CHARACTERIZATION OF SALMONELLA SEROVARS FROM SELECTED LAYER FLOCK AND DEMONSTRATION OF ANTIBACTERIAL EFFECT OF EDIBLE PLANT EXTRACTS TO SELECTED ISOLATES OF SALMONELLA SEROVARS

A THESIS

BY

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SEMESTER: JULY–DECEMBER, 2016

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MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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The author
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ABSTRACT

The present study was selected as antibacterial effect of extracts from some edible plants (Neem, Garlic and Ginger) against *Salmonella* serovars isolated from selected layer flock by using morphological, cultural and biochemical techniques. The prevalence of positive isolates was higher in Dinajpur (13.89%) in comparison to Thakurgoan (11.91%), Panchagarh (8.33%) and Nilphamari (10%) according to their study area differences. The organoleptic prevalence of positive isolates were also higher in liver (17.86%) in comparison to the heart (7.14%) and lung (10.72%) respectively. Extractions of some edible plants were performed using absolute ethanol. In this study, the antibacterial sensitivity of ethanolic extracts of Neem, Garlic and Ginger in various concentration (80, 100 and 120 mg/ml) against Multidrug resistant bacteria of layer flock (resistant to at least two antibiotics) was determined by using disc diffusion method. The higher zone of inhibition of different plant extracts (Neem, Garlic and Ginger) were 14 mm, 10 mm and 2 mm in 120 mg/ml in which the lower zone of inhibition were 10 mm, 3 mm and no zone in 80 mg/ml. In this study it was observed that ethanolic extract of Neem leaf is effective against Multidrug resistant bacteria of layer flock than Garlic and Ginger extracts. However the organisms were sensitive to Ciprofloxacin and Colistin, Intermediate to Levofloxacin and Chloramphenicol and resistant to Neomycin and Kenamycin. The isolates were also subjected to compare the efficacy of resistant antibiotics and plant extracts in vitro. In this study, it was observed that plant extracts showed higher zone of inhibition than commercial antibiotics. From this study it was concluded that the ethanolic extract of neem leaf could be used as an alternative approach to synthetic antibiotics against field isolates.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
#	:	Identifying number
%	:	Percentage
@	:	At the rate of
+	:	Positive
µg	:	Microgram
µl	:	Microlitre
^{0}C	:	Degree of celcius
Ag	:	Antigen
Assist	:	Assistant
BGA	:	Brilliant Green Agar
BER	:	Bangladesh Economic Review
BBS	:	Bangladesh Bureau of Statistics
C	:	Chloramphenicol
CIP	:	Ciprofloxacin
CL	:	Colistin
et al.	:	Associated
etc	:	Etcetera
EUCAST	:	European Committee on Antimicrobial Susceptibility Testing
FAO	:	Food and Agricultural Organization
gm	:	Gram
H_2O_2	:	Hydrogen peroxide
H_2S	:	Hydrogen sulphide
HSTU	:	Hajee Mohammad Danesh Science and Technology University
i.e.	:	That is
K	:	Kenamycin
LE	:	Levofloxacin
Ltd	:	Limited

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

M.S	:	Master of Science
MC	:	MacConkey Agar
MDR	:	Multidrug resistant
MI	:	Milliliter
MIU	:	Motility Indole Urease
MR	:	Methyl Red
N	:	Neomycin
NA	:	Nutrient Agar
NB	:	Nutrient Broth
No.	:	Number
RPM	:	Rotation Per Minute
SE	:	Standard Error
SL	:	Serial number
Sp	:	Species
SSA	:	Salmonella Shigella Agar
v/v	:	Volume by volume
VP	:	Voges-Proskauer
w/v	:	Weight by volume

CHAPTER 1

INTRODUCTION

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INTRODUCTION

The poultry production systems have led to marked increase in the production of poultry meat and eggs throughout the world (Armstrong, 1986). In Bangladesh, commercial poultry production has been growing rapidly since the early 1990 by using improved genetics, manufactured feeds and management. This dramatic growth of poultry farms throughout the country without judging feasibility of the farm in the area. The number of farms reduced to 55,000 in 2013 from 1,15,000 in 2007. Another source reported that there are about 65,902 poultry farms upto February 1013 in the country (BER, 2013). In spite of decrease in number of poultry farms, it is reported by Bangladesh Poultry Association that the country achieved self sufficiency in production of chicken meat and eggs. So the problem is excess supply of chicken meat and egg in the market.

Poultry refers to domestic birds that produce eggs, meat, manure and feathers that can be used or treated by their owners. In general, poultry provide animal protein in the form of meat and eggs. Poultry plays an important role in the national economy. A considerable amount of foreign exchange is being earned by exporting eggs and meats of poultry. Poultry husbandry also acts as a profession in unemployed young persons, landless farmers, poor, divorced women and children which can supplement their family income.

The total population of domestic fowls in Bangladesh was estimated to be 7,64,46,000 (BBS, 1991). It provides protein need in our daily food menu in terms of eggs. But it is not sufficient against need of our large population. Our food is very much deficient in protein of animal origin. In our country, one egg is needed/head/day but get only 0.2/head/day. The average quantity of protein is 9.5 gm/head/day (FAO, 1966), which is far below in comparison with developed countries.

The increasing demand of animal protein and the economic benefits obtained through chicken raising in both backyard and conventional farming systems have created a great deals of interest among the farmers in these days. But poultry farming in Bangladesh are facing various hinderances. Among these, Salmonellosis is a common problem in poultry farms of our country that causes heavy economic loss through mortality and reduced production (Khan *et al.,* 1998). The disease is most significant because the causal agents of the disease are transmitted vertically from parents to offspring.

Salmonella is an important genus of the family *Eenterobacteriaceae* (OIE Manual, 2006) and are Gram negative, short plump shaped rods, non-spore forming, non- capsulated, aerobic and facultative anaerobic organisms and inhabit the intestinal tract of man and animals (Holt *et al.,* 1994).They may be recovered from a wide range of hosts such as poultry, swine, human, foods and from the environment. Members of the genus *Salmonella* may be pathogenic to wild or domestic animals and human (Holt *et al.,* 1994).

More than 2300 serotypes of *Salmonella* have been identified, only about 10% of these have been isolated from poultry (Gast, 1997). Chickens are the natural hosts for both *S. pullorum* and *S. gallinarum* (Snoeyenbos, 1991). Pullorum disease is usually confined to the first 2-3 weeks of age and occasionally occurs in adults (Shivaprashad, 1997). Fowl typhoid is frequently referred to as a disease of adult birds and there are also reports of high mortality in young chicks (Christensen *et al.,* 1992). The epidemiology of fowl typhoid and pullorum disease in poultry, particularly with regard to transmission from one generation to the next are known to be closely associated with infected eggs (Wigley *et al.,* 2001). Contaminated eggs produced by infected laying hens are thought to be one of the main sources of human infection with *Salmonella enteritidis* (Humphrey *et al.,* 1989). Eggs may become contaminated with *Salmonella* in two main ways: (i) *Salmonella* may silently infect the ovaries of apparently healthy hens and contaminate the eggs before the shells are formed. (ii) *Salmonella* infected bird droppings contain *Salmonella* that can contaminate the outer egg shells and may penetrate when crack the shell (Deryck and Pattron, 2004).

Salmonella are vertically transmitted to the newborn chicks, therefore, regular blood testing of the parent flock and elimination of infected and carrier birds would be helpful in reducing its vertical transmission. In addition, preventing entry of rodents, vermin or other wild animals and the assurance of improved hygienic conditions would be helpful in reducing the incidence of Salmonellosis.

Generally *Salmonella* organisms are being characterized by using various cultural, morphological, biochemical, serological and molecular techniques. *Salmonella* giving rise to a large number of serotypes (more than 2300) besides leading to variation in pathogenecity of the species and may cause serious confusion in diagnosis of the disease. Antibiotic sensitivity were also performed by Gyurov and Dyako (2000); Sung *et al.,* (2002); Banani *et al.,* (2003); Khan, (2004); Rahman, (2005); Akter (2007) to select the

antibacterial agents for effective therapeutic purpose of Salmonellosis. However, antibiotics used for the treatment of Salmonellosis is problematic due to the recent emergence of multi-drug resistant of *Salmonella* strains.

Poultry is essential to the national economy of Bangladesh and the welfare of human beings. Several constraints such as the diseases, poor husbandry, low productivity and shortage of feed affect the optimal performance of this industry in Bangladesh (Haque *et al.,* 1991). Salmonellosis in poultry causes heavy economic loss through mortality and reduced production (Khan *et al.,* 1998). With great expansion of poultry rearing and farming, pullorum disease and fowl typhoid have become wide spread problem in Bangladesh (Rahman *et al.,* 1997). Age wise prevalence of avian Salmonellosis showed highest infection rate in adult layers (53.25%) in comparison to brooder (14.55%), grower (16.10%), pullet (16.10%) (Rahman *et al.,* 2004) and starter (5.71%), grower (3.33%) and layer (12.86%) were also observed by Akter *et al.,* 2007 at Dinajpur district of Bangladesh. The control of the diseases mainly relies on the use of antibiotics.

The increased usage of antibiotics has induced microorganisms to acquire resistance factors which have become a burning predicament (Abimbola *et al.,* 1993). *Salmonella* are known to carry plasmids, which encode for drug resistance. This implies that widespread use of anti-microbials may cause an increase in the frequency of occurrence of bacterial resistance to other anti-microbial as plasmids may encode resistance to additional antimicrobial agents (Salehi *et al.,* 2005). As a result there is an urgent need to find the alternative of chemotherapeutic drugs in diseases treatment particularly those of plants origin which are easily available and have considerably less side effects (Khulbe and Sati, 2009). The use of higher plants and their extract for treating the infectious diseases has long been practiced in many parts of the world (Sofowora, 1984).

Medicinal plants are natural resources, yielding valuable products, which are often used in the treatment of various ailments. Plant materials remain an important resource for combating infectious diseases and many of the plants have been investigated for novel drugs or templates for the development of new therapeutic agents (Karthy *et al.,* 2009).

Bangladesh is plentiful with many plants, among them medicinal plants as a traditional system of therapy, have been used from ancient times to cure diseases of man and animals (Akhtar *et al.,* 2000). Bangladesh, Pakistan and India bound with plants that were known to have medicinal properties. Among the medicinal plants, Neem, Garlic

and Ginger may offer a new source of antibacterial agents and from this result it is clear that the medicinal value of extracts from these plants are comparable to the present day antibiotics.

Therefore, several research work were conducted to evaluate the antibacterial effect of Garlic and Ginger extracts against *Salmonella* serovars in many countries of the world (Murugan *et al.,* 2015 and Bandna Chand *et al.,* 2013).

In Bangladesh, the information about antibacterial effect of plant extract from Neem (*Azadirachta indica*), Garlic (*Allium officinale*) and Ginger (*Zingiber officinale*) against *Salmonella* serovars isolated from layer chickens are very scanty except Kamrul *et al.,* 2014. These investigators worked on food samples from human. On the other hand Mahbuba *et al.,* 2012 worked on *Salmonella* serovars isolated from poultry but these investigators used extract of *T. arjuna* against identified isolate. However, the antibacterial effect of extracts from some edible plants (Neem, Garlic and Ginger) were not yet performed in our country. As per literature review in the context of Bangladesh no information as per mentioned earlier was recorded.

