ANTIBIOBIOTIC SENSITIVITY PROFILING OF *ESCHERICHIA COLI* AND *SALMONELLA* SPECIES ISOLATED FROM PIGEON: A PUBLIC HEALTH CONCERN

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This is to certify that the thesis entitled "ANTIBIOBIOTIC SENSITIVITY PROFILING OF ESCHERICHIA COLI AND SALMONELLA SPECIES ISOLATED FROM PIGEON: A PUBLIC HEALTH CONCERN" submitted to the department of MICROBIOLOGY AND PARASITOLOGY, faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Sher-e-bangla nagar, Dhaka-1207, in partial fulfillment of the requirements for the degree of ***Master of Science (MS) in MICROBIOLOGY****, embodies the result of a piece of bona fide research work carried out by* ***SHAH JUNGY IBNA KARIM****, Registration No.:* ***12-04957****, under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

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DEDICATED TO
MY BELOVED PARENTS

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TABLE OF CONTENTS

TITLE	PAGE NO.
ACKNOWLEDGEMENT	**i**
LIST OF CONTENTS	**ii-v**
LIST OF TABLES	**vi-vii**
LIST OF FIGURES	**vii-viii**
LIST OF APPENDICES	**ix**
LIST OF ABBREVIATION AND SYMBOLS	**ix-xi**
ABSTRACT	**xii**

LIST OF CONTENTS

NO. OF CHAPTER	TITLE NAME	PAGE NO
CHAPTER 1	**INTRODUCTION**	**1-4**
CHAPTER 2	**REVIEW OF LITERATURE**	**5-12**
2.1	Domestic & Feral Pigeon	5
2.2	Microorganisms and Birds	5-7
2.3	Bacterial Disease	7-8
2.4	Antibiotic Resistance	9
2.5	Mechanism and origin of antibiotic resistance	9-10
2.6	Antibiotic resistance in bacterial pathogens	10-11
2.7	*Escherichia coli* as the model for antibiotic resistance studies	11
2.8	Resistance gene transfer from *E. coli* of poultry and pork to human	11-12
CHAPTER 3	**MATERIALS AND METHODS**	**13-29**
3.1	Materials	13
3.1.1	Samples (Oral and Cloacal swab from pigeon)	13-14
3.1.2	Bacteriological media	14
3.1.2.1	Agar media	14
3.1.2.2	Liquid media (broth)	14
3.1.2.3	Phosphate buffer saline (PBS)	14-15
3.1.3	Chemicals and reagents	15

LIST OF CONTENT (CONT'D)

NO. OF CHAPTER	TITLE NAME	PAGE NO
3.1.4	Glass wares and other appliances	15
3.1.5	Antimicrobial discs	15
3.2	Methods	16
3.2.1	Brief description of the experimental design	16-17
3.2.2	Preparation of various bacteriological culture media	18
3.2.2.1	Nutrient Agar	18
3.2.2.2	Nutrient Broth	18
3.2.2.3	MacConkey's agar	18
3.2.2.4	Eosine Methylene Blue (EMB) agar	18
3.2.2.5	Brilliant Green agar	19
3.2.2.6	Salmonella-Shigella agar	19
3.2.2.7	Mueller Hinton Agar	19
3.2.2.8	Triple sugar iron (TSI) agar	19-20
3.2.2.9	Methyl Red and Voges–Proskauer (MR-VP) broth	20
3.2.2.10	Sugar solutions	20
3.2.3	Isolation of bacteria harboring in pigeons	20
3.2.3.1	Collection, transportation and preparation of sample	20
3.2.3.2	Serial dilution for bacterial culture (10 fold dilution method)	21
3.2.3.3	Primary culture of microorganism	21
3.2.3.4	Isolation in culture media	21-22
3.2.3.5	Microscopic study for identification of *E. coli* & *Salmonella* spp. from the suspected colonies by Gram's staining method	22
3.2.3.6	Motility test for *E. coli* & *Salmonella* spp. isolates	22
3.2.3.7	Reaction of the organism in TSI agar slant	23
3.2.4	Identification of isolated *E. coli* by using specific biochemical tests	23
3.2.4.1	Carbohydrate fermentation test	23
3.2.4.2	Catalase test	24
3.2.4.3	Methyl Red test	24

LIST OF CONTENT (CONT'D)

NO. OF CHAPTER	TITLE NAME	PAGE NO
3.2.4.4	Voges-Proskauer (V-P) test	24
3.2.4.5	Indole test	24
3.2.4.6	Molecular Identification of the isolated organism (*Escherichia coli*)	25
3.2.4.7	Bacterial DNA isolation (a) Materials , b) Procedure , c) Polymerase Chain Reaction (PCR) , d) Primer used for PCR , e) Principle of PCR , f) Preparation for a PCR mixture , g) Thermal profile , h) Electrophoresis)	25-28
3.2.7	Maintenance of stock culture	28
3.2.8	Antimicrobial sensitivity pattern of *E. coli* and *Salmonella* spp. isolated from Oral and Cloacal samples of pigeon	28-29
CHAPTER 4	**RESULTS AND DISCUSSION**	**30-60**
4.1	Total Viable Count and Total Coliform Count from the isolated sample	30-31
4.2	Prevalence of microorganism in pigeon	31
4.3	Percentage of Prevalence with specific organism from sample	31
4.4	Prevalence percentage of *E.coli* & *Salmonella* spp. in oral (20) & cloacal sample (20)	32
4.5	Results of isolation and identification of *E.coli*	32
4.5.1	Results of cultural examination	32
4.5.1.1	Culture in nutrient broth	32
4.5.1.2	Culture on MacConkey agar	32
4.5.1.3	Culture on Eosine Methylene Blue (EMB) agar	33
4.5.1.4	Culture on Brilliant Green (BG) agar	33
4.5.1.5	Results of Gram's staining technique	34
4.5.1.6	Results of Hanging drop technique	34
4.5.1.7	Results of reactions in TSI agar slant	34-35
4.5.1.8	Results of the biochemical tests	35
4.5.1.9	Sugar fermentation test	36

LIST OF CONTENT (CONT'D)

NO. OF CHAPTER	TITLE NAME	PAGE NO
4.5.1.10	Other biochemical tests	38
4.5.1.11	Molecular identification of *E.coli*	38
4.2	Results of isolation of *Salmonella* spp.	39
4.2.1	Identification of *Salmonella* spp.	39
4.2.2	Results of isolation and identification of *Salmonella* spp.	40
4.2.3	Results of cultural examination	40
4.2.3.1	Culture in nutrient broth	40
4.2.3.2	Culture on MacConkey agar	40
4.2.3.3	Culture on Eosine Methylene Blue (EMB) agar	40
4.2.3.4	Culture on Salmonella-Shigella agar	42
4.2.3.5	Results of Gram's staining technique	42
4.2.3.6	Results of Hanging drop technique	42
4.2.3.7	Results of reactions in TSI agar slant	42
4.2.3.8	Results of the biochemical tests	43
4.2.3.9	Sugar fermentation test	43
4.2.3.10	Result of Biochemical test	45
4.4	Results of drug sensitivity and resistance pattern of *E. coli* & *Salmonella* spp. Isolated from pigeon	46-60
4.5	**Discussion**	**61-66**
CHAPTER 5	**SUMMARY AND CONCLUSION**	**67-68**
	REFERENCES	**69-79**

LIST OF TABLES

NO. OF TABLE	TITLE NAME	PAGE NO.
Table 1	Oral and Cloacal swab from pigeon	13-14
Table 2	Drugs with their disc concentration for the Enterobacteriaceae family	16
Table 3	PCR primers with sequence	26
Table 4	Total Viable count and Total Coliform Count from the isolated sample	30-31
Table 5	Prevalence of microorganism in pigeon	31
Table 6	Percentage of Prevalence with specific organism from sample	31
Table 7	Prevalence percentage of *E.coli* & *Salmonella* spp. in oral & cloacal sample	32
Table 8	Demonstration of the cultural characteristics of *E. coli* isolated from oral and cloacal samples of pigeon in different agar media	33
Table 9	Demonstration of the morphology and staining characteristics, motility and reactions in TSI agar slant of *E. coli* isolated from oral and cloacal samples of pigeon in different agar media	35
Table 10	Demonstration of the biochemical reactivity pattern of *E. coli* isolated from oral and cloacal swabs of pigeon	36
Table 11	Results of isolation of *Salmonella* spp.	39
Table 12	Demonstration of the cultural characteristics of *Salmonella* spp. isolated from pigeons in different agar media	40
Table 13	Demonstration of the biochemical reactivity pattern of *Salmonella* spp. isolated from pigeon	43
Table 14	Demonstration of the sensitivity and resistance pattern of different *E. coli* isolates to different drugs in percentage	48
Table 15	Demonstration of the sensitivity and resistance pattern of different *E. coli* isolates to different drugs in percentage obtained from oral swab	50

LIST OF TABLES (CONT'D)

No. of Table	Title Name	Page No.
Table 16	Demonstration of the sensitivity and resistance pattern of different *E. coli* isolates to different drugs in percentage obtained from cloacal swab	52
Table 17	Demonstration of the sensitivity and resistance pattern of different *Salmonella* spp. isolates to different drugs in percentage obtained from swab samples of pigeon	54
Table 18	Demonstration of the sensitivity and resistance pattern of different *Salmonella* spp. isolates to different drugs in percentage obtained from oral swab samples of pigeon	56
Table 19	Demonstration of the sensitivity and resistance pattern of different *Salmonella* spp. isolates to different drugs in percentage obtained from cloacal swab samples of pigeon	58

LIST OF FIGURES

NO. OF FIGURE	TITLE NAME	PAGE NO.
Figure 1	Layout of the Experiment	17
Figure 2	Total Viable count by 10 fold dilution method	30
Figure 3	Total coliform count by 10 fold dilution method	30
Figure 4	*E.coli* in MC agar media	34
Figure 5	*E.coli* in EMB agar media	34
Figure 6	*E.coli* in BGA agar media	34
Figure 7	*E.coli* in TSI agar slant	35
Figure 8	Gram negative rod shape organism (*E.coli*)	35
Figure 9	Production of acid and gas in sugar fermentation test for *E.coli*	37
Figure 10	Production of acid and gas in sugar fermentation test positive for *E. coli*	37
Figure 11	Indole production test (positive) for *E.coli*	37
Figure 12	Urease test (negative) for *E.coli*	37
Figure 13	Citrate utilization test (negative) for *E.coli*	37

LIST OF FIGURES (CONT'D)

NO. OF FIGURE	TITLE NAME	PAGE NO.
Figure 14	Methyl red test positive for *E.coli*	37
Figure 15	Voges-proskauer test (negative) for *E.coli*	38
Figure 16	Catalase positive test for *E.coli*	38
Figure 17	Amplification of 584bp DNA from 16srRNA gene of E coli. Lane M: 100bp DNA Marker, Lane 1-10: Test sample, Lane 11: Negative control	38
Figure 18	Total salmonella count (TSC) in SS agar	39
Figure 19	*Salmonella* in McConkey agar media	41
Figure 20	*Salmonella* in EMb agar media	41
Figure 21	*Salmonella* in SS agar media	41
Figure 22	TSI agar slant positive test for *Salmonella* spp.	42
Figure 23	Sugar fermentation test of *Salmonella* spp.	44
Figure 24	Indole test positive for *Salmonella* spp.	44
Figure 25	Citrate test positive (On left) for *Salmonella* spp.	44
Figure 26	Methyl Red test positive for *Salmonella* spp.	44
Figure 27	Diagram showing the Antibiotic sensitivity pattern of *E.coli* isolated from swab samples of pigeon	49
Figure 28	Diagram showing the Antibiotic sensitivity pattern of *E.coli* isolated from oral swab samples of pigeon	51
Figure 29	Diagram showing the Antibiotic sensitivity pattern of *E.coli* isolated from cloacal swab samples of pigeon	53
Figure 30	Diagram showing the Antibiotic sensitivity pattern of *Salmonella* spp. isolated from swab samples of pigeon	55
Figure 31	Diagram showing the Antibiotic sensitivity pattern of *Salmonella* spp. isolated from oral swab samples of pigeon	57
Figure 32	Diagram showing the Antibiotic sensitivity pattern of *Salmonella* spp. isolated from cloacal swab samples of pigeon	59

LIST OF APPENDICES

NO. OF APENDIX	TITLE NAME	PAGE NO.
Apendix 1	Composition of different media	80-81

LIST OF ABBREVIATION AND SYMBOLS

ABBREVIATION	FULL WORD
AMP	Ampicillin
AMX	Amoxicillin
Approx.	Approximately
AZM	Azithromycin
BG	Brilliant Green
CFU	Colony Forming Unit
CIP	Ciprofloxacin
CNF-1	Cytotoxic necrotizing factor 1
DNA	Deoxyribonucleic acid
EMB	Eosin Methylene Blue
ERY	Erythromycin
et al.	and others
ESBL	Extended-Spectrum Beta-Lactamase
E.coli	*Escherichia coli*
etc.	Etcetra
Fig.	Figure
GM	Gentamicin
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen Sulphide
hlyA	Hemolysin A
hrs.	Hours

LIST OF ABBREVIATION AND SYMBOLS (CONT'D)

ABBREVIATION	FULL WORD
IN	Intermediate
K-1	Protein K-1
Lbs.	Pound
Ltd.	Limited
LEV	Levofloxacin
MC	MacConkey
Mg	Milligram
MH	Muller Hinton
Ml	Millilitre
Mm	Milimeter
mon.	Month
Min.	Minute
MR	Methyl Red
NA	Nalidixic Acid
NCCLS	National Committee for Clinical Laboratory Standard
NIB	National Institute of Biotechnology
NM	Non motile
No.	Number
P	Pigeon
PCR	Polymerase chain Reaction
PBS	Phosphate buffered solution
R	Resistant
Rpm	Revolutions Per Minute
S	Sensitive
SAU	Sher-e-Bangla Agricultural University
Sp	Species
SS	Salmonella Shigella
SLT	Shiga-like toxin

LIST OF ABBREVIATION AND SYMBOLS (CONT'D)

ABBREVIATION	FULL WORD
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TET	Tetracycline
TSI	Tripple Sugar Iron
UTI	Urinary tract infection
UV	Ultraviolet
V	Voltage
VP	Voges-Proskauer
yrs.	Years
^{o}C	Degree Celsius
-	Negative
%	Percentage
~	Tilde
+	Positive
®	Registard trade mark
µg	Microgram
µl	Microlitre

ABSTRACT

This study was undertaken to isolate, identify and antibiotic profiling of zoonotic bacteria harbors in pigeon, which are raised in farms and household in Sher-e-Bangla Agricultural University, Dhaka and National Institute of Biotechnology, Savar. Total bacterial load and total coliform load was determined from oral swab (n = 20) and cloacal swabs (n = 20) of pigeons. Samples were collected aseptically and inoculated onto various culture media for isolation of zoonotic bacteria. Identification of bacteria from positive samples were performed by cultural characteristics, Gram's staining, biochemical tests and molecular identification to some extent. Hundred percent prevalence with microorganism was found from all the samples. The prevalence of *E.coli* and *Salmonella* spp. in oral swab samples was (50%) and (30%) and in cloacal swab samples it was (55%) and (25%) respectively. The overall prevalence of *E.coli* and *Salmonella* spp. in pigeons was (52.50%) and (27.5%). Antibiotic sensitivity profiling of the isolated *E.coli* & *Salmonella* spp. was performed by the disc diffusion method against 9 randomly used antibiotics. The highest rate of sensitivity against *E.coli* was found with levofloxacin (67%) followed by azithromycin (63%), ciprofloxacin (62%) and gentamicin (48%). The highest rate of resistance was recorded to ampicillin (71%) followed by amoxicillin (62%), erythromycin (62%), tetracycline (52%). The highest rate of antibiotic sensitivity against *Salmonella* spp. was found with ciprofloxacin (82%) and levofloxacin, gentamicin and ampicillin showed the same result (55%). The highest rate of resistance was recorded to tetracycline (100%) followed by nalidixic acid (82%) and erythromycin (46%), amoxicillin (36%). Data from this study suggest that pigeons carry multi-drug resistant *E.coli* and *Salmonella* spp. which can be transferred to humans through direct contact or the food chain and can cause a potential public health hazard.

CHAPTER 1

INTRODUCTION

Long historical record of raising poultry under backyard system has been found in Bangladesh. For pigeon farming the weather and vast areas of crop field along with housing premises of Bangladesh are suitable. Though the pigeons provide alternative source of animal protein, the contribution of pigeon have not yet been considered in relation to the contribution of livestock sub-sector and whole poultry production. Comparatively low investment, less care, less feed and housing cost are involved, easy and economic husbandry practices, short reproduction cycle and less disease occurrence are observed in pigeon farming. For natural beautification, pigeons are used and ornamental birds as a source of recreation. They are source of palatable, delicious and easily digestible animal protein, sources of bio-fertilizer especially for family gardening and they are used as the laboratory animal in case of genetic and hormonal studies. Hence profitable pigeon farming may be an easy and reliable source of employment opportunity, way of family labor utilization and cash income. Sustainable and increasing rate of pigeon farming may enhance the rate of income; reducing the gap of animal protein deficiency; decrease the rate of poverty and it could improve the socio-economic status of the rural poor community. The ability of pigeon to carry messages has been reliably exploited in the warfare, trade, friendship maintenance and political administration. But this days, the pigeons are mainly reared for family nutrition and recreation (Asaduzzaman *et al.*, 2009). Feral pigeons (*Columba livia*) and domestic pigeons are not harmless birds. In pigeons many potential infections of humans silently exist which are not apparent, having the potentiality to transmit over 30 diseases to humans plus another ten to domestic animals. It is quite evident pigeon droppings are responsible for environmental pollution. Air pollution involves more than noxious gases from automobile exhausts and belching smokestacks. Air-borne fungi is one serious air pollutant, which are agents for infectious diseases. There is much information written about the problem, but it remains primarily in the professional journals and technical references, neatly stacked away on library shelves. Feral pigeons have been identified with mycotic, bacterial, protozoal, chlamydial, rickettsial, and parasitic diseases as well as dermatosis (Weber, 1979). Bacterial diseases identified with pigeons are listed.

