosis

The advancement of PCR technology has made it possible to amplify particular target DNA segments, enabling hybridization reactions with probes to identify salmonellae with a high level of sensitivity in tissues, environmental swabs, feces, and eggs. After enrichment culturing, PCR methods have detected initial contamination loads of less than 10 Salmonella cells in eggs and poultry environmental samples (Kim et al., 2011). Carefully chosen DNA probes can be used with PCR to detect salmonellae with particular characteristics such as genes for virulence factors, biochemical properties, or surface structures such as fimbriae. Multiplex PCR assays can simultaneously detect the presence of several serotypes (Hong et al., 2009).

2.5. Pathological findings

Liver: Golden in color and noticeably enlarged, the liver had considerable obstruction. Hepatocytes displayed hepatitis, leucocytic infiltration at perivascular locations, Kupffer cell hyperplasia, hydropic vacuolation, and a number of necrotic foci under a microscope. Only sometimes did researchers find evidence of hepatocyte necrosis and localized macrophage, lymphocyte, and heterophil aggregation. Similar degenerative, necrotic and infiltrative lesions have been reported earlier (Shivaprasad, 2000; Sujatha et al., 2003).

Heart: There was modest to severe congestion and bleeding among the heart lesions. On rare occasions, the heart would display a number of white nodules with distorted shapes. The cardiac muscle fibers had substantial deterioration and fragmentation, according to histopathological examination. Patients who had fibrous pericarditis occasionally had heterophil, lymphocyte, and macrophage infiltration.

Spleen: Similar histopathogical changes including focal necrosis, reticulo-endothelial cell hyperplasia and secondary lymphoid follicles have been reported by Shivaprasad (Shivaprasad, 2000) in spleen.

Intestine: The presence of thick, slimy mucus discharge on the mucosal surfaces of the gut lumen in some cases indicated the presence of catarrhal enteritis. The mucosal epithelium had been desquamed histopathologically, leaving denuded villi and a necrotic mass filled the lumen. Some secretory glands atrophied as a result of an extreme inflow of mononuclear cells and heterophils. There were frequently goblet cell hyperplasia and localized fibroblastic connective tissue proliferation between the glands. Giannella (Giannella, 1979) reported that Salmonella induced diarrhoea is multifactorial.

Pancreas: Hemorrhages, congestion, and mild degenerative alterations were present in the pancreas. Acinar and interlobular connective tissue frequently displayed leucocytic infiltration.

Lungs: Most individuals had pneumonic lesions that were severely obstructing their lungs. RBCs were visible in the alveoli, and a microscope revealed mild hemorrhages and congestion. Serofibrinous exudate was occasionally seen in the interlobular septa and alveoli. Similar lesions have been reported by Shivprasad (Shivaprasad, 2000).

Kidneys: The kidneys first seemed substantially enlarged, with notable lobulation and necrotic foci. The renal tubular epithelium had constricted glomeruli and degenerative and infiltrative changes under a microscope. Similar degenerative and infiltrative changes in kidneys of birds affected fowl typhoid have been described by Shivaprasad, (2000)

Bursa of Fabricius: Fabricius' bursa showed just mild congestion, which was revolting. Histopathological changes include interfollicular fibrosis and a slight decrease in lymphoid tissue in bursal follicles. Loss of lymphoid tissue from follicles

and degeneration of bursa of Fabricius has been reported by Garren and Barber (Garren and Barber, 1955).

Proventiculus: Congestion, mucosal degradation, and heterophil and lymphocyte infiltration in the mucosa, occasionally up to the serosal layer, were the hallmarks of proventiculitis. The mucosal glands occasionally atrophied as a result of leucocyte invasion. The proventricular glands were failing, and the lumen was filled with detached epithelial debris.

2.6. The Status of non-typhoidal Salmonella in Bangladesh

It is believed that chicken is a significant source of several Salmonella motile serotypes. Infected live bird booths are a reliable source for exposing day-old chicks to motile Salmonella. However, very few reports are available on the presence of motile Salmonella in Bangladesh except two studies conducted by Barua et al., (2012), Barua et al., (2013) where the prevalence of motile Salmonella in commercial layer and broiler farms were estimated 18% and 11%, respectively. In layer farms, Salmonella Kentucky was found to be the most common serotype. Along with other serotypes, this serotype was also found in broiler farms. Two additional breeder farms were revealed to have mutated Salmonella, and among the serotypes discovered there was Salmonella Enteritidis. Salmonella Virchow and Salmonella Paratyphi B var. Java. Data on the prevalence of non-typhoidal Salmonella and the frequency of antimicrobial resistance in the LBM environment in Bangladesh is not available.

2.7. The pathomicrobial studies on *Salmonella* sp infection in broiler chickens

All age groups of broiler chickens are susceptible to salmonellosis, an acute septicemic disease of avian species caused by *Salmonella* sp. All bird age groups experience high disease rates, and mortality rates can range from 10% to 90%. The sector is expanding swiftly, making it difficult to stay disease-free. This is indicatedby the fact that a number of Salmonella outbreaks reported in the world are a result of injudicious introduction of infected birds (Meeusen et al., 2007).

Thus, poultry industry is facing great setbacks due to frequent outbreaks of salmonellosis (Fatma et al., 2016). Many efforts have been undertaken since its discovery to regulate and stop the incidence in industrial poultry production. However, outbreaks of Salmonellosis still remain a serious economic problem in countries where control measures are not efficient or in those areas where the climatic conditions favor the environmental spread of these microbes (Barrow and Freitas Neto, 2011).

CHAPTER-3

MATERIALS AND METHODS

3.1. Study area

One hundred five (105) broiler farms under Chattogram district, Bangladesh (Banshkhali Upazila, Boalkhali Upazila, Fatikchari Upazila, Hathazari Upazila, Lohagara Upazila, Mirsharai Upazila, Rangunia Upazila, Raozan Upazila, Sitakunda Upazila and Chattogram Metropolitan area) were selected randomly for sample collection.

3.2. Sample collection duration

From April 2022 to November 2022, samples were taken from 105 randomly chosen broiler farms.

3.3. Biological sample collection

The cloacal swab samples were taken aseptically using cotton swabs and clean, sterile 15 ml falcon tubes that contained buffer and peptone water. Each sample was made up of five cloacal swab samples obtained from the same farm's five randomly selected birds at various sites. After assembling the combined sample, each component was divided up and brought to the clinical pathology lab of the Chattogram Veterinary and Animal Sciences University in Bangladesh. Each piece was then placed separately into a sterile plastic zipper bag.

3.4. Data collection

A standardized questionnaire was utilized to collect data over the course of the trial. A thorough literature analysis was done to identify potential causes of AMR before the questionnaire was designed. The questionnaire included questions on the location of the farm, its size, its housing and rearing arrangements, the cleanliness of its floors, the history of any diseases, and the types of drugs used etc.

3.5. Bacteriological Investigation

3.5.1. Isolation of the *Salmonella* sp

Standard bacteriological techniques were used to isolate Salmonella. In a nutshell, it was incubated at 37°C for 24-36 h following pre-enrichment of the pooled samples in buffered peptone water (Oxoid Ltd.). Later, by inoculating on Xylose Lysine Deoxycholate (XLD) agar and letting it lie for an entire night at 37°C, isolated colonies were obtained. Salmonella isolates on XLD agar developed tiny, spherical, opaque, and black colonies. The growth was first located using colony morphology and Gram's staining. The pure cultures were subjected to biochemical tests for further Salmonella infection characterization (Carter and Cole, 1990). Salmonella colonies that were suspected were grown onto blood agar and kept at 80°C for later analysis.

