DETECTION OF *ESCHERICHIA COLI* FROM WATER USED IN POULTRY FARMS AT CHITTAGONG

A THESIS BY

KAZAL KRISHNA GHOSH
ROLL NO.: 0213/01
REGISTRATION NO.: 165
SESSION: 2013-2014
SEMESTER: JULY – DECEMBER, 2014

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY AND VETERINARY PUBLIC HEALTH
FACULTY OF VETERINARY MEDICINE
CHITTAGONG VETERINARY AND ANIMAL SCIENCES UNIVERSITY
CHITTAGONG-4225

DECEMBER, 2014

DETECTION OF *ESCHERICHIA COLI* FROM WATER USED IN POULTRY FARMS AT CHITTAGONG

A Thesis

Submitted to

Department of Microbiology and Veterinary Public Health

Faculty of Veterinary Medicine

Chittagong Veterinary and Animal Sciences University, Chittagong

In partial fulfillment of the requirements for the degree of

Master of Science

In

Microbiology

By

Kazal Krishna Ghosh

Roll No.: 0213/01

Registration No.: 165

Session: 2013-2014

Semester: July-December 2014

Department of Microbiology and Veterinary Public Health

Faculty of Veterinary Science

Chittagong Veterinary and Animal Sciences University

Chittagong-4225

December, 2014

DETECTION OF *ESCHERICHIA COLI* FROM WATER USED IN POULTRY FARMS AT CHITTAGONG

A Thesis

By

Kazal Krishna Ghosh

Roll No.: 0213/01

Registration No.: 165

Session: 2013-2014

Semester: July-December 2014

Approved as to style and contents by

_____

(Prof. Dr. Abdul Ahad)

Research Supervisor

_____

(Prof. Dr. Abdul Ahad)

Chairman, Defense Committee

and

Head, Department of Microbiology and Veterinary Public Health

Chittagong Veterinary and Animal Sciences University

Chittagong

December, 2014

DEDICATED TO
MY
RESPECTED AND BELOVED PARENTS

ACKNOWLEDGEMENTS

Firstly I would like to express my deepest sense to "The Almighty God" who enables me to complete the research work and dissertation successfully for the degree of Master of Science in Microbiology.

There are lots of people I would like to thank for a huge variety of reasons. I owe my gratitude to all those people who have made this thesis possible and because of whom my graduate experience has been one that I will look back at forever. It is difficult to overstate my gratitude to my supervisor ***Prof. Dr. Abdul Ahad*** *for giving me the opportunity to do MS in Microbiology and supervising me. Thank you also for managing to read the whole thesis so thoroughly and for helpful comments on the text. Without your knowledge, perceptiveness, cracking-of-the-whip and encouragement I would never have finished. Special thanks to* ***Dr. Kazi M Komoruddin****, Director, Poultry Research and training Center (PRTC) for his valuable advice and cooperation. I would like to give special thanks to* ***Prof. Dr. Paritosh Kumar Biswas****. I also gratefully acknowledge the kind help and advices of* ***Dr. Himel Barua****. I would like to thank my respectable teacher* ***Dr. Zohorul Islam*** *for his cooperation and advices during the research work. A special thanks to* ***Dr. Md. Inkeyas Uddin****, Senior Scientific Officer, Poultry Research and Training Islam for supporting me by giving materials and the opportunity to conduct research.*

Thanks to all members of the department of Microbiology, CVSAU and PRTC for their kind cooperation. I would like to acknowledge my special thanks to my friends, junior and senior fellows for their constant help and inspiration throughout the research work. Supervisors' help and co-operation have been received from many persons during the tenure of this place of Thesis. The author is immensely grateful to all of them, although it is not possible to mention every one by name.

Finally, I am forever indebted to my parents and younger sister and all other members of my family for their understanding, endless patience and encouragement when it was most required.

Statement of Candidate

I, Kazal Krishna Ghosh, declare that this thesis is submitted in fulfillment of the requirements for the Degree of Master of Science (MS) in Microbiology, Department of Microbiology and Veterinary Public Health, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University. It is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

The Author

December, 2014

Contents

List of Tables

List of Figures

Acronyms used

AAF	Aggregative Adherence Fimbrieae
AFNOR	Association Franc aise de Normalisation (French national organization for standardization)
APEC	Avian Pathogenic *Escherichia coli*
APHA	American Public Health Association
BBA	Blood Base Agar
CFA	Colonization Factor Antigens
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CNF	Cytotoxic Necrotizing Factor
DAEC	Diffuse-Adhering *Escherichia coli*
DNA	Deoxyribo Nucleic acid
EAEC/ EAggEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EMB	Eosin Methylene Blue
EPA	Environmental Protection Agency
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
FISH	Fluorescent In Situ Hybridisation
MF	Membrane Filter
MLST	Multi Locus sequence Typing
MNEC	Meningitis/Sepsis-Associated *Escherichia coli*
MPN	Most Probable Number

MTF	Multiple-Tube Fermentation
NA	Nutrient Agar
OECD	Organization for Economic Co-operation and Development
ONPG-MUG	Ortho-Nitrophenyl-β-galactoside Methylumbelliferyl-β-D-glucuronide
PAI	Pathogenicity Islands
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
pINV	Invasion Plasmid
RAPD	Random Amplification of Polymorphic DNA
RNA	Ribonucleic Acid
stx 1	Shiga toxin 1
stx 2	Shiga toxin 2
TSI	Triple Sugar Iron
TSS	Total Suspended Solid
UPEC	Uropathogenic *Escherichia coli*
UV	Ultra Violet
WHO	World Health Organization

Abstract

An investigation was carried out to assess the microbiological safety level of water used in different poultry farms at Chittagong. All the samples were subjected to Most Probable Number (MPN) determination for observing the bacterial load. Further, culturing and biochemical test were conducted to isolate *E. coli* from MPN positive samples. Samples were also investigated for the presence of shiga toxin producing genes and antimicrobials sensitivity genes. Total 103 were found positive with MPN index count out of 150 water samples, 35 of them were positive for *E. coli*. There was a highly significant variation (P value < 0.05) in MPN index count with pH values, water source, geographical locations, farm types and water treatment. Zero coliform count was recorded in only 6% samples with lower pH value (4-5) indicating high bacterial contamination, whereas water with neutral pH values (7-7.5) was found less susceptible to bacterial contamination as 42% samples gave zero coliform count. Though all the water sources were found to be contaminated with bacteria, however, among them, high bacterial contamination was recorded from water samples of drinkers. Total 9 places were included in our study. Of them, samples from Hathazari showed highest zero coliform count (60%), in contrary, rest of the places was almost similar (20-33%) with zero coliform count. Water from broiler and layer farms were highly affected with different coliform bacteria, whereas samples of broiler farms were highly positive (46%) in coliform count (1001 and above). Water treatment with bleaching powder was only practiced in breeder farms. In case of treated water samples, 60% samples gave zero coliform count indicates lower contamination comparing with non-treatment water. Total 15 (30%) *E. coli* isolates were collected from Broiler and Layer farms, whereas lower number was observed in Breeder farms (10%). There was no significant variation (p-value > 0.05) among water sources with *E. coli* isolation. Molecular detection of all 35 *E. coli* isolates revealed no evidence of shiga toxin producing genes (*stx1*, *stx2* or both). The *E. coli* isolates were susceptible to colistin sulphate and gentamicin with an intermediate sensitivity to norfloxacin and doxycyline. The organisms showed 94.29% and 91.43% resistance to ampcillin and tetracycline, respectively. Extensive studies are recommended for the molecular study of different coliform bacteria found in water used in poultry farms and for assessing the effects of the multidrug resistance organisms on poultry population.

Keywords: Poultry, MPN, *E. coli*, PCR, Antimicrobial resistance

Chapter I: Introduction

A safe water supply is essential for healthy livestock and poultry. Contaminated water can affect growth, reproduction, and productivity of animals as well as safety of animal products for human consumption. Contaminated water supplies for livestock and poultry can also contaminate human drinking water. For these reasons, farm water supplies should be protected against contamination from bacteria, nitrates, sulfates, and pesticides (Hairston, 1995).

The use of consumption water with high physical, chemical and microbiological qualities is of fundamental importance in animal production because many animals have access to the same water source and a problem in the water quality would affect a great number of animals. This is particularly relevant in poultry production, where one single water source serves thousands of animals. Therefore, control measures must be considered as priority, in order to prevent the occurrence of diseases that are spread through water, and would certainly result in great economical losses. Although water does not provide ideal conditions for pathogenic microorganism to multiply, they will generally survive for enough time to allow waterborne transmission. Water is, therefore, an excellent transmission route of agents responsible for human and animal diseases, mainly those in which fecal-oral transmission occurs, since contamination of water supplies is still gradually increasing as a result of urban and rural activities (Amaral, 2004).

The aim of using good litter material in broiler production is to prevent the direct contact of the bird with the floor and to promote the absorption of the fecal moisture (Oliveira & Carvalho, 2002). Several materials have been used as litter, such as wood shavings and rice husks, and other materials, such as paper, peanut hulls, and sand and those have association with water contamination in poultry farms (Hernandes *et al.*, 2002; Santos *et al.*, 2000). Animals need to consume sufficient water to satisfy their requirements. However, more important than water quantity is water quality, as the hygienic and physical-chemical quality of drinking water plays a key role in ensuring efficient animal production (Amaral *et al.*, 1999; Li, 2009). Water is involved in every aspect of poultry metabolism and plays an important role in body temperature regulation, food digestion, and body waste excretion (Valias & Silva, 2001).

E. coli is used as indicator of water safety regarding fecal contamination in almost all water quality legislation in the world. It is the microbiological parameter that is most frequently monitored in drinking water and bathing water surveillance. *E. coli* is a bacterium that resides in high numbers in the intestines of warm blooded animals and has proven its value to detect fecal contamination in water (OECD, 2003). Culture techniques are routinely used for the examination of the presence of *E. coli*. These methods consist of a selective culture step followed by biochemical or genetic (Heijnen and Medema, 2006) confirmation of presumptive *E. coli* colonies or cultures methods attractive alternatives and has resulted in the development of different PCR methods to directly detect *E. coli* in water samples (Bej *et al.*, 1996; Heijnen and Medema, 2006).

The present study was therefore designed to determine the microbiological safety level of water used in poultry farms at Chittagong.

The objective of this study was, therefore,

1. To assess the bacterial load in waters of poultry farm
2. To know the prevalence of *E. coli* in poultry water
3. To find out most possible source or other risk factors associated with water contamination
4. To determine the antimicrobial susceptibility patterns of *E. coli* isolated from water

Chapter II: Review of Literature

2.1 Importance of safe drinking water in Poultry Farms

Water is the most abundant and widely distributed chemical compound in the world and also an essential nutrient for birds. Use of consumption water with high physical, chemical and microbiological qualities has great value in poultry industry. Due to access on same water source, a problem in the water quality can arise which ultimately would affect a great number of animals. This is particularly relevant in poultry production, where one single water source serves thousands of birds. Most common poultry diseases including bacterial, viral and protozoan diseases can be transmitted via drinking water. Therefore, control measures must be considered as priority, in order to prevent the occurrence of diseases that are spread through water, and would certainly result in great economical losses. Although water does not provide ideal conditions for pathogenic microorganism to multiply, they will generally survive for enough time to allow waterborne transmission. Water is, therefore, an excellent transmission route of agents responsible for human and animal diseases, mainly those in which fecal-oral transmission occurs, since contamination of water supplies is still gradually increasing as a result of urban and rural activities (Amaral, 2004).

Water is involved in every aspect of poultry metabolism. It plays important roles in regulating body temperature, digesting food, and eliminating body wastes. At normal temperatures, poultry consume at least twice as much water as feed. When heat stress occurs, water consumption will double or quadruple. A safe and adequate supply of water is therefore essential for efficient poultry production (Carter and Sneed, 1996). Diseases that can be transmitted to the bird flock through the drinking water may originate from water contamination by feces and secretions of sick birds, or by the utilization of water already contaminated by pathogenic organisms that originate from other animal species and the man, such as in the case of *Salmonella* and *E. coli*, respectively (Gama, 1995).

A safe water supply is essential for healthy livestock and poultry. Contaminated water can affect growth, reproduction, and productivity of animals as well as safety of animal products for human consumption. Contaminated water supplies for livestock and poultry can also contaminate human

drinking water. For these reasons, farm water supplies should be protected against contamination from bacteria, nitrates, sulfates, and pesticides (Hairston, 1995).

2.1.1 Water Requirement

Adequate supply of clean, good quality water is essential in order to fully utilize the potential of modern poultry genotypes selected for superior performance characteristics (Olkowski, 2009).

Table 1 - Water Consumption in various classes of poultry

Age (Weeks)	Water Consumption (ml of water per week per bird)*		
	Broiler Chickens	White Leghorn Hens	Brown Egg Laying Hens
1	225	200	200
2	480	300	400
3	725	-	-
4	1000	500	700
5	1250	-	-
6	1500	700	800
7	1750	-	-
8	2000	800	900
9	-	-	-
10	-	900	1000
12	-	1000	1100
14	-	1100	1100
16	-	1200	1200
18	-	1300	1300
20	-	1600	1500

*Based on data completed from National Research Council, 1994.

The water requirement for poultry depends on numerous environmental variables such as temperature and relative humidity, the composition of the diet, and production parameters (growth rate, egg production). Although there is large individual variability, it is generally assumed that water consumption in birds is approximately double the amount of feed consumed. Water intake can be influenced by diet form and composition. For instance, in comparison to

mash diets, poultry offered pelleted or crumbled diets will increase both feed intake and water intake. Increasing crude protein in the diet will increase water intake. Also, dietary salt content will influence water intake.

2.1.2 Different Water Sources

Different water sources may be used in the rural area for animal consumption, such as springs, shallow wells, deep and artesian wells, lakes and creeks. From a microbiological point of view, the sources of superficial water are more subjected to contamination than the underground waters, although the latter are also susceptible to this type of contamination. The most common underground waters used in the rural area are:

Springs: Places where water comes up through the ground or rock and flows naturally to the ground surface or to water masses.

Shallow wells: Vertical hole manually dug in the ground and used to withdraw water. The diameter is from 1 to 5 meters and the depth varies from 3 to 12 meters.

Deep wells: well in an unconfined aquifer, drilled using machines and generally tubular.

Artesian wells: confined aquifers that sometimes contain water with pressure enough to bring it to the soil surface. They are more protected because there are layers of impermeable material below and above them and because they are more profound than the other water sources already mentioned (Amaral, 2004).

2.1.3 Drinking Water Quality for Poultry

If the number of microorganisms found in a water sample is too high, it indicates that the water supply is contaminated. Well water is normally tested for the total bacteria level, the coliform bacteria level, and occasionally for the fecal coliform bacteria level. Coliform bacteria are organisms normally found in the digestive tracts of livestock, humans, and birds. Their presence in water is used as a sign of fecal contamination.

Standards for animal drinking water indicate that there should be fewer than 100 bacteria of all types per milliliter (ml) of water and fewer than 50 coliform bacteria per ml (Table 2). Recent field research indicates that a bacteria level of zero may be desirable to obtain optimum performance (Carter and Sneed, 1996).

Table 2 - Drinking water quality guidelines

Contaminant		Level Considered Average	Maximum Acceptable Level	Remarks
Bacteria	Total Bacteria	0/ml	100/ml	0/ml is desirable
	Coliform Bacteria	0/ml	50/ml	0/ml is desirable
Nitrogen Compounds	Nitrtate	10mg/1	25mg/1	Levels from 3 to 20 mg / l may affect performance
Acidity and Hardness	pH	6.8 - 7.5	-	a pH of less than 6.0 is not desirable. Levels below 6.3 may degrade performance.
	Total Hardness	60 – 180	-	Hardness levels less than 60 are unusually soft, those above 180, very hard.
Naturally Occurring Chemicals	Calcium	60mg/1	-	-
	Chloride	14mg/1	250mg/1	Levels as low as 14 mg / l may be detrimental
	Copper	0.002 mg / l	0.6 mg / l	Higher levels produce a bitter flavor.
	Iron	0.2 mg / l	0.3 mg / l	Higher levels produce a bitter flavor.
	Lead		0.02 mg / l	Higher levels are toxic.
	Magnesium	14 mg / l	125 mg / l	Higher levels have a laxative effect.
	Sodium	32 mg / l	-	Levels above 50 mg / l may affect performance.
	Sulfate	125 mg / l	250 mg / l	Higher levels have a laxative effect.
	Zinc	1.50 mg / l		Higher levels are toxic.

