DETECTION OF *SALMONELLA SPP.* FROM POULTRY PRODUCTION CHAIN IN DINAJPUR WITH ANTIBIOTIC SENSITIVITY TEST

A THESIS

BY

MD. AL EMRAN

Registration No.: 1505010

Semester: January-June, 2017

MASTER OF SCIENCE (MS)

IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
FACULTY OF POST GRADUATE STUDIES
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Submitted to the
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DEPARTMENT OF MICROBIOLOGY
FACULTY OF POST GRADUATE STUDIES
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JUNE, 2017

DEDICATED
TO MY
BELOVED PARENTES,
SISTERS & WIFE

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ABSTRACT

Bangladesh is an agriculture based country. Poultry rearing is considered superior to the others in agricultural sector. *Salmonella spp.* is one of the most economically significant organism in poultry sector. The aim of this study was to identify and characterize *Salmonella* species isolated from poultry production chains of Dinajpur (Kaharol and Sadar Upazila) district of Bangladesh during the period of January to June 2016. For this purpose a total of 153 samples (chick meconium, cloacal swab, poultry carcass, feed, water, transport swab and floor swab) were collected and were subjected to various cultural and biochemical techniques. Furthermore, the isolated *Salmonella* species were characterized by antimicrobial susceptibility testing. Among the samples, 23.53% (n=36) were found to be associated with *Salmonella* species. The *Salmonella* species were identified by observing on SS agar, positive to MR test and negative to VP and Indole test. Among the 36 isolates, 30.56% (n=11) belonged to serogroup B and rest of the isolates 69.44% (n=25) to serogroup D. The isolated *Salmonella* species were subjected to antimicrobial susceptibility testing with the aid of disk diffusion method using 8 antimicrobial agents. All isolates of *Salmonella* species were susceptible to ciprofloxacin, norfloxacin, streptomycin and gentamicin. Out of 36 isolates 100% *Salmonella* species were resistant to erythromycin and tetracycline. The findings of this study revealed the presence of multidrug resistant *Salmonella* species in poultry production chains of Dinajpur (Kaharol and Sadar Upazila) district of Bangladesh that posseses a serious threat to public and poultry health.

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CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Bangladesh is an agriculture based country. Poultry rearing is considered superior to the others in agricultural sector because of an almost assured in a relatively short period of time. Poultry industry which has started during 1980_s is an excellent agribusiness (Haque, 2001). Over the last decades surprising development has been occurred in this sector (Rahman, 2003). It has become a vital sector for it's generating employment, creating additional income and improving the nutritional level of the country. This sector provides fulltime employment to about 20% and partial employment to about 50% of the rural people (Alam *et al*., 2003).

The poultry population in Bangladesh is estimated to be 221.39 millions of chickens and 41.23 millions ducks (DLS, 2009). About 50,000 chicken farms and 26,000 duck farms have already been set up in private sector in addition to the Government farms. At present , there are more than 130 hatcheries producing 0.476 million day-old-chicks per week and about one million commercial layer and broiler farms supplying 0.6 million kg of poultry meat and 9.9 million table eggs per weeks (Kabir, 2005).

Development of poultry sector in Bangladesh is being hampered by a number of factors, of which the diseases are considered as the major factor causing 30% mortality of chicken per year (Das *et al*., 2005). Intestinal bacteria play an important role on health through their effects on gut morphology, nutrition, pathogenesis of intestinal diseases and immune responses (Mead *et al*., 2000*)*.

Among the bacterial diseases, Salmonellosis is major problems in the poultry industry in Bangladesh (Haider *et al*., 2008). *Salmonella* infection is one of the major constraints of

poultry farming that hindered its development in Bangladesh (Kamaruddin and Giasuddin, 2003; Das *et al.*, 2005). It causes a variety of acute and chronic diseases of poultry in Bangladesh (Bhattacharjee *et al*., 1996). Chicks can be infected with *Salmonella* spp. by vertical transmission through infected parents or by horizontal transmission through hatcheries, sexing in contaminated hatcheries, cloacal infection and transportation of equipment and feed (Opitz *et al*., 1993). There are >2500 *Salmonella* serovars distributed throughout the world (Plym and Wierup, 2006).

The genus *Salmonella* is phylogenically clustered in the family of *Enterobacteriaceae* (Bennasar *et al*., 2000; Grimont *et al*., 2000). *Salmonella* is characterized as ubiquitous, Gram-negative, intracellular, straight rod shaped, nonencapsulated, facultative, non-spore forming, and generally motile with peritrichous flagella (Gray and Fedorka-Cray, 2002; Kwang *et al*., 1996).

Salmonella spp. is potentially responsible for various pathogenic processes in man and animal including poultry (Freeman, 1985). Motile *Salmonellae* (paratyphoid group) infection cause salmonellosis in chickens and have zoonotic significance (Kabir, 2010). Affected birds appear to be depressed and can show signs of scour. It can cause diarrhea, vomition, fever, abdominal cramps in human (Johnstones, 2007). Sometimes severe diarrhea requires medical interventions such as intravenous fluid therapy. In cases, where bacteria enter into the bloodstream, symptoms include high fever, malaise, pain in the thorax and abdomen, chills and anorexia (Bell, 2002).

Antibiotics are extensively used in poultry industry either as a growth promoter or to control infectious diseases. The rise in antibiotic resistance has been reported in the past two decade in many countries including Bangladesh (Kapil, 2004).

To minimize the bacterial load in poultry farm it should be maintained strict hygienic measure, maintain proper legislation with government and social awareness. Many producers now expect Veterinarians to be an integral part of their quality assurance programs of foods of animal origin (Hubbert *et al*., 1996).

Salmonellosis status of a farm needs to be determined for its proper control and management (Ahmed *et al.*, 2008). But no work has been done yet in Bangladesh to identify the *Salmonella* spp. from different phages of poultry production chain (hatchery → farm → transport → live bird markets) at a time. Therefore, the present study was designed to isolate and identify *Salmonella* spp., as well as serogroping the isolated *Salmonella* species.

Considering the above situation the main objectives of the present research work were:

i. To isolate and identify *Salmonella* spp. from hatchery, commercial poultry farm and market using cultural and biochemical techniques with antobiotic tests.

ii. To identify the isolated *Salmonella* species by using serological tests.

iii. To observe antibiotic susceptibility and resistance patterns of identified isolate against eight antibiotics commonly used in poultry production chain.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

The present research was carried out for the isolation identification and characterization of poultry *Salmonella*. The main purpose of this chapter is to get up-to-date information regarding the research work. For obtaining the distinct evidence and key information, the review of literature has been conveniently mentioned below in pleasing manner.

2.1. Isolation of *Salmonella* spp. from poultry samples

Islam *et al*. (2016) demonstrated a study of 80 cloacal swabs comprising of 50 samples of apparently healthy broiler and 30 samples of diarrheic broiler were collected from different poultry farms at Sylhet, Bangladesh. The samples were subjected for isolation and identification of *Salmonella* spp. through a series of conventional bacteriological studies like study of morphology, staining properties, and biochemical characteristics. In results, 48% (n= 24/50) swab samples of healthy broiler and 66.7% (n= 20/30) diarrheic broiler were found to be associated with *Salmonella* spp.

Abd-Elghany *et al.* (2015) reported the presence of *Salmonella* in 200 chicken samples, collected from Mansoura, Egypt. *Salmonella* was detected in 160A (8/50), 28% (14/50), 32% (16/50) and *60%* (30/50) of whole chicken carcasses, drumsticks, livers and gizzards, respectivel[y], with an overall prevalence of 34% (68/200) among all samples.

Al-Ferdous *et al*. (2013) demonstrated a study of total of 30 samples collected from the different layers of drums of pluck shops' were subjected to bacterial isolation and identification. Among the 27 positive *Salmonella* isolates, 11.11% (n=3) were *Salmonella pullorum*, 29.83% isolates (n = 8) were *Salmonella gallinarum* and the rest 59.26% isolates (n = 16) were *Salmonella typhimurium*.

Jahan *et al.* (2013) conducted a study from dressing water, device and environmental samples collected from pluck shops (cottage poultry processors) to isolate salmonella spp. 45%(27) bacterial isolates out of 60 samples were identified as *Salmonella* spp.

Rajagopal and Mini (2013) conducted a study that, avian salmonellosis is an important disease causing serious impediment to the development of poultry industry especially in developing countries of Asia and Africa. This report sheds light on three different outbreaks of salmonellosis in three different farms in Kerala (India) describing the disease diagnosis, antibiotic resistance and the suggested control measures. All the three isolates were revealed to be *Salmonella gallinarum*.

Hyeon *et al.* (2012) isolated *Salmonella* from 118 of the 180 samples (65.5%). *Salmonella* were detected in 105 samples (88%) plated on XLD and 111 samples (94%) plated on SM-ID 2 when RVS broth was used for enrichment, and 43 samples (36.4%) plated on XLD and 67 samples (56.8%) plated on SM-ID 2 when the MKTTn broth was used. The highest sensitivity was found in the RVS-XLD combination (0.99), followed by RVS-SM-ID 2 (0.97).

Kabir (2010) reviewed an article on avian colibacillosis and salmonellosis, age wise prevalence of avian salmonellosis showed highest infection rate in adult layers (53.25%) in comparison to brooding (14.55%), growing (16.10%) and pullet (16.10%) chickens. This article provides the vital information on the epidemiolog^{y}, pathogenesis, diagnosis, control and public health concerns of avian colibacillosis and salmonellosis.

Mahendra *et al.* (2006) conducted a cross-sectional study of raw meat samples obtained from the local meat market of Kathmandu Metropolitan City, Nepal, during September 2002-May 2003, with special emphasis on isolation and identification of *Salmonella* spp.

A total 123 raw meat samples (55 chicken meat, 37 buffalo meat and 31 goat meat) were collected and analysed relative to season. *Salmonella spp*. was found in 14 (11.4%) meat samples. Eight (14.5%) samples of chicken meat, 5 (13.5%) samples buffalo meat, and in one sample (3.3%) of goat meat were positive for *Salmonella*.

Sujatha *et al*. (2003) reported that **t**he liver of chicken was found to be the most suitable organ for isolation of *S. gallinarum*. Use of pre-enrichment media was better than conventional media for the successful isolation of the bacteria. Isolates revealed moist, pin-sized, circular, non-lactose fermenting colonies on MacConkey, S-S, BGA, and BHI agar media.

2.2. Identification of *Salmonella* spp.

2.2.1. Cultural characterization

Muktaruzzaman *et al.* (2010) mentioned that *Salmonella* organisms showed different cultural characteristics in different media. These were turbidity in Tetra Thionate broth, pink white color colonies in Brilliant Green agar, gray white colony in Nutrient agar, slightly grayish color colonies in *Salmonella Shigella* agar, black color colony in Tripple Suger Iron agar, pale color colonies in MaConkey's agar, well defined glistening colonies in Blood agar and pinkish colonies in EMB agar.

Hossain (2002) isolated *Streptococcus aureus, Escherichia coli* and *Salmonella* from diarrhoeic calves. The author stated that the *Salmonella spp.* produced small round and smooth colonies on nutrient agar and opaque, translucent and colorless colonies on SS agar. The organisms produced colourless, pale, transparent colonies on MacConkey agar and small, round, low convex, translucent, pale red colour colony on BGA against pinkish background which was initially green in colour.

Freeman (1985) stated that the optimum growth temperature of *Salmonella is 37°C,* but good growth is observed at room temperature.

Cheesbrough (1985) noted that on SS and MC agar *Salmonella* produce lactose non-fermenting colony. Most strains shows blackening of the colony due to H_2S production.

2.2.2. Morphological characterization by staining techniques

Samad (2005) stated that *Salmonella* are facultatively anaerobic, Gram negative bacilli and usually enter the body via the gastrointestinal tract where they can persist for longer period of time.