3.5.2. Sub-culturing on blood agar

The preserved isolates were defrosted at room temperature following their removal from the freezer. Following plating, the isolates were grown for 24 hours at 37°C on blood agar. Blood agar (BA) was inoculated to help bacteria grow and multiply in order to support their growth. Using the streak plate approach, the colonies on parent cultures were repeatedly subculture until a pure culture with homogeneous colonies was established. Microscopic Gram's stain investigation led to the identification of pink, rod-shaped, gram-negative bacteria that were arranged in pairs or single rods. A polymerase chain reaction was performed using DNA that had been isolated from blood agar colonies after the incubation period (PCR).

3.5.3. Preservation of isolates

The Brain Heart Infusion (BHI) broth (Oxoid ltd, Basingstoke, Hampshire, UK) was used to inoculate all *Salmonella* sp. positive isolates. At 37°C, the soup was incubated overnight. A 1.5 ml Eppendorf tube containing 700 µl of BHI broth culture and 300 µl of 50 percent glycerol was then added for each isolate. The tubes were then properly labeled and stored at 80°C for further investigation.

3.6. Molecular detection of Salmonella

The Polymerase chain reaction was performed for molecular detection of Salmonella as described earlier (Dashti et al., 2009).

3.6.1. DNA extraction from the isolates

Genomic DNA was extracted by the crude boiling method (Dashti et al., 2009). The obtained isolates' DNA was extracted using the boiling technique. The process is described below in brief:

1. A loop full of fresh colonies (about 3-4) was picked from each blood agar and transferred to 1.5 ml Eppendorf tubes containing 100µl de-ionized water. The tubes were then vortexed to make a homogenous cell suspension. A ventilation hole was made on the lid of each tube.
2. The tubes were then cooked in a water bath for 15 minutes at 99°C. The tubes were placed in an ice pack for 5 minutes after boiling. The cell wall was able to disintegrate and the DNA to be released from the bacterial cell through the process of high temperature boiling and rapid cooling.
3. The suspension-filled tubes were then centrifuged for a period of time at 15000 rpm. Then, 50 µl of each tube's supernatant, which included bacterial DNA, was collected in new, sterile Eppendorf tubes and kept at -20°C until needed.

3.6.2 Identification of Salmonella by polymerase chain reaction (PCR)

Genomic DNA was extracted by the crude boiling method (Dashti et al., 2009). Later, suspected isolates were confirmed by conventional PCR assay using Salmonella genus-specific primers ST-11 (5′ -AGCCAACCATTGCTAAATTGGCGCA-3′) and ST-15 (5′-TGGTAGAAATTCCCAGCGGGTACTG-3′) (Gouws et al., 1998). Amplification was done with 25-µl total reaction volume for characteristic 429-bp PCR product by maintaining the initial denaturation at 94°C for 2 min followed by 35 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and then one final step with 10 min of extension at 72°C (Gouws et al., 1998). Escherichia coli ATCC 25922 and a Salmonella Kentucky in-house strain were used as a negative and positive control, respectively.

3.7. Antimicrobial susceptibility testing (AST)

AST of Salmonella isolates was conducted by disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Papich and Lindeman, 2018). Eight antimicrobials in all, representing six distinct groups, were used in the AST at the concentrations listed: ampicillin (10 g), amoxicillin (10 g), ceftriaxone(30g), ciprofloxacin(5g),doxycycline(10g), gentamicin (10g), sulfamethoxazole/trimethoprim (25 g), and tetracycline (30g). The results of the AST were interpreted as resistant, intermediate and sensitive according to standards provided by CLSI (Papich and Lindeman, 2018). If any isolate displayed resistance to more than two different classes of antimicrobials, it was defined as 'MDR' (Weill et al., 2006).

3.7.1. Detection of antimicrobial resistance genes

The *tetA*, *tetB*, and *tetC*, *sul-I*, *blaTEM*, and *blaCTX-M* genes were used in all Salmonella isolates using the precise sets of primers previously published (Table 3.1).

TABLE 3.1 The Primer sequences for the polymerase chain reaction (PCR) used to identify genes for antibiotic resistance

Gene	primer name	Primer sequence (5´- 3´)	Amplicon size (bp)	Reference
tetA	tetA-F	GGCGGTCTTCTTCATGC	502	(Lanz et al., 2003)
	tetA-R	CGGCAGGCAGAGCAAGTAGA		
tetB	tetB-F	CATTAATAGGCGCATCGCTG	930	(Lanz et al., 2003)
	tetB-R	TGAAGGTCATCGATAGCAGG		
tetC	tetC-F	GCTGTAGGCATAGGCTTGGT	888	(Lanz et al., 2003)
	tetC-R	GCCGGAAGCGAGAAGAATCA		
Sul-1	Sul1-F	CGGCGTGGGCTACCTGAACG	779	(Lanz et al., 2003)
	Sul1-R	GCCGATCGCGTGAAGTTCCG		
blaTEM	blaTEM F	GCGGAACCCCTATTTG	964	(Hasman et al., 2005)
	blaTEM R	TCTAAAGTATATATGAGTAA ACTTGGTCTGAC		
blaCTX-M	CTXM F	ACGCTGTTAGGAAGTG	857	(Feizabadi et al., 2010)
	CTXM R	TTGAGGCTGGGTGAAGT		

3.8. Histopathological study of *Salmonella* sp

At the CVASU Department of Pathology and Parasitology, Salmonella affected broiler chicken was taken for postmortem examination. Then the liver tissue was taken from Salmonella affected birds for histopathological study in order to identify the microscopic lesions.

For Histopathological study formalin fixed tissue samples were washed and dehydrated in graded ethanol and embedded in paraffin wax. Fixed tissues were sectioned at 5 µm thickness and stained with hematoxylin and eosin as per standard method (Luna, 1968).

3.8.1 Equipment and appliances for histopathology

1. 10% neutral buffered formalin.
2. Chloroform.
3. Paraffin.
4. Alcohol.
5. Tape Water.
6. Xylene.
7. Hematoxylin and Eosin Stain.
8. Distilled water.
9. Clean Slides.
10. Cover slips.
11. Mounting media (DPX).
12. Microscope

3.8.2 Collection of samples and processing

During tissue collection the following points were taken into consideration; the tissues were collected in conditions as fresh as possible. Normal and diseased tissues were collected side by side. The thickness of the tissues was as less as possible (5mm approximately). Formalin fixed tissues were processed by following protocol.

Fixation: The plastic container received 10% neutral buffered formalin addition. (10 folds the size and weight of the tissue) and fixed for 3-5 days.

Washing: The formalin was removed from the tissues by cutting them into thin sections and washing them in running water all night.

Dehydration: To stop cell shrinkage according to the following timetable, the tissues were dehydrated using an increasing ethanol series. The tissues were dehydrated for one hour in each of the following concentrations of ethanol: 50%, 70%, 80%, 95%, 100%, 100%.