Source: Schwartz DL, "Water Quality," VSE, 81c., Penn. State Univ. (mimeographed); and Waggoner R, Good R, and Good R, "Water Quality and Poultry Performance," in Proceedings AVMA Annual Conference, July, 1984.

The acidity or alkalinity of water is expressed as its pH level. Neutral water (that which is neither acid nor alkaline) has a pH of 7. Acidic water has a pH lower than 7 and alkaline water has a pH greater than 7. Well water normally has a pH in the range from 6.8 to 7.8, although it is not

uncommon for the pH to be either higher or lower (Carter and Sneed, 1996). Little is known about the specific pH's effect on water intake, animal health and production, or the microbial environment in the rumen. The preferred pH of drinking water for poultry is 6.5 to 8.5 (Halls, 2008). Waters with a pH outside of the preferred range may cause nonspecific effects related to digestive upset, diarrhea, poor feed conversion and reduced water and feed intake. The pH of water may impact animal health in some animals more than in others. For instance, in ruminants, consumption of water with a pH below 5.5 may contribute to metabolic acidosis, whereas alkaline water with pH greater than 8.5 may result in higher risk of metabolic alkalosis (Olkowski, 2009).

2.2 What are the coliform

The coliform group includes a broad diversity in terms of genus and species, whether or not they belong to the Enterobacteriaceae family. In standard methods for the examination of water and wastewater, coliform group members are described as all aerobic and facultative anaerobic, Gram negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35 °C (multiple-tube fermentation technique) or all aerobic and many facultative anaerobic, Gram-negative, non-spore forming, rod-shaped bacteria that develop a red colony with a metallic sheen within 24 h at 35 °C on an Endo-type medium containing lactose (membrane filter technique) (APHA, 1998).

The concept of coliform as bacterial indicators of microbial water quality is based on the premise that because coliforms are present in high numbers in the feces of humans and other warm-blooded animals, if fecal pollution has entered drinking water, it is likely that these bacteria will be present, even after significant dilution. With few exceptions, coliforms themselves are not considered to be a health risk, but their presence indicates that fecal pollution may have occurred and pathogens might be present as a result. The coliform group of bacteria, is a functionally-related group which all belong to a single taxonomic family (Enterobacteriaceae) and comprises many genera and species. Table 3 contains an example of the relationship between family, genera and species for coliforms. There are other genera in the Enterobacteriaceae family, such as *Salmonella* and *Shigella* that are not considered coliforms (Stevens *et al.*, 2003).

The US Environmental Protection Agency (EPA) has approved several methods for coliform detection: the multiple-tube fermentation technique, the membrane filter technique and the presence/absence test (including the ONPG-MUG test). AFNOR (1990) has approved the multiple-tube fermentation technique and the membrane filter technique.

Table 3 - Family, Genera and Species of some common Coliforms

Family	**Genera**	**Species**
Enterobacteriaceae	*Escherichia*	*Escherichia coli (E. coli)*
	Klebsiella	*Klebsiella penumoniae (K. penumoniae)*
	Enterobacter	*Enterobacter amnigenus (E. amnigenus)*
	Citrobacter	*Citrobacter freundii (C. freundii)*

Table 4 - Distribution of Coliform Genera in human and animal faeces.

Sample Type	**% of Total Coliform**			
	E. coli	*Klebsiella spp.*	*Enterobacter/Citrobacter spp.*	Reference
Human Faeces	96.8	1.5	1.7	Dufour (1977)
	94.1	-	5.9	Allen and Edberg (1995)
Animal	94	2	4	Dufour (1977)
	92.6	-	7.4	Allen and Edberg (1995)

Of the coliforms normally present in the gut of warm-blooded animals, *E. coli* is the most numerous and is also the only coliform which rarely grows in the environment. Table 4 shows the distribution of coliforms present in human and animal feces (Stevens *et al.*, 2003). *E. coli* is the most common coliform among the intestinal flora of warm-blooded animals and its presence

might be principally associated with fecal contamination. No *E. coli* are therefore allowed in drinking water.

2.3 Microbiological safety level of Coliform in poultry drinking water

In Brazil different research advices that water with total and fecal coliforms of 20,000/100 mL and 4,000/100 mL, respectively can be used as drinking water for animals. The observation of such values in the drinking water of larger animals may not result in health damage (Brasil, 1986). Concerning poultry production, these limits may represent sanitary problems to the flock. The birds are smaller and precocious animals, and their lower resistance may cause them to be more susceptible to infections, mainly caused by pathogens of intestinal origin that might be present in water with the fecal pollution. Therefore, Macari (1997) and Englert (1998) recommend that waters with potability levels similar to levels applicable to humans should be also used for birds.

Schwartz (1984) and Waggoner and Good, 1984 and Reddy *et al.*, 1995 considered that the number of microorganisms in the drinking water of birds should be 100 CFU/mL for total bacteria and 50 CFU/mL for coliforms. The mean levels of *E. coli* in the water of a broiler farm that used bell-type drinkers were 104 microorganisms/mL in the first week of life (Barros *et al.*, 2001), a concerning finding since this is a high fecal contamination associated to young age of the birds. Meza (1989) states that there should be a better bacteriological control of the water provided to the birds during the initial phase, since there is a fast bacterial growth and the health risk is increased for the for birds from 1 to 21 days of age.

2.4 *Escherichia coli(E. coli)*

E. coli were first described in 1885 by Theodor Escherich (Escherich, 1988). Escherich, a Bavarian pediatrician, had performed studies on the intestinal flora of infants and had discovered a normal microbial inhabitant in healthy individuals, which he named Bacterium coli commune. In 1919, the bacterium was renamed in his honour to *E. coli* (Kaper, 2005). The species *E. coli* comprises gram-negative, oxidase-negative straight cylindrical rods measuring 1.1-1.5 x 2.0-6.0 μm. They are aerobic and facultative anaerobic, rendered motile by peritrichous flagella, or non-motile. The 16S rRNA based phylogenetic tree illustrates its relatedness with other representatives of genera within the Enterobacteriaceae family. Phylogenetic analysis has

demonstrated a very close relation between *E. coli*, *Salmonella spp.* and *Citrobacter freundii* (Scheutz & Strockbine, 2005).

With the exception of *Shigella boydii* serotype 13, the four species of *Shigella*, (*S. dysentieriae*, *S. flexneri*, *S.boydi* and *S. sonnei*) show such a high degree of relatedness to *E. coli* that these five could be considered a single species. However, the distinction still prevails, for historical and medical reasons (reviewed by Scheutz & Strockbine, 2005).

The taxonomy of *E. coli* is summarized below:

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *Escherichia coli* (VetBakt, 2007)

2.4.1 Diversities of Pathogenic *E. coli*

Most *E. coli* are harmless commensals which are part of the natural gastrointestinal flora in the lower intestine of warm-blooded animals. These are considered beneficial for maintaining a healthy intestinal ecosystem and have even been candidates for probiotic treatment to counteract a variety of enteric diseases. However, some subsets of *E. coli* have acquired specific virulence attributes that render them capable of causing a variety of illnesses in healthy humans and animals (Kaper *et al.*, 2004).

Table 5 - Intestinal pathogenic *E. coli*

Pathotype	General features	Principal virulence factors
	Enteropathogenic	Pathogenicity island LEE, (McDaniel *et al.*, 1995)
EPEC	EPEC was the first pathotype of *E. coli* to be described.	Type III secretion system, intimin, Tir, EspA, EspB, EspD, EspF
ETEC	Enterotoxigenic	Colonization factor antigens (CFA)
	The organism is an important cause of childhood and travelers' diarrhea in developing countries (Nataro & Kaper, 1998)	Heat-labile toxin (LT)
		Heat-stable toxin (STa, STb)
EHEC	Enterohemorrhagic	Pathogenicity island LEE
	EHEC causes bloody diarrhea (hemorrhagic colitis) and hemolytic uremic syndrome (HUS).	Type III secretion system, intimin, Tir
		The key virulence factor for EHEC is *Stx*, which is also known as verocytotoxin The Stx family contains two subgroups *Stx1* and *Stx2* (VT)
EIEC	Enteroinvasive	Invasion plasmid (pINV)
	EIEC are responsible for diarrhea	
EAEC/ EAggEC	Enteroaggregative	Aggregative adherence fimbrieae (AAFs)
	It is a cause of persistent diarrhea in children and adults in both developing and developed countries.	EAEC flagellin
		Toxins (Pic, ShET1, EAST)
DAEC	Diffuse-adhering	Fimbrial adhesin F1845
	DAEC have been implicated as a cause of diarrhea in several studies, particularly in children >12 months of age (Nataro & Kaper, 1998)	

Interestingly, most acquired virulence factors that distinguish pathogenic *E. coli* from commensals are encoded by mobile genetic elements such as plasmids, bacteriophages and transposons. Genes coding for virulence factors are often located in the chromosome on

pathogenicity islands (PAI), large genomic regions that cannot be found in commensals. These often include genetic elements that might once have been mobile but subsequently evolved to be locked into the genome (Scheutz & Strockbine, 2005; Kaper *et al.*, 2004). The pathogenic *E. coli* are divided into different pathotypes according to virulence factors they possess. Different pathotypes of *E. coli* in humans (James *et al.*, 2004) are described in Table 5 and 6.

Table 6 - Extra-intestinal pathogenic *E. coli* (ExPEC)

Pathotype	General features	Main virulence factors
UPEC Uropathogenic *E. coli*	The subset of *E. coli* that causes uncomplicated cystitis and acute pyelonephritis.	Adhesins (typ 1, F1C, S, M, Dr) P fimbriae (Pap) Cytotoxic necrotizing factor (CNF-1) Haemolysin (HlyA)
MNEC Meningitis/sepsis-associated *E. coli*	This is the most common cause of Gram negative neonatal meningitis.	Fimbrial adhesin F1845

2.4.2 Risk Associated with *E. coli* O157

The bacteria, *E. coli* O157:H7 and the *E. coli* O157: H-non-motile variants, generally referred to as *E. coli* O157, have become a significant public health concern throughout the world. From the perspective of livestock water quality issues, these bacteria should be recognized as a potential hazard because of its ability to survive and multiply in water (Armstrong *et al.*, 1996; Coia, 1998).

Cattle are considered a primary source of these bacteria, and water contaminated with cattle feces, as well as direct or indirect contact with live cattle, are considered major routes of human infection. Cattle that carry *E. coli* O157 are asymptomatic, but in humans this pathogen creates severe disease, and in many cases is the cause of death. The risk to the general population from contaminated water sources is very high (remember Walkerton, ON).

It is noteworthy that pathogenic *E. coli* O157 can easily be disseminated among cattle through contaminated water sources (Shere *et al.*, 2002), and drinking water can be a long-term reservoir and a persistent source of cattle exposure (Lejeune *et al.*, 2001). At the herd level, *E. coli* O157 is ubiquitous in both dairy and beef cattle operations (Faith *et al.*, 1996; Hancock *et al.*, 1998; Shere *et al.*, 2002; Van Donkersgoed *et al.*, 2001; Renter *et al.*, 2003). In situations more specific to the feedlot environment, contamination of drinking water with *E. coli* O157 appears to be wide spread problem. VanDonkersgoed *et al.* (2001) reported the presence of these bacteria in 12% of water tanks from pens containing pre-slaughter cattle. A more recent study (Sargeant *et al.*, 2004) showed at least one water tank was positive for *E. coli* O157 on 60% of the feedlots.

The health hazards associated with pathogens in both humans and livestock are well documented. A contaminated water supply may introduce high numbers of organisms into a group of animals, and this scenario may create a significant 'multiplier' effect through the food chain. The potential impact of pathogens such as *E. coli* O157 must be taken seriously in the context of water quality issues. In modern agriculture, strict management of water supplies for livestock must take into consideration contamination with water-borne microbial pathogens. The effort to address these problems should be focused on protection of water sources from contamination.

2.4.3 Mode of transmission

Horizontal dissemination is the most likely method of transmission (Dho-Moulin *et al.*, 1999) via the environment, more specifically from other birds, feces, water and feed however, Barnes *et al.* (1997) suggested that rodents may be carriers of Avian pathogenic *E. coli* (APEC) and hence a source of contamination for the birds. An unfavorable housing climate, like an excess of ammonia or dust, renders the respiratory system more susceptible to APEC infections through deciliation of the upper respiratory tract (Barnes and Gross, 1997). Airsacculitis is observed at all ages in this case the bird is infected by inhalation of dust contaminated with fecal material, which may contain 106 CFU of *E. coli* per gram (Harry, 1964). This aerogenic route of infection is considered as the main origin of systemic Colibacillosis or Colisepticemia (Pourbakhsh *et al.*, 1997; Dho-Moulin, 1999).

2.4.4 Isolation and identification of *E. coli*

2.4.4.1 Culture

The MacConkey agar or Eosin methylene blue (EMB) agar (or both) are specific media for detection of *E. coli*. On MacConkey agar, deep red colonies are produced, as the organism is lactose-positive, and fermentation of this sugar will cause the medium's pH to drop, leading to darkening of the medium. Growth on EMB agar produces black colonies with a greenish-black metallic sheen (Paton and Paton, 1998).

2.4.4.2 Biochemical properties

The biochemical properties of *E. coli* are

- They are lactose positive, and grows on TSI (Triple sugar Iron test) slant with a (A/A/g+/H2S-)
- *E. coli* ferments glucose and lactose producing acid and carbon dioxide. Acid causes the phenol red indicator in the agar to turn yellow. Carbon dioxide is observed as bubbles or cracks in the agar. There is no hydrogen sulfide production, as indicated by the lack of black precipitate in the agar (Carter and Cole,1990)
- It is also, IMViC {+ + – –) (Indole positive, methyl red positive and Voges–prauskeur negative and citrate negative)
- *E. coli*; as it is indole-positive produced (red ring) in the peptone medium (Cheesbough,1985)
- Methyl red-positive (bright red) and VP-negative (no change-colorless in MR-VP(Methyl red- and Voges–prauskeur) broth (Cheesbough,1985)
- Citrate-negative (no change-green color) in citrate medium

Tests for toxin production can be done using mammalian cells in tissue culture, which are rapidly killed by shiga toxin. Although sensitive and very specific, this method is slow and expensive (Paton and Paton, 1998).

2.4.4.3 Classical methods

2.4.4.3.1 Multiple-tube fermentation technique

The technique of enumerating coliforms by means of multiple-tube fermentation (MTF) has been used for over 80 years as a water quality monitoring method. The method consists of inoculating

a series of tubes with appropriate decimal dilutions of the water sample. Production of gas, acid formation or abundant growth in the test tubes after 48 h of incubation at 35 °C constitutes a positive presumptive reaction. Both lactose and lauryl tryptose broths can be used as presumptive media, but Seidler *et al.* (1981) and Evans *et al.* (1981) have obtained interference, with high numbers of non-coliform bacteria, using lactose broth. All tubes with a positive presumptive reaction are sub-sequently subjected to a confirmation test. The formation of gas in a brilliant green lactose bile broth fermentation tube at any time within 48 h at 35 °C constitutes a positive confirmation test. The fecal coliform test (using an EC medium) can be applied to determine TC that are FC (APHA *et al.,* 1998): the production of gas after 24 h of incubation at 44.5 °C in an EC broth medium is considered as a positive result. The results of the MTF technique are expressed in terms of the most probable number (MPN) of microorganisms present. This number is a statistical estimate of the mean number of coliforms in the sample. As a consequence, this technique offers a semi-quantitative enumeration of coliforms. Nevertheless, the precision of the estimation is low and depends on the number of tubes used for the analysis: for example, if only 1 ml is examined in a sample containing 1 coliform/ml, about 37% of 1 ml tubes may be expected to yield negative results because of the random distribution of the bacteria in the sample. But, if five tubes, each with 1 ml sample, are used, a negative result may be expected less than 1% of the time (APHA *et al.,* 1998). Many factors may significantly affect coliform bacteria detection by MTF, especially during the presumptive phase. Interference by high numbers of non- coliform bacteria (Seidler *et al.*, 1981; Evans *et al.*, 1981; Means and Olson, 1981), as well as the inhibitory nature of the media (McFeters *et al.*, 1982), have been identified as factors contributing to underestimates of coliform abundance. The MTF technique lacks precision in qualitative and quantitative terms. The time required to obtain results is higher than with the membrane filter technique that has replaced the MTF technique in many instances for the systematic examination of drinking water. However, this technique remains useful, especially when the conditions do not allow the use of the membrane filter technique, such as turbid or colored waters. MTF is easy to implement and can be performed by a technician with basic microbiological training, but the method can become very tedious and labor intensive since many dilutions have to be processed for each water sample. However, it is also relatively inexpensive, as it requires unsophisticated laboratory equipment. Nevertheless, this method is

extremely time-consuming, requiring 48 h for presumptive results, and necessitates a subculture stage for confirmation which could take up to a further 48 h.