Gene (2002) reported the rod and short to long chain forming *Salmonella* organisms, which were isolated and identified from liver, spleen, intestinal contents of animals and birds.

2.2.3. Biochemical characterization and serogrouping of *Salmonella* spp.

Parvej *et al*. (2016) reported that among the 150 samples, 11 (7.33%) all were culturally and biochemically confirmed to be *Salmonella.* All possessed serovar-specific gene *SpeF* and reacted uniformly with group D antisera, suggesting that all of the isolates were *Salmonella Enterica* serovar *Gallinarum*, biovar *Pullorum* and/or *Gallinarum.*

Mahmud *et al*. (2011) reported that Serogrouping of *Salmonella* isolates was performed by slide agglutination test using commercial *Salmonella*-specific polyvalent O (A-I) antisera, *Salmonella* O group B (Factor O: 4, 5,27) antisera, and *Salmonella* O group D (Factor O: 9, 46) antisera. Among the 503 poultry samples, 106 *Salmonella* were isolated. Among the 106 isolates, 46 belonged to serogroup B (43%) and 60 isolates to serogroup D (57%) The most prevalent serogroup identified in this study was serogroup D.

Kwon *et al.* (2010) reported that the phenotypic analysis, *Salmonella gallinarum* strains (n=142) isolated during 2001 to 2007 showed the same pattern in the majority of the biochemical tests such as carbohydrate fermentation and amino acid decarboxylation. Interestingly, all of the strains could not ferment rhamnose, but SG 9R could, making rhamnose a potential biomarker to distinguish the vaccine strain.

Muktaruzzaman *et al*. (**2010)** conducted several types of biochemical media and reagents like bacteriological peptone, methyl red, phenol red, liquid paraffin wax, MR-VP media, potassium hydroxide, V-naphthol, alcohol and dulcitol were used in this study to identify salmonella isolates. In Methyl red test, Positive reaction was indicated by the persistence of red color, indication of acidity and the negative one by the yellow color. In Voges-Proskauer (V-P) test the appearance of pink color indicated positive test. In case of Indole test A red color in the reagent layer indicated indole and negative case, there was no development of red colour. In the carbohydrate fermentation test acid production was indicated by the color change from red to yellow of the medium and the gas production was noted by the appearance of gas bubbles in the inverted Durham's tube. Motility test was performed by the hanging drop slide method. The motile and non-motile organisms were identified by observing motility in contrasting with to and fro movement of bacteria.

Brooks *et al.* (2008) reported that the structure and serological specificities of the lipopolysaccharides (LPSs) from *Salmonella enterica* serovar *gallinarum* biovar *Pullorum* provided an improved basis for the distinction between antigenic types and the development of improved diagnostic tests. Several of the anti-LPS O-PS Mabs were specific for *S. pullorum a*nd other serogroup D1 *Salmonella,* and are potentially useful for the development of improved diagnostic tests for these organisms.

Sujatha *et al.* (2003) reported that all isolates of *Salmonella* showed positive reaction to M.R., citrate, nitrate, and H< sub>2</ sub>S. Sugar fermentation tests revealed acid without gas from glucose, maltose, dulcitol, galactose, trehalose, xylose, and rhamnose. All the isolates were confirmed as *S. gallinarum* with antigenic structure 9,12, by N.S.E.C.

Hossain *et al.* (2002) stated that among five basic sugars the *Salmonella* ferment dextrose, maltose and mannitol with production of acid and gas but no fermentation was observed in lactose and sucrose.

Shah *et al.* (2001) characterized *Salmonella enterica* subspecies *enterica* serovar *gallinarum* (*S. gallinarum*) strains (n=92) of avian origin isolated from Gujarat State by biotyping, antimicrobial drug resistance, bacteriocin production, and serum resistance. Variability in the phenotypic potentials associated with production of H_2S in TSI, ornithine decarboxylase activity, and fermentation of rhamnose were observed.

2.2.4. Molecular Characterization of *Salmonella* spp.

Dias *et al*. (2016) reported that samples from different steps of slaughtering and processing (n = 277) were collected from two chicken slaughterhouses (Sl1 and Sl2) located in Minas Gerais state, Brazil, and subjected to Salmonella spp. detection. The obtained isolates were subjected to serological identification and tested by PCR for specific *Salmonella* spp. genes (ompC and sifB). Also, *Salmonella* spp. isolates were subjected to XbaI macrorestriction and pulsed-field gel electrophoresis (PFGE). Sixty-eight samples were positive for *Salmonella* spp. and 172 isolates were obtained. Sl1 and Sl2 presented similar frequencies of *Salmonella* spp. positive samples during reception, slaughtering and processing ($p > 0.05$), except for higher frequencies in Sl1 for chicken carcasses after de-feathering and evisceration ($p < 0.05$).

Abd-Elghany *et al.* (2015) conducted a study to survey the presence of *Salmonella* in 200 chicken sarmples, collected from Mansoura, Egypt. *Salmonella* was detected in 160A (8/50), 28% (14/50), 32% (16/50) and *60%* (30/50) of whole chicken carcasses, drumsticks, livers and gizzards, respectivel[y], with an overall prevalence of 34% (68/200) among all samples. One hundred and sixty-six isolates were identified biochemically as *Salmonella,* and confirmed genetically by PCR, based on the presence in vA and stn genes.

Wang *et al.* (2015) demonstrated a study over one hundred and twenty six *Salmonella enteritidis* isolates recovered from 1152 retail raw poultries were characterized by antimicrobial susceptibility test, pulsed-field gel electrophoresis (PFGE), presence of quinolone resistance (Qnr) associated genes, Class I integron, extended spectrum beta-lactmases (ESBLs) encoding genes, and mutations in quinolone resistance-determining region (QRDR) of *GyrA* and *ParC.*

AL-Iedani *et al.* (2014) described that there are similarity in identification rate of *Salmonella* spp. between API 20 E system and PCR assay using *flic* gene. In this study using PCR amplification of *rfbsg* and *rfbsp* genes in differentiation of *Salmonella serovar gallinarum* into *S. gallinarum* and *S. pullorum* biovars very useful.

Barua *et al.* (2013) experimented a study that indicated local circulation of any motile *Salmonella* serovar in poultry has a wider public health impact beyond its source of origin for being dispersed elsewhere through poultry trades or human travels. The prevalence and serovar distribution of motile *Salmonella* a cross sectional survey was carried out by selecting 100 commercial broiler farms randomly. Five pooled faecal samples representing an entire housed flock of breeders or broilers were screened for presence of motile *Salmonella* following conventional bacteriological procedures.

Toboldt *et al.* (2013) demonstrated a study to investigate *Salmonella enterica* serovar strains in Germany between 2001 and 2011 from the environment, animal, food, and humans by phenotypic and genotypic methods to identify potential source of human infections and concluded that the potential sources of sporadic human infections with *S. enterica* serovar 4, 5, 12:b most likely are mushrooms, shellfish/ fish, and poultry.

Temelli *et al.* (2012) evaluated the capability of the Vitek immunodiagnostic assay system easy *Salmonella* (VIDAS ESLM) method and a specific real-time PCR system (Light Cycler), in detecting Salmonella from a total of 105 naturally contaminated samples comprised of poultry meat and poultry meat products. Twelve (33.33%), 11 (30.55%), and 18 (50.00%) out of 36 poultry meat samples were positive for Salmonella by ISO, VIDAS ESLM, and LCPCR, respectively. *Salmonella* detection rates from poultry meat products were 5.80% for ISO and 8.69% for LCPCR, whereas none of these products tested positive by VIDAS ESLM.

2.2.5. Antibiotic Susceptibility and resistance patterns of *Salmonella* spp.

Parvej *et al.* (2016) conducted a study of 150 samples, 11 (7.33%) produced characteristics pink colony with black center on XLD agar medium, and all were culturally and biochemically confirmed to be Salmonella. All possessed serovar specific gene SpeF and reacted uniformly with group D antisera, suggesting that all of the isolates were *Salmonella Enterica* serovar *Gallinarum*, biovar *Pullorum* and/or *Gallinarum*. Antimicrobial susceptibility testing revealed that 54.54% of the isolated *Salmonella Enterica* serovars were highly sensitive to ciprofloxacin, whereas the 81.81% isolates were resistant to amoxycillin, doxycycline, kanamycin, gentamycin, and tetracycline. Pulsed-field gel electrophoresis of the XbaI-digested genomic DNA exhibited identical

banding patterns, suggesting that the multidrug resistant *Salmonella Enterica* serovars occurring in commercial layers are highly clonal in Bangladesh.

Ramya *et al.* (2013) described that the sensitivity of *S. Enteritidis* was 100% for ciprofloxacin followed by chloramphenicol and amikacin (96%), gentamycin (90%), amoxicillin (82%), streptomycin (80%), tetracycline (76%), nalidixic acid (68%), ampicillin (58%) and sulfonamide (10%). The resistance was highest for sulfonamide (76%) followed by ampicillin (32%), nalidixic acid (30%) and 6-20% for gentamycin, amoxicillin and tetracycline.

Bae *et al.* (2013) reported that regarding the characteristics of their antibiotic resistance, 8 of the 11 ampicillin resistant isolates carried blaTEM only, two carried blaTEM and blaCTX-M-14 and one carried blaCTX-M-3 and only one AmR isolate with the blaCTX-M 3 β-lactamase gene *Salmonella* strain. Twenty seven *Salmonella* isolates showed nalidixic acid resistance with a mutation at amino acid codon Asp87 in gyrA and no mutation in the parC gene.

Li *et al.* (2013) identified a total of 165 *Salmonella enterica* isolates from 1382 samples taken from conventional farms, abattoirs and retail markets from 2010 to 2011 in Sichuan, China. Among these isolates, *S. enterica* serotypes *derby* (76 isolates, 46%) and *typhimurium* (16 isolates, 10%) were the most prevalent, and high antimicrobial resistance observed for tetracycline (77%), nalidixic acid (41%) and spectinomycin (41%).

De *et al.* (2012) stated that the antimicrobial susceptibility of *E. coli,* S*almonella* spp. in chicken. For *E. coli* and *Salmonella* spp. clinical resistance to newer compounds (Cefepime, cetotaxime and ciprofloxacin) was absent. Colistin sulphate resistance was absent for *E. coli* but apparent for *Salmonella* spp.

Imad *et al.* (2012) stated that antimicrobial susceptibility test of the 98 isolates of *Salmonella* revealed that 32.7% were resistant to one or more of the 24 antimicrobials tested. Generally, resistance for 13 different antimicrobial drugs was recognized. The most common resistance was to streptomycin (24/32, 75%), ampicillin (19/32, 59.4%), tetracycline (15/32, 46.9%).

Iwabuchi *et al.* (2011) described that among 452 *Salmonella* isolates, 443 (98.0%) were resistant to one or more antibiotics, and 221 (48.9%) showed multipleantibiotic resistance, thereby implying that multiple-antibiotic resistant *Salmonella* organisms are widespread in chicken meat in Japan. Resistance to oxytetracycline was most common (72.6%), followed by dihydrostreptomycin (69.2%) and bicozamycin (49.1%).

Lu *et al.* (2011) evaluated the antimicrobial resistance of *Salmonella* isolated in 2008 from a chicken hatchery, chicken farms, and chicken slaughterhouses in China. More than 80% of the *S. indiana* isolates were highly resistant to ampicillin (97.7%), amoxicillin/clavulanic acid (87.9%), cephalothin (87.9%), ceftiofur (85.7%), chloramphenicol (84.9%), florfenicol (90.9%), tetracycline (97.7%), doxycycline (98.5%), kanamycin (90.2%), and gentamicin (92.5%). About 60% of the S. *indiana* isolates were resistant to enrofloxacin (65.4%), norfloxacin (78.9%), and ciprofloxacin (59.4%). Of the *S. Indiana* isolates, 4.5% were susceptible to amikacin and 5.3% to colistin. Of the *S. enteritidis isolates*, 73% were resistant to ampicillin, 33.1% to amoxicillin/clavulanic acid, 66.3% to tetracycline, and 65.3% to doxycycline, whereas all of these isolates were susceptible to the other drugs used in the study.