Cleaning: To eliminate ethanol, the tissues were washed in chloroform for three hours (two changes; one and half hr in each).

Impregnation: Melted paraffin was used for the three-hour impregnation process (56–60°c).

Sectioning: The tissues were then cut into 5-m-thick sections using a microtome. To improve the section's adherence to the slide, a small amount of gelatin was added to the water bath. On a warm water bath set at 40 to 42°C, the portions were allowed to spread. The portions were then transferred to grease-free, transparent slides.

Drying: Slides containing sections were allowed to air dry and were maintained in a cold environment until staining.

3.8.3. The Routine hematoxylin and eosin staining procedure

The sectioned tissues were stained as described below:

1. Three xylene changes were used to deparaffinize the sectioned tissues (three minutes in each)
2. The tissues were then rehydrated using progressively lower concentrations of alcohol (three changes in absolute alcohol, each lasting three minutes; 95 percent alcohol for two minutes; 80 percent alcohol for two minutes; and 70 percent alcohol for two minutes), followed by distilled water for five minutes.
3. Harris hematoxylin was used to stain the tissues for fifteen minutes.
4. Wash in 10 to 15 minutes of running tap water.
5. The tissues were then divided by two to four dips in acid alcohol (1-part HCL and 99 parts 70 percent alcohol).
6. Afterwards, parts were given 2-4 dips in ammonia water until they turned vivid blue. Washing with tap water for five minutes.
7. Stained with eosin for one minute.
8. In alcohol, differentiated and dehydrated (95 percent alcohol: three changes, 2-4 dips each; absolute alcohol: three changes 2-3 minutes for each).
9. Cleaned in xylene: three changes (five minutes each).
10. Tissues were mounted with cover slip by using DPX.

11. The slides were inspected with low (10X) and high (40X, 100X) power objectives after drying at room temperature.

3.8. Statistical analysis

All information was entered into Microsoft Excel 2016 spreadsheet before being imported and analyzed in R 3.5.1 (Team, 2013). While taking samples from the farms, the farms' geographic coordinates were noted.

CHAPTER-4

RESULTS

4.1. Results of postmortem findings of *Salmonella* sp infection in broiler chicken

The liver displayed a variety of visible gross abnormalities, such as friable and bronze discoloration with white necrotic foci. The liver was severely enlarged, blocked, and stained with a golden sheen. There was modest to severe congestion and bleeding among the heart lesions. Sometimes the heart may have a number of white nodules with distorted shapes. Multiple surface necrotic foci and an enlarged, discolored spleen were observed. The lungs of the majority of the chicken were significantly obstructed with pneumonic lesions. On the surface, the kidneys seemed grossly enlarged, with significant lobulation and necrotic foci, slight congestion was seen in Fabricius' bursa, proventiculitis was characterized by congestion. In some cases, catarrhal enteritis was visibly present and was distinguished by thick, slimy mucus discharge on mucosal surfaces in the gut lumen. The pancreas had mild degenerative changes, hemorrhages, and congestion (Fig:2.1)

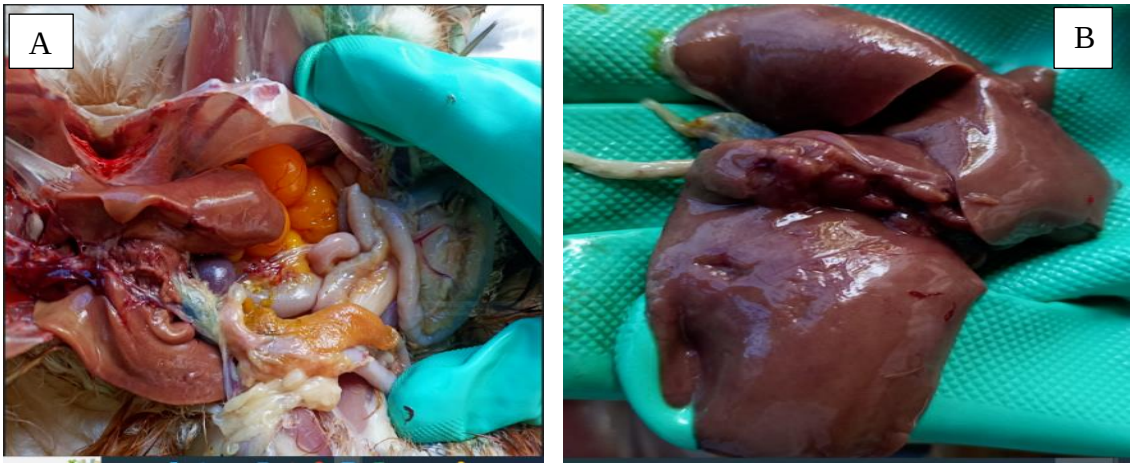


Figure 2.1: Postmortem findings of Salmonella affected broiler chicken. (A), (B)

4.2. The histopathological examination of *Salmonella* sp infection in broiler chicken

Salmonella infection caused the liver's cellular structure to be disrupted microscopically, including scrambled hepatic and sinusoidal cords as well as a dilated central vein. The hepatic tissue displayed pinpoint hemorrhages all over it. Infected birds also had severe hepatocyte vacuolation, which indicated deteriorating conditions near the central vein. In some areas, coagulation necrosis of hepatocytes was observed along with infiltration of mononuclear inflammatory cells in the interstitium of the liver. There is also presence of mild congestion of blood vessels with diffused or multifocal necrosis and damaged of the cellular structures of hepatocytes (Fig:2.2)