2.4.4.3.2 Membrane filter technique

The membrane filter (MF) technique is fully accepted and approved as a procedure for monitoring drinking water microbial quality in many countries. This method consists of filtering a water sample on a sterile filter with a 0.45 mm pore size which retains bacteria, incubating this filter on a selective medium and enumerating typical colonies on the filter. Many media and incubation conditions for the MF method have been tested for optimal recovery of coliforms from water samples (Grabow and du Preez, 1979; Rice *et al.*, 1987). Among these, the most widely used for drinking water analysis are the Endo-type media in North America (APHA *et al.*, 1998) and the Tergitol-TTC medium in Europe (AFNOR, 1990). Coliform bacteria form red colonies with a metallic sheen on an Endo-type medium containing lactose (incubation 24 h at 35 °C for TC) or yellow-orange colonies on Tergitol-TTC media (incubation 24 and 48 h at 37 and 44 °C for TC and FC, respectively).

2.4.4.4 Molecular Investigation

The following molecular methods are usually used to identify Escherichia coli

2.4.4.4.1 Polymerase chain reaction (PCR)

Candrian *et al.* (1991) developed a PCR assay specific for *E. coli* allowing rapid and unambiguous identification. It has a relatively modified internal transcribed 16S rRNA gene sequence compared to other members of Enterobacteriaceae.

2.4.4.4.2 Multi Locus sequence Typing (MLST)

Multi Locus sequence typing is a well established method for characterizing bacteria (Gordon, 2010). It is used for a variety of different bacteria to understand clonal groups and phylogenetic relatedness. In MLST seven housekeeping genes from the core genome are selected and 300-700 bp region of each of these genes are sequenced (Cooper and Feil, 2004). These sequence profiles can be used to determine the ancestry and relatedness of each strain (Gordon, 2010).

2.4.4.4.3 Pulsed field gel electrophoresis (PFGE)

It is considered as the gold standard in typing bacterial isolates (Riley, 2004; Georging 2010). According to Georging (2010) the method involves the extraction of DNA in a plug followed by restriction digestion with the appropriate restriction enzymes (Xba1 and NOTI). The large

fragments 30- 50 kbp produced as a result of restriction enzyme digestion is separated on agarose gel by altering field direction (current). This forces the small fragments to move faster than the large fragments on the agarose gel which results in a DNA pattern specific to each clone.

2.4.4.4.4 Random amplification of polymorphic DNA

Random amplification of polymorphic DNA (RAPD) is a simple PCR based genotyping method that uses short oligonucleotides of random sequence (Williams *et al.*, 1990). The random sequences bind to random priming sites and amplify DNA segments of variable lengths (Hadrys *et al.*, 1992). This results in a pattern of small and large DNA fragments (Williams *et al.*, 1990). These patterns would be identical for clonal bacterial population (Gordon, 2010) and have been proposed as alternatives for using to characterize *E. coli* isolates of avian origin (Chansiripornchai *et al.*, 2001 and de Moura *et al.*, 2001).

2.4.4.4.5 Clermont phylogenetic grouping

Clermont phylogenetic grouping is a simple and rapid method that was used by Clermont *et al.* (2007) to identify the phylogenetic groups of *E. coli*. This method uses a triplex PCR to identify the phylogenetic group by examining the presence or absence of two genes (ChuA and YiaA) and a DNA fragment (TSPE4.C2).

2.4.4.4.6 DNA Microarray Technology

This technique is based on testing water samples for the actual genetic material of a microorganism, rather than relying on growing the microbe, or using a microscope. Large amounts of known genetic information (DNA or RNA) can be stored on a very small surface and used to detect microbes in a sample by reacting with complementary DNA or RNA from the microbial population. The microarray method was first developed by Stanford University (Elkins and Chu, 1999) and was called "DNA Microarray". It is envisaged that these methods can reduce the time of analyses for fecal indicators to 4 hours and reduce the cost significantly.

2.4.4.4.7 Fluorescent in situ Hybridisation (FISH)

FISH is another genetic method for detecting microorganisms. The method uses a fluorescent marker attached to the DNA of the microorganism that is being investigated. The sample can be processed on a fixed surface, generally a microscope slide, and if the target microorganism is present, the reaction results in the microorganism "glowing". This is then viewed using a

fluorescence microscope. A number of FISH methods have been developed for the detection of total coliforms and enterococci (Fuchs *et al.*, 1998; Meier *et al.*, 1997; Patel *et al.*, 1998).

2.5 Microbiological assessment of water samples from different sources

Malaney and Weiser (1962) isolated coliform bacteria from pond water of Central North, Central Southeastern and Southwestern Ohio whereas Kroger and Noll (1969) cultured water samples of 180 wells and isolated high proportion of *E. coli* with other members of family Enterobacteriaceae.

Lin *et al.* (1974) conducted a bacteriological study of spoon river water and found 200 coliforms per 100 ml water. However, Dragas and Tratnik (1975) examined 1950 samples of drinking water and swimming pool for the presence of enteropathogenic *E. coli*. They observed that 21.5% of water samples were microbiologically unsafe and contained a high MPN of *E. coli*. Mieres and Bastardo (1975) studied the presence of enterobacteria in the water of the river Manzanares in Venezuela. They found a higher percentage of enteropathogenic organisms in water.

Badge *et al.* (1982) stated that the growth of aquatic microorganism was affected by a great variety of physical and chemical factors. The authors observed that the rate of change in bacterial density in water was related to a variety of factors such as (a) assimilable organic nutrient in water (b) organisms competing for dominance in the microbial flora, (c) water pH and (d) storage temperature.

Edberg *et al.* (1996) conducted a research to determine the numbers and types of bacteria found in three water sources-bottled water, water cooler water, and tap water-and to determine their virulence characteristics. A wide variety of water types were collected and each was analysed for Heterotrophic Plate Count (HPC) bacteria, *Pseudomonas aeruginosa*, and total coliforms. For each isolate, virulence characteristics were determined by enzyme analysis (10 associated with virulence), antibiotic susceptibility testing (natural and semi-synthetic antibiotics), acid lability (survival at pH 3.5) and cytotoxicity testing (HEp-2 cells). Results showed that all water sources had a normal bacterial content. Only 2 per cent of bottled water sources had *P. aeruginosa*. Total 6 coliforms were isolated only from bottled water that used mixed (water alternating with milk) filling lines. Environmental bacteria did not produce significant enzymes associated with

virulence, were not acid resistant, were susceptible to semi-synthetic antibiotics, and did not produce appreciable cytotoxicity. These natural aqueous bacteria were adopted to a water environment, did not grow well at conditions analogous to the human host, and did not have the characteristics associated with virulence. Future drinking water revisions and changes to the treatment processes should be directed towards the elimination of specific pathogens and to the prevention of exogenous sources of contamination rather than the elimination of natural water microbial populations.

Karn and Harada, (2001) stated that in South Asian countries such as Nepal, India, and Bangladesh, pollution of river water was more severe and critical near urban stretches due to huge amounts of pollution load discharged by urban activities. The Bagmati River in the Kathmandu valley, the Yamuna River at Delhi, and peripheral rivers (mainly Buriganga River) of Dhaka suffer from severe pollution these days. They observed that during dry season average of biochemical oxygen demand (BOD) in all these rivers is in the range of 20-30 mg/liter and total coliform are as high as 104-105 MPN/100 ml. Okagbue *et al.* (2002) analyzed sixty processed bottled water samples supplied by three companies in Zimbabwe, for microbial safety. The authors observed that four (6.7%) and seven (11.7%) samples to exceeded the recommended maximum level of total viable and coliform counts, respectively.

Johnson *et al.* (2003) worked on prevalence of *E. coli* 0157: H7 and *Salmonella spp.* in surface waters. They conducted a 2-year study to estimate the prevalence of *E. coli* 0157: H7 and *Salmonella spp.* in surface water within the basin. This study was the first of its kind to identify *E. coli* 0157: H7 in surface water collected from a Canadian watershed. They found that prevalence of *E. coli* 0157: H7 and Salmonella spp. in water samples was 0.9% (n = 1483) and 6.2% (n= 1429), respectively.

Sultana *et al.* (2009) studied the whole concepts adopted for microbiological quality is that no water intended for human consumption shall contain *E. coli* in 100 ml sample. Analysis of bacteriological quality of pump water and household's water showed that 36.36% pump water and 42.86% of household water were contaminated with fecal-coliform and coliform of non-fecal origin.

Cabral (2010) stated that water is essential to life, but many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections. In this review a general characterization of the most important bacterial diseases transmitted through water cholera, typhoid fever and bacillary dysentery is presented, focusing on the biology and ecology of the causal agents and on the diseases characteristics and their life cycles in the environment. The importance of pathogenic *E. coli* strains and emerging pathogens in drinking water transmitted diseases is also briefly discussed. Microbiological water analysis is mainly based on the concept of fecal indicator bacteria. The main bacteria present in human and animal feces (focusing on their behavior in their hosts and in the environment) and the most important fecal indicator bacteria are presented and discussed (focusing on the advantages and limitations of their use as markers). Important sources of bacterial fecal pollution of environmental waters are also briefly indicated. In the last topic it is discussed which indicators of fecal pollution should be used in current drinking water microbiological analysis. It was concluded that safe drinking water for all is one of the major challenges of the 21st century and that microbiological control of drinking water should be the norm everywhere. Routine basic microbiological analysis of drinking water should be carried out by assaying the presence of *E. coli* by culture methods. Whenever financial resources are available, fecal coliform determinations should be complemented with the quantification of enterococci. More studies are needed in order to check if ammonia is reliable for a preliminary screening for emergency fecal pollution outbreaks. Financial resources should be devoted to a better understanding of the ecology and behavior of human and animal fecal bacteria in environmental waters.

Islam *et al.* (2010) studied the bacteriological quality of treated water of different sources. It was determined by presumptive coliorm count. In source wise distribution of samples, 50% of mineral water, 87.5% of filtered water and 100% of tap water samples were exceeded the drinking water guideline value of WHO. Microorganisms in tap water comprised *Escherichia coli*(60%), *Klebsiella spp.* (40%), *Enterobacter spp.* (20%), *Pseudomonas spp.* (70%), *Proteus spp.* (10%), *Staphylococcus spp.* (40%) and *Salmonella spp.* (0%). Furthermore, there was no correlation between fecal coliform and the presence of *Salmonella* species. Results obtained from this investigation revealed that municipal tap water of Dhaka city was contaminated with a number of enteric bacteria such as *E. coli*. This organism was considered as a good bio-indicator model for surveillance studies of antimicrobial resistance.

2.6 Water treatment

Water disinfection is the usual water treatment used in bird rearing. The aim is to eliminate pathogens that might be in the water, both those originated from contamination of the water source and those incorporated in the way between the water source and the drinkers. A second objective is to leave residual levels of chlorine in the water in order to eliminate pathogens that might be added to it if infected birds have access to water in the drinkers. The use of water with controlled microbiological quality as drinking water for birds is of fundamental importance, considering that:

- Many birds have access to the water source;

- Birds intake water daily;

- Water is considered a good transmission vehicle for many pathogens;

- Residues from human and animal activities pollute a great number of water sources.

To assure that the water consumed by birds will not pose risk to the flock health, it should be disinfected. The most recommended disinfecting agent is chlorine, due to its efficiency, cost, practical use and inequity to birds when adequately applied. Water treatment in the rural area may use diverse compounds as chlorine sources, which present different levels of available chlorine (Amaral, 2004), among which two are cited below:

A. Calcium hypochloride - white powder that contains 70% of available chlorine.
B. Commercial solution of sodium hypochloride - clear liquid with 10-12% available chlorine.

In emergency situations, bleach may also be used as chlorine source; it contains available chlorine levels of 2.0%. According to Jefrey (2000), adequate chlorine dosage in drinking water for birds is 3 ppm, although birds may tolerate residual chlorine concentrations of more than 10 ppm. Concentrations of 5 ppm are indicated to control biofilm formation. The presence of organic material rapidly inactivates chlorine, therefore, drinkers should be cleaned daily to avoid accumulation of organic material. Water pH should be lower than 8.5, and optimal pH values are from 6.0 to 8.0. Chlorination should be suspended two days before any vaccination with live bacteria and virus via drinking water. Supply of chlorinated water may be resumed 4 hours after

vaccination is completed. Residual chlorine concentrations between 2 and 5 ppm has resulted in no performance impairment and has been suggested as the levels to be added in the water supplied to broilers and laying hens (Damron & Flunker, 1993). The microbiological quality of water given to broilers improved at 2 ppm of residual chlorine.

It should be remembered that chlorine reacts not only with microorganisms during chlorination, but also with organic and inorganic substances, generating the water chlorine demand (Tsai *et al.*, 1992). The presence of residual chlorine is thus important, since it will be responsible for the elimination of the microorganisms that contaminate, by different routes, the water given to the birds. Residual chlorine should be measured 30 min after the disinfectant comes into contact with the water.

2.7 Antibiotic resistance

Bacterial infections are usually treated with antibiotics. However, the antibiotic sensitivities of different strains of *E. coli* vary widely. As Gram-negative organisms, *E. coli* are resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotics which may be used to treat *E. coli* infection include amoxicillin, as well as other semi synthetic penicillins, many cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides.

Avian pathogenic *E. coli* strains are often resistant to antimicrobials approved for poultry including cephradine (Rahman *et al.*, 2004), tetracyclines (Cloud *et al.*, 1985; Irwin *et al.*, 1989; Blanco *et al.*, 1997; Bass *et al.*, 1999), chloramphenicol (Rahman *et al.*, 2004), sulfonamides (Cloud *et al.*, 1985; Blanco *et al.*, 1997; Bass *et al.*, 1999; Li *et al.*, 2007), amino-glycosides (Dubel *et al.*, 1982; Irwin *et al.*, 1989; Allan *et al.*, 1993; Blanco *et al.*, 1997; Bass *et al.*, 1999) and β-lactam antibiotics (Cloud *et al.*, 1985; Blanco *et al.*, 1997; Rahman *et al.*, 2004; Li *et al.*, 2007).

Resistance to fluoroquinolones was reported within several years of the approval of this class of drugs for use in poultry (Blanco *et al.*, 1997; Li *et al.*, 2007; White *et al.*, 2000; Van den *et al.*, 2001). There is reason for concern that genes conferring resistance to extended-spectrum β-lactams will emerge in avian pathogenic *E. coli* strains (Zhao *et al.*, 2001) and reduce the

efficacy of ceftiofur, which is currently used on a limited basis in poultry breeding flocks and hatcheries (Wooley *et al.*, 1992).

Wooley *et al.*, (1992) conducted a study at the University of Georgia. They found 97 of 100 avian pathogenic *E. coli* isolates to be resistant to streptomycin and sulfonamide and 87% of these multiple antimicrobial resistant strains contained a class 1 integron. In 1999 Bass *et al.*, reported that intI1, which carried multiple antibiotic resistance genes. Multiple antimicrobial resistance traits of avian pathogenic *E. coli* have also been associated with transmissible R-plasmids.

Antibiotic-resistant *E. coli* may also pass on the genes responsible for antibiotic resistance to other species of bacteria, such as *Staphylococcus aureus*, through a process called horizontal gene transfer. *E. coli* bacteria often carry multiple drug-resistant plasmids, and under stress, readily transfer those plasmids to other species. Thus, *E. coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance (Salyers *et al.*, 2004).

Antibiotic resistance is a growing problem. Some of this is due to overuse of antibiotics in humans, but some of it is probably due to the use of antibiotics as growth promoters in animal feed (Johnson *et al.*, 2006). Hasan *et al.*, (2011) studied the antibiotic resistance patterns in avian pathogenic *E. coli* in Bangladesh. Of the 279 dead or sick poultry of different ages, 101 pathogenic *E. coli* strains were isolated from broilers and layer hens. The strains were screened to determine phenotypic expression of antimicrobial resistance against 13 antibiotics that are commonly used in both veterinary and human medicine in Bangladesh. Of the 101 pathogenic *E. coli* isolates, more than 55% were resistant to at least one or more of the tested compounds, and 36.6% of the isolates showed multiple–drug-resistant phenotypes. The most common resistances observed were against tetracycline (45.5%), trimethoprim-sulphamethoxazole (26.7%), nalidixic acid (25.7%), ampicillin (25.7%) and streptomycin (20.8%). Resistance to ciprofloxacin (12.9%), chlormaphenicol (8.9%), nitrofurantoin (2%) and gentamicin (2%) was also observed. One isolate was resistant to cefuroxime (1%), and cefadroxil (1%). Hashem *et al.*, (2012) reported *E. coli* to be highly sensitive to colistin sulphate (100%), intermediately sensitive to ciprofloxacin (50%), resistance to amoxicillin (100%), ampicillin (75%), oxytetracycilline (50%), penicillin (25%). Resistance also found against cotrimoxazole (100%), gentamicin (75%).