By justifying the research in the context of Bangladesh and neighboring countries of the world, the present study was conducted for the evaluation of antibacterial effect of extract from some edible plants (Neem, Garlic and Ginger) against *Salmonella* serovers isolated from selected layer flock followed by using cultural, morphological, biochemical and antibiogram study, considering as entirely a new work in the field of Veterinary Microbiology in Bangladesh. Therefore, the present study was undertaken with the following specific objectives:

1. To isolate and characterize the *Salmonella* serovars from selected layer flock by using morphological, cultural and biochemical techniques.
2. To prepare crude extract of Neem, Garlic and Ginger by using ethanol.
3. To evaluate the efficacy of these plant extracts against identified isolates by using disk diffusion method.
4. To compare the efficacy of commercial antibiotics and plant extracts in vitro against field isolates.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

2.1 Isolation and identification of *Salmonella* serovars

Ellerbroek *et al.* (2010) isolated Salmonella from 400 imported chicken carcasses in Bhutan and from 178 pig carcasses in Vietnam were analyzed for antibiotic resistance on a random basis against 14 antimicrobial agents. Among the tested poultry samples, 13% were positive for *Salmonella*.

Kwon *et al.* (2010) investigated the prevalence of fowl typhoid during 2000 to 2008 and characterized the phenotype and genetic diversity of *Salmonella gallinarum* isolates.

Ahmed *et al.* (2009) isolated sixty-nine *Escherichia coli* and 10 *Salmonella*, from retail chicken meat in Hiroshima prefecture, Japan the samples were assayed for antimicrobial susceptibility, the presence of integrons and antimicrobial resistance genes.

Lestari *et al.* (2009) isolated and characterized 126 *Salmonella* isolates from conventionally raised (n=141) and organically rose (n=53) chicken carcasses obtained from 27 retail stores in Baton Rouge, Louisiana. *Salmonella* were isolated from 22% of conventional and from 20.8% of organic chicken samples. Eight *Salmonella* serovars were identified.

Dien *et al*. (2000) reported the prevalence of *S. pullorum* in 7 to 8 week old AA and ISA chicken flocks in the North Vietnam has been monitored since 1996. 5651 serum samples were examined. The seroprevalence varied from 0.788 to 13.69% depending on hygienic conditions on farms. The prevalence of *S. pullorum* infections was higher in the winter/spring season than in other seasons.

Hena and Lali-Growther (2009) stated that *Salmonella* is a Gram negative facultative rod shaped bacteria which live in the intestine of warm and cold blooded animals. Some species are ubiquitous and others are specially adaptive to a particular host. In human *Salmonella* are the cause of two diseases called Salmonellosis and enteric fever (typhoid). These bacterial zoonotic agents in the veterinary as well as medical field. The organism now named *Salmonella enteric* serotype *typhe* was discovered in 1880. Most *Salmonella* outbreaks are associated with the consumption of contaminated products of

animal origin. The widespread distribution of *Salmonella* in meat and poultry makes meat products as good source for the isolation of *Salmonella*.

Raufu *et al*. (2009) conducted to determine the prevalence of *Salmonella* serovars and the antimicrobial susceptibility in chickens and poultry meat products in rural areas in Nigeria. The study was an observational cross-sectional investigation in which the target population included exotic and local chickens in Maiduguri main markets, chickens from farms, and free-range local chickens. A total of 865 samples were collected from feces, kidney, lungs, cecum, intestine, liver, heart, gizzard and cloacal swabs from 525 different chickens. *Salmonella* was isolated from 130 of the samples.

Vigo *et al*. (2009) reported that blue and gold macaw (*Araararauna*) chicks died of fetal Salmonellosis in Buenos Aires Province, Argentina. The birds were histopathologically and microbiologically examined. *Salmonella enteric* subspecies serovars *typhimurium* was isolated from the liver, spleen, heart, lung, kidney and intestine of both birds. All strains were susceptible to Ampicillin, Cephalothin, Cefotaxime, Enrofloxacine, Nalidixic acid, Gentamicin, Streptomycin, Chloramphenicol, Fosfomycin, Tetracycline, Nitrofurantoin and Trimethoprim-sulfamethoxazole.

Kim *et al.* (2008) reported on the detection of *Salmonella gallinarum* in different commercial poultry flocks in korea. 14 field strains of *S. gallinarum* were tested for their drug resistance patterns against 18 antimicrobial agents. The biochemical patterns of all the 14 field isolates along with one standard strain of *S. gallinarum* corroborated well with the typical biochemical characteristics of classical strain of *Salmonella gallinarum*, indicating that no biochemically variant strains emerged in Korea.

Yoke-Kqueen *et al.* (2008) conducted a study to characterize the serogroups of *Salmonella* isolates and determined the relationship of antimicrobial resistance to serogroups. Multiple antimicrobial resistance (MAR) was performed on 189 *Salmonella enteric* isolates associated with 38 different serovars that were recovered from poultry and four types of indigenous vegetables. Characterization of *Salmonella* isolates based on the MAR results indicated that poultry still remains as the main reservoir for multi-drug-resistance *Salmonella*. Four isolates from the indigenous vegetables showed the highest MAR index in this study.

Akter *et al.* (2007) determined the seroprevalence of Salmonellosis in layer flocks and antibiogram study of identified isolates. In this study a total of 225 Star cross 579 brown chickens were studied with rapid serum plate agglutination test. Liver of 200 dead birds was studied for isolation and identification of *Salmonellae*. In vitro antibiotic sensitivity test of isolated *Salmonellae* were performed with commercial sensitivity discs. The overall seroprevalence was recorded 23.11%. The prevalence was varied from age to age. The highest rate was 28% in above 20 weeks of age.

Uesugi *et al.* (2007) isolated *Salmonella enteritidis* phage type (PT) 30 from drag swabs of 17 61-ha almond orchards on three farms linked to an outbreak of Salmonellosis associated with consumption of raw almonds in 2001.

Castillo *et al.* (2006) conducted a survey for the presence of *Salmonella* and *Shigella* in freshly squeezed orange juice and related samples in Guadalajara, Mexico. One hundred samples of freshly squeezed orange juice were collected from 49 street booths and 51 small food service establishments. In addition, 75 fresh orange samples, each consisting of five orange units, and 75 wiping cloths were collected from the same establishments from which juice had been collected. *Salmonella* was isolated from 14, 20 and 23% of samples of orange juice, orange surfaces and wiping cloths collected from street vendors, while *Shigella* was isolated from 6, 17 and 5% of these samples. *Salmonella enteric* serotypes *agona, typhimurium* and *anatum* were found in orange juice, fresh oranges and wiping cloth samples, while serotype Mexico was found on fresh oranges and in wiping cloths and serotypes Muenchen and Panama were found only in wiping cloth samples.

Vo *et al.* (2006) investigated epidemiologically unrelated non-typhoid *Salmonella* isolated from humans and animal origin in Vietnam. *Salmonella typhimurium, S. anatum, S. emek*, and *S. risen* were the most prevalent serovars. *S. typhimurium* phage type 90 was predominant among *S.typhimurium* isolates.

Balala (2006) identified *Salmonella* serotype from chickens and determined the antibiotic sensitivity pattern of the isolates. Meats and eggs were obtained from two local public markets whereas cloacal swabs were collected from two broiler and layer farms. Isolation and serotyping of *Salmonella* were done by using conventional method and specific typing sera, respectively. Antibiotic sensitivity test was carried out by the Kirby-Bauer method. Of the 325 samples 16 (4.9%) contained *Salmonella*. These consisted of 9.3% out of 150 meat and 2% out of 100 cloacal swabs samples. *Salmonella* was not

detected in 75 pooled egg samples. Seven serotypes were isolated with *S. weltevreden* predominating, followed by *S. derby, S. enteriditis* PT1, *S. enteriditis* phage type untypable. *S. Newport* and *S. Lexington. S. Albany* was isolated locally for the first time.

Chen *et al.* (2005) stated that *Salmonella* enteric serotype *Panama*, capable of causing severe infection in children and is often transmitted via contaminated food. The author presents the first documented case of serotype *Panama* infection that was acquired through the consumption of contaminated breast milk. The mother excreted the organism asymptomatically for at least 2 weeks.

Cherry *et al.* (2004) reported an outbreak of *Salmonella enteric* serovar *typhimurium* in a veterinary clinic in New York, United Stated of America. Confirmed cases were in one cat, two veterinary technicians, four persons associated with clinic patients and a nurse not related to the clinic. This outbreak emphasizes the importance of strong public health to the animal health community.

Bhattacharya *et al.* (2004) conducted a study to determine the presence of *Salmonella sp*. In the apparently healthy breeder flocks of organized poultry farms in Assam, Arunachal Pradesh and Meghalaya, India. A total of 832 cloacal swabs from poultry were examined for the isolation of *Salmonella*. Among the isolates, 35 (4.21%) samples were positive for *Salmonella* 15(42.9%) *S. enteriditis*, (17.1%) *S. gallinarum* and 14 (40.0%) *S. typhimurium*. All the 15 *S. enteriditis* strains belonged to a single phage type, 13a/7, whereas *S. typhimurium* were distributed between two phages, DT004 and DT193.

Sujatha *et al.* (2003) isolated and characterized *Salmonella gallinarum* from poultry in and around Hyderabad and Secunderabad cities in India. Six isolates of *S. gallinarum* were obtained from 21 clinical samples by employing both pre-enrichment and selective media. The liver was found to be the most suitable organ for isolation of *S. gallinarum*. Percentage of positive isolation was 28.67%.

Roy *et al.* (2002) isolated five hundred sixty-nine *Salmonella* out of 4745 samples from poultry products, poultry and poultry environment in 1999 and 2000 from the Pacific Northwest. These *Salmonella* were identified to their exact source and some were serogrouped, serotyped, phage typed and tested for antibiotic sensitivity.

2.2 Extraction of edible plants

Aruna *et al.* (2014) aimed to evaluate the antibacterial effects of *Z.officinale* and honey on *Salmonella spp*. isolated from cockroaches. Disc diffusion and tube dilution methods were used to determine the antibacterial activity of the ginger extract, honey and combination of both against *Salmonella spp*. The results indicated that the ginger extract, honey and its combination inhibited the growth of *Salmonella typhi*, *Salmonella paratyphi A* and *Salmonella paratyphi B*. The ginger - honey mixture had noticeable effect on examined species compared to the individual effects of ginger and honey, thus justifying their combined use in treatment for enteric infection.

Kamrul *et al.* (2014) determined the antimicrobial activity of soybean oil extract of dried ginger powder, using agar diffusion assay, against 24 isolates (4 of 6 different types) of food borne pathogens including *Escherichia coli, Pseudomonas aruginosa, Staphylococcus aureus, Vibrio cholerae, Klebsiella spp.* and *Salmonella spp*.

Bhadauria *et al.* (2004) collected leaves and stems of tulsi (*O. sanctum [O. tenuiflorum*]) and neem (*A. indica*) from different sites in Agra, Uttar Pradesh, India and evaluated for fungal infection. Infected plants showed leaf blackening, followed 15by leaf drying and shedding. Stems darkened and then dried. Decayed neem stems and leaves were infected with *Alternaria alternata*, *Cladosporium sp*. and *Fusarium moniliformis [Gibberella moniliformis*]. Tulsi leaves and stems were infected with *A. alternate, A. tenuissima* and *Mucor sp. Erysiphe sp., Chaetomium sp*. and *Stachybotrys sp*. on were also observed on neem.