Ellerbroek *et al.* (2010) isolated *Salmonella* from 400 imported chicken carcasses in Bhutan and from 178 pig carcasses in Vietnam for antibiotic resistance analyzed on a

random basis against 14 antimicrobial agents. Among the poultry samples tested, 13% were positive for Salmonella.

Wouafo *et al.* (2010) stated 150 chickens were collected from eight retail markets in Yaounde, and 90 (60%) tested positive for *Salmonella.* The isolates were tested for their susceptibilities to anioxicillin, amoxicillin/clavulanic acid, cefoxitin, cefotaxime, gentamicin, streptomycin, tetracycline, chloramphenicol, sulfonamides, nalidixic acid, ciprofloxacin, and trimethoprim/sulfamethazole by disk diffusion assay. Minimum inhibitory concentrations of ampicillin, streptomycin, tetracycline, sulfonamides, and nalidixic acid were determined for the resistant strains by agar dilution method. Eleven isolates (10.7%) of the 103 tested were susceptible to all antimicrobials. Resistance was most observed to tetracycline (84.5%), streptomycin (44.7%), and nalidixic acid (34%). Forty-one isolates (39.8%) were multidrug resistant (resistant to three or more antimicrobials from different classes), of which 68.3% were Hadar and 21.9% Enteritidis. The most frequent resistant pattern in Hadar was streptomycin-tetracycline--nalidixic acid. These results highlight once more the need for surveillance of *Salmonella* contamination in poultry.

Soomro *et al.* (2010) described that all *salmonella* isolates showed sensitivity to cefotaxime, ceftazidime, gentamicin, tobramycin, ciprofloxacin, ofloxacin, and chloramphenicol, whereas resistance to streptomycin, tetracycline, and ampicillin was also detected. A lower proportion of isolates were resistant to kanamycin and trimethoprim–sulphametoxazole.

Islam *et al.* (2008) isolated a total of 46 Salmonella spp. from 150 blood cultures. *Salmonella typhi* was predominant serotype, followed by *S. paratyphi*. A Results of Antimicrobial susceptibility pattern of *Salmonella paratyphi* against antibiotics showed

that isolates were sensitive to Amoxicillin (75%), Amoxyclave (75%), Aztreonam (100%), Amikacin (100%), Ceftriaxone (68.75%), Cephalexin (75%), Ciprofloxacin (50%), Co-trimoxazole (75%),Gentamycin (100%), Nalidixic acid (75%), Netilmicin (100%) and resistant to Amoxicillin (25%), Amoxyclave (25%), Azithromycin (100%), Ceftazidime (31.25%), Ceftriaxone (31.25%), Cephalexin (25%), Ciprofloxacin (50%).

Khan *et al.* (2005) reported the antibiogram study and plasmid profile analyses to find out the correlation of the recently isolated *Salmonella* spp. in Bangladesh. Antibiogram study revealed that the isolates were highly sensitive to ciprofloxacin, cephalexin and kanamycin but resistance to cloxacillin, erythromycin, cloxacillin.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

Study Place and Period

The present study was conducted during the period of January 2016 to June 2016 at the Department of Microbiology of Hajee Mohammad Danesh Science and Technology University, Dinajpur. The samples were collected from hatchery, different selected broiler farms and live bird markets Sadar and Kaharo Upazilla under Dinajpur district of Bangladesh.

3.1. Materials

3.1.1. Sample Selected for Study

A total of 153 samples were collected from poultry production chains of Dinajpur district of Bangladesh. The collected samples are presented in Table 1.

Table 1: Different samples were collected from poultry production chains

Samples	No. of collected samples (153)	
	Sadar	**Kaharol**
Cloacal swab	35	14
Chick meconium	25	10
Whole Carcass	22	08
Feed	10	04
Water	12	04
Transport swab	05	02
Floor swab	02	-
Total	**111**	**42**

3.1.2. Materials used for sample collection

Apron, mask, sterile hand gloves, sterile packs, sterile cotton bar, 70% ethanol, 0.85% Normal Saline and icebox were used for sample collection.

3.1.3. Solid culture media

Brilliant Green Agar (BGA), Xylose-Lysine Deoxycholate Agar (XLD), Mueller Hinton agar were used as solid culture media for this study.

3.1.4. Liquid culture media

The liquid media used in the study were nutrient broth (NB), peptone broth, methyl-red and voges-proskauer broth (MR-VP), and sugar media.

3.1.5. Chemicals and reagents

The chemicals and reagents used for the study were 70% ethanol, normal saline solution, reagents for Gram's staining (methylene blue, Gram's iodine, safranin, acetone alcohol), immersion oil, 3% hydrogen peroxide, oxidase test reagent (Tetramethyl phenylenedimine dihydrochloride), Kovac's indole reagent (4- diethylamino-benzaldehyde, concentrated HCl), mineral oil, 80% & 15% glycerin and other common laboratory reagents and chemicals.

3.1.6. Glass wares and other appliances

The glass wares and appliances were used during the whole period of the experiment are as follows: scalpel, forceps, scissors, tray, petridishes, test tubes, conical flask, pipette (1, 5, 10 and 25 ml capacities), micro pipettes (100-1000 µl, 20-200 µl, 2-20 µl, 1-10 µl, 0.1-2.5 µl), slides, hanging drop slides, glass rod spreader, test tube racks, water bath, bacteriological incubator, refrigerator, sterilizing instruments, hot air oven, centrifuge

tubes and machine, ice boxes, electronic balance, syringe and needle, compound microscope.

3.1.7. Antibiotic disc

Commercially available antibiotic discs (Himedia, India) were used to determine the antimicrobial susceptibility patterns. The antibiotics that were used against the isolated bacterial species as presented in the Table 2.

Table 2. Antimicrobial disc with their disc concentration

Name of the antibiotics	Symbol	Disc concentration (µg/disc)
Amoxycillin	AMX	30
Azithromycin	AZM	30
Ciprofloxacin	CIP	5
Erythromycin	E	30
Gentamicin	GEN	10
Norfloxacin	NOR	10
Streptomycin	S	10
Tetracycline	TE	30

3.2. Methods

3.2.1. Experimental layout

The entire laboratory works for this study were performed at bacteriology laboratory in the Department of Microbiology, HSTU, Dinajpur. The entire experiment was accomplished in following steps. The samples were collected and transported to the bacteriology laboratory in the Department of Microbiology, HSTU, Dinajpur. Randomly selected samples were used for isolation of bacteria.

Experimental layout

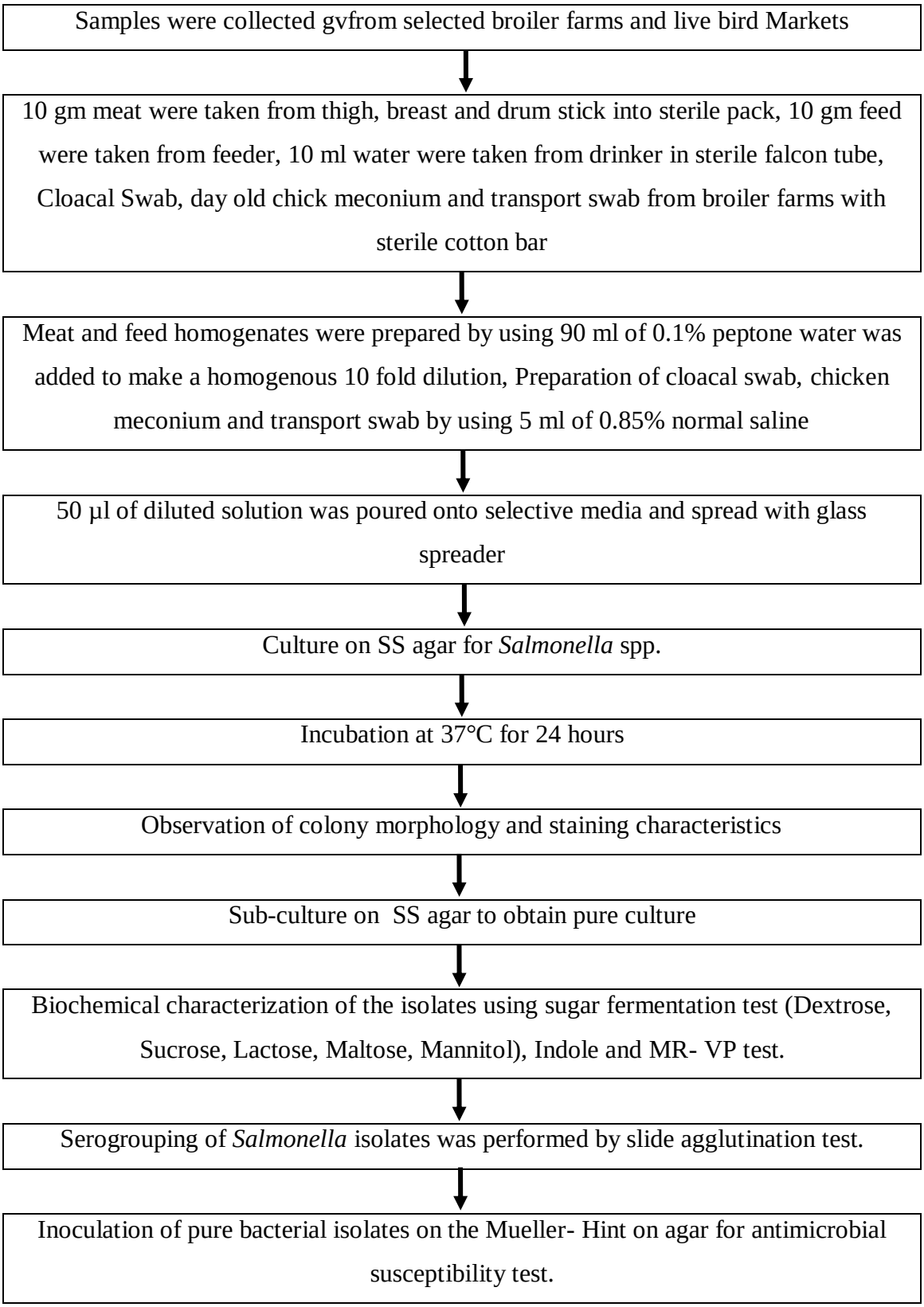


Figure 1. **Flow chart of the experimental layout**

3.2.2. Collection and transportation of samples

A total of 153 samples were collected from 2 different upazilla of Dinajpur district. During the collection of samples precautionary measures were maintained to avoid touch and ice box were used to maintain cool chain. The samples were then brought to the laboratory of the Department of Microbiology, HSTU, Dinajpur.

3.2.3. Preparation of culture media and reagents

3.2.3.1 Preparation of culture media

Salmonella-Shigella agar (SS) (Hi-media, India), Xylose Lysine Deoxycholate agar (XLD) (Hi-media, India), and Mueller Hint on agar (Hi-media, India), were used for this study. All these media were prepared according to direction of manufacturer.

3.2.4. Preparation of reagents

3.2.4.1. Sugar media

Sugar media consisted of peptone water to which fermentable sugar was added to the proportion of 1 percent. Peptone water was prepared by adding one gram of bacto peptone and 0.5 grams of sodium chloride in 100 ml distilled water. The medium was boiled for 5 minutes, adjusted to pH 7.0, cooled and then filtered through filter paper. Phenol red, an indicator at the strength of 0.2 percent solution was added to peptone water and then dispensed in 5 ml amount into cotton plugged test tubes containing a Durham's tubes, placed inversely. These were then sterilized in autoclave at 121°C for 15 minutes maintaining a pressure of 15 lbs per sq. inch (1 kg/ cm^2). The sugars used for fermentation were prepared separately as 10 percent solutions in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A gentle heat was necessary to dissolve the sugar completely and sterilized by stem sterilizer. Before use, the sterility of

each sugar medium was checked by incubating the tubes overnight at 37°C. The basic sugars dextrose, maltose, lactose, sucrose and mannitol were used to prepare sugar medium.