Chapter III: Materials and Methods

3.1 Study area

Water samples were collected from five commercial layer chicken farms, five commercial broiler farms and five breeder farms belonging to 9 places of Chittagong district (Sitakundu, Kumira, Patiya, Hat Hazari, Mirorsorai, Raojan, Pahartali, Korer Hat and Chandanaish) (Figure 1). The selected study sites were located 40-60 km away from Chittagong city, consisting of both high and low lands along with coastal areas.

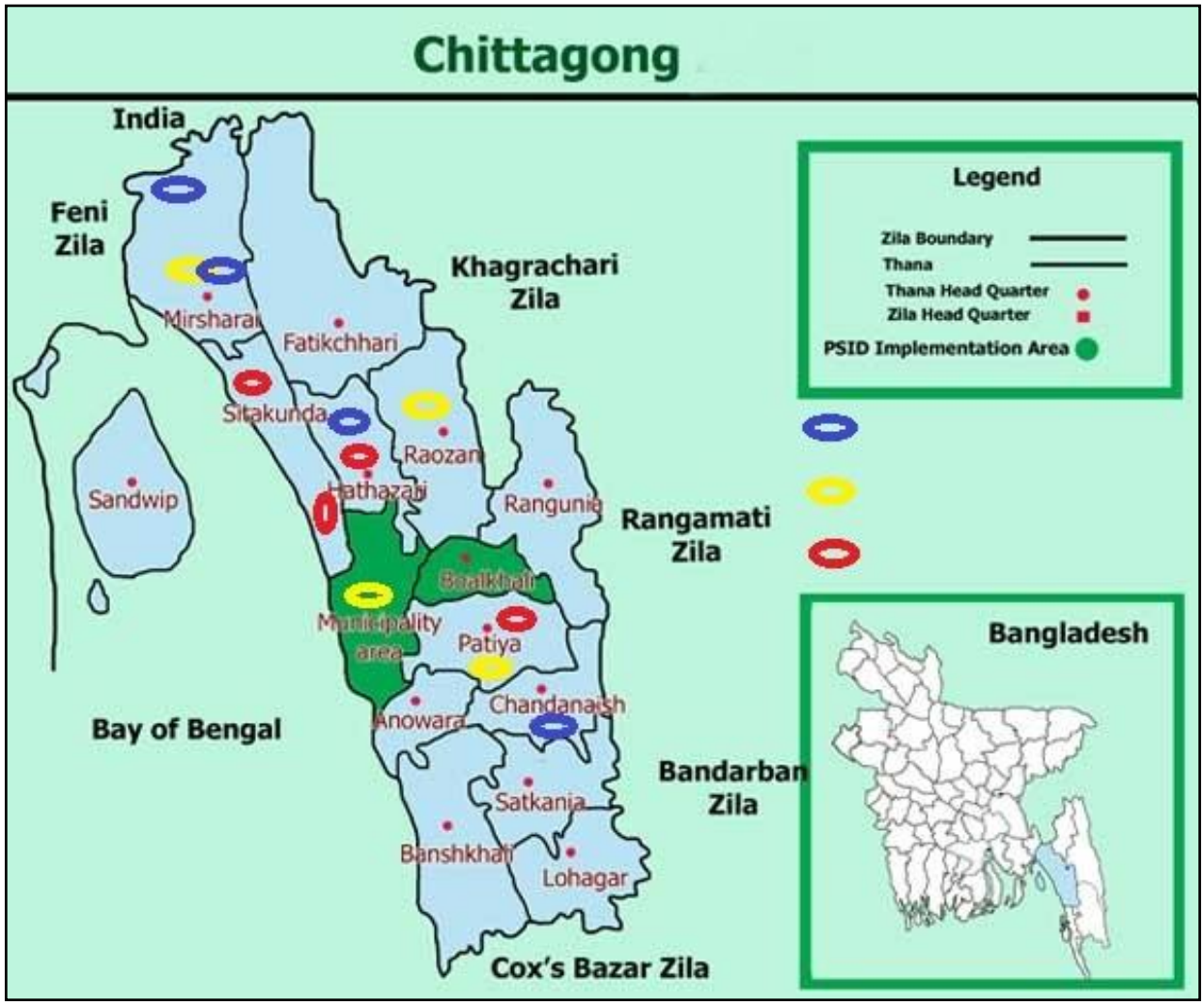

Figure 1 - Map showing the location of the study areas (Round colors)

3.2 Experimental design

Water samples used in different poultry farms were subjected to qualitative assessment including bacteriological quality. Moreover, *E. coli* were isolated from the water samples and further employed for antimicrobial susceptibility testing. The schematic representation of experimental design is presented in Figure 2.

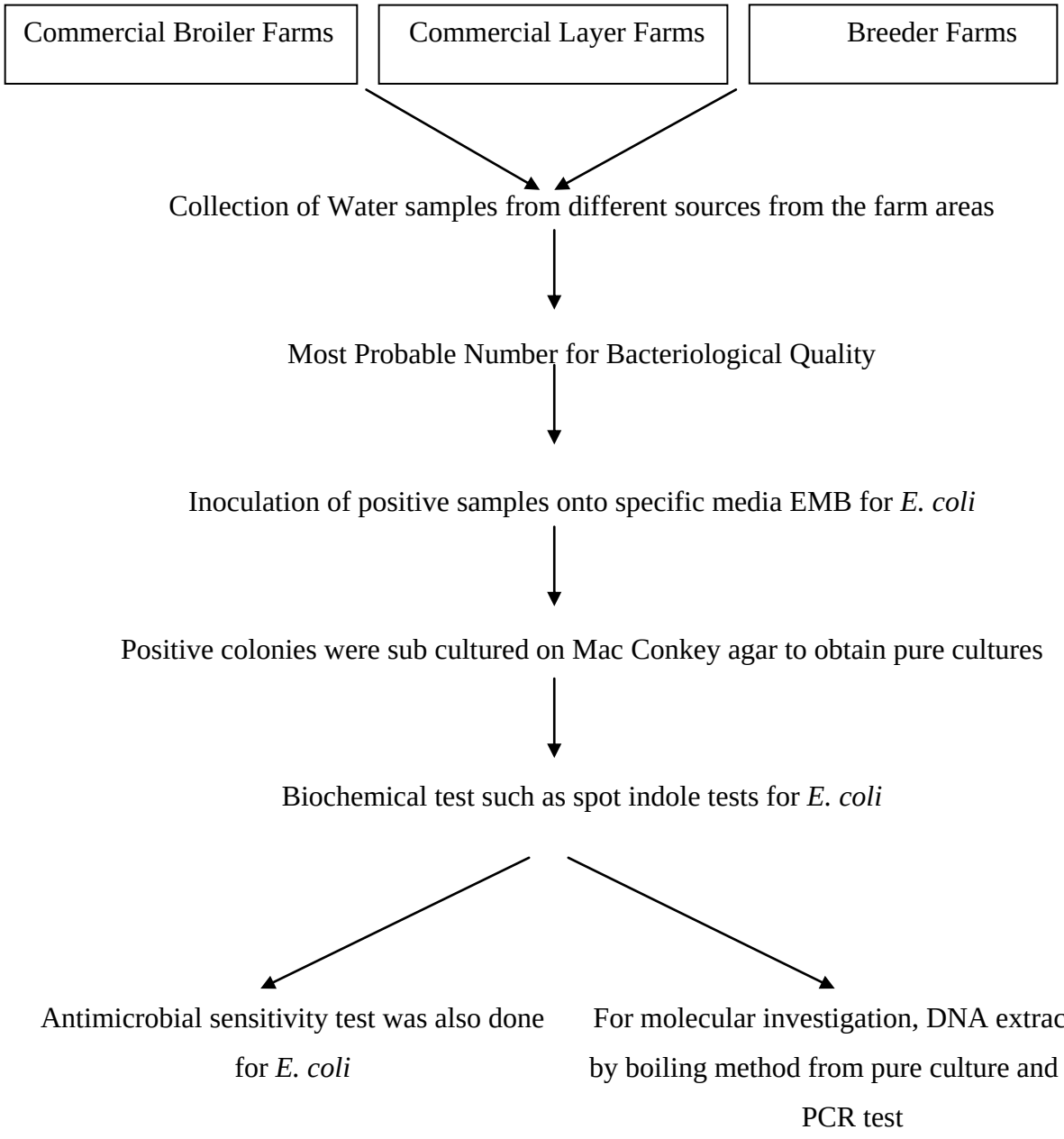

Figure 2 - Experimental Design

3.3 Study period

The study was conducted for a period of four months, starting from July 2014 - November 2014.

3.4 Sample Size

Total 150 water samples were collected from the mentioned poultry farms. From each farms, samples were collected from different sources.

3.5 Methods

3.5.1 Collection of water samples

Water samples were collected in sterile Falcon™ 50mL Conical Centrifuge Tubes from various water sources available in and around the selected broiler, layer and breeder farms of Chittagong. After collection of water samples in the Falcon Tubes the mouth was tightly covered with polyethylene flat-top screw caps and shipped the laboratory of Poultry Research and Training Centre (PRTC), CVASU for detail microbiological investigation. During transportation of samples in Falcon tubes, cool boxes were used.

3.5.2 Questionnaire for overall assessment

A prototype questionnaire was designed to collect the objectives oriented data from each farm via interviewing the farmers and observing the record book. The questionnaire has been appended in the **ANNEX 1**.

3.5.3 Preparation of different media

3.5.3.1 Mac Conkey broth

In 1000 ml purified/distilled water 34.51 grams powder of Mac Conkey broth was added. The mixture was gently heated for few minutes to dissolve the powder completely with water. Then broth was dispensed into test tubes with inverted Durham tubes. The medium was then autoclaved at 121°C for 15 minutes at 15 lbs pressure to make it sterile. After autoclaving the mixture was put into water bath adjusting temperature 50°C to cool down its temperature.

3.5.3.2 Eosin Methylene Blue (EMB) Agar Media

In 1000 ml of distilled water 36 grams powder of EMB agar base was added. The mixture was heated to boil for few minutes to dissolve the powder completely with water. The medium was then autoclaved for 30 minutes to make it sterile. After autoclaving the mixture was placed into

water bath adjusting temperature 50°C to cool down its temperature and allow the media not to solidify. From water bath 10-20 ml of medium was poured into small and medium sized sterile petridishes under safety hood to make sterile EMB agar plates. After solidifying the medium, the plates were then allowed for incubation at 37°C for overnight to check their sterility.

3.5.3.3 MacConkey Agar Media

In 1000 ml of distilled water 50 grams powder of MacConkey's agar (Difco) base was added. The mixture was heated to boil for few minutes to dissolve the powder completely with water. The medium was then autoclaved for 30 minutes to make it sterile. After autoclaving the mixture was put into water bath adjusting temperature 50°C to cool down its temperature and allow the media not to solidify. From water bath 10-20 ml of medium was poured into small and medium sized sterile petridishes under safety hood to make sterile MacConkey agar plates. After solidifying the medium, the plates were then allowed for incubation at 37°C for overnight to check their sterility.

3.5.3.4 Blood Base Agar (BBA) Media

Sixty grams of base (Hi-media) was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving. The autoclaved materials were allowed to cool to a temperature of 50°C in a water bath. Defibrinated 5% cattle blood was then added to the medium aseptically and distributed to sterile petridishes and then allowed to cool at room temperature for solidification. After solidification of the medium, the plates were allowed to incubate at 37°C for overnight to check their sterility and stored at 4°C in the refrigerator until used.

3.5.3.5 Nutrient Agar (NA) Media

In 1000 ml of distilled water 28 grams powder of nutrient agar base was added. The mixture was heated to boil for 5 minutes to dissolve all the powder completely with water. The medium was then autoclaved for 30 minutes to make it sterile. After autoclaving the mixture was put into water bath adjusting temperature 50°C to cool down its temperature and allow the media not to solidify. From water bath 10-20 ml of medium was poured into small and medium sized sterile petridish under safety hood to make sterile nutrient agar plates. After solidifying the medium in the plates, the plates were then allowed for incubation at 37°C for overnight to check their sterility.

3.6 Most Probable Number (MPN)

The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases for water examination for the presence of coliforms. An estimate of the number of coliforms (Most Probable Number) can also be done in the presumptive test. In this procedure, 9 test tubes (Large 3 and small 6 pieces) were taken and then dispensed 10 ml of dissolved Mac Conkey broth into each test tube. Autoclave was done for whole set of test tube along with control water samples. Then, each test tube was inoculated with the water samples. Three tubes received 10 ml of water, 3 tubes received 1ml of water, 3 tubes received 0.1ml of water. A count of the number of tubes showing gas production was then made after 24 hour incubation, and the figure was compared to a table developed by Jacobs and Gerstein's (1960). The number was the most probable number (MPN) of coliforms per 100 ml of the water sample. In the **ANNEX 2**, MPN table has been added.

3.7 Isolation and identification of *E. coli*

3.7.1 Culture onto different media

Sterilized platinum loop was used for streaking the lactose broth culture on EMB agar, MacConkey agar and Blood agar to get isolates in pure culture. All inoculated media were kept at 37°C for overnight in an incubator.

3.7.1.1 Eosin Methylene Blue (EMB) agar

Materials from lactose fermentation tubes were inoculated onto EMB agar plates which after incubation showed smooth circular colonies with dark centers and metallic sheen.

3.7.1.2 MacConkey agar

Positives samples were further cultured onto MacConkey agar to get the pure colonies. Colonies from EMB positive plates were inoculated onto MacConkey agar plates which after incubation, if positive for *E. coli* showed rose pink color colonies.

3.7.2 Identification of *E. coli* isolates by biochemical tests

3.7.2.1 Indole test

Two milliliter (2 ml) of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°C for 48 hours. Following, 1ml Xyline was poured into the test tube, Kovac's reagent (0.5 ml) was added, shaked well and examined after 1 minute. A red color in the reagent

layer indicated production of indole. In negative case there was no development of red color (Cheesbrough, 2006).

3.8 Detection of virulence genes by PCR

3.8.1 DNA extraction

A pure bacterial colony was mixed with 200 µl of PBS which were boiled for 10 minutes then immediately kept on ice for 10 minutes. Finally centrifugation was done at 10000 rpm for 10 minutes. The supernatant was collected and used as DNA template for PCR (Norhan *et al.*, 2014).

3.8.2 Primers used for PCR

All the probable *E. coli* isolates were investigated for the diversity based on the two virulent genes *Stx1* and *Stx2* by Polymerase Chain Reactions (PCR). The sequences of two oligonucleotide sets of primers, respectively, used for *Stx1* and *Stx2* genes are shown in Table 7.

Table 7 - Oligonucleotide Primer used to detect *Stx1* and *Stx2* gene

Primer	Primer Sequence (5' – 3')	Target gene	Anne-aling temp.(°C)	Size of product (bp)	References
***Stx1* F**	ACA CTG GAT GAT CTC AGT GG	*Stx1*	58	~614	desRosier *et al.*, 2001
***Stx1* R**	CTG AAT CCC CCT CCA TTA TG				
***Stx2* F**	CCA TGA CAA CGG ACA GCA GTT	*Stx2*	58	~779	Manna *et al.*, 2006
***Stx2* R**	CCT GTC AAC TGA GCA GCA CTT T				

3.8.3 Preparation of a PCR mixture

PCR mixture (25 µl) was prepared as follows

i. Nuclease free water = 6.5 µl
ii. 2X PCR master mixture (Promega, USA) = 12.5 µl
iii. Forward primer = 0.5 µl

iv. Reverse primer = 0.5 µl

v. DNA template = 5 µl

3.8.4 PCR reaction profile

Cycling conditions used for three sets of primers (Nadine *et al.*, 2003)

i. Initial denaturation = 95°C for 3 min

ii. Denaturation = 94°C for 30 sec

iii. Annealing = 56°C for 40 sec

iv. Extension = 72°C for 1 min

35 cycles

v. Final extension = 720C for 8 min

vi. Holding temperature = 4°C until remove from thermo-cycler

3.8.5 Electrophoresis

1% agarose gel was used for electrophoresis of the PCR products. The procedure of gel electrophoresis is given below:

a) Gel casting tray was assembled with gel comb of appropriate teeth size and number.
b) 1% agarose solution was prepared in TAE buffer by melting in a microwave oven.
c) Molten agarose was poured onto the casting tray and allowed to solidify on the bench.
d) The solid gel in its tray was transferred to the electrophoresis tank containing sufficient TAE buffer to cover the gel ≈1mm. The comb was gently removed.
e) 10 µl of each PCR product was mixed with 2µl loading buffer and the sample was loaded to the appropriate well of the gel.
f) 5 µl DNA size marker (Trackit, invitrogen, USA) was loaded in one well.
g) The leads of the electrophoresis apparatus were connected to the power supply and the electrophoresis was run at 100 V for 30 minutes
h) When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
i) The gel stained in ethidium bromide (0.5µg/ml) for 10 minutes, in a dark place.

j) The gel was destained in distilled water for 10 minutes. The destained gel was placed on the UVsolo TS imaging system (Biometra, Germany) in the dark chamber of the image documentation system.

k) The UV light of the system was switched on, the image was viewed on the monitor, focused, acquired and saved in an USB flash drive.