Hindi *et al.* (2014) Performed a study which showed Antibacterial activity of extracts of *Zingiber officinale* (ginger) against gram positive bacteria *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumonia, Streptococcus feacalis, Streptococcus mutanus, Streptococcus feacalis* and gram negative bacteria *Escherichia coli, Salmonella typhi, Moraxallia catarralis, Pseudomonas aeroginosa, Proteus mirabilis, Klebsiella pneumonia, Enterobacter spp. Acinetobacter, Serratia spp* . They used Four ginger products and Agar well diffusion method. They observed that Apple vinegar extract of fresh Ginger exhibited excellent and best antibacterial activity against both gram positive and gram negative bacteria and showed inhibition zone better than other ginger product.

Alzoreky and Nakahara (2002) screened Extracts of edible plants (26 species) from China, Japan, Thailand and Yemen for their antibacterial activity against *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *Salmonella infantis*. Buffered methanol (80% methanol and 20% PBS) and acetone extracted inhibitory substances against tested bacteria from 16 plants, as revealed by the disc assay.

2.3 Antibacterial sensitivity pattern

2.3.1 Antibacterial sensitivity pattern of edible plants

Hindi *et al.* (2014) discovered the antibacterial activity of aquatic extracts of fresh ginger, aquatic extract of powder ginger, Crude oil of Ginger and Apple vinegar extract of fresh Ginger. Agar well diffusion method was used in this study. Apple vinegar extract of fresh Ginger exhibited excellent and best antibacterial activity against both gram positive and gram negative bacteria and show inhibition zone better than other ginger product, while Aquatic extract of fresh, dry powder Ginger showed antibacterial activity against gram positive and gram negative bacteria and it show zone of inhibition better than Crude oil of Ginger. Most of ginger extracts show high antibacterial activity against both gram positive and gram negative bacteria; therefore ginger can provide protection to a certain extent against our natural enemies like bacterial pathogens.

Kamrul *et al.* (2014) showed the antimicrobial activity of the ginger extract against the all tested bacterial pathogens. In this study Soybean boil extract of ginger showed highest zone of inhibition (11.67±1.53mm) against *Salmonella spp*. and lowest zone of inhibition (8.0±1.73mm) against *Escherichia coli*. They had found that Ginger extract showed lower zone of inhibition (8.67±2.52mm) against *Staphylococcus aureus* compared to the Gram-negative bacteria and Soybean oil extract of ginger at boiling temperature has potential antimicrobial activity.

Chattopadhyay *et al.* (2009) conducted a comparative *in vitro* antibacterial potential of extracts (aqueous and ethanol) of five important medicinal plants (*Aeglemarmelos, A. indica, T. chebula, Mangifera indica and Ocimum sanctum)* were investigated using microbial growth inhibition assays against the common human pathogenic bacteria (*S. aureus, Pseudomonas aeruginosa and E. coli)* of clinical origin. All the plant materials showed varying degrees of strain specific inhibitory action and ethanol extract of the

plant materials showed higher antibacterial activity than their aqueous counterparts. Besides, *T. chebula* and *A. marmelos* had the strongest antibacterial activity out of which, *T. chebula* possessed a wider spectrum and a superior antibacterial potential over the others. The bioactive compounds of *T. chebula* might have potential as therapeutic agents for the treatment of common bacterial infections.

Mahmood *et al.* (2008) evaluated antibacterial activity of *A. indica* essential oil against five human pathogenic bacterial species *E. coli, Klebsiella sp., P. mirabulus, P. aeruginosa* and *S. aureus* by disc-diffusion method. Six mm discs were impregnated with 5 and 10 micro l of undiluted essential oil and seeded over the plates aseptically having test microorganisms. The zones of inhibition were measured after 24 hours at 37 degrees C. The essential oil exhibited significant antibacterial activity against all the test pathogens, with maximum zone of inhibition against *S. aureus*(20.0 mm & 41.5 mm) and minimum against *E. coli* (10.2 mm & 17.8 mm) for 5 and 10 micro l of essential oil, respectively. Similarly, the inhibition zones recorded in *P. mirabulus* were 15.1 mm & 26.0 mm, in *P. aeruginosa* 10.2 mm & 20.0 mm, in *Klebsiella sp.* 11.1 mm & 19.4 mm for two given concentrations of essential oil.

M. Yusha'u *et al.* (2008) discovered that fresh rhizomes of Ginger and cloves Garlic were collected, air-dried at room temperature and extracted separately using ethanol as solvent of extraction. The extracts were tested for antimicrobial activity against respiratory tract isolates of *Pseudomonas aeruginosa*, *Morganella morganii*, Providencia species, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Proteus vulgaris*. The antimicrobial activity of ginger and garlic extracts on test isolates was determined using disc diffusion method. The results of sensitivity tests indicated that ethanolic extracts of both ginger and garlic have in vitro inhibitory activity against all isolates of Gram-negative organisms tested and sensitivity of the isolates increases with increase in concentration. Ethanolic extract of Garlic showed greater in vitro inhibitory activity than that of Ginger against all isolates tested.

Alzoreky and Nakahara (2003) roported that buffered methanol (80% methanol and 20% PBS) and acetone extracts of edible plants of 26 species including neem screened for their antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella infantis* by the disc assay showed that the MIC of extracts determined by the agar dilution method ranged from 165 to 2640

mg/ml. *B. cereus* was the most sensitive microorganism to extracts from *A. indica*, *Rutagraveolens*, *Rumexnervos us*, *Thymus serpyllum* and *Zingiber officinale* with MIC of 165 to 660 mg/ml.

Alzoreky and Nakahara (2003) observed the minimum inhibitory concentrations (MICs) of extracts determined by the agar dilution method ranged from 165 to 2640 mg l _ 1. The most sensitive microorganism to extracts from *Azadirachta indica, Cinnamomum cassia, Rumex nervosus, Ruta graveolens, Thymus serpyllum and Zingiber officinale* was *B. cereus*, with MIC of 165 to 660 mg l _ 1. *E. coli* and *S. infantis* were only inhibited by *Cinnamomum cassia* extracts at the highest MIC (2640 mg l _ 1).

Sadekar *et al.* (1998) reported that neem (*A. indica*) dry leaves powder as medical herbs could be beneficial in immunosuppressant diseases of poultry. Neem leaf powder has immune stimulant effect that activates the cell mediated immune response and therefore, creates an enhanced response to any future challenges occurred by disease organisms. So, the feeding neem leaves to immunosuppressed birds increase their humoral and cell mediate immune responses.

2.3.2 Antibacterial sensitivity pattern of commercial antibiotics

Muhammad *et al*. (2010) investigated the prevalence of *Salmonella* associated mortality in three hatcheries in Joss, Central Nigeria. The author also evaluated their susceptibility to antimicrobial agents. Antimicrobial susceptibility tests showed a high prevalence of antimicrobial resistance in the study area with complete resistance to Gentamycin, Enrofloxacin, Nalidixic acid, Tetracycline and Streptomycin and substantial resistance to Triple Sulphur and Ciprofloxacin.

Dione *et al*. (2009) determined the prevalence and distribution of *Salmonella* on 57 randomly selected broiler farms in urban and periurban areas in Casamance, Senegal. They evaluated the antimicrobial resistance profiles of the *Salmonella*serovars. High levels of resistance were found to Trimethoprim-Sulfamethoxazole, Tetracycline, Trimethoprim, Streptomycin and Sulfonamides. All *Salmonella*serovars were susceptible to Fluoroquinolones and third-generation Cephalosporins. A large proportion of the isolates belonging to 11 serovars were resistance to two or more antibiotics.

Lestari *et al*. (2009) carried out a 1-year survey from October 2006 to September 2007. The author isolated and characterized 126 *Salmonella* isolates from conventionally raised

(n=141)and organically raised (n=53) chicken carcasses obtained from 27 retail stores in Baton Rouge, Louisiana. *Salmonella* was isolated from 22% of conventional and from 20.8% of organic chicken samples. Eight *Salmonella*serovarswere identified. All *Salmonella* isolates were susceptible to Amikacin, Ceftriaxone and Ciprofloxacine; however, decreased susceptibility to Quinolones (7.1%) or extended-spectrum Cephalosporins (45.2%) was observed. Resistance to multiple antimicrobials (two or more) was found among 52.4% of the *Salmonella* isolates. Antimicrobial resistance profiles differed greatly among *Salmonella*serovars and also depended on the type of chicken from which they were recovered.

Someya *et al.* (2008) examined susceptibilityof antimicrobial agents against 325 isolates of *Salmonella enteric* serotypes Cerro, Infantis,Livingstone and Montevideo isolates from layer houses on a commercial egg-production farm in the western region of Japan between 1997 and 2002. No antimicrobials were used for therapeutic purposes on the farm during this period. From 1.8 to 3.1% of the isolates were resistance to Ampicillin, Chloramphenicol and Tetracycline. Resistance to Streptomycin and Sulfisoxazole was found in 52.9 and 65.5%, respectively of *Salmonella montevideo* isolates and in 0 to 13.2% of the isolates of the other serotypes. All the streptomycin-resistant isolates of *Salmonella montevideo* also exhibited resistance to Sulfisoxazole. *Salmonella Montevideo* isolates were first isolated in 1998, and 80.0% of the isolates obtained in this year were resistance to Streptomycin and Sulfisoxazole.

Kobayashi *et al.* (2007) collected a total of 328 cloacal swabs and 163 footpads of wild birds which were investigated for the presence of *Salmonella*. All 19 isolates from cloacal swabs were serotyped as *Salmonella typhimurium* susceptible to all five conventional antimicrobial agents (Ampicillin, Chloramphenicol, Streptomycin, Oxytetracycline and Nalidixic acid) tested. In contrast, 15 *Salmonella* isolated from footpads included *S. muenhen*, *S. Virchow*, *S. bareily* and *S. bovisrnorbificans*, including *S. typhimurium*; these non-Salmonella *typhimurium* isolates showed multiple drug resistance.

Akter *et al.* (2007) determined the antibiogram study revealed that the isolates were sensitive to Ciprfloxacin (80%), Nitrofurantoin (100%), Sulphamethoxazole/Trimeoprim and Amoxycilline (50%), Tetracycline (60%) but resistance to Penicillin-G and Erythromycin. The author suggest for further studies should be conducted on serotyping

of the isolated *Salmonellae*, isolation and identification of *Salmonellae* from different feed and environmental samples.

Balala (2006) identified that isolates (cloacal swabs) were sensitive to Norfloxacin, Gentamicin, Cephalothin but were resistant to Nitrofurantoin. All serotypes were also resistant to at least one antibiotic while 9.7% showed multi-drug resistance to Nitrofurantoin, Tetracycline and Trimethoprim-Sulfamethoxazole.

Zaidi *et al.* (2006) carried out a research and staled that resistance to oral drugs used for the treatment of Salmonellosis was observed for Ampicillin (14.6% of isolates were resistant), Chloramphenicol (14.0 ofisolates) and Trimethoprim-Sulfamethoxazole (19.7% of isolates). Resistance to Ceftriaxone emerged in 2002 and was limited to the serotype *S. typhimurium*. Twenty-seven percent of the isolates were resistant to Nalidixic acid and none were resistant to ciprofloxacin. Multidrug resistance was most common among isolates of serotypes *S. typhimurium and S. anatum*.