3.2.4.2. MR-VP broth

A quantity of 3.4 gm of bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the tubes were autoclaved at 121°C for 15 minutes maintaining a pressure of 15 lbs per sq. inch (1 kg/ cm2). After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and then stored in a refrigerator for future use.

3.2.4.3. Methyl-Red solution

The indicator methyl-red (MR) solution was prepared by adding 0.1 gm of Methyl red powder in 300 ml of 95% alcohol and diluting this to 500 ml with the adding of 200 ml of distilled water.

3.2.4.4. Voges-Proskauer solution

3.2.4.5. Alpha- naphthol solution

Alpha- naphthol solution was prepared by dissolving 5 gm of α-naphthol in 100 ml of 95% ethyl alcohol

3.2.4.6. Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by dissolving 40 gm of Potassium hydroxide crystals in 100 ml of cold distilled water.

3.2.4.7. Normal saline solution

Normal saline solution was prepared by adding 0.85 gm of crystalline sodium chloride in 100 ml of distilled water in a sterilized flask and autoclaved at 121°C for 15 minutes maintaining a pressure of 15 lbs per sq. inch (1 kg/ cm^2).

3.2.5. Procedure for bacterial isolation and identification

3.2.5.1. Method for obtaining pure culture

Pure culture of *Salmonella* spp. were obtained as per the methods described by Corry *et al*. (1995). 10 gm of samples were homogenized with 90 ml of 0.1% peptone water and 50 µl of homogenized sample was poured on to selective agar media and spread with glass spreader and incubated at 37°C for 24 hours. Single colony appeared on the selective media was further streaked onto selective media to obtain pure cultures.

3.2.5.2. Staining of isolated bacteria

3.2.5.2.2. Gram's staining

The representative bacterial colonies were characterized morphologically using Gram's stain according to the method describe by Merchant and Packer (1967). The procedure was as follows: A small colony was picked up from agar plates with a bacteriological loop, smeared on separate glass slide and fixed by gentle heating. Crystal violate was then applied on each smear to stain for two minutes and then washed with running water. Few drops of Gram's iodine were then added to act as mordent was then added (acts as decolorizer) for few seconds. After washing with water, Safranin was added as counter stain and allowed to stain for 2 minutes. The slides were then washed with water, blotted and dried in air and then examined under microscope with high power objective (100X) using immersion oil.

3.2.5.3. Motility test

The motility test was performed according to the method described by (Cowan, 1985) to differentiate motile bacteria from non-motile one. A single colony was picked up and placed into an eppendorf tube containing normal salaine solution. Homogenous solution was prepared by mixing by vortex. One drop of mixed solution was placed on the cover slip and was placed inverted over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the concave depression of the hanging drop slide for better attachment of the cover slip to prevent air current and evaporation of the fluid, the hanging drop slide was then examined carefully under 100X power objective of a compound microscope using immersion oil. The motile and non-motile bacteria were identified by observing motility in contrasting with Brownian movement of bacteria.

3.2.5.4. Biochemical tests

3.2.5.4.1. Sugar fermentation test

The sugar fermentation test was performed by inoculating a loop full of NB culture of the isolated bacteria into each tube containing five basic sugars (e. g., dextrose, sucrose, lactose, maltose, and mannitol) separately and incubated for 24 hours at 37°C. Acid production was indicated by the color change reddish to yellow in the medium and the gas production was noted by the appearance of gas bubbles inside inverted Durham's tube.

3.2.5.4.2. Catalase test

This test was used to differentiate bacteria, which produce the enzyme catalase, such as *Salmonella*, *Staphylococci*, *Campylobacter* from that non-catalase one such as,

Streptococci. To perform this test, a small colony of good growth pure culture of isolated bacteria was smeared on a slide. Then one drop of catalase reagent (3% H_2O_2) was added on the smear. The slide was observed for bubble formation. Formation of bubble within few seconds was the indication of positive test while the absence of bubble formation indicated negative result (Cheesbrough, 1985).

3.2.5.4.3. Indole test

Two milliliter of peptone water was added with the 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) was added, shaked well and examined after 1 minute. A red color in the reagent layer indicated indole. In negative case no development of red color was observed (Cheesbrough, 1985).

3.2.5.4.4. Methyl red test

The test was conducted by inoculating a colony of the isolated bacteria in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of methyl red solution was added. A red coloration was positive and indicates an acid PH resulting from the fermentation of glucose. A yellow coloration indicated negative result (Cheesbrough, 1985).

3.2.5.4.5. Voges-Proskauer test

Two milliliter of sterile glucose phosphate peptone water was inoculated with the 5 ml of isolated bacteria. It was incubated at 37°C for 48 hours. A very small amount of creatine was added and mixed. Three milliliter of sodium hydroxide was added and shaked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink color for positive cases. In case of negative cases there was no development of pink color (Cheesbrough, 1985).

3.2.7. Serogrouping of *Salmonella* by O-antigen test

One drop of saline (0.85% NaCl) was added as control on a glass slide by the use of a wire. A loop full of culture from the Nutrient agar (NA) was plate transferred onto the glass slide and mixed with the drop of saline. Agglutination within 30 seconds indicated that it's rough strains. The strains can't be used for serotyping. Proceeded serotyping with antisera if no agglutination were recorded. Added one drop of salmonella agglutinating antisera Poly A-I on each test area on the slide. Added a loop full of culture from NA plate to each spot of antiserum. Mixed carefully the culture with the O-serum. Rocked the glass slide gently for one minute. Agglutination with the antisera indicated that the strain has an O-antigen. It was a screen procedure. Then tested with O group B and O group D. Some strain agglutinated with O group B (O:4,5,27) and Some strain agglutinated with O group D (O:9,46). Concerning to O factor of each group referred as Antigenic formulas of the Salmonella Serovers 9th edition, 2007, WHO Collaborating centre for Reference and Research on Salmonella, Institute Pasteur, Paris, France.

3.2.8. Antimicrobial susceptibility test

All isolates that are selected were tested for antimicrobial drug susceptibility against eight commonly used antibiotics by disc diffusion method (Bauer *et al.*, 1966) then followed the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2007).

The procedure of disc diffusion method is presented below:

- One well isolated colony was selected from the XLD agar plates.
- Colony was picked up with a sterile loop and streaked onto nutrient broth and incubated overnight at 37°C.
- After overnight incubating bacterial suspension was mixed with PBS.

- The bacterial suspension was compared with 0.5 MacFarland standards. The comparison was made by viewing this tube against a sheet of white paper on which black lines were drawn.
- A sterile cotton bud was dipped into the bacterial suspension. The excess fluid of swab was removed by pressing firmly against the inside of the tube just above the fluid level.
- The bud was streaked over the entire surface of Mueller-Hinton agar (Himedia, India) medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums.
- The antimicrobial discs were placed individually using sterile forceps and then gently press down onto the agar.
- The plates were inverted and incubated at 37°C for overnight. After incubation the diameter of the zone of complete inhibition (including diameter of the discs) was measured in millimeters with slide calipers.

3.2.8.1. Recording and interpreting results

The zones of inhibition was compared with the zone size interpretative tables (Table 3) provided by Clinical and laboratory Standards Institute (CLSI, 2007). Antimicrobial testing results were recorded as susceptible, intermediately susceptible and resistant according to zone diameter interpretative standards provided by (CLSI, 2007).

Table 3. Zone diameter interpretive standards for *Salmonella* spp.

Antimicrobial agents	Zone diameter standard		
	Resistant	**Intermediate**	**Susceptible**
Amoxycillin	≤13	14-16	≥17
Azithromycin	≤13	14-17	≥18
Ciprofloxacin	≤15	16-20	≥21
Erythromycin	≤13	14-22	≥23
Gentamicin	≤12	13-14	≥15
Norfloxacin	≤12	13-16	≥17
Streptomycin	≤12	-	≥13
Tetracycline	≤14	15-18	≥19

3.2.9. Maintenance of stock culture

During the experiment period, it was necessary to preserve the isolated bacterial species for long period. For this purpose, pure cultures of the isolated bacteria were kept as stock culture and this was done by using 80% buffered glycerin. 0.5 m1 80% glycerin and 1 ml bacterial culture were taken in an eppendrof tube, mixed well and then stored at -80°C. The isolated bacteria were given code name for convenience.

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

4.1. Results of isolation and identification of *Salmonella* spp.

A total of 153 samples [comprises 30 meat samples from composite of thigh, breast and drumstick, 49 cloacal swabs, 35 chick meconium, 7 transport swabs, 14 feed samples, 16 water samples, 2 floor swabs] were subjected to isolation and identification of *Salmonella* spp. A total of 36 *Salmonella* like organisms were selected for identification of *Salmonella* spp. as presented in Table 12.

4.1.1. Results of cultural examination

Broth

Nutrient broth inoculated with the samples revealed the growth of *Salmonella* after 24 hours of incubation at 37°C. The growth of *Salmonella* was indicated by the presence of turbidity.

Solid media

Salmonella spp. shown translucent, black smooth round colonies on Xylose-Lysine Deoxycholate (XLD) and Brilliant Green Agar (BGA) agar media after 24 hours of incubation at 37ºC as presented in Table 6 and Plate 1.

4.1.1.2. Results of Gram's staining

In Gram's staining, organism revealed as pink colored short rod shaped bacteria arranged in single or paired as presented in Table 6 and Plate 2.

4.1.1.3 Results of motility test

The motile and non-motile *Salmonella* spp. were isolated by observing motility in contrasting with Brownian movement of *Salmonella*. The results of motility test are presented in Table 6.

Table 4. Results of cultural, morphological and motility characteristics of isolated *Salmonella* spp.

Colony morphology		Staining characteristics	Motility
Xylose-Lysine Deoxycholate agar	Salmonella-Shigella agar	Pink short rod shaped, gram negative bacteria arranged in single or paired.	+ve or -ve
Black centered colony.	Translucent, black smooth round colonies.		

Legends: "-Ve"= negative, "+Ve" = positive.

4.1.2. Results of different biochemical test

The isolates were checked and confirmed by their purity using XLD and SS agar media. Then a series of selective biochemical tests for identification of *Salmonella* spp. were performed as presented in Table 7.

4.1.2.1. Results of catalase test

All the isolates of *Salmonella* spp. were found positive as the isolates produced bubbles in catalase test. The results are presented in Plate 2.

4.1.2.2. Results of sugar fermentation test

All the isolates of *Salmonella* spp. were fermented dextrose, maltose and mannitol with acid and gas production but did not fermented sucrose and lactose as presented in Table 7 and Plate 3.

4.1.2.3. Results of methyl red test

Salmonella spp. were positive to MR test. The test was conducted by inoculating a colony of the isolated *Salmonella* in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of methyl red solution was added. A red coloration was produced and indicated an acid PH resulting from the fermentation of glucose as presented in Table 7 and Plate 3.

4.1.2.4. Results of Voges-Proskauer and Indole test

All the isolates of *Salmonella* spp. were negative to VP and Indole test as no development of pink or red color was observed. The results of Voges-Proskauer and Indole test are presented in Table 7 and Plate 4.

Table 5. Biochemical reaction patterns of *Salmonella* spp.

Bacteria	Sugar fermentation properties					MR	VP	Indole
	Dextrose	Maltose	Sucrose	Lactose	Mannitol			
Salmonella spp.	AG	AG	-ve	-ve	AG	+ve	-ve	-ve

Legends: MR= Methyl red, VP = Voges- proscaure reaction. , AG = Acid and Gas, "-Ve"= negative, "+Ve" = positive.