3.9 Maintenance of Stock Culture

Stock culture was prepared by adding 1 ml of 80% sterilized glycerol in 1 ml of pure culture in nutrient broth and it was stored at -20˚ C for further use.

3.10 Antimicrobial Susceptibility Test

Susceptibility and resistance of different antibiotics was measured by disc diffusion or Kirby-Bauer method (Bauer *et al.*, 1966). 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. One well isolated colony was selected from the MS agar plates and EMB agar plate. Colony was touched with a sterile loop and streaked onto nutrient agar and incubated overnight at 37 ºC. 4 or 5 well isolated colonies were transferred into a tube of sterile physiological saline and vortex thoroughly. 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 dawn. A sterile cotton swab 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 broth was streaked homogenously on the medium. Antibiotic discs were applied aseptically to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile forceps on Mueller-Hinton agar plates. The plates were then inverted and incubated at 37 ºC for 24 hours. The diffusion discs with antimicrobial drugs were placed on the plates and incubated for 18 hours at 37 ºC. After incubation the diameter of the zone of inhibition (including diameter of the discs) was measured in millimeters with a ruler.

3.10.1 Antimicrobial discs

The list of commercially available antimicrobial disc (Oxoid, UK) used in this study with their concentration is given in Table 8. After placing of the discs on the plate, the plates were inverted

and incubated at 37°C for 16 to 18 hours. 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.

Table 8 - Antimicrobial agents with their disc concentration

Antimicrobial agents	Symbol	Disc concentration (µg/disc)
Ampicillin	AMP	10
Amoxicillin-clavulanic acid	AML	20/10
Ciprofloxacin	CIP	5
Enrofloxacin	ENR	10
Norfloxacin	N	10
Gentamicin	GEN	10
Doxicycline	DO	30
Tetracycline	TE	30
Colistin Sulphate	CT	10

3.10.2 Recording and interpreting results

The zones of growth inhibition was compared with the zone-size interpretative table (Table 9) provided by Clinical and Laboratory Standards Institute (CLSI, 2007). Antimicrobial testing results were recorded as susceptible, intermediate and resistant according to zone diameter interpretive standards provided by CLSI (2007).

3.11 Statistical analysis

Prevalence of *E. coli* in broiler, layer and breeder farms along with MPN index count, at different parameters of bird and poultry farm compared for statistical significance using chi square test. A p value of ≤ 0.05 was considered to be statistically significant.

Table 9 - Zone diameter interpretive standards for *E. coli*.

Group of Antimicrobials	Antimicrobial agents	Zone diameter (millimeter)		
		Resistant	Intermediate	Sensitive
Penicillin	Ampicillin	≤13	14-16	≥17
β-lactamase inhibitor combination	Amoxicillin-clavulanic acid	≤13	14-17	≥18
Fluoroquinolones	Ciprofloxacin	≤15	16-20	≥21
	Enrofloxacin [a]	≤16	17-19	≥20
	Norfloxacin	≤12	13-16	≥17
Aminoglycosides	Gentamicin	≤12	13-14	≥15
Tetracyclines	Doxicycline	≤10	11-13	≥14
	Tetracycline	≤11	12-14	≥15
Lipopeptides	Colistin Sulphate [b]	≤10	11-13	≥13

Source: CLSI, 2007; [a] Seol *et al.*, 2005; [b] Lo-Ten-Foe *et al.*, 2007

Chapter IV- Results

4.1. Overall pH values from the water samples

All 150 water samples were subjected to pH measurement. Overall, pH value ranged from 4.02 to 9.04. The highest and lowest pH was recorded in drinkers. However, samples were collected from drinkers in highest number. The pH values of different water samples were given below in Figure 3.

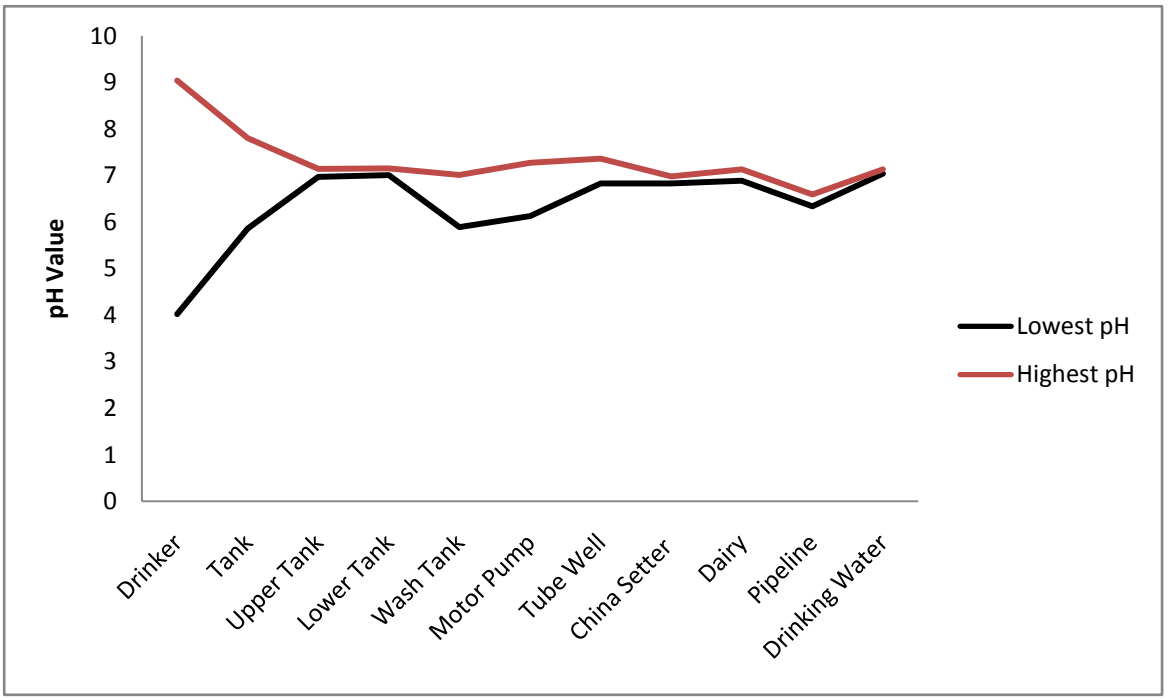

Figure 3 - pH value of water samples collected from different sources at different poultry farms of Chittagong.

4.2 Most Probable Number (MPN) from collected samples

All 150 collected water samples from five broilers, five layer and five breeder farms were tested for MPN to obtain the coliform load. Of them, 103 samples produced positive results by changing the color of the media and gas production. The MPN index count has been grouped into four categories according to coliform number (0, 1-200, 201-1000, 1001 and above).

4.2.1 Relationship of MPN with pH value of water samples

The pH values were categorized into four groups (A, B, C and D). The group A, B, C and D indicates the pH ranges of 4-5, 6-6.99, 7-7.5 and >7.6, respectively. The highest (60%) number of MPN index (1001 and above) was counted in group D (7.6 and above), whereas lowest (14%) was in group C (7-7.5). There was a significant variation ($P < 0.05$) of MPN with pH values was observed in the study area (Table 10). However, in case of Group A (4-5), only 6% samples were found with no coliform bacteria or zero coliform count, while the highest (42%) number of zero coliform count (0) was recorded in Group C (7-7-5).

Table 10 - Relationship of MPN with pH values

pH Category	Total Number of Observation	Most Probable Number/100 ml No of Positive (n) (%)#				Chi Square Value	P – Value*
		0	1-200	201-1000	1001<		
A (4-5)	18	1(6)	5 (28)	6 (33)	6(33)		
B (6-6.99)	73	23(32)	24(33)	6(8)	20(27)	24.0906	0.004
C (7-7.5)	52	22(42)	20(38)	3(6)	7(14)		
D (>7.6)	5	1(20)	1(20)	0(0)	3(60)		

* Values at P<0.05 are statistically significant; #Number in parenthesis indicates percentage.

4.2.2 Bacterial load in different sources of water in the selected farms

Water samples were collected from total 11 sources. The sources have been grouped into four categories. Group 1 contained Tank, Upper Tank, Lower Tank and Wash Tank. Group 2 contained Drinker only. Group 3 contained Motor Pump and Tube Well whereas Group 4 was

for China Setter, Dairy farm, Pipeline and Drinking water. However, the sources were not same for each farm. China setter was only found in case of Breeder farms.

In this present study, a significant relationship was found between water source and the MPN (Table 11). Samples from Group 1 showed the highest (42%) zero coliform count (0) which indicates lower contamination, in contrast the higher bacterial contamination was found in Group 2 and 4 where only 23% samples were recorded for zero coliform count (0), respectively. The highest number of observation (56%) were found into MPN index (1-200) in Group 2, while lowest number (4%) of observation was recorded in MPN index (1001 and above) in Group 1. In case of MPN index (1001 and above), highest (34%) was detected in Group 3 samples. It indicates that Group 2 samples revealed high bacterial load, while more number of coilform (1001 and above) were high in Group 3 samples.

Table 11- Bacterial load in different sources of water in the selected farms

Water Source	Total Number of Observation	Most Probable Number/100 ml No of Positive (n) (%)#				Chi Square Value	P – Value*
		0	1-200	201-1000	>1001		
1	24	10 (42)	10 (42)	1 (4)	3 (13)		
2	39	9 (23)	22 (56)	3 (8)	5 (13)		
3	74	27 (36)	12 (16)	10 (14)	25 (34)	24.5824	0.003
4	13	3 (23)	6 (46)	1 (8)	3 (23)		

* Values at P<0.05 are statistically significant; #Number in parenthesis indicates percentage.

1= Tank; 2= Drinker; 3= Motor Pump and Tube well; 4= Dairy Farm, China setter, Pipe line and Drinking Water

4.2.3 Bacterial load in different farms at different zones of Chittagong

In our study, we collected water samples of different poultry farms from 9 places. Here, we divided the places into five zones (1, 2, 3, 4 and 5) according to their distance. There was a significant variation in the bacterial load in water samples with the selected zones (Table 12).

The highest (60%) percentage of samples with no bacterial load was observed in Zone 3, in contrary only 20% samples were found with zero coliform count in Zone 2 indicating a heavy bacterial contamination in zone 2. In case of coilform number (1001 and above), highest (34%) percentage of observation was detected on Zone 4, while no observation (0%) was found in Zone 1. In Zone 2, a total of 10 samples were collected, 8 were found positive, where 60 % were positive on 1-200 MPN index and 20% were on 1001 and above. It indicates that Zone 2 is the highest coliform prevalent zone (Total 80%). In contrast to this, Zone 3 is the lowest prevalent zone.

Table 12 - Coliform load vs. Farm areas

Farm Areas#	Total Number of Observation	Most Probable Number/100 ml No of Positive (n) (%)#				Chi Square Value	P – Value*
		0	1-200	201-1000	>1001		
1	20	6 (30)	14 (70)	0 (0)	0 (0)		
2	10	2 (20)	6 (60)	0 (0)	2 (20)		
3	20	12 (60)	3 (15)	1 (5)	4 (20)	32.9930	0.001
4	70	19 (27)	18 (26)	9 (13)	24 (34)		
5	30	10 (33)	9 (30)	5(17)	6 (20)		

* Values at P<0.05 are statistically significant; #Number in parenthesis indicates percentage.

#Here, 1= Raojan, Chandanaish; 2= Pahartali; 3= hathazari; 4= sitakunda, kumira, Mirsaria, Korer hat; 5= Patiya

4.2.4 Differences in Coliform load in different types of poultry farms

In the study, water samples were collected from Broiler, Layer and Breeder farms. Among those types of farms, Breeder farms were highly free from bacterial contamination as because highest (60%) percentage of samples was found with zero coliform count, On the other hand, 0 coliform counts was recorded 16 and 19%, respectively in broiler and layer farms (Table 13). In case of coliform count (1001 and above), samples of broiler farms were highly positive (46%).

Table 13 - Differences in coliform load in different types of poultry farms

Farm Type	Total Number of Observation	Most Probable Number/100 ml No of Positive (n) (%)#				Chi Square Value	P – Value*
		0	1-200	201-1000	>1001		
Broiler	50	8 (16)	13 (26)	6 (12)	23 (46)		
Layer	50	9 (19)	24 (50)	7 (15)	8 (16)	42.5531	0.000
Breeder	50	30 (60)	13 (26)	2 (4)	5 (10)		

* Values at P<0.05 are statistically significant; #Number in parenthesis indicates percentage.

4.2.5 Comparison of Coliform count between bleaching powder treated and non-treated water

In our present study, we observed that water treatment was only practiced by breeder farms, however there was no such water treatment in case of layer and broiler farms. In case of treated water samples, 60% samples gave zero coliform count, on the other hand, lowest (17%) percentage of samples was counted for no bacterial load (Table 14). In all group of MPN index (1-200, 200-1000, 1001 and above), treated water samples were in lower percentage comparing with non treated water and this variation was found as statistically significant (P<0.005).

Table 14 - Relationship between water treatment and MPN

Water Treatment with bleaching powder Category	Total Number of Observation	Most Probable Number/100 ml No of Positive (n) (%)#				Chi Square Value	P – Value*
		0	1-200	201-1000	>1001		
A (Treated)	50	30 (60)	13 (26)	2 (4)	5 (10)	29.495	0.000
B (Non treated)	150	17 (17)	37 (38)	13 (13)	31 (32)		

* Values at P<0.05 are statistically significant; #Number in parenthesis indicates percentage.

4.3 Identification of *E. coli*

All the 150 water samples collected were inoculated into MacConkey broth and among them a total of 103 samples (69%) were found to be positive for coliform growth. The rest 47 samples did not show any growth in the MacConkey broth. The samples showing bacterial growth in the MacConkey broth were then inoculated onto EMB agar specific for *E. coli*. Among the isolates, 49 were positive in EMB agars (Figure 4). Then positive colonies from EMB agar were inoculated on MacConkey agar to obtain pure colonies and 40 samples were found positive via observing pink colonies on MacConkey agar (Figure 5). Finally, the positive colonies from MacConkey agar were subjected to indole test and 35 were revealed the positive result against *E. coli* (Figure 6).