Wilson (2004) isolated from raw, chilled, retail chickens (n=434) sampled between 1998 and 2000 for resistance to 12 antibiotics. Of 23 *Salmonella* isolated, 30% were susceptible and 30% were resistant or intermediately resistant to one antibiotic, 26% to two and 13% to four or more. One *Salmonella* Saint-paul and two *Salmonella typhimurium* isolates were resistant to more than four antibiotics. Highest resistance rates were Sulfonamide (52%), Streptomycin (26%), Tetracycline (22%) and Ampicillin (17%). Isolates (n=27) from frozen chicken portions (n=150) imported from Brazil and Thailand were also tested. Brazilian *Salmonella* showed no multiple resistances, but an isolate of *Salmonella virchow* from Thai chickens was resistant to two antibiotics.

Xu *et al*. (2003) investigated respiratory and alimentary microflora, in cases of distemper and parvovirus infection. *E. coli*, *Salmonella* and *Staphylococcus* existed in 45 nasal secretion samples from distemper cases with diarrhea. *Staphylococcus* and *Klebsiella pneumonia* in 36 nasal secretion samples from distemper cases mainly with respiratory symptoms. In an antibiotic sensitivity test, bacteria from faeces and nasal secretion cultures showed the highest sensitivity rates of 83.33% and 86.36% Amikacin (from 8 antibiotics tested). In a united antibiotic sensitivity test, Chloramphenicol and Amikacin showed the highest synergism rates of 58.82% and 63.4% to bacteria from feces and nasal secretion cultures respectively.

Pumshothanian *et al.* (2003) performed an antibiogram testing using 65 local isolated *Salmonella spp*. from poetry environment. They conducted the experiment on 33 different antibiotics and antibacterial agents and found that all of the isolates were susceptible to Cotrimoxazole, Cephaloridine and Cephalexin.

Anzai *et al.* (2003) examined sixteen strains of *S. enteric* serotype *typhimurium* (*S. typhimurium*) isolated from foals from 1981 Until 1996 in Hokkaido Prefecture, Japan, for their biotype, drug resistance, plasmid profile, virulence plasmid and virulence- in mice and foals. Nine strains isolated from through bred foals in Hidaka district in1981 were shown to possess similar properties. Likewise, two strains isolated from crossbred foals in a farm in Sorachi district in 1996, although district from the Hidaka isolates, were similar to one another. These 11 strains were all shown to contain a virulence plasmid and to be virulent in mice and foals. However five other strains isolated from through bred foals that were bred in different farms in Hidaka between 1990 and 1993 demonstrated variable properties and did not possess the virulence plasmid nor show virulence mice. The author suggested that large outbreaks of *S. typhimurium* infection ill foals were likely to be caused by virulent strains possessing the virulence plasmid, although sporadic infections may also be non-virulent *S. typhimurium*.

Gita *et al.* (2003) observed pathogenicity test of *S. worthinglon* isolated from Japanese quails was conducted in while albino mice, India. All the mice died within 2448 h suggesting that the organism were virulent. The pathological changes included congestion of the liver, intestine and spleen. *S. worthinglon* were re-isolated from dead mice. Aimblograril of *S. worthinglon* showed them to be sensitive to most antibiotics except Gentamicin.

Sung *et al.* (2002) performed an antibiogram study of 72 isolated *Salmonella* front in South Korea, against 13 antimicrobial drugs available 3market. About 57% of the isolates were resistant to Nalidixic acid (NA), 38.9% to Ampicillin (AM), 34.7% to Streptomycin (SM), 27.8% each to Carbenicillin (CB) and Tetracycline (TE) and 18.1% to Kanamycin (KA). There were less than 10% of the strains that were resistant to Sulfamethoxazole/Trimethoprim (ST) Andcefalitin (CF). The most frequent multiple resistant pattern was resistance to AM, CB, KA, SM, TE and NA (26.4%).

Roy *et al.* (2002) isolated a total of 569 *Salmonella spp*. out of 47, 15 samples from poultry products poultry and poultry environments in 1999 and 2000 from the Pacific

Northwest (USA). Among the isolates 92 *Salmonella spp*. were tested for antibiotic sensitivity. All of the isolates were resistant to Erythromycin, Lincomycin and Penicillin except for one sample, *S. berta*, which was moderately sensitive to penicillin. All of the tested *Salmonella spp*. was susceptible to Sarafloxacin the percentage of *Salmonella spp*. susceptible to Sultamethoxazole-Trimethoprim, Gentamicin triple sulfa and tetracycline were 97.83, 92.39, 86.96 and 82.61% respectively.

Takahashi *et al.* (2001) isolated thirty-six strains of *Salmonella typhimurium* from skin surfaces and cecal contents of broilers at chicken processing plants Prefecture between 1997 and 1999 were examined for drug resistance. Pattern of drug resistance showed that 6 strains were resistant to 1 drug and 30 strains resistant too2 or more antibiotics. One strain *S. typhimurium* definitive pillage type 104 (DT104) was found to be multidrug-resistant.

Hui and Das (2001) isolated 280 *Salmonella spp*. from different internal organs of poultry. They found that all the isolates were sensitive to Gentamicin (100%), followed by Ciprofloxacin and Ofloxacin (93.33% each), Chloramphenicol and Norfloxacin (86.66% each), Cefotaxime (80%), Kanamycin (73.34%), Cotrimoxazole and Nalidixic acid (66.67% each) and Cloxacilline (93.33%) followed by Tetracycline (96.66%) and Penicillin G (Benzyl penicillin) (80%).

Bau *et al.* (2001) isolated 13 *Salmonella spp.* from poultry products in Brazil and on antibiogram study found that all isolates were resistant to Penicillin G and susceptible to the other antimicrobial drugs tested.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

The research work was conducted during the period from July to December, 2016 at the bacteriological laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. The detailed outline of the materials and methods are given below:

3.1 Materials used

3.1.1 Study area

The samples were collected from the selected layer flocks at different areas of Rangpur division (Dinajpur, Thakurgoan, Panchagahr, Nilphamari District) and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University for laboratory analysis.

3.1.2 Research design

Completely Randomized Design (CRD) and descriptive cross-sectional survey were used to isolate and identify the *Salmonella* serovars from selected layer flocks. The design was chosen because the study was concerned with identification of *Salmonella* serovars from selected layer flock by using morphological, cultural and biochemical techniques.

3.1.3 Sample size determination

Sample size was determined by using prevalence rate of 12.86% from previous studies (Akter *et al.,* 2007) at 5% level of significance and the following formula was employed (Zelalem *et al.,* 2011).

$$N = \frac{Z^2pq}{d^2}$$

Where,

N= is the desired sample size

Z = is the standard normal deviation that provide 95% confidence interval (1.96)

P = is the prevalence rate of *Salmonella* in layer from previous studies (Akter *et al.,* 2007)

q = 1-p

d = is the absolute precision (error bound 0.05)

$$N = \frac{(1.96)^2 \times (.13) \times 0.87}{(0.05)^2}$$

$$= \frac{3.84 \times 0.87}{(0.05)^2}$$

$$= \frac{0.4224}{0.0025} = 168.96 = 169 \approx 168$$

3.1.4 Data collection procedure

The structured questionnaire was used for the data collection by using face to face farmer interview. Farmer interview were done using a structured questionnaire on participants who rear the layer flocks.

3.1.5 Sample collected from dead birds

A total of 168 field samples comprising liver, heart, lung of the study areas were aseptically collected and carried to the Microbiology laboratory for isolation and characterization of *Salmonella* serovars (Table 1).

Table 1: Samples from suspected birds based on spatial differences

Name of district	No. of farm	Types of samples	No. of sample tested
Dinajpur	15	Liver	24
		Heart	24
		Lung	24
Thakurgaon	6	Liver	14
		Heart	14
		Lung	14
Panchagarh	4	Liver	8
		Heart	8
		Lung	8
Nilphamari	5	Liver	10
		Heart	10
		Lung	10
Total	30		168

3.1.6 Collection and preparation of edible plant extracts

The Neem leaves were collected from HSTU campus and fresh garlic and ginger were also collected from local market of Dinajpur town. Garlic and ginger were sliced with sterile sharp knife. The sliced pieces and Neem leaves were cleaned and washed using sterile distilled water. For the preparation of dust, the collected samples were sun dried for a week and followed by oven at 55-60°C for 2 days. The dried samples were pulverized in electric grinder to get extractable powder. The dusts were preserved in airtight plastic container until they were directly used for screening and preparation of ethanol extract. Ten (10) gram of each powder were added to 80ml of ethanol and was shaking overnight at room temperature, then the suspensions were filtered. After that filtrates were concentrated using Rotary-evaporating machine to get viscous substance. These were transferred to a beaker and taken on a water bath for further drying at room temperature. Finally a solid mass were obtained and stored at 4°C until use. The extracts were considered as the 100% concentration of the extract. The concentrations 80%, 100% and 120% were made by diluting the concentrated extract with appropriate volumes of 100% ethanol.

3.1.7 Preparation of Ethanolic extract disc

Disc having different concentration (80%, 100% and 120%) of ethanolic extract of Neem, Garlic and Ginger were prepared using filter paper and it was allowed to dry for 30 minutes.

3.1.8 Experimental birds

The birds were divided in to three groups categorized as group A, B and C on the basis of their age by following way-

Group A: 0-8 weeks
Group B: 9-20 weeks
Group C: Above 20 weeks

3.1.9 Materials used for sample collection

3.1.9.1 Glassware and appliances

The glassware's and appliances were used during the whole period of the experiment are as follows: scalpel, forceps, scissors, tray, petridishes, test tubes, conical flask, pipette, micro pipette, slides, 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, spirit lamps, match lighter, bacteriological loop, inoculum loop, autoclave machine, filter paper.

3.1.9.2 Chemicals and reagents

The chemicals and reagents used for the study were Gram's stains (Gram's iodine, safranin, acetone alcohol, immersion oil), Methyl Red-Vogesproskaur (MR-VP) solution, Kovac's indole reagent, alcohol, glycerin and other common laboratory reagents and chemicals.

3.1.10 Media for culture

3.1.10.1 Liquid media

3.1.10.1.1 Nutrient broth (NB)

Nutrient broth (NB) was used to grow the *Salmonella* organisms from the samples collected from the samples collected from the study areas before performing biochemical test and disinfectant efficacy test.

3.1.10.1.2 Bacto selenite broth (BSB)

Bacto selenite broth (BSB) is a useful fluid medium which allows the growth of *Salmonella* but inhibits other enteric organisms.

3.1.10.2 Solid Media

3.1.10.2.1 Nutrient agar (NA)

Nutrient agar (NA) medium was used to grow the *Salmonella* organisms from the collected samples.

3.1.10.2.2 Salmonella-Shigella agar (SS)

Salmonella-Shigella (SS) agar medium was used as a selective medium for *Salmonella* organism which causes enhancement of the growth of *Salmonella* while inhibiting the growth of other contaminating organisms and shows typical colony characteristics.

3.1.10.2.3 Brilliant green agar (BGA)

Brilliant green agar (BGA) was used as a selective medium for the isolation and identification of *Salmonella* organisms.

3.1.10.2.4 MacConkey agar (MC)

MacConkey agar (MC) was used for the identification of organisms under the family *Enterobacteriaceae* through studying fermentation characteristics.

3.1.10.2.5 Eosin methylene blue (EMB) agar

Eosin methylene blue (EMB) agar medium was used for the purpose of observing differential growth of *Salmonella spp.* and *Eschrichia coli*.

3.1.10.3 Media used for biochemical test

In order to identify *Salmonella* the following media were used for biochemical tests: Sugar media (dextrose, maltose, lactose, mannitol and sucrose), Simmons citrate agar, Triple sugar iron (TSI) agar, MIU, Indole test, Methyl Red-Vogesproskaur (MR-VP) test.

3.1.11 Hexisol hand rub

Hexisol hand rub (100 ml bottle) was used for antisepsis prior to swab sample collection from the selected species.

3.1.12 Data analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS version 16.0).

3.2 Methods

3.2.1 Experimental lay out

The layout of the experiment is schematically presented in figure 1, figure 2 and figure 3.The first step includes the isolation and identification of *Salmonella* serovars from selected layer flock (dead birds) by using morphological, cultural and different biochemical techniques. The second step is antibacterial sensitivity pattern of medicinal plants against identified field isolates and the third step is antibacterial sensitivity pattern of some commercial antibiotics.

Layout of the experiment:

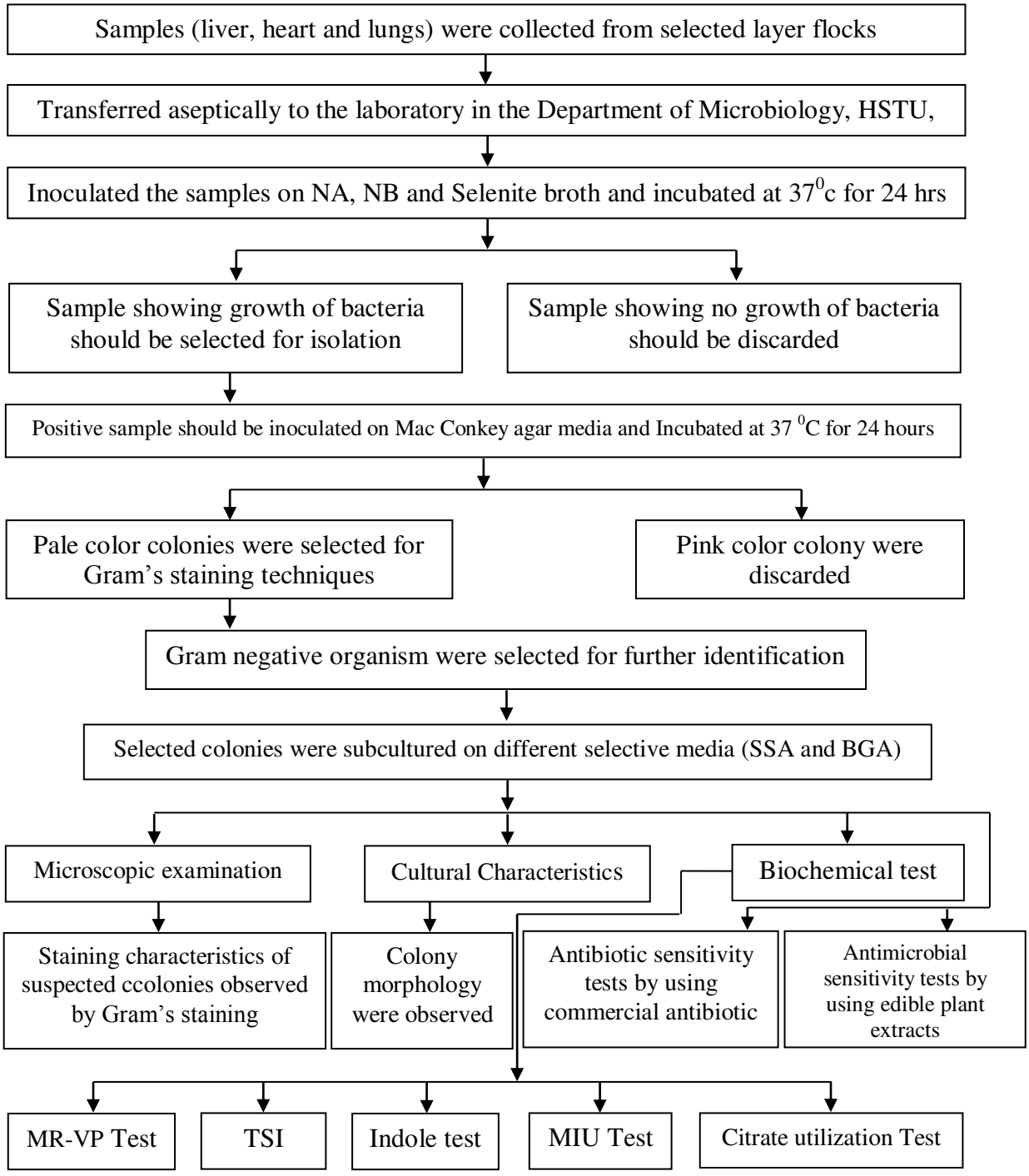

Fig. 1: Schematic Illustration of the isolation and identification of *Salmonella* serovars from selected layer flocks

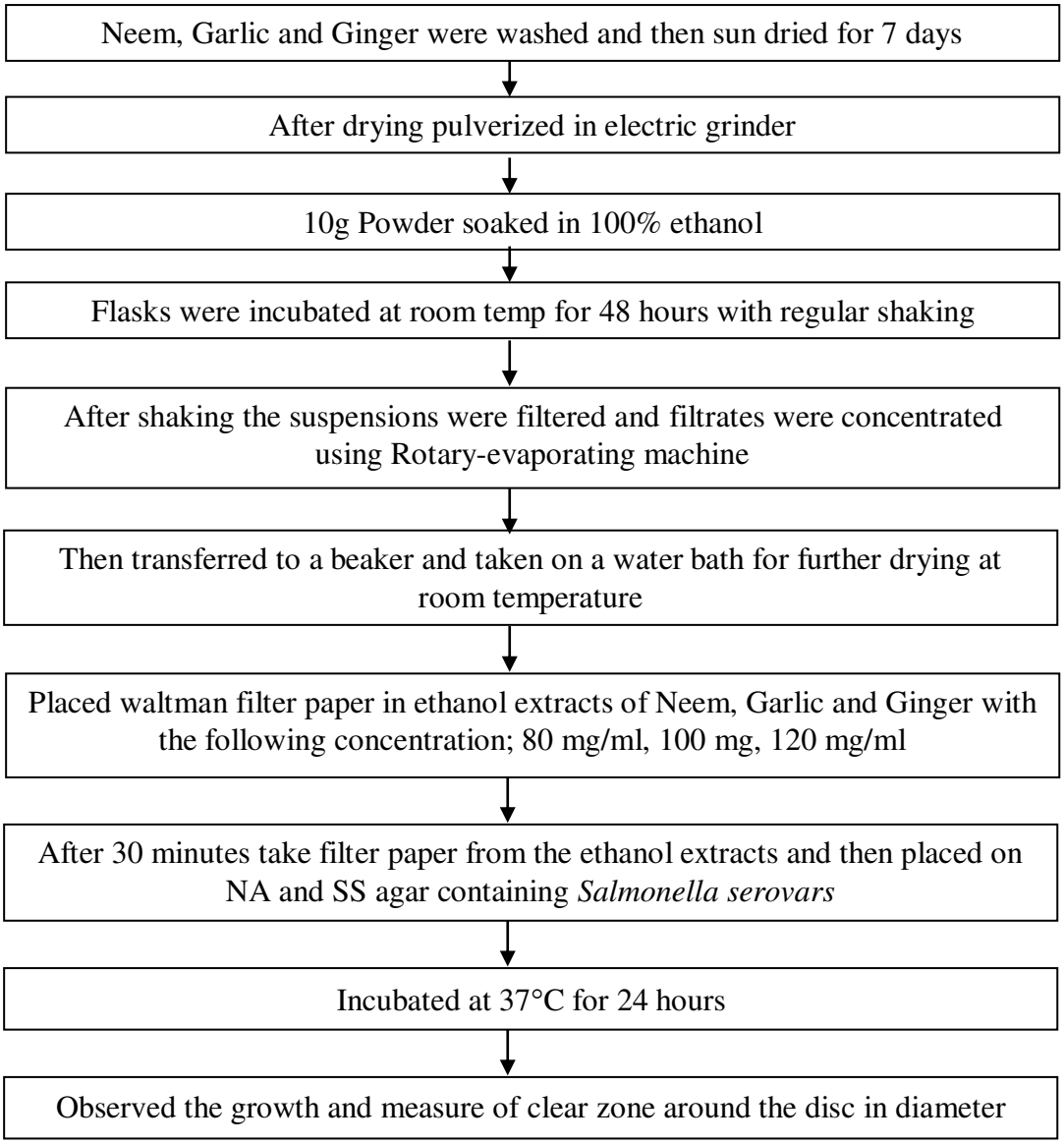

Fig. 2: Schematic Illustration of the extraction and antibacterial sensitivity pattern of medicinal plant

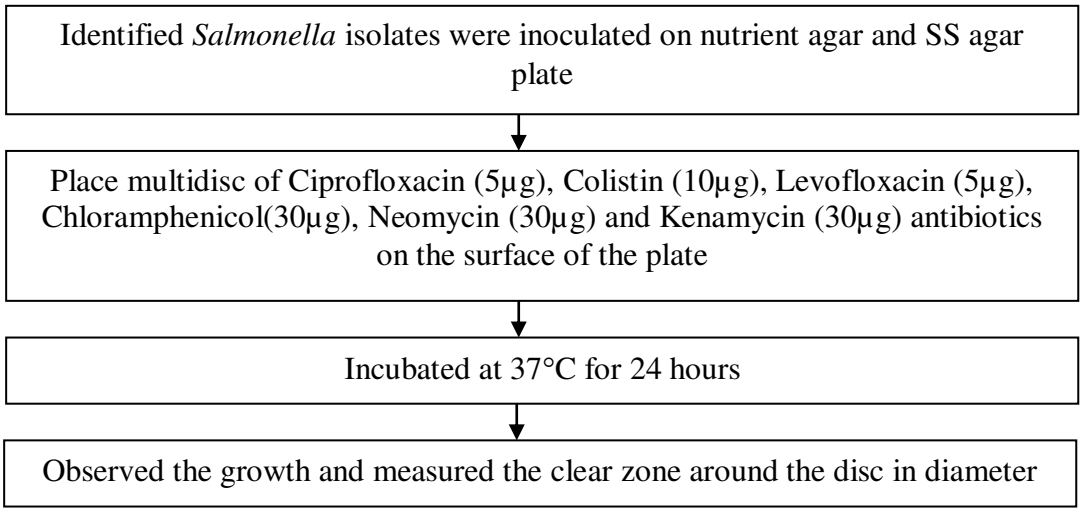

Fig. 3: Antibacterial sensitivity pattern of some commercial antibiotics

3.2.3 Preparation of culture media and reagents

3.2.3.1 Culture media

3.2.3.1.1 Liquid media

3.2.3.1.1.1 Nutrient broth media

For preparation of NB, 13 gms of dehydrated NB base (Hi Media, India) was dissolved in 1000 ml of distilled water, heated gently by an electric heater & then sterilized by autoclaving at 121^0C for 15 lbs pressure per square inch for 15 minutes. The broth was transferred to sterile test tubes in 5ml quantities and incubated at 37^0C over night to check the sterility of broth and then stored at 4^0C in the refrigerator until used.

3.2.3.1.1.2 Bacto selenite broth

In 100 ml of cold water, 2.3 grams of dehydrated Bacto selenite broth (Difco) was added and heated up to boiling to dissolve the medium. It was then shaken well and distributed in 5 ml quantities to each of the sterile test tubes stoppered with cotton plugs and sterilized in the autoclave for cultural characterization or stored at 4^0C in refrigerator for future use.

3.2.3.1.2 Solid media

3.2.3.1.2.1 Nutrient agar media

28gms of Bacto-Nutrient agar (Hi Media, India) was suspended in1000ml distilled water and boiled to dissolve completely. The solution was sterilized by autoclaving at l21^{0}C for 15 minutes at 15 lbs pressure. After autoclaving, the medium was poured in 20ml quantities in sterile petri dishes to form a thick layer and was allowed to solidify. It was kept incubation overnight at 37^{0}C to check the sterility of the medium. The sterile nutrient agars were stored at refrigerator at 4^{0}C until used.

3.2.3.1.2.2 Salmonella-Shigella agar media

An amount of 60gms powder of SS agar base (Hi-media, India) was added to 1000 ml distilled water in a flask and heated to boil for dissolving the medium completely. The medium was then sterilized by autoclaving at 12^{0}Cmaintaining a pressure of 15 lb pressure/sq. inch for 15 minutes. After autoclaving, the medium was put into a water bath at 45^{0}C to cool down its temperature. Then 20 ml of medium was poured into each sterile petridishes and allowed to solidify. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37^{0}C for overnight to check their sterility and then stored at 4^{0}C in a refrigerator for future use.

3.2.3.1.2.3 Brilliant green agar

According to the direction of manufacturer 52 gm of BGA powder (Oxoid, England) was suspended in 1000 ml of distilled water in a conical flask. It was then gently heated with gentle agitation and brought just to the boil to dissolve the medium completely. After sterilization by autoclaving, the medium was cooled to 50^{0}C, mixed properly and poured into sterile petridishes (10 ml each petridish) and allowed to solidify. Then the petridishes were incubated at 37^{0}C for overnight to check their sterility and used to culture the organism or stored at 4^{0}C in refrigerator for future use.

3.2.3.1.2.4 MacConkey agar media

An amount of 51.5 gms of Bacto-MacConkey agar (Hi Media India) suspended in 1000 ml of distilled water were taken in a flask. The suspension was heated up to boiling to dissolve the medium completely and then sterilize by autoclaving at 121^{0}C under 15 Ibs pressure per square inch for 15 minutes. The media was then poured into sterile

petridishes (75 mm diameter) in 20 ml quantities to form thick layer. The sterile of the media was checked by incubating at 37^0C over-night and stored at 4^0C.

3.2.3.1.2.5 Eosine Methylene Blue agar

36 gms of EMB agar base (Hi-Medium Laboratories Pvt. Ltd) was added to 1000ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in 10 ml quantites in sterile glass petridishes (medium size) and 15 ml quantites in sterile glass petridishes (larger size) t form a thick layer. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of petridishes partially removed. The sterility of the medium was judged and used to store at 4^0C in refrigerator for further use.

3.2.3.2 Reagents preparation

3.2.3.2.1 Simmons Citrate agar

24.28 grams of Simmons Citrate agar base (Hi-Media Laboratories Pvt. Ltd) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium size) and in 15 ml quantities in sterile petridishes (larger size) to form a thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of petridishes partially removed. The sterility of the medium was judged and used to store at 4^0C in refrigerator for further use.

3.2.3.2.2 Procedure of Indole test

2 ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours.0.5 ml Kovac's indole reagent was added, shaked well and examined after 1 minute. A red color ring at the top of the reagent indicated positive test. In negative case there is no development of red ring.

3.2.3.2.3 Procedure of Motility Indole Urease Test (MIU)

MIU was prepared in a test tube. Then the organism was inoculated into the media by stabbing method with the help of sterile straight wire. Then the test tube was incubated

37^0C overnight. Single stick that is no turbidity throughout the medium indicate gram negative organism (non motile) and turbidity throughout the medium indicate gram positive case.

3.2.3.2.4 Procedure of Triple Sugar Iron Test (TSI)

Triple sugar irons contain three sugars (Glucose, Sucrose and Lactose). At first TSI agar slant was prepared in a test tube. Then the organism was inoculated into the butt with a sterilized wire and on the slant with a wire loop producing zigzag streaking. The tube was incubated for 24 hours at 37^0C.Yellow color of butt and slant of the test tube indicate fermentation of Glucose, Sucrose and Lactose fermentation and butt shows blacking indicate H_2S production.

3.2.3.2.5 Methyl Red-Voges Proskaure test

3.2.3.2.5.1 Preparation of MR-VP broth

A quantity of 17 gms of Bacto MR-VP medium (Hi Media, India) was dissolved in 1000 ml distilled water dispensed in 2 ml amount in each test tube and then the tubes were autoclaved at 121^0C maintaining a pressure of 15 pounds/sq. Inch for 15 minutes. After autoclaving, the tubes containing medium were incubated at 37^0C for overnight to check their sterility and then stored in a refrigerator for future use.

3.2.3.2.5.2 Preparation of MR solution

The indicator methyl red solution was prepared by dissolving 0.1 gm of Bacto methyl-red (Difco) in 300 ml of 95% alcohol and diluting to 500 ml with the addition of 200 ml of distilled water.

3.2.3.2.5.3 Voges-Proskauer solution

3.2.3.2.5.3.1 Alpha-naphthol solution

Alpha-naphthol solution was prepared by dissolving 5gms of 1-naphthol in 100ml of 95% ethyl alcohol.

3.2.3.2.5.3.2 Potassium hydroxide solution

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

3.2.3.2.6 Procedure of MR test

The test was performed by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37^0C, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of a bright red color. A yellow or orange color was a negative test.

3.2.3.2.7 Procedure of VP test

2 ml of sterile glucose phosphate peptone water were inoculated with the 5 ml of test organisms. It was incubated at 35-37^0C for 48 hours. A very small amount (knife point) of creatine was added and mixed. 3 ml of 40% potassium hydroxide were added and shacked 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 negative cases there was no development of pink color.

3.2.3.2.8 Gram's staining method for Morphological characterization

The representative *Salmonella* colonies were characterization morphologically using Gram's stain according to the method described by Merchant and Packer (1967).

Briefly, a small colony was picked up from SS and BG 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 was then added to act as mordant for one minute and then again washed with running water. Acetone alcohol was then added (acts as decolorizer) for few seconds. After washing with water, safranin was added as counter stain and allowed to stain for two 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.

3.2.3.2.9 Antibiotic sensitivity tests

All bacterial isolates subjected to antibiotic sensitivity test by Kirby-Bauer Disc diffusion method according to the guidelines of The European Committee on Antimicrobial Susceptibility Testing standard (EUCAST).The antimicrobial discs were applied to the plates. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the undersurface of the plate without opening the lid. The zones of

growth inhibition were compared with the zone size interpretative table provided by EUCAST (Table 1). Antimicrobial testing results were recorded as sensitive, intermediate and resistant according to zone diameter interpretative standards provided by EUCAST.

3.2.3.2.10 Antibacterial activity of medicinal plant extracts

Susceptibility and resistance of different antibiotics was measured in vitro by employing the modified Kirby-Bauer (Bauer *et al.,* 1966) method. This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the disc.

3.2.3.2.10.1 Disk diffusion method

Filter paper discs of 6mm in diameter were prepared and sterilized. The cultures were enriched in sterile nutrient broth for 6-8 hours at 37^0C. Using sterile cotton swabs, the cultures were aseptically swabbed on the surface of sterile nutrient agar plates, the different concentration of ethanolic extract of neem, garlic and ginger discs were aseptically placed over the seeded agar plates sufficiently separated from each other to avoid overlapping of inhibition zones. The plates were incubated at 37^0C for 24 hours and the diameter of the inhibition zone was measured in mm (Bandna Chand, 2013).

Table 2: List of plants with their concentration

Edible plants	Symbol	Disc concentration (mg/ml)		
Neem	N	80%	100%	120%
Garlic	Ga	80%	100%	120%
Ginger	Gi	80%	100%	120%

3.2.3.2.11 Maintenance of stock culture

During the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose the organisms from pure culture were inoculated into the tubes of nutrient agar slants and incubated at 37^0C for 24 hours. After the growth of organisms the tubes were sealed with paraffin wax and kept in the refrigerator at 4^0C.

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

The current study was performed as per experimental layout mentioned in page no. 23-25. As per experimental layout the antibacterial effect of plant extracts against *Salmonella* serovars were studied for the first time in layer flock with isolation and identification of 168 samples from suspected birds infected with Salmonellosis. The isolates were confirmed by using morphological (staining), cultural and biochemical techniques and positive isolates were also used for the detection of antibacterial effects by using some edible plants extract namely Neem, Garlic and Ginger. The results of above mentioned all experiments were presented below:

4.1 Isolation and identification of *Salmonella* serovars

Salmonella serovars were isolated and identified from the layer bird samples (liver, heart and lung) after cultivation on NA, MC agar, BGA and SS agar medium. The results of morphological study and cultural characteristics are presented in (Table 5, Page 38). *Salmonella* serovars was detected from seventy six out of two hundred samples. Among the positive samples, 15 farms were from Dinajpur, 6 farms were from Thakurgoan, 4 farms from Panchagarh and 5 farms were from Nilphamari. The percentages of positive isolates from different samples (liver, heart and lung) of above mentioned areas were 13.89%, 11.91%, 8.33% and 10% respectively and the average prevalence of *Salmonella* serovars in the study area was 11.9% (Table 3, Page 36). In case of internal organs, (liver; 10, heart; 04, lung; 06) the prevalence of *Salmonella* were also 17.86%, 7.14% and 10.72% respectively (Table 4, Page 36). Each of the positive samples was treated as an isolates.

Table 3: Prevalence of *Salmonella* serovars based on spatial (study area) differences

Name of district	Types of samples	No. of sample tested	No. of positive isolates	Prevalence of isolates (%)	χ^2 value	Level of significance
Dinajpur	Liver	24	4	13.89	0.666	0.881 (NS)
	Heart	24	3			
	Lung	24	3			
Thakurgoan	Liver	14	2	11.91		
	Heart	14	1			
	Lung	14	2			
Panchagarh	Liver	8	2	8.33		
	Heart	8	-			
	Lung	8	-			
Nilphamari	Liver	10	2	10		
	Heart	10	-			
	Lung	10	1			
Total		**168**	**20**	**11.9**		

Table 4: Organoleptic prevalence of *Salmonella* infection in dead birds

Name of organ	No. of sample tested	No. of positive isolates	Prevalence of isolates (%)	χ^2 value	Level of significance
Liver	56	10	17.86	3.18	0.20 (NS)
Heart	56	04	07.14		
Lung	56	06	10.72		
Total	**168**	**20**	**11.9**		

4.2 Identification of field isolates by cultural and morphological characteristics

4.2.1 Results of cultural examination

4.2.1.1 Nutrient broth

Nutrient broth inoculated separately with the samples for observing the growth of bacteria and incubated at 37^0C for 24 hours. After 24 hours of incubation the presence of turbidity indicate the positive result (Plate 1, Page 45, Table 5).

4.2.1.2 Nutrient agar

Nutrient agar plates streaked separately with the samples revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and was indicated by the growth of circular, smooth, opaque, translucent colonies (Plate 2, Page 45, Table 5).

4.2.1.3 MacConkey (MC) agar

On MacConkey agar the organisms showed pale, colorless smooth, transparent raised colonies (Plate 3, Page 46, Table 5).

4.2.1.4 Selenite broth

Bacto Selenite broth inoculated separately with the colonies from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and positive result was indicated by the presence of brick red coloration in selenite broth and also confirmed by streaking on BGA or SS agar plate (Plate 4, Page 46, Table 5).

4.2.1.5 Salmonella-Shigella (SS) agar

The organisms exhibited opaque, translucent colorless, smooth, round colonies with black center when cultured on SS agar (Plate 5, Page 47, Table 5).

4.2.1.6 Brilliant green agar (BGA)

The organisms produced pale pink color colonies against a pinkish background when cultured on BGA, which was green in color before growth of the organisms (Plate 6, Page 47, Table 5).

4.2.2 Results of Gram's staining technique

In Gram's staining the organisms revealed Gram-negative, Pink colour, small rod shaped appearance, arranged in single and paired when observed under microscope (Plate 7, Page 48, Table 5).

Table 5: Identification of *Salmonella* serovars by cultural and morphological characteristics

Sample (organs)	Colony Characteristics				
	Staining characters	**NA**	**MC**	**SS**	**BGA**
Liver	Gram negative, short rod shaped	Circular, smooth, opaque, translucent colonies	Pale, colorless smooth, transparent raised colonies	Opaque, translucent colorless, smooth, round colonies with black center	Pale, pink color colonies against a pinkish background
Heart					
Lungs					

Legends

NA = Nutrient agar; **MC** = MacConkey agar; **SS** = Salmonella-Shigella agar; **BGA** = Brilliant Green agar.

4.2.3 Characterization of field isolates by biochemical tests

4.2.3.1 Methyl red test

In Methyl Red test the appearance of red color in the media after the addition of appropriate reagent (Methyl red) with the cultural growth was observed and thus indicating the isolated *Salmonellae* were positive for MR test (Plate 8, Page 48, Table 6).

4.2.3.2 Voges-Proskauer test

In Voges-Proskauer (VP) test yellow coloration of the media was observed after the addition of appropriate reagent (Alpha-naphthol, Potassium hydroxide) with cultural growth was observed and thus indicating the isolated *Salmonellae* were negative for VP test (Plate 9, Page 49, Table 6).

4.2.3.3 TSI slant reaction

In the stab culture of TSI agar, the isolated *Salmonella* organisms produced an alkaline reaction (red) in slant and acid reaction (yellow) in the butt and slightly black color due to H_2S gas production were also recorded (Plate 10, Page 49, Table 6).

4.2.3.4 Motility Indole Urea (MIU) tests

The isolated *Salmonella spp*. produced turbidity throughout the medium was indicated that the organism is non-motile, no red color in the neck (Indole negative) and no urease production (Plate 11, Page 50, Table 6).

4.2.3.5 Indole test

All of the isolates were indole negative (Plate 11, Page 50, Table 6).

4.2.3.6 Simmons citrate agar

In the citrate agar utilization test the color of green medium turned to deep blue color indicating the isolates were positive for *Salmonella spp*. (Plate 12, Page 50, Table 6).

Table 6: Organoleptic characterization of *Salmonella* serovars by biochemical tests

Isolates	Indo	MR	VP	SC	MIU	TSI		
						Butt	Slant	H_2S
Salmonella	_	+	_	+	_	Y	R	+
	_	+	_	+	_	Y	R	+
	_	+	_	+	_	Y	R	+

Legends

Indo = Indole; **MR** = Methyle Red; **VP** = Voges-Proskauer; **SC** = Simmons citrate utilization; **MIU** = Motility Indole Urea; **TSI** = Triple Sugar Iron

4.2.4 Antimicrobial activity using disc diffusion method

4.2.4.1 Antimicrobial sensitivity of plant extracts using disc diffusion method

The results of the antibacterial activity of plant extracts using the disc diffusion method were given in Table 7, 8, 9. It indicates that *Salmonella* serovars showed different levels of sensitivities in the tested samples of Neem, Garlic and Ginger extracts. The zone of inhibition of the neem and garlic extracts was higher than that of the ginger extract for the corresponding concentrations (80%, 100%, 120%). In neem extract, the zone of inhibition varried from 9mm to 14mm in diameter. The greater zone of inhibition was observed in 120mg/ml of concentration (14mm) and the lower zone of inhibition in 80mg/ml of concentration (9mm) (Plate 13, Page 51, Table 7). In garlic extract, the zone of inhibition varried from 2mm to 10mm in diameter. The greater zone of inhibition was observed in 120mg/ml of concentration (10mm) and the lower zone of inhibition in 80mg/ml of concentration (2mm) (Plate 14, Page 51, Table 8). In ginger extract, the zone of inhibition varried from 2mm to 3mm in diameter. The greater zone of inhibition was observed in 120mg/ml of concentration (3mm) and the lower zone of inhibition in 100mg/ml of concentration (2mm) (Plate 15, Page 52, Table 9).

Table 7: Antimicrobial activity of Neem extract on *Salmonella* serovars

Tested sample	Concentration (%)	Zone of inhibition (mm)			Average
		T1	T2	T3	
Neem	80%	10	10	9	9.67
	100%	12	12	11	11.67
	120%	14	13	13	13.34

Table 8: Antimicrobial activity of Garlic extract on *Salmonella* serovars

Tested sample	Concentration (%)	Zone of inhibition (mm)			Average
		T1	T2	T3	
Garlic	80%	3	2	3	2.67
	100%	7	6	7	6.67
	120%	9	10	10	9.67

Table 9: Antimicrobial activity of Ginger extract on *Salmonella* serovars

Tested sample	Concentration (%)	Zone of inhibition (mm)			Average
		T1	T2	T3	
Ginger	80%	_	_	_	_
	100%	_	2	2	1.33
	120%	2	3	2	2.33

Table 10: Antimicrobial sensitivity pattern of different plant extracts against identified field isolates

Name of Farm	Sample no. (Positive)	Zone of inhibition (mm)								
		Neem			Garlic			Ginger		
		80%	100%	120%	80%	100%	120%	80 %	100%	120%
Robi, Nil	-	-	-	-	-	-	-	-	-	-
Mos, Nil	-	-	-	-	-	-	-	-	-	-
Rej, Nil	-	-	-	-	-	-	-	-	-	-
Sel, Nil	18(2)	10	12	14	3	7	10	-	-	3
Moj, Nil	12(1)	10	11	13	3	6	10	-	-	3
Ash, Panch		-	-	-	-	-	-	-	-	-
Kris, Panch	-	-	-	-	-	-	-	-	-	-
Kud, Panch	15(2)	10	11	14	3	7	9	-	-	2
Shah, Panch	9(0)	-	-	-	-	-	-	-	-	-
Haz, Thaku	12(0)	-	-	-	-	-	-	-	-	-
Mot, Thaku	18(3)	9	12	14	3	6	10	-	2	3
Mas, Thaku	12(2)	8	10	13	2	6	10	-	-	3
Sad, Thaku		-	-	-	-	-	-	-	-	-
Joy, Thaku		-	-	-	-	-	-	-	-	-
Ani, Thaku		-	-	-	-	-	-	-	-	-
Has, Dinj		-	-	-	-	-	-	-	-	-
SPP,Dinj	18(2)	9	11	14	3	6	10	-	2	3
Naz,Dinj		-	-	-	-	-	-	-	-	-
Mot,Dinj		-	-	-	-	-	-	-	-	-
Sor,Dinj		-	-	-	-	-	-	-	-	-
Abd,Dinj		-	-	-	-	-	-	-	-	-
Arif,Dinj		-	-	-	-	-	-	-	-	-
Mah,Dinj		-	-	-	-	-	-	-	-	-

Agro,Dinj	12(0)	-	-	-	-	-	-	-	-	-
Mono,Dinj		-	-	-	-	-	-	-	-	-
Vai,Dinj		-	-	-	-	-	-	-	-	-
Rai,Dinj	12(3)	9	12	14	3	6	10	-	-	3
Ras,Dinj		-	-	-	-	-	-	-	-	-
SR,Dinj	18(3)	9	11	14	3	5	10	-	-	2
Lot,Dinj	12(2)	10	12	14	3	6	10	-	2	3
mean±SD	168(20)	9.33±0.70	11.33±0.70	13.77±0.44	2.89±0.44	6.11±0.6	9.9±0.33		0.75±1.03	2.75±0.46
Lev. of Sig.		P<0.001			P<0.001			P<0.001		
Mean±SD		11.48±1.95			6.30±2.95			1.15±1.35		
Lev. of Sig.		P<0.001								

Legends (District Name)

Nil = Nilphamari

Panch = Panchagarh

Thaku = Thakurgaon

Dinj = Dinajpur

All are significant at 0.1% level

In this present study Table 10 showed the antibacterial sensitivity pattern of different plant extracts against identified field isolates. It was observed that there was significant ($p< 0.01$) differences among the three plant extracts.

4.2.4.2 Antimicrobial sensitivity pattern of commonly used antibiotics

Antibiotic sensitivity pattern of isolated *Salmonella* serovars were performed against 6 commonly used antibiotics belongings to different groups. After incubation, plates were examined and diameters of the zones of inhibition for individual antimicrobial agents were designated as sensitive, intermediate and resistance as per EUCAST (The European Committee on Antimicrobial Susceptibility Testing) standard (Table 11).

Table 11: Zone size interpretative chart (EUCAST standard)

Antibiotics	Symbol	Disc content	Zone of inhibition (mm)		
			Sensitive	Intermediate	Resistance
Ciprofloxacin	CIP	5	21	16-20	15
Colistin	CL	10	11	-	10
Levofloxacin	LE	5	17	14-16	13
Chloramphenicol	C	30	18	13-17	12
Neomycin	N	30	19	14-18	13
Kenamycin	K	30	18	14-17	13

From the study, the *Salmonella* serovars isolated from chicken sensitive to Ciprofloxacin, Colistin intermediate to Levofloxacin, Chloramphenicol and resistance to Neomycin and Kenamycin (Table 12).

Table 12: Antibiotic sensitivity pattern of *Salmonella* serovars

Antibiotics	Zone of inhibition (mm)		
	Sensitive	Intermediate	Resistance
Ciprofloxacin	21	-	-
Colistin	11	-	-
Levofloxacin	-	15	-
Chloramphenicol	-	16	-
Neomycin	-	-	13
Kenamycin	-	-	13

Legends:
CIP = Ciprofloxacin; **CL** = Colistin; **LE** = Levofloxacin; **CH** = Chloramphenicol; **N** = Neomycin; **KENA** = Kenamycin.

Table 13: Comparison between resistant antibiotics and plant extracts

Name of Antibacterial agents	Zone of inhibition(mm)
Neem	13-14
Garlic	9-10
Ginger	2-3
Neomycin	7
Kenamycin	8

CHAPTER 5

DISCUSSION

CHAPTER 5

DISCUSSION

The research topic entitled as characterization of salmonella serovars from selected layer flock and demonstration of antibacterial effect of edible plant extracts to selected isolates of salmonella serovars were selected with the main objectives covering isolation and identification of *Salmonella* serovars, preparation of crude extract from different edible plants and determination of efficacy of plant extracts against identified isolates.

Salmonellosis is an infectious and vertically transmitted disease of chicken. The diseases is characterized by the genus *Salmonella* under the family *Eenterobacteriaceae* (OIE Manual, 2006) and are Gram negative, short plump shaped rods, non-spore forming, non- capsulated, aerobic and facultative anaerobic organisms and inhabit the intestinal tract of man and animals (Holt *et al.,* 1994). Salmonellosis in poultry causes heavy economic loss through mortality and reduced production (Khan *et al.,* 1998). With great expansion of poultry rearing and farming, pullorum disease and fowl typhoid have become wide spread problem in Bangladesh (Rahman *et al.,* 1997).

The present study was reflected on the antibacterial effect of some edible plant extracts against *Salmonella* serovars. The collected samples were subjected to morphological, cultural and biochemical study. In addition the identified isolates were subjected to antibacterial sensitivity study with plant extracts prepared from Neem, Garlic and Ginger were compared with the commercially used available antibiotics by using disc diffusion method and EUCAST standard.

In our present study, a total of 168 samples comprising liver (56), heart (56), lung (56) were collected from dead birds. Out of 168 samples 20 samples were found to be positive for *Salmonella* serovars by cultural and morphological properties. In this study all positive isolates were showed negative reaction to lactose fermentation on MacConkey agar plate, opaque, translucent and colorless colonies on Salmonella-Shigella agar, Pale pink color colonies against a pinkish background on Brilliant Green agar and deep blue color from green color on Simmons citrate agar. This findings supported by other authors (Ahmed *et al.,* 2009; Hossain, 2002 and Islam *et al.,* 1998).

In Gram's staining, the morphology of the isolated bacteria was small rod shape, gram negative, single or paired which was supported by several authors (Ahmed *et al.,* 2009; Freeman, 1985 and Jones, 2002).

In this present study, specific enriched media and biochemical tests were used for the isolation and identification of *Salmonellae*, which was also used by a number of researchers (Lee *et al.,* 2003 and Dhruba *et al.,* 1999). In this study, colony characteristics of *Salmonella* serovars on MC agar, SS agar and BG agar were similar to the findings of other authors (Ahmed *et al.,* 2009 and Hossain, 2002).

In our present findings, the prevalence of Salmonella serovars were recorded as per information received from the farmers by using a structured questionnaire and the prevalence was higher in Dinajpur (13.89%) in compairing with Thakurgoan (11.91%), Panchagarh (8.33%) and Nilphamari (10%) according to their study area differences and organoleptic prevalence were also higher in Liver (17.86%) in comparing with the heart (7.14%) and lung (10.72%) respectively. This findings were similar to the Lee *et al.,* 2001.

In our present study, the observed variation in prevalence of Salmonella infection could be related with several factors such as geoclimatic situation, positive immunity level, infecting dose, simultaneous infection with other disease, stress, managemental practice, biosecurity failure and different locations of the study areas.

Poultry farming is a rapidly growing enterprise in Bangladesh due to huge demand of its meats and eggs (Raha, 2007). Indiscriminate uses of antibiotics are reported in the poultry production as treatment and feed supplements. Residual effects of antibiotics present in the meat and eggs may cause public health problems such as development of drug resistant bacteria in humans (Diarra and Malouin, 2014). MDR (Multi Drug Resistance) bacteria are reported in poultry in Bangladesh due to indiscriminate use of antibiotics. Poultry veterinarians are facing challenges to treat MDR bacteria and cost of poultry production has been increased. Mortality has been increasing since most antibiotics are inactive against pathogenic MDR bacteria considering the above mentioned problem, our present study were extended to determine the effectiveness of plant extracts against field isolates.

In the present study, the antibacterial sensitivity of different plant extracts revealed that all of the field isolates were sensitive to all extracts used in this study at varying levels. All the tested isolate were found highly sensitive to Neem extracts than Garlic and Ginger extracts and suggesting that these plant extract could be the first choice of drug as an alternative approach to synthetic antibiotic. These results are similar to the findings of Beuth *et al.,* 2006 and Akihisa *et al.,* 2009. Variation in antibacterial sensitivity of plant extracts showed the necessity of in vitro antibacterial sensitivity test prior to the treatment. It also emphasizes to have judicious selection of antibacterial agents for effective treatment.

The present study was also subjected to compare the efficacy of commercial antibiotics with plant extracts against field isolates. From this study, it was also observed that most of the isolates were resistance to synthetic antibiotics and only Ciprofloxacin and Colistin were sensitive. In addition, the resistant antibiotics were also compared with the plant extracts. In this study it was also observed that plant extracts were highly sensitive to field isolates.

In conclusion, the plant extracts results revealed that all extracts were found to be effective and safe for the treatment of Salmonella infection against field isolates. However, the recommendation for experimentally used some edible plant extracts remain unresolved until and unless further characterization regarding the clarification of serotype or serovars by using molecular techniques and also covering field trial in a broader scale.

In communication of the present study further following research work should be performed:

1. Characterization of field isolates covering different serotype by using molecular techniques like PCR.
2. The targeted gene PCR product should be further studied after performing sequence of the PCR product.
3. Type specific plant extracts against indigenous *Salmonella* serovars might be developed, monitored and evaluated for proper control of *Salmonella* infection in poultry industry of Bangladesh.

CHAPTER 6

SUMMARY AND CONCLUSION

CHAPTER 6

SUMMARY AND CONCLUSION

Among economically important diseases of poultry, Salmonellosis is one of the most well recognized bacterial diseases in poultry industry causing heavy economic losses through mortality and reduced production. It is now targeted as a silent killer to our poultry industry, as the causal organisms transmit both vertically and horizontally.

To prevent the spread of Salmonellae in poultry farm, disease management strategies could be undertaken by introducing a continuous monitoring of organism, culling of infected and carrier bird and implementation of good husbandry practice with biosecurity plan.

A total of 168 samples from dead birds were tested for the presence of *Salmonellae*. Of which 20 samples were found to be positive for *Salmonellae*. From the present study, it was found that all the tested isolates were highly sensitive to neem extracts suggesting that these plant extract could be the first choice of drug as an alternative to synthetic antibiotic. Antibacterial sensitivity of plant extracts showed the necessity of in vitro antibacterial sensitivity test prior to treatment. It also emphasizes to have judicious selection of antibacterial agents for effective treatment.

Concluding Remarks:

1. A concise informative prevalence of *Salmonella* serovars in the context of Northern part of Bangladesh considering study area and organoleptic differences was established for the effective treatment.

2. Isolation and identification technique of *Salmonella* serovars were successfully performed by using staining, cultural and biochemical techniques in general. However, the strain characterization of field isolates could not be readily used with molecular techniques like PCR.

3. Antibacterial sensitivity study conducted that Neem extracts would be the first choice of drug followed by Garlic extracts and Ginger extracts respectively.

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APPENDICES

APPENDICES

APPENDIX 1

Composition of Different Media

1. Nutrient agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25^{0}C)	7.4 ± 0.2

2. Nutrient broth

Ingredients:	**g/L**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final p^H	7.2

3. Eosine methylene blue Agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	10
Lactose	5.0
Sucrose	5.0
Dipotassium phosphate	2.0
Eosin - Y	0.40
Methylene blue	0.065
Agar	20.0
Final pH (at 25^{0}C)	7.2 ± 0.2

4. MacConkey agar (Mreck)

Ingredients:	**g/L**
Peptic digest of animal tissue	17.0
Protease peptone	3.0
Lactose monohydrate	10
Bile salt	1.5
Sodium chloride	5.0
Agar-agar	15.0
Neutral red	0.03
Final pH (at 25^{0}C)	7.1 ± 0.2

5 .TSI agar (Hi Media)

Ingredients:
g/L

Peptic digest of animal tissue	10.00
Casein enzymic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00
Final pH(at 25°C)	7.4 ± 0.2

6. Simmons Citrate Agar

Ingredients:
Gms / Litre

Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000
Final pH (at 25°C)	6.8±0.2

7. MIU medium base (Hi Media)

Ingredients:	**g/L**
Casein enzymic hydrolysate	10.00
Dextrose	1.00
Sodium chloride	5.00
Phenol Red	0.01
Agar	2.00
Final pH(at 25°C)	6.8 ±0.2

8. MR-VP medium (Hi Media)

Ingredients:	**g/L**
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00
Final pH (at 25°C)	6.9 ± 0.2

APPENDIX 2

Preparation of reagents

Kovacs reagent

P-dimethyl aminobenzal dehyde	5 gm
Amyl alcoho	l75gm
Conc.HCL	25 ml

1. V-P reagent 1

5% alpha –naptholin absolute ethyl alcohol

2. V-P reagent 2

40% potassium hydroxide containing 0.3 creatine. The ingredients were dissolved by heating gently over steam bath. When in solution add 0.052 gm of cotton blue dye.

3. Methyl red solution

Methyl red	0.05 gm
Ethanol (absolute)	28 ml
Distilled water	22 ml

4. Phenol red solution

0.2% aqueous solution of phenol red

5. Potassium hydroxide solution

40% aqueous solution of KOH

6. Gram stain solution

 Stock crystal violet

Crystal violet	10 gm
Ethyl alcohol (95%)	1000 ml

 Stock oxalate solution

Ammonium oxalate	1 gm
Distilled water	1000 ml

 Lugols iodine solution

Iodine crystal	1 gm
potassium iodide	2 gm

 Ethyl alcohol 250 ml

 Acetone 250 ml

 Counterstain

Safranine	2.5 ml
Ethyl alcohol (95%)	100 ml

Safranine working solution

The stock safranine is diluted 1:4 with distilled water.

APPENDIX 3

Questionnaire for the surveillance/investigation of *Salmonella* infection in layer chicken

1. Particulars of the farm owner:

i) Name of the farm:.....ii) Owner name:.....iii) Village……

iv) Upazila:..... v) District:.....

2. i) Type of farm: Commercial- Small ☐ Medium ☐ Large ☐

Native- Scavenging ☐ Semi-Scavenging ☐

ii) Total No. of birds in a flock:.....

iii)Age of bird:……..

3. Source of egg/chicks:

Name:……..Breeder ☐ Shop ☐

4. Management System:

i) Housing:

Type of housing: Litter ☐ Cage ☐

ii) Diet history:

a) Food item: Ready feed: Yes ☐ No ☐

Loose feed: Yes ☐ No ☐

b) Time of feeding.....times/per day

iii) Biosecurity and sanitary condition:

a) Hygienic condition of the house: Poor ☐ Good ☐ Very good ☐

b) Cleaning time of the house.....days/ per week

c) Washing of house with: Antiseptic powder ☐ Disinfectant ☐ Water ☐

d) People close in contact with poultry: Visitors ☐ Workers ☐ Others ☐

e) Waste disposal time: Regular ☐ One day after ☐

iv) Hatchability (Per day egg production):…….....

v) Deworming: Yes ☐ No ☐

5. Disease history:

i) History: 1.
2.
3.

ii) Treatment given: Yes ☐ No ☐

iii) Medication:

Name of medicine used	Remarks	Name of medicine used	Remarks

iv) Post-mortem lesion (if possible): 1..... 2.....
3..... 4.....

v) Mortality rate:……

6. Sample Collection:

i) Number of collected sample……

ii) Name of sample……..

7. Vaccination:

Name of vaccine	Age (days)	Name of vaccine	Age (days)

_____

Signature of investigator