4.1.4. Results of *Salmonella* spp. obtained from poultry production chains

4.1.4.1. Results of *Salmonella* spp. in chick meconium

Results of isolation and identification of *Salmonella* spp. in chick meconium are presented in Table 8. A total of 35 samples were collected and 4 samples were positive for *Salmonella* spp.

Table 6. Results of *Salmonella* spp. in collected sample of chick meconium (CM)

Type of Sample	Placement	Sample No.	Sample ID	Result
				***Salmonella* spp.**
Chick meconium	Sadar Dinajpur.	1	08Apr031030115CM1	Negative
		2	08Apr031030115CM2	Negative
		3	08Apr031030115CM3	Negative
		4	08Apr031030115CM4	Negative
		5	08Apr031030115CM5	Negative
		6	13Apr03103011CM1	**Positive**
		7	13Apr031030111CM2	Negative
		8	13Apr031030111CM3	Negative
		9	13Apr031030111CM4	Negative
		10	13Apr03103011CM5	Negative
	Kaharol Dinajpur	11	22Mar021020104CM1	Negative
		12	22Mar021020104CM2	Negative
		13	22Mar021020104CM3	Negative
		14	22Mar021020104CM4	Negative
		15	22Mar021020104CM5	Negative
		16	23Mar021020105CM1	Negative
		17	23Mar021020105CM2	**Positive**
		18	23Mar021020105CM3	**Positive**
		19	23Mar021020105CM4	Negative
		20	23Mar021020105CM5	Negative
		21	10Apr021020109CM1	Negative
		22	10Apr021020109CM2	Negative
		23	10Apr021020109CM3	Negative
	Sadar, Dinajpur	24	10Apr021020109CM4	Negative
		25	10Apr021020109CM5	Negative
		26	11 Mar021020312CM1	Negative
		27	11 Mar021020312CM2	Negative
		28	11 Mar021020312CM3	Negative
		29	11 Mar021020312CM4	Negative
		30	11 Mar021020312CM5	**Positive**
		31	28 Mar021020302CM1	Negative
		32	28 Mar021020302CM2	Negative
		33	28 Mar021020302CM3	Negative
		34	28 Mar021020302CM4	Negative
		35	28 Mar021020302CM5	Negative

4.1.4.2. Results of *Salmonella* spp. in cloacal swabs

Results of isolation and identification of *Salmonella* spp. in cloacal swabs are presented in Table 9. A total of 49 samples were collected and 10 samples were positive for *Salmonella* spp.

Table 7. Results of *Salmonella* spp. in collected sample of cloacal swabs (CS).

Type of Sample	Placement	Sample No.	Sample ID	Result
				***Salmonella* spp.**
Cloacal swabs	Sadar, Dinajpur	1	12Mar021020104CS1	**Positive**
		2	12Mar021020104CS2	Negative
		3	12Mar021020104CS3	**Positive**
		4	12Mar021020104CS4	Negative
		5	12Mar021020104CS5	**Positive**
		6	12Mar021020104CS6	Negative
		7	12Mar021020104CS7	Negative
		8	14Mar021020302CS1	Negative
		9	14Mar021020302CS2	Negative
		10	14Mar021020302CS3	Negative
		11	14Mar021020302CS4	Negative
		12	14Mar021020302CS5	**Positive**
		13	14Mar021020302CS6	Negative
		14	14Mar021020302CS7	**Positive**
		15	24Mar021020110CS1	Negative
		16	24Mar021020110CS2	Negative
		17	24Mar021020110CS3	Negative
		18	24Mar021020110CS4	Negative
		19	24Mar021020110CS1	**Positive**
		20	24Mar021020110CS2	**Positive**
		21	24Mar021020110CS3	Negative
	Kaharol, Dinajpur	22	13Mar021020102CS1	Negative
		23	13Mar021020102CS2	Negative
		24	13Mar021020102CS3	Negative
		25	13Mar021020102CS4	Negative
		26	13Mar021020102CS5	Negative
		27	13Mar021020102CS6	**Positive**
		28	13Mar021020102CS7	**Positive**
		29	27Mar021020311CS1	Negative
		30	27Mar021020311CS2	**Positive**

4.1.4.3. Results of *Salmonella* spp. in feed

Results of isolation and identification of *Salmonella* spp. in feed are presented in Table 10. A total of 14 samples were collected and 4 samples were positive for *Salmonella* spp.

Table 8. Results of *Salmonella* spp. in collected feed (F) samples.

Type of Sample	Placement	Sample No.	Sample ID	Result
				***Salmonella* spp.**
Feed	Sadar, Dinajpur	1	12Mar021020104F1	Negative
		2	12Mar021020104F2	**Positive**
		3	14Mar021020302F1	Negative
		4	14Mar021020302F2	Negative
		5	24Mar021020110F1	Negative
		6	24Mar021020110F2	Negative
	Sadar, Dinajpur	7	13Mar021020102F1	**Positive**
		8	13Mar021020102F2	Negative
		9	27Mar021020311F1	Negative
		10	27Mar021020311F2	Negative
	Kaharol, Dinajpur	11	17Mar031030111F1	**Positive**
		12	17Mar031030111F2	**Positive**
		13	18Mar031030115F1	Negative
		14	18Mar031030115F2	Negative

4.1.4.4. Results of *Salmonella* spp. in transport swabs

Results of isolation and identification of *Salmonella* spp. in transport swabs are presented in Table 11. A total of 7 samples were collected and 2 samples were positive for *Salmonella* spp.

Table 9. Results of *Salmonella* spp. in collected samples of transport swabs.

Type of Sample	Placement	Sample No.	Sample ID	Result
				***Salmonella* spp.**
Transport swabs	Sadar, Dinajpur	1	14Mar02T	**Positive**
		2	23Mar02T	Negative
		3	24Mar02T	Negative
	Kaharol, Dinajpur	4	27Mar02T	Negative
		5	13Mar02T	Negative
	Sadar, Dinajpur	6	17Mar03T	**Positive**
		7	18Mar03T	Negative

4.1.4.5. Results of *Salmonella* spp. in water sample

Results of isolation and identification of *Salmonella* spp. in water as presented in Table 12. A total of 14 water samples were collected and 1 samples shown positive result for *Salmonella* spp.

Table 10. Results of *Salmonella* spp. in collected water samples

Type of Sample	Placement	Sample No.	Sample ID	Result
				***Salmonella* spp.**
Water	Sadar, Dinajpur	1	12Mar021020104W_1	Negative
		2	12Mar021020104W_2	Negative
		3	14Mar021020302W_1	Negative
		4	14Mar021020302W_2	Negative
		5	24Mar021020110W_1	Negative
		6	24Mar021020110W_2	Negative
	Kaharol, Dinajpur	7	13Mar021020102W_1	Negative
		8	13Mar021020102W_2	Negative
		9	27Mar021020311W_1	Negative
		10	27Mar021020311W_2	Negative
	Sadar, Dinajpur	11	17Mar031030111W_1	Negative
		12	17Mar031030111W_2	Negative
		13	18Mar031030115W_1	**Positive**
		14	18Mar031030115W_2	Negative

4.1.4.6. Results of *Salmonella* spp. in broiler meat

Results of isolation and identification of ***Salmonella*** spp. in broiler meat were presented in Table 13. A total of 28 samples (thigh, breast and drumstick) were collected and 9 samples were positive for *Salmonella* spp.

Table 11. Results of *Salmonella* spp. in collected broiler meat samples

Type of Sample	Placement	Sample No.	Sample ID	Result
				***Salmonella* spp.**
Broiler meat	Bahadur Bazar	1	12Mar021020104WC1	**Positive**
		2	12Mar021020104WC2	Negative
		3	12Mar021020104WC3	Negative
		4	12Mar021020104WC4	**Positive**
		5	14Mar021020302WC1	Negative
		6	14Mar021020302WC2	**Positive**
		7	14Mar021020302WC3	Negative
		8	14Mar021020302WC4	**Positive**
		9	24Mar021020110WC1	Negative
		10	24Mar021020110WC2	Negative
		11	24Mar021020110WC3	Negative
		12	24Mar021020110WC4	Negative
	Railbazar	13	13Mar021020102WC1	**Positive**
		14	13Mar021020102WC2	**Positive**
		15	13Mar021020102WC3	Negative
		16	13Mar021020102WC4	Negative
		17	27Mar021020311WC1	Negative
		18	27Mar021020311WC2	Negative
		19	27Mar021020311WC3	Negative
		20	27Mar021020311WC4	Negative
	Bahadur Bazar	21	17Mar031030111WC1	Negative
		22	17Mar031030111WC2	Negative
		23	17Mar031030111WC3	Negative
		24	17Mar031030111WC4	Negative
		25	18Mar031030115WC1	**Positive**
		26	18Mar031030115WC2	**Positive**
		27	18Mar031030115WC3	Negative
		28	18Mar031030115WC4	**Positive**

Legends: F= Feed, W= Water, WC=Whole carcass (broiler meat), CS = Cloacal swabs, T= Transport swabs and CM= Chick meconium.

4.1.4.7. Results of *Salmonella* spp. obtained from live bird markets

A total of 6 samples collected from live bird markets and all 6 sample were positive for *Salmonella* spp. Results of *Salmonella* spp. in different samples collected from 2 live bird markets as presented in Table 14.

Table 12. Results of *Salmonella* spp. in collected samples from live bird markets.

Type of Sample	Placement	Sample No.	Sample ID	Result
				***Salmonella* spp.**
Broiler meat	Bahadur Bazar	1	14Mar02LWC	**Positive**
	Railbazar	2	12Mar02LWC	**Positive**
Floor swabs	Sadar, Dinajpur	3	14Mar02FS	**Positive**
	Kaharol, Dinajpur	4	12Mar02FS	**Positive**
Water	Sadar, Dinajpur	5	14Mar02W	**Positive**
	Kaharol, Dinajpur	6	12Mar02W	**Positive**

Legends: FS = Floor swabs, W= Water and LWC= Live whole carcass (broiler meat).

4.1.5. Summary of the results of *Salmonella* spp. from poultry production chains

From sadar of Dinajpur District a total of 111 samples were collected from 3 broiler farms and 25 samples were positive for *Salmonella* spp. (Table 16). The positive (%) samples of isolated *Salmonella* spp. in collected cloacal swabs, feed, chick meconium, transport swabs and broiler meat were 5 (23.8%), 1 (16.6%), 2 (13.3%), 1 (33.3%) and 4 (33.3%) respectively (Table 15). There were absence of *Salmonella* spp. in water samples in selected broiler farms of Sadar, Dinajpur.

So, A total of 105 samples were collected from 5 broiler farms of Dinajpur district and 19 (18.09%) samples were shown positive for *Salmonella* spp. (Table 15). Out of 105 samples, 35 cloacal swabs, 10 feed, 10 water, 20 broiler meat, 25 chick meconium and 5 transport swabs were subjected for *Salmonella* spp. (Table 15). Out of 35 cloacal swabs

samples 7 (20%) shown positive, out of 10 feed samples 2 (20%) shown positive, out of 25 chick meconium samples 3 (12%) shown positive, out of 20 broiler meat samples 6 (30%) shown positive and out of 5 transport swab samples 1 (20%) shown positive for *Salmonella* spp. (Table 15). The highest percentages of *Salmonella* spp. were observed in collected samples of cloacal swabs 7 (20%) from Kaharol of Dinajpur district (Table 15).

In Kaharol Upazila under Dinajpur district, a total of 42 samples were collected from 2 broiler farms and 11 (26.19%) samples were positive for *Salmonella* spp. (Table 15). The positive (%) samples of isolated *Salmonella* spp. in collected cloacal swabs, feed, water, chick meconium, transport swabs and broiler meat were 3 (21.4%), 2 (25%), 1 (25%), 1 (10%), 1 (50%) and 3 (37.5%) respectively (Table 15).