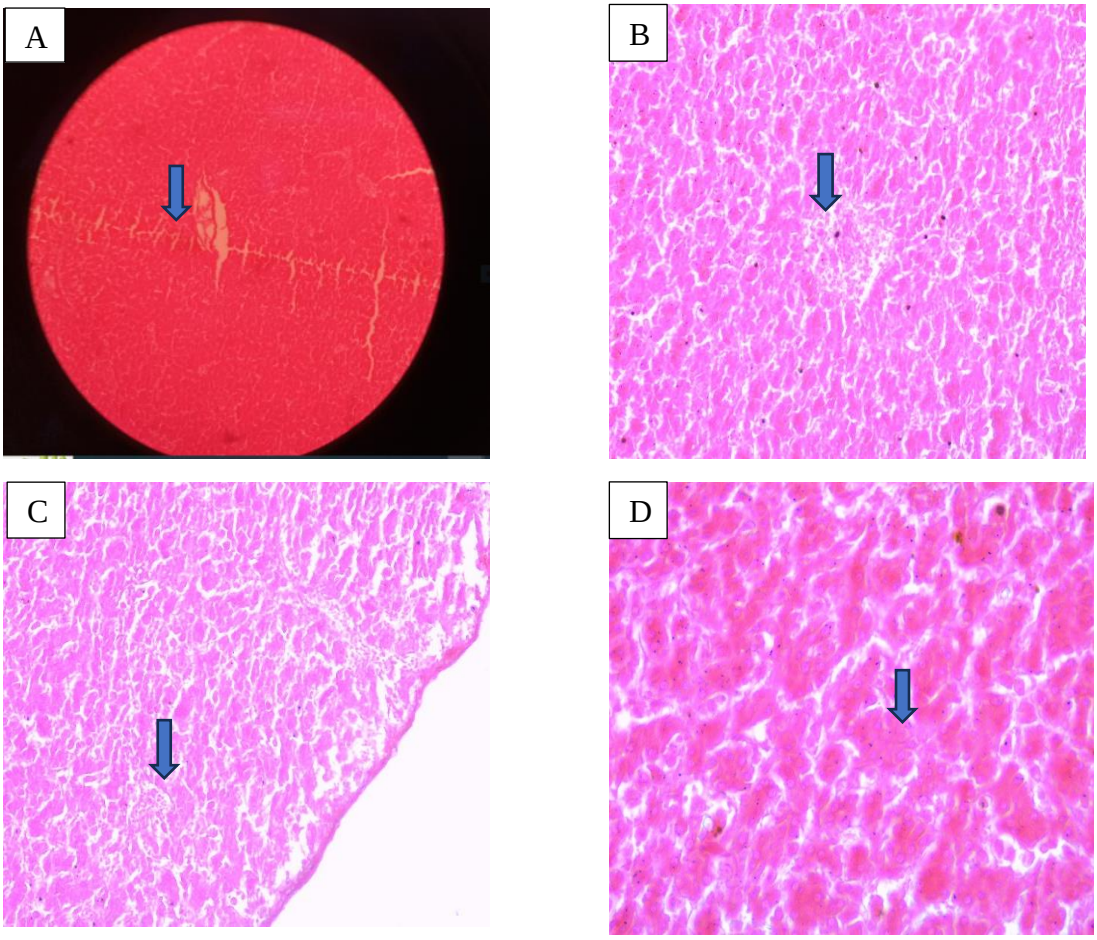


Figure 2.2: Diffuse necrosis in liver (A) and congestion and infiltration of lymphocyte (B, C, D).

4.3. Prevalence of *Salmonella* sp infection in farm level

To isolate and identify Salmonella sp., 105 pooled samples (cloacal swabs) total were gathered from various parts of Bangladesh's Chattogram district. The number of 8 isolates (7.62%) (95 percent CI: 3.35 percent -14.16 percent) tested positive for *Salmonella* sp.

4.4. Analysis of risk factors

4.4.1. Prevalence of *Salmonella* sp according to potential explanatory variables in different upazila in Chattogram

Isolation percentage of *Salmonella* sp in cloacal samples according to different location, number of chickens per flock, number of shed, floor type, flock age and water source,

disposal system, disinfection, presence of rodents, other diseases history, antibiotic use are shown in Table 4.1 The fisher-exact test revealed no statistically significant connection (p> 0.1) between any of the parameters and the presence of *Salmonella* sp in the broiler cloacal sample.

Table 4.1 Prevalence of Salmonella infection according to different factors at farm level

Variable	Category	Positive	95% CI	Prevalence	p value
Area	Fotikchhari (42)	2	0.6-16.2	4.76	0.819
	Raozan (24)	2	1.0-27.0	8.33	
	Banshkhali (21)	2	1.2-30.4	9.52	
	Hathazari (18)	2	1.4-34.7	11.11	
Number of chickens	Min-1300 (45)	5	3.7-24.1	11.11	0.243
	1301-max (60)	3	1.0-14.0	5.00	
Number of shed	1 (86)	6	2.6-14.6	6.98	0.598
	2-4 (19)	2	1.3-33.1	10.53	
Water supply	Tube well (102)	8	3.4-14.9	7.84	0.614
	Pond (3)	0	0	0	
Establishment of house	2017 and after (91)	7	3.1-15.2	7.69	0.942
	Before 2017 (14)	1	0.2-33.9	7.14	
Floor type	Concrete (84)	7	3.4-16.4	8.33	0.581
	Mud (21)	1	0.1-23.8	4.76	
Flock Age (days)	21 (38)	2	0.6-17.7	5.26	0.493
	After 21 (67)	6	3.4-18.5	8.96	
Number of dead birds per flock	0-25 (26)	2	0.9-25.1	7.69	0.987
	more than 25 (79)	6	2.8-15.8	7.59	
Dead bird's disposal system	Yes (4)	0	0	0	0.558
	No (101)	8	3.5-15.0	7.92	
Disinfection before restock	Yes (101)	8	3.5-15.0	7.62	0.558
	No (4)	0	0	0	
	Yes (71)	5	2.3-15.7	7.04	0.748

Presence of rodents in the poultry house (PR)	No (34)	3	1.9-23.7	6.88	
Have Knowledge about Salmonellosis	Yes (2)	0	0	0	0.682
	No (103)	8	3.4-14.7	7.77	
Previous history of other diseases	Yes (82)	6	2.7-15.2	7.32	0.826
	No (23)	2	1.0-28.0	6.98	
Antibiotic use	Yes (88)	6	2.5-14.2	6.82	0.482
	No (17)	2	1.4-36.4	11.76	
Complete antibiotic course	Yes (72)	5	2.3-15.5	6.94	0.700
	No (33)	3	1.9-24.3	9.09	
Maintain withdrawal period	Yes (8)	2	3.2-65.0	25	0.054
	No (97)	6	2.3-13.0	6.19	

4.4.2. Univariable association of risk factors with the occurrence of *Salmonella* sp in broiler chickens at farm level

Table 4.2 displays the prevalence of Salmonella sp. in broilers in relation to various farm-level variables. Though none of the association were statistically significant, we found that the odds ratio of *Salmonella* sp isolation was higher where the flocks had maximum (≤1300) number of chickens (OR=2.4, 95% CI,0.5%-10.5% and P=0.254). The prevalence of *Salmonella* sp was higher where the flock age more than 21 days, (OR=1.8, 95% CI, 0.3%-9.2%, P=0.498) than the flock age 21 days (≤21). The prevalence of *Salmonella* sp was higher at farms where presence of rodents in the broiler shed, (OR=1.3, 95% CI, 0.3%-5.7%, P=0.748) than at farms where no rodents in the poultry farms. Poultry farms that were affected by other diseases, (OR = 1.2,95% CI,0.2%-6.4%, P=0.826) *Salmonella* sp colonization was higher than the farms that was

not affected by other diseases. The broiler farm where there was no use of antibiotics, showed higher prevalence of *Salmonella* sp than the farm whereas the farmer used antibiotic. On the other hands, where the farmers used to complete the antibiotic course had lower prevalence than the farms did not follow the complete antibiotic course, (OR=1.3, 95% CI, 0.3%-6.0%, P=701). On the other hands the prevalence of *Salmonella* sp at farms that had more than 1300 birds per flock and the farms that had 1300 and less birds per flock per shed was (0.5% vs. 10.5%; OR = 2.4) and the prevalence of *Salmonella* sp was higher at farms that had 2-4 sheds per farm than the farms that had number of shed only one in the farm (0.2% vs. 8.4%; OR = 1.6). There was no significant association with maintaining the withdrawal period of antibiotic with the Salmonella isolation.