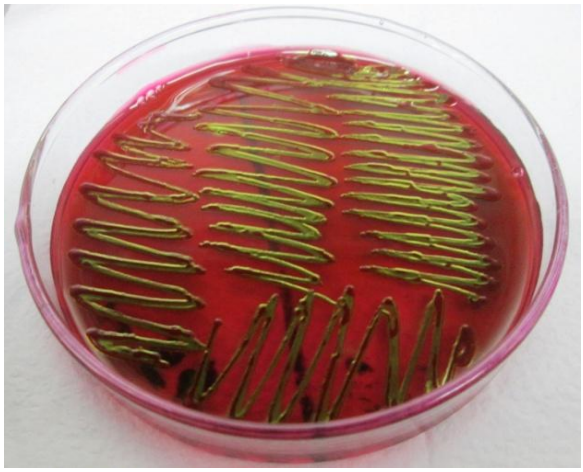

Figure 4 - Growth of *E. coli* on EMB agar

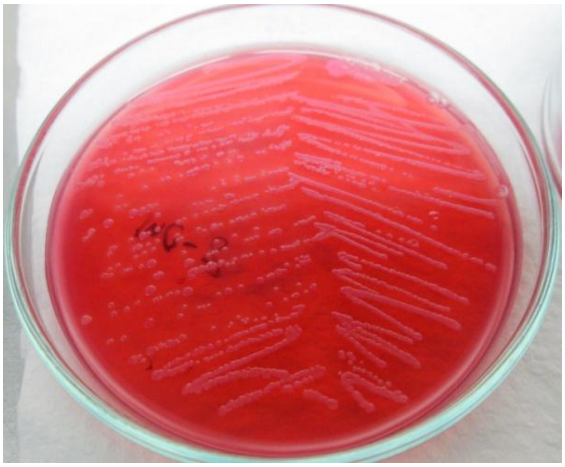

Figure 5 - Growth of *E. coli* on MacConkey agar

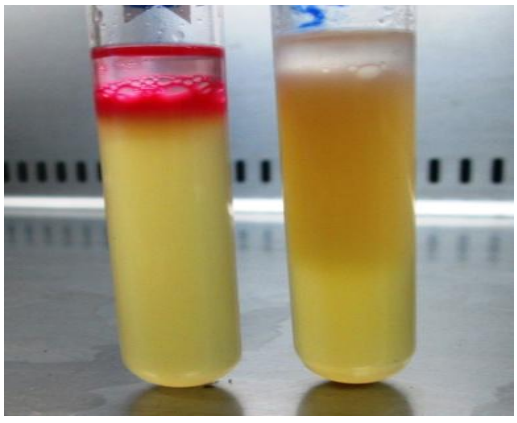

Figure 6 - Indole test for identification of *E. coli*

4.3.1 Prevalence of *E. coli* in water samples of different farms

We found similar prevalence of *E. coli* in Broiler and Layer farms which was 30% respectively (Table 15). In case of Breeder farms, only 5 samples were positive out of 50 and placed into lowest prevalence group (10%). A significant variation was observed in water samples of different farms.

Table 15 - Prevalence of *E. coli* in different farms.

Water Source	Total Number of Observation	*E. coli* (n) (%)#		Chi Square Value	P –Value*
		Positive	Negative		
Broiler	50	15 (30)	35 (70)		
Layer	50	15 (30)	35 (70)	7.4534	0.024
Breeder	50	5 (10)	45 (90)		

* Values at P<0.05 are statistically significant; #Number in parenthesis indicates percentage.

4.3.2 Prevalence of *E. coli* in different water sources

The groups of different water sources have been described earlier. The *E. coli* was prevailed in highest rate (31%) in group 4, whereas Group 1 samples revealed lowest (8%) prevalence of *E. coli*. However, no significant variation was found in *E. coli* prevalence at different water sources.

Table 16 - Water sources vs. Prevalence of *E. coli*

Water Source	Total Number of Observation	*E. coli* (n) (%)#		Chi Square Value	P –Value*
		Positive	Negative		
1	24	2 (8)	22 (92)		
2	39	7 (18)	32 (82)	5.7450	0.125
3	74	22 (30)	52 (70)		
4	13	4 (31)	9 (69)		

* Values at P<0.05 are statistically significant; #Number in parenthesis indicates percentage.

1= Tank; 2= Drinker; 3= Motor Pump and Tube well; 4= Dairy Farm, China setter, Pipe line and Drinking Water

4.4 Detection of virulence genes

For molecular detection from among the culturally and biochemically positive samples 35 isolates from each of either *E. coli* were selected for DNA extraction. PCR amplification of specific DNA fragments was then conducted using specific primer sets for shiga toxin producing *E. coli* (targeting *stx1* and *stx2* genes for *E. coli*). The PCR products were then run in 1% agarose gel with ethedium bromide, incorporating 100bp size markers and visualized under UV light.

However, none of the sample showed containing *stx1* and *stx2* genes (Figure 7).

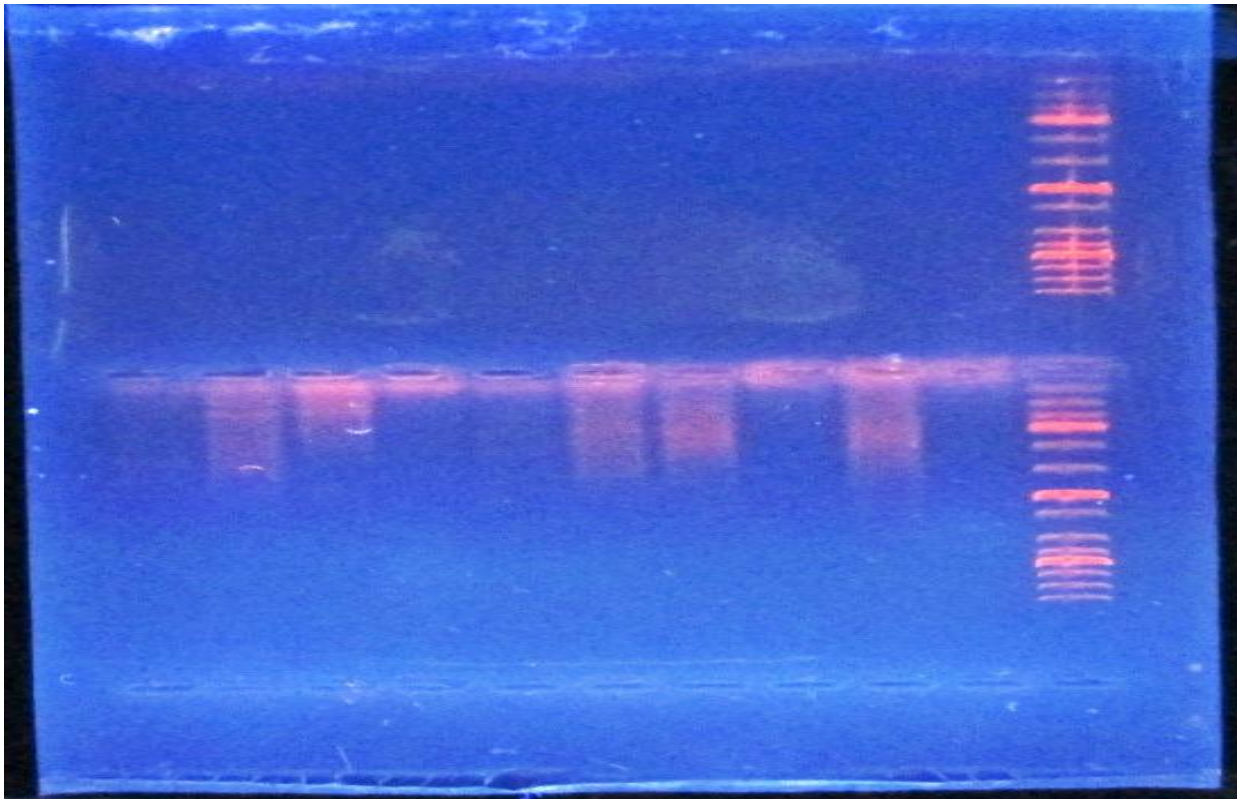

Figure 7 - Results of PCR for *stx1* and *stx2* genes of *E. coli*

4.5 Results of Antibiogram Study

All the 35 isolates were tested for susceptibility to 9 different antimicrobial agents. The frequencies of isolates showing sensitive, intermediately resistant and resistant to the antimicrobials tested are shown in Figure 8. Of the tested isolates 94.29% and 82.86% were sensitive to gentamicin and colistin sulphate, respectively; 94.29% isolates were resistant to tetracycline, 91.43% to ampicillin and 88.57% to amoxicillin.

More than 90% resistance observed in ampicillin and tetracycline. Very close to 90% (88.57%) resistance was shown in amoxicillin. The least percentage resistance was found in gentamicin. More than 80% sensitivity was found only with two antibiotics (gentamicin and colistin sulphate).

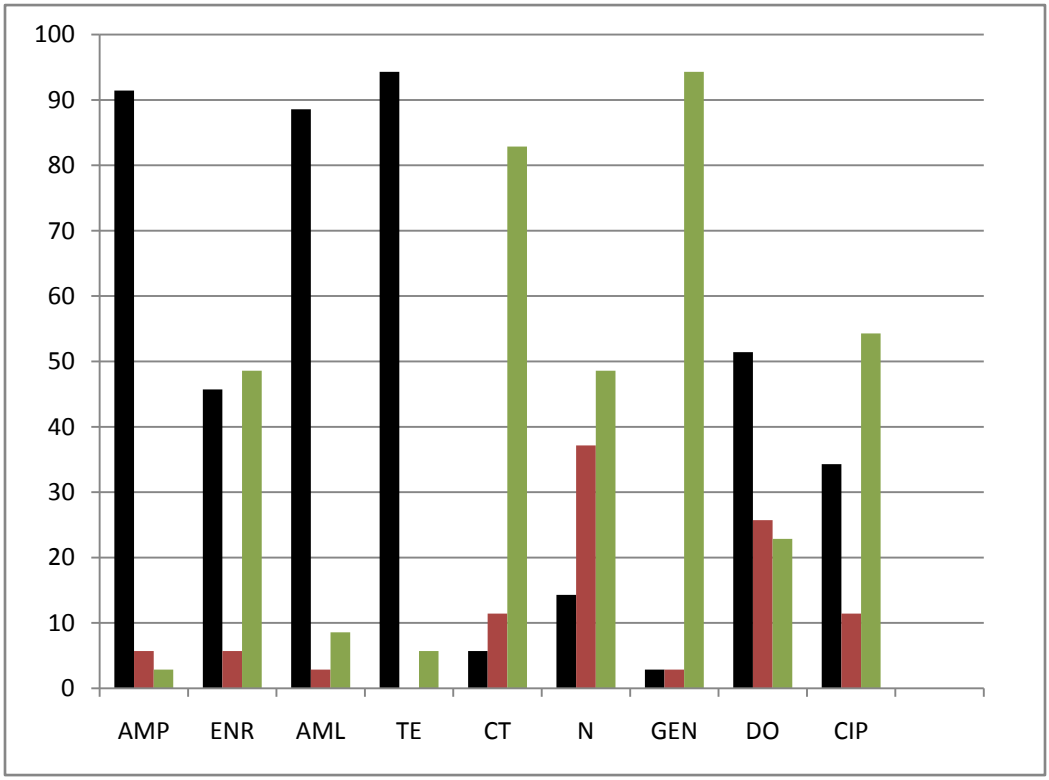

Figure 8 - Results of antimicrobial susceptibility testing; S, I, R = proportional representations of sensitive, intermediately-resistant and resistant isolates, respectively, against the antimicrobials tested; AML, Amoxicillin; CIP, Ciprofloxain; TE, Tetracyclin; AMP,Ampicillin; DO, Doxicyclin; GEN, Gentamicin; N, Norfloxacin; ENR, Enrofloxacin; CT, Colistin Sulphate.

Chapter V- Discussion

The results of the study revealed that, bacterial load was found in 103 out of total 150 water samples by observing the change in color of MacConkey broth and gas production after incubation. After culturing this positive samples into EMB agar, MacConkey agar and Indole test (biochemical), only 35 (15%) were found to be *E. coli* and rest were probably not *E. coli*. Coliform count (1001 and above) and *E. coli* load was found highest in broiler farms accounting 46% and 30%, respectively; whereas lowest (10%) was recorded in breeder farms.

Lowest pH value (4.02) was recorded in drinkers from different poultry farms. It might be due to using the same drinkers for large number of birds. Carter and Sneed (1996) stated that well water normally should have pH in the range from 6.8 to 7.8, although it is not uncommon for the pH to be either higher or lower which is almost similar with our present study (6.13 to 7.36). The water pH value was almost neutral in breeder farms. They treated water with chlorine before using to birds and it might be a reason for neutral pH value. There was a significant variation in MPN index with pH values in the current study. Water samples with lower pH group A (4-5) showed only 6% zero coliform counts which indicates high bacterial load, while the highest (42%) number of zero coliform count (0) was recorded in Group C (pH Value, 7-7-5) which indicates lower bacterial contamination. Number of coliforms above 1001 was found highest (60%) in group D (pH value, 7.6 and above) and lowest (14%) in group C (pH value, 7 – 7.5). It indicates that neutral water harbors less coliforms than acidic water. From the current study, a high contamination of fecal coliforms was found in water samples collected from drinkers as because only 23% samples showed no coliform. In contrast, water samples from Tanks; and Motor Pump and Tube well showed 42% and 36% zero coliform count, respectively. All water samples presented MPN values were higher than the international standards (Amaral *et al.*, 2001; Bettega *et al.*, 2006; Carter *et al.*, 2010). This contamination may be due to the use of bell drinkers. Studying the water quality in different types of drinkers, Barros *et al.* (2001) and Valias & Silva (2001) found that bell drinkers may reduce the biological quality of the water provided to broilers, posing a high risk of contamination. In our study, we found higher contamination in drinkers which is almost similar with the findings of the above researchers. And, higher contamination in drinkers in studied farms might be occurred via shedding of intestinal flora of

birds during watering. Or, due to using same drinker for large number of birds where water can get contaminated from sick birds.

As the hygienic status of drinking water varies greatly among farms, methods and strategies to identify critical contamination points need to be identified and updated, and measured to control water-borne diseases at farm level need to be applied. Work on water samples from open wells in Conakry was carried out by Gelinas *et al.* (1996) and reported widespread well water contamination for nitrate and fecal bacteria. Reason for this was linked to insufficient well maintenance, high permeability of the soil and shallow water table of the area sampled. Lewis *et al.* (1981) reported the pit latrine sanitation and shallow underground water exploitation for drinking purposes conflict one another because of the facilitated migration of contaminants, both bacteriological and chemicals, through the soil.

A significant difference found among bacterial load and farm locations. Bacterial load was found in almost all places, however the ranges are not same for all places. The highest (60%) percentage of samples with no bacterial load was observed in Zone 3 (Hathazari), in contrary only 20% samples were found positive with zero coliform count in Zone 2 (Pahatali) indicating a heavy bacterial contamination in zone 2. Interestingly, there were no water samples with number of coliform above 201 in case of Zone 1 (Raojan and Chandanaish). This might be due to influence of geographical locations on bacterial loads. Hassan *et al.* (2009) conducted a research on fifteen (15) different water sources in some rural communities of Zaria, Nigeria for microbial index of water quality in relation to seasonal influence and found that the microbial quality of the water sources located near point sources of fecal contamination (pit latrine, sanitary drainage) raised some points of concern particularly during the rainy season when the water sources become extensively contaminated. Whereas, Ferguson *et al.* (1996) studied the relationship between indicators and water quality in an estuarine system and associated rainfall and sewage overflows with significant increases in MPN index count. Though, we did not include this parameter in our study. But, it might have influence along with geographical locations in increased MPN index count.

Present study showed zero coliform count in Broiler farms, Layer farms and Breeder farms were 16%, 19% and 60% respectively which states the highest contamination of water in Broiler farms. Also, In case of coliform count (1001 and above), samples of broiler farms were

highly positive (46%). Koelkebeck *et al.* (1999) studied the effect of water quality using different water sources for layers, and the experimental results indicated that the quality of drinking water may greatly affect layer performance. Considering that water consumption (both daily and per cycle) is a key indicator of bird welfare, appropriate water supply and management is highly desirable (Manning *et al.*, 2007). Total bacteria and coliform bacteria concentrations should not exceed 100 and 50 colony-forming units/100 ml, respectively, in the drinking water of broilers (Carter *et al.*, 2010; Valias & Silva, 2001).

In our study, we found water treatment only practiced in Breeder farms and bacterial load was lowest (60%) there comparing with non-treatment water. In order to avoid diseases transmission, attention must be paid for the sources of infection inside farms and hygienic construction of such troughs and disinfection of drinking water in order to avoid diseases transmission (Draz *et al.*, 1996). In case of Broiler and Layer farms, we found same number of *E. coli* positive samples (30% for both), whereas lowest *E. coli* positive samples was found in Breeder farms (10%). Concerning *E. coli*, it was observed that the obtained incidence in case of broiler farms (10.2 %) was higher to that recorded by Sotohy *et al.* (1989) (9.19%), Basha *et al.* (1997) (10.93%) and Mohammed (2005) (11.43%), while it was similar with that recorded by Draz *et al.* (1996) (36.7 %), Abd El-Haleem (2000) (20.8 %), El-Zarka *et al.* (2003) (25%), and Shita *et al.* (2009) (21.3 %). Samaha *et al.* (2013) examined 25 water samples collected from broiler farms and layer farms respectively and found 10.2 % and 12.6 % respectively. But, in our study we found higher prevalence rate than this. It might be due to different biosecurity and management practices in the farms.