In this study, a total of 153 samples were collected from 3 broiler farms of Sadar, Dinajpur; 2 broiler farms of Kaharol upazilla of Dinajpur district and 2 broiler farms from Sadar of Dinajpur district in Bangladesh. Out of 153 samples, 36 (20.4%) samples have shown positive for *Salmonella* spp. (Table 16).

In this study, a total of 6 samples were collected from 2 live bird markets of Dinajpur district. All 6 (100%) samples were positive positive for *Samonella* spp. in collected broiler meat, floor swab and water samples of Kaharol upazilla in Dinajpur district as presented in Table 15.

Table 13. Summary of isolated *Salmonella* spp. from poultry production chains

Placement (no. of farms)	No. of samples collected							*Salmonella* spp. No. of isolates (%)						
	CS	F	W	WC	T	CM	Total	CS	F	W	WC	T	CM	Isolates
Sadar, Dinajpur (3)	21	6	6	12	3	15	63	5 (23.8)	1 (16.6)	0 (0)	4 (33.3)	1 (33.3)	2 (13.3)	13 (20.6)
Kaharol, Dinajpur (2)	14	4	4	8	2	10	42	2 (14.2)	1 (25)	0 (0)	2 (25)	0 (0)	1 (10)	6 (14.2)
Total (Dinajpur) (5)	35	10	10	20	5	25	105	7 (20)	2 (20)	0 (0)	6 (30)	1 (20)	3 (12)	19 (18.09)
Sadar, Dinajpur (2)	14	4	4	8	2	10	42	3 (21.4)	2 (25)	1 (25)	3 (37.5)	1 (50)	1 (10)	11 (26.19)
Gross Total (7)	49	14	14	28	7	35	147	3 (21.4)	2 (25)	1 (25)	3 (37.5)	1 (50)	1 (10)	11 (26.19)
Live bird markets (2)	**LWC**		**W**		**FS**		6	**LWC**		**W**		**FS**		**Isolates**
	2		2		2			2 (100%)		2 (100%)		2 (100%)		6 (100%)

Legends: F= Feed, FS = Floor swabs, W= Water, WC=Whole carcass (broiler meat), LWC= Live whole carcass, CS = Cloacal swabs, T= Transport swabs and CM= Chick meconium.

Table 14. Total *Salmonella* spp. isolated from poultry production chains of Kaharol and Sadar, Dinajpur districts.

District (No. of samples)	No. of *Salmonella* spp. isolated samples (%)
Sadar Dinajpur (111)	25 (22.52)
Kaharol (42)	11 (26.19)
Total (153)	**36 (23.53)**

4.1.6. Result of *Salmonella* spp. serogrouping

Serogrouping of *Salmonella* isolates was performed by slide agglutination test using commercial *Salmonella* specific polyvalent O (A-I) antisera, *Salmonella* O group B (Factor O: 4,5,27) antisera and *Salmonella* O group D (Factor O: 9,46) antisera (S&A Reagent Lab).The test was performed according to the protocol supplied by the manufacturer. All isolates were positive to *Salmonella* Poly A-I antisera and then some were positive to *Salmonella* O group B antisera and some were positive to *Salmonella* O group D antisera as presented in Table 17, 18 and Plate 6, 7.

Table 15. Serogrouping of *Salmonella* isolates by slide agglutination test

Isolate no.	Result		
	Poly A-I	**Group B (O:4,5,27)**	**Group D (O:9,46)**
1	Positive	Negative	Positive
2	Positive	Negative	Positive
3	Positive	Negative	Positive
4	Positive	**Positive**	**Negative**
5	Positive	**Positive**	**Negative**
6	Positive	Negative	Positive
7	Positive	Negative	Positive
8	Positive	Negative	Positive
9	Positive	**Positive**	**Negative**
10	Positive	Negative	Positive
11	Positive	Negative	Positive
12	Positive	Negative	Positive
13	Positive	**Positive**	**Negative**
14	Positive	Negative	Positive
15	Positive	**Positive**	**Negative**
16	Positive	**Positive**	**Negative**
17	Positive	Negative	Positive
18	Positive	Negative	Positive
19	Positive	**Positive**	**Negative**
20	Positive	Negative	Positive
21	Positive	Negative	Positive
22	Positive	Negative	Positive
23	Positive	Negative	Positive

24	Positive	Negative	Positive
25	Positive	**Positive**	**Negative**
26	Positive	Negative	Positive
27	Positive	Negative	Positive
28	Positive	**Positive**	**Negative**
29	Positive	**Positive**	**Negative**
30	Positive	**Positive**	**Negative**
31	Positive	Negative	Positive
32	Positive	Negative	Positive
33	Positive	Negative	Positive
34	Positive	Negative	Positive
35	Positive	Negative	Positive
36	Positive	Negative	Positive

Table 16. Summary of *Salmonella* spp. serogrouping

Isolates	**No. (%) of *Salmonella* spp. isolates**		
Salmonella spp. (n=36)	Poly A-I	Group B (O:4,5,27)	Group D (O:9,46)
	36 (100)	11 (30.56)	25 (69.44)

4.1.7. Results of antimicrobial study

4.1.7.1. Results of antimicrobial susceptibility of *Salmonella* spp.

All 36 *Salmonella* isolates were subjected to antimicrobial susceptibility testing against 8 selected antibiotics. The results of susceptibility analysis showed that all the 36 (100%) *Salmonella* isolates were susceptible to gentamicin, norfloxacin, ciprofloxacin and streptomycin. All isolates of *Salmonella* spp. 100% (n=36) were resistant to tetracycline and erythromycin, whereas 17 (47.22%) isolates were susceptible, 5 (13.89%) isolates were intermediate and 14 (38.89%) isolates were resistant to amoxicillin. Another 7(19.44%) isolates were susceptible, 12 (33.33%) isolates were intermediate and 17 (47.22%) isolates were resistant to azithromycin. Results are presented in Table 19 and in Plate 8a, 8b.

Table 17. Antimicrobial susceptibility pattern of *Salmonella* spp. by disk diffusion method

Antimicrobial agents	No. (%) of *Salmonella* spp. isolates		
	S	I	R
Amoxicillin	17 (47.22)	5 (13.89)	14 (38.89)
Azithromycin	7 (19.44)	12 (33.33)	17 (47.22)
Ciprofloxacin	36 (100)	0 (0)	0 (0)
Erythromycin	0 (0.0)	0 (0)	36 (100)
Gentamicin	36 (100)	0 (0)	0 (0.0)
Norfloxacin	36 (100)	0 (0)	0 (0.0)
Streptomycin	36 (100)	0 (0)	0 (0)
Tetracycline	0 (0.0)	0 (0)	36 (100)

Legends: S= Susceptible; I= Intermediate; R= Resistance

4.1.7.2. Results of antimicrobial resistance pattern of *Salmonella* spp.

The results of antimicrobial resistance patterns of *Salmonella* spp. are summarized in Table 20. Out of 36 *Salmonella* spp. 13 (36.11%) were resistant to 2 agents E-TE. 6 (16.67%) were resistant to 3 agents E- AMX-TE. 9 (25%) were also resistant to 3 agents E-AZM-TE. Another 8 (22.22%) were resistant to 4 agents AMX-AZM-E--TE. The isolated *Salmonella* spp. were detected as multidrug resistant isolates as presented in Table 20 and Plate 8a, 8b.

Table 18. Results of antimicrobial resistance pattern of *Salmonella* spp.

Isolates	Resistance profiles	No. of isolates (%)
Salmonella spp. (n=36)	No resistance demonstrated	–
	Resistant to 2 agent (E-TE)	13 (36.11)
	Resistant to 3 agents (E- AMX-TE)	6 (16.67)
	Resistant to 3 agents (E-AZM-TE)	9 (25)
	Resistant to 4 agents (AMX-AZM-E--TE)	8 (22.22)
	Total resistant isolates	**36 (100)**

Legends: AMX=Amoxicillin, AZM=Azithromycin, E=Erythromycin, GEN=Gentamicin, CIP=Ciprofloxacin, NOR=Norfloxacin, TE=Tetracycline, S=Streptomycin.

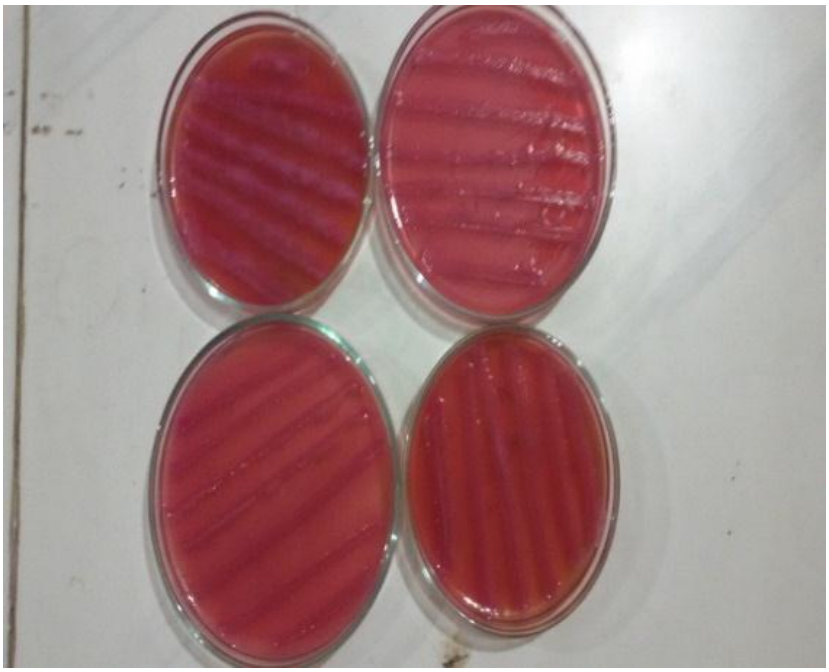


Plate 1. Growth of *Salmonella spp* in Brilliant Green Agar showing pale pink colour colonies