Table 4.2 Univariable logistic regression of Salmonella infection in farm level

Variable	Category	Odds Ratio	95% CI	p value
Area	Fotikchhari	Ref		
	Raozan	1.8	0.2-13.8	0.563
	Banshkhali	2.1	0.3-16.1	0.473
	Hathazari	2.5	0.3- 19.3	0.380
Number of chickens	Min-1300	2.4	0.5- 10.5	0.254
	1301-max	Ref		
Number of shed	1	Ref		
	2 – 4	1.6	0.2-8.4	0.600
Establishment of house	2017 and after	1.08	0.1-9.5	0.943
	Before 2017	Ref		
Floor type	Concrete	1.8	0.2-15.6	0.586
	Mud	Ref		
Flock Age (days)	21	Ref		
	After 21	1.8	0.3-9.2	0.498
Number of dead birds per flock	0-25	1.01	0.2-5.4	0.987
	more than 25	Ref		
Presence of rodents in the poultry house	No	Ref		
	Yes	1.3	0.3-5.7	0.748
	No	Ref		

Previous history of other diseases	Yes	1.2	0.2-6.4	0.826
Antibiotic use	Yes	Ref		
	No	1.8	0.3-9.9	0.487
Complete antibiotic course	Yes	Ref		
	No	1.3	0.3-6.0	0.701
Maintain withdrawal period	Yes	5.05	0.8-30.6	0.078
	No	Ref		

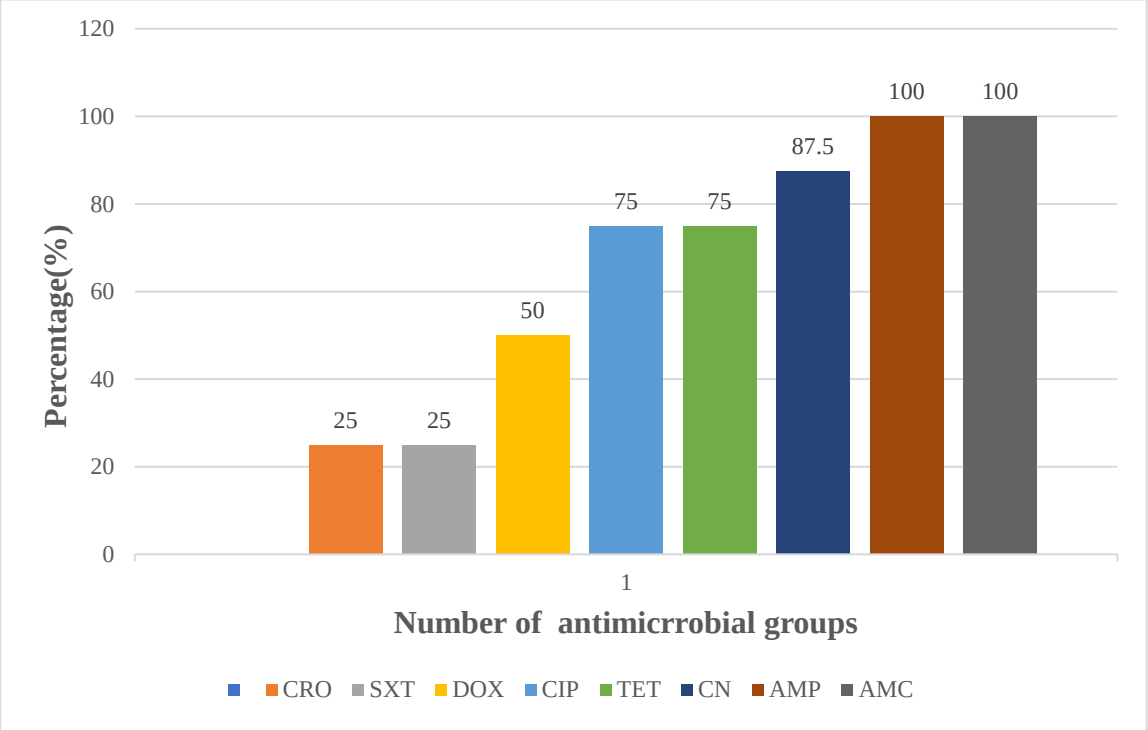
4.4.3. The antimicrobial resistance profile and percentage of multidrug resistance to *Salmonella* sp. isolates

The antimicrobial susceptibility pattern of eight *Salmonella* sp isolates revealed that each strain was resistant to ampicillin and amoxicillin (100%) followed by gentamicin 87.5% (95% CI: 63.05.–100%; 47.34-99.68%), ciprofloxacin and tetracycline (75%; 95% CI: 34.91–96.81%), sulfamethoxazole/trimethoprim and ceftriaxone (25%; 95% CI: 3.18–65.08%), and finally doxycycline (50%; 95% CI: 15.70-84.30), (see Fig 4.1). Majority of the positive samples (7) were multidrug-resistant (MDR) in *Salmonella* sp infection in broiler chicken at farm level. We observed that one isolate was resistant to three antibiotics, four isolates were resistant to four antimicrobials and two positive samples were resistant to five antimicrobials (see Table:4.3).

Table 4.3. The percentage of multidrug resistance to *Salmonella* spp. isolates (Broiler chicken, N=8)

No of multidrug resistance antimicrobial (MDR)	n	%	95% CI
MDR, **yes**	7	87.5	47.3-99.7
MDR, **no**	1	12.5	0.3-52.7
Three antimicrobial resistances	1	14.3	0.4-57.9
Four antimicrobial resistances	4	57.1	18.4-90.1
Five antimicrobial resistances	2	28.6	3.7-70.9

Figure 4.1: The antimicrobial resistance pattern of Salmonella isolates [n = 8]



4.4.4. The distribution of antimicrobial resistance genes

Among the isolates tested 100% (95% CI, 54.07%-100%) carried the *tetA* gene followed by 33.3% (95% CI, 4.33%-77.72%) the *tetB* gene and 16.67% (95% CI, 0.42%-64.12%) the *tetC* gene. Table 4.4 displays the prevalence of the tetracycline, sulfamethoxazole/trimethoprim, ampicillin, and ceftriaxone resistance genes in Salmonella isolates, out of the 8 isolates, 100% (95% CI, 15.81%-100%) were found positive for the presence of the *Sul-I* gene. The *blaTEM* gene was detected in 87.5%% (95% CI, 47.35%-99.68%) isolates, whereas the *blaCTX-M* gene in 50 % (95% CI, 1.26%-98.74%).

TABLE 4.4. The occurrence of antimicrobial resistance genes among Salmonella isolates [n = 8] from broiler chicken

Antimicrobial agents	Resistance genes	No. of resistant isolates	Prevalence (95% CI)
Sulfonamide	*sul1*	2	100 (15.81-100)
Ampicillin	bla_{TEM}	7	87.5 (47.35-99.68)
Tetracycline	*tetA*	6	100 (54.07-100)
	tetB	2	33.3(4.33-77.72)
	tetC	1	16.67 (0.42- 64.12)
Ceftriaxone	*blaCTX-M*	1	50 (1.26-98.74)

4.4.5. The results of growth characteristics of *Salmonella* sp in XLD and Blood agar

Salmonella presented pink colonies with a black center on XLD agar in the current investigation, displaying cultural traits. After that, XLD agar and blood agar were used to subculture the suspicious colonies. It's interesting to note that *Salmonella* sp. emerged on a blood agar plate as small, round, smooth, gray colonies that were whitish, big, spherical, and somewhat rough. In our investigation, microscopic Gram's stain analysis revealed rod-shaped bacteria that were arranged in pairs or single rods (see Fig: 4.2 ,4.3 ,4.4).

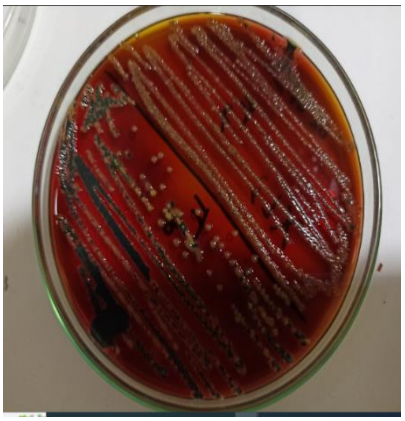


Fig. 4.2: *Salmonella* sp on Xylose Lysine Deoxycholate agar (XLD)

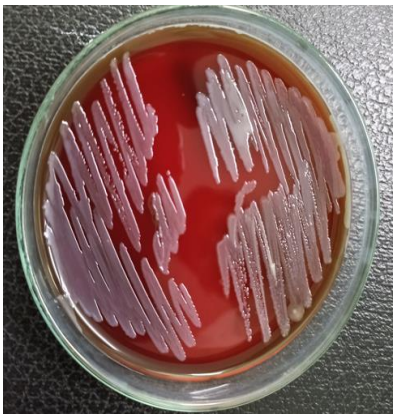


Fig. 4.3: *Salmonella* sp on blood agar media

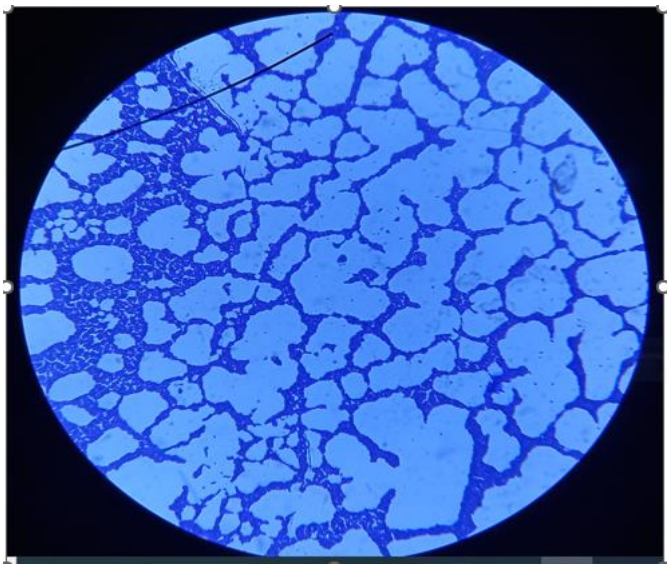


Fig. 4.4: Gram's staining properties of *Salmonella* sp

4.4.6. The result of DNA extraction, PCR and culture sensitivity test of *Salmonella* sp

In the current investigation, the isolated colonies were first tested for antimicrobial susceptibility, DNA extraction, and molecular confirmation using PCR. To evaluate the antimicrobial profile and estimate the diameter of each antimicrobial agent's zone of growth inhibition, all isolated *Salmonella* spp. underwent antimicrobial susceptibility testing using the disk diffusion method (see Fig 4.6, 4.7,4.8, 4.9). Then the boiling procedure for extracting DNA was depicted in the figure (see Fig. 4.5). The PCR products were then separated on a 1.5 percent agarose gel, stained with ethidium bromide, and captured using a Gel documentation system. Next, PCR amplification was carried out using a thermocycler. The expected width of the gel band was used to monitor the positive sample, and photos were taken using a UV transilluminator.

Fig. 4.5: DNA extraction for the detection of *Salmonella* sp.

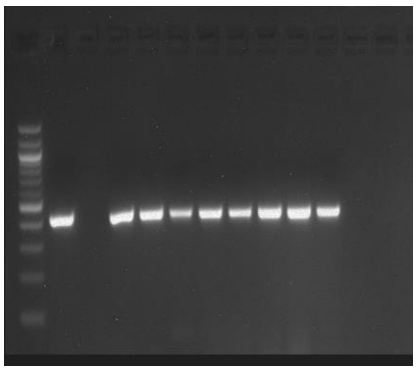


Fig. 4.6: PCR assay for the detection of *Salmonella* sp.

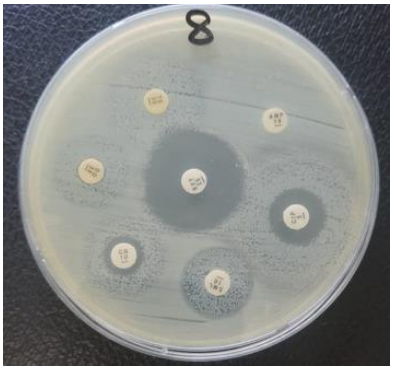


Fig. 4.7: Bacterial zone of inhibition.

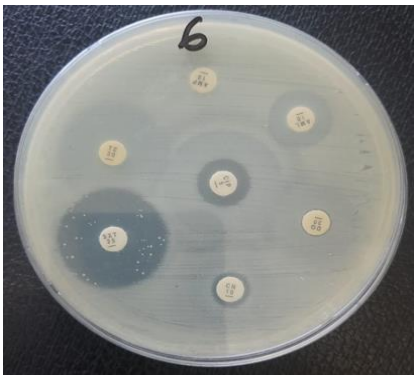


Fig. 4.8: Bacterial zone of inhibition

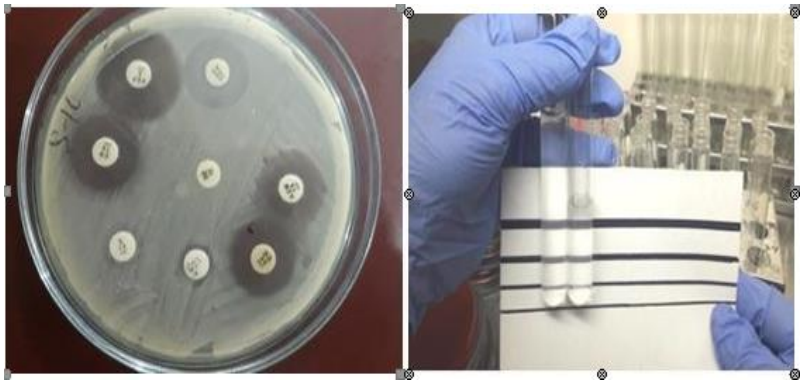


Fig. 4.9: Comparing inoculum with McFarland Standard

CHAPTER-5

DISCUSSION

Salmonellosis in birds poses a serious danger to the poultry industry since it can result in significant financial losses through death and decreased output. On the other hands, avian salmonellosis poses a substantial threat to both human health and the poultry industry, resulting in significant financial losses. Worldwide, salmonella has a significant negative economic impact. It typically passes from animal to human and has an impact on the poultry industry worldwide. Financial losses are a result of expensive management and treatment, lost output, and mortality. AMR is an escalating global health problem (Dahlen et al., 2012). AMR can affect sustainable development goals (SDGs), especially those targeting hunger, health and economic growth (Clifford et al., 2018a). MDR Salmonella has emerged as a major public health issue worldwide (Marshall and Levy, 2011). This research work was conducted to isolate *Salmonella* sp, detection of antimicrobial resistance pattern as well as antimicrobial resistant gene of broiler chickens from different areas of Chattogram, Bangladesh. By using motility, biochemical analysis, PCR, and culture staining, the isolates were identified as Salmonella. Finally, the isolates of broiler chickens found in this investigation were characterized for their antibiotic sensitivity, resistance, and resistance gene. Among 105 isolates, 8 isolates were positive for Salmonella. Despite the importance of the poultry sector in the National Economy of Bangladesh, insufficient disease data brings bottlenecks toward understanding the disease burden like its true prevalence, spatial and temporal distribution, and economic impact (Hamid et al., 2017). Among the different types of bacterial and viral origin diseases in Bangladesh, Salmonella infection is cogitated to be one of the major problems nowadays (Rahman et al., 2017; Al Mamun et al., 2019). According to the results of the current investigation, there are commercial broiler poultry farms in the Chattogram district that are the source of Salmonella strains that are circulating, with an overall prevalence of 7.62 percent in broiler chicken. Similar results was observed by another study where 7.33% Salmonella prevalence in healthy broiler chicken (Parvej et al., 2016) was recorded. Our estimated prevalence was a little higher to the findings that was reported an overall prevalence of 6.88% in Kashmir Valley, India (Mir et al., 2010). However, the prevalence rate was lower than that in other studies conducted in other parts of India (Kumar et al., 2012; Kaushik et

al., 2014). The changes in farm management systems (biosecurity, hygiene, sanitary, etc.), sample size, sample kinds, geographical and seasonal distributions, and method-related factors may all be related to the observed variances in the prevalence of *Salmonella* spp. In contrast, few studies in broiler and layer poultry farms of the same region showed a bit higher prevalence (Barua et al., 2012; Barua et al., 2013; Hossain et al., 2015). The prevalence estimates of Salmonella in broiler farms were reportedly variable from as low as 10% to as high as 37% or even higher irrespective of geographical variation (Salles et al., 2008; Snow et al., 2008; Dione et al., 2009; Elgroud et al., 2009; Samanta et al., 2014; Asif et al., 2017). Similarly, (Alam et al., 2003) reported 23.8% prevalence of Salmonella infection in poultry in the Dinajpur district of Bangladesh. The majority may be affected by the methodology, the context in which it was used, or a number of environmental factors. Research has shown that changes in feed by modifying ingredients and composition of nutrients have an effect on the sensitivity of chickens to Salmonella infection (Vandeplas et al., 2010). Another study (Bari et al., 2012) reported a variation in the prevalence of Salmonella in different areas, such as in Gazipur (20%), Manikgonj (16%) and Saver (15%) of Bangladesh. In another study, Al Mamun et al. (2017) reported the prevalence of Salmonellosis in poultry as 23.53%, which is lower than the previous findings, and also Mahmud et al., (2011) reported a prevalence of 37.9% in Bangladesh. Previously a study in Bangladesh, showed the highest rate of Salmonella occurrence in cloacal swabs (32%) among different samples of poultry (Karim et al., 2017) and another study found 48% in cloacal swabs (Islam et al., 2016). The presence of *Salmonella* spp. in cloacal swabs of healthy broiler chicken provides the evidence of persistent intestinal colonization of *Salmonella* spp. of the individual bird (Saelinger et al., 2006). According to our research, flock size may have an effect on how often Salmonella infections occur. Due to the higher flock density, which facilitates the propagation of any pathogen, larger flocks may have a higher infection rate It is significant to remember that the success of a detection depends on the sensitivity of the culture method and the selected sampling plan. Besides, intermittent shedding and non-uniform distribution in poultry houses may also be responsible for variability in results (Proux et al., 2002).

Salmonella is one of the MDR bacteria, showing resistance to ampicillin, streptomycin, chloramphenicol, sulfonamides and tetracycline (Guilfoile and Alcamo, 2007). Salmonella antimicrobial resistance has reached alarming levels globally. It has been observed to occur primarily in hosts who take the antimicrobial medications and is linked to incorrect antimicrobial agent use. In our study total 8 isolates were investigated for susceptibility and resistance patterns by disc diffusion method using 8 commonly used antibiotics belonging to different groups. Among the variety of antibiotics tested, the highest resistance was found with Ampicillin, Amoxicillin, Gentamycin, Tetracycline and ciprofloxacin. According to the current study, the isolated Salmonella strains exhibited resistance to the following regularly used antibiotics: ampicillin, amoxicillin, tetracycline, trimethoprim/sulfamethoxazole, gentamicin, doxycycline, ciprofloxacin, and ceftriaxone in a different range. The antibiotic sensitivity patterns in our study showed that the Salmonella isolates were 100% resistant to ampicillin and amoxicillin, followed by gentamicin (87.5%), ciprofloxacin (75%), tetracycline (75%), doxycycline (50%) and sulfamethoxazole/trimethoprim (25%) and ceftriaxone (25%). Strong selective pressure by exposure to regularly used antibiotics could be one of the main causes for the emergence of such antibiotic-resistant Salmonella strains (Wright, 2007). The improper dosages and regimens of antibiotics used excessively and illogically in commercial chicken raising may be a factor in the emergence of resistance. Plasmid-mediated horizontal transfer of antimicrobial resistance gene(s) may play important role in developing such a high rate of drug resistance among the isolates (Carattoli, 2003). Another reason could be that feed firms and farmers frequently utilize these antibiotics as a growth booster and add them to the water and chicken feed, respectively. A study showed that, Salmonella strains isolated from poultry sources were commonly resistant against ampicillin, tetracycline and chloramphenicol and susceptible to nalidixic acid and gentamicin as found in several studies in Bangladesh (White et al., 2001; Hasan, 2004; Mayrhofer et al., 2004; Sisak et al., 2006; Ribeiro et al., 2007; Van et al., 2007). Resistance against penicillin, ampicillin, tetracycline and erythromycin was often observed due to low cost, ready availability and for drug abuse (Van et al., 2007). Moreover, all the isolates exhibited multidrug resistance against more than five antibiotics. Similar findings on multidrug resistance among Salmonella strains have been reported from Bangladesh and various parts of the world (White et al., 2001;

Hasan, 2004; Mayrhofer et al., 2004; Okoli et al., 2006; Sisak et al., 2006). It's possible that bacteria have the capacity to acquire antibiotic resistance genes and transfer those genes to a variety of different bacterial species. Additionally, it's possible that readily available and inexpensive antibiotics like ampicillin, penicillin, tetracycline, and erythromycin are frequently used as growth promoters, feed additives, or preservatives to poultry flocks and animals that produce food to meet Bangladesh's expanding food needs. A previous study detected resistance against amoxicillin and doxycycline suggesting overuse or misuse of these antibiotics (Antunes et al., 2016; Clifford et al., 2018b). Previous studies have reported that Salmonella isolated from poultry in Bangladesh were sensitive to ciprofloxacin (Temml et al., 2014; Faruque et al., 2019; Mridha et al., 2020). However, another study showed that, Salmonella isolates were found to be susceptible to ampicillin, piperacillin/tazobactam, nitrofurantoin, imipenem and amikacin, as was previously reported (Zhang et al., 2018). The fact that these antimicrobial medicines are frequently utilized for therapeutic purposes in veterinary care rather than for feed supplementation or growth promotion in Bangladesh may account for the isolates in the current study's resistance to such antimicrobial agents. A high number of the isolates showed MDR to tetracycline, ciprofloxacin, and sulphonamides, which are antimicrobial agents commonly used in veterinary medicine. Zhao et al., (2017) reported high resistance against tetracycline (72.0%) and ampicillin (69.4%) in Salmonella isolates of diverse origin. (Zishiri et al., 2016) also found high tetracycline (93.0%) resistance in Salmonella isolated from chickens; however, resistance against ampicillin was 47%. Previously, another study reported (Alam et al., 2020) 100% MDR *Salmonella* spp. from broilers in Bangladesh. The unrestricted use of more potent antibiotics in poultry farms for quick growth and disease prevention may be the cause of the high MDR in this investigation. Bangladesh doesn't have any rigorous restrictions preventing the use of antibiotics off-label in poultry farms, though. As a result of the regular use of antibiotics in chicken farms, germs are under pressure to evolve resistance in order to survive. Another study showed that there was a rise in the number of studies reporting emergence of ciprofloxacin resistance in nontyphoidal Salmonella around the world (Hsueh et al., 2004; Mulvey et al., 2013). A study in India where the focus was mainly on ciprofloxacin resistance in S. Typhi and S. Paratyphi (Gaind et al., 2006) nonetheless, our current investigation revealed that 75% of *Salmonella* sp. were resistant to ciprofloxacin. It may be the cause of the widespread

use of this antibiotic in the treatment of bacterial infections in broiler chickens. In addition, it might be possible reason that the unethical use of some antibiotics in chicken feed and water by feed millers and farmers, respectively, is another factor contributing to the development of multidrug resistance.

In our present study we identified the presence of *tetA* (100%), *tetB* (33.3%), *tetC* (16.67%), *blaTEM* (87.5%), *sul1* (100%) and *blaCTX-M (*50%) that are responsible for specific antibiotic resistance. Another study showed that the prevalence of *tetA, tetB and tetC* among the isolates was 81.4%, 19.8% and 10.5%, respectively, where *tetA* was found as the most prevalent tetracycline resistance gene, an agreement with the findings of some previous studies (Adesiji et al., 2014; McDermott et al., 2016). Around 37.21% isolates harboured the *sul-I* gene responsible for sulfonamides resistance (Alam et al., 2020) which was lower than our present study. Ahmed and Shimamoto, (2012) also found only tetA gene among the six genes screened (*tetA*, *tetB*, tetC, *tetD, tetE and tetG*) in MDR *S. Typhimurium*. (Adesiji et al., 2014)detected the presence of four tetracycline resistance genes (*tetA, tetB, tetC,* and *tetG*) in MDR Salmonella from retail meat, poultry feces and clams with *tetA* being the most frequent (100%). Another study reported that sulfonamide resistance is encoded by *sul1* and *sul2* genes. *Sul1* gene was detected in 82.35% isolates compared to sul2 gene in only 8.82%. Randall et al., (2004) also reported predominance of sul1 gene in *S. Typhimurium*. Resistance to tetracycline was comparable to findings of (Akbar and Anal, 2013) but less than that of (Ellerbroek et al., 2010) who reported 100% resistance in their study. The inappropriate use of tetracycline as a growth booster in chicken feed has perhaps been the cause of tetracycline resistance. Another study showed that, the presence of *blaTEM* gene in the Salmonella isolates was 95.4%, which was the highest among all the resistance genes studied, and the results revealed that almost all ampicillin-resistant isolates possessed the *blaTEM* gene (Olesen; Adesiji et al., 2014). On the other hand, our current study demonstrates that the ceftriaxone resistance gene, specifically blaCTX-M, was circulating in a low frequency (50%) among the isolates isolated. The reason for this low prevalence of the gene may be related to its low or non-existence in Bangladeshi poultry farming.

The finding of MDR Salmonella in broiler chicken, which was one of the significant findings of the current study, is extremely concerning for public health. The majority of the farms surveyed shared similar general features throughout the study area and study time, including flock size, raising method, management techniques, etc. The majority of the farms under investigation had minimal biosecurity measures in place, making it simple for people, wild animals, birds, and rats to gain access. Studies showed that wild birds and rodents play a pivotal role in the transmission and spillover of Salmonella within and in between farms as they act as the carrier of Salmonella (Kinde et al., 2005; Bouzidi et al., 2012). The findings suggested that broiler chickens play a significant role as reservoirs of Salmonella that is multidrug resistant. Drug-resistant salmonellosis has become a serious problem due to the usage of antibiotics to treat and prevent illness, which increases the need for greater regulation over the monitoring of antimicrobial agent resistance. Therefore, the poultry industry should employ cautious management by implementing more effective disinfection guidelines in order to reduce the population of antibiotic-resistant bacteria. Moreover, a moderate use of antibiotics may help prevent the occurrence of antibiotic resistance in pathogens (Chen and Jiang, 2014). Therefore, it is essential to adopt strong controls over antibiotic usage, especially in food animals. Antibiotic non-judicial use must be examined under proper scientific and public health laws. Additionally, after doing in vitro tests for antibiotic susceptibility, any treatment plan should be followed. That will reduce the emergence of microbial bugs which are spreading worldwide and responsible for fatal disease outcome in different parts of world (Aarestrup et al., 2001).

CHAPTER-6

CONCLUSION

This work offers important background knowledge and empirical support for the existence of MDR Salmonella strains in Bangladeshi poultry. The results of this study indicate that multidrug-resistant *Salmonella* sp. is common among Bangladeshi commercial broilers. As a result, it is proposed that more intervention studies be carried out to better understand the risk factors connected to *Salmonella* sp in poultry and to develop methods to diminish them in order to manage Salmonellosis across the entire chicken industry.

CHAPTER-7

LIMITATION: RECOMMENDATIONS AND FUTURE PERSPECTIVES

- Because of time and budget constraints, the study was carried out on a small scale. The study can be done with a larger sample size in the future.
- The resistant isolates' minimum inhibitory concentration (MIC) was not tested because to time and resource limitations.
- A deeper knowledge of the origin and dissemination of the described genes might have been obtained by their sequencing.

Appendix: Questionnaire survey

Base level for farms Information:

Study Area:

ID no of farm:

Date:

Season of sample collection:

Owner's information

Name of Farmer:

Farms and Farmer's contact address:

Farm and flock information:

Farm Establishment year:

Number of Farm:

Length of the house:

Width of the house:

Number of Chicken:

Flock age:

Floor type:

Type of litter used:

Number of people work:

Water supply:

Number of dead birds per flock:

Dead bird disposal system:

General observational information and checklist:

Presence of rodents in the poultry farm: Yes/ No

Practice of all-in all-out system: Yes/No

Disinfection of farm before restock: Yes/No

Any knowledge about Salmonellosis:Yes / No

Previous history of any diseases? Yes/No

Elimination of dead birds every day: Yes/No

What types of drugs usually used? Yes/No

Do you complete the antibiotic course? Yes/No

Do you still allow withdrawals? Yes/No

Do you maintain actual dose according to prescription? Yes/No

Do you have knowledge about AMR? Yes/No

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