In case of Tank, only 8% samples were found positive for *E. coli*, whereas more positive isolates were found in motor China setter, pipeline, dairy farms and drinking water. Interestingly, no significant variation amount was found among the different water sources. This difference in *E. coli* percentage in water samples that collected from both broiler farms and layer farms may be due to the variety of watering systems as layer farms use nipple watering system versus automatic and manual drinkers in broiler farms which are exposed to contamination from birds and litter particles.

The identities of 35 bacterial isolates that yielded red color ring in Indole test were subjected to PCR to detect two genes, *stx1* and *stx2* (Shiga toxin producing), however none of the isolates

yielded any of the two genes. Exact cause is unknown. Whether the samples really did not have any shiga toxin producing gene (*stx1*, *stx2*) or it might be due to not optimization of PCR condition for detecting the genes. Due to time constraint, enough time was not found for optimizing PCR condition.

E. coli is able to acquire resistance easily; therefore it is a good bioindicator model for surveillance studies of antimicrobial resistance. Antimicrobial resistance testing was performed by disc diffusion method using 9 different antibiotics. For *E. coli*, doxicycline (25.71%) and norfloxacin (37.14%) showed intermediate sensitivity but, tetracycline, ampicillin, and amoxycillin showed 94.29%, 91.43% and 88.57% resistance respectively. This finding was supported by Cloud *et al.* (1985), Irwin *et al.* (1989), Blanco *et al.* (1997), Bass *et al.* (1999) and Li *et al.* (2007). For colistin sulphate and gentamicin the organism showed 94.29% and 82.86% sensitivity respectively. Nazir *et al.* (2005) and Hashem *et al.* (2012) also got similar result who reported *E. coli* to be highly sensitive to colistin sulphate (100%) and ciprofloxacin to be intermediately (50%) sensitive. However, Rahman *et al.* (2004) reported *E. coli* to be highly sensitive to ciprofloxacin but, they opined that it may be due to that ciprofloxacin was then newly introduced in the treatment of poultry in Bangladesh when the organism did not have enough time to grow resistance against the drug.

The major limitation of this study was time limit; seasonal variation was also not included. Secondly, the study was conducted with only limited number of samples. So, the study should also be performed at different location of Chittagong Division along with the consideration of total suspended solid (TSS), turbidity, seasonal and temperature variation.

Chapter VI- Conclusion

Total 103 out of 150 water samples were found positive with MPN index count. Samples from Broiler farms showed higher (46%) MPN index (1001 and above) than Layer and Breeder farms. The bacaterial load was more in Drinkers than other sources. And, there was a significant variation in MPN index count with water treatment and pH value. In case of lower pH value, the bacterial load was more comparing with higher pH values. Similar observation found in case of non-treated water where maximum bacterial load was counted. However, water treatment was only performed in Breeder farms in our current findings. Geographical locations were also associated with variation in bacterial load. Out of 103 positive samples from MPN, only 35 were found positive for *E. coli*. Among them, highest number of *E. coli* isolates (30% respectively) was obtained from Broiler and Layer farms. However, no significant variation was found in *E. coli* prevalence at different water sources. None of the isolates yielded any Shiga toxin producing gene (*stx1*, *stx2*). The *E. coli* isolates were susceptible to Colistin Sulphate and Gentamycin with an intermediate sensitivity to norfloxacin and doxycyline. The organisms showed 94.29% and 91.43% resistance to ampcillin and tetracycline, respectively. This knowledge of antibiotic sensitivity could be used in prescribing antibiotics for the treatment of colibacillosis in the Chittagong region. However, for detecting virulence genes of *E. coli* further work is needed for establishing a molecular diagnostic method.

Chapter VII - References

Allan BJ, van D, Hurk JV, Potter AA 1993: Characterization of Escherichia coli isolated from cases of avian colibacillosis. *Canadian Journal of Veterinary Research*. 57: 146-151.

Allen MJ, Edberg SC 1995: The public health significance of bacterial indicators in drinking water. *The Royal Society of Chemistry*. Special Publication. Athenaeum Press, UK.

Amaral LA 2004: Drinking Water as a Risk Factor to Poultry Health. *Brazilian Journal of Poultry Science*. 6: 191-199.

Amaral LA, Nader FA, Isa H, Barros LSS 2001: Qualidade higiênicosanit ária e demanda de cloro da água de dessedentação de galinhas de postura coletadas em bebedouros tipo nipple e taça. *Revista Brasileira de Ciência Avícola*. 3: 249-255.

Amaral LA, Rossi Junior OD, Cardoso V (1999). Qualidade higienicosanitária da água de bebedouros pendular e nipple utilizados na criação de frangos de corte. Revista Brasileira de Ciência Avícola. 1: 145-148.

APHA, AWWA, AEF 1998: Standard Methods for the Examination of Water and Wastewater (20th ed.), Washington, DC.

Armstrong GL, Hollingsworth J, Morris JG 1996: Emerging foodborne pathogens: Escherichia coli O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiologic Reviews*. 18: 29-51.

Association Franc aise de Normalisation (AFNOR) 1990: Eaux- me ´thodes d'essais. Recueil de Normes Franc aises (4th ed.) la De ´fense, Paris, pp: 735.

Badge US, Khan AM, Varma AK 1982: Influence of physiochemical factors of the coliform bacteria in closed lake water. *International Journal of Environmental Studies*. 18: 237-241.

Barnes HJ, Gross WB 1997: Colibacillosis in disease of poultry (10th ed.), Calnek BW, Barnes HJ, Beard CW, Mcdougald LR, Saif YM.eds., Iowa state university press , Ames, IA,USA, pp: 131-141.

Barros LSS, Amaral LA, Rossi Junior OD 2001: Aspectos microbiológicos e demanda de cloro de amostras de água de dessedentação de frangos de corte coletadas em bebedouros pendulares. *Revista Brasileira de Ciência Avícola*. 3: 193-198.

Basha OA 1997: Sources of contamination with certain pathogens inside poultry houses Ph.D. Thesis (Animal Hygiene). Faculty of Veterinary Medicine, Alexandria University.

Bass L, Liebert CA, Lee MD, Summers AO, White DG, Thayer SG, Maurer JJ 1999: Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 43: 2925-2929.

Bauer AW, Kirby WMM, Shrris JC, Truck M 1966: Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology*. 45: 493-496.

Bej AAK, Ng WWY, Morgan SS, Jones DDD, Mahbubani MMH 1996: Detection of viable *Vibrio cholerae* by reverse-transcriptase polymerase chain reaction (RT–PCR). *Molecular Biotechnology*. 5: 1-10.

Bettega JMPR, Machado RM, Presibella M, Baniski G, Almeida, Barbosa CA 2006: Analytical methods for water microbiological control for human consumption. *Ciência Agrotecnica*. 30: 950-954.

Blanco JE, Blanco M, Mora A, Blanco J 1997: Prevalence of bacterial resistance to quinolones and other antimicrobials among avian *Escherichia coli* strains isolated from septicemic and healthy chickens in Spain. *Journal of Clinical Microbiology*. 35: 2184-2185.

Brasil 1986: Conselho Nacional do Meio Ambiente. Conama. Diario Oficial da União de.

Cabral JPS 2010: Water microbiology: bacterial pathogens and water. *International Journal of Environmental Research and Public Health*. 7: 3657-3703.

Candrian UB, Furrer C, Hofelein R, Meyer M, Jermini, Luthy J 1991: Detection of Escherichia coli and identification of enterotpxigenic strains by primerdirected enzymatic amplification of specific sequences. *International Journal of Food Microbiology*. 1239-352.

Carter GR, Cole JRJ (1990). Diagnostic procedure in Veterinary Bacteriology and Mycology (5th ed.), Academic press Inc., Harcourt, Brace Jovanovich, Publishers, New York.

Carter TA, Sneed RE 1996: Drinking water quality for poultry. Published by: North Carolina Cooperative Extension Service, Available at: http://www.bae.ncsu.edu/programs/ extension/evans/ps&t-42.html (accessed on 24 November, 2014).

Carter TA; Ronald E. Sneed RE 2010: Drinking water quality for poultry. Available from: http://www.ces.ncsu.edu/depts/ poulsci/tech_manuals/drinking_water_quality.html (accessed on 24 November, 2014).

Chansiripornchai N, Ramasoota P, Sasipreyajan J, Svenson SB 2001: Differentiation of avian *Escherichia coli* (APEC) isolates by random amplified polymorphic DNA (RAPD) analysis. *Veterinary Microbiology*. 80: 77-83.

Cheesbough M 1985: Medical Laboratory Manual for Tropical countries. *Microbiology*. 3: 400-480.

Cheesbrough M 2006: Medical laboratory manual for tropical countries (2nd ed.), Microbiology, London, United Kingdom, pp: 400-480.

Clermont O, Johnson JR, Menard M, Denamur E 2007: Determination of the *Escherichia coli* O types by allele specific polymerase chain reaction, application to the O types involved in human septicemia. *Diagnostic Microbiology and Infectious Diseases*. 57: 129-136.

Clinical and Laboratory Standards Institute (CLSI) 2007: Performance standards for antimicrobial susceptibility testing. 17th Informational Supplement document M100-S17: 1, Wayne, Pennsyslvania, pp: 32-50.

Cloud SS, Rosenberger JK, Fries PA, Wilson RA, Odor EM 1985: In vitro and in vivo characterization of avian *Escherichia coli*. I. Serotypes, metabolic activity, and antibiotic sensitivity. *Avian Diseases*. 29: 1084-1093.

Coia JE 1998: Clinical, microbiological and epidemiological aspects of *Escherichia coli* O157 infection. *FEMS Immunology and Medical Microbiology*. 20: 1-9.

Cooper JE, Feil EJ 2004: Multilocus Sequence typing-what is resolved. *Trends in Microbiology*. 12: 373-377.

Damron BL, Flunker K 1993: Broiler chick and laying hen tolerance to sodium hypochlorite in drinking water. *Poultry Science*, 72: 1650-1655.

DesRosiers A, Fairbrother JM, Johnson RP, Desautels C, Letellier A, Quessy S 2001: Phenotypic and genotypic characterization of Escherichia coli verotoxin-producing isolates from humans and pigs, *Journal of Food Protection*. 64: 1904-1911.

Dho-Moulin M, Fairbrother JM 1999: Avian pathogenic *Escherichia coli* (APEC). *Veterinary Research*. 30: 299-316.

Dragas AZ, Tratnik M 1975: On the value of examination of drinking water and swimming pools for the presence of entero-pathogenic *E. coli*. *Microbial Abstract*. 10:10878.

Draz AA, El-Gohary AH, Samaha HA 1996: Environmental pollution with certain bacterial pathogens of zoonotic importance in some poultry farms.7th Scientific Congress, Faculty of Veterinary Medicine, Assuit University, Assuit, Egypt, 63-70.

Dubel JR., Zink DL, Kelley LM, Naqi SA, Renshaw HW 1982: Bacterial antibiotic resistance: frequency of gentamicin-resistant strains of *Escherichia coli* in the fecal microflora of commercial turkeys. *American Journal of Veterinary Research*. 43: 1786-1789.

Dufour AP 1977: *Escherichia coli*: the fecal coliform. ASTM Special Technical Publication 635, Philadelphia, pp: 48-58.

Edberg SC, gall O, Kontnick C 1996: Analysis of the virulence characteristics of bacteria isolated from bottled, water cooler and tap Water. *Microbial Ecology in Health and Disease*. 9: 67-77.

El-Haleem AYF 2000: Some epidemiological studies on *E. coli* in poultry farms. M.V.Sc Thesis, (Animal Hygiene), Faculty of Veterinary Medicine, Zagazeg University.

Elkins R, Chu FW 1999: Microarrays: their origins and applications. *Tibtech*. 17: 217-218.

El-Zarka RS 2003: The role of environment inside poultry houses in disease occurrence. Ph.D. Thesis (Animal Hygiene), Faculty of Veterinary Medicine, Alexandria University.

Englert S 1998: Produção de frangos de corte. In: Englert, S. Avicultura: tudo sobre raças, manejo e alimentação (7th ed.), Guaíba: Agropecuária, pp: 94-151.

Escherich T 1988: The intestinal bacteria of the neonate and breast-fed infant. *Reviews of Infectious Diseases*. 10: 1220-5.

Evans TM, Waarvick CE, Seidler RJ, LeChevallier MW 1981: Failure of the most-probable-number technique to detect coliforms in drinking water and raw water supplies. *Applied and Environmental Microbiology*. 41: 130-138.

Faith NG, Shere JA, Brosch R, Arnold KW, Ansay SE, Lee MS, Luchansky JB, Kaspar CW 1996: Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. *Applied and Environmental Microbiology*. 62: 1519-1525.

Ferguson CM, Coote BG, Ashbolt NJ, Sterasim IM 1996: Relationship between Indicators, Pathology and Water Quality in an Estuarine System, *Water Research*. 30: 2045-2054.

Fuchs BM, Wallner G, Beisker W, Schwippl I, Ludwig W, Amann R 1998: Flow cytometric analysis of the in situ accessibility of Escherichia coli 16S rRNA for fluorescently labelled oligonucleotide probes. *Applied and Environmental Microbiology*. 64: 4973-4982.

Gama MSQ 1995: Água, que cura, que nutre, que mata. Aves & Ovos, pp: 30-33.

Gelinas Y, Randall H, Robidoux L, Schmit J 1996: Well Water Survey in two Districts of Conackry (Republic of Guinea) and Comparison with the Pipe City Water. *Water Research*. 30: 2017-2026.

Georging RV 2010: Pulse field gel electrophoresis: A review of Application and investigation in the molecular epidemiology of infectious diseases. *Infection Genetics and Evolution*. 10: 866-875.

Gordon DM 2010: Strain typing and the Ecological structure of *Escherichia coli*. J*ournal of AOAC International*. 93: 974-984.

Grabow WOK, Preez M 1979: Comparison of m-Endo LES, MacConkey, and Teepol media for membrane filtration counting of total coliform bacteria in water. *Applied and Environmental Microbiology*. 38: 351-358.

Hadrys H, Balick M, Schiewater B 1992: Application of random amplified polymorphic DNA(RAPD) in molecular ecology. *Molecular Ecology*. 1: 55-63.

Hairston JE 1995: Water Quality: Managing Drinking Water Quality. Agriculture and Natural Resources. *The Alabama Cooperative Extension System*. ANR-790-2.6.

Halls AE 2008: Water Quality For Poultry. Nutreco Canada Inc.

Hancock DD, Besser TE, Rice DH, Ebel ED, Herriott DE, Carpenter LV 1998: Multiple sources of *Escherichia coli* O157 in feedlots and dairy farms in the northwestern USA. *Preventive Veterinary Medicine*. 35: 11-19.

Harry EG 1964: The survival of *E. coli* in the dust of poultry houses. *Veterinary Record*. 76: 466-470.

Hasan B, Faraque R., Drobni M, WaldenStrom J, Sadique A, Ahmed KU, Islam Z,Parvez MB, Olsen B, Alam M 2011: High prevalence of antibiotic resistance in pathogenic *Escherichia coli* from large and small scale poultry farms in Bangladesh. *Avian Diseases*. 55: 689-692.

Hashem MA, Elahi MF, Mannan MA, Mannan MF, Kabir MHB, Kashem MA, Pallab MS 2012: Isolation, identification and Antibiogram of *Escherichia coli* from broiler at Chittagong district Bangladesh. *Wayamba Journal of Animal Science*. 312-316.

Hassan AS 2009: Seasonal influence on Microbial Quality of water sources in some rural communities of Zaria, Nigeria. *Science World Journal*. 4: 23-27.

Heijnen L, Medema G 2006: Quantitative detection of *E. coli*, *E. coli* O157 and other shiga toxin producing *E. coli* in water samples using a culture method combined with real-time PCR. *Journal of Water Health*. 4: 487-498.

Hernandes R, Cazetta JO, Moraes VMB 2002: Frações nitrogenadas, glicídicas e ammonia liberada pela cama de frangos de corte em diferentes densidades e tempos de confinamento. *Revista Brasileira de Zootecnia*. 31: 1795-1802.

Hernandes R, Cazetta JO, Moraes VMB 2002: Frações nitrogenadas, glicídicas e ammonia liberada pela cama de frangos de corte em diferentes densidades e tempos de confinamento. *Revista Brasileira de Zootecnia*. 31: 1795-1802.

Irwin RJ, McEwen SA, Clarke RC, Meek AH 1989: The prevalence of verocytotoxin-producing *Escherichia coli* and antimicrobial resistance patterns of nonverocytotoxin-producing *Escherichia coli* and *Salmonella* in Ontario broiler chickens. *Canadian Journal of Veterinary Research*. 53: 411-418.