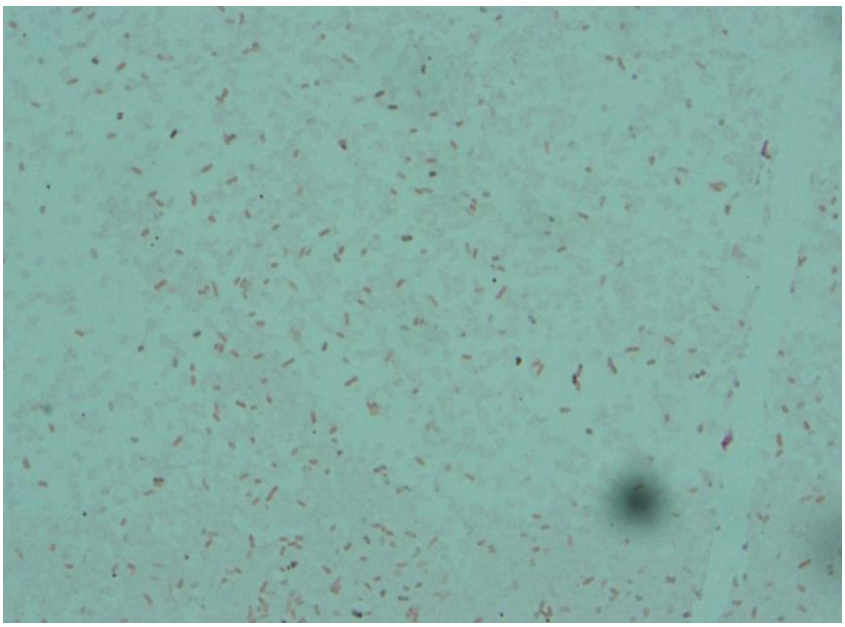
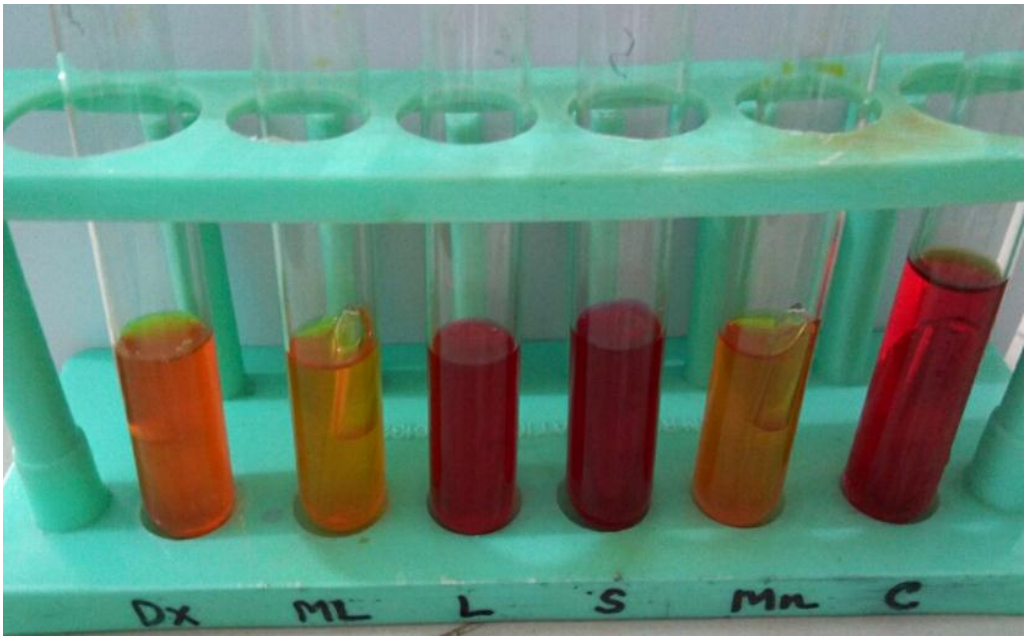


Plate 2. Gram-negative, pink color, small rod shaped *Salmonella* spp. arranged in single or paired under the microscope.



Legends: D = Dextrose, ML= Maltose, L= Lactose, S= Sucrose, MN= Mannitol.

Plate 3. Sugar fermentation test of *Salmonella* spp. where *Salmonella* fermented dextrose, maltose and mannitol with acid and gas production.

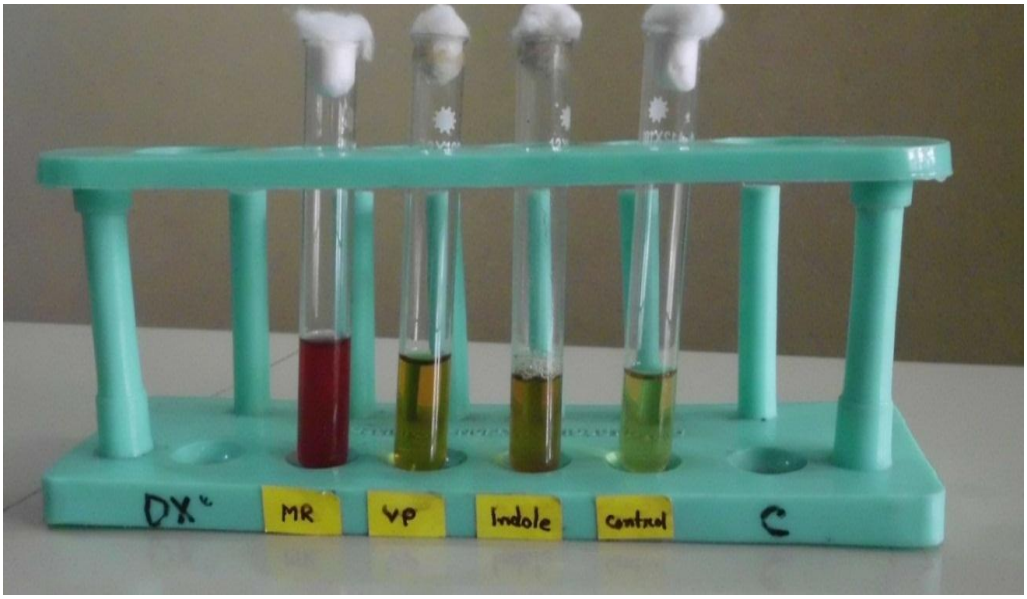


Plate 4. Results of Methyl Red, Voges-Proskauer and Indole test of *Salmonella.* Where MR test (+ve), VP and Indole test (-ve).

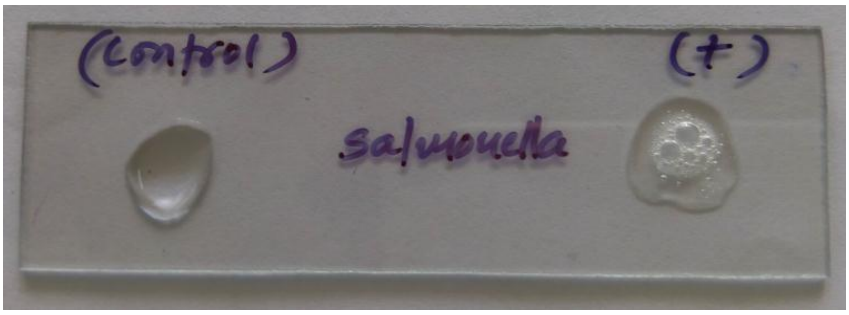


Plate 5. Catalase test that was carried out as per the method described in materials and methods section. One isolate of *Salmonella* spp. showing bubble formation, no bubble formation indicating negative or control.

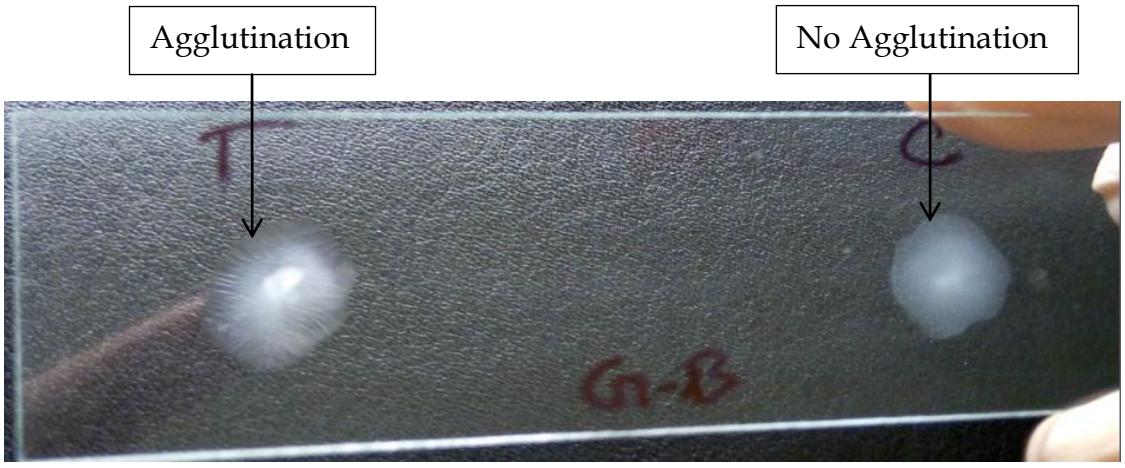


Plate 6. Serogrouping of *Salmonella* spp. by slide agglutination test with Group B antisera (agglutination indicates positive, no agglutination indicates negative).

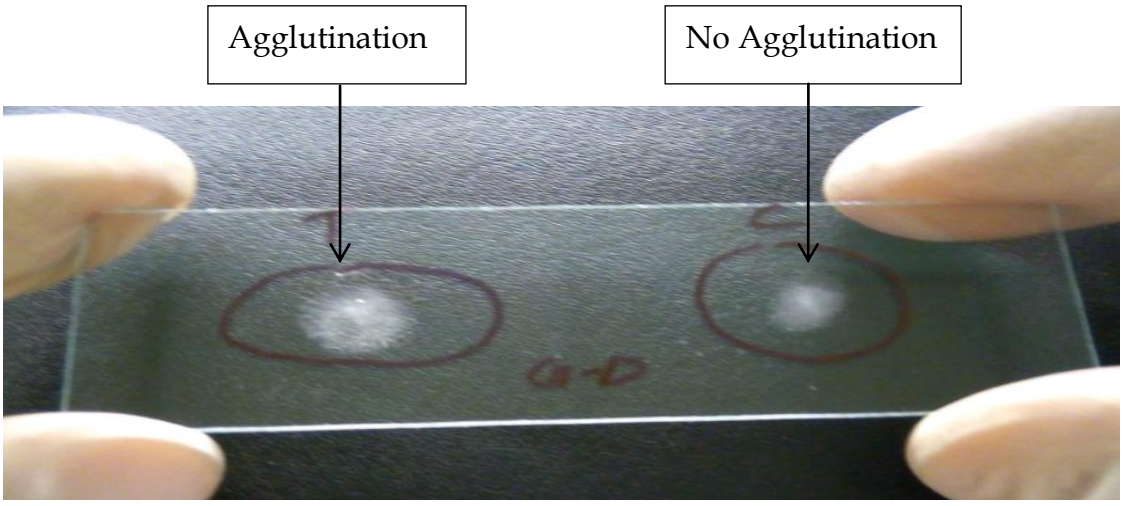


Plate 7. Serogrouping of *Salmonella* spp. by slide agglutination test with Group D antisera (agglutination indicates positive, no agglutination indicates negative).

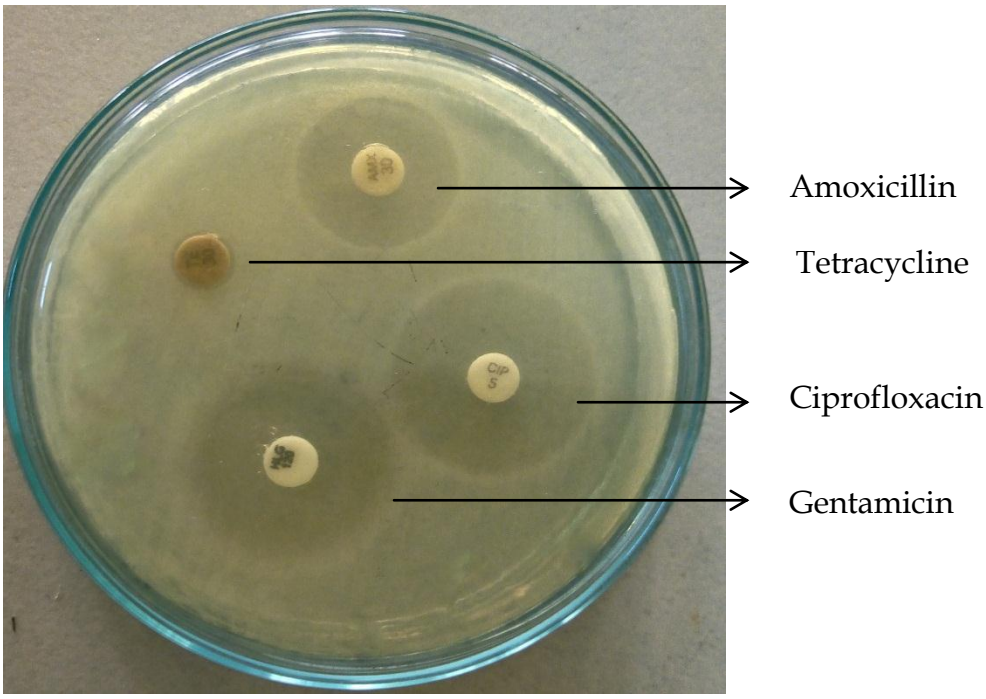


Plate 8 a. Antimicrobial susceptibility and resistance pattern of *Salmonella* spp.