Erysipeloid generally starts in a break in the skin and is accompanied with a sensation of burning, throbbing pain, and intense itching. *Erysipelothrus insidiosis* is the causal agent. For the transmission of the organism pigeons are responsible. *Listeria monocytogenes* causes Listeriosis. Changes in the cells of the nervous system is caused by this organism. It may cause conjunctivitis, endocarditis, and skin infections. It also responsible for the occurrence of meningitis in newborns, abortions, premature delivery, stillbirths, and death. From pigeons the organism has been isolated. Parteurellosis is caused by a highly contagious bacteria, *Pasteurella multicida*. The disease may be divided in four groups of syndromes: 1) infection of the upper respiratory tract as nasal discharge or conjuntivitis, 2) infection of the lower respiratory tract as bronchitis or pneumonia, 3) infecton of internal organs as appendicitis or inflammation of the urinary tract, 4) abscessed wound infections. Pigeons can spread the bacteria through their droppings or nasal discharge. A dead pigeon persist the organism for three months and can live as long as a month in pigeon manure. Salmonellosis is more than food poisoning. Gastroenteritis is the most common manifestation. Enteric fever or septicemia may follow several weeks later as a relapse. Septicemias often terminate fatally. Persistent infections are less common but very important. There may be an abscess or local infection as arthritis, bronchopneumonia, endocarditis, meningitis, osteomyelitis, or pyelonephritis (Weber, 1979). Pigeons are important in the spread of salmonellae, wherever the pigeons defecate the bacteria are left (Müller, 1965). They trample back and forth through their copious excretion on window ledges and air intake vents. Dusts to contaminate food or homes enter through air conditioners and ventilators. *Salmonella typhimurium* var. Copenhagen is the most common salmonella isolated from pigeon, which is found in about 2% of pigeon feces (Müller, 1965). For human and animal health, members of the genus *Salmonella* are a risk. These pathogens are considered the most commonly transmitted bacteria from poultry products to humans, and severe economic losses are associated with this (Hafez, 2005). The diseases caused by these microorganisms are called salmonellosis, differentiated as: typhoid fever, which affects only humans and superior primates and it is caused by *S.* serovar Typhi; fowl typhoid, caused by *S.* serovar Gallinarum, specific of birds; and pullorum disease, caused by *S.* serovar Gallinarum biovar Pullorum, also specific of birds. Paratyphoid infections are caused by the remaining serotypes and they are zoonotic,

affecting animals and humans (Berchieri and Freitas Neto, 2009). *Escherichia coli* is another microbial species that is frequently isolated from pigeons, which colonizes the intestine of individuals soon after hatching without necessarily causing infection. The role of this species as a member of the intestinal microbiota of birds is not fully understood. *E. coli* may act as a source of vitamins and promote a competition for colonizing sites in the intestinal epithelium against invading pathogenic microorganisms (Ferreira and Knöbl *et al.,* 2009). The pathogenicity of *E. coli* strains that cause colibacillosis is related to virulence factors, which may be used as diagnostic tools to differentiate pathogenic from non-pathogenic strains (Johnson, 1991). From free-living pigeons in Brazil *E. coli* strains have been isolated which is Diarrheagenic, which generates a possible zoonotic risk and a role in the transmission of these pathogens (Silva *et al.,* 2009). Avian Pathogenic *E. coli* (APEC) is an extra-intestinal pathotype that is composed by strains that are a frequent cause of economic losses on poultry. However, a possible zoonotic risk from these strains has been suggested due to the genetic similarity with Uropathogenic *E. coli* (UPEC) strains, which cause urinary infections in human (Rodriguez-Siek *et al.,* 2005; Ewers *et al.,* 2007 and Mora *et al.,* 2013). Although there are no reports of direct transmission of *E. coli* from pigeons to humans, the proximity with these birds reinforced the possibility of a zoonotic risk. In addition to pathogen dissemination, free-living birds may host strains with antimicrobial resistance, which could be a risk to humans, considering that resistance to antibiotics is a serious problem from clinical and public health perspectives (Gutiérrez *et al.,* 1990). Antimicrobial resistance is one of the main causes of failure in antimicrobial therapy. This mechanism of survival presented by the microorganisms occur naturally or can be acquired. However, acquired resistance is more important due to the fact that it limits viable options of drugs. This form is originated from mutation or gene transference, which may be chromosomic or extra-chromosomic (Spinosa, 2006). The control of colibacillosis mainly relies on the use of antimicrobial drugs. Recently, the prevalence of antimicrobial resistance has been increasing in major bacterial pathogens (Parry and Threlfall, 2008). Bacteria have developed strategies for survival within the host during an infection and one of these strategies is the resistance of isolates to the antimicrobial drugs. Antimicrobial resistance is a serious problem because it limits the therapeutic possibilities in the treatment of bacterial diseases in domestic animal species in general and poultry in

particular (Williams and Heymann, 1998 and Nicole *et al.*, 2000). The number of multi-drug resistant *E. coli* are continuously increasing although various antimicrobial agents are being used (Hussain *et al.*, 1982). Uncontrolled use of antibiotics in medicine and animal husbandry for both treatment and prevention of bacterial diseases over the course of decades has fostered the selection of resistant bacteria (Tomasz, 1994 and Singer *et al.*, 2003). Antimicrobial-resistance E. coli occurred in diseased pigeons in various results as described by (Kimpe *et al.*, 2002; Ibrahim, 2007; Farghaly and Mahmoud, 2011 and Dutta *et al.*, 2013). The E. coli strains in particular had acquired resistance against the most commonly used antimicrobial in pigeons, (Kimpe *et al.*, 2002). A survey of epidemiological data revealed that pigeons may host 60 species of human pathogens. From these pathogens, 45 are fungi, five are viruses, nine are bacteria and one is a protozoan. However, only five of them are routinely transmitted for humans (Haag-Wackernagel and Moch, 2004). In addition, ectoparasites found in these birds may infest humans temporarily, causing skin irritation and itch. Systemic symptoms may also occur, such as fatigue, weakness, dizziness, tachycardia, thoracic oppression and sleepiness (Haag-Wackernagel and Bircher, 2010).

Objective of the Investigation:

1. To Isolate and identify bacteria harbors in pigeon, which are raised in farms and household around Dhaka city.
2. To investigate the antibiotic sensitivity pattern of the isolated bacteria.

CHAPTER 2
REVIEW OF LITERATURE

Isolation, characterization and drug sensitivity determination of the bacteria isolated from oral and cloacal swabs of different pigeon was performed using the information gained from the following related review of literature.

2.1 Domestic & Feral Pigeon

While several of the approximately 300 species of pigeons and doves (family Columbidae) are kept as pets, domestic pigeon usually refers to breeds of *Columba livia*, also called the rock pigeon. Breeding of pigeon is a popular hobby worldwide, and over 350 different breeds are currently recognized. Their ubiquitous association with humans over the millennia pervades literature, visual arts, and religious symbolism and during war and peacetime they have also served as steadfast messengers. Thousands of years ago as with dogs, chickens, and other domesticated animals, the huge variety of domestic pigeons represents a large-scale selection experiment was started. Domestication involves intensive directional selection for particular phenotypes, followed by stabilizing or purifying selection; similar evolutionary processes operate during selective sweeps and sexual selection in natural populations. In pigeons, these processes have produced arguably the greatest phenotypic diversity within any single avian species. For example, facial morphology in pigeons ranges from the tiny beak of the African owl to the massive, recurved beak of the Scandaroon. Many breeds have elaborate feather ornaments, such as the Jacobin breed favored by Queen Victoria. And extremes in body mass among breeds differ by an order of magnitude. Except Antarctica, the current geographic range of the rock pigeon extends to all continents. Rock pigeons are feral outside the native range and even many places within it. That is, they are free-living descendants of escaped domestics, and some Old World feral populations are probably thousands of years old. Molecular evidence suggests that the racing homer, a breed that has regular opportunities to fly outside the loft and potentially escape (Michael and Eric, 2013).

2.2 Microorganisms and Birds

The complex relationships between birds and microorganisms are increasingly becoming the subject of ecological research (Maul *et al*., 2005). Microbial interaction with birds can happen in many different ways. Among them some are commensals, living *in vivo* as part of the normal feather or gut flora without apparently affecting their host. Some are avian pathogens, either obligatorily (e.g., *Chlamydia psittaci*) or opportunistically (e.g., *Pseudomonas aeruginosa*). Other microbes, particularly fungi such as *Cladosporium* and *Epicoccum*, have the potential to be allergens (Hubalek, 1995). Both pathogenic and allergenic species can act to reduce fitness, making individuals more susceptible to competition and predation, while severe infections/reactions are significant causes of mortality (Nuttall, 1997). On the other hand, the presence of microbes can be beneficial; for example, *Enterococcus faecium* has been found to 14 increase the fitness of pied flycatcher (*Ficedula hypoleuca*) nestlings (Moreno *et al*., 2003), while *Eupenicillium javanicum* contains the cyclic depsipeptide, eujavanicin A, the antifungal properties of which are effective against *Aspergillus fumigatus*, a cause of avian aspergillosis (Nakadate *et al*., 2008). The members of the family *Enterobacteriaceae* including *Salmonella* species, *Escherichia coli*, *Enterobacter aerogenes* and *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus mirablis*, and *Providencia alcalifaciens* pathogenic to humans (Vilcins *et al*., 2002). Free-living pigeons are potential reservoirs for several pathogenic microorganisms, including *Chlamydophila psittaci* and bacteria belonging to the genus *Salmonella*. In Japan, *S. typhimurium* and *C. psittaci* have been isolated with a high frequency from feral pigeons (Casanovas *et al*., 1995, Pasmans *et al.*, 2004). *Chlamydophila psittaci* DNA also has been detected in the faeces (16%) of feral pigeons in north-eastern Italian towns (Magnino *et al*., 2009). *Chlamydophila psittaci* was detected by in two pigeon guano samples in an Urban Setting in the City of Bari (Apulia Region) Italy. *Salmonella* species were not found (Tarsitano *et al*., 2010). *Campylobacter jejuni* was isolated *from* 106 of 200 pigeon feces samples tested (Megraud, 1987). *Salmonella* was isolated in an incidence of (7.04%) from fecal samples in purebred pigeons (Methner and Lauterbach, 2003). It was recorded that *Salmonella* spp. were isolated from (5.7%) of the cloacal swabs of free-living pigeons in the city of Ljubljana, Slovenia (Dovc *et al*., 2004). Isolation of (22.8%) of *S. Typhimurium var. Copenhagen* from pooled faecal

samples from pigeon lofts from the city of Ghent (Belgium) (Pasmans *et al*., 2004). pigeons are considered as the possible reservoir of Shiga toxin 2f producing *E. coli* associated with human disease (Sonntag *et al*., 2005). Detection of *Campylobacter jejuni* in fresh faecal samples of six out of 200 feral pigeons while all samples were negative for salmonellae (Lillehaug *et al*., 2005). Isolation of salmonellae from (3.9%) of faecal samples from feral pigeons in Japan (Tanaka *et al*., 2005). *Campylobacter* and *Salmonella* spp. were isolated from live bird to prepackaged carcass for 3 flocks of squab (McCrea *et al*., 2006). *Salmonella enterica* was detected in an incidence of (3.2%) from pigeons captured in Fort Collins, Colorado (Pedersen *et al*., 2006). Wild birds are common environmental reservoir of Salmonellae, but the incidence of the organism in wild birds in general tends to be very low (Kirk *et al*., 2002; Reehe *et al*., 2003 and Abulreesh *et al*., 2007). Salmonella species are either completely absent or exhibit very low prevalence in pigeons (Cizek *et al*., 1994; Casanovas *et al*., 1995; Hubalek *et al*., 1995; Kirk *et al*., 2002; Refsum *et al*., 2002; Reehe *et al*., 2003; Vlahovic *et al*., 2004; Dovc *et al*., 2004; Tanaka *et al*., 2005; Lillehaug *et al*., 2005; Pedersen *et al*., 2006 and Kobayashi *et al*., 2007). 400 fresh faecal samples of rock pigeons in Makkah city were analyzed in western Saudi Arabia and found 2.5% and 2% of samples to be positive for shigatoxin producing *E.coli* 0157 and *Salmonella* respectively (Abulreesh, 2011). Shiga –toxin producing *E. coli* and *Salmonella* species were found in faecal droppings and/or cloacal swabs of pigeons that live in urban and rural areas around the world (Morabito *et al*., 2001; Haag and Moch, 2004; Kobayashi *et al*., 2007 and Wani *et al*., 2004). The prevalence rate (28.16%) of bacterial isolates of public health importance in pigeons was reported by. The incidence of bacterial pathogens differed according to health status of examined pigeons and ages either squabs or adults, as it gave the higher incidence in freshly dead squabs (33.33%) and in adults (28.57%) followed by diseased squabs (31.03%) and adults (26.67%) then finally slaughtered pigeons (25.56%). There was a wide range of bacterial pathogens isolated from nasal and cloacal swabs of diseased pigeons including *Camylobacter jejuni, Citrobacter freundii, Diplococcus pneumoniae, E.coli, K. oxytoca, K. pneumoniae, Mannheimia haemolytica, P. aeruginosa, Salmonella* sp., *S. aureus*and *Y. enterocolitica* (Ibrahim, 2007).

2.3 Bacterial Disease

Thirty-three *Streptococcus gallolyticus*, 60 *Escherichia coli* and 18 *Salmonella enterica* serotype was isolated from pigeons (*Columba livia*). Observation of antimicrobial susceptibility in pigeons was done and found. Aminoglycosides (gentamicin and kanamycin), trimethoprim and flumequine relatively inactive against the streptococci tested. Acquired tetracycline resistance amounted to 85%, and lincomycin and macrolide (erythromycin) resistance to 48% and 45%, respectively. Fluoroquinolone (enrofloxacin) resistance was found in four *S. gallolyticus* strains. All strains were susceptible to ampicillin with E. *coli* strains were resistant to all antibiotics tested (Kimpe *et al.*, 2002).It was reported from bacterial contaminations in fecal samples from feral pigeons in 7 prefectures. Isolation of *Salmonella typhimurium* and *S. cerro* from 17 (3.9%) of 436 samples, as well as *Mycobacterium* spp. including *M. aviumintracellulare* complex from 29 (19.0%) of 153 samples. The polymerase chain reaction detected *Chlamydia psittaci* and *C. pecorum* in 106 (22.9%) of 463 samples, but *E. coli* O-157 was not isolated from any of the samples (Tanaka *et al.,* 2005). An experimental study was conducted to determine the prevalence of shiga toxin– producing *Escherichia coli* (STEC) and *S. enterica* in pigeons. They estimated the prevalence of STEC and *S. enterica* by bacteriologic culture of cloacal swabs collected from pigeons trapped at urban and dairy locations in and around Fort Collins, Colorado. Presumptive E. *coli* isolates were tested for the presence of virulence genes SLT-1, SLT-2, eae, hlyA, K1, CNF-1, CNF-2, and LT using polymerase chain reaction. They suggested that pigeons may acquire S. enteric from cattle and play a role in recirculation and persistence of the microorganism at dairies (Pedersen *et al.,* 2006). Observation of paratyphoid infection as a main bacterial disease in pigeons caused by *Salmonella enterica* subsp. *enterica* serovar Typhimurium. It was suggested that the persisting ability of *Salmonella* serovar Typhimurium intracellularly inside pigeon macrophages to development of chronic carriers is responsible for the maintainance the infection in the flock (Pasmans *et al.*, 2007). It was suggested that antimicrobial-resistant pathogenic *E.coli* is present in pigeons around greater Guwahati. Out of 150 pigeons subjected to microbiological investigation, they found 91(60.67 %) samples were positive for *E. coli.* The most frequently occurring serotypes were O157 (9.89%), followed by O68, O121 (7.69%), O9, O75, O131 (5.49%), O2, O13, O22

(3.30%). Antibiogram investigation of the isolates revealed that 91 isolates (100%) exhibited resistanceagainst Ampicillin followed by Nitro-furantoin (73.62%), Tetracycline (65.93 %), Oxytetracycline (62.63 %) and Streptomycin (61.54). Gross changes of some pigeons showed fibrinous pericarditis and perihepatitis and coligranuloma in different organs like liver and serosal surface of intestine. Microscopically, they observed severe congestion and haemorrhages in different organs such as liver, kidney, lung and intestine. In some cases thick layer of fibrinous exudates with large number of heterophills over the surface of liver and heart with early degenerative changes as well as focal necrosis (Dutta *et al.*, 2013).549 samples were collected from pigeon droppings from 14 locations in Kyushu, Japan, to isolate Shiga toxin 2f-producing Escherichia coli (STEC2f) and to investigate characteristics of the isolates. They detected shiga toxin stx2f gene fragments by PCR in 16 (2.9%) of the 549 dropping samples across four of the 14 locations. They also obtained 23 STEC2f-isolates from seven of the original samples and from three pigeon dropping (Murakami *et al.*, 2014).

2.4 Antibiotic Resistance

"Antibiotics" are group of antimicrobial agents synthesized by microorganisms like bacteria or fungi and have the property of inhibiting the growth of other microorganism (bacteria). Though the term antibiotics and antimicrobials are often used interchangeably, antibiotics actually refer to naturally occurring biomolecules, while the term antimicrobials encompass both naturally occurring and synthetically derived molecules. Antimicrobials include all compounds that act against all types of microorganisms, such as bacteria (antibacterial), viruses (antiviral), fungi (antifungal) and protozoa (antiprotozoal). Antibiotics are widely used for preventing and treating various infections in humans and animals. Antibiotics are also used as growth promoters in animal food production sectors, where its addition in feed enhances animal growth and improves the quality of products (Cheng *et al*., 2014). Thus antibiotics are extensively used in intensive and large-scale farming industry. But their indiscriminate and irrational use in different fields like agriculture, fisheries, livestock industry, etc., has given rise to development of resistant bacteria (Aarestrup, 2005) and this results in the spread of resistance by transfer of its resistant determinants to other bacteria (Stanton, 2013). The driving force that induces

resistance in microorganisms to particular agent is the selective evolutionary pressure by the environment or antimicrobial agents and it has been observed that there is an association between the indiscriminate use of antimicrobial agents and the occurrence of resistance. The resistance developed in pathogenic microorganisms makes the antimicrobial agents not only ineffective but also leads to difficulty in treating 6 many of the bacterial infections that were easily treatable formerly. Hence, to overcome the increased rate of mortality and morbidity due to antibiotic resistance, a number of alternatives /replacements have been proposed (Seal *et al*., 2013).

2.5 Mechanism and origin of antibiotic resistance

Though the antibiotics were more successful as therapeutics against many bacterial infection in the history of medicine, their irrational and indiscriminate use has created enormous pressure resulting in the development of antibiotic resistance in bacteria (Witte, 1998). Antibiotic resistance can be an intrinsic property of bacteria themselves or it can be acquired later. In natural or intrinsic resistance to a drug occurs without any additional changes in their genetic elements, whereas acquired resistance results through random mutations or acquisition of foreign genetic material carrying resistance determinants (Hollenbeck and Rice, 2012), The antimicrobial agent becomes effective against a target bacterial species only when a susceptible antibiotic target site exists in the cell, the antibiotic reaches the target in sufficient quantity and the antibiotic is not inactivated or modified by the bacterial cell wall (Sutcliffe *et al*., 1999). So, the unavailability or any change in these conditions trigger the cells to acquire resistance. The mode of acquiring resistance to an antimicrobial drug in bacterial species is categorized broadly into two groups based on biochemical and genetic aspect (Senka *et al*., 2008).

2.6 Antibiotic resistance in bacterial pathogens

Escherichia coli, a member of the normal gut flora of humans and animals, possess many beneficial functions. Nonetheless, their pathogenic role is also well recognized as they cause many bacterial infections including urinary tract infection (UTI), diarrhea, meningitis and pneumonia (Lim *et al*., 2009). There are several reports on resistance of *E.coli* to several antibiotics such as tetracycline, nalidixic acid, cefotaxime, chloramphenicol, gentamicin, ampicillin, kanamycin, trimethoprim / sulfamethoxazole, etc

(Adzitey, 2011; Sukhumungoon *et al*., 2011 and Lim *et al*., 2009;). The traditional method of treatment for *E. coli* infections was a combination of an aminoglycoside and ampicillin but has developed extreme resistance to many drugs (beta lactams, tetracycline, and aminoglycosides) (Enne *et al*., 2001 and Sunde *et al*., 1998). *Salmonella* is a Gram negative bacteria capable of causing disease in humans as well as in domestic animals. Salmonellosis is one of the common foodborne disease caused by *Salmonella* spp. It is a significant pathogen of food producing animals and these animals are the primary source of salmonellosis (Forshell and Wierup, 2006). As *Salmonella* is an intracellular pathogen, the effective way to eradicate this organism is to use antibiotics that have intracellular activity. The earliest groups of drugs used in the treatment of *Salmonella* infections were neomycin and colistin. This was followed by the use of absorbable drugs such as ampicillin, amoxicillin, chloramphenicol, tetracycline, and co-trimoxazole, which unfortunately do not have substantial intracellular activity. But strains of *Salmonella* resistant to several antimicrobial agents have been reported worldwide (Angulo and Griffin, 2000 and Breuil *et al*., 2000). Several studies have reported the prevalence of multi resistance genes in different serotypes of *Salmonella* (Aarts *et al*., 2001). Among the members of the genus *Vibrio* many are pathogenic to humans and are implicated in food-borne diseases (Tavakoli *et al.,* 2012).

2.7 *Escherichia coli* as the model for antibiotic resistance studies

Role of *E. coli* in relation to its hosts is diverse varying from commensalism to an established pathogen. *E. coli* is classified on the basis of pathogenesis into three major groups: commensal strains, intestinal pathogenic strains, and extra-intestinal pathogenic strains (Russo and Johanson, 2000). It is amongst the first organisms to populate and colonize the gut micro-flora in the neonates (Lozupone *et al.,* 2012 and Tenaillon *et al.,* 2010). The primary habitat of *E. coli* is the lower intestinal tract with which it typically establishes commensal associations. It has been estimated that half of the living *E. coli* cells are outside their host, in their secondary habitat (Savageau, 1983). Beside these habitats, certain strains have the potential to cause a wide spectrum of intestinal and extra-intestinal diseases such as urinary tract infection, septicemia, meningitis, and pneumonia in humans and animals. With its large range of pathologies, *E. coli* is the major cause of

morbidity and mortality around the world. Each year *E. coli* causes more than two million deaths due to infant diarrhoea (Kotloff *et al*., 1999 and Kosek *et al*., 2003) and extra-intestinal infections (mainly septicemia derived from urinary tract infection), and is also responsible for approximately 150 million cases of uncomplicated cystitis (Russo and Johnson, 2003). Resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine (Tadesse *et al*., 2012**)**. The past 2 decades have witnessed major increase in emergence and spread of multidrug-resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and certain cephalosporins (Levy and Marshall, 2004).

2.8 Resistance gene transfer from *E. coli* of poultry and pork to human

As commensal bacteria constitute a reservoir of resistance genes for (potentially) pathogenic bacteria, their level of resistance is considered to be a good indicator for selection pressure by antibiotic use and for resistance problems to be expected in pathogen. A progressive global spread of *Enterobacteriaceae* in pork and chicken meat expressing plasmid-mediated enzymes that inactivate β-lactam antibiotics, suggest that the food chain might play a role in the epidemiology and the transmission of extended-spectrum cephalosporin-resistant *enteric* bacteria to humans (Aarestrup *et al*., 2008; Abgottspon *et al*., 2014 and Hammerum *et al*., 2014). In The Netherlands observations were made regarding transfer of ESBL resistance genes from poultry and pork to the humans (Overdevest *et al*., 2011; Leverstein-van Hall *et al*., 2011and Kluytmans *et al*., 2013). Further, antimicrobial resistant *E. coli* may be selected in the intestines of patients, treated with several antimicrobial agents or from heavy use of antimicrobial agents in food animal production. Bacteria originating from food animals frequently carry resistance to antimicrobial agents, including those commonly used in humans (Hammerum and Heuer, 2009). Such resistant strains from animals can colonize or infect the human population via contact (occupational exposure) or via the food chain. Moreover, resistance genes can be transferred from bacteria of animals to human pathogens in the intestinal flora of humans (Van den Bogaard and Stobberingh, 1999). The same antimicrobial resistance genes have been detected both in animals and/or meat and in humans, which indicate horizontal spread. Different animal model studies have demonstrated that the intestine is a hot spot for

horizontal transfer of resistance genes between *E. coli* (Licht *et al*., 2003; Duval-Iflah *et al*., 1994 and Hart *et al*., 2006). A chicken and mouse intestine model was used to detect the transfer of a tetracycline resistance gene from *E. coli* of animal origin to *E. coli* of human origin (Hart *et al.*, 2006). Such experiments have also been used by other workers showing the transfer of genes (Johnson *et al*., 2005 and Ramchandani *et al*., 2005). The transfer of antimicrobial resistance genes has been detected in the intestine of humans (Smith, 1969 and Trobos *et al*., 2008). It is reported that Quinolone and Fluoroquinolone resistant *E. coli* isolates from broilers, broiler meat and humans are closely related and show clonal links, indicating that poultry and their food products can be a source of resistant *E. coli* in humans (Thorsteinsdottir *et al*., 2010 and AbdiHachesoo *et al*., 2013). In a study it was reported that Drug-resistant human isolates were similar to poultry isolates and drug-susceptible and drug-resistant poultry isolates were largely indistinguishable (Johnson *et al*., 2007). Many drug-resistant human faecal *E. coli* isolates may originate from poultry, whereas drug-resistant poultry source *E. coli* isolates likely originate from susceptible poultry (Johnson *et al*., 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples

Table 1. Oral and Cloacal swab from pigeon

Sample No. and Name	Collection zone	Sample source	Gender	Age	Sample type
1. P1	Savar, Dhaka	pigeon	Male	1yrs.	Oral swab
2. P2	Savar, Dhaka	pigeon	Male	8 mon.	Oral swab
3. P3	Savar, Dhaka	pigeon	Male	8 mon.	Oral swab
4. P4	Savar, Dhaka	pigeon	Male	1yrs.	Oral swab
5. P5	Savar, Dhaka	pigeon	Male	1.4 yrs.	Oral swab
6. P6	SAU, Dhaka	pigeon	Male	1.5 yrs.	Oral swab
7. P7	SAU, Dhaka	pigeon	Female	3 mon.	Oral swab
8. P8	SAU, Dhaka	pigeon	Female	1.5 yrs.	Oral swab
9. P9	SAU, Dhaka	pigeon	Male	2.4 yrs.	Oral swab
10. P10	SAU, Dhaka	pigeon	Female	1.8 yrs.	Oral swab
11. P11	Savar, Dhaka	pigeon	Male	1.1 yrs.	Oral swab
12. P12	Savar, Dhaka	pigeon	Female	1.3 yrs.	Oral swab
13. P13	Savar, Dhaka	pigeon	Male	1.8yrs.	Oral swab
14. P14	Savar, Dhaka	pigeon	Female	1.4yrs.	Oral swab
15. P15	Savar, Dhaka	pigeon	Male	1.2yrs.	Oral swab
16. P16	SAU, Dhaka	pigeon	Female	1.5 yrs.	Oral swab
17. P17	SAU, Dhaka	pigeon	Male	1.6 yrs.	Oral swab
18. P18	SAU, Dhaka	pigeon	Male	1yrs.	Oral swab
19. P19	SAU, Dhaka	pigeon	Male	1.6 yrs.	Oral swab
20. P20	SAU, Dhaka	pigeon	Female	1.3 yrs.	Oral swab
21. P21	Savar, Dhaka	pigeon	Male	1yrs.	Cloacal swab
22. P22	Savar, Dhaka	pigeon	Male	8 mon.	Cloacal swab
23. P23	Savar, Dhaka	pigeon	Male	8 mon.	Cloacal swab
24. P24	Savar, Dhaka	pigeon	Male	1yrs.	Cloacal swab
25. P25	Savar, Dhaka	pigeon	Male	1.4 yrs.	Cloacal swab
26. P26	SAU, Dhaka	pigeon	Male	1.5 yrs.	Cloacal swab
27. P27	SAU, Dhaka	pigeon	Female	3 mon.	Cloacal swab
28. P28	SAU, Dhaka	pigeon	Female	1.5 yrs.	Cloacal swab
29. P29	SAU, Dhaka	pigeon	Male	2.4 yrs.	Cloacal swab
30. P30	SAU, Dhaka	pigeon	Female	1.8 yrs.	Cloacal swab
31. P31	Savar ,Dhaka	pigeon	Male	1.1 yrs.	Cloacal swab

Table1 (cont'd)					
32. P32	Savar ,Dhaka	pigeon	Female	1.3 yrs.	Cloacal swab
33. P33	Savar, Dhaka	pigeon	Male	1.8yrs.	Cloacal swab
34. P34	Savar, Dhaka	pigeon	Female	1.4yrs.	Cloacal swab
35. P35	Savar, Dhaka	pigeon	Male	1.2yrs.	Cloacal swab
36. P36	SAU, Dhaka	pigeon	Female	1.5 yrs.	Cloacal swab
37. P37	SAU, Dhaka	pigeon	Male	1.6 yrs.	Cloacal swab
38. P38	SAU, Dhaka	pigeon	Male	1 yrs.	Cloacal swab
39. P39	SAU, Dhaka	pigeon	Male	1.6 yrs.	Cloacal swab
40. P40	SAU, Dhaka	pigeon	Female	1.3 yrs.	Cloacal swab

Collection of oral and cloacal sample was done from the same bird. (P1, P21), (P2, P22),(P3, P23), (P4, P24), (P5, P25), (P6, P26), (P7, P27), (P8, P28), (P9, P29), (P10, P30), (P11, P31), (P12, P32), (P13, P33), (P14, P34), (P15, P35), (P16, P36), (P17, P37), (P18, P38), (P19, P39), (P20, P40). Though they were collected from the same bird, they were regarded as two different sample as they were collected from oral cavity and cloaca of pigeon.

3.1.2 Bacteriological media

3.1.2.1 Agar media

Agar media used for bacteriological analysis were, MacConkey (MC) agar, Eosin Methylene Blue (EMB) agar, Brilliant Green (BG) agar, Salmonella shigella (SS) agar, and Muller Hinton (MH) agar.

3.1.2.2 Liquid media (broth)

The liquid media used for this study were Nutrient broth, Peptone broth, Methyl-Red and Voges-Proskauer broth (MR-VP broth) and Sugar media (dextrose, maltose, lactose, sucrose and mannitol).

3.1.2.3 Phosphate Buffered Saline (PBS)

For preparation of phosphate buffered saline, 8 gm of sodium chloride (NaCl), 2.89 gm of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), 0.2 gm of potassium chloride (KCl) and 0.2 gm of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled

water. The solution was heated to dissolve completely and p^H was adjusted with the help of p^H meter. The solution was then sterilized by autoclaving and stored at 4ºC for future use.

3.1.3 Chemicals and reagents

The chemicals and reagents used for this study were 0.1% Peptone water, Phosphate buffered saline (PBS), reagents for Gram's staining (Crystal Violate, Gram's iodine, Safranin, Acetone alcohol), 3% Hydrogen peroxide, Phenol red, Methyl red, 10% Potassium hydroxide, Kovac's indole reagent (4-dimethylamino-benzaldehyde, concentrated HCL), Mineral oil, Normal saline and other common laboratory chemicals and reagents.

3.1.4 Glass wares and other appliances

The following glass wares and appliances were used during the course of the experiment. Test tubes (with or without Durham's fermentation tube and stopper), petridishes, conical flask, pipette (1 ml, 2 ml, 5 ml, 10 ml) & micro-pipettes (1ml, 200µl, 100µl, 10 µl) slides and cover slips, hanging drop slides, immersion oil, compound microscope, bacteriological loop, sterilized cotton, cotton plug, test tube stand, water bath, bacteriological incubator, refrigerator, sterilizing instruments, thermometer, ice carrier, hand gloves, spirit lamp, match lighter, laminar air flow, hot air oven, centrifuge tubes and machine, PCR machine, thermo scientific nanodrop spectrophotometer, UV transilluminator, Gel documentation machine electronic balance, syringe and needle, tray, forceps, scalpel, scissors etc.

3.1.5 Antimicrobial discs

Commercially available antimicrobial discs (OXOID Limited, Canada) were used for the test to determine the drug sensitivity and resistance pattern and to interpret their disease potential. This method allowed for the rapid detection of the efficacy of drugs against the test organisms by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the discs inhibiting the growth of the organisms. The following antimicrobial agents with their disc concentration were used to

test the sensitivity and resistance pattern of the selected *E. coli and Salmonella sp.* isolates from oral and cloacal swab of pigeon.

Table 2. Drugs with their disc concentration for the Enterobacteriaceae family

Name of drugs	**Disc concentration (µg /disc)**	**Zone Diameter Interpretive Standard (mm)**		
		Resistant	**Intermediate**	**Susceptible**
Gentamycin (GM)	10	≤13	14--17	≥18
Azithromycin (AZM)	15	≤13	14--17	≥18
Erythromycin (ERY)	15	≤13	14--17	≥18
Levofloxacin (LEV)	5	≤13	14--16	≥17
Tetracycline (TET)	30	≤14	15--18	≥19
Amoxicillin (AMX)	10	≤13	14--17	≥18
Ampicillin (AMP)	10	≤13	14--16	≥17
Nalidixic acid (NA)	30	≤13	14--18	≥19
Ciprofloxacin (CIP)	5	≤15	16--20	≥21

Legend: µg = micro gram

3.2 Methods

3.2.1 Brief description of the experimental design

The entire study was divided into two major steps: The first step included selection of sources, collection of samples, isolation, identification and characterization of microorganisms on the basis of their colony morphology, staining properties, motility and biochemical characteristics & molecular identification. In the second step, the current status of drug sensitivity and resistance pattern of a total of 40 isolates of microorganism isolated from pigeons was determined.

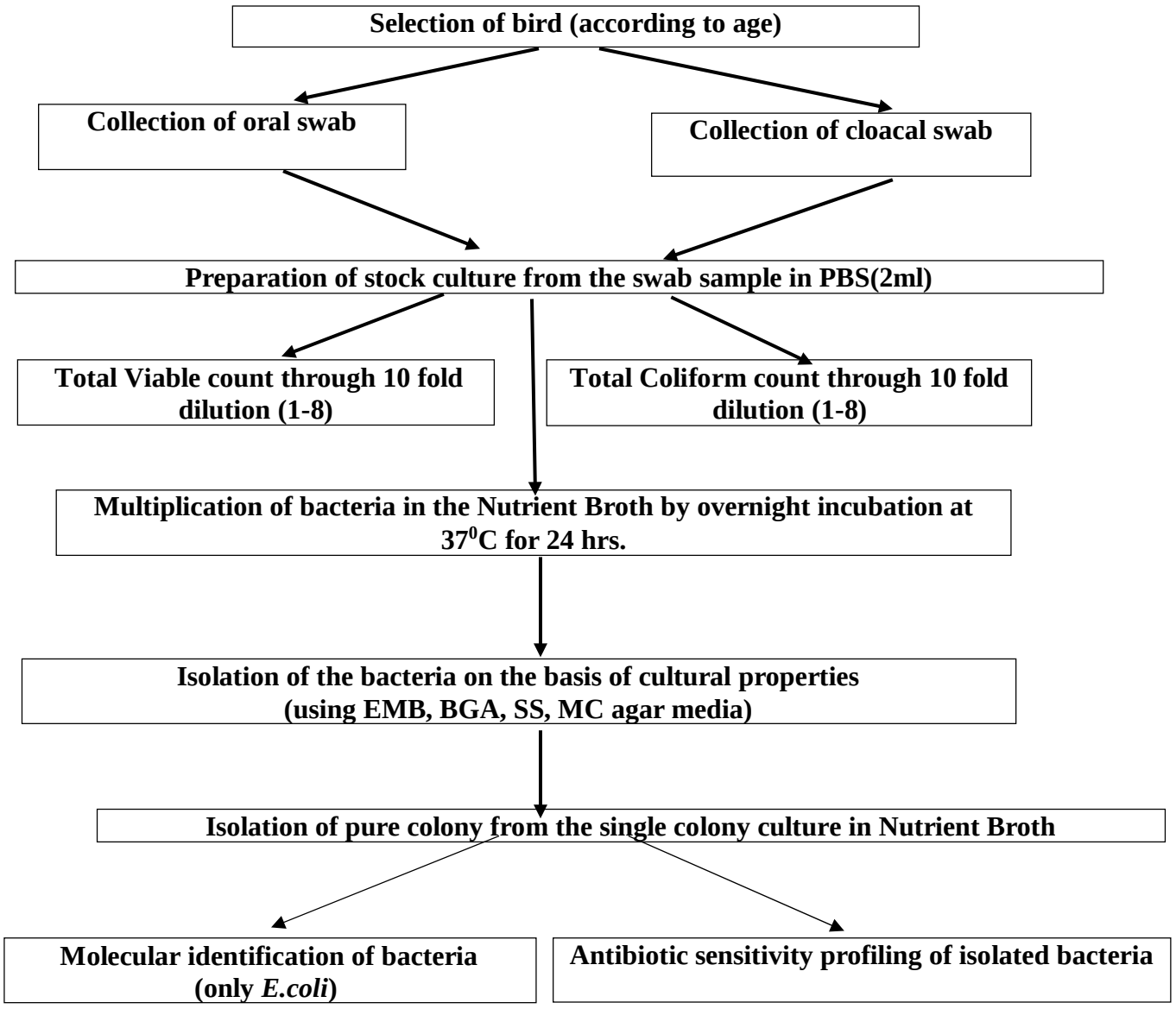

Figure 1: Layout of the Experiment Design

3.2.2 Preparation of various bacteriological culture media

3.2.2.1 Nutrient Agar

Nutrient agar was prepared by dissolving 28 grams of dehydrated nutrient agar (HiMedia, India) in to 1000 ml of distilled water and was sterilized by autoclaving at 121ºC under 15 lb pressure per square inch for 15 minutes. Then the agar was dispensed into petridish (90 mm and 100 mm) and was incubated at 37ºC for overnight to check their sterility and stored at 4ºC in the refrigerator until used.

3.2.2.2 Nutrient Broth

Nutrient Broth was prepared by Suspended 25 grams in 1000 ml purified/distilled water. Heat if necessary to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 30 minutes. The broth was filled in test tubes & incubated at 37ºC for overnight to check their sterility and stored at 4ºC in the refrigerator until used.

3.2.2.3 MacConkey's agar

49.53 grams of Bacto MacConkey agar (HiMedia, India) was suspended in to 1000 ml of cold distilled water and was heated for boiling to dissolve the medium completely. It was then poured in to sterile petridishes and allowed to solidify. After solidification of the medium in the plates, the plates were then incubated at 37ºC for overnight to check their sterility.

3.2.2.4 Eosine Methylene Blue (EMB) agar

Thirty six grams powder of EMB agar base (HiMedia, India) was suspended in 1000 ml of distilled water. The suspension was heated to boil for few minutes to dissolve the powder completely with water. The medium was autoclaved for 30 minutes to make it sterile. After autoclaving the medium was put in to water bath at 45ºC to cool down its temperature at 40ºC. From water bath 10-20 ml of medium was poured in to small and medium sized sterile petridishes to make EMB agar plates. After solidification of the medium in the plates, the plates were incubated at 37ºC for overnight to check their sterility.

3.2.2.5 Brilliant Green agar

According to the direction of manufacturer (HiMedia, India) 58 grams of dehydrated medium was suspended in 1000 ml distilled water and heated for boiling to dissolve the medium completely. The medium was sterilized by autoclaving. After autoclaving the medium was put in to water bath of 45ºC to decrease its temperature. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37ºC for overnight to check their sterility and then stored at 4ºC in a refrigerator for future use.

3.2.2.6 Salmonella-Shigella agar

According to the direction of manufacturer (HiMedia, India) 60 grams of dehydrated medium was suspended in 1000 ml distilled water and heated for boiling to dissolve the medium completely. The medium was sterilized by autoclaving. After autoclaving the medium was put in to water bath of 50ºC to decrease its temperature. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37ºC for overnight to check their sterility and then stored at 4ºC in a refrigerator for future use.

3.2.2.7 Mueller Hinton Agar

Suspended 38.0 grams in 1000 ml distilled water & Heated to boiling to dissolve the medium completely. After the sterilization by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooling was done to 45-50°C.Then it was mixed well and poured into sterile Petridishes. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37ºC for overnight to check their sterility and then stored at 4ºC in a refrigerator for future use.

3.2.2.8 Triple Sugar Iron (TSI) agar

Sixty five grams of dehydrated medium (Difco, USA) was mixed with 1000 ml cold distilled water in a flask and heated for boiling to dissolve the medium completely. The solution was distributed in tubes which were plagued with cotton. The tubes were then sterilized by autoclaving and slanted in such a manner as to allow a generous butt. After

solidification tubes were incubated at 37ºC for overnight to check sterility. The sugar solutions were sterilized in Arnold's steam sterilizer at 100ºC for 30 minutes for three consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water. The sugar solutions were incubated at 37ºC for 24 hours to check sterility. These solutions were used for biochemical test.

3.2.2.9 Methyl Red and Voges–Proskauer (MR-VP) broth

A quantity of 3.4 gm of MR-VP medium (HiMedia, India) was dissolved in 250 ml of distilled water, distributed in 2 ml quantities in test tube and then autoclaved. After autoclaving, the tubes containing medium were incubated at 37ºC for overnight to check their sterility and then stored at 4ºC for future use.

3.2.2.10 Sugar solutions

The medium consists of 1% peptone water to which fermentable sugars were added. Peptone water was prepared by adding 1 gram of Bacto peptone (Difco, USA) and 0.5 grams of sodium chloride in 100 ml distilled water, boiled for 5 minutes, adjusted to pH 7.6 by phenol red (0.02%) indicator, cooled and then filtered through filter paper. The solutions were then dispensed in 5 ml amount into cotton plugged test tubes containing invertedly placed Durham's fermentation tubes. Then the sugars, dextrose (MERCK, India), maltose (s.d. fiNE-CHEM Ltd.), lactose (BDH, England), sucrose (MERCK, India) and mannitol (PETERSTOL TENBEG) used for fermentation were prepared separately as 10 percent solutions in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A little heat was necessary to dissolve the sugar. These were then sterilized by autoclaving for 15 minutes. The sugar solutions were sterilized in Arnold's steam sterilizer at 100ºC for 30 minutes for three consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water. The sugar solutions were incubated at 37ºC for 24 hours to check sterility. These solutions were used for biochemical test.

3.2.3 Isolation of bacteria

3.2.3.1 Collection, transportation and preparation of sample

(a) **Oral swab**: A number of 20 samples of oral swab were also collected through using sterile swab stick in 2 ml Eppendorf tube filled with PBS from the pigeon from Sher-e-Bangla Agricultural University campus and pigeon farm, Ashuliya, Savar, Dhaka. The collected samples were immediately carried to the laboratory in an ice box containing ice and processed for isolation and characterization of bacteria.

(b) Cloacal swab

A number of 20 samples of cloacal swab were also collected through using sterile swab stick in 2 ml Eppendorf tube filled with PBS from the pigeon from Sher-e-Bangla Agricultural University campus and pigeon farm, Ashuliya, Savar, Dhaka. The collected samples were immediately carried to the laboratory in an ice box containing ice and processed for isolation and characterization of bacteria.

3.2.3.2 Serial dilution for bacterial culture (10 fold dilution method)

Serial dilution of the stock sample was done to lowering the bacterial count for the total viable count (TVC) and total coliform count (TCC). It was done by taking 8 (1-8) Eppendorf tube filled with 900µl of PBS. 100µl of stock sample was transferred from the stock tube (2ml) to the Eppendorf tube next to the stock tube. Then 100µl of diluted sample is transferred from the first Eppendorf tube to the next. Successive dilution should be made in the same way to the last tube and from the last tube 100µl of diluted sample should be discarded. From the last tube 25µl of liquid sample should be transferred to the nutrient Agar media and MacConkey agar to elucidate the total viable count & total coliform count. Enumeration of *Salmonella* was done by transferring same amount of liquid sample in the Salmomella –Shigella agar.

3.2.3.3 Primary culture of microorganism

Primary growth of all kinds of bacteria present in the collected samples was performed in nutrient broth. The samples were inoculated in nutrient broth and incubated for overnight at 37^0C for the growth of the organisms.

3.2.3.4 Isolation in culture media

After primary culture of the organism, a small amount of inoculums from Nutrient broth was streaked on the MacConkey agar and Brilliant green agar & Salmonella-Shigella agar to observe the colony morphology of the isolates. Characteristic colony morphology of the organisms indicating *E.coli* was selected for subculture on selective media such as EMB agar and *Salomonella* on Salmonella-Shigella agar. Morphological characteristics (shape, size, surface texture, edge and elevation, color, opacity etc) of the suspected colonies on different agar media developed within 18 to 24 hours of incubation were carefully recorded.

3.2.3.5 Microscopic study for identification of *E. coli* & *Salmonella sp* from the suspected colonies by Gram's staining method

Gram's staining was performed as per recommendation of Merchant and Packer (1976) to determine the size, shape and arrangement of bacteria. The procedure was as follows- A small colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gentle heating. Crystal violet solution was then applied on the smear to stain for two minutes and then washed through running tap water. Gram's iodine was then added to act as a mordent for one minute and then again washed with running water. Acetone alcohol was then added, which act as a decolorizer. After washing with water, safranine was added as counter stain and allowed to stain for 2 minutes. The slide was then washed with water, blotted and air dried and then examined under microscope with high power objective (100X) using immersion oil. In positive case the organism *E. coli* was revealed gram negative, pink colored, rod shaped appearance, arranged in single or in pair.

3.2.3.6 Motility test for *E. coli* & *Salmonella* isolates

The motility test was performed according to the method described by Cowan, 1985 to differentiate the motile bacteria from the non-motile one. Before performing the test, a pure culture of the test organism was allowed to grow in nutrient broth. One drop of cultured broth was placed on the cover slip and was placed inverted condition over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the concave depression of the hanging drop slide for better attachment of the cover slip to prevent air current and evaporation of the fluid. The hanging drop slide was then examined carefully under 100X power objective of a compound microscope using immersion oil. The motile and non-motile organisms were identified by observing motility in contrasting with to and from movement of bacteria.

3.2.3.7 Reaction of the organism in TSI agar slant

After isolation of the organisms on the selective media, differential screening media such as TSI agar was used for further characterization. The test organisms were cultured into TSI agar slant by stab or streak method. In TSI agar slant, if the organism ferments only glucose and the tube will turn yellow within a few hours. The bacteria quickly exhaust the limited supply of glucose and start oxidizing amino acid for energy, produce ammonia as an end product. Oxidation of amino acids increases the pH and the indicator in the slanted portion of the tube will turn red. If the organism in the TSI agar slant ferments lactose and/or sucrose, the slant will turn yellow and remain yellow for several days due to the increased level of acid production. Gas production of the organisms can be ascertained by the appearance of bubbles in the agar. TSI agar can also be used to indicate whether Hydrogen Sulphide (H_2S) has been produced due to the sulpher containing compounds. H_2S reacts with the ferrous sulphate of the medium producing ferric sulphide, which will appear as a black precipitate.Yellow slant, yellow butt, presence of gas bubbles and absence of black precipitate in the butt is positive for *E. coli* & black precipitate in the butt is identical for *Salmonella* sp. .

3.2.4 Identification of isolated *E. coli & Salmonella* spp. by using specific biochemical tests

Several biochemical tests were performed for confirmation of *E. coli & Salmonella* isolates.

3.2.4.1 Carbohydrate fermentation test

The carbohydrate fermentation test was performed by inoculating 0.2 ml of nutrient broth culture of the isolated organisms into the tubes containing different sugar media (five basic sugars such as dextrose, maltose, lactose, sucrose and mannitol) and incubated for 24 hours at 37ºC. Acid production was indicated by the color change from red to yellow and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube (Cheesbrough, 2006).

3.2.4.2 Catalase test

For this study 3 ml of catalase reagent (3% H2O2) was taken in a test tube. Single colony from the pure culture of E. coli was taken with a glass rod and merged in the reagent. The tube was observed for bubble formation. All of the isolates were catalase positive; formation of bubble within few seconds was the indication of the positive test, while the absence of bubble formation indicated negative result (Cheesbrough, 2006).

3.2.4.3 Methyl Red test

The test was conducted by inoculating single colony from the pure culture of the test organism in 5 ml sterile MR-VP broth. After 5 days incubation at 37ºC, 5 drops of methyl red solution was added and observed for color formation. Development of red color was positive and indicated an acid pH of 4.5-6 resulting from the fermentation of glucose. Development of yellow color indicated negative result (Cheesbrough, 2006).

3.2.4.4 Voges-Proskauer (V-P) test

The test *E. coli* organisms were grown in 3 ml of sterile MR-VP broth at 37ºC for 48 hours. Then 0.6 ml of 5% alpha-napthol and 0.2 ml of 40% potassium hydroxide containing 0.3%

creatine was added per ml of broth culture of the test organism. Then shaking well and allowed to stand for 5-10 minutes to observe the color formation. Positive case was indicated by the development of a bright orange red color. In negative cases there was no development of pink color (Cheesbrough, 2006).

3.2.4.5 Indole test

The test organisms were cultured in test tubes having 3 ml of peptone water containing tryptophan at 37ºC for 48 hours. Then 1 ml of diethyl ether was added, shaked well and allowed to stand until the ether rises to the top. Then 0.5 ml of Kovac's reagent was gently run down the side of the test tube so that it forms a ring in between the medium and the ether layer and observed for the development of color of the ring. Development of a brilliant red colored ring indicated indole production. In negative case there is no development of red color (Cheesbrough, 2006).

3.2.4.6 Molecular identification of the isolated organism (*Escherichia coli*):

3.2.4.7 Bacterial DNA isolation:

a) Materials:

TE buffer
10% (w/v) sodium dodecyl sulfate (DSS)
20 mg/ml proteinase K (stored in small single-use aliquots -20^{0}C)
3 M sodium acetate, pH 5.2
25:24:1 phenol/chloroform/Isoamyl alcohol
Isopropanol
70% ethanol
90% ethanol
1.5 ml microcentrifuge tube

b) Procedure:

Inoculate a 25 ml of liquid culture with the bacterial strain of interest. Grow in conditions appropriate for that strain until the culture is saturated.

Spin 1.0 ml of overnight culture in a microcentrifuge tube for 5 minutes at 10000 rpm.

Discard the supernatant.

Repeat the step.

Resuspended the supernatant in 467µl TE Buffer by pipetting. Add 30 µl of 10% SDS & 3µl of proteinase K to give a final concentration of 100µg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 30min. to 1hr. at 37^0C.

Add and approximately equal volume of (500µl) of phenol/chloroform/Isoamyl alcohol. Mix thoroughly but carefully to avoid the mixing of DNA, by inverting the tube until the phases are completely mixed.

Then the centrifuge the tubes at 12000 rpm for 10 minutes.

Remove aqueous, viscous supernatant (~450 µl) to a fresh microcentrifugetube, leaving the interface behind. Add an equal volume of phenol/chloroform/Isoamyl alcohol, extract thoroughly and spin in a micro centrifuge at 10000 rpm for 5 min.

Transfer the supernatant to a fresh tube (~400 µl).

Add $1/10^{th}$ volume of 3m sodium acetate and mix.

Add 0.6 volumes of isopropanol to precipitate the nucleic acids, keep on ice for 10 minutes.

Centrifuge at 13500 rpm for 15 minutes.

Decant the supernatant.

Wash the obtained pellet with 1ml of 95% ethanol for 5 minutes. Then centrifuge at 12000 rpm for 10 minutes.

Decant the supernatant.

Dry the pellets as well as there are no alcohol.

Resuspend the pellet in 50µl of TE and stored DNA at 4^0C for short time and -20^0C for long term.

c) Polymerase Chain Reaction (PCR):

d) Primer used for PCR

Agenus specific PCR was to perform to amplify 16S rRNA of *E.coli* using previously published primers (Tsen et al., 1998) the list of primers are shown in table The reverse primer TTCCCGAAGGCACATTCT is used with the forward GGGAGTAAAGTTAATCCTTTGCTC to identify the pathogenic organism.

Table 3: PCR primers with sequence

Primer	Sequence(5´-3´**)**	Size(bp)	Reference
E.coli 16E1(F)	GGGAGTAAAGTTAATCCTTTGCTC	584bp	Tsen et al., 1998
E.coli 16E2(R)	TTCCCGAAGGCACATTCT		

F= Forwar R= Reverse bp= Base pair

e) Preparation for a PCR mixture

PCR mixture (25 µl) was prepared as follows:

Nuclease free water: 12.5µl

2x master PCR mix: 9.5µl

Forward primer: 0.5µl

Reverse primer: 0.5µl

DNA template: 2.0µl

f) Thermal profile

PCR reaction profile was prepared as follows:

Initial denaturation: 95^0C for 5 minute

Denaturation: 94^0C for 30 sec

Annealing: 60^0C for 30 sec

Extension: 72^0C for 30 sec

35 cycle

Final extension: 72^0C for 5 min

g) Electrophoresis

2% agarose (Sigma) gel was used for electrophoresis of the PCR products. The procedure of gel electrophoresis is given below:

Gel casting tray was assembled with gel comb of appropriate teeth size and number.

2% agarose solution was prepared in TBE buffer by melting in a microwave oven.

Molten agarose was poured on to the casting tray and allowed to solidify on the bench.

The hardened gel in its tray was transferred to the electrophoresis tank containing sufficient TBE buffer to cover the gel ~ 1mm.The comb was gently removed.

7 µl of each PCR product was mixed with 2-3 µl loading buffer and the sample was loaded to the appropriate well of the gel.

5 µl of DNA marker was loaded in a well.

The leads if the electrophoresis apparatus were connected to the power supply and the electrophoresis was run at 100V.

When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.

The stained in ethidium bromide (0.5 µg/ml) for 10 minutes, in a dark place.

The gel was destained in distilled water for 10 minutes.

The destained gel was placed on the UV transilluminator in the dark chamber of the image documentation system.

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

3.2.7 Maintenance of stock culture

Stock culture was prepared by adding 1ml of 80% sterilized glycerol in 1 ml of pure culture in nutrient broth and it was stored in -20^0C.

3.2.8 Antimicrobial sensitivity pattern of *E. coli* and *Salmonella* spp. isolated from oral and cloacal samples of pigeon

A total of 21 *E. coli* isolates & 11 *Salmonella* spp. Collected from 20 oral and 20 cloacal samples of pigeon were used for disc sensitivity testing. The antimicrobial sensitivity testing of each isolate was carried out by the Kirby-Bauer disc diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) procedures. Antibiotic sensitivity discs used were gentamicin (GM), azithromycin (AZM), levofloxacin (LVX), tetracycline (TE), ampicillin (AP), ciprofloxacin (CIP), erythromycin (E), amoxicillin (A), nalidixic acid (NA) and an antiprotozoal drug metronidazole (MET). This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent in the medium surrounding the disc. The suspension of the test organism was prepared in a test tube containing 5 ml nutrient broth by overnight incubation in shaking incubator. By micropipette 100µl of broth culture of the test organism was poured on Muller-Hinton agar plate. Sterile glass spreader was used to spread the culture homogenously on the medium. Inoculated plates were closed and allowed to dry for approximately 3-5 minutes. Then the antibiotic discs were applied aseptically to the surface of the inoculated agar plates at a special arrangement with the help of a sterile forceps. The plates were then inverted and incubated at 37ºC for 24hours.

After incubation the plates were examined and the diameter of the zone of complete inhibition was measured by mm scale. The zone diameters for individual antimicrobial agents were translated in to sensitive, intermediate and resistant categories by referring to an interpretation table.

CHAPTER 4

RESULTS & DISCUSSION

The results presented below demonstrated the isolation and identification of bacteria isolates from oral and cloacal swab samples of pigeon from different farm and households around Dhaka district. The results also the sensitivity and resistance pattern of the isolates to different drugs.

4.1 Total Viable Count and Total Coliform Count from the isolated sample

Table 4: Total Viable count and Total Coliform Count from the isolated sample

Serial no & Name of Sample	Total Viable Count (TVC)(CFU/ml)	Total Coliform Count (TCC) (CFU/ml)
1. P1	1.6×10^5	Nil
2. P2	1.8×10^5	Nil
3. P3	4.1×10^5	3.2×10^5
4. P4	2.3×10^5	2.1×10^5
5. P5	2.8×10^5	0.8×10^5
6. P6	3.6×10^9	Nil
7. P7	3.4×10^9	Nil
8. P8	2.4×10^9	2.96×10^9
9. P9	2.9×10^9	Nil
10. P10	2.7×10^9	6.5×10^9
11. P11	3.5×10^9	3.5×10^9
12. P12	1.1×10^9	Nil
13. P13	3.2×10^7	1.75×10^9
14. P14	2.7×10^7	Nil
15. P15	3.8×10^9	6.8×10^9
16. P16	3.1×10^7	4.1×10^9
17. P17	2.9×10^9	1.7×10^9
18. P18	0.7×10^5	Nil
19. P19	1.3×10^5	Nil
20. P20	1.4×10^5	Nil

Table 4 (cont'd)

21. P21	1.8×10^7	3.6×10^6
22. P22	2.0×10^7	5.7×10^9
23. P23	1.4×10^7	Nil
24. P24	2.6×10^7	3.1×10^6
25. P25	2.3×10^7	Nil
26. P26	2.2×10^9	3.7×10^9
27. P27	3.5×10^9	4.1×10^9
28. P28	3.7×10^9	Nil
29. P29	2.2×10^9	1.22×10^9
30. P30	2.5×10^9	Nil
31. P31	2.7×10^9	Nil
32. P32	3.4×10^9	Nil
33. P33	1.5×10^9	2.96×10^9
34. P34	1.8×10^5	Nil
35. P35	3.9×10^7	3.3×10^7
36. P36	2.8×10^7	3.1×10^7
37. P37	1.4×10^5	Nil
38. P38	4.6×10^7	2.9×10^7
39. P39	3.7×10^7	3.9×10^7
40. P40	1.3×10^5	Nil

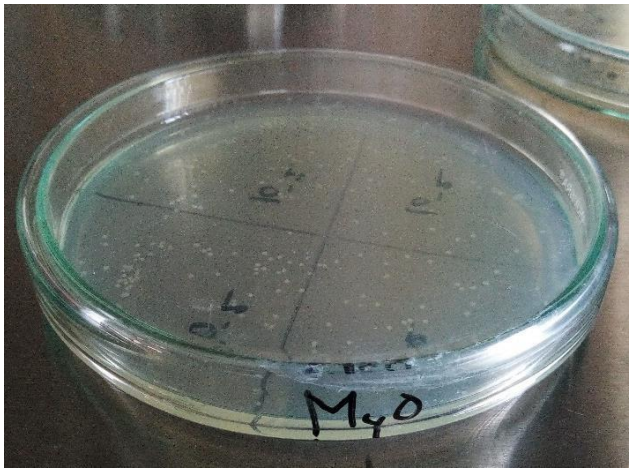

Figure 2: Total Viable count by 10 fold dilution method

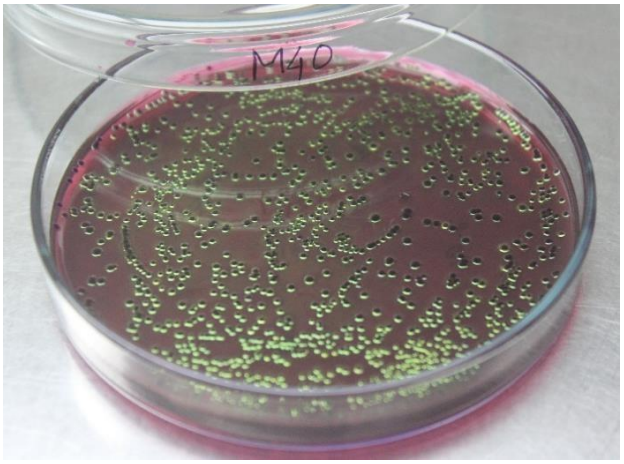

Figure 3: Total coliform count by10 fold dilution method

4.2 Prevalence of microorganism in pigeon:

Table 5: Prevalence of microorganism in pigeon

No. of sample investigated	No. of sample containing organism	Percentage (%) of prevalence
40	40	100

4.3 Percentage of Prevalence with specific organism from sample:

table 6: Percentage of Prevalence with specific organism from sample

No. of sample investigated	**No. of *E.coli* isolates with (Prevalence)**	**No. of *Salmonella* spp. isolates (with (Prevalence)**	**Non identified (P9, P12, P14, P18, P23, P25, P28, P32)**
40	21 (52.5%)	11 (27.5%)	8 (20%)

4.4 Prevalence percentage of *E.coli* & *Salmonella* sp. in oral (20) & cloacal sample (20):

Table 7: Prevalence percentage of *E.coli* & *Salmonella* sp. in oral & cloacal sample

Isolation or organism from oral (20) cloacal (20) sample	**Isolated organism**	**Prevalence percentage (%)**
Oral sample (P3, P4, P5, P8, P10, P11, P13, P15, P16, P17)	*E.coli*	50
Oral sample (P1, P2, P6, P7, P19, P20)	*Salmonella* spp.	30
Cloacal sample (P21, P22, P24, P26, P27, P29, P33, P35, P36, P38, P39)	*E.coli*	55
Cloacal sample (P30, P31, P34, P37, P40)	*Salmonella* spp.	25

4.5* Results of isolation and identification of *E.coli

4.5.1 Results of cultural examination

4.5.1.1 Culture in nutrient broth

All the *E. coli* isolates produced turbidity in nutrient broth.

4.5.1.2 Culture on MacConkey agar

Bright pink colored colonies on MacConkey agar produced by the organisms after overnight incubation were presumptively selected as *E. coli* (shown in Table 8, Figure 4).

4.5.1.3 Culture on Eosine Methylene Blue (EMB) agar

Greenish colonies with metallic sheen produced by the organisms on EMB agar after overnight incubation were tentatively confirmed as *E. coli.* Tentatively confirmed *E. coli* isolate from cattle produced Greenish red colonies with faint metallic sheen (shown in Table 8, Figure 5).

4.5.1.4 Culture on Brilliant green (BG) agar

Yellowish green colonies surrounded by an intense yellow green zone on BG agar produced by the organisms after overnight incubation were tentatively chosen as *E. coli* (shown in Table 8, Figure 6).

Table 8. Demonstration of the cultural characteristics of *Escherichia coli* isolated from oral and cloacal samples of pigeon in different agar media

Sources of *E. coli*	**Colony characteristics in different agar media**		
	MC agar	**EMB agar**	**BG agar**
Oral swab samples (P3, P4, P5, P8, P10, P11, P13, P15, P16, P17)	Bright pink colored colonies	Greenish colonies with metallic sheen	Yellowish green colored colonies
Cloacal swab samples(P21, P22, P24, P26, P27, P29, P33, P35, P36, P38, P39)	Bright pink colored colonies	Greenish colonies with metallic sheen	Yellowish green color colony

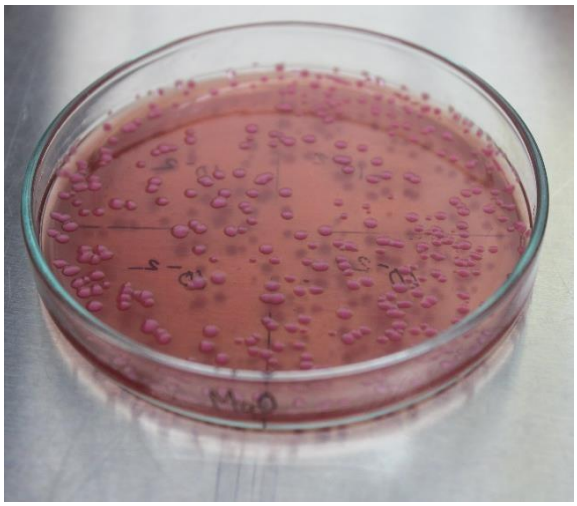

Figure 4: *E.coli* in MC agar media

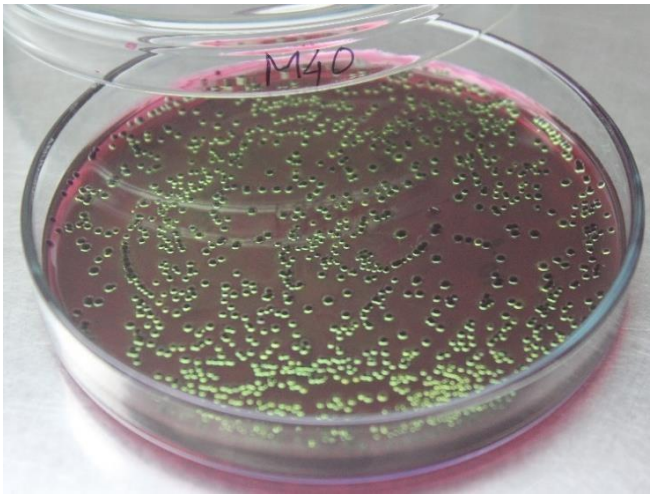

Figure 5: *E.coli* in EMB agar media

Figure 6: *E.coli* in BG agar media

4.5.1.5 Results of Gram's staining technique

Light microscopic examination after Gram's staining revealed Gram-negative, pink colored, rod shaped organisms arranged as single or in pair (shown in table 9, Figure 8).

4.5.1.6 Results of Hanging drop technique All the *E. coli* isolates were found to be motile with hanging drop slide preparation under microscopy.

4.5.1.7 Results of reactions in TSI agar slant

Yellow colored slant and yellow colored butt with accumulation of gas bubbles in the butt were found after inoculation and overnight incubation of the suspected *E. coli*

organisms in TSI agar slant. There was no Hydrogen Sulphide production hence no blackening of the butt (shown in Table 9, Figure 7).

Table 9. Demonstration of the morphology and staining characteristics, motility and reactions in TSI agar slant of *Escherichia coli* isolated from oral and cloacal samples of pigeon in different agar media

Sources of *E. coli*	Morphology and staining characteristics	Motility	Reactions in TSI agar slant
Oral swab samples (P3, P4, P5, P8, P10, P11, P13, P15, P16, P17)	Gram negative rod shaped organism arranged as single or in pair	+	Yellow slant and butt with gas but no H_2S production
Cloacal swab samples(P21, P22, P24, P26, P27, P29, P33, P35, P36, P38, P39)	Gram negative rod shaped organism arranged as single or in pair	+	Yellow slant and butt with gas but no H_2S production

Figure 7: *E.coli* in TSI agar slant

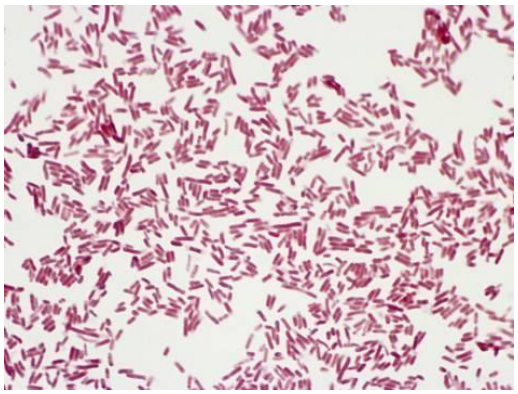

Figure 8: Gram negative rod shape organism (*E.coli*) at 100X magnification

4.5.1.8 Results of the biochemical tests

Tentatively confirmed *E. coli* isolates by colony characteristics, morphology and staining characteristics and by motility and reaction in TSI agar slant were subjected to different biochemical tests for identification.

4.5.1.9 Sugar fermentation test

All the *E. coli* isolates fermented five basic sugars with the production of acid and gas .Decreased production of acid and gas was observed in pigeon swab isolates during sucrose fermentation Acid production was indicated by the color change of the sugar

media from reddish to yellow and the gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube (shown in Table 10, Figure 9&10).

Table 10. Demonstration of the biochemical reactivity pattern of *E. coli* isolated from oral and cloacal swabs of pigeon

Sources of *E. coli*	**Fermentation properties with five basic sugars**					**M R test**	**V- P Test**	**Indole production test**	**Catalase test**	**Urease test**	**Citrate test**
	DX	**ML**	**L**	**S**	**MN**						
Oral swab samples	AG	AG	AG	A↓G↓	AG	+	–	+	+	–	–
Cloacal swab samples	AG	AG	AG	A↓G↓	AG	+	–	+	+	–	–

Legends: DX = Dextrose; ML = Maltose; L = Lactose; S = Sucrose; MN=Mannitol; A = Acid production; G = Gas production; A↓ = Less acid production; G↓ = Less Gas production; + = Positive reaction; - = Negative reaction.

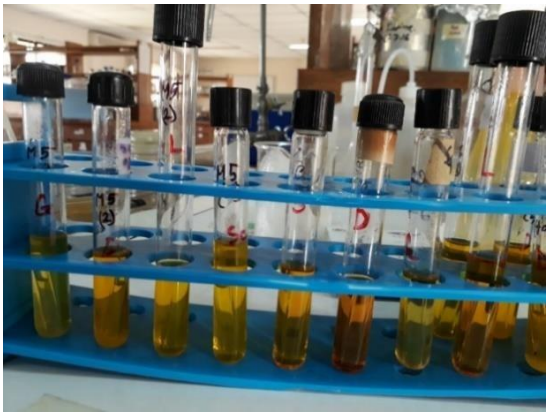

Figure 9: Production of acid and gas in sugar fermentation test for *E.coli*

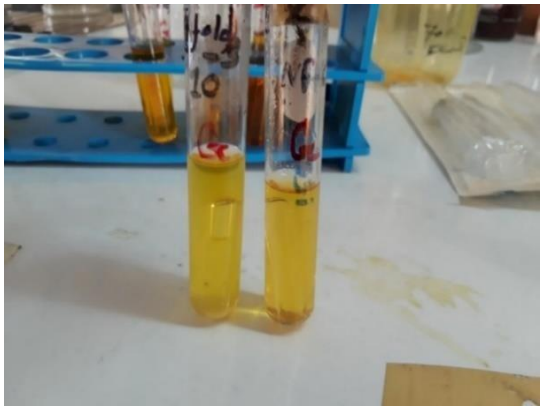

Figure 10: Production of acid and gas in sugar Fermentation test positive for *E. coli*

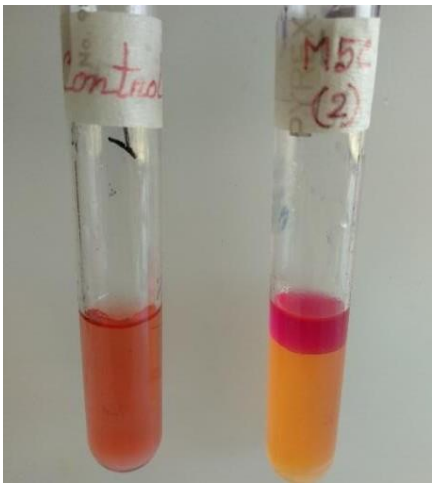

Figure 11: Indole production test (positive) for *E.coli*

Figure 12: Urease test (negative) for *E.coli*

Figure 13: Citrate utilization test (negative) for *E.coli*

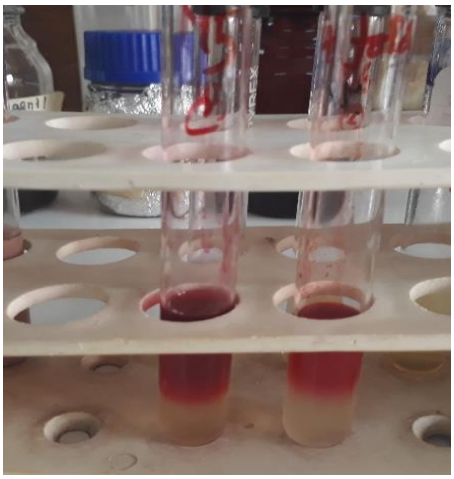

Figure 14: Methyl red test positive for *E.coli*

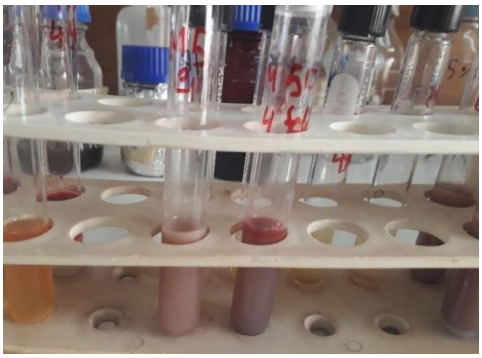

Figure 15: Voges-proskauer test (negative) for *E.coli*

4.5.1.10 other bioche

All the isolates were Catalase test positive (Table 10, Figure 16), Indole test positive (Table 10, Figure 11), Urease test (Table 10, Figure 12), Citrate test (Table 10, Figure 13), Methyl red (MR) test positive (Table 10, Figure 13,), Voges- Proskauer (V-P) test negative (Table 10, Figure 15) and The above mentioned pattern of biochemical reactions were considered as *E. coli*.

4.5.1.11 Molecular identification of *E. coli*

For molecular identification, DNA was extracted from isolated *E coli*. Then PCR was performed followed by electrophoresis in agarose gel. About 584bp DNA was amplified from 16S rRNA gene using the primer GGGAGTAAAGTTAATCCTTTGCTC as forward and TTCCCGAAGGCACATTCT as reverse for the identification of pathogenic E.coli organism All the isolates were found positive in PCR. A representative figure is shown below. Positive result found for the pathogenic organism and negative result found for the other reverse primer.

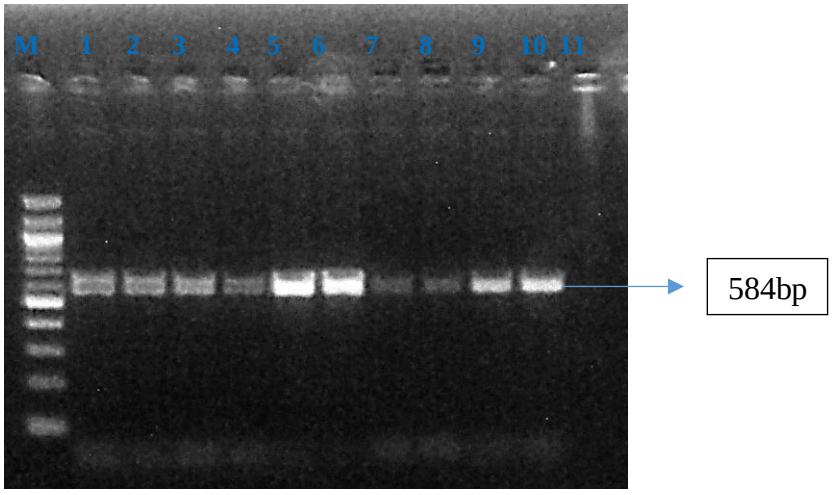

Figure 17: Amplification of 584bp DNA from 16S rRNA gene of *E coli*. Lane M: 100bpDNA Marker, Lane 1-10: Test sample, Lane 11: Negative control

4.2 Results of isolation of *Salmonella* spp.

Table 11: Results of isolation of *Salmonella* spp.

Name of the samples	Total *Salmonella* count(TSC) (CFU/ml)
1. P1 (Oral swab)	1.6×10^5
2. P2 (Oral swab)	1.8×10^5
3. P6 (Oral swab)	3.6×10^9
4. P7 (Oral swab)	3.4×10^9
5. P19 (Oral swab)	1.3×10^5
6. P20 (Oral swab)	1.4×10^5
7. P30 (Cloacal swab)	2.5×10^9
8. P31 (Cloacal swab)	2.7×10^9
9. P34 (Cloacal swab)	1.8×10^5
10. P37 (Cloacal swab)	1.4×10^5
11. P40 (Cloacal swab)	1.3×10^5

4.2.1 Identification of *Salmonella* spp.: The isolated samples are arranged as follows. The total *Salmonella* count was same as the total viable count

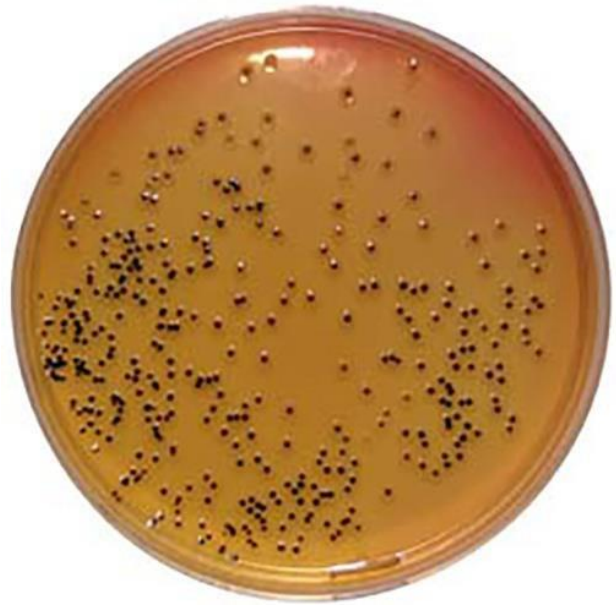

Figure 18: Total *Salmonella* count (TSC) in SS agar media

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

4.2.3 Results of cultural examination

4.2.3.1 Culture in nutrient broth

All *Salmonella* spp. isolates produced turbidity in nutrient broth.

4.2.3.2 Culture on MacConkey agar

Red to pink-white colonies surrounded by brilliant red zones after overnight incubation were presumptively selected as *Salmonella* spp. (shown in Table 12, figure 19).

4.2.3.3 Culture on Eosine Methylene Blue (EMB) agar

Grey color colonies produced by the organisms on EMB agar after overnight incubation were tentatively confirmed as *Salmonella* spp. (shown in table 12, Figure 20).

Table 12. Demonstration of the cultural characteristics of *Salmonella* Isolated from pigeons in different agar media

Sources of *Salmonella* spp.	**Colony characteristics in different agar media**		
	MC agar	**EMB agar**	**SS agar**
Oral swab samples (P1, P2, P6, P7, P19, P20)	Red to pink-white colonies surrounded by brilliant red zones	Grey colour colony	Colonies with black centers
Cloacal swab samples (P30, P31, P34, P37, P40)	Red to pink-white colonies surrounded by brilliant red zones	Grey colour colony	Colonies with black centers

Figure 19: *Salmonella* spp. in MacConkey agar media

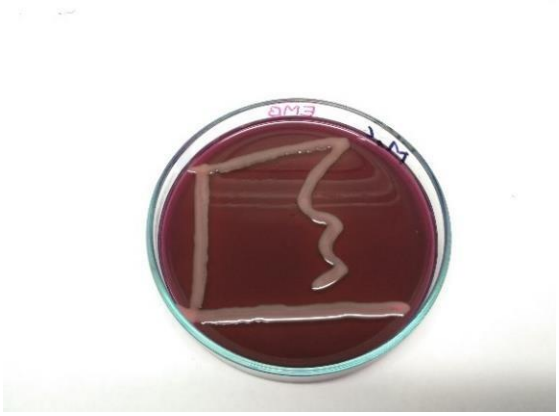

Figure 20: *Salmonella* spp. in EMB agar media

Figure 21: *Salmonella* spp. in SS agar media

4.2.3.4 Culture on Salmonella-Shigella agar

Colonies with Hydrogen Sulphide production after overnight incubation. Produced by the *Salmonella* spp. isolated from oral and cloacal samples (shown in Table 12, Figure 21).

4.2.3.5 Results of Gram's staining technique

Light microscopic examination after Gram's staining revealed Gram-negative, pink colored, rod shaped organisms arranged as single or in pair.

4.2.3.6 Results of Hanging drop technique

All the *Salmomella* spp. isolates were found to be motile with hanging drop slide preparation under microscopy.

4.2.3.7 Results of reactions in TSI agar slant

Yellow colored slant and black colored butt with accumulation of gas bubbles in the butt were found after inoculation and overnight incubation of the suspected *Salmonella* spp. organisms in TSI agar slant. There was presence of Hydrogen Sulphide production that's why blackening of the butt (Figure 22).

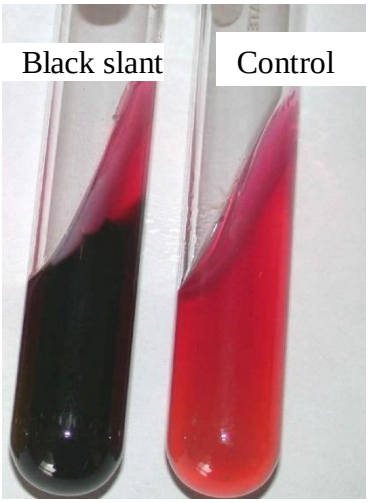

Figure 22: TSI agar slant positive test for *Salmonella* spp.

4.2.3.8 Results of the biochemical tests

Tentatively confirmed *Salmonella* spp. isolates by colony characteristics, morphology and staining characteristics and by motility and reaction in TSI agar slant were subjected to different biochemical tests for identification.

Table 13. Demonstration of the biochemical reactivity pattern of *Salmonella* spp. isolated from pigeon.

Sources of *Salmonella* spp.	**Fermentation properties with five basic sugars**					**M R test**	**V- P Test**	**Indole production test**	**Citrate**
	DX	**ML**	**L**	**S**	**MN**				
Oral swab samples	AG	AG	NF	NF	A↓G	+	–	–	+
Cloacal swab samples	AG	AG	NF	NF	AG	+	–	–	+

Legends: DX = Dextrose; ML = Maltose; L = Lactose; S = Sucrose; MN=Mannitol; A = Acid production; G = Gas production; A↓ = Less acid production; NF =No Fermentation G = Gas production; + = Positive reaction; - = Negative reaction.

4.2.3.9 Sugar fermentation test

The result carbohydrate fermentation test of *Salmonella* spp. was performed by inoculating a loopful of thick bacterial culture into the tubes containing five basic sugars (dextrose, maltose, sucrose, lactose, and mannitol) and incubated at 37^0C for 24 hours. Acid production was indicated by the change of media from pink to yellow color and gas production was indicated by the appearance of gas bubbles in the inverted Durham's fermentation tubes (Table 13, Figure 23).

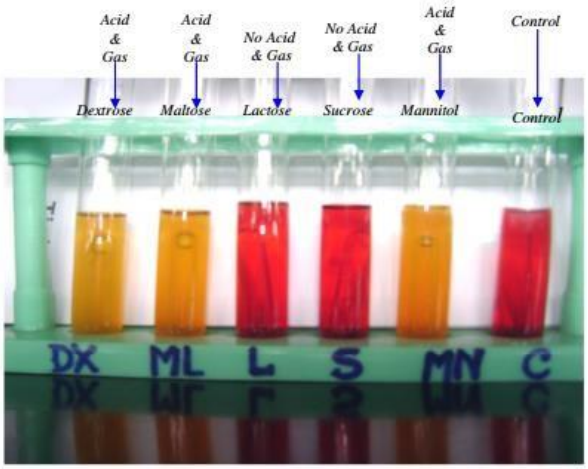

Figure 23: Sugar fermentation test of *Salmonella* spp.

Figure 24: Indole test positive for *Salmonella* spp.

Figure 25: Citrate test positive (On left) for *Salmonella* spp,

Figure 26: Methyl Red positive for *Salmonella* spp.

4.2.3.10 Result of Biochemical test

All the isolates were, Indole test positive (Table 13, Figure 24), Citrate test (Table 13, Figure 25), Methyl red (MR) test positive (Table 13, Figure 26).The above mentioned pattern of biochemical reactions were considered as *Salmonella* spp.

4.4 Results of drug sensitivity and resistance pattern of *E. coli & Salmonella* spp. isolated from pigeon sample

A total of 21 different *E. coli* isolates collected from 40 different samples from pigeon (oral and cloacal swab) were used for drug sensitivity testing. Nine different drugs were used for disc diffusion method test.

A large number of *E. coli* isolates from pigeon samples were found sensitive to LEV (67%), AZM (63%), CIP (62%) and GM (48%). A little number was sensitive to AMX & NA (19%) and AMP & TE (15%). None of the isolate showed sensitivity to ERY. The highest resistance was against ERY (62%) Without any kinds sensitivity (either complete or intermediate). They showed comparatively higher resistance against AMP (71%), AMX (62%) & TET (52%). Comparatively lower resistance was showed by them against NA, AZM, and GM 24%, 23%, and 29%, respectively. Most of the isolates showed intermediate sensitivity against NA (57%). Against ERY & CIP they have shown the equal intermediate sensitivity about (38%) and GM, TET and LEV against (33%). Against AMP & AZM they shown lower intermediate sensitivity (14%) and against AMX the Intermediate sensitivity was 19% (Table 14, Figure 27).

In comparison between the *E.coli* from oral and cloacal swab samples, *E.coli* from the oral swab sample showed higher sensitivity against LEV (70%) and the cloacal samples shows against AZM (82%). The oral isolates of *E.coli* showed comparatively higher sensitivity against CIP (60%) & cloacal isolates showed against CIP, GM and LEV was (64%). Complete sensitivity against ERY was (0%) in both isolates. Lower sensitivity found in case of oral isolates to AZM, GM, AMX, NA, AMP, TET was 40, 30, 20, 20, 10, 10% respectively. (Table 15, Figure 28) In case of cloacal isolates lower sensitivity was found

against TET, NA, AMP and AMX was 46, 20, 18 and 18%, respectively. Complete & intermediate sensitivity found against CIP and LEV in isolates from both type of samples. Oral isolated has shown higher resistance against TET (70%) & comparatively higher against AMP (60%) and ERY (50%) and lower resistance against NA, AZM, AMX which was 40% and GM which was 20%. On the other hand cloacal isolates showed higher resistance against AMX, AMP and ERY was 82, 82 and 72%, respectively. NA, TET, and AZM relatively in lower percentage viz. 40, 36, 9, respectively. (Table 16, Figure 29) Intermediate sensitivity showed by oral isolates which was 50% against ERY and GM, 40% against NA, CIP and AMX, 30% against AMP & LEV, 20% against AZM & TET. The cloacal samples showed no intermediate sensitivity AMX & AMP, 36% was intermediate sensitive against CIP & LEV, 18% was intermediate sensitive against GM & TET. NA showed 40% intermediate sensitivity against cloacal sample and 28% against ERY.

A total of 11 different *Salmonella* isolates collected from 40 different samples from pigeon (oral & cloacal swab).

A large number of *Salmonella* isolates from pigeon samples were found sensitive to CIP (82%), AMP (55%), LEV (55%) and GM (55%), AMX (46%), AZM (46%). A little number was sensitive to ERY 19%. No resistance was found against NA & TET. The highest resistance was against TET (100%) & NA 82%. none of the isolates showed Intermediate sensitivity & complete sensitivity. They showed comparatively lower resistance against ERY, AMX, AMP, AZM, CIP, LEV which was 46, 36, 27, 27, 52, 18% respectively. Most of the isolates showed intermediate sensitivity against GM (45%) & intermediate sensitivity against ERY was 36%. Against AZM & LEV they have shown the equal intermediate sensitivity about (27%). Intermediate sensitivity against AMX, AMP and NA was 18, 18 & 18% respectively. Against CIP & LEV they didn't shown any intermediate sensitivity. (Table 17, Figure 30)

In comparison between the *Salmonella* sp. from oral and cloacal swab samples, *Salmonella* sp. from the oral sample showed higher sensitivity to CIP (83%) and the cloacal samples shows against AZM (80%). The oral isolates of *Salmonella sp.* showed comparatively

higher sensitivity against GM, AMX, LEV, AMP, ERY and AZM was 67, 50, 50, 50, 50, 20 & 33.33% respectively. Complete sensitivity of cloacal isolates against AMP, AZM and LEV was 60, 60 & 60% respectively. The sensitivity against NA & TET was 0% and 0% in both isolates. Oral isolates showed lower sensitivity to AZM & ERY which was 33.33% & 20%. (Table 18, Figure 31).

Cloacal isolates showed lower sensitivity against GM, AMX and ERY which was 40, 40 & 20% respectively. Complete sensitivity found against CIP & GM in oral samples and in cloacal sample it was GM & LEV. Oral isolated has shown 100% resistance against TET. Oral isolates showed higher sensitivity against NA (83%) and lower resistance against AZM, AMP, AMX, and LEV was 33.33, 33, 30, 17% respectively. On the other hand the cloacal isolates showed higher resistance against TET & NA was 100% & 80%. Relatively lower resistances of cloacal sample against AMP, AZM, CIP, AMX, ERY was 20, 20, 20, 40, 40% relatively. (Table 19, Figure 32) Intermediate sensitivity found against oral isolates was 17-33 % overall. GM showed 60% intermediate sensitivity against cloacal sample and rest was around 20-40 %.

Table 14: Demonstration of the sensitivity and resistance pattern of different *E. coli* isolates to different drugs in percentage

Name of the sample / Different drugs	P3	P4	P5	P8	P10	P11	P13	P15	P16	P17	P21	P22	P24	P26	P27	P29	P33	P35	P36	P38	P39	Percentage (%) of resistance to different drugs		
																						R	IN	S
AMX	R	IN	IN	R	IN	IN	R	S	S	R	R	S	S	R	R	R	R	R	R	R	R	62	19	19
AMP	R	R	IN	R	R	IN	R	S	IN	R	R	S	S	R	R	R	R	R	R	R	R	71	14	15
AZM	IN	R	S	S	R	S	IN	R	R	S	IN	R	S	S	S	S	S	S	S	S	S	23	14	63
ERY	R	R	IN	R	IN	IN	IN	IN	R	R	IN	R	R	R	R	IN	R	R	R	R	IN	62	38	0
NA	IN	R	S	IN	R	S	R	R	IN	IN	S	R	IN	IN	IN	IN	IN	IN	IN	IN	S	24	57	19
CIP	S	S	S	S	IN	S	IN	IN	IN	S	IN	S	S	IN	S	S	IN	S	IN	S	S	0	38	62
GM	IN	R	IN	IN	R	IN	IN	S	S	S	R	S	S	S	S	S	S	S	IN	R	IN	19	33	48
TET	R	R	R	R	IN	IN	R	R	S	R	R	R	S	R	IN	R	IN	IN	IN	S	IN	52	33	15
LEV	IN	S	S	S	S	S	IN	S	IN	S	S	IN	IN	S	S	S	IN	S	S	IN	S	0	33	67

Legends: GM = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; TET = Tetracycline; AMP = Ampicillin; ERY = Erythromycin; AMX = Amoxicillin; NA = Nalidixic Acid; CIP = Ciprofloxacin; S = sensitive; IN = intermediate; R = resistant; P3, P4, P5, P8, P10, P11, P13, P15, P16, P17= Oral swab; P21, P22, P24, P26, P27, P29, P33, P35, P36, P38, P39= Cloacal swab

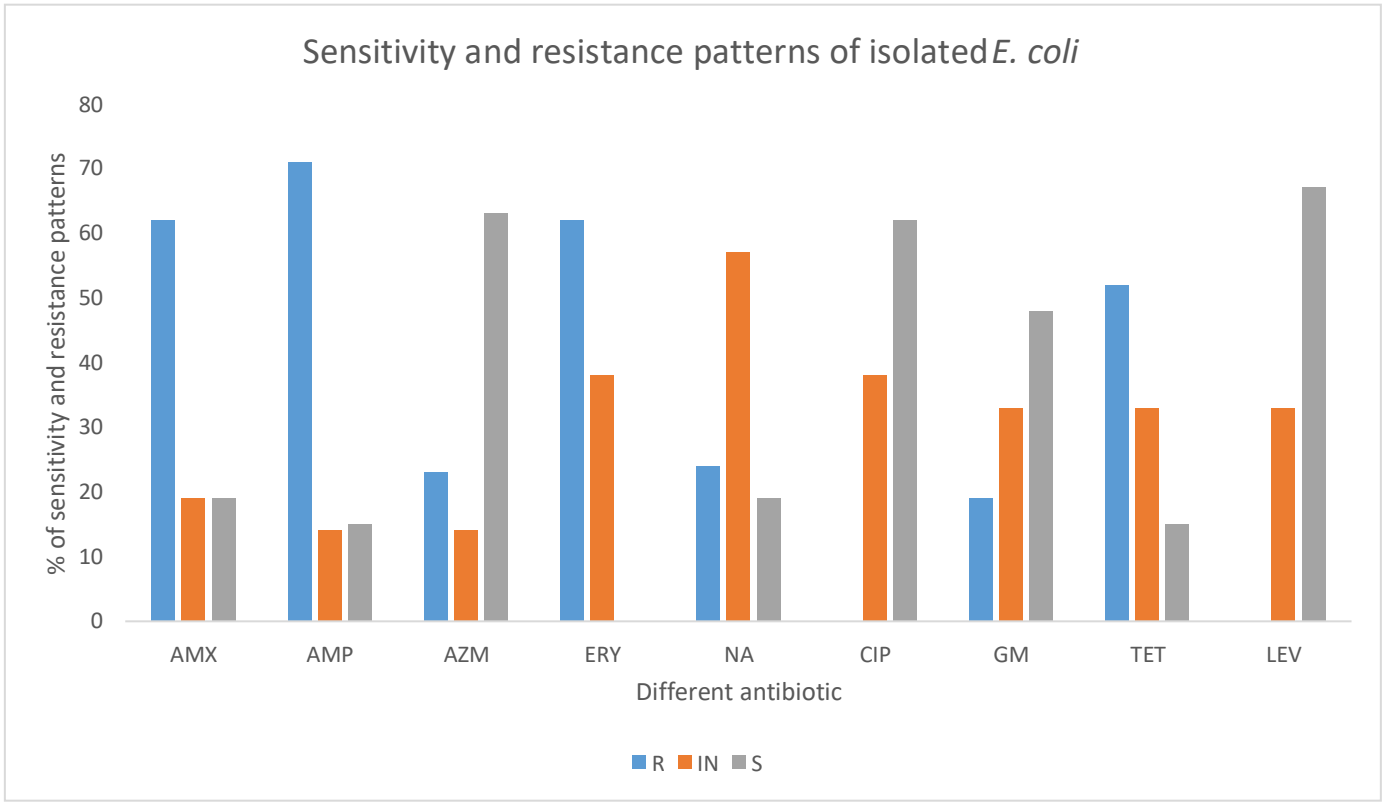

Figure 27: Diagram showing the antibiotic sensitivity pattern of *E.coli* isolated from swab samples of pigeon

Table 15: Demonstration of the sensitivity and resistance pattern of different *E. coli* isolates to different drugs in percentage obtained from oral swab

Oral swab / Different drugs	P3	P4	P5	P8	P10	P11	P13	P15	P16	P17	Percentage (%) of resistance to different drugs		
											R	IN	S
AMX	R	IN	IN	R	IN	IN	R	S	S	R	40	40	20
AMP	R	R	IN	R	R	IN	R	S	IN	R	60	30	10
AZM	IN	R	S	S	R	S	IN	R	R	S	40	20	40
ERY	R	R	IN	R	IN	IN	IN	IN	R	R	50	50	0
NA	IN	R	S	IN	R	S	R	R	IN	IN	40	40	20
CIP	S	S	S	S	IN	S	IN	IN	IN	S	0	40	60
GM	IN	R	IN	IN	R	IN	IN	S	S	S	20	50	30
TET	R	R	R	R	IN	IN	R	R	S	R	70	20	10
LEV	IN	S	S	S	S	S	IN	S	IN	S	0	30	70

Legends: GM = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; TET = Tetracycline; AMP = Ampicillin; ERY = Erythromycin; AMX = Amoxicillin; NA = Nalidixic Acid; CIP = Ciprofloxacin; S = sensitive; IN = intermediate; R = resistant; P3, P4, P5, P8, P10, P11, P13, P15, P16, P17= Oral swab

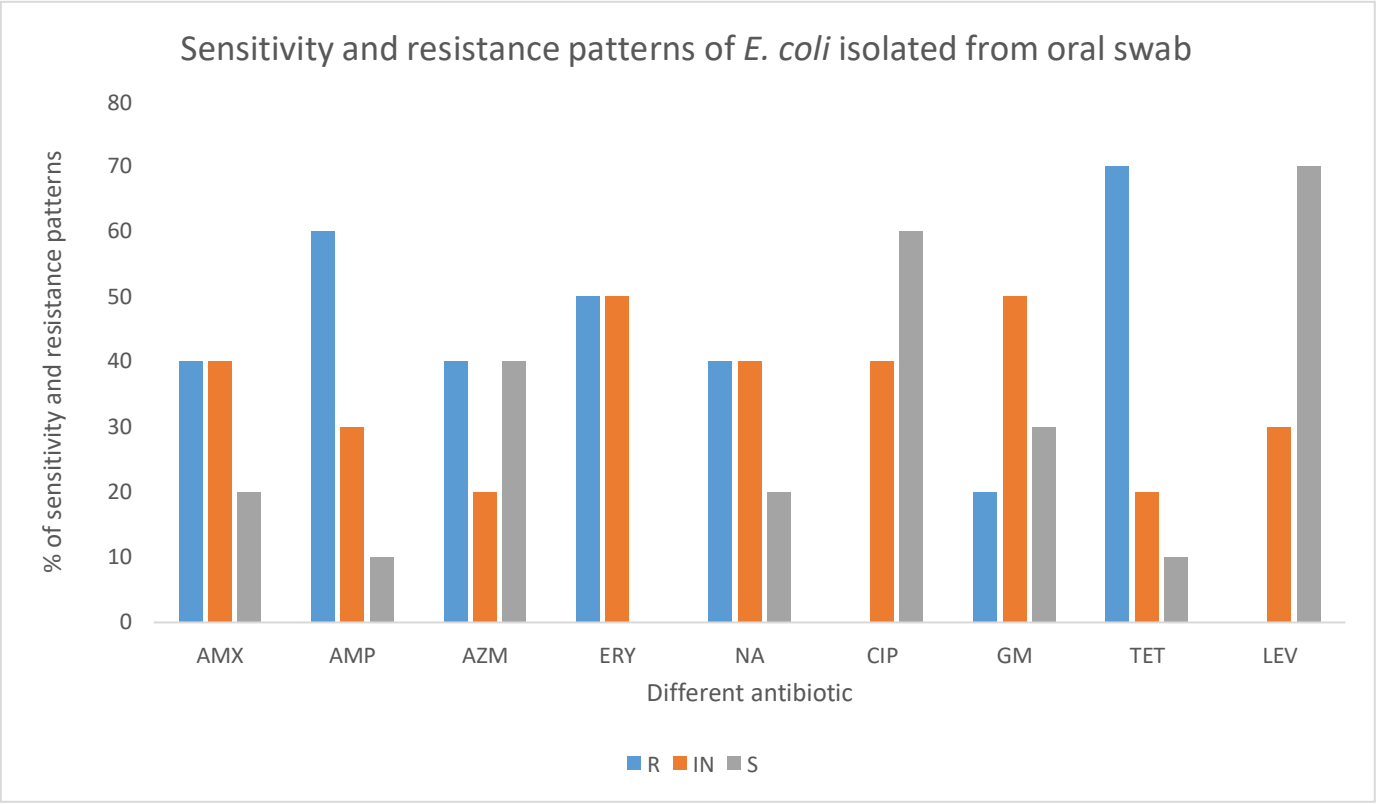

Figure 28: Diagram showing the antibiotic sensitivity pattern of *E.coli* isolated from oral swab samples of pigeon

Table 16: Demonstration of the sensitivity and resistance pattern of different *E. coli* isolates to different drugs in percentage obtained from cloacal swab

Cloacal swab / Different drugs	P21	P22	P24	P26	P27	P29	P33	P35	P36	P38	P39	Percentage (%) of resistance to different drugs		
												R	IN	S
AMX	R	S	S	R	R	R	R	R	R	R	R	82	0	18
AMP	R	S	S	R	R	R	R	R	R	R	R	82	0	18
AZM	IN	R	S	S	S	S	S	S	S	S	S	9	9	82
ERY	IN	R	R	R	R	IN	R	R	R	R	IN	72	28	0
NA	S	R	IN	IN	IN	IN	IN	IN	IN	IN	S	40	40	20
CIP	IN	S	S	IN	S	S	IN	S	IN	S	S	0	36	64
GM	R	S	S	S	S	S	S	S	IN	R	IN	18	18	64
TET	R	R	S	R	IN	R	IN	IN	IN	S	IN	36	18	46
LEV	S	IN	IN	S	S	S	IN	S	S	IN	S	0	36	64

Legends: GM = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; TET = Tetracycline; AMP = Ampicillin; ERY = Erythromycin; AMX = Amoxicillin; NA = Nalidixic Acid; CIP = Ciprofloxacin; S = sensitive; IN = intermediate; R = resistant; P21, P22, P24, P26, P27, P29, P33, P35, P36, P38= Cloacal swab

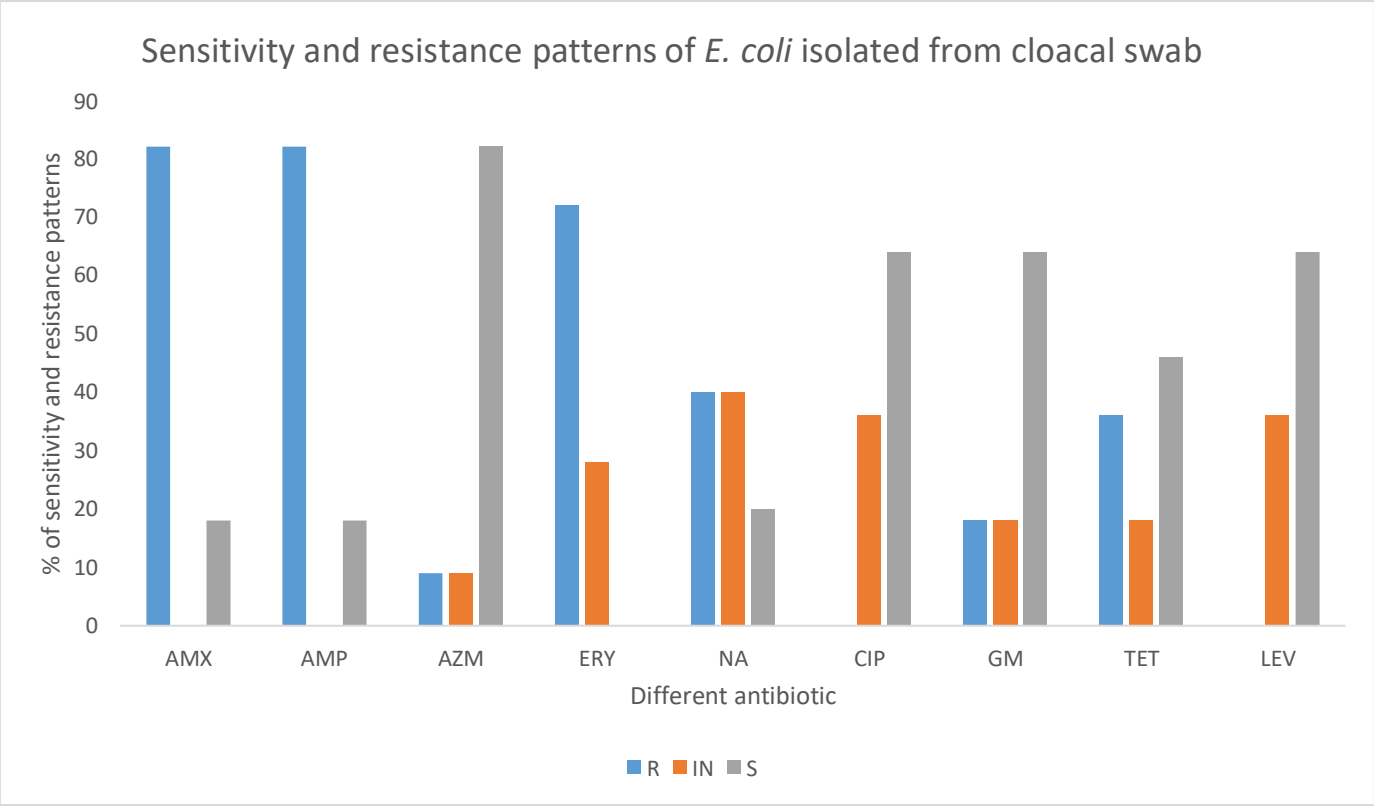

Figure 29: Diagram showing the antibiotic sensitivity pattern of *E.coli* isolated from cloacal swab samples of pigeon

Table 17: Demonstration of the sensitivity and resistance pattern of different *Salmonella* spp. isolates to different drugs in percentage obtained from swab samples of pigeon

Name of the sample / Different drugs	P1	P2	P6	P7	P19	P20	P30	P31	P34	P37	P40	Percentage (%) of resistance to different drugs		
												R	IN	S
AMX	S	S	IN	R	R	S	S	S	R	R	IN	36	18	46
AMP	S	R	R	S	S	IN	S	IN	S	S	R	27	18	55
AZM	S	S	IN	R	IN	R	S	R	IN	S	S	27	27	46
ERY	R	IN	S	R	R	IN	IN	R	R	S	IN	46	36	18
NA	R	R	R	IN	R	R	R	R	IN	R	R	82	18	0
CIP	S	S	S	S	S	IN	S	IN	S	S	S	18	0	82
GM	S	S	S	IN	IN	S	IN	S	IN	S	IN	0	45	55
TET	R	R	R	R	R	R	R	R	R	R	R	100	0	0
LEV	S	R	IN	S	S	R	IN	S	S	IN	S	18	27	55

Legends: GM = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; TET = Tetracycline; AMP = Ampicillin; ERY = Erythromycin; AMX = Amoxicillin; NA = Nalidixic Acid; CIP = Ciprofloxacin; S = sensitive; IN = intermediate; R = resistant; P1, P2, P6, P7, P19, P20= Oral swab, P30, P31, P34, P37, P40 = Cloacal swab

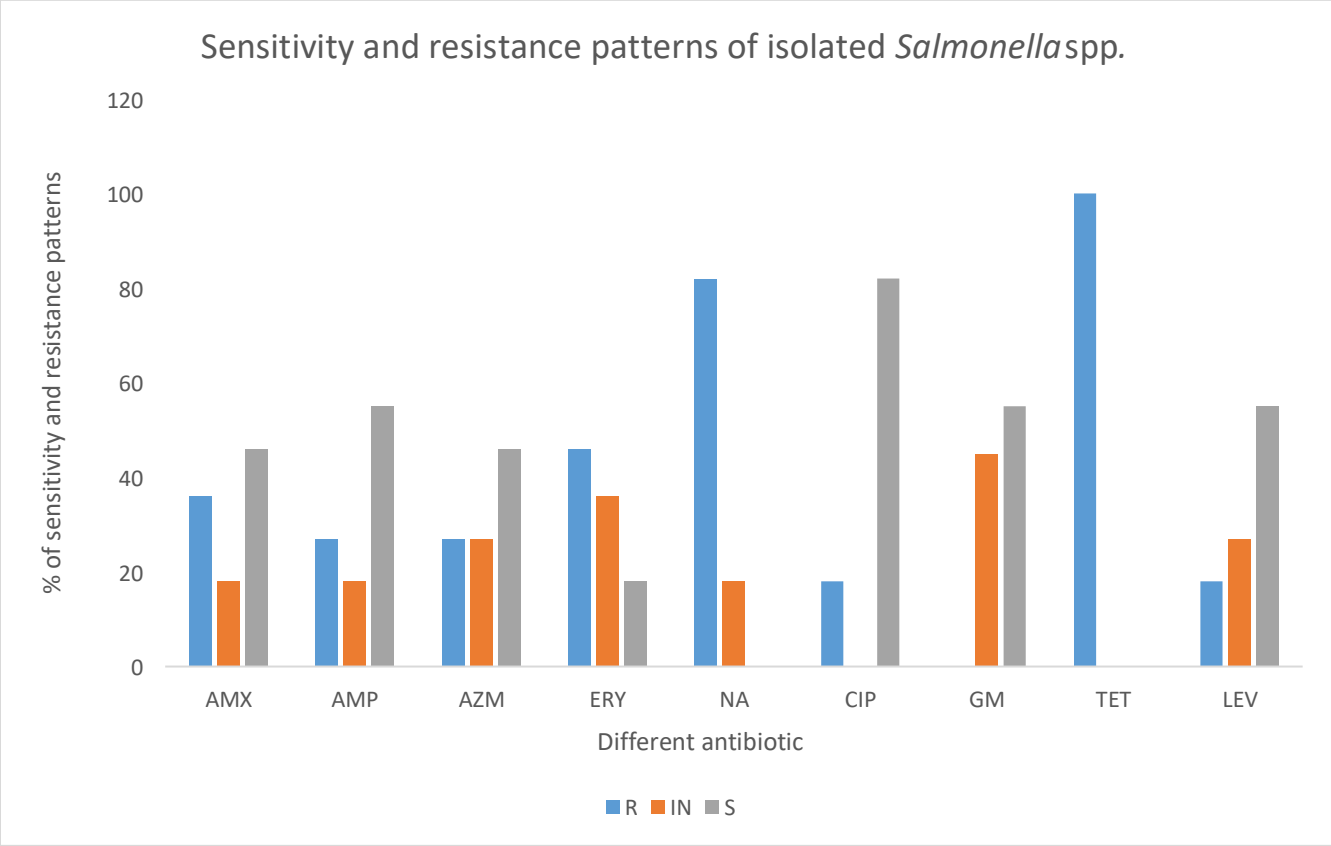

Figure 30: Diagram showing the antibiotic sensitivity pattern of *Salmonella* spp. isolated from swab samples of pigeon

Table 18: Demonstration of the sensitivity and resistance pattern of different *Salmonella* spp. isolates to different drugs in percentage obtained from oral swab samples of pigeon

oral swab samples / **Different drugs**	P1	P2	P6	P7	P19	P20	Percentage (%) of resistance to different drugs		
							R	IN	S
AMX	S	S	IN	R	R	S	33	17	50
AMP	S	R	R	S	S	IN	33	17	50
AZM	S	S	IN	R	IN	R	33.33	33.33	33.33
ERY	R	IN	S	R	R	IN	50	30	20
NA	R	R	R	IN	R	R	83	17	0
CIP	S	S	S	S	S	IN	0	17	83
GM	S	S	S	IN	IN	S	0	33	67
TET	R	R	R	R	R	R	100	0	0
LEV	S	R	IN	S	S	R	17	33	50

Legends: GM = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; TET = Tetracycline; AMP = Ampicillin; ERY = Erythromycin; AMX = Amoxicillin; NA = Nalidixic Acid; CIP = Ciprofloxacin; S = sensitive; IN = intermediate; R = resistant; P1, P2, P6, P7, P19, P20= Oral swab, P30, P31, P34, P37, P40 = Cloacal swab

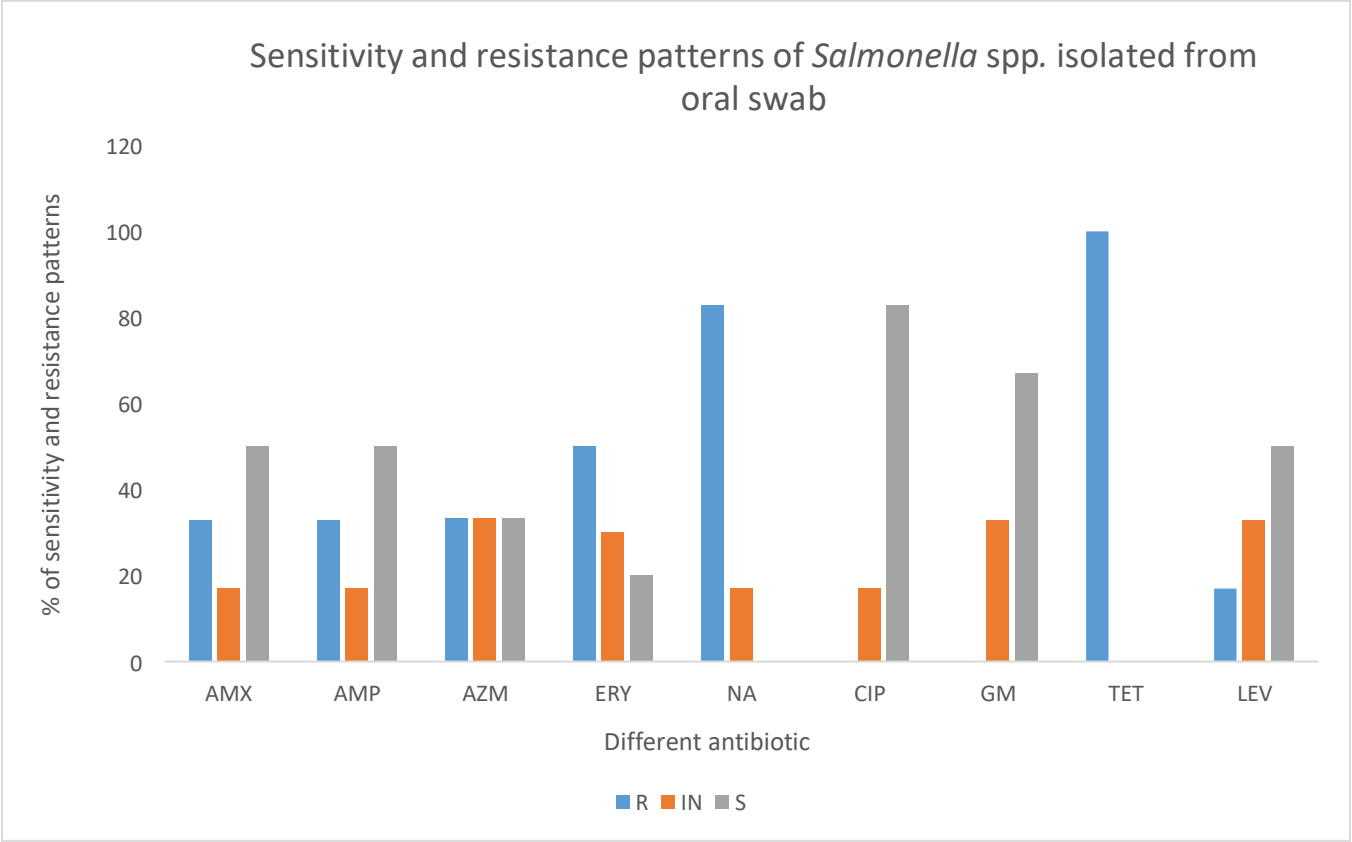

Figure 31: Diagram showing the antibiotic sensitivity pattern of *Salmonella* spp. isolated from oral swab samples of pigeon

Table 19: Demonstration of the sensitivity and resistance pattern of different *Salmonella* spp. isolates to different drugs in percentage obtained from cloacal swab samples of pigeon

Cloacal swab samples / **Different drugs**	P30	P31	P34	P37	P40	Percentage (%) of resistance to different drugs		
						R	IN	S
AMX	S	S	R	R	IN	40	20	40
AMP	S	IN	S	S	R	20	20	60
AZM	S	R	IN	S	S	20	20	60
ERY	IN	R	R	S	IN	40	40	20
NA	R	R	IN	R	R	80	20	0
CIP	S	IN	S	S	S	20	0	80
GM	IN	S	IN	S	IN	0	60	40
TET	R	R	R	R	R	100	0	0
LEV	IN	S	S	IN	S	0	40	60

Legends: GM = Gentamicin; AZM = Azithromycin; LEV = Levofloxacin; TET = Tetracycline; AMP = Ampicillin; ERY = Erythromycin; AMX = Amoxicillin; NA = Nalidixic Acid; CIP = Ciprofloxacin; S = sensitive; IN = intermediate; R = resistant; P1, P2, P6, P7, P19, P20= Oral swab, P30, P31, P34, P37, P40 = Cloacal swab

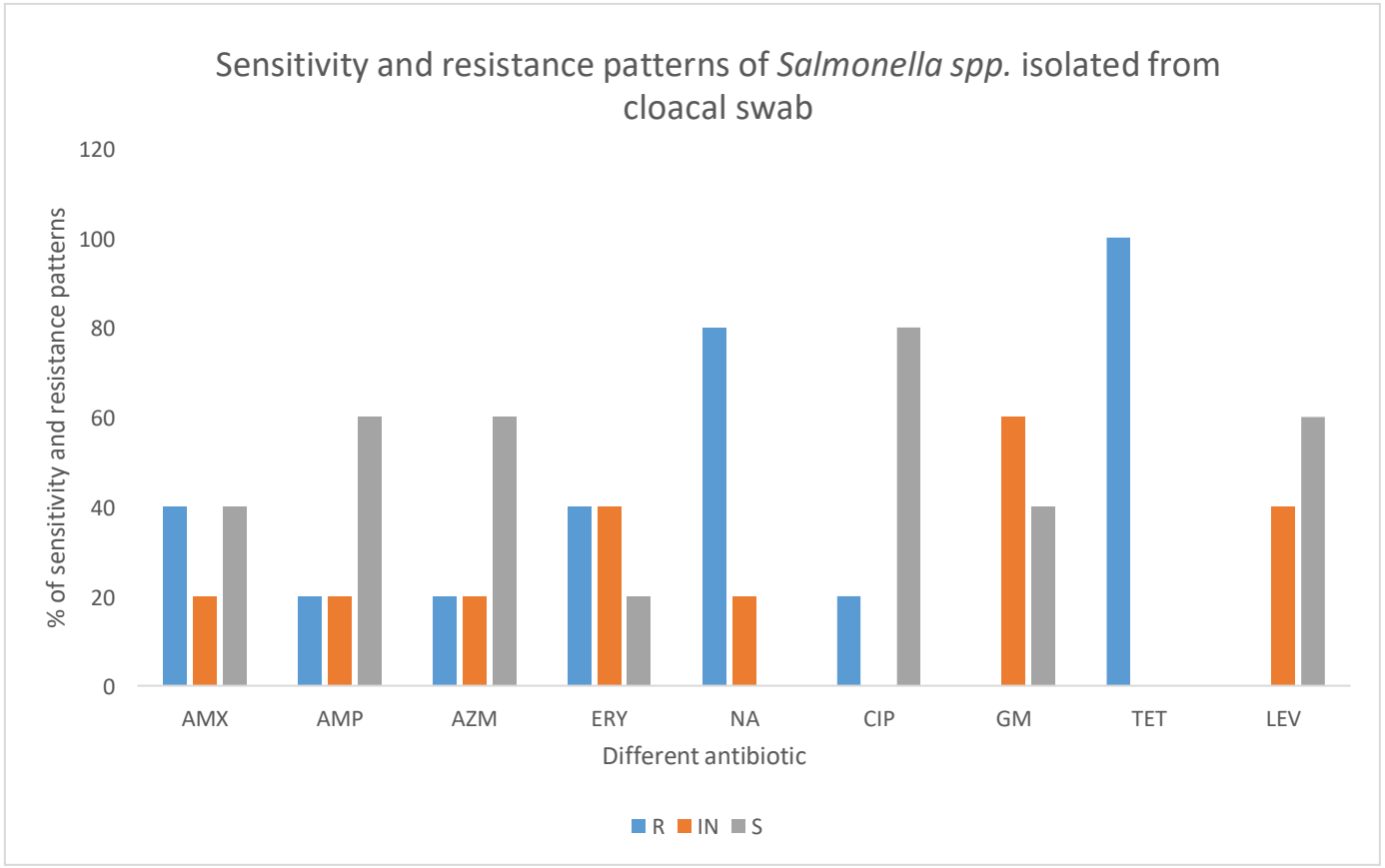

Figure 32: Diagram showing the antibiotic sensitivity pattern of *Salmonella* spp. isolated from cloacal swab samples of pigeon

4.5 Discussion

The present study was conducted primarily for the isolation and identification of the *E.coli* and *Salmonella* spp. isolated from swab samples of pigeon in Dhaka and around Dhaka city and also to determine the current status of drug sensitivity and resistance pattern of the isolates to determine the drug of choice for therapeutic use against infection caused by these organisms.

Isolation and identification results of the study indicated that the selected samples contained Gram negative and motile organisms (*E.coli* & *Salmonella* spp.). Colony characteristics of *E.coli* in three different agar media and fermentation ability with five basic sugars were similar with a bit exception. Interesting findings of the colony characteristics of the isolates were also observed. All the *E. coli* isolates were able to produce characteristic metallic sheen colony on the EMB agar, bright pink colony on MacConkey agar, yellowish green colonies surrounded by an intense yellow green zone on BG agar. In Gram's staining, the morphology of the isolated bacteria exhibited pink, small rod shaped Gram negative bacilli and in the hanging drop technique all the isolates revealed motile. Molecular identification was done for *E.coli* identification showed positive result 16S rRNA identification band at 584bp. similar result was found in a previous study by (Rawal *et al.,* 2013) from lake water. All the *Salmonella* isolates were able to produce Red to pink-white colonies surrounded by brilliant red zones in MacConkey agar media, grey color in EMB agar & Colonies with black centers in SS agar. In Gram's staining, the morphology of the isolated bacteria exhibited pink, small rod shaped Gram negative bacilli and in the hanging drop technique all the isolates revealed motile. These findings were supported by several authors such as (Buxton and Fraser, 1977), (Freeman, 1985) and (Jones *et al.,* 1987). Another fundamental basis for the identification of *E. coli* & *Salmonella* organism was determining the ability or inability of fermentation of five basic sugars with acid and gas production. However, species identification and differentiation by fermentation reaction was difficult (Freeman, 1985) and showed similar reactions in different sugars (OIE Manual, 2000). All the *E.coli* isolates from pigeon revealed a complete fermentation of five basic sugars as stated by (Mckec *et al.,* 1995), (Shandhu and Clarke, 1996) and (Beutin *et al.,* 1997). However differentiation of *Salmonella* into species

level was difficult based on their sugar fermentation pattern. All the isolates of this study fermented dextrose, maltose and mannitol and produced acid and gas but did not ferment sucrose and lactose which satisfied the statement of (Buxton and Fraser, 1977), (Hossain, 2002) and (Han *et al.*, 2011). In the present study, the isolated *E. coli* organisms fermented dextrose, maltose, lactose, sucrose and mannitol with the production of both acid and gas. Less production of acid and gas during sucrose fermentation was found. (Ewing *et al.*, 1973) and (Ali *et al.*, 1998) also studied the biochemical characteristics of the different strains of *E. coli* isolated from different sources. They reported a little or no difference in these biochemical characters and stated that such similarity among the isolates might be due to presence of some common genetic materials.

The results of Catalase, MR and indole test of the *E. coli* isolates were positive but V-P test was negative as reported by (Buxton and Fraser, 1977).The result of Indole test for *Salmonella* was negative (formation of yellow ring), MR positive & result of V-P test was negative which satisfy the statement of (Buxton and Fraser, 1977).

In present study, the prevalence of microorganism in pigeon was 100% among 40 samples (20 oral & 20 cloacal) all were infected with different microorganisms. 21 samples among 40 samples were infected with *E.coli*, 11 samples were infected with Salmonella and rest 8 samples were unidentified. The infection with E.coli was (52.5%). Infection with E.coli for oral & cloacal sample was respectively 50% & 55%. In a previous study (Dey *et al.*, 2013) found overall prevalence of *E. coli* in pigeon was 69.64%, (Dutta et. al., 60.67 %) samples were found positive for *E. coli* infection, (Zigo *et. al.*, 2017) found *E. coli* (50.8%) from cloacal swab & (25.3%) from oral swab. On the other hand (Abulreesh, 2011) found only 2.5% were positive to shiga-toxin producing *E. coli* from rock pigeon. The prevalence of *Salmonella* in pigeon was (27.5%) & Infection with *Salmonella* for oral & cloacal sample was respectively 30% & 25%. In previous studies (Rahman *et al.*, 2016) found *Salmonella* spp. could be isolated from 39.58% duck and 28.57% pigeon samples, (Akbarmehr, 2010) found 15.55% prevalence of *Salmonella* in pigeon & (Hosain *et al.*, 2012) found 35.71% prevalence. The rest 20% sample was unidentified for any specific organism.

Bacteria can overcome the effect of drugs used for the treatment purpose by producing various enzymes and metabolites that either degrade the antimicrobial agents or help the bacteria to survive through various mechanisms. So, the current status of sensitivity and resistance pattern of the *E. coli* & *Salmonella* isolates to different drugs should be determined to choose the best drug for treatment purpose. To perform this study a total of 21 *E. coli* isolates from 40 pigeon (oral + cloacal) samples, were subjected to antimicrobial sensitivity test which was carried out by disc diffusion method. Nine different drugs were used for this study. The sensitivity test revealed that most of the *E. coli* isolates, from pigeon oral and cloacal samples were sensitive to LEV and AZM followed by GM and CIP. In terms of resistance, most of the isolates were resistant to AMP, AMX & ERY followed by TET, NA, AZM and GM. In the present study a high percentage of *E. coli* isolates, from pigeon samples were sensitive to LEV (67%) and AZM (63%) followed by CIP and GM showing 62% and 48% sensitivity respectively. On the other hand, 71% of the isolates were resistant to AMP and 62% to AMX followed by TET (52%), NA (24%), AZM (23%) and GM (19%). A previous study of (Zigo *et al.,* 2017) showed increased frequency of tetracycline (82.3%), ampicillin (48.1%) and amoxicillin (45.1%), Gentamicin (6.3%) resistant strains of *E. coli.* Previous study about antimicrobial resistant *E. coli* strains in domestic pigeons were studied by (Kimpe, 2002), in Belgium the high levels of resistance were found among *E. coli* isolates, while resistance to tetracycline (detected in 50% of the isolates) was the most prevalent. In another study conducted by (Ishiguro, 1978) in domestic pigeons in Japan were isolated strains of *E. coli* resistant to tetracycline, streptomycin, sulfonamides and quinolones and concluded that high antimicrobial resistance. (Hassan *et al.,* 2008) the most strains were frequently highly resistance to trimethoprim-sulphmethoxazol (89.5%) followed by ampicillin (84.2%), tetracycline (78.9%), erythromycin (78.9%) and doxycycline (73.6%).

In this study a number of isolates also showed intermediate reaction to NA (57%), CIP & ERY (38%), GM, TET & LEV (33%), AMX (19%) and AMP & AZM (14%). Intermediate Sensitivity drugs could not be compared due to lack of relevant literature.

In the present study, all the *E. coli* isolates from pigeon oral found to be sensitive to LEV (70%), CIP (60%) followed by AZM (40%), GM (30%), NA (20%), AMP (10%) and TET (10%) showing marked resistance to TET (70%), AMP (60%) & ERY (50%). Specific

sensitivity pattern of oral swab sample cannot be compared due to lack of relevant literature.

In the present study, all the *E. coli* isolates from pigeon cloacal found to be sensitive to AZM (82%) and CIP, LEV, GM was (60%) followed by TET (46%), NA (20%), AMX (18%) and AMP (18%). showing marked resistance to AMX, AMP & ERY was 82%, 82% & 72% respectively which were in a previous study (Zinnah *et al.,* 2008) as sensitive to LVX (90%) and CIP (80%) followed by GM (60%) and AZM (50%) but highly resistant to other drugs such as MET (100%), NA (90%), TET (80%), AMX (80%), ERY (90%) and AMP (70%).

The intermediate sensitivity shown by the oral isolates of pigeon was against ERY & GM (50%), AMX (40%), NA (40%), CIP (40%), AMP (30%), LEV (30%), AZM (20%), and TET (20%). This may be an intermediate phage for the conversion of *E. coli* isolates from sensitive to resistant form. Intermediate sensitivity drugs could not be compared due to lack of relevant literature.

11 *Salmonella* isolates from 40 pigeon (oral + cloacal) samples were subjected to antimicrobial sensitivity test which was carried out by disc diffusion method. Nine different drugs were used for this study. The sensitivity test revealed that most of the *Salmonella* isolates, from pigeon oral and cloacal samples were sensitive to CIP followed by GM, LEV, AMP, AMX and AZM. In terms of resistance, most of the isolates were resistant to TET, NA & ERY followed by AMX, AMP, AZM, CIP and LEV.

In the present study a high percentage of *Salmonella* isolates, from pigeon samples were sensitive to CIP (82%) followed by AMP, GM and LEV showing 55%,55% and 55% sensitivity respectively. On the other hand, 100% of the isolates were resistant to TET and 82% to NA followed by ERY (46%), AMX (36%), AMP (27%), AZM (27%), CIP (18%) and GM (18%). In a previous study (Saifullah *et al.,* 2016) revealed multi-drug resistance *Salmonella*. The highest resistance was found against Ampicillin (88.23%) followed by Cephalexin (82.35%). The rate of sensitivity of the isolates was higher to Ciprofloxacin (100%) followed by Azithromycin (82.35%) Gentamicin (76.47%) and Nalidixic acid (76.47%). In this section, the variation was found in the sensitivity pattern of *Salmonella* isolates against AMP, AZM, GM and AZM.

A number of isolates also showed intermediate reaction to GM (45%), ERY (36%), AZM & LEV (27%), AMX (18%) and AMP (18%) & NA (18%).

In the present study, all the *Salmonella* isolates from pigeon oral found to be sensitive to CIP (83%), GM (67%) followed by LEV, AMX & AMP (50%), AZM (33.33%) and ERY (20%) showing marked resistance to TET (100%), NA (83%) & ERY (50%) which were contradictory with the findings of (Saifullah *et al.,* 2016). They showed that the isolates were 12.5%, 62.5%, 37.5%, 25% and 0% resistant to GEN, AMP, CN (Cephalexin), NA and AZM respectively.

In the present study, all the *Salmonella* isolates from pigeon cloacal found to be sensitive to CIP (80%) and AMP, AZM, LEV was (60%) followed by AMX (40%), GM (40%) and ERY (20%) .In a previous study (Hosain *et al.,* 2012) revealed that 80% of the *Salmonella* isolates were sensitive to ciprofloxacin followed by sulphamethoxazole (70%), chloramphenicol (60%), kanamycin (60%), gentamicin (60%) and nalidixic acid (60%).. On the other hand 90% of the Salmonella isolates were found resistant to amoxicillin (90%), followed by ampicillin (80%), erythromycin (80%) and tetracycline (60%).

The intermediate sensitivity shown by the cloacal isolates of pigeon was GM (60%), ERY (40%), LEV (20%), AMX (20%), AMP (20%), and NA (20%). This may be an intermediate phage for the conversion of *Salmonella* isolates from sensitive to resistant form.

The significance of occurrence of antibiotic resistance in food-borne pathogens has increased sharply and probably linked with the extensive use of antimicrobial agents in veterinary medicine and human (Bronzwaer *et al.,* 2002). Several species of *Salmonella* spp. are known to carry multi drug resistant genes (Gebreyes and Altier, 2002) which have been a matter of concern. Based on the present study, it may be concluded that LVX and CIP will be the first drugs of choice and GM and AZM will be the second drugs choice to resist the infections caused by *E. coli* & *Salmonella* in pigeon and as well as human, cattle, sheep, goat, chicken and duck.

It may be noted that the determination of drug sensitivity and resistant pattern may be valuable as background information for the use of future therapeutics to control the bacterial diseases effectively. Otherwise, indiscriminate use of antimicrobial drugs may

lead to the development of drug resistant mutants causing serious health hazards of different animals and birds including human being. However, routine laboratory isolation and drug sensitivity determination of the organisms is impracticable. So, periodical checking of the drug sensitivity and resistance pattern of the organisms remains more important to select the best drug of choice for the treatment of diseases caused by the infectious diseases.

CHAPTER 5

SUMMARY AND CONCLUSION

The present study was conducted for isolation and identification of *E.coli* and *Salmonella* spp. microorganisms from pigeon and also to perform a comparative study to determine the sensitivity and resistance pattern of the isolates to different antimicrobial agents.

After collection, the samples were subjected to various tests and experiments for isolation and identification of zoonotic organism in pigeon. It is reported that *E.coli* and *Salmonella*. Primary isolation was performed by propagating the organisms in nutrient broth followed by culture on different agar media such as MacConkey agar, EMB agar, BG agar and SS agar for the determination of their colony characteristics. A total of 40 from 20 birds where oral and cloacal samples were regarded as two different sample. Total Viable Count (TVC) and Total Coliform Count (TCC) was done by 10 fold dilution method. Isolates was found among them 21 was *E.coli* and 11 was *Salmonella* and the rest 8 couldn't be identified in this study. They were identified on the basis of colony morphology. Gram's staining technique and hanging drop technique was performed and reaction in TSI agar slant was also observed. Biochemical properties of the isolates were studied by fermentation test with five basic sugars and also by Catalase test, MR test, V-P test and Indole production test.

The study was also extended to investigate in vitro sensitivity and resistance pattern of the *E. coli* & *Salmonella* spp. isolates to different drugs. Study revealed that there were considerable variations among the isolates of different sources in respect of drug sensitivity and resistance pattern.

A high percentage of *E. coli* isolates from the pigeons were sensitive to LVX and CIP followed by GM and AZM while most of the *E. coli* isolates were resistant to AMP, AMX, ERY, TET and NA.

In case of *Salmonella* isolated good sensitivity found against CIP followed by AMP, GM, and LEV while most of the *Salmonella* spp. isolates were resistant to NA, ERY, AMX and complete resistance was found against TET.

It is assumed that one or more drug resistant clones have gradually acquired resistance to other drugs by conjugation with multi-drug resistant strains.

From the present study it may be concluded that

(a) Oral and Cloacal samples collected from the pigeons from the farm and markets near Dhaka are infected with *E.coli* and *Salmonella* spp. . Identified bacteria from the pigeons was *E.coli* and *Salmonella*.

(b) *E. coli* infections of different animals and birds and also of human being may be treated effectively with LVX and CIP followed by GM and AZM. Infection with *Salmonella* spp. can be treated with CIP followed by AMP, GM and LEV.

Indiscriminate use of antimicrobial agents should be avoided in order to eliminate health hazards in man and animals caused by *E. coli* & *Salmonella* through preventing the development of multi-drug resistant mutants in nature.

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APPENDIX I

Composition of different media

1. Nutrient broth

peptic digest of animal tissue	5.0 gm
Sodium chloride	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Distilled water	1000 ml
Final pH (at 25ºC)	7.4 ± 0.2

2. Nutrient Agar

Peptone	5.000 gm
Sodium chloride	5.000 gm
HM peptone B#	1.500 gm
Yeast extract	1.500 gm
Agar	15.000 gm
Final pH (at 25°C)	7.4±0.2

3. MacConkey Agar

Peptones (meat and casein)	3.000 gm
Pancreatic digest of gelatin	17.000 gm
Lactose monohydrate	10.000 gm
Bile salts	1.500 gm
Sodium chloride	5.000 gm
Crystal violet	0.001 gm
Neutral red	0.030 gm
Agar	13.500 gm
pH after sterilization(at 25°C)	7.1±0.2

4. Eosin Methylene Blue Agar

Peptic digest of animal tissue	10.000 gm
Dipotassium phosphate	2.000 gm
Lactose	5.000 gm
Sucrose	5.000 gm
Eosin - Y	0.400 gm
Methylene blue	0.065 gm
Agar	13.500 gm
Final pH (at 25°C)	7.2±0.2

5. Brilliant Green Agar Medium

Peptone	5.000 gm
Tryptone	5.000 gm
Yeast extract	3.000 gm
Lactose	10.000 gm

Sucrose	10.000 gm
Sodium chloride	5.000 gm
Phenol red	0.080 gm
Brilliant green	0.0125 gm
Agar	20.000 gm
pH after sterilization (at 25°C)	6.9±0.2 gm

6. Salmonella-Shigella agar

Proteose peptone	5.000 gm
Lactose	10.000 gm
Bile salts mixture	8.500 gm
Sodium citrate	8.500 gm
Sodium thiosulphate	8.500 gm
Ferric citrate	1.000 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	13.500 gm
Final pH (at 25°C)	7.0±0.2

7. Mueller Hinton Agar

HM infusion B from	300.000
Acicase	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.4±0.1

8. Methyl Red Indicator

Methyl red	0.200 gm
Ethyl alcohol	60.000 ml
Distilled water	40.000 ml

9. Voges–Proskauer (MR-VP) broth

Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9±0.2

10. Phosphate buffer saline

Sodium chloride	8.0 gm
Disodium hydrogen phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water to make	1000 ml