Islam S, Begum HA, Nili NY 2010: Bacteriological safety assessment of municipal tap water and quality of bottle water in Dhaka City: health hazard analysis. *Bangladesh Journal of Medical Microbiology*. 4: 9-13.

Jacobs MB, Gerstein MJ, Nostrand, Priceton NJ 1960: Handbook of Microbiology (6^{th} ed.). Princeton, NJ: Van Nostrand.

James BK, James PN, Harry LTM 2004: Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*. 2: 123-140.

Jefrey JS 2000: Sanitation-Disinfection. Basics for Poultry Flocks. Davis: University of California, Veterinary Medicine Extension. Available at: http:// www.vetmed.ucdavis. edu/vetex/Home.html (accessed on November 24, 2014).

Johnson J, Kuskowski M, Menard M, Gajewski A, Xercavins M, Garau J 2006: Similarity between human and chicken *Escherichia coli* isolates in relation to ciprofloxacin resistance status. *Journal of Infectious Diseases*. 194: 71–80.

Johnson JYM, Thomas JE, Graham TA, Townshend I, Byrne J, Selinger LB, Gannon VPJ 2003: Prevalence of *Escherichia coli* 0157:H7 *Salmonella spp.* in surface waters of southern Alberta and its relation to manure sources. *Canadian Journal of Microbiology*. 49: 326-335.

Kaper JB 2005: Pathogenic *Escherichia coli*. *International Journal of Medical Microbiology*. 295: 355-6.

Kaper JB, Nataro JP, Mobley HL 2004: Pathogenic *Escherichia coli*. *Nature Reviews Microbiology.* 2: 123-40.

Karn SK, Harada H 2001: Surface water pollution in three urban territories of Nepal, India and Bangladesh. *Environmental Management*. 28: 483 - 96.

Koelkebeck KW, McKee JS, Harrison PC, Parsons CM 1999: Performance of laying hens provided water from two sources. *Journal of Applied Poultry Research*. 8: 374-379.

Koelkebeck KW, McKee Valias APGS, Silva EN 2001: Comparative study of systems of drinkers in the microbiological quality of the water consumed by broiler chickens. *Revista Brasileira de Ciência Avícola*. 3: 83-89.

Kroger E, Noll J 1969: Result of well water examination in a second marsh community. *Microbial Abstract.* 39: 4445-4453.

Lejeune J, Besser TE, Hancock DD 2001: Cattle water troughs as reservoirs of *Escherichia coli* O157. *Applied and Environmental Microbiology*. 67: 3053-3057.

Lewis WJ, Foster SSD, Read GH, Scertenleib R 1981: The need for an integrated approach to Water Supply and Sanitation in Developing Countries. *Science Total Environment*. 21: 53-59.

Li L 2009: Clean drinking water is crucial in enhancing animal productivity. Proceedings of the 17th Annual ASAIM SEA Feed Technology and Nutrition Workshop, Vietnam. pp: 1-6.

Li XS, Wang GQ, Du XD, Cui BA, Zhang SM, Shen JZ 2007: Antimicrobial susceptibility and molecular detection of chloramphenicol and florfenicol resistance among *Escherichia coli* isolates from diseased chickens. *Journal of Veterinary Science.* 8: 243–247.

Lília P, Lisete F, Catarina M, Isabel G 2007: Adaptive Mutations in Bacteria: High Rate and Small Effects. *Science*. 317: 813–815.

Lin S, Evans RL, Beuscher DB 1974: Bacteriological assessment of spoon river water quality. *Applied Microbiology*. 28: 288-297.

Macari M 1997: Qualidade da água e bebouros para frangos de corte: tipos, vantagens e desvantagens. In: Conferência Apinco'97 de Ciência e Tecnologia Avícolas, São Paulo. Brasil. *Campinas: Facta*. pp: 121-143.

Malaney GW, Weiser HH 1962: Coliform, enterococci, thermodurics, thermophiles and psychrophiles in untreated farm pond waters. *Applied Microbiology*. 10: 44-51.

Manna SK, Brahmane MP, Das R, Manna C, Batabyal S 2006: Detection of *Escherichia coli* O157 in foods of animal origin by culture and multiplex polymerase chain reaction. *Journal of Food Science and Technology*. 43: 77-79.

Manning L, Chadd SA, Baines RN 2007: Key health and welfare indicators for broiler production. *World's Poultry Science Journal*. 63: 46-62.

McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB 1995: A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of National Academy of Sciences*. 92: 1664-1668.

McFeters GA, Cameron SC, LeChevallier MW 1982: Influence of diluents, media, and membrane filters on detection of injured waterborne coliform bacteria. *Applied and Environmental Microbiology*. 43: 97-103.

Means EG, Olson BH 1981: Coliform inhibition by bacteriocin- like substances in drinking water distribution systems. *Applied and Environmental Microbiology*. 42: 506-512.

Meier H, Koob C, Ludwig W, Amann R, Frahm E, Hoffmann S, Obst U, Schleifer, KH 1997: Detection of enterococci with rRNA targeted DNA probes and their use for hygienic drinking water control. *Water Science and Technology*. 35: 437-444.

Melita S, Nicholas A, David C 2003: Review of Coliforms As Microbial indicators of drinking water quality. Production by Biotext Pty Ltd, Canberra, National health and medical research council.

Meza H 1989: Controle de qualidade na produção de frangos de corte. *Avicultura & Suinocultura Industrial*, 80: 38-44.

Mieres RL, Bastardo JW 1975: Enterobacteria in the waters of the river Manzanares at cumana (Venezuela). *Microbial Abstract*. 10: 11822.

Mohamed AMH 2005: Evaluation of some disinfectants on microbial contents of air and water in some poultry farms. Ph.D. Thesis, (Animal Hygiene), Faculty of Veterinary Medicine, Alexandria University.

Moura AC, Irino K, Vidotto MC 2001: Genetic variability of avian *Escherichia coli* strains evaluated by enterobacterial repetitive intergenic consensus and repetitive extragenic palindromic polymerase chain reaction. *Avian Diseases*. 45: 173-181.

Nadine B, Marc H, Nancy R, Lieve H 2003: Detection and characterization of verotoxigenic *E. coli* by a VTEC/EHEC multiplex PCR in porcine feces and pig carcass swabs. *Research in Microbiology*. 154: 97-104.

Nataro JP, Kaper JB 1998: Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*. 11: 142–201.

National Research Council 1994: Nutrient Requirements of Poultry (9th ed.), National Academy Press, Washington, DC.

Nazir KHMNH, Rahman MB, Nasiruddin KM, Akhtar F, Khan MFR, Islam MS 2005: Antibiotic sensitivity of *E. coli* isolated from water and its relation with plasmid profile analysis. *Pakistan Journal of Biological Science*. 8: 1610-1613.

Norhan KAE, Ibrahim EE, Ahmed MA, Sabry IE, Yousreya HM 2014: Molecular studies on *M. gallisepticum* and avian pathogenic *E. coli* induced infections in broilers. *European Journal of Veterinary Medicine*. 2014: 4.

Okagbue RN, Dlamini NR, Siwela M, Mpofu F 2002: Microbiological quality of water processed and bottled in Zimbabwe. *African Journal of Health Science*. 9: 99-103.

Oliveira MC, Carvalho ID 2002: Rendimento e lesões em carcaças de frangos de corte criadosem diferentes camas densidadespopulacionais. *Ciência Agrotécnica*. 26: 1076-1081.

Olkowski A (2009). Livestock Water Quality, A Field Guide for Cattle, Horses, Poultry, and Swine. *Canadian Council of Ministers of the Environment*.

Organisation for Economic Co-operation and Development (OECD) 2003: Assessing Microbial Safety of Drinking Water. *WHO*, ISBN 92-94-09946-8.

Patel R, Piper KE, Rouse MS, Steckelberg JM, Uhl JR, Kohner P, Hopkins MK, Cockerill FR, Kline BC 1998: Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. *Journal of Clinical Microbiology*. 36: 3399-3407.

Paton JC, Paton AW 1998: Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clinical Microbiology Reviews*. 11: 450–79.

Pourbakhsh SA, Boulianne M, Martineau-Doizé B, Dozois CM, Desautels C, Fairbrother JM 1997: Dynamics of *Escherichia coli* infection in experimentally inoculated chickens. *Avian Diseases*. 41: 221-233.

Rahman MA, Samad MA, Rahman MB, Kabir SML 2004: In vitro antibiotic sensitivity and therapeutic efficacy of experimental *Salmonellosis, Colibacillosis* and *Pasteurellosis* in broiler chickens. *Bangladesh Journal of Veterinary Medicine*. 2: 99–102.

Reddy MR, Raju MVLN, Chawak MM, Rama Rao SV 1995: Importance of water in poultry health. *Poultry Adviser*. 28: 31-37.

Renter DG, Sargeant JM, Oberst RD, Samadpour M 2003: Diversity, frequency, and persistence of *Escherichia coli* O157 strains from range cattle environments. *Applied and Environmental Microbiology*. 69: 542-547.

Rice EW, Fox KR, Nash HD, Read EJ, Smith AP 1987: Comparison of media for recovery of total coliform bacteria from chemically treated water. *Applied and Environmental Microbiology*. 53: 1571-1573.

Riley LW 2004: Distinguishing Pathovars from nonpathovars: *Escherichia coli* Molecular Epidemiology of infectious Diseases Principles and Practices. Washington, DC: ASM press.

Salyers AA, Gupta A, Wang Y 2004: Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology*. 12: 412-6.

Samaha HA, Haggag YN, Nossair MA, Donia GR, Heba F 2013: Microbiological evaluation of water used in different animal farms. *Alexandria Journal of Veterinary Sciences*. 39: 82-90.

Santos EC, Cotta JTB, Muniz JA, Fonseca RA, Torres DM 2000: Avaliação de alguns materiais usados como cama sobre o desempenho de frangos de corte. *Ciência Agrotécnica*. 14: 1024-1030.

Sargeant JM, Sanderson MJ, Griffin DD, Smith RA 2004: Factors associated with the presence of *Escherichia coli* O157 in feedlot–cattle water and feed in the Midwestern USA. *Preventive Veterinary Medicine*. 66: 207-237.

Scheutz F, Strockbine NA 2005: Genus I. *Escherichia*. In: Brenner DJ, *et al.* (Eds.) The Protebacteria Part B The Gammaproteobacteria. Springer. *Bergey's Manual of Systematic Bacteriology*. 2: 607-623.

Schwartz DL 1984: "Water Quality," VSE, 81c., Penn. State University (mimeographed).

Seidler RJ, Evans TM, Kaufman JR, Warvick CE, LeCheva- lier MW 1981: Limitations of standard coliform enumeration techniques. *Journal of American Water Works Association*. 73: 538-542.

Shere JA, Kaspar CW, Bartlett KJ, LindenSE, Norell B, Francey S, Schaefer DM 2002: Shedding of *Escherichia coli* O157:H7 in dairy cattle housed in a confined environment

following waterborne inoculation. *Applied and Environmental Microbiology*. 68: 1947-1954.

Shita HMA 2009: Epidemiological Studies on Some Pathogenic Bacteria inside Poultry Farms. M.V.Sc. Thesis (animal hygiene), Faculty of Veterinary Medicine, Alexandria University, Egypt.

Silva EN 1998: Passo a passo contra *Escherichia coli*. *Avicultura Industrial*. 88: 24-27.

Sotohy AS 1989: Hygienic significance of some microbial isolates from broiler houses. M. V. Sc. Thesis, Veterinary Hygiene, Assuit University.

Stevens M, Ashbolt M, Cunliffe D 2003: Recommendations to change the use of coliforms as microbial indicators of drinking water quality. National Health and Medical Research Council, Australian Government.

Sultana T, Rahman HM, Ara N, Mamun SH, Hasan NM 2009: Bacteriological quality of drinking water supplies in Khulna city, Bangladesh. *Journal of Innovation and Development Strategy*. 3: 31-34.

Thomas A, Carter, Sneed RE 1996: Drinking wtarer quality for poutry. North Carolina Cooperative Extension Service, http://www.ces.ncsu.edu/depts/poulsci/tech_manuals/drinking_water_quality.html (accessed on Nov 20, 2014).

Tsai S, Schade JE, Molyneux BT 1992: Chlorination of poultry chiller water: chlorine demand and disinfection efficiency. *Poultry Science*. 71: 188-196.

Valias APGS, Silva EN 2001: Comparative study of systems of drinkers in the microbiological quality of the water consumed by broiler chickens. *Revista Brasileira de Ciência Avícola*. 3: 83-89.

Van D, Bogaard AE, London N, Driessen C, Stobberingh EE 2001: Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *Journal of Antimicrobial Chemotherapy*. 47: 763-77.

Van DJ, Berg J, Potter A, Hancock D, Besser T, Rice D, LeJeune J, Klashinsky S 2001: Environmental sources and transmission of *Escherichia coli* O157 in feedlot cattle. *Canadian Veterinary Journal*. 42: 714-720.

VetBakt 2007: *Escherichia coli*. Available from: http://www.vetbakt.se (accessed on Nov 16, 2014).

Waggoner R, Good R 1984: Water Quality and Poultry Performance. In Proceedings AVMA Annual Conference.

White DG, Piddock LJ, Maurer JJ, Zhao S, Ricci V, Thayer SG 2000: Characterization of fluoroquinolone resistance among veterinary isolates of avian *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 44: 2897-2899.

Williams JGK, Kubelik AR, Livak KJ, Rafolski JA, Tingey SV 1990: DNA Polymorphisms amplified by arbitrary Primers are useful as genetic markers. *Nucleic Acid Research*. 18: 6531-6535.

Wooley RE, Spears KR, Brown J, Nolan LK, Dekich MA 1992: Characteristics of conjugative R plasmids from pathogenic avian *Escherichia coli*. *Avian Diseases*. 36: 348-352.

Zhao S, White DG, McDermott PF, Friedman S, English L, Ayers S, Meng J, Maurer JJ, Holland R, Walker RD 2001: Identification and expression of cephamycinase blacmy genes in *Escherichia coli* and *Salmonella* isolated from food animals and ground meats. *Antimicrobial Agents and Chemotherapy*. 45: 3647-3650.

ANNEX 1

Questionnaire relating to water sample collection to detect *E. coli*

Sample ID	……
Farm Name	……
Location of the farm	……
Total Bird	……
Bird type	…….
Source of Water	……
Washing Frequency of Tank or Pipeline	……
Water treatment with bleaching powder	……
Previous history of Colibacillosis in Farm	……
Information about recent anitibiotic use	……
pH value of the sample	……
Additional Comment	……

ANNEX 2

Most Probable Number per 100 ml using three tubes each inoculated with 10, 1.0 and 0.1ml of sample.

Tubes Positive			M.P.N	Tubes Positive			M.P.N	Tubes Positive			M.P.N
10 ml	**1.0 ml**	**0.1 ml**		**10 ml**	**1.0 ml**	**0.1 ml**		**10 ml**	**1.0 ml**	**0.1 ml**	
0	0	1	**3**	1	2	0	**11**	2	3	3	**53**
0	0	2	**6**	1	2	1	**15**	3	0	0	**23**
0	0	3	**9**	1	2	2	**20**	3	0	1	**39**
0	1	0	**3**	1	2	3	**24**	3	0	2	**64**
0	1	1	**6**	1	3	0	**16**	3	0	3	**95**
0	1	2	**9**	1	3	1	**20**	3	1	0	**43**
0	1	3	**12**	1	3	2	**14**	3	1	1	**75**
0	2	0	**6**	1	3	3	**29**	3	1	2	**120**
0	2	1	**9**	2	0	0	**9**	3	1	3	**160**
0	2	2	**12**	2	0	1	**14**	3	2	0	**93**
0	2	3	**16**	2	0	2	**20**	3	2	1	**150**
0	3	0	**9**	2	0	3	**26**	3	2	2	**210**
0	3	1	**13**	2	1	0	**15**	3	2	3	**290**
0	3	2	**16**	2	1	1	**20**	3	3	0	**240**
0	3	3	**19**	2	1	2	**27**	3	3	1	**460**
1	0	0	**4**	2	1	3	**34**	3	3	2	**1100**
1	0	1	**7**	2	2	0	**21**	3	3	3	**1100+**
1	0	2	**11**	2	2	1	**28**				
1	0	3	**15**	2	2	2	**35**				
1	1	0	**7**	2	2	3	**42**				
1	1	1	**11**	2	3	0	**29**				
1	1	2	**15**	2	3	1	**36**				
1	1	3	**19**	2	3	2	**44**				

From Jacobs and Gerstein's Handbook of microbiology, csopyright, 1960