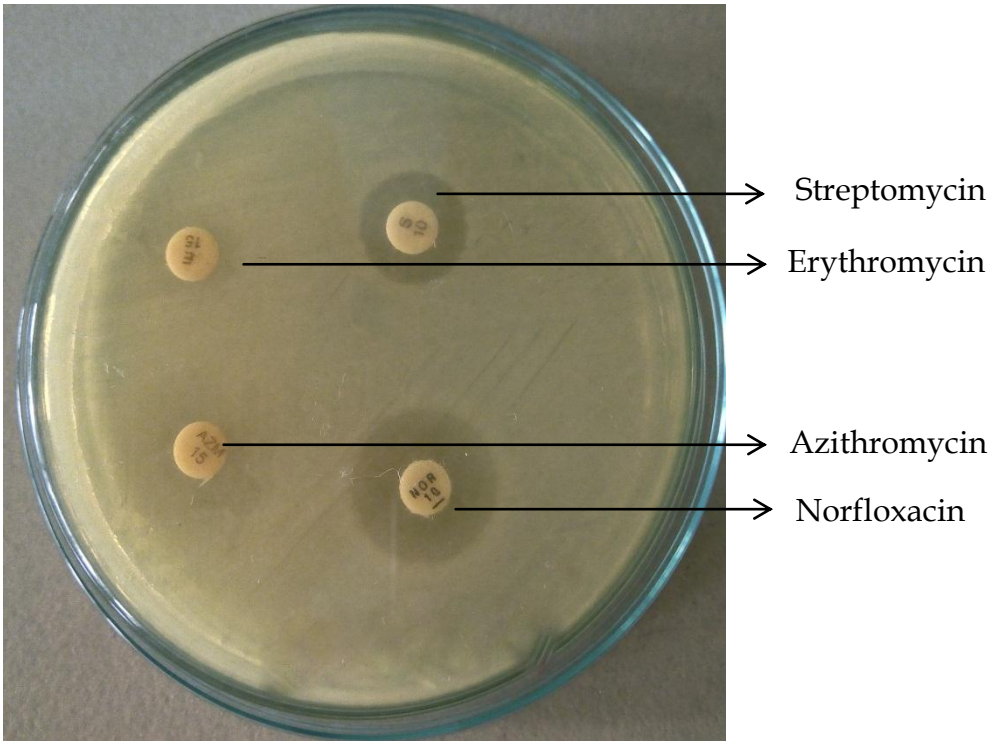


Plate 8 b. Antimicrobial susceptibility and resistance pattern of *Salmonella* spp.

CHAPTER 5

DISCUSSION

CHAPTER 5

DISCUSSION

The present study was carried out for the isolation, identification and characterization of *Salmonella* spp. from poultry production chains of kaharol and Sadar, Dinajpur district of Bangladesh. The samples (broiler meat, cloacal swab, chick meconium, transport swab, feed, water and floor swab) were collected and immediately brought to the microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for the laboratory analysos. Serogrouping of *Salmonella* isolates were performed by slide agglutination test using commercial *Salmonella*-specific polyvalent O (A-I) antisera, *Salmonella* O group B (Factor O: 4, 5, 27) antisera, and *Salmonella* O group D (Factor O: 9, 46) antisera (S&A Reagent Lab). Finally, antibiogram study was done to evaluate the sensitivity and resistance patterns of the *Salmonella* spp. with available and mostly used antibiotics.

For the cultural examination several selective media such as BGA was used simultaneously to culture the organism and isolation of *Salmonellae* which was also used by a number of researchers (Hyeon *et al.,* 2012; Muktaruzzaman *et al.,* 2010; Habrun and Mitak, 2003). The colony characteristics of *Salmonella* spp. found in this study was translucent, black smooth, small round colonies on SS agar, Pink color colony with black centre in XLD agar, were similar to the findings of other authors (Muktaruzzaman *et al.,* 2010; Sujatha *et al*., 2003; Habrun and Mitak 2003; Sarker *et al.,* 2009; Rahman *et al.,* 2009; Khan *et al.,* 2005).

In Gram's staining, the morphology of the isolated *Salmonella* spp. exhibited Gram negative small rod arranged in single or paired which was supported by several authors Freeman (1985), Buxton and Fraser (1977), Merchant and Packer (1967).

In this study, biochemical tests were performed for the identification of *Salmonella* spp. which were also used by several researchers (Muktaruzzaman *et al.,* 2010; Lee *et al*., 2003). In carbohydrate fermentation test, the isolates that fermented glucose, maltose and produced acid and gas but did not ferment lactose those indicated positive for *Salmonellae* as was stated by Buxton and Fraser (1977). The isolates were positive for Methyl Red test but negative for VP test indicating characteristics of *Salmonella* spp. test which was similar with the statement of Muktaruzzaman *et al.* (2010). In indole test, all the test isolates (n=36) did not develop any red color that indicated the *Salmonella* spp. The isolates were also negative to indole test and this was similar with the findings of Lee *et al*. (2003).

In this study 28.57% (n=7) of *Salmonella* spp. were positve in transport swabs samples (n=7). The result was supported by several authors (Akond *et al.*, 2012). 28.57% (n=4) of *Salmonella* spp. were positive in feed samples (n=14). The prevalence of *Salmonella* in feed sample (29.16%) was closely similar to the findings of Islam *et al*. (2014). There was also 18.75% (n=3) *Salmonella* spp. were present in water samples (n=16). The results of this study was closely related with the results of several authors (Saha *et al.*, 2012*;* Samanta *et al*., 2014).

Out of 49 cloacal swab 10 samples (20.41%) were positive for *Salmonella* spp. The higher percentage of Salmonella was observed in Kaharol (21.4%) in comparing with Sadar, Dinajpur. This findings supported by several researchers (Islam *et al*., 2016; Parvej *et al*., 2016; Sarker *et al*., 2012). Cloacal swabs have been used to provide evidence of persistent intestinal colonization by *Salmonellae* in individual birds reported by Gast *et al*. (1997).

In our present study, it was observed that out of 153 samples 23.53% were identified as Slmonella sp.. The highest prevaence of Salmonella was observed in Kaharol (26.19%) and lowest in Sadar (22.52%), Dinajpur. This findings supported by (Al-Ferdous *et al*., 2013; Kabir, 2010).

From the collected 153 samples, 36 *Salmonella* spp. were isolated . Among the 36 isolates, 30.56% (n=11) belonged to serogroup B and 69.44% (n=25) isolates belonged to serogroup D. The most prevalent serogroup identified in this study was serogroup D. These findings were in agreement with the result reported by several researchers (Mahmud *et al*., 2011; Arroyo and Arroyo, 1995).

In the limited attempt, samples were collected from only two districts for isolation and identification of *Salmonella* spp. So, investigations on other districts will be required to identify the *Salmonella* spp. associated with commercial poultry production chain.

In relation to the present study, further study might be performed on the following aspects:

i. Genomic studies about genes responsible for pathogenicity and drug resistence of *Salmonella* spp.

ii. Molecular characterization of the isolated *Salmonella* spp. by pulsed field gel electrophoresis (PFGE).

CHAPTER 6

SUMMARY AND CONCLUSIONS

CHAPTER 6

SUMMARY AND CONCLUSIONS

The present study was undertaken during the period of January to June 2016 for the isolation and characterization of *Salmonella* spp. from poultry production chains of Kaharol and Sadar upazilla under the Dinajpur district of Bangladesh.

For this purpose cloacal swab, meat samples (composite of thigh, breast and skin), chick meconium, feed, water, transport swab and floor swab samples were collected and were subjected to inoculation onto different selective culture media. The isolated *Salmonella* like organism were then subjected to gram's staining. After presumptive diagnosis of the *Salmonella* spp., biochemical tests were performed for the identification of the *Salmonella*. Then all positive isolates were subjected to motility test to differentiate motile and nonmotile S*almonella*. Then basic sugar test was done where *Salmonella* fermented dextrose, maltose and mannitol with acid and gas production**.** The results of isolated *Salmonella* spp. in Methyl Red was positive indicated an acid P^H resulting from the fermentation of glucose. Voges-Proskauer and Indole test were negative.

Among the 153 samples of poultry production chains (hatchery → farm → transport → live bird markets), 36 *Salmonella* like organism were isolated by using various cultural and biochemical techniques.

Out of 36 *Salmonella* spp. 22.52% (n=25) were isolated from poultry production chains of Sadar, Dinajpur district and 26.19% (n=11) were isolated from poultry production chains of kaharol, Dinajpur district. The highest occurrence of *Salmonella* spp. 36.67% (n=11) were observed in meat samples (composite of thigh, breast and skin). The second

highest occurrence of *Salmonella* spp. 20.4% (n=10) were observed in cloacal swab samples.

In this study serogrouping was done by *Salmonella* Poly A-I antisera, *Salmonella* O group B and O group D antisera. Among the 36 isolates, 11 were belonged to serogroup B and 25 isolates were belonged to serogroup D. The most prevalent serogroup identified in this study was serogroup D, 69.44% (n=25).

The antibiogram study was undertaken to assess the susceptibility and resistance pattern of *Salmonella* spp. against eight antibiotics commonly used for poultry production. The results of susceptibility analysis showed that all of the 36 *Salmonella* isolates were susceptible to gentamicin, norfloxacin, ciprofloxacin and streptomycin. Out of the 36 isolates 13 (36.11%) isolates were resistant to erythromycin and tetracycline. 6 (16.67%) isolates were resistant to erythromycin, amoxicillin and tetracycline. 9 (25%) were resistant to erythromycin, azithromycin and tetracycline. Another 8 (22.22%) were resistant to amoxicillin, erythromycin, azithromycin and tetracycline. The findings of the present study was revealed the prevalence of *Salmonella* spp. as well as presence of multidrug resistant *Salmonella* spp. in the samples of the study area.

Considering the findings of this research work, it may be concluded that:

i. The presence of *Salmonella* spp. in different phages of poultry production chains were observed.

ii. The most prevalent *Salmonella* serogroup identified in this study was *Salmonella* serogroup D.

iii. The findings of the present study revealed the presence of multidrug resistant *Salmonella* spp. in poultry production chain.

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APPENDICES

APPENDICES

Appendix I. Questionnaire for exposure assessment of *Salmonella* spp. in broiler farm

Date:

Information for sample collection from farm and hatchery

Name and addresses of the farm/farm owner:

Farm code:

Date of DOC received:

Source of DOC (hatchery name):

Source of Feed (feed mill):

Type of feed: Starter/Grower/Finisher

Date of arrival of feed at farm from where feed samples were collected:

Expected date of poultry sale:

Any medicine/chemicals was there given to the flock while sampling? : Y/N

If yes, please mention the name of medicine and dose:

Sample ID:

Sample type	Sample ID	Remarks
Feed		
Water		
Cloacal swabs		
Chick meconium		
Whole bird		

Appendix II. Questionnaire for exposure assessment of *Salmonella* spp. from transport swabs

Date:

Information for sample collection from transport

Sample ID:

Name of transporter	
Name of the transporter	
Vehicle no.	
Type of vehicle	Track/van/others
Cage used in vehicle	Y/N
Type of cage	Plastic/Bamboo/iron/other
C&D of vehicle	Yes/partial/No
C&D of vehicle	Before/ after reaching the farm

Appendix III. Questionnaire for exposure assessment of *Salmonella* spp. in live bird markets (LBM)

Date:

Information for sample collection from live bird markets (LBM)

Name of LBM:	
a) Road #:	b) Village/Area/Ward:
c) Union/city corporation/Pouroshava:	d) Upazila/PS:
e) Zila/District:	f) Division:
g. No of Whole carcass :	Sample ID:
h. No of water sample:	Sample ID:
i. Birds selling/storage floor swab No :	Sample ID: