

**ANTIBIOTIC RESIDUES IN CHICKEN MEAT AND STATUS OF
ANTIBIOTIC RESISTANCE IN *E. COLI* ISOLATED FROM
DIFFERENT ECOSYSTEMS OF CHHATTISGARH**

Ph.D. THESIS

By

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**DEPARTMENT OF VETERINARY PUBLIC HEALTH AND EPIDEMIOLOGY
COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDARY, ANJORA
DAU SHRI VASUDEV CHANDRAKAR KAMDHENU
VISHWAVIDYALAYA, DURG (C.G.)**

2021

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Thesis

**Submitted to the
DAU SHRI VASUDEV CHANDRAKAR KAMDHENU
VISHWAVIDYALAYA, DURG**

By

Choodamani Chandrakar

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF**

**DOCTOR OF PHILOSOPHY
IN
VETERINARY PUBLIC HEALTH**

March, 2021

ROLL NO. -201705004

I.D. NO. – K140117003

VITA

The author of this thesis, Choodamani Chandrakar was born on December 28th, 1987 at Gunderdehi block, District Balod (C.G.). He passed his higher secondary examination in the year 2005 with first division from Govt. higher secondary school Anda, District Durg (C.G.). In July 2009 he started his career in veterinary profession for his B.V. Sc. and A.H. from College Of Veterinary Science and Animal Husbandry Anjora, Durg (C.G.) and completed it in July 2014 with OGPA of 7.92/10.00 and in year 2016 he completed his master degree in Veterinary Public Health by securing first division with distinction from College Of Veterinary Science and Animal Husbandry Anjora, Durg (C.G.). Due to his keen interest in research work towards public health, he joined doctoral degree programme in the Department of Veterinary Public Health and Epidemiology in August 2017. He has completed the requisite course works for Ph.D. program.

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CERTIFICATE-I

This is to certify that the thesis entitled “**Antibiotic residues in chicken meat and status of antibiotic resistance in *E. coli* isolated from different ecosystems of Chhattisgarh**” submitted in partial fulfillment of the requirements for the degree of “**Doctor of Philosophy**” of Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Durg, is a record of bonafide research work carried out by **Choodamani Chandrakar**, under my guidance and supervision. The subject of the thesis has been approved by Student’s Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (certificate awarded etc.) or has been published/published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by him.

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CERTIFICATE-II

This is to certify that the thesis entitled “**Antibiotic residues in chicken meat and status of antibiotic resistance in *E. coli* isolated from different ecosystems of Chhattisgarh**”. Submitted by **Choodamani Chandrakar** to the Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Durg, in partial fulfillment of the requirements for the degree of “**Doctor of Philosophy**” in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science and Animal Husbandry, Anjora, Durg, has been approved by the Student’s Advisory Committee after oral examination in collaboration with the external examiner.

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ACKNOWLEDGEMENT

First of all I bow my head towards "**The Almighty**", the one above all of us, God, for answering all my prayers, for giving me energy, endurance and showered many blessings to accomplish my endeavours.

With Regardful memories.....

It is a matter of pleasure to glance back and recall the path one traverses during the days of hard work and pre-perseverance. It is still great at this juncture to recall all the faces and spirit in the form of teachers, friends, near and dear ones. I would consider this work nothing more than incomplete without attending to the task of acknowledging the overwhelming help I received during this endeavour of mine.

I would like to express my deepest sense of appreciation and regards to my major advisor **Dr. Sanjay Shaky**, Professor, Department of Veterinary Public Health and Epidemiology and Chairman of Advisory Committee, for his patience, motivation, enthusiasm, constructive criticism, moral support, discipline, simplicity, immense knowledge, meticulous supervision, friendly nature and continuous unstoppable encouragement during the entire course of research work. I'm privileged to have guide like him.

In this regard, I wish to prompt my profound sense of gratitude to **Dr. S. K. Tiwari**, Dean, College of Veterinary Science and Animal Husbandry, Anjora, Durg for providing necessary facility to conduct this study successfully.

I am very thankful to members of my advisory committee, **Dr. O. P. Mishra**, Professor and Head, Department of Veterinary Physiology and Biochemistry, **Dr. S. D. Hirpurkar**, Professor and Head, Department Of Veterinary Microbiology, **Dr. S. L. Ali**, Professor and Head, Department of Clinical Medicine, **Dr. Anil Patyal**, Assistant Professor, Department of Veterinary Public Health and Epidemiology for their generous help, cooperation, suggestions and support during the entire course of my research work. I shall ever stay indebted to them.

I extend my amiable thanks to, **Dr. Tripti Jain**, Assistant professor, Animal Biotechnology Center and **Dr. Asit Jain**, Assistant Professor, Department of Animal Breeding and Genetics, for providing their time, knowledge and continuous appreciation, boosted me to complete my work efficiently.

I can't express my gratitude in words to the person's helped me in collection of samples from different regions. I would also like to thanks **Dr.D. Bhonsle**, Associate Professor Department of Livestock Production Management, **Dr. N. Kumbhakar**, Principal, Veterinary Polytechnic, Jagadapur, **Dr. D. Suryam Dora**, Subject Matter Specialist, KVK, Kanher, **Dr. A.K. Pandey**, Kananpendari Zoo, Bilaspur, **Dr. Pankaj Sai**, Veterinary Assistant Surgeon, Manora, Jashpur, **Dr. Upendra Washist**, Veterinary Assistant Surgeon, Kavardha and **Dr. Tarun Ramteke**, Veterinary Assistant Surgeon, Rajnandgaon, help a lot in sample collection during this Covid pandemic situation.

I am greatly thankful to Dr. Nitin Gade, Assistant Professor, Department Of Veterinary Physiology And Biochemistry and Dr. Smita, Veterinary Assistant Surgeon, Rajnandgaon (In service Ph.D. Scholar, VPH&E) for their valuable suggestions, encouragement and constructive help throughout the course of study.

I would like to thanks Dr. Chandrahas Sannat, Assistant Professor, Department Of Veterinary Microbiology and Dr. Nidhi Rawat, Assistant Professor, Department Of Veterinary Microbiology, Dr. Amit Gupta Ph.D. Scholar, Department Of Veterinary Microbiology for their valuable suggestion and time to time advice.

I am heartily thankful to my seniors Dr. Ajay Chaturvedani, and Dr. Sudhir Jaisawal for his consistent support, encouragement every time and during my work, I can't forget to acknowledge, help of my beloved brothers Jagendra Kumar Chandrakar, Moolchand Chandrakar and Ravikant Chandrakar.

I am pleased to thank my seniors, Dr. Bhumika and Dr. Vivek Kumar Naik for their encouragement, suggestions, help and care.

My sincere thanks to my juniors Dr. Rizwan, Dr. Diksha, Dr. Ankit Shukla, Dr. Gamini, Dr. Tripti, Dr. Siddhant and Dr. Kavita.

The technical assistance provided by Mr. Lalit Deshmukh, Mr. Rajendra Yadav and Mr. Sunderlal Dewangan, from the Department of Veterinary Public Health and Epidemiology and Late Mr. Thakur (Electrician, VCA) were highly acknowledged.

I am thankful to Sahu bhaiya for preparing this manuscript nicely.

I can't imagine to complete this work without the support of my family. My profound regards to my Mummy-Papa, Mama-Mami and other family members.

Finally, I express my sincere thanks to all those who helped me either directly or indirectly at various stages during the tenure of this study.

Anjora, Durg

Choodamani Chandrakar

March, 2021



*Dedicated To My
Beloved Family & Respected Guide*

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LIST OF ABBREVIATIONS

Abbreviations	Full forms
ADI	acceptable daily intake
α	alpha
AMX	amoxycillin
AMP	ampicillin
ANOVA	analysis of variance
~	approximately
@	at the rate
bp	base pair
β	beta
<i>bla</i>	beta-lactamase
CFM	cefexime
CTX-M	cefotaxime hydrolysing
CAZ	ceftazidime
CTC	chlortetracycline
CIP	ciprofloxacin
CAC	codex alimentarius commission
$^{\circ}\text{C}$	degree celsius
dNTP	deoxy-ribose nucleotide tri-phosphate
edn.	edition
ENR	enrofloxacin
EMB	eosin methylene blue agar
EDI	estimated daily intake
<i>et al.</i>	et alia (and other)
EDTA	ethylene-diamine tetra-acetic acid
EU	european union
ESBL	extended-spectrum beta-lactamase
Fig.	figure
g	gram
HPLC	high performance liquid chromatography
hr	hour
H ₂ S	hydrogen sulphide
i.e.	id est (that is)
I.U.	international unit
kb	kilo base
λ	lambda
<	less than
$\leq$	less than or equal to
MgCl ₂	magnesium chloride
MRL	maximum residue limit
MR	methyl red
μg	microgram
μl	micro-liter
μ	micron
mg	milligram

ml	milliliter
mm	millimeter
min	minute
>	more than
$\geq$	more than or equal to
MH	mueller hinton
MDR	multi-drug-resistance
X	multiple
viz.	namely
ng	nano gram
No.	number
OTC	oxytetracycline
ppb	parts per billion
/	per
%	per cent
PDA	photo diode array detector
PCR	polymerase chain reaction
$\pm$	positive or negative
PCA	principal component analysis
rpm	revolution, per minute
sec (s)	second (s)
etc	so on (latin et cetera)
SPE	solid phase extraction
SD	standard deviation
SPSS	statistical package for social science
SHV	sulphydal variable
SMZ	sulphamethazine
TEM	temoniera
TC	tetracycline
<i>Taq</i>	<i>thermus aquaticus</i>
TBE	tris-boric acid-edta
UV	ultraviolet
VP	voges- proskauer
V	voltage
w/v	weight by volume



INTRODUCTION



CHAPTER-I

INTRODUCTION

With world's second-largest human population of 1.25 billion and about 250 million economically strong potential consumers, the domestic demand for meat and livestock products in India is increasing rapidly and this demand often exceeds the supply. The Food and Agriculture Organization (FAO) of the United Nations has predicted that by 2030 India's poultry consumption will increase by "a staggering 850 percent (Robinson and Pozzi, 2011). In order to meet the rising demand of animal food and growing export potential, the poultry industry is rapidly intensifying along with increasing administration of antimicrobial agents in food-producing animals to prevent and treat diseases (Khachatourians, 1998; Manna *et al.*, 2006). The food-producing animals may get exposed to antimicrobials by many direct and indirect routes. The direct route involves addition of low doses of antimicrobials to animal feed as growth promoter for improving feed conversion efficiency and increasing lean to fat ratio (Hao *et al.*, 2014). Further antibiotics are also used in prophylactic and therapeutic measures in animals. However, indirect routes are minor routes of exposures to antimicrobials and usually occur from contaminated environment (antibiotics treated human/animal waste, effluents from antimicrobial production units) (Kummerer, 2009; Moudgil *et al.*, 2018).

The drawback of excessive use of antimicrobial drugs is that they get accumulated in the tissues and organs of treated animals as residues and eventually become part of the food pyramid (Goetting *et al.*, 2011; Rokka *et al.*, 2012)

Organisation for Economic Cooperation and Development (OECD) estimate that the global average annual consumption of antimicrobials for per kilogram of chicken meat production was $148 \text{ mg}\cdot\text{kg}^{-1}$ (Van Boeckel *et al.*, 2015). In 2010, the five countries with the largest shares of global antimicrobial consumption in food animal production were China (23%), the United States (13%), Brazil (9%), India (3%), and Germany (3%). By 2030, this ranking is projected to be China (30%), the United States (10%), Brazil (8%), India (4%), and Mexico (2%) (Laxminarayan *et al.*, 2015; Van Boeckel *et al.*, 2015). The veterinary drug residues in poultry products can potentially be transmitted to humans via consumption of contaminated edible tissues and may lead to several pathological implications that are considered as major health issues. For instance, residual betalactams, including cephalosporin and penicillin, have been reported to cause dermatitis, cutaneous eruptions, anaphylaxis, and gastrointestinal symptoms in humans via ingestion of contaminated poultry products (Paige *et al.*, 1997). Penicillin residues are considered as the most problematic because of the vulnerability of a large proportion of people, many are allergic (Babapour *et al.*, 2012). Moreover, penicillin residues in poultry can lead to severe anaphylactic reactions, while eggs containing residues of sulfonamides in higher concentrations cause skin allergies upon consumption (Idowu *et al.*, 2010; Kabir *et al.*, 2004).

The residual levels of tetracycline in a diet intended for human consumption have been reported to lead to poor development of fetuses, staining of teeth in young children, gastrointestinal disorders, and pro-inflammatory, cytotoxic, and immunopathological effects (Idowu *et al.*, 2010; Lawal *et al.*, 2015; Palmieri *et al.*, 2014). Likewise, the persistence of sulphamethazine, oxytetracycline, and furazolidone

residues in humans is known to pose immuno-pathological effects (such as autoimmunity, carcinogenicity), while that of gentamicin and chloramphenicol may be mutagenic, nephropathic, and hepatotoxic or they may lead to reproductive abnormalities or bone marrow toxicity (Nisha, 2008). While some drug residues such as nitroimidazole and 3-nitrofurantoin cause different cancers in humans (Anderson *et al.*, 2003; Novais *et al.*, 2010). Continuous exposure of ciprofloxacin residues to humans is also known to cause toxicity, impede cytochrome (CYP1A2) mediated metabolism, and can result in increased concentration of drug in systemic circulation due to reduced renal clearance (Khan *et al.*, 2015). The veterinary drug residues in poultry products can potentially be transmitted to humans via consumption of contaminated edible tissues and may lead to several pathological implications that are considered as major health issues. Chicken meat contaminated with drug residues may pose serious public health hazards in the form of antibiotic resistant bacterial formation, allergic manifestations, or alteration of useful microflora of digestive tract to no microflora and/or harmful or non-healthy microflora (Mund *et al.*, 2017).

The widespread use of antibiotics in human and veterinary medicine for therapeutic or prophylactic purposes is the main driver of the acquisition and selection of antibiotic resistant bacteria (FAO, 2016; Hogeia *et al.*, 2014; Landers *et al.*, 2012). The resulting release of active or unmetabolised antibiotics from human and animal waste, from aquaculture, and from pharmaceutical companies into the environment further increases the risk of selection of antibiotic resistance (Wellington *et al.*, 2013; Taylor *et al.*, 2011). Evidences are growing now a day's indicating that aquaculture also plays an important role in the transfer and emergence of resistant

bacteria. India is the second largest fish producer in the world with a production of 11.41 million tonnes (MT) (2016–17), which however accounts only for 6.6% of the global production. Cultured fish production of 6.5 MT (freshwater: 5.8 MT; brackishwater: 0.7 MT) accounts for 57% of the total fish production in India (Anand *et al.*, 2020). Various aquaculture systems exist in the region including traditional aquaculture systems such as simple culturing fish ponds and shrimp ponds, and integrated aquaculture system which often consist of livestock and aquaculture. Because small and marginal farmers are the core of the Indian rural economy constituting 85% of the total farming community, thus to empower them, concept of integrated farming system was emerged. Integrated farming system refers to agricultural systems that integrate livestock and crop production or integrate fish and livestock and may sometimes be known as integrated bio systems (Kumar *et al.*, 2018). Also to obtain better productivity in aquaculture pond fertilization is a common practice in aquaculture aimed at increasing the production of natural food for farmed fishes. Inorganic fertilizers are expensive and their use by smallholder farmers may be limited. Animal wastes like cow dung and poultry litter are widely used in many countries to sustain pond productivity at a low cost (Gangadhar *et al.*, 2017). In the integrated aquaculture, the manure from animals (often ducks, chickens and pigs, in cages located above or adjacent to aquaculture ponds) is directly excreted into the ponds as fish food or as fertilizer to stimulate the growth of planktonic organisms eaten by the fish (Zhang *et al.*, 2013). Pond has a special place in Indian village community because these ponds are used by villagers for their day to

day activity e.g. taking bath, washing their clothes and their culinary utensils as well as washing their cattle and other pet animals (Singh, 2016).

In present situation, to increase the production of fish, these village ponds are also used for aquaculture. Antibiotics are extensively used in aquaculture in order to increase production, control bacterial infections and promote fish growth (Cabello, 2006). The use of antibiotics in the aquaculture industry has become an increasing concern for the general public due to the presence of antibiotic residues and development of antibiotic-resistant bacteria. The associated antibiotic resistance genes in the aquatic environment have been recognized as emerging environmental contaminants (Pruden *et al.*, 2006). Among various antibiotic classes, tetracyclines, sulfonamides and betalactams are widely used in aquaculture to cure bacterial infections due to their broad-spectrum antimicrobial activity. Therefore, these type of community ponds which are used for aquaculture and integrated aquaculture pond due to interaction with different species may work as mixing vessels which leads to emergence of antibiotic resistance because of activity like pond fertilization with animal waste and rampant application of antibiotics to treat fish diseases. Also it provides space for horizontal transmission and transfer of bacteria in different host like human, animal and water ecosystem. Therefore, it is essential to understand antibiotic resistance profiles in aquaculture environments in order to protect microbial ecosystems and human health (Chereau *et al.*, 2017).

It is frequently recognised that the widespread use of antibiotics in human health, for veterinary use, in aquaculture and agriculture, represents an important

reason for the increase in antibiotic resistance (Fatta-Kassinos *et al.*, 2011). The widespread use of antibiotics in non-humans use also contributes to development of resistance and has implications for humans, as we share the same environment. This has become an important arm in recently emerged 'One Health Concept'. The objective of the one health concept is to understand the interface of human-animal-environment. To combat antibiotic resistance, combined efforts of human and animal healthcare professionals, as well as environmentalists along with policy makers, are necessary. Therefore, an overall concept of 'One World-One Health-One Medicine' is promoted, to foster collaborative efforts of multiple disciplines, working locally, nationally and globally, to reach a goal of optimal health for people, animals and a clean environment (Mersha and Tewodros, 2012).

The association of environmental factors with antibiotic resistance is an emerging issue (Bush *et al.*, 2011). The components of the environment are natural, physical as well as social and behavioural and they all play an important role in modifying community health and effectiveness of medicines. A number of studies document the effect of social and behavioural factors on antibiotic resistance, while little information is available on relationships between the biophysical environment and antibiotic resistance. *E. coli* which present in the intestinal tracts of both humans and animals, is released into the environment through faecal material and is therefore used as an indicator of faecal contamination (Ishii and Sadowsky, 2008). *E. coli* is also a reservoir for antibiotic resistance genes. Some virulent strains can be harmful to the host animal, typically being the cause of diarrhea, which is unpleasant for healthy adults and often lethal to children in the developing world (Shtawa, 2016). In

the natural environment, the resistant bacteria and resistance genes from animal or environmental origin might transfer to humans and may cause severe illness that may require hospitalization. Among the antibiotics commonly used both in human and veterinary medicine are the fluoroquinolones, with ciprofloxacin being the most consumed fluoroquinolone worldwide. As a consequence of this, bacterial fluoroquinolone resistance has been reported both in humans and animals (Hordijk *et al.*, 2012).

Housefly (*Musca domestica*) is known as carrier of a large number of bacteria and is involved in the transmission of important bacterial agents causing human and animal infections. Its feeding and reproductive habits make it an important mechanical and biological vector for several human and animal pathogens including those causing blindness, nosocomial, enteric and anthro-po-zoonotic infections (Hemmatinezhad *et al.*, 2015). It also serves as reservoir and disseminator of metazoan parasites of both medical and veterinary significance. The flies, having close association with different forms of excreta and decaying organic matter, represent a substantial public health risk whenever they have access to human food.

In tropical countries such as India, where summer temperatures range from 40 to 45°C, housefly proliferation is favored. This occurs because at temperatures around 35°C, the female starts oviposition two days after copulation, while in temperatures around 15°C it takes 9 days; additionally, in temperatures higher than 25°C, the evolution from maggots to adult takes from 5 to 10 days, while in temperatures below 16°C, 40 to 50 days are needed. In such settings, housefly control

is of special concern as they can disperse up to 25 km (Chakrabarti *et al.*, 2010), live for about two or three months and some of the microorganisms living in the body or on the body surface of these flies can remain viable for as long as 35 days (Nasaj *et al.*, 2016). Adult house flies are considered mechanical vectors involved in the dissemination of foodborne diseases, such as *E. coli* diarrhea, Shigellosis, Salmonellosis, Cholera, Hepatitis A and E, Amoebic dysentery and parasitic worms.

In the view of above, the present study was undertaken with the following objectives:-

- 1. To assess the status of antibiotic residues in retail chicken meat samples in Chhattisgarh and its public health significance**
- 2. To assess the antibiotic resistance status in *E. coli* isolated from different ecosystems**
- 3. To assess the possible role of house fly in spread of antimicrobial resistant bacteria**

*REVIEW OF
LITERATURE*

CHAPTER-II

REVIEW OF LITERATURE

Broiler chickens are considered among the largest sources of meat across the world. The demand for chicken meat has increased in every region of the world, particularly in developing and Asian countries. Chicken is the most consumed meat as it is cheaper than other meats and is subject to fewer religious prohibitions or cultural taboos. Poultry farmers use disease preventive and growth promoter antibiotics for faster growth of chickens in the shortest possible time to increase the rate of feed assimilation and to lower the incidence of mortality caused by a pathogen attack. Antibiotics may result in dysfunctionality of beneficial gut microbiota and increase resistance among microbial pathogens in poultry (Muaz *et al.*, 2018) . However, all drugs that are administered to food producing animals may lead to residues in the edible tissues, milk, eggs and their products. The presence of these residues may pose potential health risks, including allergic reactions, direct toxic effects, cancer and a change in the resistance of bacteria exposed to antibiotics (Mastovska, 2011; Phillips, 2007).

Antimicrobial resistance (AMR) is recognized as one of the major Global Health challenges of the 21st century. Widespread antimicrobial use is considered to be the main factor associated with resistance in bacterial populations (Aarestrup *et al.*, 2008; Acar *et al.*, 2012). The use of antimicrobials in health care, agriculture, horticulture, aquaculture and industrial settings has an impact on the expression,

selection, persistence and transfer of resistance traits in bacterial populations (Aminov and Mackie, 2007; Mathew *et al.*, 2007). Chhattisgarh is the 6th largest fish producer state in the country. It is noted that nowadays use of antibiotics in aquaculture increasing day by day and different aquaculture production practices and community practices like integrated aquaculture farming and ponds are simultaneously used by human, animal and aquaculture is a contributing factor for emergence of resistance and transfer of resistance bacteria and antibiotic resistant gene among different species.

Antimicrobial-resistant bacteria can spread through many routes. Recently, the role of flies in spreading antimicrobial-resistant bacteria was reported (Blaak *et al.*, 2014). With continuing urban expansion into areas zoned for agricultural use, the public health community has become increasingly concerned about flies associated with animal production. Flies can move freely between livestock and human residences and may play an important role as vectors of antimicrobial-resistant bacteria, not only within a farm, but also over large distances to the meat and other livestock product market (Usui *et al.*, 2015). This is a problematic condition for human health point because it is an agent which can easily disseminate the resistant bacteria from any other source to the food.

2.1 Antibiotic residues and food safety

Now a days development of a range of agrochemicals and veterinary drugs, the intensification of agricultural production and the increase in industrialization and associated environmental contamination points towards an increased exposure of the

consumer to chemical residues from food and other sources (O'Keeffe and Kennedy, 1998). Yet the consumer looks increasingly for “pure” food, even for food produced according to the systems of yester year, and food producers, processors and retailers in their advertising and in their systems strive to deliver such “pure” food to the customer. In respect of residues in food, the emphasis is increasing towards natural or “organic” systems for food production but, paradoxically, even if this goes some way to satisfy the concerns on agrochemical, veterinary drug and, possibly, industrial contaminants, it may not address the complete range of chemical contaminants, such as mycotoxins and different drugs used in animal production system (Kennedy and O'keeffe, 1996).

To meet the growing demand for animal products in world, food animal production is increasingly dominated by concentrated animal feeding operations, which are more efficient and economically more competitive compared to the traditional family farming and “free-range” farming. However, pigs, chickens, cows, and fishes raised at high stocking densities in close confinement have high levels of stress, and such conditions favor occurrence and spread of infectious diseases. Consequently, antimicrobials are routinely administered to animals, via feed or drinking water, or by injection, for treatment and prevention of diseases. In addition, antimicrobials at low and sub-therapeutic doses are often used as growth promoters to improve feed efficiency and promote animal growth (Hu and Cheng, 2016).

2.2 Human health risks associated with drug residues

The veterinary drug residues in poultry products can potentially be transmitted to humans via consumption of contaminated edible tissues and may lead to several

pathological implications that are considered as major health issues. Chicken meat contaminated with drug residues may pose serious public health hazards in the form of antibiotic resistant bacterial formation, allergic manifestations, or alteration of useful microflora of digestive tract to no microflora and/or harmful or non-healthy microflora (Mumtaz *et al.*, 2000).

For instance, residual β -lactams, including cephalosporin and penicillin, have been reported to cause dermatitis, cutaneous eruptions, anaphylaxis, and gastrointestinal symptoms in humans via ingestion of contaminated poultry products. Penicillin residues are considered as the most problematic because of the vulnerability of a large proportion of people, many are allergic (Babapour *et al.*, 2012). Moreover, penicillin residues in poultry can lead to severe anaphylactic reactions while eggs containing residues of sulfonamides in higher concentrations cause skin allergies upon consumption (Idowu *et al.*, 2010). The residual levels of tetracycline in a diet intended for human consumption have been reported to lead to poor development of fetuses, staining of teeth in young children, gastrointestinal disorders, and pro-inflammatory, cytotoxic, and immuno-pathological effects (Lawal *et al.*, 2015; Palmieri *et al.*, 2014). Similarly, residual amounts of tilmicosin affect hematological and biochemical parameters such as total protein, albumen, cholesterol, and triglyceride concentrations (Elsayed *et al.*, 2014). Likewise, the persistence of sulphamethazine, oxytetracycline, and furazolidone residues in humans is known to pose immuno-pathological effects (such as autoimmunity, carcinogenicity), while that of gentamicin and chloramphenicol may be mutagenic, nephrotoxic, and hepatotoxic or they may lead to reproductive abnormalities or bone

marrow toxicity. Similarly, intake of nitrofurans metabolites as residues via poultry products may produce toxic, mutagenic, or carcinogenic side effects and may transmit antibiotic resistance among human microflora, hence the application of nitrofurans in poultry has been banned (Khan *et al.*, 2015 ; Nisha, 2008). While some drug residues such as nitroimidazole and 3-nitrofurans cause different cancers in humans. Continuous doses of ciprofloxacin residues to humans is also known to cause toxicity, impede cytochrome (CYP1A2) mediated metabolism, and can result in increased concentration of drug in systemic circulation due to reduced renal clearance. Moreover, the consumption of drug residues *via* chicken products may result in the production and proliferation of drug-resistant bacteria in human beings, which could lead to therapeutic failures among such infected individuals (Mehdi *et al.*, 2018; Mund *et al.*, 2017).

2.3 Impact on environment

The indiscriminate and abusive use of antibiotics can result in higher concentrations of antibiotics in the environment, which can be termed as antibiotic pollution. The sources via which antibiotics can be released into the environment are diverse, including the human waste streams, and wastes from veterinary use and livestock farming (Gillings, 2013). Antibiotics used for prophylaxis or therapy in humans contaminate the human waste streams, likewise, the antibiotics used in animals for growth promotion, prevention, and treatment equally contaminate the animal's waste streams. Thus, these are considered as prime sources of antibiotic release into the environment (Dolliver and Gupta 2008). This is because the administered antibiotics are not fully metabolized, and are released unchanged into

the environment, i.e., water, manure or soils. The amount and rate at which the antibiotics are being released into the environments depends on the specific antibiotic and its administered dosage, as well as the species and the age of the animals (Zhao *et al.*, 2010). Nevertheless, these waste streams will contain both the antibiotics and resistance genes; both considered as pollutants, and their fate in the environment differ. Furthermore, antibiotics and their metabolites contained in stockpiled animal manure may seep through the pile to surface and groundwater, and also into the soil. This is especially so for antibiotics with high water affinity or which are water soluble, thus making their spread and eco toxicity in the environment faster, and widely with the aid of water fluidity. In the same view, antibiotics can be introduced into the environment via soil fertilization with raw animal manure, irrigation with wastewater generated from farm activities, or via accidental release by runoffs from farms (Manyi-Loh *et al.*, 2018).

Also, the farmers disposed of their used antibiotic containers by merely throwing them into drains, refuse dumps, or onto bare ground, instead of burying them as recommended. The author further mentioned that these antibiotics were stored under suboptimal environmental conditions, vulnerable to temperature fluctuations that could accelerate their decomposition, thereby causing a reduction in their concentration and efficacy during administration. Such circumstances promote antibiotic resistance of bacteria living in the gastrointestinal tracts of the animals, due to constant exposure to sub lethal levels of these antibiotics, or could even cause prompt administration of an overdose of the antibiotics which is noted to be inefficient. More especially, in commercial and intensive poultry farming, antibiotics

may be administered to the entire animal population in feed or water, rather than targeting only the diseased animals. Thus, resistance becomes unavoidable. Interestingly, antibiotics produced naturally by environmental microorganisms, to deter competitors from living space and food, are gradually accumulating in the environment. Seemingly, antibiotics are released from their production facilities in high concentrations into the environment (Criswell, 2004; Li *et al.*, 2010). Sahoo *et al.* (2010) noted that antibiotics could be found in the natural environment via improper disposal of out-of-date drugs from pharmaceutical shops, and unwanted, expired household pharmaceuticals.

Accordingly, these antibiotics released usually consist of different types, and consequently, they do not degrade, all at the same time, i.e., they degrade at different rates in the environment over time by the main elimination processes, including sorption, photo degradation, biodegradation, and oxidation (Li *et al.*, 2014; Liu *et al.*, 2016). Albeit, other applied methods, such as adsorption, filtration, coagulation, sedimentation, advanced oxidation processes have been implemented (Homem and Santos, 2011). Specifically, several findings have demonstrated the use of composting, and anaerobic and aerobic digestion to cause the reduction of the antibiotic's level in manure, wastewater, and sludge, but these processes vary in efficiency with the category of the antibiotics, the conditions employed for composting, as well as the type of livestock manure (Manyi-Loh *et al.*, 2018). None the less, the presence of these antibiotics in the environment may create selective pressure resulting in antibiotic resistance and also the removal processes, reduce the concentrations of these antibiotics, allowing time for the exposed bacteria to develop

resistance which may be presented as stress adaptation, co-selection, cross-resistance, and cross-protection.

According to Manzetti and Ghisi (2014), the most vulnerable ecosystems to antibiotic contamination are confined aquatic ecosystems such as ponds and lakes and soils close to urban sites. Aquatic compartments, such as water and sediments, can thus play an important role in the transfer, evolution and ecology of antibiotic resistance genes. Large amounts of antibiotics administered to animals are excreted into the environment via urine and faeces. After metabolic changes in animals, up to 30-90% of the dose consumed is found in the urine and feces as parent compounds and/or metabolite compounds (Carvalho and Santos, 2016). This makes sewage disposal systems one of the most important routes by which antibiotics can enter in the environment (Ronquillo and Hernandez, 2017) and contaminate even coastal waters (Chen *et al.*, 2015). Antibiotics' risks in the aquatic environment and sediments are important because they can influence aquatic life behavior. Antimicrobials have qualitative and quantitative effects on the microbial community residing in sediments, which in turn can affect the degradation of organic matter. Residues of antibiotics present in the water contribute strongly to the maintenance, emergence and dissemination of bacterial populations with a low level of resistance and are ready to evolve towards resistance.

The aquatic environment is considered as an important point for acquisition and spread of antibiotic resistance genes by bacteria. Devarajan *et al.* (2017) reported multi-resistance profiles in *Pseudomonas* spp. in aquatic sediment samples, which is potentially transferable to humans. Laroche *et al.* (2009) reported resistance genes in

estuary samples, mainly carried by *Enterococcus* spp. and *E. coli*. In addition, a study Furtula *et al.* (2013) carried out on samples collected from 2 poultry farms showed that 58% of *Enterococcus* spp. isolates in surface waters and 100% of isolates in groundwater were resistant to more than an antibiotic. According to Carvalho and Santos (2016), the toxic effects of antibiotics in the aquatic environment increased when combined with other antibiotics.

In the soil, antibiotic's behavior differs according to their physicochemical properties, soil characteristics, as well as climate conditions. Acid rain accelerates the antibiotics accumulation in animal manure and soil surface while long-lasting rains foster antibiotics' migration in deeper parts of the soil. According to Pan and Chu (2017), antibiotics leaching are higher in sandy soils than in clay and silty soils. Norfloxacin and tetracycline tend to persist in the soil surface while sulfamethazine and erythromycin pose a higher risk for deeper soil layers and groundwater. The soil can be also contaminated by antibiotics in litter. Animal bedding contains residues of antimicrobial compounds. Residues of bacitracin, salinomycin, penicillin and virginiamycin were detected in chicken litter at concentrations ranging from 0.07 to 66 mg/L (Furtula *et al.*, 2010). When this bedding material is used as nitrogen amendment, the resistant bacteria can live in the soil for several months. According to De Liguoro *et al.* (2003), biotransformation and biodegradation of antibiotics on agricultural sites can take up to 150 days. In addition, antibiotic by-products in the environment remain bioactive and can be potentially more toxic, stable and mobile than their parent compounds (Carvalho and Santos, 2016).

2.4 Environmental antimicrobial resistance a burning issue

AMR is inextricably linked to the three basic domains of “One Health” that is, human, animal, and environmental health. While AMR affects human, animal, and environmental health equally, human health and to some extent animal health get the spotlight. Environmental health is the least-explored domain of One Health. While our overall knowledge of soil microbes is limited, genes encoding antibiotic resistance are not a new phenomenon in response to extensive use of antimicrobials in humans or animals. Within the 1% of all soil microbes that have been identified, there appears to be a global “resistome” (Kahn, 2016; Perry and Wright, 2013). Selective pressures placed on environmental bacteria forced these bacteria to express resistance genes to survive. Resistance genes are known to persist in nature and can be transmitted that way among bacteria in sewage, soil, water, and waste. Perhaps humans can learn from the environment since antibiotic resistance genes are ancient, predating the selective pressure of modern antibiotic use. Such genes have been identified in Alaskan permafrost sediments that date back to the Late Pleistocene era. Microbes that are metabolically active readily have the potential for evolving or acquiring resistance genes (D’Costa *et al.*, 2011).

In most low and middle-income countries, the use of antibiotics for animal growth promotion and treatment is unregulated, which results in the high proportion of antimicrobial-resistant bacteria, antimicrobial resistance genes (ARGs), and antimicrobial residues in foods of animal origin and farm waste. Resistant bacteria and genes emanating from food animal farms are a public health threat. These

resistance genes could enter the human food chain through water, soil, and manure. In particular, manure is known to harbor various AMR bacteria and genes. Despite the huge volumes of antimicrobials used on food animal farms, very little information is available regarding the presence of AMR genes in manure and soil around these farms. The dissemination of manure on soil increases the risk of resistance gene exposure to crops, surface water bodies, groundwater, and human populations living close to the farms. Few studies have explored the presence of AMR bacteria or ARGs in soil/manure samples and their persistence in the outside environment (Kumar *et al.*, 2018; Pornsukarom and Thakur, 2016). In one such study, various *Salmonella* serotypes were found to persist in the soil outside swine farms for up to 21 days post-manure application (Pornsukarom and Thakur, 2016). The authors confirmed the role of manure as a reservoir in the dissemination and persistence of antimicrobial-resistant bacteria in the environment. Recently, another study detected 149 unique resistance genes at three large-scale (10,000 animals per year) commercial swine farms in China. The 63 most prevalent ARGs were enriched 192 fold up to 28,000 fold compared to the antibiotic-free manure or soil controls. The diversity and abundance of ARGs reported in this study is alarming and clearly indicates that unmonitored use of antibiotics and metals on swine farms has expanded the diversity and abundance of the antibiotic resistance reservoir in the farm environment (Zhu *et al.*, 2011). Another major environmental reservoir of ARGs is the wastewater effluents from drug industries and municipalities. It is known that a large proportion of the antimicrobials consumed by humans are excreted in their bioactive form in feces and urine (Zhang *et al.*, 2015). These biologically active components in

wastewaters exert selective pressure and force bacteria to develop resistance. The presence of antibiotic resistance genes and antibiotics in wastewater effluent selects for novel combinations of AMR. These resistance determinants can be transferred to susceptible microorganisms by horizontal gene transfer via plasmids, integrons, and transposons (Devarajan *et al.*, 2016; Xu *et al.*, 2015; Kniel *et al.*, 2018).

2.5 Analytical methods for monitoring of antibiotic residues

The antibiotic residues which are present in different food commodities like milk, meat, egg and other edible offal may pose toxicities to consumers. Thus rapid, reliable, specific and sensitive methods for early and rapid detection of veterinary drugs in foods of animal origin are indispensable. In general, analytical methods for monitoring antibiotic residues can be classified in two groups (i.e. confirmatory and screening). Confirmatory methods are mostly based on liquid chromatography (LC) coupled to mass spectrometry (MS) (Blasco *et al.*, 2009; Kokkonen *et al.*, 2009) to quantify analyte concentrations, however many researches are devoted to detect antibiotics by means of LC with UV detection technique (Benito-Pena *et al.*, 2009).

In general, the reported methods (from the Web of Knowledge database) are grouped depending on the type of analytical technique applied, and the corresponding percentages for each one are used for detection purpose as can be seen, LC-MS is the most employed analytical method (38%), followed by LC-UV (18%) and ELISA (18%). However, the development of other screening, methods (12%) and biosensors (8%) is increasing considerably (Chafer-Pericas *et al.*, 2010).

2.5.1 Screening methods of antibiotic-residue determination

The screening methods of antibiotic-residue detection in foods of animal origin are primarily semi-quantitative in nature. The ideal characteristics of these screening methods are low possibilities of false positive data, ease of use, high throughput, short analysis time, cost effectiveness and good selectivity. The screening tests can be categorized into microbiological inhibition assay, immunoassays and biosensor.

2.5.1.1 Microbial assay

The most widely used method for the detection of antibiotic residues in animal originated food-stuffs is the microbial inhibition method, this method is not only cost effective but also able to cover a large number of different antibiotics in a single test run (Mitchell *et al.*, 1998). Two main test formats can be distinguished: the tube test and the plate test. A tube (or vial, or ampoule) test consists of a growth medium inoculated with (spores of) a sensitive test bacterium, supplemented with a pH or redox indicator. At the appropriate temperature, the bacteria start to grow and produce acid, which will cause a color change. The presence of antimicrobial residues will prevent or delay bacterial growth, and thus is indicated by the absence or delay of the color change (Pikkemaat, 2009). The pioneer workers in the field microbial inhibition test used *Sarcina lutea* (Nouws *et al.*, 1979) and *Bacillus subtilis* (Schothorst, 1969) in Netherland and Germany respectively. (Vishnuraj *et al.*, 2016).

2.5.1.2 Immunoassay

Immunoassays are semi-quantitative methods characterized by their high specificity, high sensitivity, simplicity and cost effectiveness, which make them particularly useful in routine work. They are based on the specific reaction between

antibody and antigen. As Immunoassays are semi-quantitative methods characterized by their high specificity, high sensitivity, simplicity and cost effectiveness, which make them particularly useful in routine work. They are based on the specific reaction between antibody and antigen. Immunoassays applied to antibiotic determination have been classified depending on the detection label [i.e. enzyme-linked immunosorbent assay (ELISA), fluoroimmunoassay (FIA) and time-resolved fluoroimmunoassay (TRFIA)]. ELISA is a widely used method for the detection of antibiotic residue in all kinds of tissue samples (Mungroo and Neethirajan, 2014). ELISA based techniques have the advantage of high sensitivity, broad specificity and ability to handle large number of small volume samples in short time (Wang *et al.*, 2009; Vishnuraj *et al.* 2016).

FIA's have been employed to overcome some ELISA problems (e.g., high background absorption, large number of incubation and wash steps, and enzyme degradation). Few FIA's have been applied directly in plates. Their limited sensitivity, due to the high background fluorescence levels, results in a decreasing signal-to-noise ratio. In a recent work, Pyun *et al.* (2008) employed a commercial solid-phase FIA (SPFIA) test to monitor antibiotic residues in pork; it was simple and fast, and the limit of detection (LOD) achieved were 100 µg/kg and 150 µg/kg for streptomycin and dihydrostreptomycin, respectively.

2.5.1.3 Biosensors

Among the latest generation of screening methods, biosensors represent an interesting approach because they can operate fully automatically. According to the established biosensor definition, they contain a biological recognition element

(enzymes, proteins, nucleic acids, cells, tissues) coupled to a signal transduction element. Some advantages of biosensors are that they are as specific as the biorecognition element used and that they allow rapid, continuous control and in-situ applications. In spite of these advantages, biosensor design suffers from two main limitations. The first is the instability of the biological sensing component, which can lose its activity in hours or days depending on the nature of the molecule and exposure to environmental stresses, such as pH, temperature or ionic strength. The second limitation is on the size of the physico-chemical transducers used in biosensors. Several classifications exist depending on the parameter evaluated (Koyun *et al.*, 2012). Therefore, the biosensors can be classified according to the biological element (enzymatic, immunosensor and microbiological). Biosensors always provide opportunity for automation, in situ analysis and development of large number of commercial detection kits. Any biosensor system comprise two main components namely, a transducing device and a recognition element. The advantage of biosensors includes, it can detect non polar molecules which are not receptive to most devices, high specificity, quick and real time application for industry. But the drawbacks include chances of contamination of biosensor and it cannot be heat sterilized (Huet *et al.*, 2010; Vishnuraj *et al.*, 2016).

2.5.2 Confirmatory methods

Quantitative and/or confirmatory methods, include gas chromatography with electron capture, flame ionization, or mass spectrometry detection, as well as LC with ultraviolet (UV), fluorometric or electrochemical detection, or MS. Almost all antibiotics are LC-amenable compounds and can be analyzed by LC techniques,

although some compounds such as chloramphenicol, florfenicol, and thiamphenicol were historically determined by gas chromatography with mass spectrometry or electron capture detection. (Wang and Leung, 2012). The antibiotics are LC amenable compounds and can be analyzed by LC techniques.

The use of high performance liquid chromatography (HPLC) was expanded during the 1990s. Its usage is increasing day by day in the field of residue analysis. The variety of mobile phases, the availability of wide range of column packings and the variation in modes of operation are the reason for the high demand (Kebede *et al.*, 2014). It is a separative technique and its ability to detect compounds depends on the type of detector used. It is an automated process with high specificity, sensitivity, accuracy and taken short time to produce results. The results produced are of a high resolution and easy to read. Nowadays, the LC-MS/MS is most frequently used analytical tool for detection of a large number of multiclass veterinary drug residues in food. But main drawback is high cost and necessity of technically expertise person.

2.6 Detection of antibiotic residues in chicken meat

2.6.1 Prevalence of tetracycline residues in chicken meat

Shahid *et al.* (2007) determined the status of oxytetracycline residues in chicken meat. Preliminary screenings were performed by a microbial assay using *Bacillus subtilis* as test organism. Oxytetracycline (OTC) in positive samples was detected and quantified using high performance liquid chromatography. Linear calibration curve was obtained with correlation coefficient of 0.9981 while average recoveries were greater than 91% with RSD values between 1.64 to 2.07%, while the

LOD was 0.01 µg/ ml. Out of 29 meat samples analysed for OTC residues, 13 (44.8%) had detectable residue level for OTC and 6 (20.7%) had higher residue levels than the recommended maximum residue level (0.2, 0.6 and 1.2 µg/g) for muscles, liver and kidney respectively.

Nikolaidou *et al.* (2008) developed and validate HPLC method for determination of seven tetracycline antibiotic residues in chicken muscle. Overall recoveries ranged from 92-110.1% and 89-106% for chicken tissue and eggs respectively. All RSD values were lower than 11%.

Gajda and Posyniak (2009) simultaneously determined tetracycline (TC), OTC, chlortetracycline (CTC) with their 4-epimers and doxycycline (DC) residues by using HPLC. Chromatographic separation was achieved on C18 analytic column using a mobile phase consisting of acetonitrile, methanol and 0.02 M oxalic acid in gradient mode. Detection was carried out by UV detector at 355 nm. The recoveries of the analyte from spiked samples were 50%- 80%. The decision limits were from 110 to 125 µg/gm and detection capabilities were from 120-155 µg/gm, depending on analytes. The prepared method was successfully verified in the National Residue Control Programme. The prepared procedure is used for the control of TCs residue in the animal tissue samples, including chicken, pork and beef muscles, as well as the kidneys and liver.

Shalaby *et al.* (2011) optimized and validated liquid chromatographic method with diode-array detection for determination of tetracycline residue in chicken meat and liver. Validity study of the method revealed that all obtained curves showed good linearity ($r^2 > 0.999$) over the range of 50-5000 ng. Sensitivity was found to be 1.44,

1.90, 0.95 and 1.23 ng for OTC, TC, CTC and DC, respectively. Accuracy was in the range of 71.88-92.44.3% for meat and liver respectively. Precision was lower than 10% in all cases indicating that the method can be used as a validated method. LOD was found to be 4.4, 5, 13 and 10 ng for OTC, TC, CTC and DC, respectively. The corresponding values of limit of quantification (LOQ) were 10, 13, 27 and 22 ng.

Adewuyi *et al.* (2011) developed and validated a method for detection of residual oxytetracycline in livers and tissues of broilers using HPLC technique has been established and validated. Tetracycline was extracted from livers and tissues by standard procedures and clean-up by liquid liquid extraction after which extracts were analyzed by HPLC. From the result, the recovery values for samples analyzed ranged between 76.4 to 85 % for all samples analyzed. Oxytetracycline residue level in breast tissue and liver ranged from 670.00 ± 3.00 to 1816 ± 49.33 and 1551.16 ± 10.6 to 2697.53 ± 1.89 $\mu\text{g/kg}$. The limit of detection for oxytetracycline was 13.0 while the limit of quantification was 42.0 $\mu\text{g/kg}$.

Salama *et al.* (2011) reported the incidence of tetracyclines' (TCs) residue (OTC, TC, CTC and DC) in fresh chicken samples (meat and liver) collected during one year period. TC residues were analysed using an HPLC method. The limit of detection for meat was 4.4, 5, 10 and 7ng/g for OTC, TC, CTC and DC, respectively, compared with 5.5, 6, 12 and 9ng/g stated for liver. The recovery of the method ranged from 91% to 70% depending upon both matrix type and tetracycline kind. The results revealed that 66 (44%) samples contained TC residues including 21 (42%) breast, 19 (38%) thigh and 26 (52%) liver samples. The corresponding contaminated ranges were 124–5812, 107–6010 and 103–8148mg/kg. A total of 12 (8%), 13

(7.33%) and 20 (13.33%) samples of breast, thigh and liver, respectively, had TC residues above the Codex maximum residue limit (MRL). Liver samples had a higher incidence and level than those found in breast or thigh samples.

Cetinkaya *et al.* (2012) detected tetracycline residues in chicken meat by liquid chromatography tandem mass spectrometry. Overall, the recoveries for TCs ranged from 56.9% to 101.2%, with standard deviations of 4.5–13.2%. Detection limits ranged from 7.9 to 14.6 µg/kg. In four of 60 samples, doxycycline was detected in a range from 19.9 to 35.6 µg/kg and in one sample tetracycline was detected at 17.2 µg/kg. CTC and OTC were not detected in any of the tested samples.

Abdel-Mohsein *et al.* (2015) evaluated occurrence of OTC, TC, DC and CTC in 282 samples (muscle, liver and kidney samples) collected from broiler farms (Small and Large) in Upper Egypt using HPLC. They detected OTC residue in 50, 100 and 93 % of muscle, liver and kidney samples, respectively, while TC residue was detected in 69, 72 and 86 % samples, respectively. None of the samples were positive for DC and CTC. They detected OTC residue level in liver, 8.48 µg/g and 6.88 µg/g in samples from small farms and large farms, respectively. They found higher DC residue level (24.24 µg/g) in liver samples from large farms however, the differences between the levels in small and large farms were not significant. They further observed highest antibiotic residue for DC in liver and kidney representing 20.43 µg/g and 14.4 µg/g, respectively.

Ibrahim *et al.* (2015) screened eighty seven chicken meat samples collected from different poultry slaughter houses and marketing centers in Khartoum State, Sudan, for the presence of residues of Tetracycline by microbiological assay and

confirmed positive samples by using HPLC. Their results showed that more than 5% (n=87) of the meat samples have noticeable oxytetracycline antibiotic residues with concentration levels between 150 to 500 µg/kg. They indicated that antibiotics may be improperly used by producers, and suggested that there is a strong need for surveillance through regular mandatory testing of chickens sold for human consumption.

Ahmed and Gareib (2016) examined 40 random fresh chicken breast and chicken luncheon samples (20 of each) from different retail markets in Ismailia Governorate, Egypt using HPLC. They obtained mean values of oxytetracycline 0.5 ± 0.1 and 1.4 ± 0.2 µg / g in chicken breast and chicken luncheon respectively, while the mean values of ciprofloxacin observed 0.02 ± 0.01 and 0.03 ± 0.01 µg/g., respectively. The study concluded that oxytetracycline residue was higher than MRL while ciprofloxacin residue was lower than MRL.

Moon *et al.* (2018) antibiotic residue level in chicken muscle samples collected in and around the Ludhiana city by high performance liquid chromatography. Amongst 150 samples examined for tetracycline residues 14, 2, and 6% samples were found positive for oxytetracycline, tetracyclines, and chlortetracycline residues, respectively. Similarly, residues of enrofloxacin and ciprofloxacin were detected in 22.66 and 11.33% samples, respectively when other 150 samples were examined. Out of positive samples 4.66, 0.66, 1.33, 12 and 1.33% samples were above maximum residual limit for oxytetracycline, tetracyclines, chlortetracycline, enrofloxacin and ciprofloxacin, respectively. Based on Hazard

Quotient values it can be concluded that risk assessed is negligible since the calculated Hazard Quotient values are potentially safe.

2.6.2 Prevalence of Sulfonamide in chicken meat

Cheong *et al.* (2010) detected four common SAs, Sulfadiazine, Sulfamethazine (SMZ), Sulfamethoxazole and Sulfaquinoxaline, in chicken breast and liver samples using reverse phase HPLC using UV detector at 266 nm. The concentration of SAs detected in samples from 11 states in Peninsular Malaysia ranged from 0.006-0.062 µg/g in breast meat samples and 0.08-0.193 µg/g in liver samples. Except for sample from Johor, concentration of SAs in all the samples were lower than MRLs established by Malaysia (0.1 µg/g). Exposure of sulfonamides in Malaysian consumers ranged from 0.002-0.088 µg/kg body wt. /day. The highest value of sulfonamides exposure was found in Johor with an estimated daily intake (EDA) of Sulfamethoxazole (SMX) in Johor.

Mehtabuddin *et al.* (2012) determined the residual level of sulfonamides in poultry meat and eggs. Detection of sulfonamide residues were made by HPLC with UV detector set at 265 nm using C 18 column (25 cm×0.46, 5 µm) under isocratic conditions and using 0.01 M potassium di-hydrogen phosphate (KH₂PO₄) buffer and methanol (70:30 v/v) as a mobile phase with a flow rate of 1 ml/min. The limit of detection was 0.02 µg/g and 0.025 µg/ml for meat and eggs, respectively. It was noted that 43% meat and 30% egg samples had detectable levels of sulfonamide residues whereas 23% meat and 10% egg samples exceeded recommended maximum residual level and were unfit for human consumption. The study revealed the

presence of sulfonamide residues in poultry meat and eggs because of indiscriminate use of sulfonamides in commercial broilers and layers without observing withdrawal period of this drug.

Awaisheh *et al.* (2019) surveyed 120 poultry meat samples, 20 samples (16.7%) were sulfamethazine (SMZ) violative positive and exceeded the European Union maximum limit (100 µg/kg) and accordingly were unfit for human consumption. Whereas, 51 samples (42.5%) were with SMZ concentrations of 10-100 µg/kg. The average SMZ concentration was 235.58 µg/kg with a range of 11.47-800 µg/kg poultry meat. It is also noteworthy the high EDI of SMZ by Jordanian adults, 0.286 µg SMZ/kg bw./day. Moreover, results prevailed that the highest SMZ incidence rate and contamination level were for imported poultry samples followed by domestic poultry samples, which may indicate that SMZ contamination in poultry meat is an international issue. The study reported high SMZ incidence rate, contamination level, and EDI values, which is likely due to indiscriminate use of SMZ in poultry production.

2.6.3 Prevalence of fluoroquinolone residues in chicken meat

Salehzadeh *et al.* (2007) screened two hundred and seventy chicken muscle, liver and kidney samples from 90 broiler farm in Tehran province of Iran collected over a period of one year starting from August 2001. HPLC was used for separating, detecting and analyzing enrofloxacin residues in samples. All samples showed enrofloxacin residues and samples from 22 (24% of farms showed residues of enrofloxacin above MRLs (Maximum Residue Limits). 8 (8.88%), 12 (13.33%) and 22 (24.44%) of muscle, liver and kidney samples showed residues of enrofloxacin

above MRLs respectively. The mean concentrations of enrofloxacin in muscle, liver and kidney samples were 18.32 ± 32.29 SD, 18.34 ± 12.36 SD and 26.06 ± 19.52 SD ng/g respectively. This study confirmed widespread misuses of enrofloxacin in farms and lack of implementation of recommended withdrawal times.

Pena *et al.* (2010) analyzed a total of 98 poultry samples, including chicken and turkey muscle using a sensitive and reliable analytical method based on liquid chromatography with spectrofluorometric detection for simultaneous determination of four fluoroquinolone antibiotics, namely enrofloxacin, ciprofloxacin, norfloxacin, and sarafloxacin. The method involved extraction with 0.15 mol L^{-1} HCl and clean-up by solid-phase extraction using Oasis HLB cartridges. The limits of quantification, expressed as the lowest fortification level with acceptable precision were $15 \text{ } \mu\text{g kg}^{-1}$ for enrofloxacin, ciprofloxacin, and norfloxacin, and $30 \text{ } \mu\text{g kg}^{-1}$ for sarafloxacin, these values are in compliance with requirements for monitoring of maximum residues levels. Overall recoveries from spiked samples ranged from 80% to 92% with relative standard deviations lower than 6.1%. Of the chicken and turkey samples analysed, 44.2% and 37.8%, respectively, were contaminated.

Er, B., Onurdag *et al.* (2013) developed a novel analytical procedure and analyzed 127 chicken and 104 beef meat samples collected randomly from local markets of Ankara, Turkey. Extraction and determination of quinolones were made by ELISA procedure. One hundred eighteen of 231 (51.1%) examined chicken meat and beef samples were found to contain quinolone antibiotic residue. Among the chicken meat and beef samples, 58 (45.7%) of chicken meat samples and 60 (57.7%)

of beef meat samples were positive for quinolones, respectively. The mean levels ($\pm$ SE) of quinolones were found to be 30.81 ± 0.45 $\mu\text{g/kg}$ and 6.64 ± 1.11 $\mu\text{g/kg}$ in chicken and beef samples, respectively.

Omotoso and Omojola (2015) designed to screen residues of three fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin) in raw meat. Microbiological assay, followed by HPLC was used to screen three hundred and twenty samples of beef, chicken, pork and chevon purchased from open markets. Initial screening by microbiological assay revealed that 50%, 55%, 40% and 40% of beef, chicken, pork and chevon, respectively were positive for residues of antibiotics. Further analysis by HPLC with UV detection revealed the presence of ciprofloxacin, norfloxacin and ofloxacin at varying concentrations in the meat samples. Ofloxacin was the least in frequency and abundance in all meat types.

2.6.4 Prevalence of multiresidue antibiotics in chicken meat sample

Yamaguchi *et al.* (2015) conducted a study in Vietnam from 2012 to 2013. Meat samples were collected from slaughterhouses and retail stores in Ho Chi Minh City and Nha Trang. A total of 28 antibiotics were analyzed using a LC-MS/MS screening method. Sulfonamides, fluoroquinolones, and tilmicosin were detected in some of the samples. Sulfaclozine and fluoroquinolones were mainly detected in chicken samples, and sulfamethazine was mainly detected in pork samples. High levels of sulfonamide residues, ranging between 2500 and 2700 $\mu\text{g/kg}$ sulfaclozine and between 1300 and 3600 $\mu\text{g/kg}$ sulfamethazine, were present in two chicken and three pork samples, respectively. Tilmicosin was detected at ranges of

150–450 µg/kg in 10 chicken samples. Positive percentages were 17.3, 8.8, and 7.4% for chicken, pork, and beef, respectively, for an average of 11.9%. The results suggest an appropriate withdrawal period after drug administration had not been observed in some livestock.

Jammoul and Darra (2019) screened antibiotic residues from 80 chicken samples collected from farms located in different regions of Lebanon. The evaluation of antibiotics residues in 80 chicken muscles samples has shown that 77.5% of samples were at least contaminated with antibiotics residues, out of which 53.75% were exposed to co-occurrence of multidrug residues. The screening of the four antibiotics families has shown that ciprofloxacin (quinolones) represents the highest occurrence percentage (32.5%), followed by amoxicillin (β-lactams) (22.5%) and then tetracyclines (17.5%). Means of sarafloxacin, amoxicillin, and penicillin G residues levels were above the MRL recommended limit of the European Union.

Oyedeki *et al.* (2019) employed a solid-phase extraction method using liquid chromatography tandem mass spectrometry in the positive and negative electrospray ionisation and the multiple reaction monitoring modes were employed. Residues of nineteen (19) antibiotics were found in the three different matrices at different levels with varying detection frequencies ranging between 2 and 4% (sulfamoxole, penicillin-G, albendazole and phebedazole) and 14–54% for all the other antibiotics. The highest number of violative samples was found in the turkey gizzard and chicken muscle. Sulfixosazole had the highest percentage violation of 80.00% in turkey gizzard while sulfamethoxazole, notwithstanding its lower frequency in chicken

muscle had highest maximum concentration and 100% violation. The presence of these drugs, however, does not pose any immediate health risk.

Oyedemi *et al.* (2020) used high performance liquid chromatography with diode array detection method for the simultaneous determination of six antibiotic residues (enrofloxacin, sulfadimethoxine, sulfamerazine, sulfamethoxazole, sulfamoxole, and tylosin) in 111 samples of fresh and frozen poultry meats. The limit of detection ranged from 5.37–55.4 ng/g, while the quantification limit was in the range of 17.9–184 ng/g, respectively, with minimal matrix effect. The calibration curves obtained exhibited a good linear response with the coefficient of determination, $R^2 > 0.996$. Some concentrations exceeded their maximum residue limits in most samples. These findings indicated elevated levels of antibiotic residues in tissues of locally produced and illegally imported poultry meat samples.

2.7 Human health risk assessment

Pena *et al.* (2010) analysed a total of 98 poultry samples, including chicken and turkey muscle using a sensitive and reliable analytical method based on liquid chromatography (LC) with spectrofluorimetric detection, for simultaneous determination of four fluoroquinolone antibiotics, namely enrofloxacin, ciprofloxacin, norfloxacin, and sarafloxacin. The EDI for the worst contamination level in this study was also calculated. A chicken sample was found to be contaminated with 114.2 and 50.7 µg/kg enrofloxacin and norfloxacin, respectively, which corresponds to estimated daily intake (EDI) of 170 and 75.7 ng kg⁻¹ body weight per day, respectively and 246 µg/kg body weight per day for total enrofloxacin and norfloxacin. The microbiologically acceptable daily intake (ADI)

for enrofloxacin is 6.2 µg/kg body weight per day. This intake was not exceeded by the EDI for enrofloxacin for the most contaminated sample, which represents approximately 3% of the microbiological ADI established.

Wang *et al.* (2017) screened 20 common antibiotic (three tetracyclines, four fluoroquinolones, three macrolides, three betalactams, four sulfonamides, and three phenicols) residues in 125 samples from common types of livestock and poultry meat, milk and aquatic products in Shanghai by ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry in 2016 and assessed their role in human exposure by Monte Carlo Simulation. The estimated daily exposure dose by Monte Carlo Simulation was less than 1 µg/kg/day. Antibiotic residues in aquatic products and their consumption accounted for 74.71% and 70.35% of overall variance of estimated antibiotic exposure for men and women, respectively. These findings indicated a high level of antibiotic residues in meat milk and aquatic products were an important source for exposure of human to antibiotics.

Pereira *et al.* (2018) have identified, quantifies and estimated the daily intake in adult and 3-year-old populations of the fluoroquinolones, norfloxacin, ciprofloxacin and enrofloxacin through poultry muscle consumption. The results showed detection frequencies of 78% and 62% in supermarket and school canteen samples, respectively. Of the 182 analysed samples, 4 did not comply with enrofloxacin maximum residue level, and 9 were contaminated with norfloxacin, not allowed as a veterinary medicine of food-producing animals. The highest estimated daily intake value was obtained for the 3-year-old population regarding the sum of enrofloxacin

and ciprofloxacin ($0.46 \mu\text{g kg}^{-1}\text{day}^{-1}$); value substantially lower than the established acceptable daily intake ($2.0 \mu\text{g kg}^{-1}\text{day}^{-1}$). Although the low risk found, the high detection frequencies support the apprehension of the different international organizations, towards the emergence of human bacterial resistances to fluoroquinolone originated from poultry production.

Oyedeji *et al.* (2019) examined imported frozen poultry products for antibiotic residues considering their health implications. Residues of 19 antibiotics were detected in the three different matrices at different levels with varying detection frequencies ranging between 2 and 4% (sulfamoxole, penicillin-G, albendazole and phebenadazole) and 14–54% for all the other antibiotics. The highest number of violative samples was found in the turkey gizzard and chicken muscle. The calculated EDI for the different poultry parts was below $1\mu\text{g/kg}$ body weight per day, with broiler muscle having the highest value of $0.017 \mu\text{g/kg bw/day}$.

2.8 Antibiotic resistance in aquaculture and pond ecosystem

Su *et al.* (2011) have isolated *Enterobacteriaceae* from four integrated fish farms in Zhongshan, South China and tested for antibiotic resistance, tetracycline resistance genes, sulfonamide resistance genes, and class 1 integrons. Relatively high antibiotic resistance frequencies were found, especially for ampicillin (80%), tetracycline (52%), and trimethoprim (50%). Out of 203 *Enterobacteriaceae* isolates, 98.5% were resistant to one or more antibiotics tested. Multiple antibiotic resistance (MAR) was found highest in animal manures with a MAR index of 0.56. Tetracycline resistance genes (*tetA*, *tetC*) and sulfonamide resistance genes (*sul2*) were detected in more than 50% of the isolates. The *Int1* gene was found in 170 isolates (83.7%).

Sahoo *et al.* (2012) investigated the antibiotic resistance pattern of *E. coli* isolated from child stool samples, cow-dung and drinking water from the non-coastal (230 households) and coastal (187 households) regions of Odisha, India. Susceptibility testing of *E. coli* isolates ($n = 696$) to the following antibiotics tetracycline, ampicillin/sulbactam, cefuroxime, cefotaxime, cefixime, cotrimoxazole, amikacin, ciprofloxacin, norfloxacin and nalidixic acid was performed by the disk diffusion method. Ciprofloxacin minimum inhibitory concentration (MIC) values were determined for ciprofloxacin-resistant isolates ($n = 83$). Resistance to at least one antibiotic was detected in 90% or more of the *E. coli* isolates.

Varghese and Roymon (2013) isolated thermotolerant *E. coli* ($n=50$) from municipal water, ground water, lake (Talpuri talab), and river Shivnath. Resistance to three or more antibiotics (multidrug resistance) was shown by 24.4% of isolates. MAR index of isolates ranged from 0.11 to 0.55. 61% of isolates in this study had an index of 0.22, 14.63% had 0.44, 7.23% had 0.33 and 2.44% had 0.55. A high index of 0.79 was shown by river Shivnath followed by 0.2 by Talpuri talab. The MAR index >0.2 is considered to originate from high risk source of contamination. High MAR indices of individual isolates and sampling sites in Durg-Bhilai region showed that water sources are contaminated with antibiotic resistant *E. coli* arising from high risk sources of contamination.

Zhang *et al.* (2013) investigate antibiotic resistance frequencies, resistance genes and resistance patterns in *E. coli* strains isolated from traditional and integrated aquaculture systems in South China by using antibiotic susceptibility testing and real

time polymerase chain reaction (PCR) technique. The *E. coli* isolates were found to be resistant to at least one antibiotic among 12 antibiotics. Higher resistance frequencies to ampicillin, sulfamethoxazole, trimethoprim, streptomycin and tetracycline were found comparable to the rest antibiotics. Among the 10 tetracycline resistance genes detected in the resistant isolates, the most prevalent tetracycline resistance genes were *tetA*, *tetW* and *tetB* with the frequency of 69.7%, 63.5% and 21.9%, respectively. Four resistance genes mainly encoding extended-spectrum beta-lactamases (ESBLs) were detected in these resistant isolates, with the detection frequencies of *bla*_{TEM} (28.4%) > *bla*_{OXA} (9.7%) > *bla*_{CTX} (9.3%) > *bla*_{CARB} (5.2%) > *bla*_{SHV} (0.0%). It was found that the integrated aquaculture system exhibited generally higher prevalence of antibiotic resistance than the traditional aquaculture system.

He *et al.*, (2014) examined the occurrence, abundance and variation of ARGs conferring resistance to chloramphenicols, sulfonamides and tetracyclines in the environments of two representative types of broiler feedlots (free range and indoor) by quantitative PCR, and assessed their dissemination mechanisms. The ARG abundances in the samples from indoor broiler feedlots were generally different to the free range feedlots, suggesting the importance of feeding operations in ARG dissemination. Pearson correlation analysis showed significant correlations between ARGs and mobile genetic element genes (*Int1* and *Int2*), and between the different classes of ARGs themselves, revealing the roles of horizontal gene transfer and coselection for ARG dissemination in the environment. Further regression analysis

revealed that *fexA*, *sul1* and *tetW* could be reliable indicator genes to surrogate anthropogenic sources of ARGs in boiler feedlots.

Huang *et al.* (2017) analyzed water and sediment from eight culture ponds (integrated culture: duck-fish pond; monoculture: duck pond and fish pond) and a control pond (without any aquaculture activity) in Zhongshan, South China. Seventeen types of ARGs were detected in all ponds, which conferring resistance to four classes of antibiotics including tetracycline (*tetA*, *tetB*, *tetC*, *tetE*, *tetG*, *tetL*, *tetA-P*, *tetM*, *tetO*, *tetS*, *tetW* and *tetX*), AmpC beta-lactamase products (*EBC* and *FOX*), sulfonamide (*sul1* and *sul2*) and erythromycin (*ermA*), with class 1 integron (*Int1*) as motility gene. The total concentrations of detected ARGs in culture pond water were much higher than control (about 1.6e4.0 times). Integrated culture showed lowest absolute abundance of P ARGs in water and the highest in sediment. Monoculture ponds showed higher relative abundance of P ARGs both in water (fish pond: 0.5149) and sediment (duck pond: 0.4919). As the main contributor to the ARGs abundance and significant correlations with P *tet*, P ARGs and *Int1* ($P < 0.01$), *tetA* was suggested to be a potential indicator for the abundance of tetracycline resistance genes in these classes of aquaculture modes in the Pearl River Delta. This study provides a case for the ARGs abundance in aquaculture and as a reference for the upcoming health risk assessment in aquatic environment.

Zhou *et al.* (2020) investigated the effects of duck manure, As and cefotaxime on the bacterial community, and the evolution and distribution of ARGs and metal (loid) resistance genes (MRGs) in water and biofilm. Five groups of fish tanks

included a control and four test groups. The experimental period lasted for 100 days. Six ARGs (*CIT*, *DHA*, *EBC*, *FOX*, *MOX*, *TEM*), two MRGs (*arsB*, *arsC*), and two integron genes (*intI1*, *intI2*) were tracked and detected in water and biofilm. The results showed that duck manure brought ARGs and MRGs into fish tanks. Stress factors (cefotaxime, As) increased the relative abundance of resistance genes, and this was positively correlated with stress concentrations. The biofilm was visible significantly at the end of stage 3, and the total relative abundance of resistance genes in biofilm was higher than water from stage 5 onwards. Evolution of AmpC β -lactamase resistance genes was more obvious than MRGs, especially for *MOX*, which increased by 3 orders of magnitude.

2.9 Antibiotic resistance in commensal *E. coli*

2.9.1 Prevalence of resistant *E. coli* in humans

Cho *et al.* (2011) studied the prevalence of antibiotic resistance in fecal *E. coli* isolates from healthy persons and patients with diarrhoea. *E. coli* isolates (n = 428) were obtained from fecal samples of apparently healthy volunteers and hospitalized patients with diarrhea. Susceptibility patterns of isolates to 16 antimicrobial agents were determined by agar disc diffusion. Most *E. coli* isolates exhibited less than 10% resistance against imipenem, cefotetan, aztreonam, cefepime, cefoxitin, amikacin and netilmicin, although greater than 65% were resistant to ampicillin and tetracycline. However, antibiotic resistance rates to cefoxitin, cefotaxime, amikacin, and netilmicin were significantly higher in the isolates of men than those of women ($p < 0.05$) in isolates from patients with diarrhoea.

Dureja *et al.* (2014) investigated distribution of phylotypes and presence of genes encoding integrons, extended beta-lactamases and resistance to other antimicrobials in the commensal *E. coli* isolates from healthy adults in Chandigarh, India. Integron-encoded integrases were detected in 25 isolates with class 1 integron-encoded *intI1* integrase being in the majority (22 isolates). The gene cassettes identified were those for trimethoprim, streptomycin, spectinomycin and streptothricin. Phenotypic assay for screening the potential ESBL producers suggested 16 isolates to be ESBL producers. PCR detection using gene-specific primers showed that 15 out of these 16 ESBL-producing *E. coli* harboured the *bla*_{CTX-M-15} gene.

Purohit *et al.* (2017) investigated commensal coliforms from stool samples from children aged 1–3 years and their environment (animals, drinking water from children's households, common source and waste-water) were studied for antibiotic susceptibility and plasmid-encoded resistance genes. *E. coli* isolates from human (n = 127), animal (n = 21), waste (n = 12), source (n = 10), and household drinking water (n = 122) carried 70%, 29%, 41%, 30%, and 30% multi-drug resistance, respectively. ESBL producers were 57% in human and 23% in environmental isolates. Co-resistance was frequent in penicillin, cephalosporin, and quinolone. Antibiotic-resistance genes *bla*_{CTX-M-9} and *qnrS* were most frequent. Group D-type isolates with resistance genes were mainly from humans and waste water.

Antony *et al.* (2018) collected faecal samples of 154 healthy volunteers were screened for *Enterobacteriaceae* resistant to commonly used antibiotics by standard methods. Polymerase chain reaction was done to detect *bla*_{CTX-M}, *bla*_{CIT}, *bla*_{KPC-1} and

*bla*_{NDM-1} genes coding for ESBL, AmpC, KPC and NDM, respectively. Colonisation rate of enteric bacteria with multiple drug resistance (MDR) genes in the community was 30.1%. However, phenotypically, only ESBL (3.2%) and NDM (0.65%) were detected. While the genes coding for ESBL, AmpC and NDM were detected in 35.6%, 17.8% and 4.4% of the MDR isolates, respectively. Carriage of MDR strains with a potential to express multidrug resistance poses a threat of dissemination in the community. Awareness for restricted use of antibiotics and proper sanitation can contain the spread of resistant bacteria.

Bok *et al.* (2018) conducted the comparison of genetic diversity of commensal *E. coli* isolates from two age group adults and young children. *E. coli* strains were isolated on MacConkey agar and identified by biochemical tests. Phenotypic analysis of resistance was based on disc-diffusion method. The prevalence of virulence genes was significantly higher among isolates from adults than from young children. Phylogroup B2 predominated among *E. coli* from adults, whereas phylogroup A was the most common in isolates from young children. The analyses of antimicrobial resistance revealed that resistance to at least one antimicrobial agent and multidrug-resistance were detected significantly more frequent in the isolates from adults than from young children.

Purohit *et al.* (2019) determined the prevalence of antibiotic resistance in commensal *E. coli* in young children in rural setting of central India and search for its correlations with demographic and behavioral factors. They isolated six isolates of *E. coli* per stool sample and subjected them to antibiotic susceptibility testing. We found resistance to ampicillin, quinolones, cephalosporins, sulfamethoxazole, co-

trimoxazole, in at least one isolate from 89% to 100% of children. Extended spectrum beta lactamase producing *E. coli* were identified in all but one child and multidrug resistance was identified in isolates from all children. Female gender ($p=0.04$) and higher wealth ($p=0.03$) was significantly correlated with less antibiotic resistance. Thus, the high prevalence of antibiotic resistance in commensal *E. coli* in rural community from India needs urgent measures to control the growing antibiotic resistance crisis.

2.9.2 Prevalence of resistant *E. coli* in animals

Manna *et al.* (2006) studied occurrence of *E. coli* serotype O157 in cattle stool in West Bengal, India, and the virulence properties and antimicrobial resistance of the *E. coli* isolates. Following enrichment in modified EC broth and plating onto HiCrome MS.O157 agar, a total of 14 strains of *E. coli* serotype O157 was isolated from faecal samples from two (2.04%) slaughtered cattle and six (7.59%) diarrhoeic calves. By multiplex PCR, Shiga toxin genes were detected in all the isolates. Among 14 strains, ten were resistant to at least one of the antimicrobial agents tested. The study showed that occurrence of Shiga toxin-producing and multiple antibiotic-resistant *E. coli* O157 among cattle population in this region of India is significance.

Mahanti *et al.* (2013) isolated 363 *E. coli* from 165 faecal samples of healthy buffaloes in West Bengal, India. Twenty-four of these isolates (6.61%) were found to carry at least one gene characteristic for Shiga toxin-producing *E. coli* (STEC). These STEC strains belonged to 13 different O-serogroups. The isolated STEC strains were resistant most frequently to erythromycin (95.83%), cephalothin (62.5%), amikacin

(54.17%), kanamycin (45.83%) and gentamicin (41.67%) group of antibiotics. No ESBL-producing (*bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}) or quinolone resistance gene (*qnrA*) was detected in the STEC isolates.

Mainda *et al.* (2015) focused on the use of antibiotics on small, medium and commercial-sized dairy farms in the central region of Zambia and its relationship to antibiotic resistance in *E. coli*. An *E. coli* isolate was obtained from 98.67% (371/376) of the sampled animals and tested for resistance to six classes of antibiotics. The estimated prevalence of resistance across the different farming systems was: tetracycline (10.61; 95%CI: 7.40–13.82), ampicillin (6.02; 95%CI: 3.31–8.73), sulfamethoxazole/ trimethoprim (4.49; 95%CI: 2.42–6.56), cefpodoxime (1.91; 95%CI: 0.46–3.36), gentamicin (0.89; 95%CI: 0.06–1.84) and ciprofloxacin (0%).

Islam *et al.* (2016) collected a total of 150 rectal swab samples from selected goats. The samples were subjected for the isolation of *E. coli* through a series of conventional bacteriological and biochemical techniques. The isolated *E. coli* were used for assessing antimicrobial susceptibility by disk diffusion method. The overall prevalence of *E. coli* in the rectal swabs of goats was 52% (n=78/150). The young goats were highly susceptible (65.0%; n=52/80) to *E. coli* infection as compared to adults (37.1%; n=26/70). The prevalence was higher (66.7%; n=42/63) in the goats that was reared in poor hygienic condition as compared to the goats that were reared under good hygienic condition.

Ndegwa *et al.* (2019) collected fecal samples from a mixed age cohort over a one-year period. No antibiotic had been used on the study animals one year prior to and during the study period. Resistant isolates were detected in all age groups and prevalence in goat kids was significantly higher than adults; 43–48% vs. 8–25% respectively. The proportion of resistant isolates was higher when animals were congregated near handling facility than on pasture. Most isolates were resistant to tetracycline (51%) and streptomycin (30%), but also to antibiotics that had never been used on the farm; ampicillin (19%). *TetB*, *bla*_{TEM}, (*aadA* and *strpA/strpB*) genes were detected in 70%, 43%, (44% and 24%) of tetracycline, ampicillin, and streptomycin resistant isolates respectively. Environmental sources may play a role in acquisition of resistant bacteria in pastured goats.

Shabana and Enazi (2020) investigate the plasmid-mediated resistance in *E. coli* strains isolated from healthy and diarrhoeaic sheep and goats. A total of 159 *E. coli* strains harbored plasmids. The isolates antibiogram showed different patterns of resistance in both healthy and diarrheic animals. A total of (51.5%) *E. coli* strains were multidrug-resistant. *rmtB* gene was detected in all Aminoglycoside-resistant *E. coli*, and the ESBL-producing *E. coli* possessed different *CTX-M* genes. Similarly, fluoroquinolone-resistant *E. coli* possessed different *qnr* genes. On the analysis of the *gyrB* gene sequence of fluoroquinolone-resistant *E. coli*, multiple point mutations were revealed.

Subbiah *et al.* (2020) investigated that bacterial transmission are responsible for the emergence, spread and persistence of antimicrobial-resistant bacteria, but

their relative contribution likely differs across varying socio-economic, cultural, and ecological contexts. Household-level data (n=425) was collected and bacteria isolated from people, livestock, dogs, wildlife and water sources (n= 62,376 isolates). The relative prevalence of different resistance phenotypes is similar across all sources. Multi-locus tandem repeat analysis (n= 719) and whole-genome sequencing (n= 816) of *E.coli* demonstrate no evidence for host-population subdivision. Multivariate models show no evidence that veterinary antibiotic use increased the odds of detecting AR bacteria, whereas there is a strong association with livelihood factors related to bacterial transmission, demonstrating that to be effective, interventions need to accommodate different cultural practices and resource limitations.

2.10 Role of house flies in the spread of antimicrobial resistance

Usui *et al.* (2013) investigated the role of flies in spreading the ESBL gene from food-producing animals to humans. Cephalosporin resistant strains were isolated from 14.3% (13/91) of houseflies, 10.3% (7/68) of false stable flies, and 7.5% (7/93) of cattle feces. Twenty-seven cephalosporin-resistant strains were tested for the presence of antimicrobial resistance genes of the 27 samples, 22 isolates from 11 houseflies, 5 false stable flies, and 6 cattle feces samples harbored the *bla*_{CTX-M-15} gene. All *bla*_{CTX-M-15} harboring isolates belonged to phylogenetic group D and the ST38 clonal group. Analysis of pulsed-field gel electrophoresis showed that these isolates were divided into two clusters, indicating that flies carried several of the same clones that were detected in cattle feces. All *bla*_{CTX-M-15} gene-harboring plasmids were transferable and were members of incompatibility group FIB. These results

suggested that transferable plasmids encoding ESBL were prevalent among flies and cattle.

Blaak *et al.* (2014) reported the isolation of ESBL-producing *E. coli* from house flies and blow flies caught at two poultry farms, offering a potential alternative route of transmission of ESBL-producing *E. coli* from poultry to humans. Overall, 87 flies were analyzed in 19 pools. ESBL-producing *E. coli* bacteria were detected in two fly pools (10.5%) a pool of three blow flies from a broiler farm and a pool of eight house flies from a laying-hen farm. From each positive fly pool, six isolates were characterized and compared with isolates obtained from manure (n= 53) sampled at both farms and rinse water (n=10) from the broiler farm. Among six fly isolates from the broiler farm, four different types were detected with respect to phylogenetic group.

Sola-Gines *et al.* (2015) evaluate the presence of *E. coli* strains resistant to cephalosporins in flies captured in the areas surrounding five broiler farms. The presence of ESBL genes, their plasmid location, and the mobile genetic elements involved in their mobilization were studied. Out of 682 flies captured, 42 yielded ESBL-producing *E. coli*. Of these isolates, 23 contained *bla*_{CTX-M-1}, 18 contained *bla*_{CTX-M-14}, and 1 contained *bla*_{CTX-M-9}. ESBL genes were associated mainly with the presence of the IncI1 and IncFIB replicons. Additionally, all the strains were multidrug-resistant, and five of them also harbored *qnrS*. The entrance of flies into broiler houses constitutes a considerable risk for colonization of broilers with multidrug-resistant *E. coli*.

Songe *et al.* (2017) collected flies found on fish in markets in Mongu and Lusaka districts of Zambia. For the entire study, a total of 418 fly samples were analyzed in the laboratory and *Salmonella* spp. and enteropathogenic *E. coli* were isolated from the flies. Further laboratory screening revealed that overall, 17.2% (72/418) (95% CI; 43.2%–65.5%) of total samples analyzed contained ESBL producing *E. coli*. These significant findings call for a strengthening of the antibiotic administering policy in Zambia and the development of sustainable interventions to reduce fly numbers in food markets and improve food safety and hygiene.

Sobur *et al.* (2019) detected antibiotic-resistant pathogenic *E. coli* in house flies captured from various environmental settings. Isolation and identification of *E. coli* was performed based on culture and PCR. Antimicrobial susceptibility testing was conducted using disk diffusion test. Detection of virulence and antimicrobial resistance genes was carried out using PCR methods. The prevalence of *E. coli* in flies was 61% with highest in dairy farms (70.67%) followed by food centers (65.33%), dustbins (64%) and area close to hospital (44%). The prevalence rates of *tetA*, *tetB*, *ereA*, *CITM*, *SHV*, *mcr-3* and *catA1* resistance determinants were 88.75, 48.52, 41.67, 37.17, 27.77, 18.37 and 14.81%, respectively. Multidrug-resistant pathogenic *E. coli* are widely distributed in house flies, which require a one-health approach to delineate their control.

*MATERIALS AND
METHODS*

CHAPTER III

MATERIALS AND METHODS

The rampant application of the antibiotics as feed additive and extra label use for treatment in poultry production is currently regarded as a serious problem for public health and it can pose hazards to the health of consumers because residues of these drugs are present in meat of these poultry birds. Antibiotic resistance is one of the biggest threats to global health and aquatic ecosystems are one of the most important areas and play role in the horizontal transfer of resistance gene between different bacterial communities. Chhattisgarh is the sixth largest producer of inland fish and the fifth largest producer of fish seed in the India. The state has 59591 rural/village ponds covering 0.75 lakh hectares which is used as aquaculture unit and rural people of Chhattisgarh depend entirely on pond water for taking bath, washing their clothes and their culinary utensils as well as washing their cattle and other pet animals. All these livelihood, day to day practices can lead to resistance development and transfer of resistance among bacteria. House fly is known as vector for different pathogen, can be involved in the transmission of antibiotic resistance bacteria and spread the resistant bacteria to different animal originated foods. The present study was undertaken to study the status of antibiotic residues in marketed chicken meat from districts of Chhattisgarh and to know the status of antibiotic resistance in *E. coli* isolated from different aquatic ecosystems and flies collected from milk and meat shops.

The present study was conducted at the Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences and Animal Husbandry, Anjora, Durg, Chhattisgarh.

3.1 Sampling plan

3.1.1 Description of Study Area

This study was carried out in Chhattisgarh, a state geographically situated in central India between 17°46' N to 24°5' North Latitude and 80°15' E to 84°20' East Longitude. The state is divided into three agroclimatic zones viz., Northern hill, Central plains and Bastar plateau and subdivided into 27 revenue districts with 146 blocks and 19720 villages. The climate of state is tropical dry sub-humid type with around 1400 mm average annual rainfall and temperature between 0-25°C in winter and between 30-45°C in summer. Due to the part of tropical country state support three well defined seasons summer, winter and monsoon. The total geographical area of the state is around 135,192 Km² with 25.5 million human population (Jain *et al.*, 2019) of which about 80% of the population is living in rural areas.

The Chhattisgarh state produced 60.88 thousand tonnes of meat and 18928 lakhs of eggs in financial year 2018-2019(AHDC, 2020). It is the 6th largest fish producer of inland fisheries in the country. Available water in the state is 1.57 lakh ha out of which 1.47 lakh ha water area has been developed under fish farming i.e., 94% of the total water area (Anand *et al.*, 2020).

3.2 Collection of samples

3.2.1 Marketed chicken meat samples

Chicken meat samples were collected from freshly slaughtered poultry birds from retail markets, and butcher shops of different districts located in three agro climatic zones of Chhattisgarh state. The samples were collected in a sterile self-sealing polythene bags, which was neatly labelled, packed and brought in insulated sample collection box in ice to the laboratory for further analysis.

3.2.2 Collection of water and fecal samples from community and aquaculture ponds

The study was conducted in plain zone of Chhattisgarh in which water and fecal sample were collected from Drug and Kanker districts. In study area many ponds are used as aquaculture farms, comprising of traditional aquaculture ponds, integrated aquaculture ponds and village community pond, where human, animal and aquaculture activities are undertaken simultaneously. To study the bacterial resistance pattern in water ecosystem, three ponds were selected for the collection of water samples: village community pond (W1), traditional fish pond (W2), and duck-fish-goat integrate pond (W3). The fecal samples were also collected from: cattle and buffalo (M1), human utilizing village community pond water; five samples (M2), human not utilizing village community pond water; five samples (M3), duck from the duck-fish-goat integrated pond (M4), goat from the duck-fish-goat integrated pond (M5).

The water samples were collected in 500 ml sterile polypropylene bottles at 0–20 cm below the water surface, while the feces samples were collected in 100 mL

sterile sampling box. All samples were stored in a cooler, transported to the laboratory, and processed within 12 h after collection.

3.2.3 Collection of house flies from meat and milk shops

Milk and meat shops located in Durg and Raipur were the places of choice for the present study. A total of 150 house flies (*Muska domestica*) were collected from these markets by using sticky tapes. Approximately 9-12 pieces of the sticky tapes (~ 15 cm per piece) were set up on each shop on card board and placed at area of flies congregation. Approximately 25-30 flies stucked within 10–60 min, were collected from each shop and brought to the laboratory as early as possible in refrigerated condition using sample collection box filled with ice packs.

3. 3 Materials

3.3.1 Equipments

The equipments used for the present study were Merck millipore water purification system (Direct-Q3TM, MerckKGaA, Darmstadt, Germany), pH/Tutor pH/Temperature bench meter (Eutech instruments, Thermo Scientific), Vortex Shaker (Tarsons), Refrigerated Centrifuge (Thermo Fisher), Electronic balance (Sartorius), -20 °C Deep freezer (Remi), -80 °C Deep freezer (Thermo Scientific) Hot air oven (Unitech), Autoclave (Obromax), Gel documentation system (Bio-Rad), Horizontal gel electrophoresis unit (Biometra), Hot air oven (Unitech), Incubator (Mac), UV spectrophotometer (Biometra TI3), Water bath (Rivotek), Laminar flow (Klenz flow), Refrigerator (LG), Micropipette (Thermo Scientific), Ultrasonic bath (PCi Analytics) and filtration chamber with pump for vacuum creation were used during the course of

present study. The remaining instruments used in this study were described in concerned section.

3.3.2 Glass wares and plastic wares

The glassware from Borosil Glass Works Ltd. (India) and plastic wares comprising polypropylene tubes (Tarsons, India), microfuge tubes, micropipette tips (Tarsons, India), disposable syringes (Dispovan), parafilm (E- Merk, Germany) and PVDF syringe filters (Merk, Millipore) were used in the present study. Chromatographic auto sampler glass vials were purchased from Waters Technologies Ltd. (USA). The glasswares were thoroughly cleaned with hot water and rinsed with deionized milli-Q water followed by drying in hot air oven at 80 °C. Before use, glasswares were again rinsed with HPLC grade acetone to make them free from any residual contamination.

3.3.3 Reagents solvents and biologicals

All the reagents used in the present study were of analytical grade and procured from Merck life science (EMPARTA[®]) and Himedia. Solvents used in the study were of HPLC grade and purchased from Merck. The molecular biology grade buffers and other reagents were purchased from Sigma-Aldrich and Merck.

All the bacteriological media, chemicals and reagents used in the present study were obtained from HiMedia, (India), Thermo Scientific, (USA) and Genei, (India) and prepared according to the instructions provided by the manufacturing firms.

3.4 Detection of antibiotic residues in chicken meat samples

3.4.1 High performance liquid chromatography (HPLC) system

The Alliance[®] – e2695 analytical high performance liquid chromatography with Waters[®] 2998 Photodiode Array (PDA) detector was used. It was an integrated, pre-configured, pre-plumbed system controlled by Empower[™] 3 software. The system carries quaternary gradient pump to deliver pulse free solvent flow for accurate and highly reproducible gradient formation. The Specifications of the HPLC system are summarized in Table 1.

3.4.2 HPLC columns

For separation of antibiotics by HPLC system, Xbridge columns, (C18, 3.5 μm ; 150 mm x 4.6 mm) and ZORBAX Eclipse XDB column (C18, 5 μm ; 250 mm x 4.6 mm) were procured from Waters Corporation and Agilent Technologies USA, respectively. These columns had the high mass loading capability, excellent low pH stability, high efficiencies and ability to produce superior peak shapes.

Table 1: Performance specifications of Waters Alliance® – e2695 HPLC system

Solvent management by quaternary pump	
Number of solvents	One To Four
Degasser	4 channel, vacuum, built in
Maximum operating Pressure	5000psi
Flow rate range	0.000 to 10.000 ml/min
Flow Accuracy	±1% or 10µl
Auto Sampler	
Injection volume range	0.1 to 100 µl
Injection accuracy	±1 µl (±2%) (50 µl, N=6)
Sample carry over	≤0.01% for caffeine
Column heater/cooler	
Temperature range	Ambient -15 to 65 °C
Temperature Accuracy	±0.6 °C
PDA Detector	
Detection type	Photo diode array detection
Light source	Deuterium arc lamp
Wavelength range	190 to 800nm
Wavelength accuracy	±1nm

3.4.3 Solid phase extraction unit

To remove specific interferences from the sample and minimize problems in detection and quantitation of analytes of interest, solid phase extractor was used. It was connected to the vacuum pump for suction of samples under negative pressure.

3.4.3.1 Solid phase extraction cartridges

Oasis HLB (60 mg, 3 cc): Hydrophilic-lipophilic-balanced reversed-phase sorbent for acids, bases and neutrals was used for clean-up of the sample extracted solution. These cartridges were supplied by Waters Corporation, USA. These extraction cartridges have better efficiency to remove co-extracts from main analyte solution.

3.4.4 Standards

The antibiotics used as analytical standards of antibiotics (Table 2) (tetracyclines, sulphonamides and fluoroquinolones) were procured from Sigma-Aldrich. Purities of antibiotic standards were in the range of 98-99%. The standard operating procedures were followed for obtaining, labelling, storing and handling of antibiotic standards. Reference standards were initially stored in deep freezer at -20 °C under dry storage conditions and were brought to room temperature prior to use. The standards working solutions of different antibiotics were prepared in volumetric glass flask using different solvents (Table 2). These standard solutions were diluted further by using same solvent to make working standard solution with different concentrations. The working standards solutions were stored in deep freezer at -20°C. Particular attention was given to compound toxicity and likelihood of exposure to the compound during handling.

Table 2 List of antibiotic standards used during the study

Group of antibiotic	Antibiotics	Purity	Solvent used for making standard stock solution
Tetracyclines	Oxytetracycline (OTC)	98-99%	Methanol
	Tetracycline (TC)	98-99%	Methanol
	Chlortetracycline (CTC)	98-99%	Methanol
Sulphonamide	Sulfamethazine (SMZ)	98-99%	Methanol
Fluoroquinolones	Ciprofloxacin (CIP)	98-99%	1 ml acetic acid + Methanol
	Enrofloxacin (ENR)	98-99%	1 ml acetic acid + Methanol

3.4.5 Preparation of standard solutions

Standard stock solution (0.1mg/ml) of oxytetracycline, tetracycline, chlortetracycline, Sulfamethazine, and ciprofloxacin, enrofloxacin were prepared by dilution of appropriate volume of the standards in respective solvent. The stock solution containing glass vials were wrapped in aluminium foil and stored under -20°C until used. Working solution in the concentration ranging from 50 µg/kg to 500 µg/kg were prepared daily by appropriate dilution of aliquots of the standard stock solutions in HPLC grade water. The working solutions were used directly to produce calibrations standards in the range of 50,100,200,300 and 500 µg/kg concentrations and also used for sample fortification. All the stock solutions were stored for a period of 90 days under -20°C storage condition.

3.4.6 Preparation of reagents

3.4.6.1 Preparation of 0.01 M Sodium EDTA-McIlvaine buffers

Sodium EDTA-McIlvaine buffer (0.01M) was prepared by mixing 11.8g of citric acid monohydrate, 13.72g of Na_2HPO_4 and 33.62g of sodium ethylene diaminetetraacetate in 1L HPLC grade water and pH was adjustment to 4. The prepared buffer was stored at 4°C until used.

3.4.6.2 Preparation of 20 % Trichloroacetic acid (TCA)

Twenty gram of crystalline trichloroacetic acid was dissolved in 100ml of HPLC grade acetonitrile. The prepared acid was stored at 4°C until used.

3.5 Standardization and validation of HPLC method for detection of antibiotic residues in chicken meat

For the detection of OTC, TC, CTC, SMZ, CIP and ENR belonging to three different groups of antibiotics, two different HPLC protocols were standardised. However all antibiotics were efficiently extracted and cleaned up from meat matrix by using a single method.

3.5.1 Standardization of HPLC conditions

The main aim was to separate and detect OTC, TC, CTC, SMZ CIP and ENR, belonging to three different groups, by using HPLC with photodiode array detector and measuring each analyte at its best detection condition, i.e. maximum absorption wavelength. The optimum wavelengths were: 280nm (SMZ), 280 nm (ENR and

CLP) and 360 nm (OTC, TC and CTC).The mobile phase employed by Zhou *et al.* (2009) and Moema *et al.* (2012) were used for standardization of HPLC condition in the present study (Table 3) .

A 50µl/15µl aliquot of each antibiotic standard (OTC, TC, CTC, SMZ,CIP, and ENR) solution at a concentration of 500 µg/kg (prepared from stock solution in water/methanol) was injected into the HPLC system. The chromatographic separations of antibiotics were performed on C18 Xbridge and Zorbax Eclipse XDB C18 columns. The retention time of standards were recorded.

3.5.2 Standardization of extraction procedure for antibiotic residues from meat

The method for simultaneous extraction of OTC, TC, CTC,SMZ, CIP and ENR from meat was developed. The method consisted of extraction of antibiotics from meat matrix followed by clean-up of extracts on SPE cartridges.

3.5.2.1 Liquid-liquid extraction of antibiotic residues from meat

Five gram chicken meat is homogenized and transferred into 50 ml polypropylene centrifuge tube. Then 4 ml of 20 % TCA in acetonitrile was added. The sample is then vortexed for 5 min and 20 ml of McIlvaine-EDTA buffer (pH 4) was added and sample was again vortexed and sonicated for 10 min. The sonicated sample was centrifuged at 7000 rpm for 15 min at room temperature.The supernatant was separated and filtered through Whatman # 42 filter paper. The filtrate was then collected in a clean beaker and processed for further clean-up of sample.

Table 3 HPLC conditions for detection of antibiotic residues in chicken meat

Antibiotic classes	Tetracyclines (OTC,TC,CTC)	Sulfonamide (SMZ)	Fluoroquinolones (CIP, ENR)
HPLC Conditions			
Injection volume	50µl	50 µl	15 µl
Flow rate	1ml/min	1ml/min	0.82 ml/min
Detection wavelength	360 nm	280 nm	280 nm
Column	ZORBAX XDB-C18 (C18, 5 µm; 250 mm x 4.6 mm)	ZORBAX XDB-C18 (C18, 5 µm; 250 mm x 4.6 mm)	Xbridge- C18 (3.5 µm; 150 mm x 4.6 mm)
Column Temperature	30 °C	30 °C	40°C
Analysis Time	10 min	10 min	17 min
Mobile Phase	0.01 M oxalic acid (pH 4.0), methanol and acetonitrile (70:10:20, v/v/v), (Isocratic)	0.01 M oxalic acid (pH 4.0), methanol and acetonitrile (70:10:20, v/v/v), (Isocratic)	A (acetonitrile) and B (0.1% formic acid in water) at pH 2.74. (Gradient)*

* Gradient elution mode: initially A was at 13% and was gradually increased to 15% in 7 min, decreased to 10% in another 7 min and finally decreased to 13% in 3 min.

3.5.2.2 SPE clean-up of antibiotic residues from of meat extract

Clean up of extract was done by employing Solid Phase Extraction (SPE) unit. The sample was applied to SPE column (Oasis HLB 3cc, 60 mg cartridge) preconditioned with 3 ml methanol and 3 ml of water under gravity. After sample loading and passing the sample through the cartridge under vacuum, the cartridges were washed with 2 ml of 5 % methanol. The putative antibiotic residues were finally eluted with 3 ml of methanol and 2 ml of 1% TFA in acetonitrile. Elute was collected in a clean beaker and concentrated to dryness at 45°C under vacuum concentrator. The residues were dissolved in 1 ml solvent (MeOH : water,20:80) and filtered through 0.22 µm syringe filter and stored in vials for further analysis. A standardised injection volume of 50µl/15 µl was injected into the HPLC system for detection of respective antibiotic residues.

3.5.3 HPLC analysis of antibiotic residues

The processed meat samples (blank or spiked) were analysed in the HPLC system as mentioned in Table 3. Mobile phase containing an aqueous phase and organic phase was used for the analysis of processed samples. The flow rate was fixed according to antibiotics and quantification was performed by using PDA detector.

3.5.3.1 Preparation of calibration curves for antibiotics

Plotting of calibration curves, were done with known antibiotic standard solutions (OTC, TC, CTC, SMZ, CIP and ENR) at five fortification levels: 50,100,200,300 and 500 µg/kg were prepared from stock antibiotic solutions of

0.1mg/ml after appropriate dilutions. The analysis was done in triplicate for each concentration of antibiotic. The calibration curves were plotted separately for each antibiotics targeted in the present study.

The spiked meat samples were extracted, clean-up and analysed by injecting 50µl/15 µl of aliquot into the HPLC system. The retention times (RTs) and peak areas of spiked samples were recorded. The calibration curves were plotted by least- square linear regression analysis of the peak area of analyte (y-axis) against the corresponding analyte concentration (x-axis). From calibration curves, the linear equation was obtained *i.e.* $y = mx+c$

Where,

y = peak area of analyte

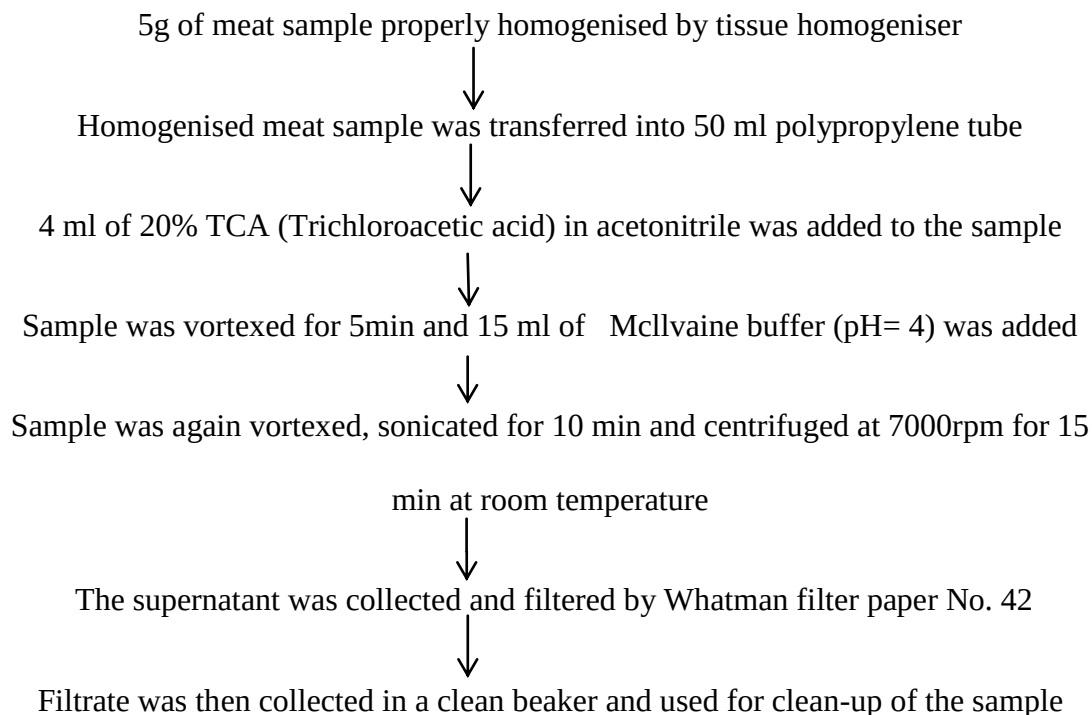
x = concentration (ng/ml)

m = slope of calibration curve

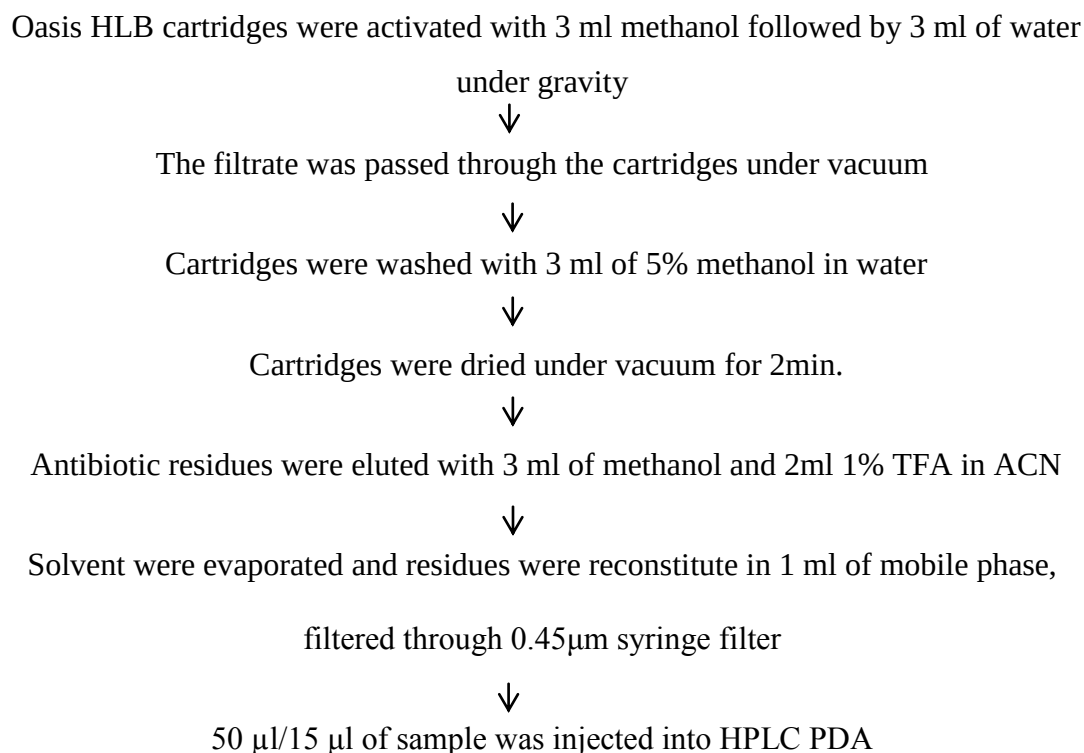
c = intercept

The linear regression equation and the coefficient of determination (r^2) were also determined by calibration curves.

Flow diagram 3.1: Extraction of antibiotic residues from meat sample



Flow diagram 3.2: Clean-up of sample for antibiotic residues



3.5.4 Validation of HPLC method

The method was validated according to the European Commission decision 2002/657/EC using spiked meat samples (European Commission, 2002; European Commission, 2010). The following performance criteria were determined: linearity, specificity, intra-assay and inter-assay precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

3.5.4.1 Linear range, LOD and LOQ

The Linear range, LOD and LOQ were calculated from the calibration curves by triplicate analysis of OTC, TC, CTC, SMZ, CIP and ENR at five dilution levels (50,100, 200, 300 and 500 µg/kg) in solvent as mentioned above.

The linear range for all the above mentioned antibiotics were evaluated by plotting calibration curves in the range of 50-500 µg/kg. LOD and LOQ were calculated from the standard deviation (σ) of y-intercepts of regression analysis and the slope of calibration curve (m). The detection limit of method is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. It was calculated using following formula:

$$\text{LOD} = 3.3 \times \frac{\text{Standard deviation of intercept } (\sigma)}{\text{Slope of calibration curve } (m)}$$

The quantification limit of method is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Following formula was used:

$$\text{LOQ} = 10 \times \frac{\text{Standard deviation of intercept } (\sigma)}{\text{Slope of calibration curve } (m)}$$

3.5.4.2 Specificity

The Specificity of the method was assessed by analysis of ten different blank samples to determine any interference from endogenous compounds.

3.5.4.3 Accuracy

The accuracy of an analytical method expresses the nearness between the expected value and the value found. It is obtained by calculating the percent recovery (R%) of the analyte recovered. To evaluate the accuracy of the developed method, successive analysis (n = 5) for three different concentrations (300 µg/kg, 200 µg/kg and 100 µg/kg) of all standard antibiotic (OTC, TC, CTC, SMZ CIP and ENR) solutions were performed using the developed method. The data of the experiment were statistically analyzed using the formula [% Recovery = (Recovered conc. / Injected conc.) × 100] to study the recovery and validity of the developed method. The mean recovery within 80–110% was accepted.

3.5.4.4 Precision

Precision of a measurable technique is the degree of agreement among individual test, when the technique is applied repetitively to analyze multiple replicates in different occasions. The precision of the method consists of intraday precision and inter-day precision. The intraday precision was assessed by analyzing the five replicates of same concentrations of 200 µg/kg within the same day. The

inter-day precision was determined by analyzing five replicates of three different concentrations (100,200,300 µg/kg) of antibiotics on five different days. The total precision of the method was expressed as the relative standard deviation (%RSD).

3.5.5 Risk assessment of dietary exposure to antibiotic residues through meat

The risk assessment of dietary intake of antibiotic residues through meat was estimated based on amount of residues in meat samples. The mean level of antibiotic concentration in meat samples was evaluated. Average daily consumption of meat based on 60 kg body weight for an adult and 15 kg body weight for children were taken into account (JECFA, 2006). A risk analysis was determined using the hazard quotient (HQ).

$$\text{Hazard quotient} = \frac{\text{Estimated daily intake (EDI)}}{\text{Acceptable daily intake (ADI)}}$$

EDI for antibiotic residues was calculated using formula given by Juan *et al.* (2010)

$$\text{EDI} = \frac{(\text{Mean of } \mu\text{g of antibiotics per kg of food}) \times (\text{Daily intake of food})}{\text{Body weight (kg)}}$$

ADI is determined as a conservative estimate of the safety ingestion levels by the human population, based on the lowest “no observed adverse effect level” among a battery of toxicological safety studies. ADI values for antibiotic residues established by WHO were taken into account while calculating the HQ.

As there are no official data on the daily poultry consumption in the study area, the mean annual poultry intake per person outlined in the report of the Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture and Farmers welfare, Government of India (DADF, 2017) was used. As per the report per capita availability of chicken meat in India was estimated to be 5 Kg/year up to 2022-23.

3.6 Isolation and identification of *E.coli*

3.6.1 Isolation of *E. coli* from pond water sample

Isolation of *E. coli* from pond water sample was performed following the protocol of USEPA (2006). Each water sample was ten-fold serially diluted (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6}) with a phosphate-buffered saline (PBS) solution and 100 ml of each dilution was then filtered through a cellulose nitrate filter (0.45 μ m pore size, 50 mm diameter) in order to obtain 6 colonies per filter. The filters were placed on to M-TEC Agar and incubated at 35 °C for 2 h, followed by incubation at 44 °C for 24 h. The red colonies on each M-TEC agar plate were assumed as *E. coli*. In order to ensure the isolation of *E. coli*, colonies were randomly inoculated onto EMB agar plate and incubated at 37 °C for 24 h. Isolates with green-metallic sheen were confirmed as *E. coli*.

3.6.2 Isolation of *E. coli* from human and animal fecal samples

Approximately 1g of the faecal sample was diluted in 100 ml of sterile phosphate buffer saline (PBS) by vortexing for 1 min and 100 μ l diluted liquid was again diluted into 10ml PBS. Again 100 μ l diluted sample was further diluted in 10ml

PBS. For proper mixing each step of dilution process was vortexed for 1 min. Finally 20µl of the aliquot was spread on M-TEC Agar using a sterile spreader and incubated at 37°C for 24 hours. The pinkish-red colony was randomly picked and subcultured once more on EMB agar under same conditions. The isolate showed green-metallic sheen was confirmed as *E. coli*. From each sample five to six isolates of *E. coli* were selected for future analysis.

3.6.3 Antibiotic susceptibility testing

Antibiotic sensitivity test was performed as per the method of Bauer and Kirby (1966). Commercially available antibiotic discs (HiMedia laboratories Limited, Mumbai) were used to test susceptibility of the isolates against different antibiotics. (Table 4).

For determining the susceptibilities of bacterial isolates to different beta-lactam and non beta-lactam antibiotics disc diffusion method was performed on Mueller-Hinton agar (MHA) plates and results were interpreted as per clinical and laboratory standards institute (CLSI, 2012) guidelines. Pure culture from the nutrient agar slant was transferred into a tube containing 5 ml Luria-Bertani broth. The broth culture was incubated at 37°C. After incubation, a sterile cotton swab was dipped into the suspension. The swab was swirled several times and pressed firmly on the clear wall of the tube to remove excess inoculum. The inoculum in swab was then inoculated on the dried surface of a Mueller–Hinton agar plates by rubbing the swab over the entire sterile agar surface. This procedure was repeated two times, rotating the plates approximately 60° each time to ensure an even distribution of inoculum. The plate was left for 3 to 5 min to allow any excess surface moisture to be absorbed before applying the antibiotic impregnated discs.

The predetermined set of antimicrobial discs viz. Oxytetracycline (30mcg), Ampicillin/Cloxacillin (10mcg), Ampicillin (10mcg), Ciprofloxacin (5mcg), Gentamicin (30mcg), Cefotaxime (30mcg), Aztreonam (30mcg), Ceftazidime (30mcg), and Cefixime (5mcg), were placed onto the surface of the inoculated agar plates. The discs were distributed evenly with minimum gap of 24 mm from centre to centre. The plates were placed in inverted position in an incubator at 37°C within 15 min of the placements of discs. After 16 to 18 hrs. of incubation, each plate was examined for the zones of inhibition. The diameter of the zones of complete inhibition was measured and compared with the zone size interpretation chart provided by supplier and were graded as sensitive, intermediate, and resistant.

Table: 4 Antibiotic discs used in present study

S. N.	Antibiotic discs	Symbol	Concentration (mcg)	Interpretative criteria diameter of zone of inhibition (mm)		
				R	I	S
01.	Oxytetracycline	O	30	<11	12-14	>15
02.	Ampicillin/Cloxacillin	AX	10	<11	12-14	>15
03.	Ampicillin	AMP	10	<13	14-16	>17
04.	Ciprofloxacin	CIP	5	<15	16-20	>21
05.	Gentamicin	GEN	10	<12	13-14	>15
06.	Cefotaxime	CTX	30	<22	23-25	>26
07.	Aztreonam	AT	30	<17	18-20	>21
08.	Ceftazidime	CAZ	30	<17	18-20	>21
09.	Cefixime	CFM	5	<15	16-18	>19
10.	Cefotaxime/clavulanic acid	CEC	30/10	≥5 mm than the cefotaxime alone		

R= Resistant, I =intermediate, S= sensitive

3.6.3.1 Multiple antibiotic resistances (MAR) index

The multiple antibiotic resistance (MAR) index of each sample was calculated using the equation (Krumperman, 1983): $a/(b \times c)$, where a is the aggregate antibiotic resistance score of all isolates from the sample, b is the number of antibiotics, and c is the number of bacterial isolates obtained from the sample.

3.6.4 Isolation of Template DNA

3.6.4.1 Isolation of Genomic DNA

Template DNA incorporated in PCR reaction was prepared by boiling and snap chill method. Culture from nutrient agar was inoculated into 5 ml of Luria-Bertani broth (LB) and incubated for 12-16 hrs. at 37°C. Cells from 1.5 ml of the culture were harvested by centrifugation at 10000 g in a microcentrifuge for 2 min at room temperature. The supernatant was decanted and pellet in microcentrifuge tube was added with 1.5 ml PBS and mixed by vortexing, the suspension was then centrifuged for 2 min and supernatant was discarded. The obtained pellet was mixed with 300µl molecular grade water and then kept in the water bath for 15 min at 100°C followed by immediate chilling on crushed ice for at least 20 min. Finally tubes were centrifuged at 12000 g for 2 min and clear supernatant was collected and stored at -20°C until further use.

3.6.4.2 Isolation of Plasmid DNA

Selected bacterial isolates which showed reduced susceptibility to any one or combination of antibiotics were selected for isolation of plasmid DNA using HiPurA® Plasmid DNA Miniprep kit (Cat. No. MB508, Himedia) following the

manufacturer's protocol. The extracted plasmid DNA was stored at -20°C for further use.

3.6.5 Measurement of DNA concentration and purity

The concentrations of DNA were measured with Nanodrop 2000C (Thermo Scientific, USA), and adjusted to 100ng/μl for further PCR studies. Pure DNA samples (with an optical density ratio of 1.8 to 2 at 260/280 nm) were stored at -20°C, until further use.

3.6.6 Molecular characterization and detection of antibiotic resistant genes

3.6.6.1 Molecular characterization of *E.coli*

The *E.coli* isolates were further confirmed by PCR using following *E. coli* 16s rRNA specific primers quoted by Sundong-bo *et al.* (2011).

F:ATCAACCGAGATTCCCCCAGT

R: CACTATCGGTCAGTCAGGAG.

3.6.6.2 PCR conditions for detection of *E.coli* (16S rRNA)

PCR reactions were carried out in Applied Biosystems thermal cycler (Veriti™). The amplification conditions were 3 min of denaturation at 94 °C, followed by 35 cycles of 94°C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds, and a final extension step of 72 °C for 10 min (Sundong-bo *et al.*, 2011).

3.6.7 Detection of antibiotic resistance genes

The major resistance genes for tetracycline (*tetA* and *tetB*), ESBL (*bla_{SHV}*, *bla_{TEM}*, *bla_{CTX-M}*), fluoroquinolone (*gyrA* and *parC*) as well as integrons class 1 (*Int 1*) were detected by using following primers (Table 5).

Table: 5 Details of the primers used in present study

Antimicrobial family	Target gene	Primer	Product size (bp)	Reference
Beta-lactams	bla _{SHV}	F ATGCGTTATATTCGCCTGTG	747	(Paterson <i>et al.</i> , 2003)
		R TGCTTTGTTAT CGGGCCAA		
	bla _{TEM}	F TCGCCGCATACACTATTCTCAGAATGA	445	(Apaka <i>et al.</i> , 2010)
		R ACGCTCACCGGCTCCAGATTTAT		
	bla _{CTX™}	F ATGTGCAGYACCAGTAARGTK ATG GC	593	(Boyd <i>et al.</i> , 2004)
		R TGGGTRAARTARGTSACCAGAAYCAGC GG		
Tetracycline	tetA	F GCTACATCCTGCTTGCCTTC	210	(Titilawo <i>et al.</i> , 2015)
		R CATAGATCGCCGTGAAGAGG		
	tetB	F TTGGTTAGGGGCAAGTTTTG	359	
		R GTAATGGGCCAATAACACCG		
Fluroquinolone	gyrA	F CGTCGCGTACTTTACGCCATGAACG	586	(Dasgupta <i>et al.</i> , 2018)
		R ATACCTTGCCGCGACCGGTACGG		
	parC	F TGTATGCGATGTCTGAACTG	265	
		R CTCAATAGCAGCTCGGAATA		
Transposones	Int 1	F CCTCCCGCACGATGATC	280	(Khoramrooz <i>et al.</i> , 2016)
		R TCCACGCATCGTCAGGC		
Genotyping	ERIC	F ATG TAA GCT CCT GGG GAT TCA	variable	(Ibrahim <i>et al.</i> , 2016)
		R AAG TAA GTG ACT GGG GTG AGC G		

Table6: Composition of PCR reactions for detection of various genes

Gene	10X PCR buffer with 20nM Mgcl ₂ (µl)	DNTP mix (10mM mix) (µL)	Forward/ Reverse primer (10 pico Moles/µl) (µl)	Taq DNA Poymerase (3U/ µl) (µl)	Template (µl) (Genomic /plasmid DNA)	NFW (µl)	Total Reaction volume (µl)
<i>bla</i> _{SHV}	1	1	0.5/0.5	0.2	2 plasmid	2.8	10
<i>bla</i> _{TEM}			0.5/0.5				
<i>bla</i> _{CTX-M}			0.5/0.5				
<i>tetA</i>	1	1	0.25/0.25	0.2	2 plasmid	4.8	10
<i>tetB</i>			0.25/0.25				
<i>gyrA</i>	1	1	0.25/0.25	0.2	0.5 Genomic	6.3	10
<i>parC</i>			0.25/0.25				
<i>Int 1</i>	1	1	0.25/0.25	0.2	2 plasmid	5.3	10
ERIC	1	1	0.5/0.5	0.2	1 Genomic	5.8	10
16S rRNA	1	1	0.25/0.25	0.2	0.5 Genomic	6.8	10

Table 7: PCR conditions employed for various genes

PCR steps	PCR conditions	Target genes								
		<i>bla</i> _{SHV}	<i>bla</i> _{TEM}	<i>bla</i> _{CTX-M}	<i>tetA</i>	<i>tetB</i>	<i>gyrA</i>	<i>parC</i>	<i>Int 1</i>	ERIC
Initial denaturation	Temperature (°C)	95	95	95	95	95	95	95	94	94
	Time (m)	10	10	10	5	5	2	2	4	3
Denaturation	Temperature (°C)	94	94	94	95	95	95	95	94	94
	Time (m/s)	30s	30s	30s	30s	30s	25s	25s	50s	30s
Annealing	Temperature (°C)	60	60	60	59	59	52	52	57	52
	Time (m)	30s	30s	30s	30s	30s	1	1	30s	1
Extension	Temperature (°C)	72	72	72	72	72	72	72	72	65
	Time (m)	2	2	2	1	1	1.2	1.2	1	4
Cycles		30	30	30	35	35	32	32	30	35
Final Extension	Temperature (°C)	72	72	72	72	72	72	72	72	65
	Time (m)	10	10	10	5	5	7	7	10	8

3.6.8 Agarose gel electrophoresis

To analyse the amplified products, the submarine agarose gel electrophoresis was performed as described by Sambrook and Russell (2001). Agarose gel (1.6%) was prepared by putting 0.8 gm agarose in 50 ml 1X Tris-Borate EDTA (TBE) buffer and subjected to heat until the agarose was completely dissolved and appeared as a clear transparent solution. The agarose solution was allowed to cool to 60°C and then ethidium bromide (0.5µg/ml) dye was added to it. Thereafter the gel was poured into the gel casting tray held within the gel holding tray and the comb was placed into the slots on the tray in such a manner that a gap of 0.5 mm was left between the tips of comb teeth and the floor of casting tray and thus completely sealed wells were formed. It was allowed to solidify for 20-30 min and then the comb was gently removed. The casting gel along with the running tray was submerged into the electrophoresis tank containing 1X TBE buffer. A total volume of 4 µl DNA samples were taken and loaded carefully into the wells of agarose gel. To determine the size of the amplified PCR product 100 bp DNA ladder was loaded in one well. Electrophoresis was performed at 70 V for 45 min and the mobility was monitored by the migration of the dye in the gel. After appropriate migration, the gel was visualised under UV transilluminator.

3.6.9 Visualization of the gel in Gel Doc[™] XR

The amplified PCR products were visualized under transilluminator and the gel were documented by Gel Doc[™] XR.

3.6.10 The multiple antibiotic gene (MAG) index

The multiple antibiotic gene (MAG) index of each sample was calculated by the equation: $d/(e \times f)$, where d is the aggregate antibiotic resistance genes of all isolates from the sample, e is the number of antibiotic resistance genes, and f is the number of bacterial strains isolated from the sample.

3.6.11 Genetic diversity analysis by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

Phylogenetic analysis of *E.coli* isolates was performed by ERIC-PCR as described by Ibrahim *et al.* (2016) using forward (5'-ATGTAAGCTCCTGGGGATTCAC-3') and reverse (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers. The PCR mixture included 1 µl of 10x Taq buffer (Tris HCL with 20mM MgCl₂), 1µl of 2.5 mM concentration of mix dNTPs, 0.5 µl of 10 pmol/µl of each primer, 0.6 U Taq Polymerase and 1 µl of DNA template. Nuclease-free water was added to make the final volume to 10 µl. Sterile nuclease-free water (5 µl) was used as negative control. The cycling conditions included initial denaturation (94°C, 3 min) followed by 35 cycles of denaturation (94°C for 30 s), primer annealing (52°C for 1 min), extension (65°C for 4 min) and final extension (64°C for 8 min). The PCR products were electrophoresed on a 1 % agarose gel at 80 V for 1.5 hr. The agarose gel was stained with ethidium bromide (0.5 µg/ml). A separate well charged with 100 bp - 10 kb DNA ladder was run concurrently. The amplified products were visualised and confirmed over a gel documentation system. The similarity in banding pattern of different isolates was analyzed with gel

documentation system (Gel Doctm XR) and clustering was studied employing the unweighted pair group method with arithmetic mean (UPGMA).

3.7 Isolation of *E. coli* from house fly samples

E. coli from house fly samples was isolated as per method described by Gupta *et al.* (2012). The flies in sample bags were crushed using a pestle to expose their inner tissues to the 5 ml MacConkey's lactose broth (HiMedia, India) and incubated at 37°C for 24 hrs. Thereafter, a loop-full culture from enrichment broth was taken and streaked onto eosin methylene blue agar (HiMedia, India) and incubated at 37°C for 24 hrs. Dark-centered and flat colonies with metallic sheen were considered as *E. coli* and used in study.

3.7.1 Biochemical screening of bacterial isolates

The bacterial isolates were subjected to various biochemical tests. Isolated colonies from the selective agar plates were streaked onto nutrient agar slants and incubated at 37°C for 24 ± 2 hrs and were subjected to various biochemical tests viz. Methyl red, Voges Proskauer, Indole test and Citrate utilization test following the procedure described by Agarwal *et al.* (2003).

3.7.1.1 Indole test

Bacterial isolate was inoculated in a medium containing 3ml of sterile trypton water. Tube was incubated for 48 hrs. at 35-37°C. After incubation 0.5 ml Kovac's indole reagent was added and observed for 10 min. for production of red color at the top of the broth which indicate positive test.

3.7.1.1.2 Methyl red test

Bacterial isolate was inoculated in 0.5 ml sterile glucose phosphate broth. Tube was incubated overnight at 35-37°C and thereafter a drop of methyl red solution was added and observed for red color production which indicates positive result.

3.7.1.1.3 Voges- Proskauer test

Bacterial isolate was inoculated in 2 ml of sterile glucose phosphate peptone water and incubated at 35-37°C for 48 hrs after that 0.6 ml of Barritt's reagent A (α naphthol) and 0.2 ml of Barritt's reagent B (40% potassium hydroxide) were added. Tube was shaken vigorously and kept for 1 hr at room temperature. Tube was observed for development of pinkish red color in positive test.

3.7.1.1.4 Citrate utilization test

Bacterial isolate was streaked on a slope of Simmons citrate agar in tubes with the help of sterile loop, first slope was streaked and then butt was stabbed. Tube was then incubated at 35°C for 48 hrs. Tube was observed for development of bright blue colour in positive test.

3.7.2 Antibiotic susceptibility testing

Antibiotic sensitivity test was performed as per the method of Bauer and Kirby (1966). Commercially available antibiotic discs (HiMedia laboratories Limited, Mumbai) were used to test susceptibility of the isolates against different antibiotics (Table 4). One extra antibiotic Cephalexin (30mcg) was used, procedure was similar as described earlier in 3.6.3.

The *E. coli* isolates were further characterized using molecular techniques in different sections (3.6.3, 3.6.4, 3.6.5, 3.6.6, 3.6.7, 3.6.8, 3.6.9 and 3.6.11) of this chapter.

3.7.3 Multiple antibiotic resistances (MAR) index

The multiple antibiotic resistance (MAR) index was calculated as per the method described by Krumperman (1983), by applying a/b where “a” is the number of antibiotics to which an isolate was resistant and “b” is the number of antibiotics to which the isolates were exposed. MAR index more than 0.2 indicated injudicious use of antibiotics.

3.7.4 Detection of ESBL producing isolates

The isolate with a zone of inhibition diameter of ≤ 17 mm for aztreonam, ≤ 17 mm for ceftazidime, and ≤ 22 mm for cefotaxime in disc diffusion susceptibility testing were considered further for screening for ESBL phenotypes.

3.7.4.1 Phenotypic confirmation test for ESBL

The phenotypic confirmatory test for ESBL producers was performed as per CLSI (2012) guidelines. For this purpose, cefotaxime (30 μ g), cefotaxime-clavulanic acid (30/10 μ g), were used.

Discs were placed 25 mm apart on a MHA plate inoculated with 0.5 McFarland suspension of the test isolate. Plates were incubated at 35°C for 18 hrs. After incubation the zone diameter around each of disc was measured. A difference of ≥ 5 mm between the zone diameters of either of the cephalosporin

discs and their respective cephalosporin/ clavulanic acid discs were considered as positive phenotypic confirmatory test for ESBL producers.

3.8 Statistical analysis

The data were presented in tables and graphs using Excel spreadsheets. IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., New York, USA) was used to describe the nature and the characteristics of the data using descriptive statistics. The mean, standard deviation, quartiles, maximum as well as the minimum concentration of residues in positive samples were calculated. The mean residue concentrations among different zones was compared using one way analysis of variance (ANOVA) with post hoc Tukey's test at 95% mean confidence interval. T-test was used to compare mean residue concentrations among broiler chicken meat and backyard chicken meat.

Further ANOVA with post-hoc Tukey's test was used to evaluate the statistical significance in the bacterial resistance against different antibiotic agents and different sample sources water and fecal *E. coli* isolates resistance was compared. Principal component analysis (PCA) and clustering was employed for analysing the relationship among group of variables in the data set.

*RESULTS AND
DISCUSSION*

CHAPTER IV

RESULTS AND DISCUSSION

In the present study meat samples were analyzed for the presence of antibiotic residues, for this a sensitive, confirmatory HPLC multi residue method was optimized and validated. This method efficiently determined the presence of oxytetracycline, tetracycline, chlortetracycline, sulphamethazin, ciprofloxacin and enrofloxacin residues in chicken meat samples. The above mentioned antibiotics belong to tetracycline, sulphonamide and fluroquinolone group of antibiotics. The validation was performed according to the European Commission Decision 2002/657/EC (European Commission, 2002) and performance criteria involving linear range, specificity, intraday-assay and interday-assay precision, accuracy, LOD and LOQ were determined. The optimized and validated method was applied for the detection of antibiotic residues in marketed chicken meat samples in different agroclimatic zones of Chhattisgarh. Human health risk assessment based on EDI and HQ was performed to evaluate hazards associated with consumption of contaminated chicken meat sample.

To assess the antibiotic resistance in *E. coli* from aquatic ecosystem of three different type of aquaculture practice prevalent in Chhattisgarh and related animal and human fecal samples were used for the isolation of *E. coli*. Membrane filtration method for water sample and spread plate method for fecal sample was used for isolation of *E. coli*. A total of 232 *E. coli* isolates from water and fecal sample were isolated. These isolates were screened for antimicrobial resistance following antimicrobial susceptibility test (AST). Multidrug resistant *E. coli* isolates from

different group of water and fecal samples were selected and genomic and plasmid DNA was extracted. All selected isolates were screened for 16sRNA gene of *E. coli*, antibiotic resistant genes *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, *tetA*, *tetB*, *gyrA*, *parC* and class 1 integron gene *Int1*. ERIC PCR genotyping was performed for all the selected isolates.

The house fly, *Musca domestica* predominantly present in different environmental settings and is a potent vector of disease transmission. To identify the possible role of house flies in antibiotic resistant *E. coli* transmission, flies were captured from milk and meat shops. A total of 45 *E. coli* isolates were recovered from 150 fly samples. AST pattern and phenotypic ESBL production were detected by using disc diffusion method. Heat map and hierarchical clustering was performed by using antibiotic susceptibility data. Genotyping of all the multidrug resistant and ESBL producing isolates were done by using ERIC PCR.

4.1 Detection of antibiotic residues in chicken meat samples

The analytical method for detection of antibiotic residues in chicken meat by applying HPLC-PDA method as well as the sample preparation methods were optimized and validated for application to retail broiler chicken meat and backyard chicken meat.

4.1.1 Optimization of HPLC conditions and method validation for extraction and detection of antibiotic residues from meat samples

The HPLC method for the detection of OTC, TC, CTC, SMZ, CIP, and ENR as well as procedure for simultaneous extraction and clean-up of antibiotics from chicken meat was standardized and validated.

4.1.1.1 Optimization of HPLC conditions for detection of tetracycline and sulphonamide antibiotics in meat

In the present study, the simultaneous detection of antibiotics belonging to two different groups i.e. tetracyclines (oxytetracycline, tetracycline, chlortetracycline), and sulphonamides (sulphamethazine) in meat was carried out. The mobile phase employed by Zhou *et al.* (2009) for simultaneous detection of oxytetracycline, tetracycline, chlortetracycline, and Doxycycline in propolis was used for standardization of HPLC conditions. The use of Zorbax Eclipse XBD - column and isocratic elution of 0.01 M oxalic acid (pH 4.0), methanol and acetonitrile (70:10:20, v/v/v), resulted in effective separation of all the targeted antibiotics. The separation was best achieved by using flow rate of 1 ml/min and column temperature at 30°C. The obtained detection wavelength of 360 nm for OTC, TC and CTC and 280 nm for SMZ was found best for maximum absorption for these compounds. The retention time for different antibiotic standards are presented in Table 8 and chromatograms are presented in Fig.1 and Fig.2.

Table 8: Retention time of tetracyclines and sulphamethazine standards

Antibiotic standards	Retention Time (min)
Oxytetracycline	3.94
Tetracycline	4.45
Chlortetracycline	8.52
Sulphamethazine	5.54

In, present study HPLC method was standardized and validated for simultaneous detection of antibiotics belonging to different groups i.e. tetracyclines and sulphonamides. Cinquina *et al* (2003) and Yu *et al* (2012) developed and validated an HPLC confirmatory method for determination of tetracycline and multiresidues in meat, respectively. The simultaneous determination of multi-residues of antibiotics belonging to different groups had also been carried out using liquid chromatography coupled to tandem mass spectrometry (Giusepponi *et al* 2019, Lu *et al* 2019 and Zhang *et al* 2018).

4.1.1.2 Optimization of HPLC conditions for detection of fluoroquinolone antibiotic in meat

Simultaneous detection of antibiotics belonging to fluoroquinolone group i.e. ciprofloxacin and enrofloxacin in meat was carried out as per method described by Moema *et al.* (2012) with little modification. In this study new Xbridge column was used thus different separation related parameters like injection volume, flow rate, column temperature and maximum absorption wavelength were optimized to obtain good separation with the 15µL injection volume, flow rate to 0.82mL/min and column temperature to 40 °C, excellently separate the targeted antibiotics standard on X-Bridge column. The retention time of standards is presented in Table 9 and chromatogram is shown in Fig.3

Table 9: Retention time of ciprofloxacin and enrofloxacin standards

Antibiotic standards	Retention Time (min)
Ciprofloxacin	7.10
Enrofloxacin	9.45

4.1.1.3 Extraction of antibiotics from meat

Two different HPLC detection method were used but the very crucial sample extraction was done by the using single extraction procedure for all the studied antibiotics without affecting their chemical structures and physicochemical properties. The main experimental steps i.e. protein precipitation, extraction, clean-up using solid-phase cartridges and concentration of elute were evaluated. Figure 4a, 4b, 5a, 5b and 6a, 6b comprise the result of extraction of antibiotics from the meat sample, by standardized sample extraction protocol.

In the present study, 20% and 30% solution of trichloroacetic acid (TCA) in acetonitrile was evaluated for precipitation of meat protein with best results obtained from 20% TCA in acetonitrile. TCA was also used as meat protein precipitant in a study conducted by Cinquina *et al* (2003) in which they used 20% TCA in ultrapure water for precipitation of meat protein. Literature revealed that, different solutions such as formic acid, TCA, acetic acid, acetonitrile, methanol etc. were used for the precipitation of meat proteins (Saxena *et al.*, 2018, Tolgyesi *et al.*, 2014, and Cinquina *et al.*, 2003).

For preliminary extraction of antibiotics from meat matrix, various extraction buffers such as phosphate buffer (pH 7) phosphate citrate EDTA buffer (pH 4 and 7) and sodium EDTA-McIlvaine buffer (pH 4 and 7) were evaluated. The best results were obtained with sodium EDTA-McIlvaine buffer at pH 4 that resulted in simultaneous extraction of all targeted antibiotics from meat with good recoveries. The sodium EDTA-McIlvaine buffer has been used by many researchers for the extraction of antibiotic residues from meat (Cinquina *et al.*, 2003, Lu *et al.*, 2019 and Tolgyesi *et al.*, 2014).

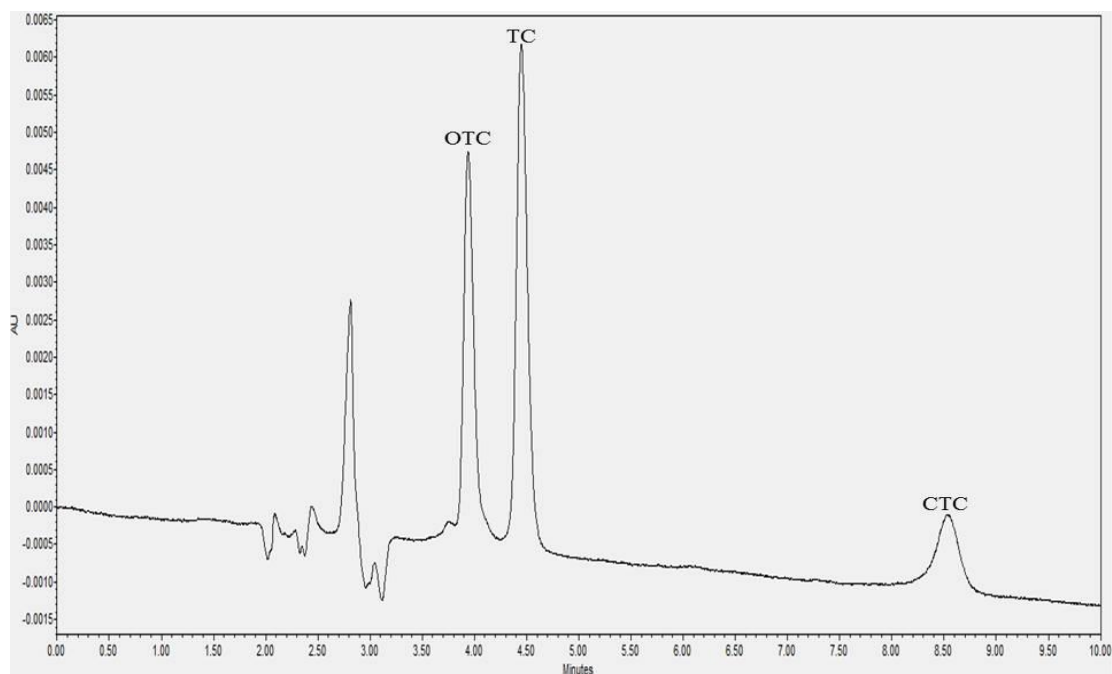


Fig. 1: Chromatogram of tetracycline (OTC, TC and CTC) standards (500 µg/kg) at 360 nm

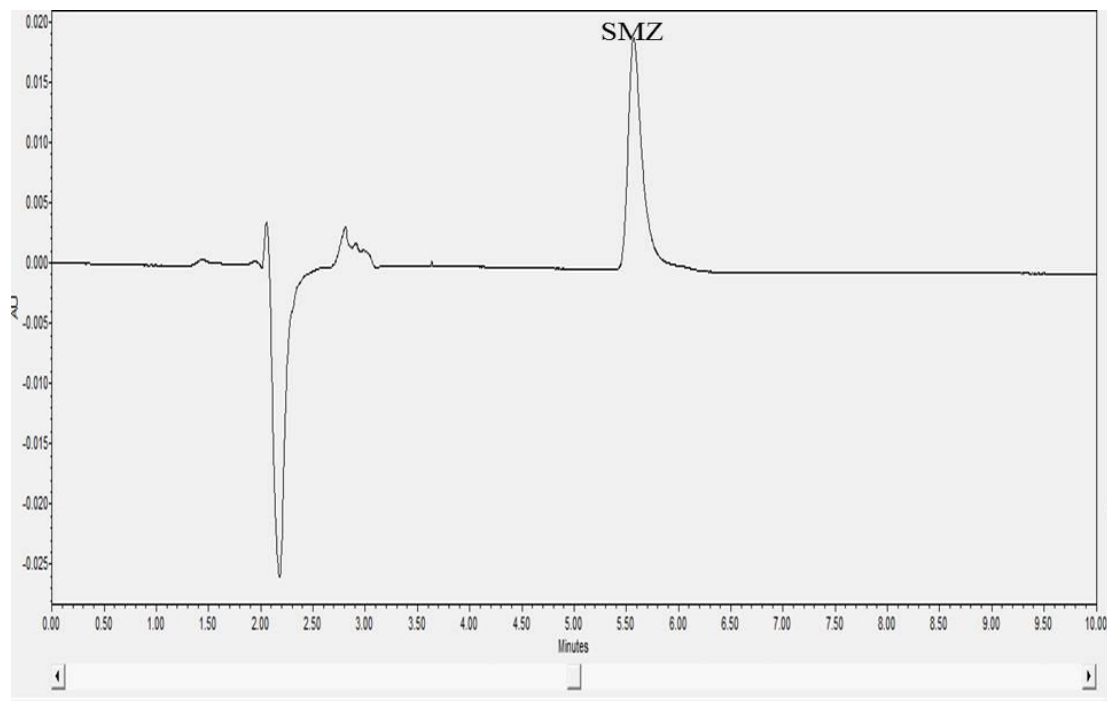


Fig. 2: Chromatogram of sulphamethazine (SMZ) standard (500 µg/kg) at 280 nm

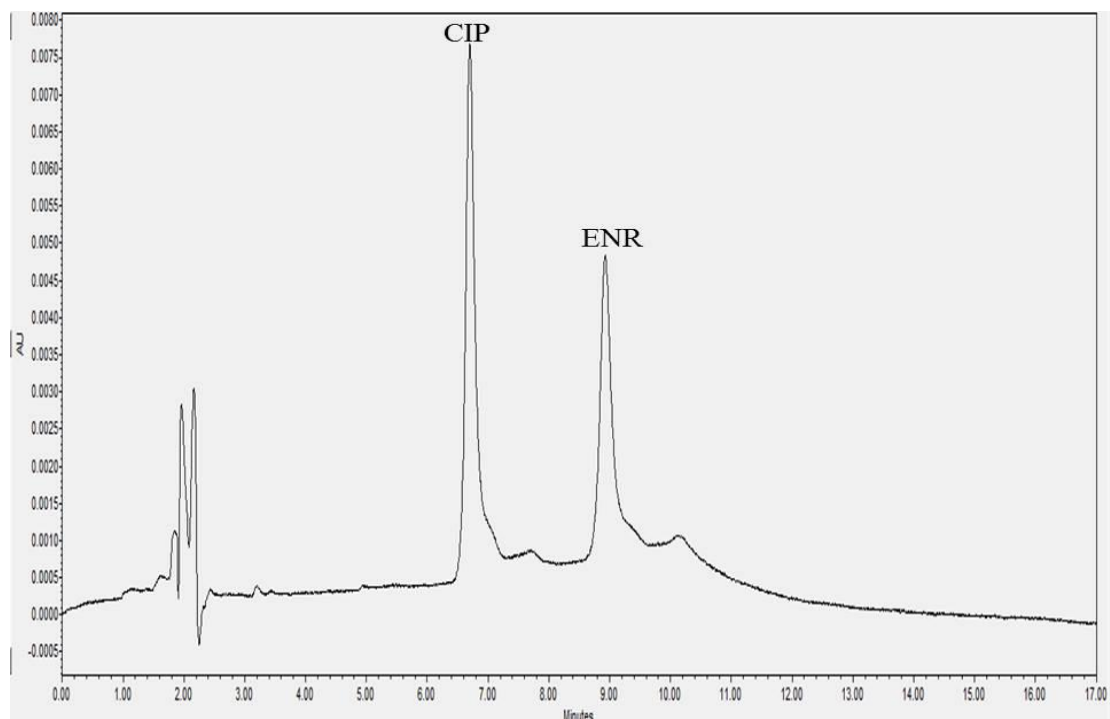


Fig. 3: Chromatogram of ciprofloxacin (CIP) and enrofloxacin (ENR) standards (500 µg/kg) at 280 nm

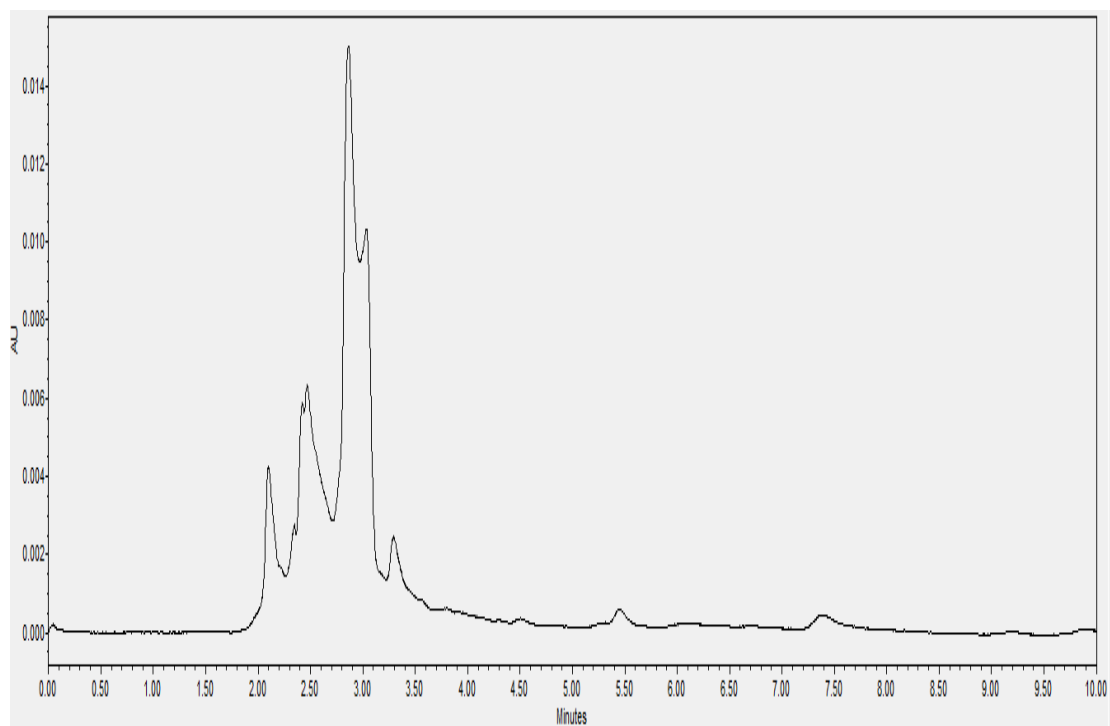


Fig. 4a: Chromatogram of blank meat sample at 360 nm

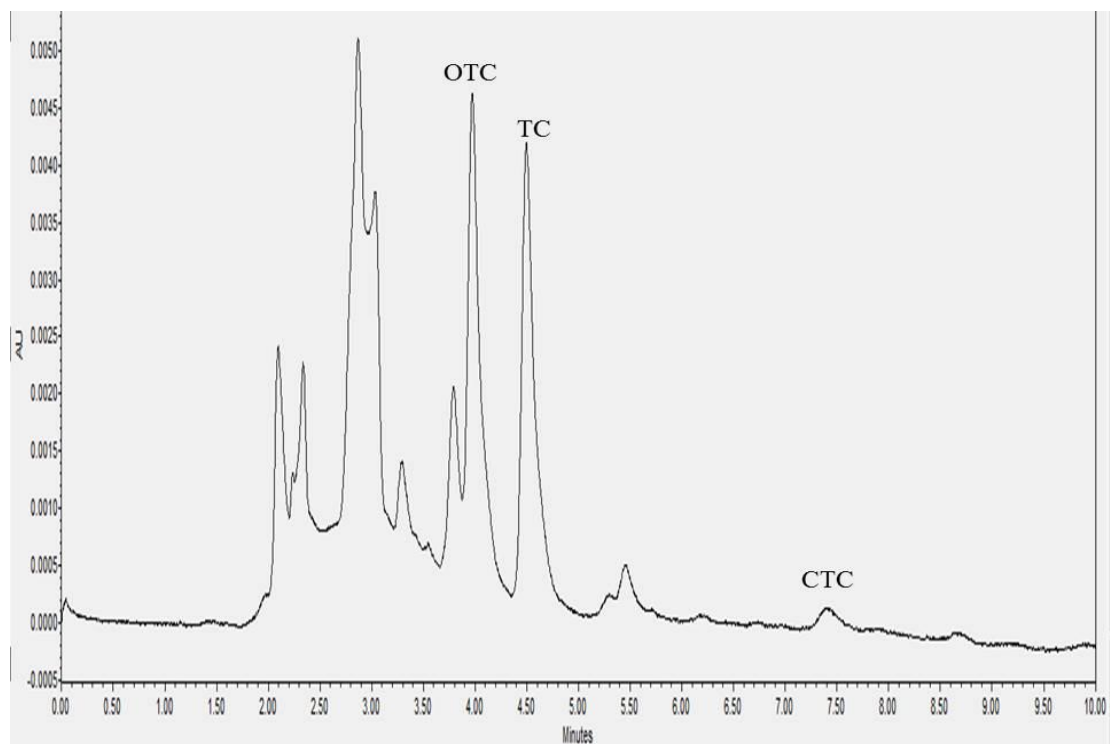


Fig. 4b: Chromatogram of meat sample spiked with OTC, TC and CTC (300 µg/kg) at 360 nm

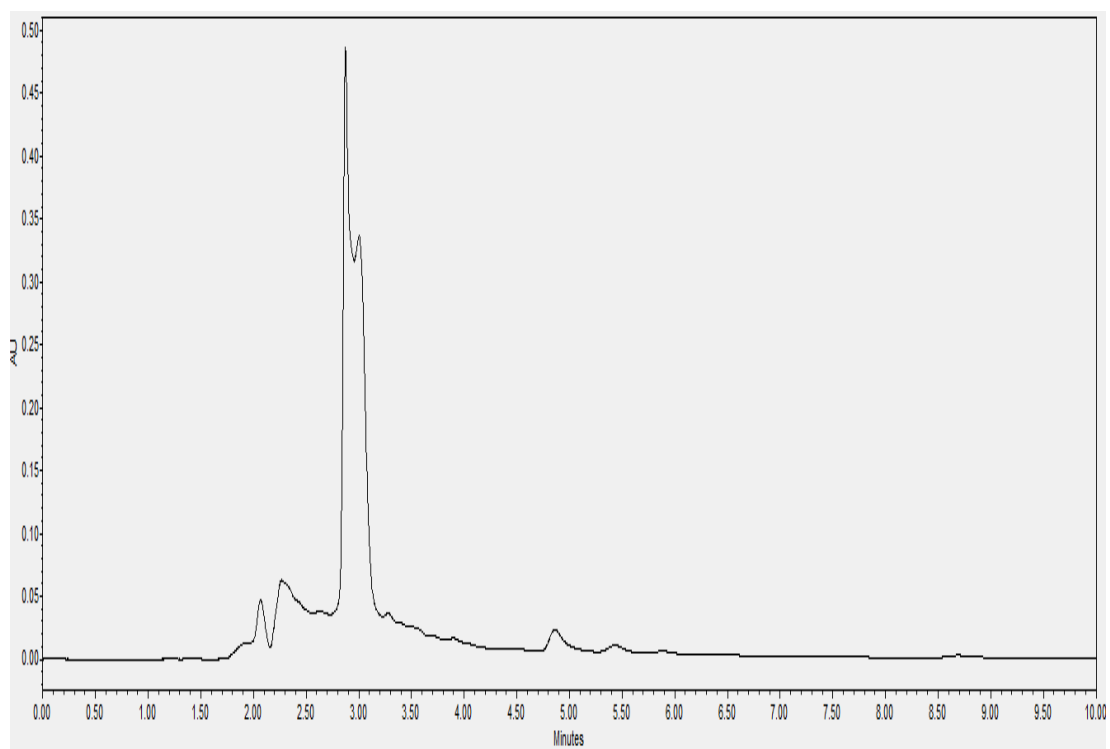


Fig. 5a: Chromatogram of blank meat sample at 280 nm

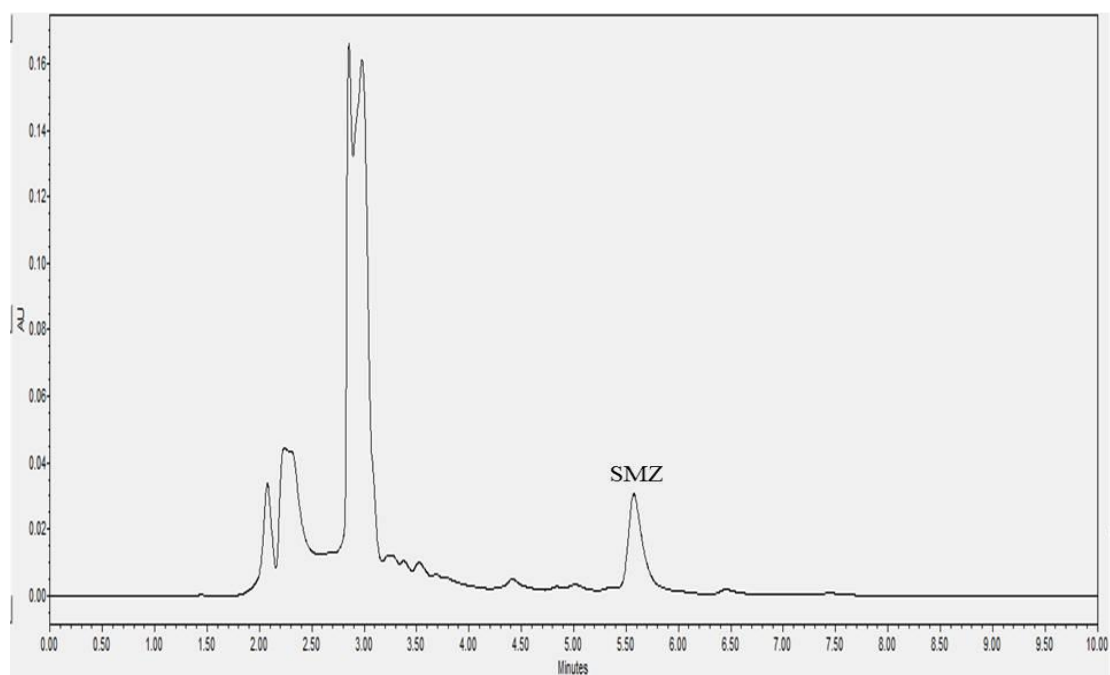


Fig. 5b: Chromatogram of meat sample spiked with SMZ (300 µg/kg) at 280 nm

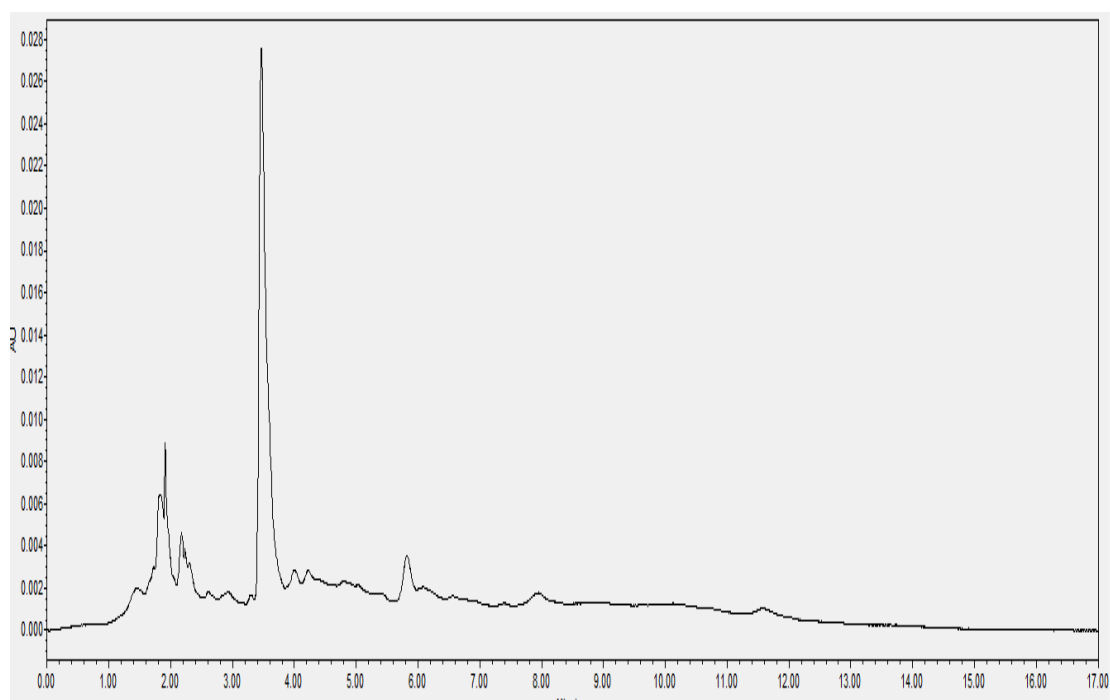


Fig. 6a: Chromatogram of blank meat sample at 280 nm

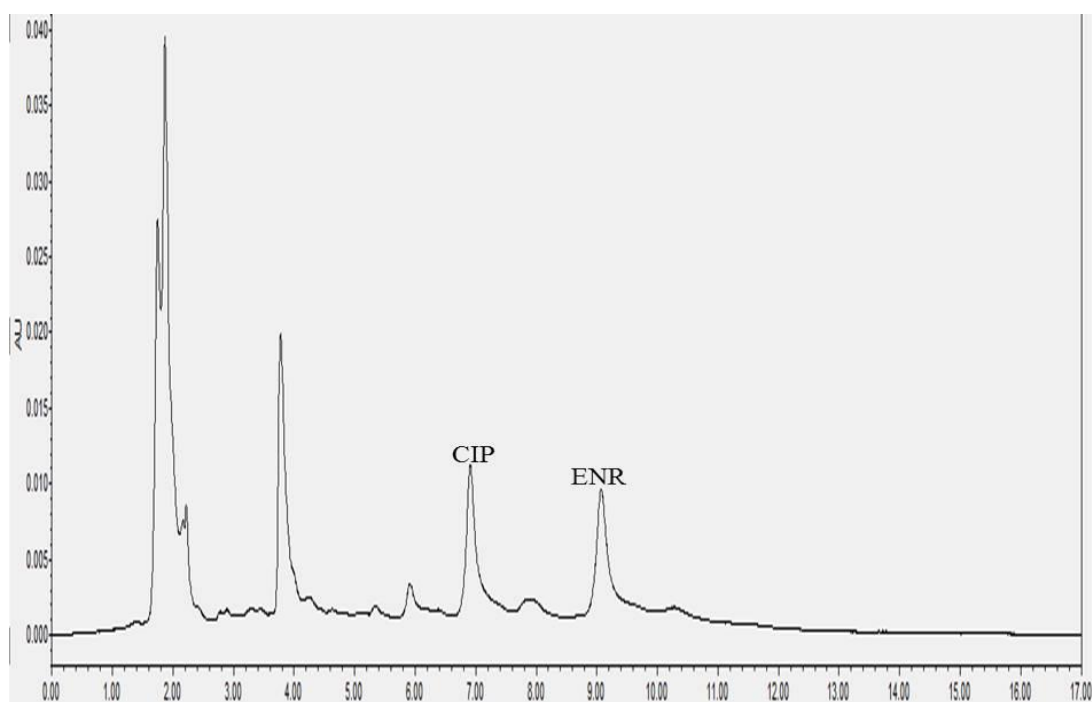


Fig. 6b: Chromatogram of meat sample spiked with CIP and ENR (300 µg/kg) at 280 nm

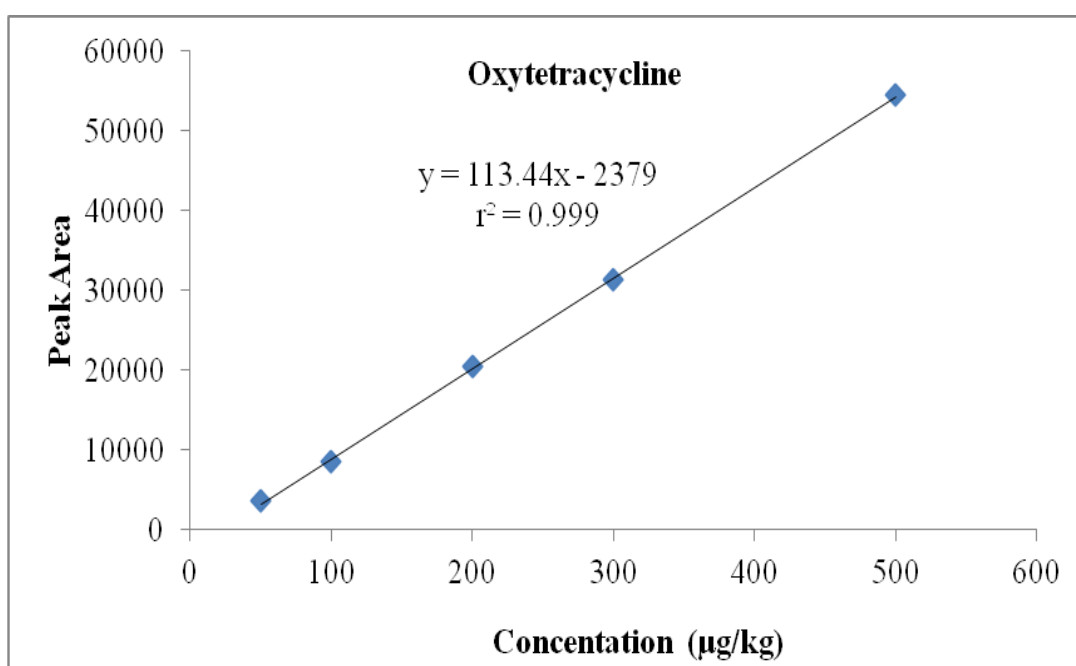


Fig. 7: Standard calibration curve for oxytetracycline (OTC)

The extracts obtained after protein precipitation and extraction were cleaned up using solid phase extraction cartridges. The extract cleanup was obtained with Oasis HLB cartridges which allowed higher recovery values and lower interferences near the retention time of each analyte. Various researchers have reported higher recovery values using Oasis HLB cartridges for clean-up of extracts (Cinquina *et al.*, 2003 and Lu *et al.*, 2019). The elution of antibiotics was carried out with methanol as it has high elution strength because of high polarity. The representative chromatograms of blank meat samples and samples spiked with 300 µg/kg of antibiotic standards are shown in Fig. 4a, 4b (OTC, TC CTC), 5a, 5b (SMZ) and 6a, 6b (CIP, ENR) .

4.1.1.4 Method Validation

The standardized method was validated for the determination of oxytetracycline, tetracycline, chlortetracycline, sulphamethazin, ciprofloxacin and enrofloxacin by evaluating the following validation parameters: linear range, specificity, intraday-assay and interday-assay precision, accuracy, LOD and LOQ.

4.1.1.4.1 Method validation for tetracyclines

The method was validated for the determination of OTC, TC and CTC in chicken meat using spiked samples.

4.1.1.4.1.1 Oxytetracycline

The linearity, LOD and LOQ were assessed from calibration graphs by constructing five point solvent matched calibrations curve by using OTC standard at five dilution levels: 50,100,200,300,500 µg/kg in triplicate analysis (Fig.7).

The calibration curve for OTC was linear in the range of 50-500 µg/kg with correlation coefficient (r^2) of 0.999. The LOD and LOQ were calculated

from the standard deviation of Y-intercepts of regression analysis and the slope of calibration curve. The LOD and LOQ for OTC were found to be 17.22 $\mu\text{g/kg}$ and 52.18 $\mu\text{g/kg}$, respectively. The values of LOD and LOQ were below the MRL (100 $\mu\text{g/kg}$) established by EU/CAC (2017) for OTC in meat.

The application of the method to ten different blank meat samples in order to verify the method specificity demonstrated that no interferences from endogenous compounds were detected at 360 nm at the retention time of OTC. All the standardized parameter of validation was under the compliance of 2002/657/EC (European Commission, 2002).

The precision of the method was evaluated using the results obtained over 1 day of operation under the same conditions (intraday -assay) and during 5 days (inter-assay precision). The intra-assay precision was evaluated through the analysis in one single day with five blank meat samples fortified with 200 $\mu\text{g/kg}$ of OTC. The intra-assay precision expressed as % RSD (relative standard deviation) was found to be 5.8%. The interday-assay precision was evaluated through the analysis of fortified blank meat samples at three fortification levels (100, 200 and 300 $\mu\text{g/kg}$) for five different days. The interday-assay precision also expressed as % RSD was found to be 11.2, 6.8 and 7.1% at spiking concentration of 100, 200 and 300 $\mu\text{g/kg}$, respectively.

According to European Commission Decision 2002/657/EC (European Commission, 2002), the intraday-assay precision and interday-assay precision should be lower than 15% and 23%, respectively and the observed values were in agreement with EU guidelines.

The accuracy of the method was assessed by recovery tests, analyzing fortified blank meat samples at three fortification levels (100, 200 and 300 µg/kg). The average recoveries of OTC at the concentration of 100, 200 and 300 µg/kg were found to be 84.1, 85.5 and 86.1%, respectively and were in accordance with the EU guidelines, which established a range of 80-120%(European Commission, 2002). The assay validation parameters for OTC are presented in Table 10.

Similarly, in another study by Gajda and Posyniak (2009) reported the average recoveries for the extraction of oxytetracycline residues from spiked meat samples between 80% and 86% with LOD and LOQ of 9.8 µg/kg and 27.2 µg/kg, respectively, whereas the present study revealed comparatively equal recoveries with ability of standardized method to detect and quantify more or less equal concentration.

Table 10: Method validation parameters for oxytetracycline

Method Validation Parameters	Values
Linear range (µg/kg)	50–500
Linearity (r^2)	0.99
Linear regression (Equation)	$y = 113.44x - 2379$
LOD (µg/kg)	17.22
LOQ (µg/kg)	52.18
Intraday assay precision, n = 5 (%RSD) 200 µg/kg	5.82%
Inter day assay precision, n = 5 (%RSD)	
100 µg/kg	11.27%
200 µg/kg	6.87%

300 µg/kg	7.12%
Recovery %	
100 µg/kg	84.1
200 µg/kg	85.5
300 µg/kg	86.1

4.1.1.4.1.2 Tetracycline

The linear range, LOD and LOQ were obtained from five point solvent matched calibrations curve by using TC standard at five dilution levels: 50, 100, 200, 300 and 500 µg/kg with triplicate analysis (Fig. 8).

The calibration curve for TC was linear in the range of 50-500 µg/kg with correlation coefficient (r^2) of 0.99. The LOD and LOQ for TC were found to be 14.0 µg/kg and 42.3 µg/kg, respectively and were below the MRL (100 µg/kg) established by EU/CAC (2017) for TC in meat.

The application of the method to ten different blank meat samples in order to verify the method specificity demonstrated that no interferences from endogenous compounds were detected at 360 nm at the retention time of TC.

The intraday-assay precision was found to be 5.46% at spiking concentration of 200 µg/kg whereas interday-assay precision was found to be 10.8, 12.2 and 7.4% at spiking concentration of 100, 200 and 300 µg/kg, respectively. The observed values were in accordance with EU guidelines (intraday-assay precision < 15% and interday-assay precision < 23%). The average recoveries of TC at the concentration of 100, 200 and 300 µg/kg were found to be 83.8, 88.0 and 86.4%, respectively and were in accordance with the EU guidelines. The assay validation parameters for TC are presented in Table 11.

In a study conducted by Salama *et al.* (2011) HPLC method for the determination of tetracycline residues in meat was optimized and validated with recoveries ranging from 72-75% which was lower to recoveries recorded in the present study. Contrary to this, method validated by Cinquina *et al.* (2003), indicated recovery rate ranging between 80.3-81.4% which were less as compared to recoveries observed in the current study.

Table 11: Method validation parameters for tetracycline

Method Validation Parameters	Values
Linear range (µg/kg)	50–500
Linearity (r^2)	0.99
Linear regression (Equation)	$y = 125.58x + 12.52$
LOD (µg/kg)	14.00
LOQ (µg/kg)	42.30
Intraday assay precision, n = 5 (%RSD) 200 µg/kg	5.46%
Inter day assay precision, n = 5 (%RSD)	
100 µg/kg	10.88%
200 µg/kg	12.26%
300 µg/kg	7.44%
Recovery %	
100 µg/kg	83.8
200 µg/kg	88
300 µg/kg	86.4

4.1.1.4.1.3 Chlortetracycline

The linear range, LOD and LOQ were obtained from five point solvent matched calibrations curve by using CTC standard at five dilution levels: 50, 100, 200, 300 and 500 µg/kg with triplicate analysis (Fig. 9).

The calibration curve for CTC was linear in the range of 50-500 µg/kg with correlation coefficient (r^2) of 0.998. The LOD and LOQ for CTC were found to be 50.9 µg/kg and 154.4 µg/kg, respectively. The values of LOD was good but LOQ was not below the MRL (100 µg/kg) established by EU/CAC (2017) for CTC in meat. The application of the method to ten different blank meat samples in order to verify the method specificity demonstrated that no interferences from endogenous compounds were detected at 360 nm at the retention time of CTC.

The intraday-assay precision was found to be 4.2% at spiking concentration of 200 µg/kg whereas interday-assay precision was found to be 12.2, 8.6 and 9.9% at spiking concentration of 100, 200 and 300 µg/kg, respectively. The observed values were in accordance with the EU guidelines. The average recoveries of CTC at the concentration of 100, 200 and 300 µg/kg were found to be 84.8, 86.8 and 88.0%, respectively and were in accordance with EU guidelines. The assay validation parameters for CTC are presented in Table 12.

The method validated by Cinquina *et al.* (2003), for recovery established the mean recoveries for chlortetracyclins from spiked meat samples ranged from 85.3-86.9% which were comparable to recoveries recorded in the present study. On the other hand, Salama *et al.* (2011) during the validation of standardised method for the determination of chlortetracycline in meat the rate of recoveries

which ranged from 82-83% whereas LOD and LOQ were 10 µg/kg and 27 µg/kg, respectively. The method validation in the present study revealed comparatively better recoveries but detected higher concentration and limit of quantification was high as compared to study carried out by Cinquina *et al.* (2003).

Table 12: Method validation parameters for chlortetracycline

Method Validation Parameters	Values
Linear range (µg/kg)	50–500
Linearity (r^2)	0.99
Linear regression (Equation)	$y = 37.716x + 10.898$
LOD (µg/kg)	50.95
LOQ (µg/kg)	154.40
Intraday assay precision, n = 5 (%RSD) 200 µg/kg	4.25%
Inter day assay precision, n = 5 (%RSD)	
100 µg/kg	12.21%
200 µg/kg	8.64%
300 µg/kg	9.97%
Recovery %	
100 µg/kg	84.8
200 µg/kg	86.8
300 µg/kg	88

4.1.1.4.2 Method validation for sulphonamides

The method was validated for the determination of SMZ in meat using spiked samples.

4.1.1.4.2.1 Sulphamethazine

The linear range, LOD and LOQ were obtained from five point solvent matched calibrations curve by using SMZ standard at five dilution levels: 50, 100, 200, 300 and 500 µg/kg with triplicate analysis (Fig.10).

The calibration curve for SMZ was linear in the range of 50-500 µg/kg with correlation coefficient (r^2) of 0.999. The LOD and LOQ for SMZ were found to be 12.8 µg/kg and 38.8 µg/kg, respectively. The LOD and LOQ values were below the MRL (100 µg/kg) established by EU for SMZ in meat.

The application of the method to ten different blank meat samples in order to verify the method specificity demonstrated that no interferences from endogenous compounds were detected at 280 nm at the retention time of SMZ.

The intraday-assay precision was found to be 6.6% at spiking concentration of 200 µg/kg whereas interday-assay precision was found to be 7.2, 6.6 and 6.7% at spiking concentration of 100, 200 and 300 µg/kg, respectively. The values observed were in agreement with EU guidelines. The average recoveries of SMZ at the concentration of 100, 200 and 300 µg/kg were found to be 81.8, 81.6 and 84% respectively and were in accordance with the EU guidelines. The assay validation parameters for SMZ are presented in Table 13.

The average recoveries for sulphamethazine from spiked meat samples were found to be ranging 60-90% Mehtabuddin *et al.* (2012). These recovery values were less as compared to recoveries detected in the present study. However, the studies conducted by Cheong *et al.* (2010) the mean recoveries

detected were ranging from 82-90% which were comparable to the recoveries found in the present study.

Table 13: Method validation parameters for sulphamethazine

Method Validation Parameters	Values
Linear range ($\mu\text{g/kg}$)	50–500
Linearity (r^2)	0.99
Linear regression (Equation)	$y = 167.75x - 809$
LOD ($\mu\text{g/kg}$)	12.80
LOQ ($\mu\text{g/kg}$)	38.80
Intraday assay precision, n = 5 (%RSD) 200 $\mu\text{g/kg}$	6.68 %
Inter day assay precision, n = 5 (%RSD)	
100 $\mu\text{g/kg}$	7.20%
200 $\mu\text{g/kg}$	6.63%
300 $\mu\text{g/kg}$	6.74%
Recovery %	
100 $\mu\text{g/kg}$	81.8
200 $\mu\text{g/kg}$	81.6
300 $\mu\text{g/kg}$	84

4.1.1.4.3 Method validation for fluroquinolones

The method was validated for the determination of CIP and ENR in meat using spiked samples.

4.1.1.4.3.1 Ciprofloxacin

The linear range, LOD and LOQ were obtained from five point solvent matched calibrations curve by using CIP standard at five dilution levels: 50, 100, 200, 300 and 500 µg/kg with triplicate analysis (Fig. 11).

The calibration curve for CIP was linear in the range of 50-500 µg/kg with correlation coefficient (r^2) of 0.999. The LOD and LOQ for SMZ were found to be 17.4 µg/kg and 52.8 µg/kg, respectively. The values of LOD and LOQ were below the MRL (100 µg/kg) established by EU for CIP in meat.

The application of the method to ten different blank meat samples in order to verify the method specificity demonstrated that no potential interferences from endogenous compounds were detected at 280 nm at the retention time of CIP since these compounds eluted from meat matrix at lower retention times.

The intraday-assay precision was found to be 5.8% at spiking concentration of 200µg/kg whereas interday-assay precision was found to be 10.6, 10.5 and 6.5% at spiking concentration of 100, 200 and 300 µg/kg, respectively. The values were in accordance with EU guidelines. The mean recoveries of CIP at the concentration of 100, 200 and 300 µg/kg were found to be 82.6, 86.2 and 88.4%, respectively and were in accordance with the EU guidelines. The assay validation parameters for CIP are presented in Table 14.

Various HPLC based methods have been developed and validated in the past for the determination of ciprofloxacin residues in meat. Pena *et al.* (2010) developed an LC method with UV detection for the determination of ciprofloxacin residues in meat with mean recoveries ranging from 80-92%. During the present study, comparatively equal recoveries were obtained for

ciprofloxacin . Similarly, another study carried out by Moema *et al.* (2012), the mean recovery range was found to be 89-96% which was comparatively equal to the recovery obtained in the present study.

Table 14: Method validation parameters for ciprofloxacin

Method Validation Parameters	Values
Linear range (µg/kg)	50–500
Linearity (r^2)	0.99
Linear regression (Equation)	$y = 129.18x - 536.18$
LOD (µg/kg)	17.44
LOQ (µg/kg)	52.87
Intraday assay precision, n = 5 (%RSD) 200 µg/kg	5.87%
Inter day assay precision, n = 5 (%RSD)	
100 µg/kg	10.64%
200 µg/kg	10.55%
300 µg/kg	6.53%
Recovery %	
100 µg/kg	82.6
200 µg/kg	86.2
300 µg/kg	88.4

4.1.1.4.3.2 Enrofloxacin

The linear range, LOD and LOQ were obtained from five point solvent matched calibrations curve (Fig.12) by using ENR standard at five dilution levels: 50, 100, 200, 300 and 500 µg/kg with triplicate analysis .

The calibration curve for ENR was linear in the range of 50-500 µg/kg with correlation coefficient (r^2) of 0.99. The LOD and LOQ for ENR were found to be 15.9 µg/kg and 48.3 µg/kg, respectively. The values of LOD and LOQ were below the MRL (100 µg/kg) established by EU for ENR in meat.

The application of the method to ten different blank meat samples in order to verify the method specificity demonstrated that no potential interferences from endogenous compounds were detected at 280 nm at the retention time of ENR since these compounds eluted from meat matrix at lower retention times.

The intraday-assay precision was found to be 6.1% at spiking concentration of 200µg/kg whereas interday-assay precision was found to be 6.0, 8.2 and 7.0% at spiking concentration of 100, 200 and 300 µg/kg, respectively. The values were in accordance with EU guidelines. The mean recoveries of ENR at the concentration of 100, 200 and 300 µg/kg were found to be 83.8, 85.0 and 88.2%, respectively and were in accordance with the EU guidelines. The assay validation parameters for ENR are presented in Table 15.

The study of, Meoma *et al.* (2012) developed and validated a HPLC confirmatory method for the determination of enrofloxacin residues in meat with LOD and LOQ of 5 µg/kg and 23 µg/kg respectively which were comparatively higher than values observed in the current study. However lower mean recovery for enrofloxacin from spiked meat samples (75%) was detected by Bailac *et al.* (2006) .

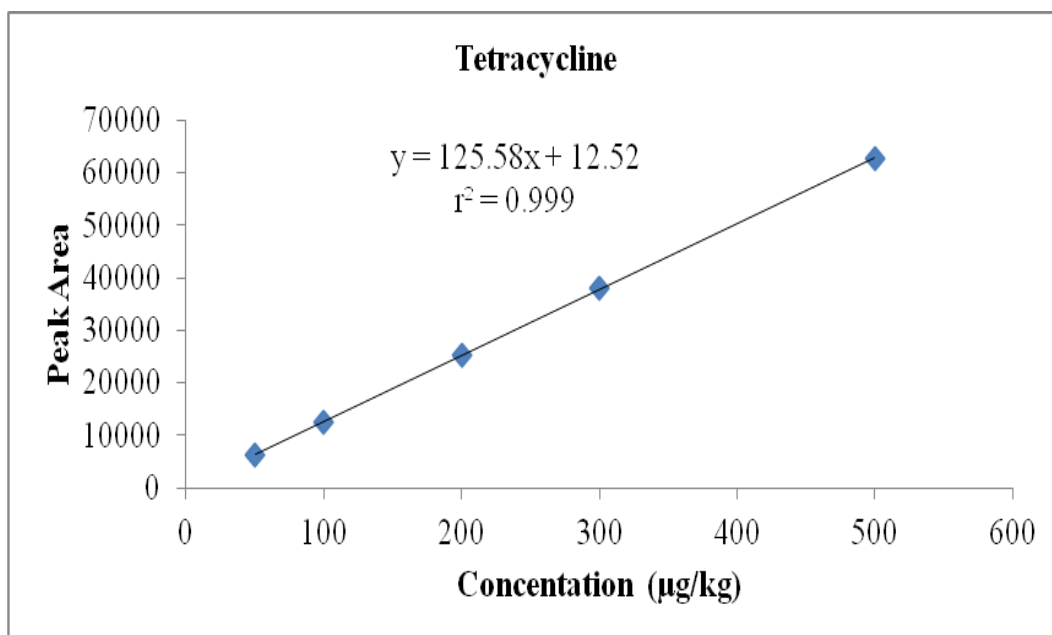


Fig. 8: Standard calibration curve for tetracycline (TC)

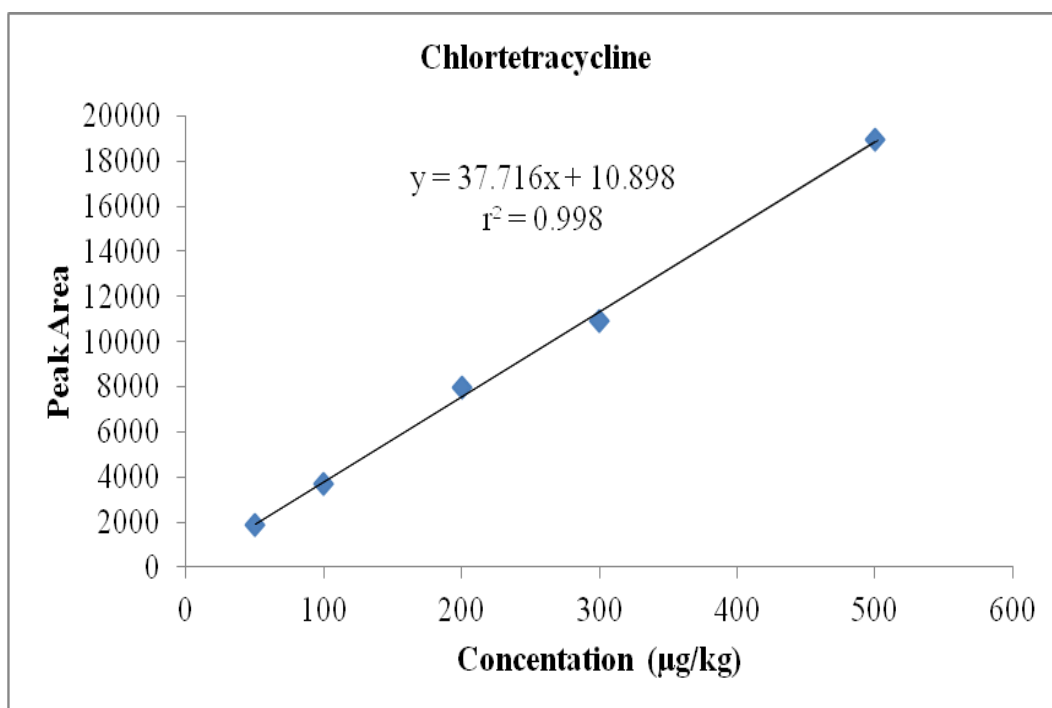


Fig. 9: Standard calibration curve for chlortetracycline (CTC)

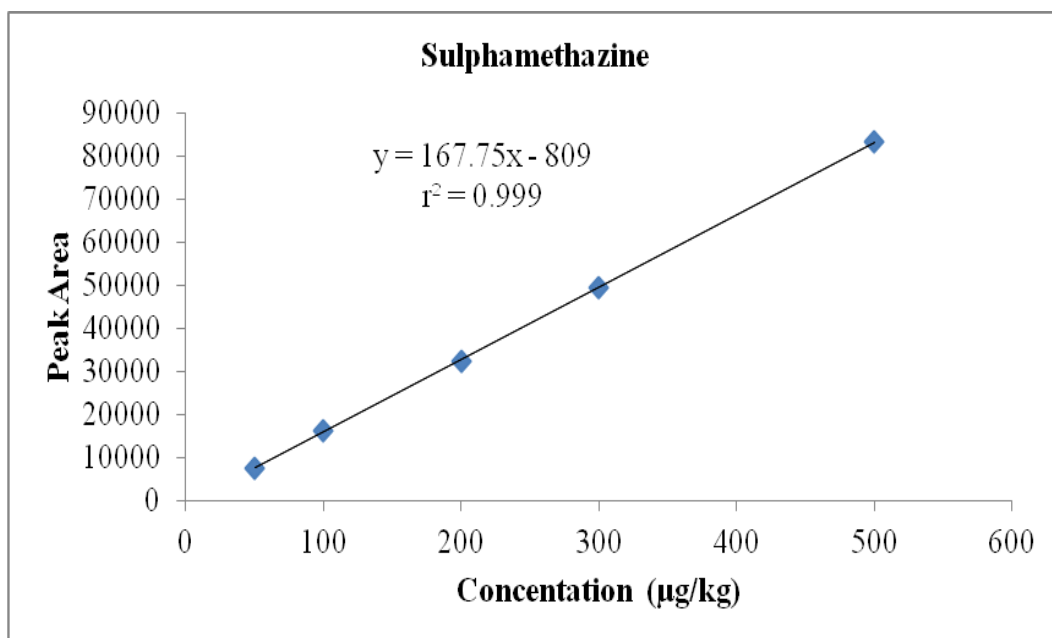


Fig. 10: Standard calibration curve for suphamethazine (SMZ)

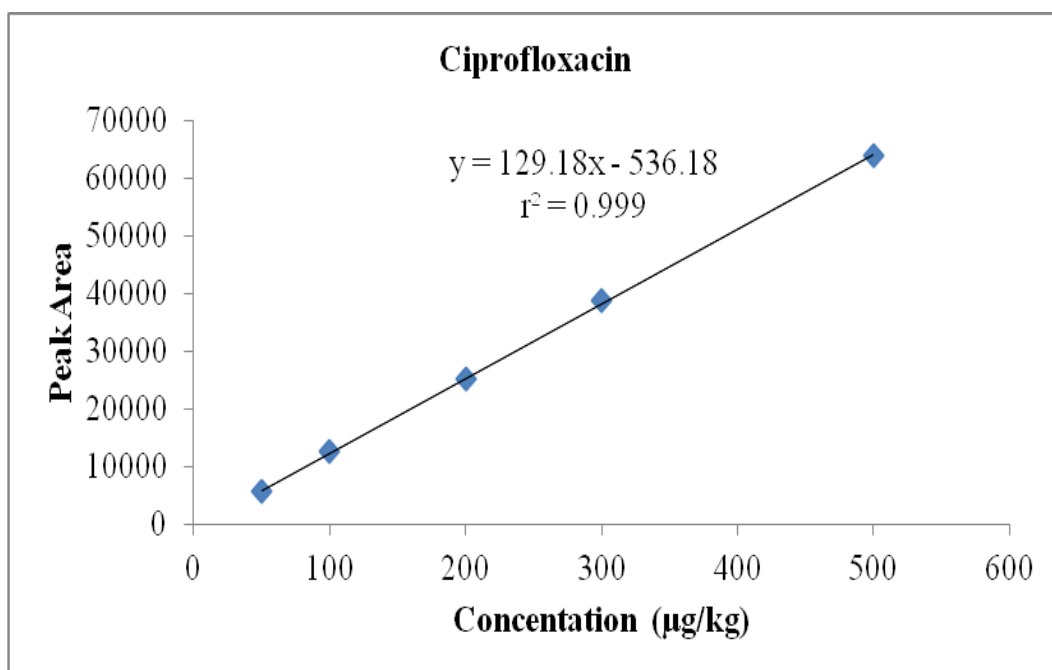


Fig. 11: Standard calibration curve for ciprofloxacin (CIP)

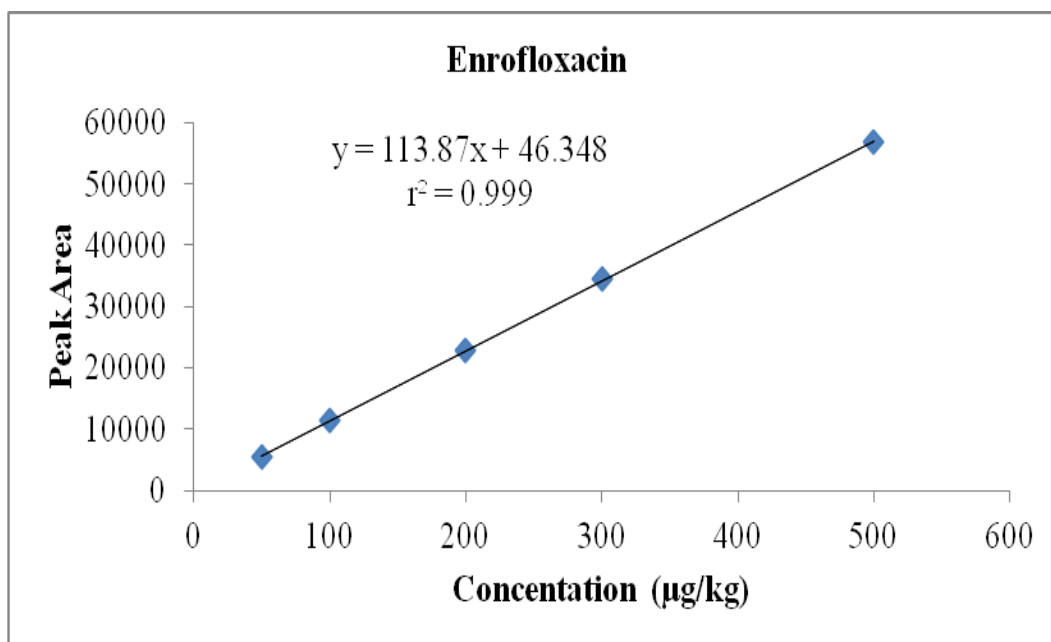


Fig. 12: Standard calibration curve for enrofloxacin (ENR)

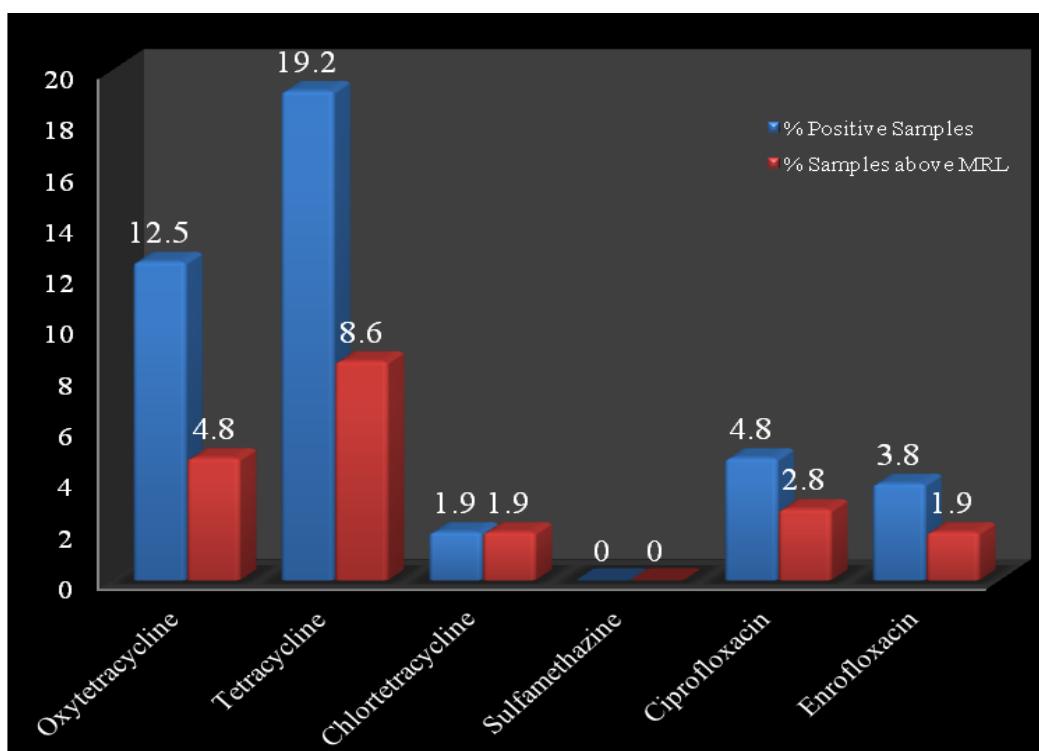


Fig. 13: Antibiotic residues detected in meat samples collected from Plain agroclimatic zone

Table 15: Method validation parameters for enrofloxacin

Method Validation Parameters	Values
Linear range (µg/kg)	50–500
Linearity (r^2)	0.99
Linear regression (Equation)	$y = 129.18x - 536.18$
LOD (µg/kg)	15.96
LOQ (µg/kg)	48.39
Intraday assay precision, n = 5 (%RSD) 200 µg/kg	6.17%
Inter day assay precision, n = 5 (%RSD)	
100 µg/kg	6.05%
200 µg/kg	8.24%
300 µg/kg	7.05%
Recovery %	
100 µg/kg	83.8
200 µg/kg	85
300 µg/kg	88.2

4.1.2 Application of optimized and validated method for detection of antibiotic residues in meat samples collected from different agroclimatic zones of Chhattisgarh

A total of 249 meat samples (214 from broiler and 35 from backyard chicken meat) were collected from individual birds during visit to different agroclimatic zone (Plain zone, Northern hill and Bastar plateau) of Chhattisgarh. These samples were processed for the analysis of antibiotic residues using method standardized and validated during the present study.

4.1.2.1 Screening of meat samples from Plain agroclimatic zone for the presence of antibiotic residues

A total of 104 meat samples collected from individual birds slaughtered at different chicken meat shops located in different districts of Plain agroclimatic zone of Chhattisgarh were processed for analysis of antibiotic residues. Out of these, 44 (42.3%) samples were found positive for antibiotic residues with mean $\pm$ SD (standard deviation) of 126 $\pm$ 79.7 μ g/kg. Out of 44 positive samples, 13 (12.5%) were positive for oxytetracyclin, 20 (19.2%) for tetracycline, 2 (1.9%) for chlortetracycline, 5 (4.8%) for ciprofloxacin and 4 (3.8%) for enrofloxacin (Fig. 13). None of the sample from Plain zone was found positive for sulphamethazine, residues. A total of 21 (20.1%) samples [5 (4.8%) for oxytetracycline 9 (8.6%) for tetracyclin, 2(1.9%) for chortetracyclin, 3 (2.8%) for ciprofloxacin and 2(1.9%) for enrofloxacin] exceeded the established MRL. The range and mean $\pm$ SD of oxytetracycline ,tetracycline, chlortetracycline, ciprofloxacin, enrofloxacin, residues detected were 50.9-170.4 μ g/kg and 93.7 $\pm$ 38.4 μ g/kg; 48.0-343.4 μ g/kg and 144.8 $\pm$ 100.8 μ g/kg; 135.9-294.8 μ g/kg and 215.4 $\pm$ 112.3 μ g/kg; 70.1-144.4 μ g/kg and 111 $\pm$ 31.1 μ g/kg; 57-170.4 μ g/kg and 110 $\pm$ 49, respectively. The antibiotic residues detected along with their mean, minimum and maximum concentrations as well as samples above MRL are summarized in Table 16 and Fig. 13.

Table 16: Antibiotic residue detected in meat samples collected from Chhattisgarh Plain zone

Antibiotic residues	n/N	%Positive	Mean $\pm$SD^a (μg/kg)	Minimum (μg/kg)	Maximum (μg/kg)	MRL^b (μg/kg)	Samples above MRL	% Samples above MRL
Oxytetracycline	13/104	12.5	93.7 $\pm$ 38.4	50.9	170.4	100	5	4.8
Tetracycline	20/104	19.2	144.8 $\pm$ 100.8	48	343.4	100	9	8.6
Chlortetracycline	2/104	1.9	215.4 $\pm$ 112.3	135.9	294.8	100	2	1.9
Sulphamethazine	0/104	-	-	-	-	100	Nil	Nil
Ciprofloxacin	5/104	4.8	111 $\pm$ 31.1	70.1	144.4	100	3	2.8
Enrofloxacin	4/104	3.8	110 $\pm$ 49	57	170.4	100	2	1.9
Overall	44/104	42.3	-	-	-	-	21	20.1

Note. N, total number of samples analyzed; n, number of positive samples; MRL, maximum residue limit.

^aMean $\pm$ SD of positive samples.

^bMRL established by European Union.

4.1.2.2 Screening of meat samples from Northern hill zone for the presence of antibiotic residues

A total of 55 meat samples were collected from northern hill agroclimatic zone and processed for analysis of antibiotic residues. Out of these, 28 (50.9%) samples were found positive for antibiotic residues with mean $\pm$ SD of 148.9 $\pm$ 68.1 μ g/kg . Among the 28 positive samples, 5(9.0%) were positive for oxytetracycline, 7(12.7%) for tetracycline and 16(29.0%) for sulphamethazine (Fig. 14). None of the sample from norther hill zone was found positive for chlortetracycline, ciprofloxacin and enrofloxacin residues. A total of 17 (30.9%) samples [1(1.8%) for oxytetracycline and 16 (29.0%) for sulphamethazine] exceeded the established MRL whereas none of the sample was found to have tetracycline residues above MRL. The range and mean $\pm$ SD of oxytetracycline, tetracycline and sulphamethazine residues detected were 60.4-170.6 μ g/kg and 112.8 $\pm$ 47.8 μ g/kg; 50.4-105 μ g/kg and 73.4 $\pm$ 20.2 and 131.2-276.1 μ g/kg and 193.1 $\pm$ 49 μ g/kg, respectively. The antibiotic residues detected along with their mean, minimum and maximum concentrations as well as samples above MRL are summarized in Table 17 and Fig.14.

Table 17: Antibiotic residue detected in meat samples collected from Northern hill

Antibiotic residues	n/N	%Positive	Mean $\pm$SD^a (μg/kg)	Minimum (μg/kg)	Maximum (μg/kg)	MRL^b (μg/kg)	Samples above MRL	% Samples above MRL
Oxytetracycline	5/55	9	112.8 $\pm$ 47.8	60.4	170.6	100	1	1.8
Tetracycline	7/55	12.7	73.4 $\pm$ 20.2	50.4	105	100	Nil	Nil
Chlortetracycline	0/55	-	-	-	-	100	Nil	Nil
Sulphamethazine	16/55	29	193.1 $\pm$ 49	131.2	276.1	100	16	29
Ciprofloxacin	0/55	-	-	-	-	100	Nil	Nil
Enrofloxacin	0/55	-	-	-	-	100	Nil	Nil
Overall	28/55	50.9					17	30.9

Note. N, total number of samples analyzed; n, number of positive samples; MRL, maximum residue limit.

^aMean $\pm$ SD of positive samples.

^bMRL established by European Union.

4.1.2.3 Screening of meat samples from Bastar plateau zone for the presence of antibiotic residues

A total of 55 meat samples collected from Bastar plateau zone were processed for analysis of antibiotic residues. Out of these, 24 (43.6%) samples were found positive for antibiotic residues with mean $\pm$ SD of 80.1 $\pm$ 23.9 μ g/kg. Out of 24 positive samples, 12 (21.8%) were positive for oxytetracycline, 5 (9.0%) for sulphamethazine, 6(10.9%) for ciprofloxacin and 1 (1.8%) for enrofloxacin (Fig. 15). None of the sample was found positive for tetracycline and chlortetracycline residues. A total of 5 (9.0%) samples [1 (1.8%) oxytetracycline, 2 (3.6%) for sulphamethazine and 2 (3.6%) for ciprofloxacin] exceeded the established MRL whereas none of the sample was found above MRL for enrofloxacin residues. The range and mean $\pm$ SD of oxytetracycline, sulphamethazine, ciprofloxacin and enrofloxacin residues detected were 50.2-110.2 μ g/kg and 71.5 $\pm$ 18.9 μ g/kg; 70.4-115.2 μ g/kg and 92.3 $\pm$ 18.2 μ g/kg; 60.2-150.3 μ g/kg and 89.1 $\pm$ 37.7 μ g/kg; 67.7 μ g/kg and 67.7 $\pm$ 0, respectively. The antibiotic residues detected along with their mean, minimum and maximum concentrations as well as samples above MRL are summarized in Table 18 and Fig. 15.

Table 18: Antibiotic residue detected in meat samples collected from Bastar plateau

Antibiotic residues	n/N	%Positive	Mean $\pm$SD^a (μg/kg)	Minimum (μg/kg)	Maximum (μg/kg)	MRL^b (μg/kg)	Samples above MRL	% Samples above MRL
Oxytetracycline	12/55	21.8	71.5 $\pm$ 18.9	50.2	110.2	100	1	1.8
Tetracycline	0/55	-	-	-	-	100	Nil	Nil
Chlortetracycline	0/55	-	-	-	-	100	Nil	Nil
Sulphamethazine	5/55	9	92.3 $\pm$ 18.2	70.4	115.2	100	2	3.6
Ciprofloxacin	6/55	10.9	89.1 $\pm$ 37.7	60.2	150.3	100	2	3.6
Enrofloxacin	1/55	1.8	67.7 $\pm$ 0	67.7	0	100	Nil	Nil
Overall	24/55	43.6	-	-	-	-	5	9

Note. N, total number of samples analyzed; n, number of positive samples; MRL, maximum residue limit.

^aMean $\pm$ SD of positive samples.

^bMRL established by European Union.

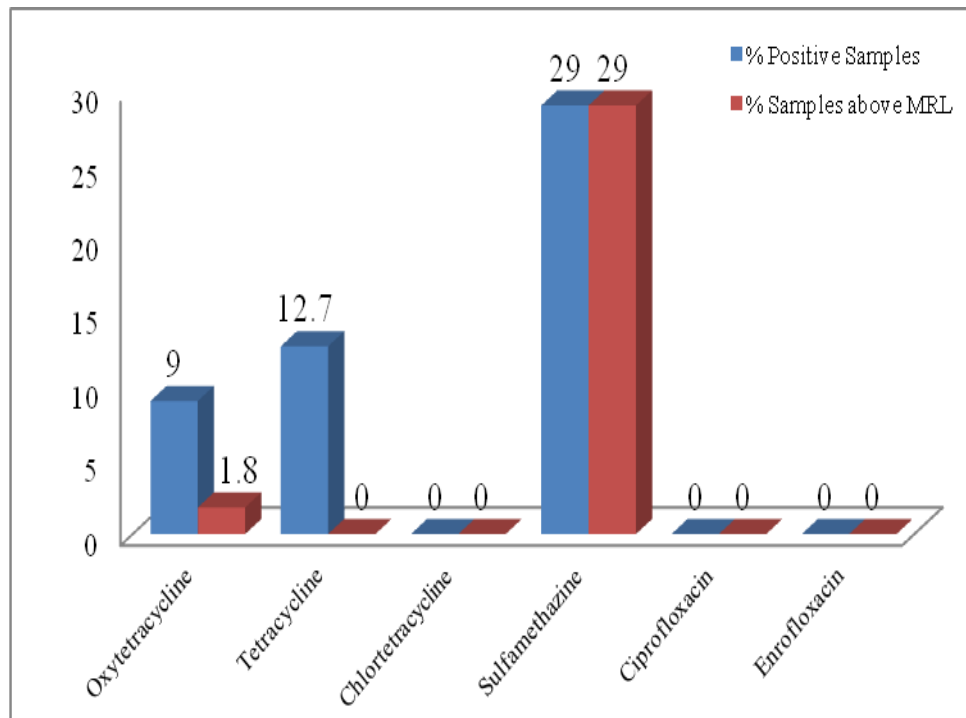


Fig. 14: Antibiotic residues detected in meat samples collected from Northern hill agroclimatic zone

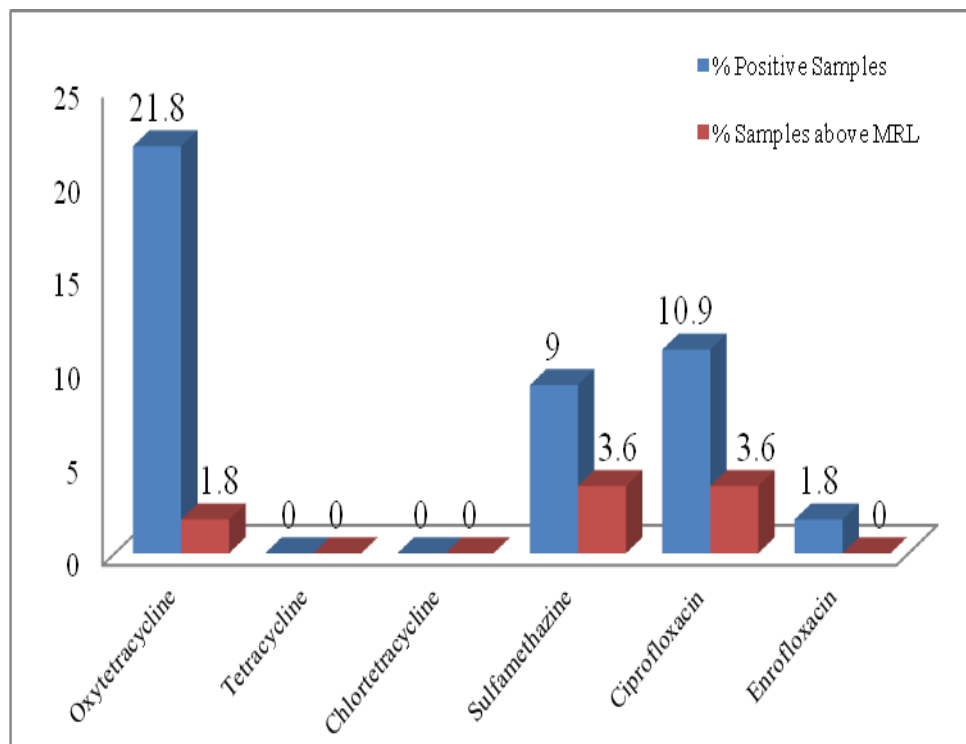
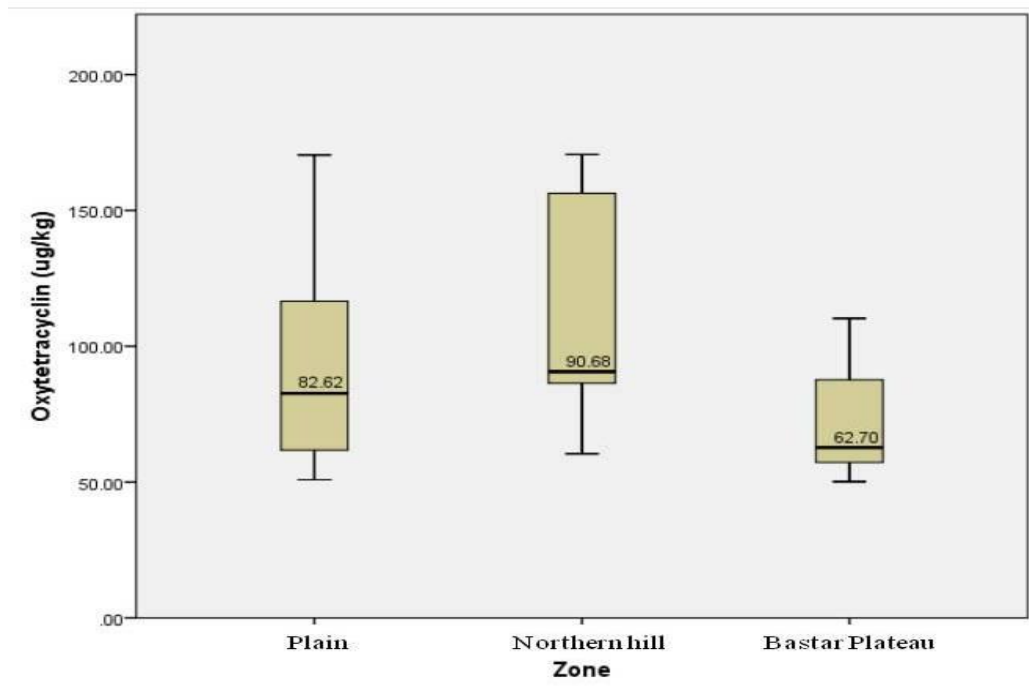
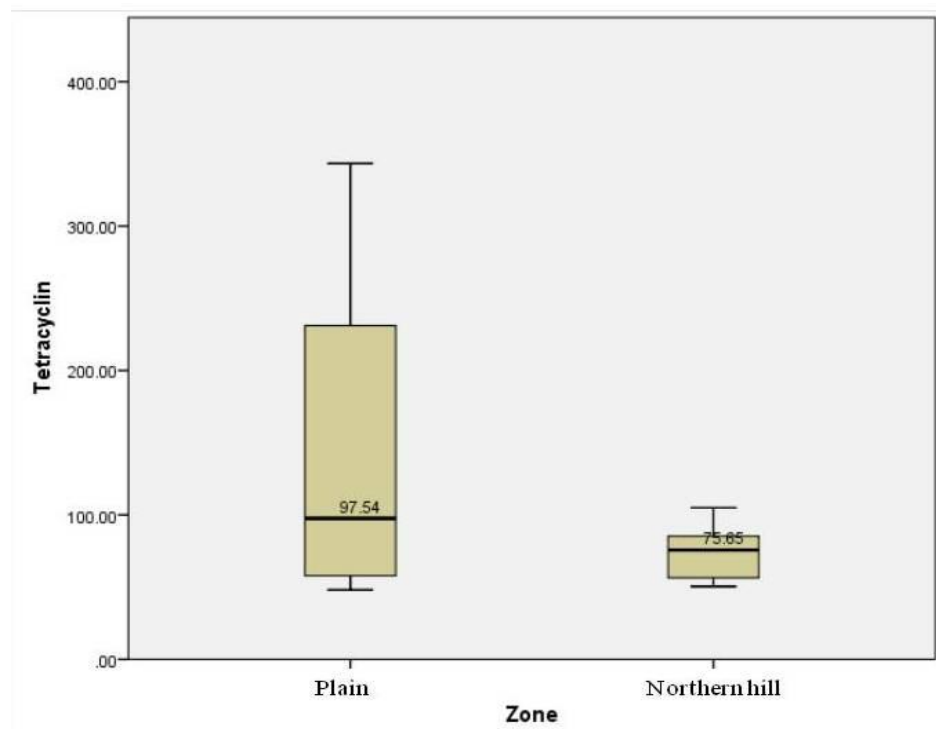


Fig. 15: Antibiotic residues detected in meat samples collected from Bastar plateau agroclimatic zone

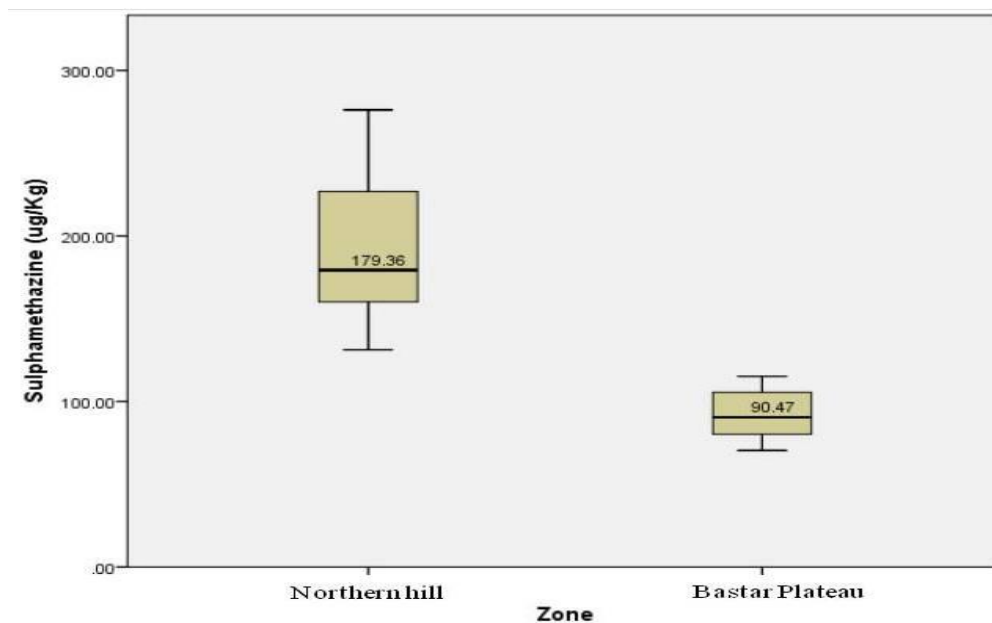
(a)



(b)



(c)



(d)

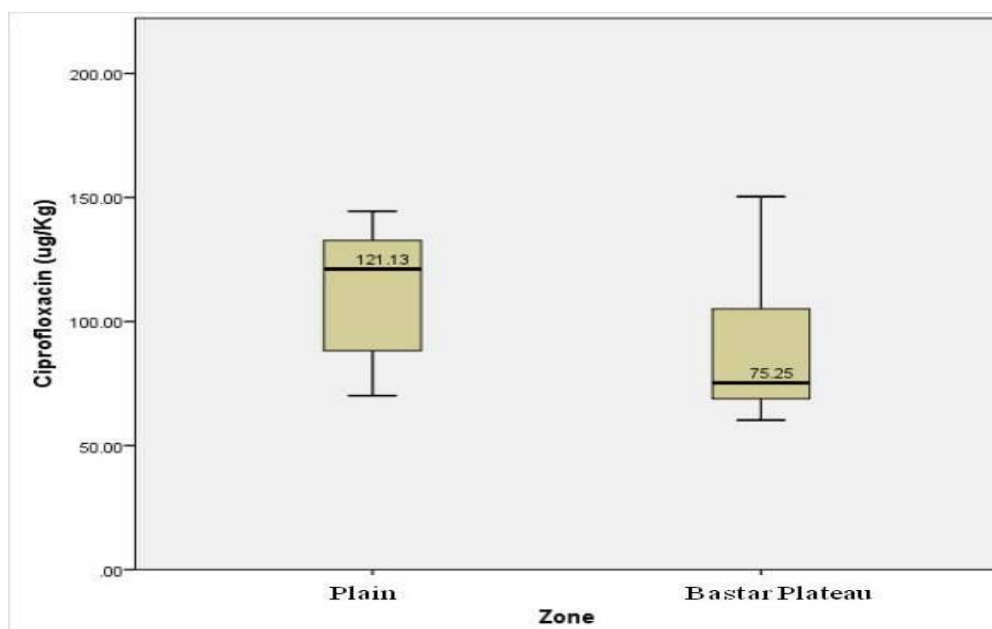


Fig. 16: Box and whisker plots for the residues of (a) oxytetracycline, (b) tetracycline, (c) sulphamethazine and, (d) ciprofloxacin in meat samples from Plain, Northern hill, and Bastar plateau

The horizontal line in the box represents the median value and the lower and upper edge of the box mark the first and third quartiles. The whiskers extending from the box show the highest and lowest values.

4.1.2.4 Screening of backyard chicken meat samples for the presence of antibiotic residues

A total of 35 marketed backyard chicken meat samples from three agroclimatic zones were processed for analysis of antibiotic residues. Out of these, 13 (37.1%) samples were found positive for antibiotic residues with mean $\pm$ SD of 86.3 $\pm$ 26.8 μ g/kg. 12 (34.2%) samples were positive for oxytetracycline residues while 1 (2.8%) sample was positive for ciprofloxacin residues. Larger proportion of backyard chicken meat samples were found positive for oxytetracycline but only one sample was found contaminated with ciprofloxacin residues with concentration below the established MRL. The range and mean $\pm$ SD for oxytetracycline residues detected were 53.3-137.57 μ g/kg and 89 $\pm$ 26.2 μ g/kg while for ciprofloxacin residues mean $\pm$ SD was 54.4 $\pm$ 0 μ g/kg. Three (8.5%) sample exceeded the established MRL for oxytetracyclin residues. The antibiotic residues detected along with their mean $\pm$ SD, minimum and maximum concentrations as well as samples above MRL are summarized in Table 19.

The most commonly found antibiotic residues in chicken meat samples from three agroclimatic zones were oxytetracycline, tetracycline, sulphamethazine and ciprorofloxacin. The sulphamethazine residues were not detected in meat samples from Plain region. In Northern hill region, sulphamethazine was predominantly present as compared to other agroclimatic zone and it was devoid of ciprofloxacin and enrofloxacin residues. In Bastar region chicken meat samples were mainly contaminated with oxytetracycline and ciprofloxacin but in these meat samples were not detected the residues of tetracycline and chlortetracycline

Table 19: Antibiotic residue detected in meat samples of backyard poultry

Antibiotic residues	n/N	%Positive	Mean $\pm$SD^a (μg/kg)	Minimum (μg/kg)	Maximum (μg/kg)	MRL^b (μg/kg)	Samples above MRL	% Samples above MRL
Oxytetracycline	12/35	34.2	89 $\pm$ 26.2	53.3	137.57	100	3	8.5
Tetracycline	0/35	-	-	-	-	100	Nil	Nil
Chlortetracycline	0/35	-	-	-	-	100	Nil	Nil
Sulphamethazine	0/35	-	-	-	-	100	Nil	Nil
Ciprofloxacin	1/35	2.8	54.4 $\pm$ 0	54.4		100	Nil	Nil
Enrofloxacin	0/35	-	-	-	-	100	Nil	Nil
Overall	13/35	37.1	-	-	-	-	3	8.5

Note. N, total number of samples analyzed; n, number of positive samples; MRL, maximum residue limit.

^aMean $\pm$ SD of positive samples.

^bMRL established by European Union.

also, the mean concentration of antibiotic residues in positive samples were compared statistically among Plain, Northern hill and Bastar plateau using one way ANOVA with Post hoc Tukey's test at 95% mean confidence interval (Table 20). There was a statistical significant difference in the means of residues detected in meat samples collected from Plain and as well as Northern hill and Bastar plateau ($p < 0.05$). However, no statistical significant difference was observed in the means of residues detected in plain and northern hill ($p > 0.05$) region. Box and whisker plots for antibiotic residues in chicken meat samples from different agroclimatic zone were plotted (Fig. 16). Similarly mean concentration of antibiotic residues in positive samples of backyard chicken and broiler chicken was compared by using t-test. Statistically significant difference was observed in the means of residues detected in backyard and broiler chicken meat ($p < 0.05$).

Table 20: Comparison of mean residues detected among three agroclimatic zone by one way ANOVA

Zones (A)	Zones (B)	Mean difference (A and B)	p-Value
Plain Zone	Northern Hill Zone	-22.9	0.21
	Bastar Plateau	45.9*	0.007*
Northern Hill Zone	Plain Zone	22.9	0.21
	Bastar Plateau	68.8*	0.0002*
Bastar Plateau Zone	Plain Zone	-45.9*	0.007*
	Northern Hill Zone	-68.8*	0.0002*

* $p < 0.05$ —The mean difference is significant at 5% level.

Abbreviation: ANOVA, analysis of variance.

The findings of the present study indicated that among the antibiotic residues in chicken meat (broiler and backyard) samples in all three agroclimatic zones of Chhattisgarh, oxytetracycline was the leading antibiotic detected in the meat samples

and it contributed 38% of the total antibiotic residue detected in meat samples, followed by tetracycline, Sulphamethazine, ciprofloxacin, enrofloxacin and chlortetracycline residue in meat samples which contributed 25%,19%, 11%, 5% and 2% respectively. (Table 21 and Fig.17)

Table 21: Overall prevalence of antibiotic residue detected in meat samples

Antibiotic residues	n/N	%Positive	Mean $\pm$ SD ^a (μ g/kg)	Minimum (μ g/kg)	Maximum (μ g/kg)
Oxytetracycline	42/249	16.8	88.3 $\pm$ 33.2	50.2	170.6
Tetracycline	27/249	10.8	126.3 $\pm$ 92.4	48.0	343.4
Chlortetracycline	2/249	0.8	215.4 $\pm$ 112.3	135.9	294.8
Sulphamethazine	21/249	8.4	169.1 $\pm$ 61.7	70.4	276.1
Ciprofloxacin	12/249	4.8	95.5 $\pm$ 34	54.4	150.3
Enrofloxacin	5/249	2	101.9 $\pm$ 46.5	57	170.5
Overall	109/249	43.7	-	-	-

Note. N, total number of samples analyzed; n, number of positive samples

^aMean $\pm$ SD of positive samples

Considering the prevalence of tetracyclines in chicken meat samples, the result obtained in present study were comparable to those reported by Sahu and Saxena (2014), Moon *et al.* (2018) and Jammoul and Dara (2019). On contrary, prevalence of tetracyclines found in present study was significantly lower than the ones reported by Salma *et al.* (2011), Aman *et al.* (2017), Sarker *et al.* (2018) and Baghani *et al.* (2019).

Prevalence of sulphamethazine in chicken meat samples obtained in current study was almost similar to those obtained by Cheong *et al.* (2010) and Jammoul and Dara (2019) but lower than that reported by Awaisheh *et al.* (2019).

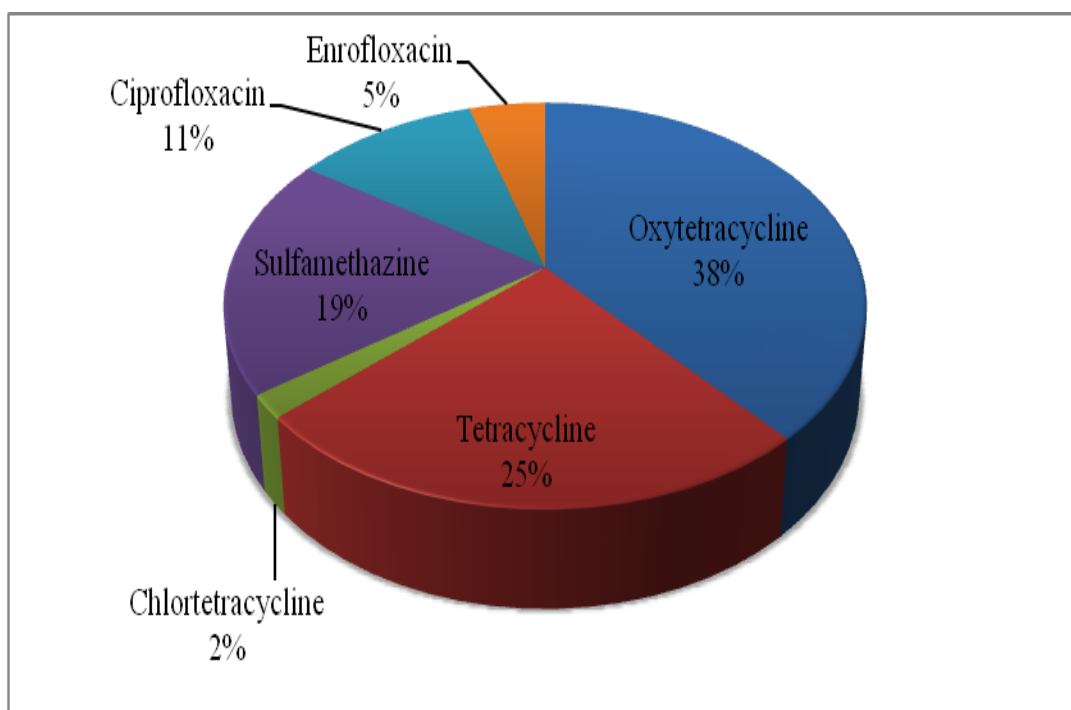


Fig. 17: Overall distribution of studied antibiotic residues in chicken meat

Prevalence of 4.8% for ciprofloxacin and 2% for enrofloxacin was found in present study, which was comparable to the findings of Yamaguchi *et al.* (2015) and Prajapati *et al.* (2018) but significantly higher than the those reported by Moghadam *et al.* (2018), Jammoul and Dara (2019), Verma *et al.* (2020). In contrast, lower prevalence rate was reported by Meena *et al.* (2018).

The residues of antibiotics recovered in the respective studies could easily be related to the group of antibiotics being preferred or prescribed by the veterinarian/physician for the treatment of poultry birds in the respective areas/region/state or country of the study. Many studies carried out in India and abroad revealed higher concentration of antibiotic residues in poultry feed which ultimately led to occurrence of these residues in chicken meat (Cornejo *et al.*, 2017; Waghamare *et al.*, 2020).

The above findings highlighted that the percentage of positive samples, mean concentration of residues in positive samples as well as percentage of samples above MRL were higher in meat samples collected from plain region and northern hill region as compared to Bastar plateau (Table 20). These findings could be attributed to address the issue unawareness and easy access of veterinary drugs to farmers for use as growth promoting agents as well as for preventive and therapeutic purpose.

4.2.5 Estimation of human health risk assessment

The risk assessment for meat samples from broiler chicken meat of different agroclimatic zone and backyard chicken meat was carried out to determine the hazards to consumers associated with consumption of meat containing antibiotic residues. The mean concentration of oxytetracycline and ciprofloxacin residues in backyard poultry meat samples was 89 and 54.5 µg/kg, respectively. The mean

concentrations of antibiotic residues detected in meat from broiler chicken meat are presented in Tables 16-18. HQ for antibiotic residues detected in meat samples from different agro climatic zones was calculated for determining any health risks to consumers (Table 22). HQ is the ratio of the potential exposure to a substance and the level at which no adverse effects are observed. If HQ is equal to or less than 1, the risk is not considered significant to human health as a result of exposure, whereas if HQ is more than one, the consumer is exposed to the risk. As the HQ for antibiotic residues detected in meat samples from broiler chicken meat and the backyard chicken was less than 1, thus it could be assumed that there were no significant adverse effects on the health of consumers associated with the consumption of the chicken meat. The similar findings were reported by Pena *et al.* (2010) and Wang *et al.* (2017) who estimated the dietary exposure to antibiotic residues detected in meat samples. Both the studies reported no toxicological risk to consumers associated with the consumption of the analyzed meat samples with regards to examined antibiotics. Similar findings were also reported by Pereira *et al.* (2018) and Oyedeleji *et al.* (2019), where the estimated dietary exposure to antibiotic residues via meat was below the toxicological reference value.

The present study analyzed the occurrence of antibiotic residues in chicken in Chhattisgarh, India and assessment of human health risk due to the consumption of meat containing residues. Risk assessment based on per capita availability of chicken meat in humans revealed that the current levels of antibiotic residues in meat pose no significant adverse effects on the health of consumers. However, the occurrence of residues in meat above established MRL is certainly a matter of concern and exposure of consumers to these residues through meat consumption could result in

significant adverse effects on their health on a long-term basis. The occurrence of antibiotic residues in chicken meat indicates a need for implementing appropriate regulatory legislations and regular monitoring of residues to prevent the nonprudent use of antibiotics in treatment as well as feed additive in poultry farming as growth promoter and thus preventing unacceptable health risk to consumer.

Table 22: Risk assessment based on hazard quotient for antibiotic residues *via* marketed chicken meat intake in adults and children

Antibiotic	Meat samples	Age group	Body weight^a (kg)	EDI (µg/kg/day)	ADI^b (µg/kg/day)	Hazard quotient
Oxytetracycline	Plain Zone	Adult	60	0.0212	30	7.1×10^{-4}
		Children	15	0.0849	30	28.3×10^{-4}
	Northern Hill	Adult	60	0.0255	30	8.5×10^{-4}
		Children	15	0.1022	30	34.1×10^{-4}
	Bastar Plateau	Adult	60	0.0162	30	5.4×10^{-4}
		Children	15	0.0648	30	21.6×10^{-4}
	Backyard Poultry	Adult	60	0.0201	30	6.7×10^{-4}
		Children	15	0.0806	30	26.9×10^{-4}
Tetracycline	Plain Zone	Adult	60	0.0328	30	10.9×10^{-4}
		Children	15	0.1312	30	43.8×10^{-4}
	Northern Hill	Adult	60	0.0166	30	5.5×10^{-4}
		Children	15	0.0665	30	22.2×10^{-4}
Chlortetracycline	Plain Zone	Adult	60	0.0488	30	16.3×10^{-4}
		Children	15	0.1952	30	65.1×10^{-4}
Sulphamethazine	Northern Hill	Adult	60	0.0437	50	8.7×10^{-4}
		Children	15	0.1750	50	35×10^{-4}
	Bastar	Adult	60	0.0209	50	4.2×10^{-4}

	Plateau	Children	15	0.0836	50	16.7×10^{-4}
Ciprofloxacin	Plain Zone	Adult	60	0.0251	7.1	35.4×10^{-4}
		Children	15	0.1006	7.1	141.7×10^{-4}
	Bastar	Adult	60	0.0201	7.1	28.4×10^{-4}
	Plateau	Children	15	0.0807	7.1	113.8×10^{-4}
	Backyard	Adult	60	0.0123	7.1	17.4×10^{-4}
	Poultry	Children	15	0.0493	7.1	69.5×10^{-4}
Enrofloxacin	Plain	Adult	60	0.0249	6.2	40.2×10^{-4}
	Zone	Children	15	0.0997	6.2	160.8×10^{-4}
	Bastar	Adult	60	0.0153	6.2	24.7×10^{-4}
	Plateau	Children	15	0.0613	6.2	99×10^{-4}

Abbreviations: ADI, acceptable daily intake; EDI, estimated daily intake.

^a Body weights for different age groups were derived from FAO/WHO guidelines.

^b ADI (EMEA, 1998; JECFA, 1995, 2004, Hanna *et al.*, 2018).

4.2 .1 Antibiotic resistance in *E. coli* isolates

A total of 223 isolates of *E. coli* from the water and fecal samples, were isolated and evaluated for resistance against nine antibiotics (Fig.18-21A-B). The results showed that 53.2±20.7% of the *E. coli* isolates were resistant to AMP, 76.4±16.9% to CTX, 55.8±27.0% to CAZ, 38.3±24.7% to CFM, 47.9±16.6 to AX. In contrast, a lower incidence of bacterial resistance was found against the other antibiotics with frequencies of 24.2±23.7% to GEN, 20.2±20.1% to O, and 11.3±7.4 to CIP. Similar bacterial antibiotic resistance profiles for the water and fecal samples were found with a significantly higher resistance incidence to beta-lactamase group of antibiotics AMP, CTX, CAZ, CFM, and AT than to CIP, GEN and O (One-way ANOVA, $P < 0.05$) (Table 23 and Fig. 22).

The antibiotic resistance frequencies of *E. coli* isolates from the different aquaculture ponds are presented in Table 23. A total of 87 *E. coli* were isolated from the pond water samples (W1, W2 and W3), with the average resistance frequencies of 61.3±33.5% to AMP, 84.7±7.9% to CTX, 66.6±13.3% to CAZ, 48.6±22% to CFM, 50.8±24.4% to AX, 31.5±21.9% to GEN, 41.4±26.7% to AT, 10.3±6.6% to CIP and 16.1±8.5% to O. Higher resistance frequencies to AMP, CTX, CAZ, CFM and AX were detected in the *E. coli* isolated from the pond water samples. Figure 23 shows the highest resistance frequencies in W1 and the lowest resistance frequencies in W2 for these antibiotics (AMP, CTX, CAZ, CFM and AT). The *E. coli* isolates from pond W2, as well as from the pond W1 and pond W3 were resistant to all 9 antibiotics, whereas all the *E. coli* isolates from pond showed low resistance frequencies to GEN, CIP and O in comparison to other betalactamase antibiotic tested

in the present study (Table 23). Analysis of the overall mean percentage of resistance showed variations based on the different pond and their related activity for *E. coli* isolates by ANOVA ($p < 0.05$), despite low overall resistance for W2 (27.3 ± 28.9) and W1(58.8 ± 26) showed high resistant to different antibiotics tested in the present study (Fig. 24).

A total of 136 *E. coli* were isolated from the fecal samples (M1, M2, M3, M4 and M5), with the average resistance frequencies of $48.4 \pm 10.7\%$ to AMP, $71.4 \pm 19.7\%$ to CTX, $49.3 \pm 32.4\%$ to CAZ, $32.2 \pm 26.5\%$ to CFM, $46.2 \pm 13.3\%$ to AX, $19.8 \pm 26\%$ to GEN, $22.8 \pm 12.9\%$ to AT, $11.9 \pm 8.5\%$ to CIP and $22.6 \pm 25.5\%$ to O. The *E. coli* isolates from the sample of M3 origin exhibited resistance to only five antibiotics (AMP, CTX, CAZ, AX, and AT), with no resistance to the other four antibiotics (CFM, GEN, CIP and O). All the *E. coli* isolated from the fecal samples of human using community pond, cattle fecal sample from community pond and duck and goat fecal samples of the integrated aquaculture ponds (M1, M2, M4 and M5) showed resistance to all tested 9 antibiotics (Table 23). The *E. coli* isolated from the fecal samples of three ponds showed higher resistance to most of the tested antibiotics as against the *E. coli* from the fecal samples of humans not using the community pond (Fig. 25). Overall mean percentage analysis of the resistance showed difference among the human and animal fecal *E. coli* isolates associated with different ponds by ANOVA ($p < 0.05$). Overall higher resistance in fecal *E. coli* was recorded in M4 (61.4 ± 25.1) followed by M5 (46.8 ± 23.6), M1 (27.3 ± 15.3), M2 (24.7 ± 15.3) and M3 (20.3 ± 25.09) (Fig. 26).

The MAR index ranged between 0.40 to 0.56 in the *E. coli* isolated from the water samples and from 0.25 to 0.53 in the fecal sample isolates (Table 23). The highest MAR index was shown in W1 (0.56), followed by M4 (0.53), W3 (0.51), M5 (0.43), W2 (0.40), M2 (0.35), M3 (0.28), and M1 (0.25) (Table 23). MAR index values >0.2 are regarded as an indicator of anthropogenic contamination. MAR index values <0.2 indicate that the environment of isolation has no history or very low use of antibiotics (Krumperman, 1983). Present study showed that more than 0.2 MAR index for both pond and fecal *E. coli* isolates, indicative of anthropogenic contamination. The haphazard use of antibiotics may be responsible of higher resistance among the isolates obtained during the present study.

Table 23: Percentages of *E. coli* isolates resistant to different antibiotics.

Resistance frequencies of <i>E.coli</i> isolates (%)											
	Water sample				Fecal sample						Total Water and Fecal Resistance
Antibiotics	W1	W2	W3	Average Resistance	M1	M2	M3	M4	M5	Average Resistance	
AMP	86.6	23.3	74	61.3±33.5	42.8	54	41.6	64.7	39.1	48.4±10.7	53.2±20.7
CTX	83.3	93.3	77.7	84.7±7.9	54.2	54	66.6	100	82.6	71.4±19.7	76.4±16.9
CAZ	80	53.3	66.6	66.6±13.3	22.8	27	29.1	94.1	73.9	49.3±32.4	55.8±27.0
CFM	63.3	23.3	59.2	48.6±22	22.8	18.9	0	58.8	60.8	32.2±26.5	38.3±24.7
AX	70	23.3	59.2	50.8±24.4	25.7	48.6	41.6	58.8	56.5	46.2±13.3	47.9±16.6
GEN	40	6.6	48.1	31.5±21.9	2.85	2.7	0	58.8	34.7	19.8±26	24.2±23.7
AT	66.6	13.3	44.4	41.4±26.7	20	21.6	4.1	29.4	39.1	22.8±12.9	29.8±19.8
CIP	16.6	3.3	11.1	10.3±6.6	14.2	13.5	0	23.5	8.69	11.9±8.5	11.3±7.4
O	23.3	6.6	18.5	16.1±8.5	17.1	5.4	0	64.7	26	22.6±25.5	20.2±20.1
Isolates	30	30	27		35	37	24	17	23		
MAR Index	0.56	0.4	0.51		0.25	0.35	0.28	0.53	0.43		

W1, water sample of the village community pond; W2, water sample of the traditional fish pond; W3, water sample of the integrated duck-fish-goat pond; M1, fecal sample of cattle and buffalo regularly enter village community pond; M2, fecal sample of human using village community pond; M3, fecal sample of human not using village community pond; M4, fecal sample of duck of the integrated duck-fish-goat pond and M5, fecal sample of goat of duck-fish-goat integrated pond.

AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CFM, cefixime; AX, ampicillin/loxacin; GEN, gentamicin; AT, aztreonam; CIP, ciprofloxacin; O, oxytetracycline.

Isolates, total isolates of *E.coli* from each sample type MAR, multiple antibiotic resistance index

The results of present study showed that the high antibiotic resistances were detected in all of the ponds, which indicates that pond water can serve as an environmental reservoir of antibiotic resistance. A high prevalence of antibiotic resistant *E.coli* has previously been documented in various studies conducted in integrated fish farm of China (Su *et al.*, 2011; Zhang *et al.*, 2013).

The antibiotic resistance dissemination sources should be investigated further in the future, but two potential sources of the AR detected in the ponds can be postulated. Especially in some developing countries, antibiotics are used in aquaculture as a preventive measure to reduce the occurrence of costly disease outbreaks a practice that has been linked to the emergence of antibiotic resistant bacteria (Cabello, 2006). Previous studies found that fish farmers in developing countries, including China and India commonly used antibiotics (amoxicillin, enrofloxacin, ciprofloxacin, oxytetracycline, penicillin, sulfadiazine, sulfamethoxazole, sulfadimidine, and trimethoprim) (Seiler and Berendonk, 2012; Sinha and Khurana, 2016; Mo *et al.*, 2017). Antibiotics are also still used in animal husbandry for similar purposes in many countries and antibiotic resistant bacteria may be present in the animal feces used to fertilize these fishponds and runoff or leaking from human waste storage. A study in Guangdong province found a high prevalence of antibiotic resistance bacteria in the feces of ducks and pigs raised on integrated fish farms (Su *et al.*, 2011). In Chhattisgarh, the community ponds are influenced with direct human and animal activities, moreover most of them are located near to human habitat thus receive the human waste run off. These ponds are

now a days used for aquaculture activity which is utilizing the antibiotics and animal manure. All these factors are the possible cause of high resistance in *E. coli* of such type of ponds. These types of integrated activity run community pond are present in most of the villages of Chhattisgarh.

Fecal sample *E. coli* isolates from integrated pond ecosystem showed very high resistance than the human and cattle fecal *E. coli* isolates of community pond. Even the high resistant *E. coli* isolates were detected in human using pond water for bathing and other day to day activity than the person not using pond water for bathing. Findings suggested that more closely involvement with pond water may promote the transfer of these resistant bacteria and pond water work as reservoir or mixing vessels for transmission and sharing of resistant bacteria. Zhang *et al.* (2013) reported that *E. coli*, isolated from the manure samples of three integrated aquaculture ponds showed higher resistance to most tested antibiotics than from the free range duck manure sample.

Findings of present study showed higher resistance for the ampicillin than the tetracycline which corroborates the findings of Su *et al.*, (2011) who reported higher resistance especially for ampicillin (80%), tetracycline (52%), and trimethoprim (50%) with MAR index of 0.56 in integrated fish farm isolates. Varghese and Roymon (2013) reported resistance pattern of *E. coli* isolates from water samples collected in and around Durg district and recorded that 85.4% of isolates were resistance to 2 or more antibiotics. MAR index of isolates ranged from 0.11 to 0.55. 61% which is comparable to the range of MAR index observed during the present study.

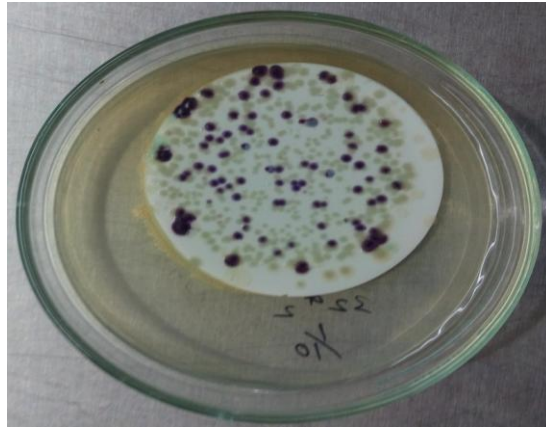


Fig. 18: *E. coli* isolated from water sample showing pink colonies on M-TEC agar



Fig. 19: *E. coli* isolated from fecal sample showing pink colonies on M-TEC agar (spread plate)

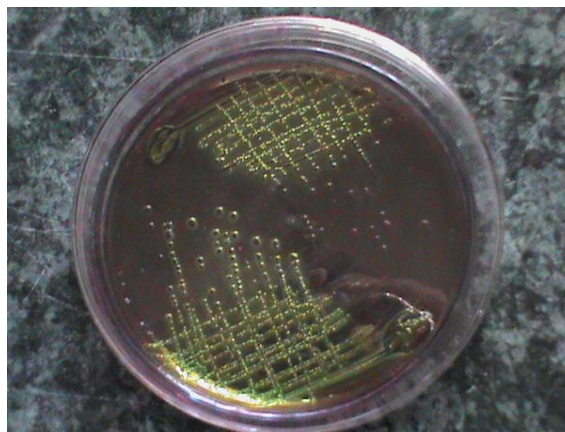


Fig. 20: *E. coli* isolates showing metallic sheen around the colonies on Eosin Methylene Blue agar (EMB) plate

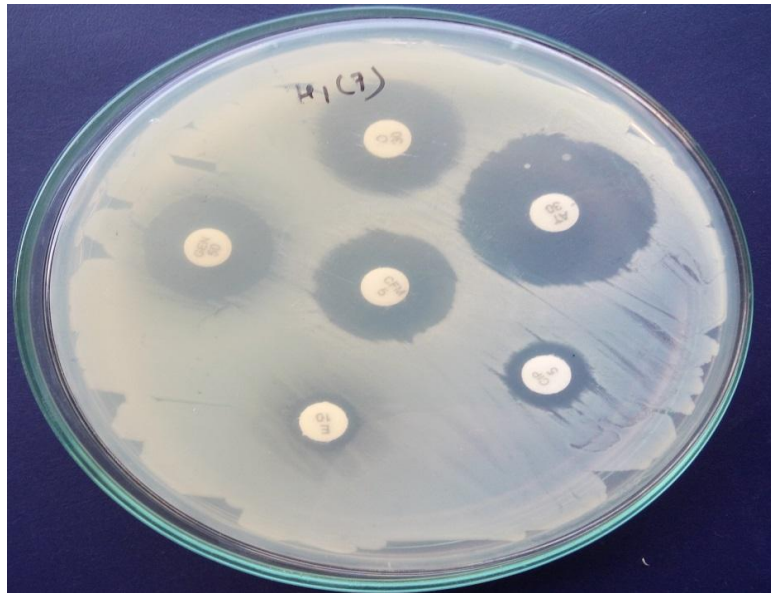


Fig. 21 A: Isolate showing zone of inhibition against different antibiotics

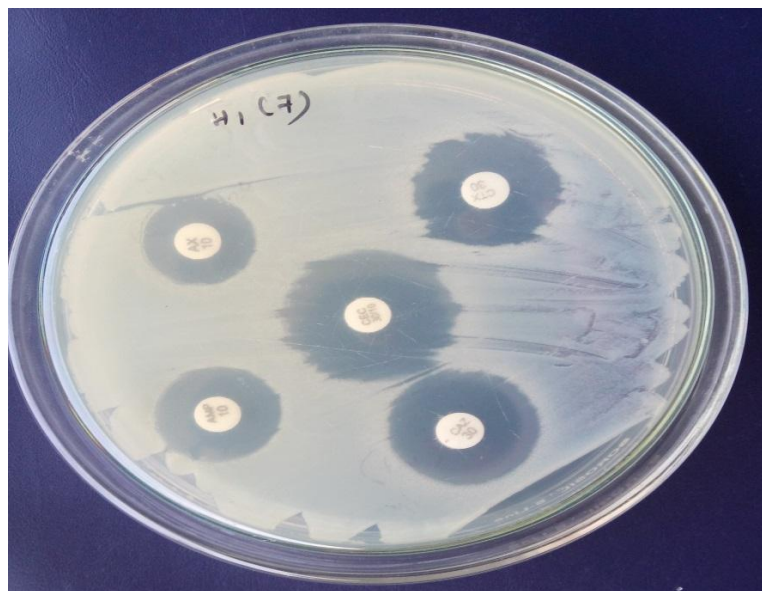


Fig. 21 B: Isolate showing zone of inhibition against different antibiotics

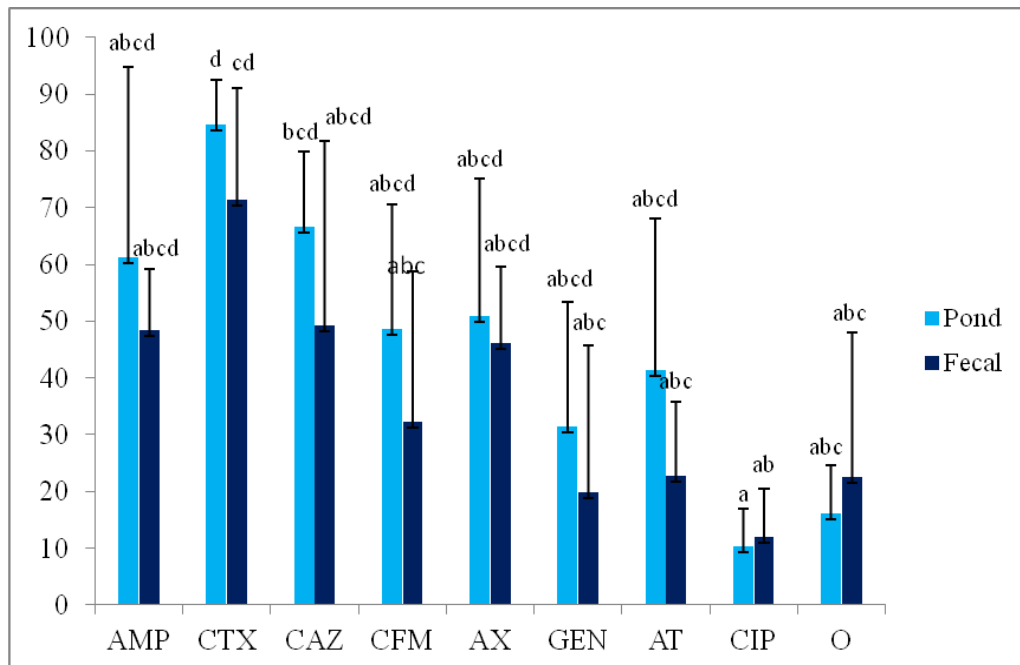


Fig. 22: Total resistance frequencies against the nine antibiotics in *E. coli* isolated from the various water and fecal samples

One-way ANOVA Turkey test, $P < 0.05$. Error bars are standard deviations of the resistance frequencies of 223 isolates. Bars sharing the same letter (a, b, c) are not significantly different.

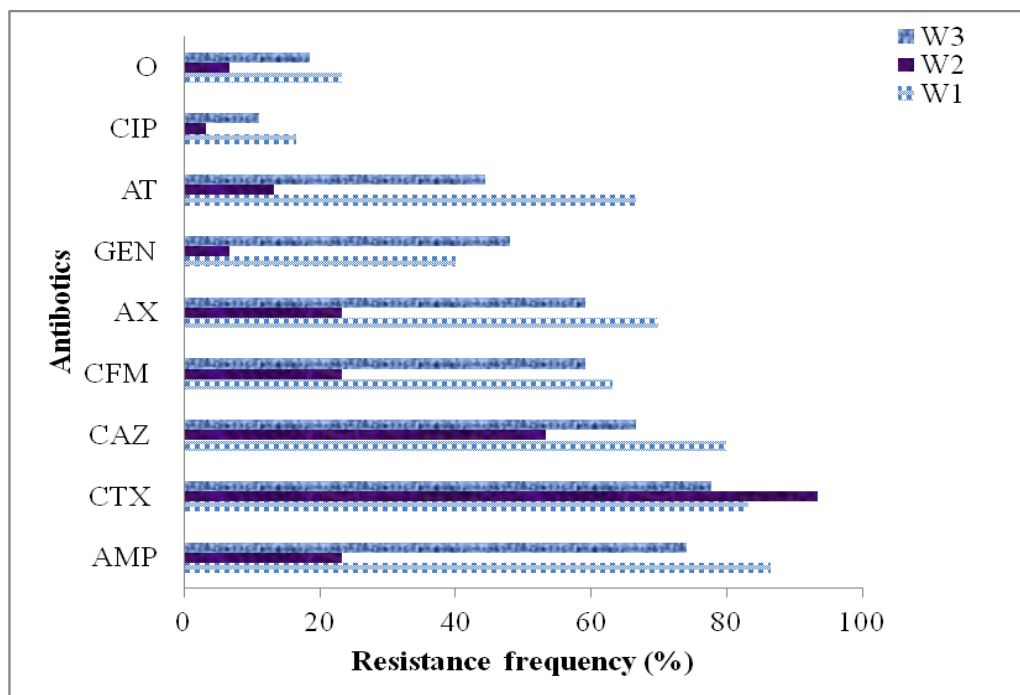


Fig. 23: Antibiotic resistance profile of *E. coli* isolated from the water samples

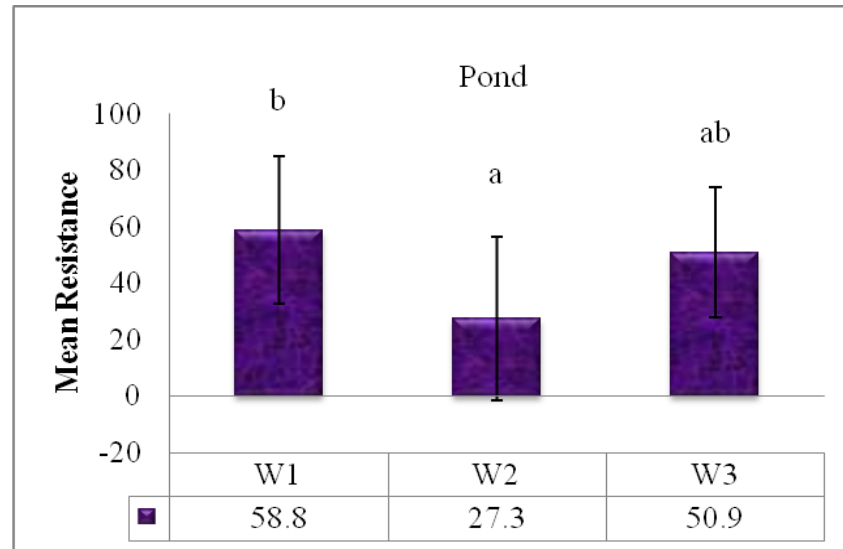


Fig. 24: Mean percentage resistance in *E. coli* isolates of different pond
 Tukey's test performed using a $p < 0.05$ criterion for statistical significance; bars sharing the same letter (a, b, c) are not significantly different.

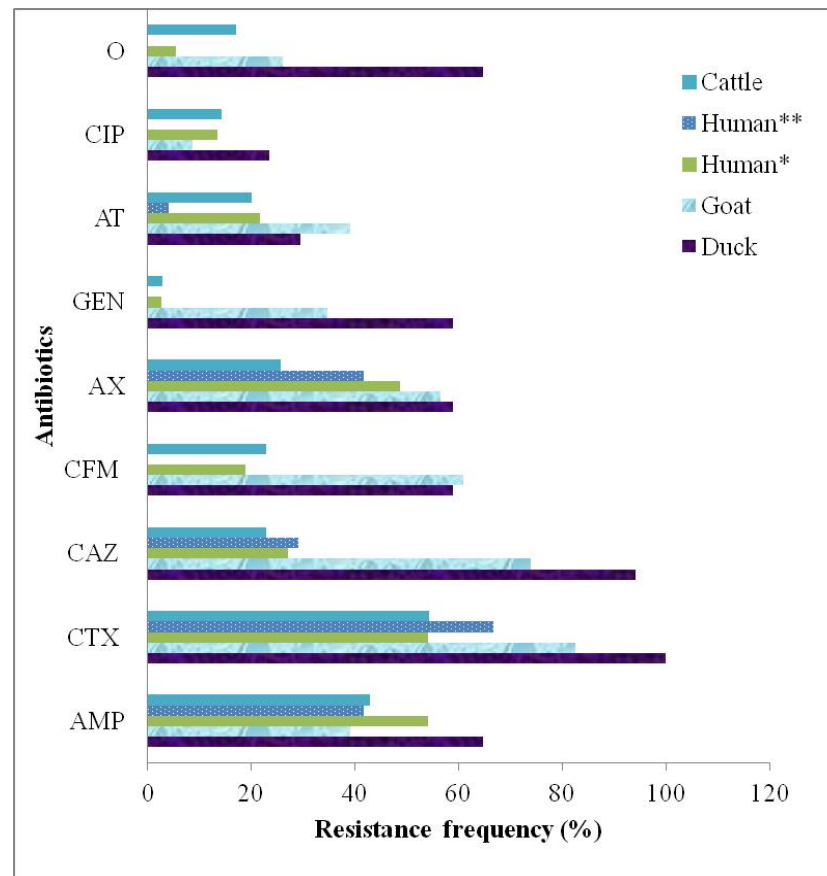


Fig. 25: Antibiotic resistance profile of *E. coli* isolated from the fecal samples

*Human utilizing village community pond

** Human not utilizing village community pond

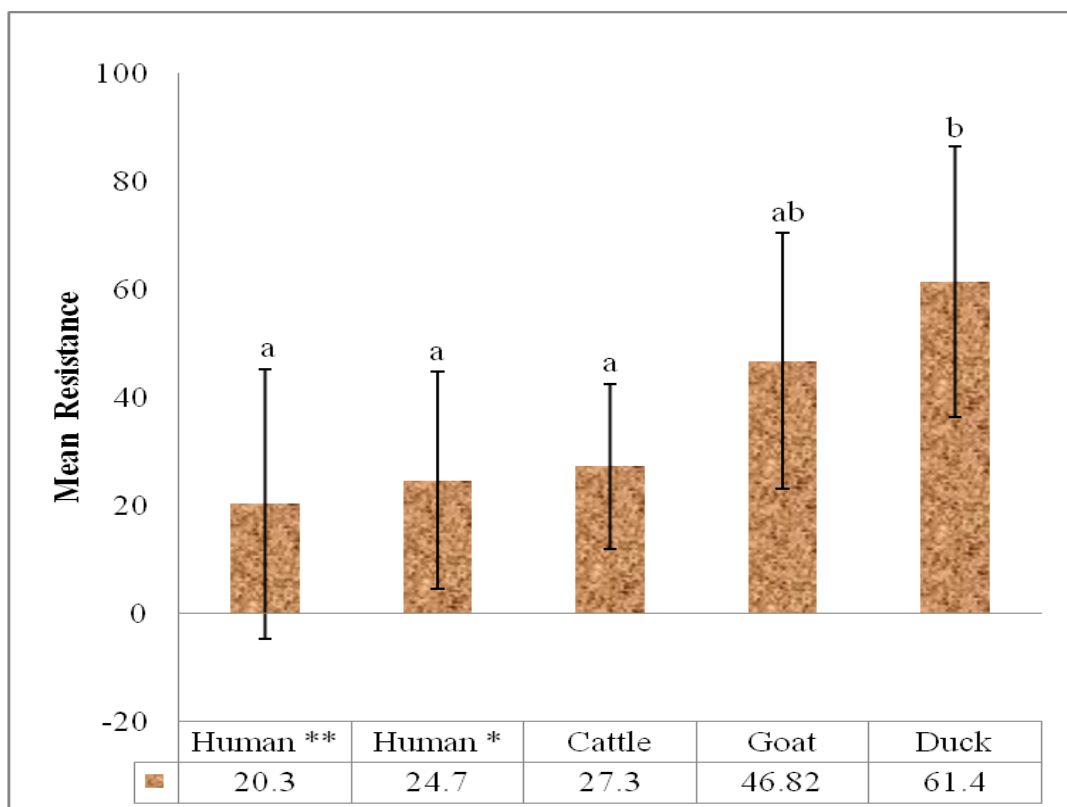


Fig. 26: Mean percentage resistance in *E. coli* isolated from various fecal samples
 Tukey's test performed using a $p < 0.05$ criterion for statistical significance; bars sharing the same letter (a, b, c) are not significantly different.

The *E. coli* isolated from healthy animals during the study showed significant resistance, many studies also reported the presence of antibiotic resistant in *E. coli* of healthy farm animals and assumed that environmental sources may play role in the acquisition of resistant bacteria (Mahanti *et al.*, 2013; Islam *et al.*, 2016).

4.2.2 Antibiotic resistance patterns

A total of 502 different resistance phenotype patterns of 9 antibiotics were tested based on the combination theoretical analysis software. Among these 223 *E. coli* isolates, 14.3% were not found resistant to any antibiotics. Only 4.0% and 1.7% were resistant to eight or nine type of antibiotics, respectively, and 52.4% showed resistance to three to seven type of antibiotics (Fig. 4). In other words, only 49 phenotype patterns in the water samples and 54 phenotype patterns in the fecal samples were observed among all *E. coli* isolates from the three ponds and five types of fecal samples.

Among the 49 phenotype patterns for the water samples, 41 patterns had a low frequency with only one or two *E. coli* isolates. The most common multiple antibiotic resistance pattern was CTX.CAZ and CTX (7 isolates each), followed by AMP.CTX.CAZ.CFM.AX.AT (6 isolates), AMP.AX and AMP.CTX.CAZ.AX (5 isolates each), AMP.CTX.CAZ.CFM.AX.GEN.AT and CTX.CAZ.AT (3 isolates each) (Tables 24 and Fig. 27).

Among the 54 resistance phenotype patterns for the fecal samples, 45 patterns had a low frequency with only one or two *E. coli* strain. The most common antibiotic resistance pattern was CTX (14.9%, 13 isolates) followed by AMP.CTX.AX and CTX.CAZ (6.6%, 9 isolates), AMP.CTX.CAZ.CFM.AX.AT (4 isolates),

AMP.CAZ.AX, AMP.CTX.CFM.AX, AMP.CTX.CAZ.CFM.AX.GEN.AT.O, and AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP.O (3 isolates) (Table 25 and Fig.28). The *E. coli* isolates exhibited 16 phenotype patterns for W1, 14 patterns for W2, 22 patterns for W3, 14 patterns for M1, 21 patterns for M2, 7 patterns for M3, 15 patterns for M4, and 23 patterns for M5.

Pattern determinations have benefit in identification of multidrug resistant isolates and their characteristics to the particular antibiotic. It also gives idea about the type of pattern commonly prevalent in environment. Zhang *et al.*(2013) reported 89 phenotype patterns in the water samples and 46 phenotype patterns in the animal manure samples *E. coli* isolates from the two aquaculture systems.

Table 24: Phenotype patterns of the *E. coli* isolates from water

S.N.	Phenotype pattern	Isolates	phenotype pattern of water samples		
			W1	W2	W3
1	CTX	7	0	7	0
2	CAZ	2	0	1	1
3	GEN	1	0	0	1
4	CTX.CAZ	7	0	7	0
5	CTX.GEN	1	0	1	0
6	AMP.AX	5	5	0	0
7	CTX.CAZ.AT	3	3	0	0
8	CTX.CAZ.AX	1	0	1	0
9	CTX.CFM.AT	1	0	1	0
10	AMP.CTX.AX	2	0	2	0
11	CTX.CAZ.CFM	1	0	1	0
12	CFM.AX.GEN	1	0	0	1
13	AMP.CAZ.CFM	1	0	0	1
14	AMP.CTX.CAZ	1	0	0	1
15	CTX.CAZ.GEN.AT(1)	1	1	0	0
16	AMP.CTX.CAZ.AX	5	1	3	1
17	AMP.CTX.CAZ.O	1	1	0	0
18	AMP.CTX.CAZ.AT	1	1	0	0
19	CTX.CAZ.CFM.AT	1	0	1	0
20	CTX.CAZ.CFM.O	1	0	1	0
21	CTX.CFM.GEN.AT	1	0	1	0

22	AMP.CTX.CFM.AX	1	0	1	0
23	AMP.CTX.GEN.O	1	0	0	1
24	AMP.CTX.CAZ.GEN	1	0	0	1
25	AMP.CTX.CAZ.CFM.AT	1	1	0	0
26	AMP.CTX.CAZ.AX.O	2	1	0	1
27	AMP.CTX.AX.GEN.O	1	1	0	0
28	AMP.CTX.CAZ.GEN.AT	2	2	0	0
29	AMP.CTX.CFM.AX.AT	1	0	0	1
30	AMP.CTX.AX.AT.CIP	1	0	0	1
31	AMP.CAZ.CFM.AX.GEN	1	0	0	1
32	AMP.CTX.CAZ.CFM.GEN	1	0	0	1
33	AMP.CTX.CAZ.AX.GEN	1	0	0	1
34	AMP.CTX.CAZ.CFM.GEN.AT	1	1	0	0
35	AMP.CTX.CAZ.CFM.AX.AT	6	3	0	3
36	AMP.CTX.CFM.AX.CIP.O	1	1	0	0
37	AMP.CTX.CAZ.CFM.AT.O	1	0	0	1
38	AMP.CTX.CFM.GEN.AT.O	1	0	0	1
39	AMP.CTX.CAZ.CFM.AX.GEN	1	0	0	1
40	CTX.CAZ.CFM.AX.GEN.AT	1	0	0	1
41	CTX.CFM.AX.GEN.AT.O	2	0	0	2
42	AMP.CTX.CAZ.CFM.AX.AT.O	1	1	0	0
43	AMP.CTX.CAZ.CFM.AX.GEN.AT	3	2	0	1
44	AMP.CTX.CAZ.CFM.AX.AT.CIP	1	0	0	1
45	AMP.CTX.CAZ.AX.GEN.AT.CIP	1	0	0	1

46	AMP.CTX.CAZ.CFM.AX.GEN.AT.O	1	1	0	0
47	AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP	2	2	0	0
48	AMP.CTX.CAZ.CFM.AX.AT.CIP.O	1	0	1	0
49	AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP.O	1	1	0	0

AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CFM, cefixime; AX, ampicillin/cloxacillin; GEN, gentamicin; AT, aztreonam; CIP, ciprofloxacin; O, oxytetracycline.

Isolates, total isolates having each resistance pattern from pond ecosystem

W1, water sample of the community pond; W2, water sample of the traditional fish pond; W3, water sample of the integrated duck-fish-goat pond.

Table 25 Phenotype patterns of the *E. coli* isolates from fecal sample

	Phenotype pattern	Isolates	Phenotype pattern of samples				
			M1	M2	M3	M4	M5
1	AX	1	0	0	0	0	1
2	CAZ	2	0	0	2	0	0
3	GEN	1	0	1	0	0	0
4	CIP	1	0	1	0	0	0
5	AMP	3	1	1	1	0	0
6	CTX	13	4	6	2	0	1
7	CTX.O	1	0	0	0	1	0
8	CTX.AX	1	0	0	1	0	0
9	AMP.AX	1	0	1	0	0	0
10	CTX.CAZ	9	1	2	4	2	0
11	AMP.CTX	2	1	1	0	0	0
12	CTX.CIP	1	0	1	0	0	0

13	CTX.AX. GEN	1	0	0	0	0	1
14	CTX.CAZ. GEN	2	0	0	0	0	2
15	CTX.CAZ.O	1	0	0	0	1	0
16	AMP.AX.O	1	0	1	0	0	0
17	AMP.AX.AT	1	0	1	0	0	0
18	AMP.AX.CIP	1	0	1	0	0	0
19	AMP.CAZ.AX	3	0	3	0	0	0
20	AMP.CTX.AX	9	0	1	8	0	0
21	CTX.CAZ.CFM.AX	1	0	0	0	0	1
22	AMP.CTX.AX.AT	1	0	1	0	0	0
23	CTX.CAZ.CFM.GEN	2	0	0	0	1	1
24	CTX.CAZ.GEN.O	2	0	0		2	0
25	CTX.CAZ.CFM.O	1	0	0	0	1	0
26	AMP.CFM.AT.CIP	1	0	1	0	0	0
27	AMP.CTX.CFM.AT	1	1	0	0	0	0
28	AMP.CTX.CFM.AX	3	2	1	0	0	0
29	AMP.CTX.CIP.O	2	2	0	0	0	0
30	AMP.CTXCAZ.CFM	1	1	0	0	0	0
31	AMP.CTX.CAZ.AX	2	1	1	0	0	0
32	CTX.CAZ.CFM.GEN.AT	2	0	0	0	0	2
33	CTX.CAZ.CFM.GEN.O	1	0	0	0	0	1
34	AMP.CTX.CAZ.AX.AT	2	1	0	1	0	0
35	AMP.CTX.CAZ.AX.CIP	1	0	1	0	0	0
36	CTX.CAZ.CFM.AX.AT	1	0	0	0	0	1
37	AMP.CTX.CAZ.CFM.AT	1	0	0	0	1	0
38	AMP.CTX.CAZ.CFM.AX	1	0	0	0	1	0

39	AMP.CTX.CFM.AX.AT	2	0	2	0	0	0
40	AMP.CAZ.CFM.AX.AT	1	1	0	0	0	0
41	AMP.CTX.AX.CIP.O	1	1	0	0	0	0
42	AMP.CTX.CAZ.CFM.AX. GEN. CIP	2	0	0	0	0	2
43	AMP.CTX.CAZ.CFM.AX.AT	4	2	2	0	0	0
44	AMP.CTX.CAZ.AX.GEN.O	2	0	0	0	2	0
45	AMP.CTX.CAZ.CFM.AX. GEN	1	0	0	0	0	1
46	AMP.CTX.CAZ.CFM.AX. GEN.AT	2	0	0	0	0	2
47	AMP.CTX.CAZ.CFM.AX.AT.O	1	0	1	0	0	0
48	AMP.CTX.CAZ.AX.GEN.CIP.O	1	0	0	0	1	0
49	AMP.CTX.CAZ.CFM.AX.GEN.O	1	0	0	0	1	0
50	CTX.CAZ.CFM.AX.GEN.CIP.O	1	0	0	0	1	0
51	AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP	1	0	0	0	1	0
52	AMP.CTX.CAZ.CFM.AX.GEN.AT.O	3	0	0	0	1	2
53	AMP.CTX.CAZ.CFM.AX. GEN. CIP.O	1	0	0	0	0	1
54	AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP.O	3	1	0	0	1	1

AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CFM, cefixime; AX, ampicillin/cloxacillin; GEN, gentamicin; AT, aztreonam; CIP, ciprofloxacin; O, oxytetracycline.

Isolates, total isolates having each resistance pattern from fecal sample

M1, fecal sample of cattle and buffalo regularly enter village community pond; M2 fecal sample of human using village community pond; M3, fecal sample of human not using village community pond; M4, fecal sample of duck of the integrated duck-fish-goat pond and M5, fecal sample of goat of duck-fish-goat integrated pond.

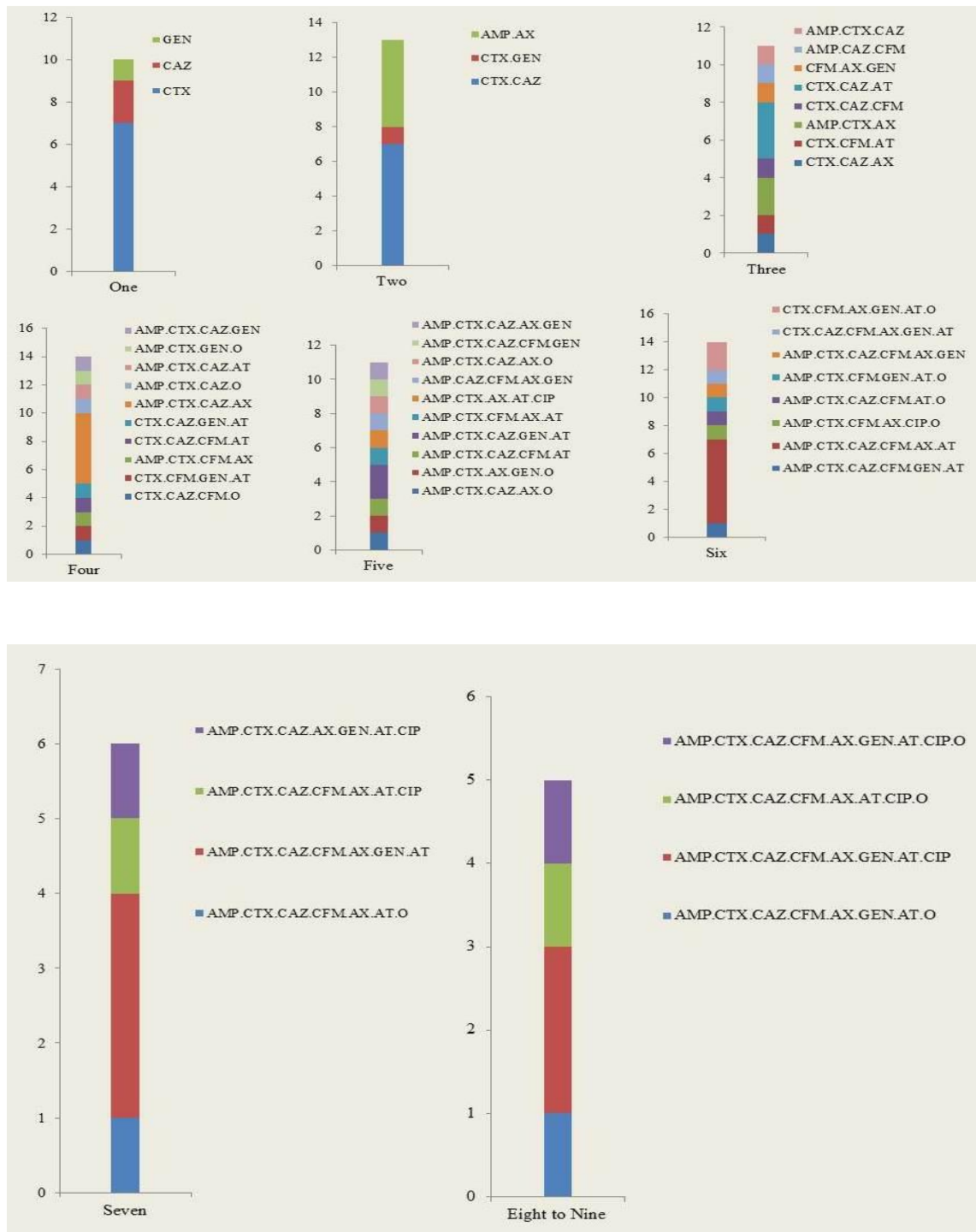


Fig. 27: Antibiotic resistance phenotype patterns for the *E. coli* isolates from water sample
 AMP, Ampicillin; CTX, Cefotaxime; CAZ, ceftazidime; CFM, cefixime; AX, ampicillin/cloxacillin; GEN, gentamicin; AT, aztreonam; CIP, ciprofloxacin; O, oxytetracycline.

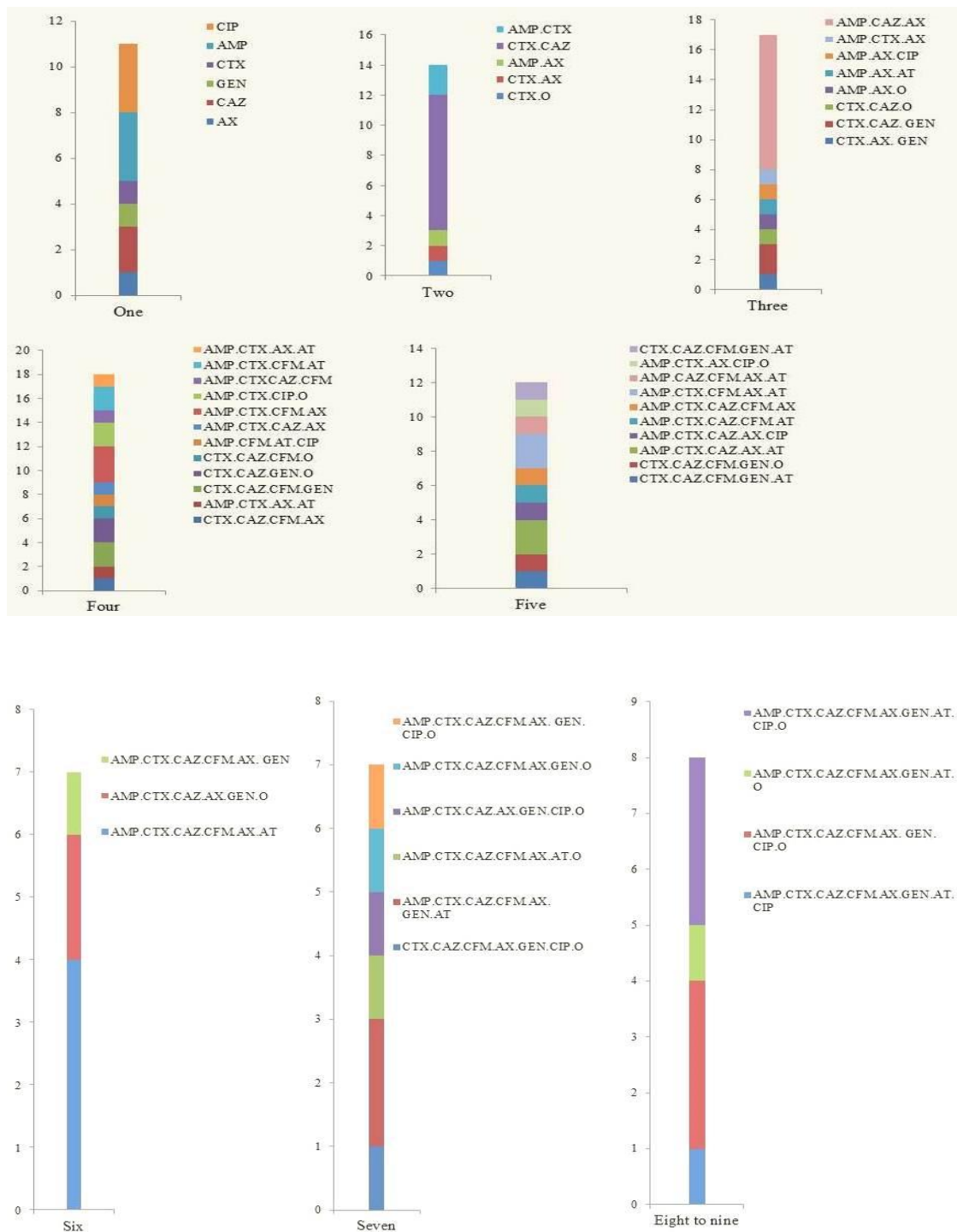


Fig. 28: Antibiotic resistance phenotype patterns for the *E. coli* isolates from fecal sample

AMP, Ampicillin; CTX, Cefotaxime; CAZ, ceftazidime; CFM, cefixime; AX, ampicillin/cloxacillin; GEN, gentamicin; AT, aztreonam; CIP, ciprofloxacin; O, oxytetracycline.

4.2.3 Antibiotic resistance genes and class 1 integrons

Out of 223 isolated *E. coli*, 191 isolates were resistant to one or more antibiotics. From each pond and fecal sample group six or seven multidrug resistant isolates were selected for detection of antibiotic resistance genes. Seven antibiotic resistance genes (beta-lactamase resistant genes: *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV} ; plasmid mediated tetracycline efflux genes: *tetA*, *tetB*; fluroquinolone resistant genes: *gyrA* and *parC*) were detected in these resistant *E. coli* isolates. The target genes *bla*_{TEM} and *bla*_{SHV} were not detected in these isolates. The distribution of resistant genes in these 44 resistant isolates is given in Table 26. The highest detection frequency was found for *gyrA* gene followed by *parC*, *tetB*, *Int1*, *bla*_{CTX-M} and *tetA*, in water origin *E. coli* isolates, for the fecal origin *E. coli* isolates the order of resistant gene detection frequencies was *gyrA* > *parC* > *Int1* > *tetA* > *tetB* and *bla*_{CTX-M} (Table 26 and Fig. 29-33).

The antibiotic resistance gene frequencies of *E. coli* isolates from the different ponds ecosystem are presented in Table 26. A total of 20 multidrug resistant *E. coli* isolates were selected from the pond water samples (W1, W2 and W3), with the average resistance gene and integron frequencies of 30.9% to *bla*_{CTX-M}, 95.2% to *gyrA*, 72.2% to *parC*, 16.6% to *tetA*, 63.4% to *tetB* and 46.8% to *Int1*. Higher resistance gene frequencies to *gyrA*, *parC*, *tetB*, and *bla*_{CTX-M} were detected in the *E. coli* isolated from the pond water samples. W1 pond showed highest resistance to *bla*_{CTX-M} and the lowest resistance frequencies in W2. The multidrug resistance *E. coli* isolates from pond W1, W2 and W3 showed absence of *bla*_{TEM} and *bla*_{SHV} resistant genes, whereas *tetA* gene was not detected in pond W1 and pond

W3 in the present study.

A total of 24 multidrug resistant *E. coli* isolates, six of each group, were selected from the fecal samples of M1, M2, M4 and M5 and screened for resistance genes. The average resistance gene frequencies of 12.49 % to *bla*_{CTX-M}, 95.8% to *gyrA*, 74.9% to *parC*, 66.6% to *tetA*, 62.4% to *tetB* and 83.3% to *Int1* for class1 integron gene were detected. Higher resistance gene frequencies to *gyrA*, *parC*, *tetA*, *tetB* and integron gene *Int1* were detected in the *E. coli* isolated from the fecal samples (Table 26). Only M1 and M2 fecal sample isolates showed the presence of *bla*_{CTX-M} antibiotic resistant genes. M1 showed highest frequencies of presence of resistance genes in comparison to M2, M4 and M5 representative isolates. The selected multidrug resistance *E. coli* isolates from different fecal samples origin represents the presence of multidrug resistant genes except only *bla*_{TEM}, *bla*_{SHV} were not detected in the present study.

Table 26: Detection frequencies of antibiotic resistance genes in *E. coli* isolated from the water and fecal samples from different ecosystems

Resistance gene frequencies of <i>E. coli</i> isolates from different Sampling sites ^b (%)									
	Water sample				Fecal sample				
ARGs ^a	W1	W2	W3	Average frequencies	M1	M2	M4	M5	Average frequencies
<i>bla</i> _{CTX-M}	42.85	16.66	33.33	30.94	33.33	16.66	0	0	12.49
<i>bla</i> _{TEM}	0	0	0	0	0	0	0	0	0
<i>bla</i> _{SHV}	0	0	0	0	0	0	0	0	0
<i>gyrA</i>	85.71	100	100	95.23	100	100	83.33	100	95.83
<i>parC</i>	100	33.33	83.33	72.22	100	83.33	100	16.66	74.99
<i>tetA</i>	0	50	0	16.66	83.33	16.66	66.6	100	66.64
<i>tetB</i>	83.3	57.14	50	63.48	83.33	50	50	66.66	62.49
<i>Int1</i>	57.14	16.66	66.66	46.82	100	100	66.66	66.66	83.33
MAG Index^c	0.46	0.47	0.4	-	0.5	0.38	0.42	0.4	-

^aARGs, antibiotic resistance gene.

^bSampling sites: W1, water sample of the community pond; W2, water sample of the traditional fish pond; W3, water sample of the integrated duck-fish-goat pond; M1, fecal sample of cattle and buffalo regularly enter village community pond; M2 fecal sample of human using village community pond; M4, fecal sample of duck of the integrated duck-fish-goat pond; and M5, fecal sample of goat of duck-fish-goat integrated pond.

^c MAG, multiple antibiotic resistance gene index.

It is assumed that the unregulated use of antimicrobials in animal husbandry and integrated fish farm practices with subsequent use of animal and poultry waste with entry of antibiotic residues into fish ponds has created a pool of resistance genes in the aquaculture facilities. Subinhibitory concentrations of antimicrobials have, been reported to select antibiotic resistant genes (ARGs) and ARGs may persist in aquatic environmental bacteria even in the absence of sufficient selection pressure.

Beta-Lactamase antimicrobials are widely used in Asian countries, for the treatment of both human and animals. Anthropogenic as well as use of animal originated fecal matter for pond manuring may lead to contamination of the aquatic environment is considered the major cause of betalactam resistance genes in *Enterobacteriaceae* worldwide. On the Indian subcontinent, the *bla*_{NDM-1} gene has been reported on plasmids of varying size in various members of *Enterobacteriaceae* in clinical isolates as well as in bacteria from drinking and sewage water in New Delhi (Walsh *et al.*, 2011).

Limited reports on the prevalence of antibiotic resistant genes among bacteria isolated from the environment are available. Bajaj *et al.* (2015) reported that *bla*_{TEM} was the most widespread (100%) beta-lactamase gene, followed by *bla*_{CTX-M} (16%), among 61 *E. coli* isolates of river in the northern part of India. Further mentioned selection of *E. coli* isolates was not according to their antimicrobial resistance. The difference in beta-lactamase gene prevalence might be due to the difference in the methodology of selection of *E. coli* isolates. *bla*_{CTXM-15} was also the most prevalent

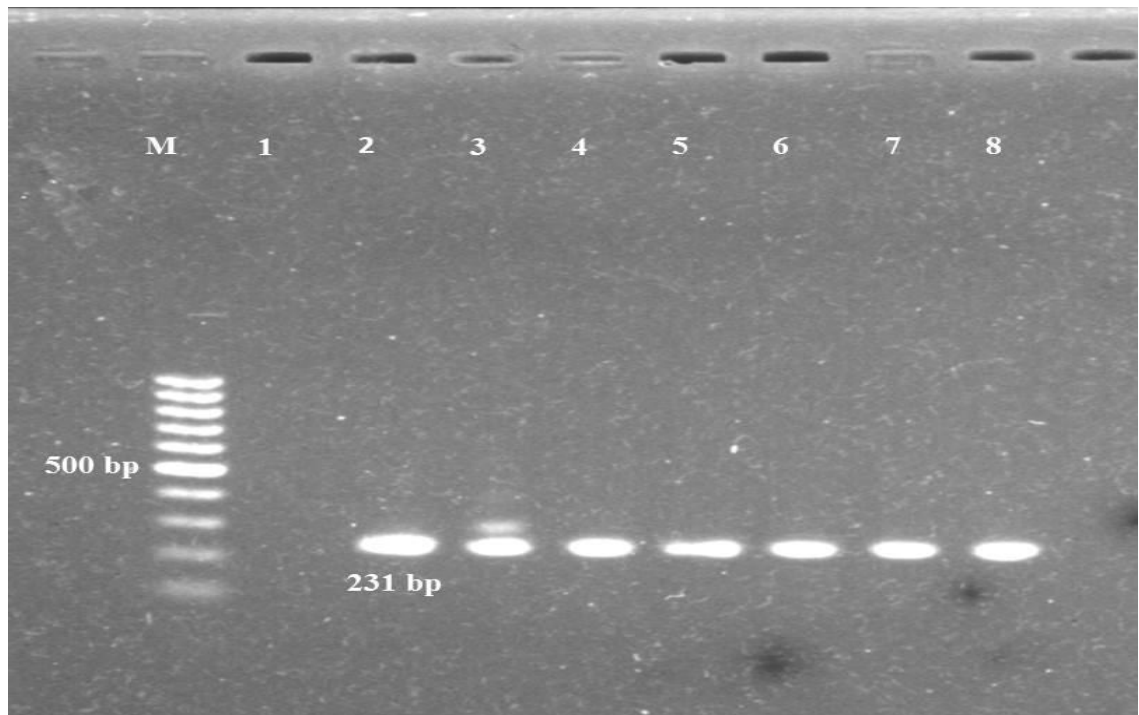


Fig. 29: Agarose gel electrophoresis showing amplified PCR products of 16 S rRNA genes for *E. coli* isolates, Lane M 100 bp ladder, Lane 2 positive control, Lane 3- 8 positive isolates

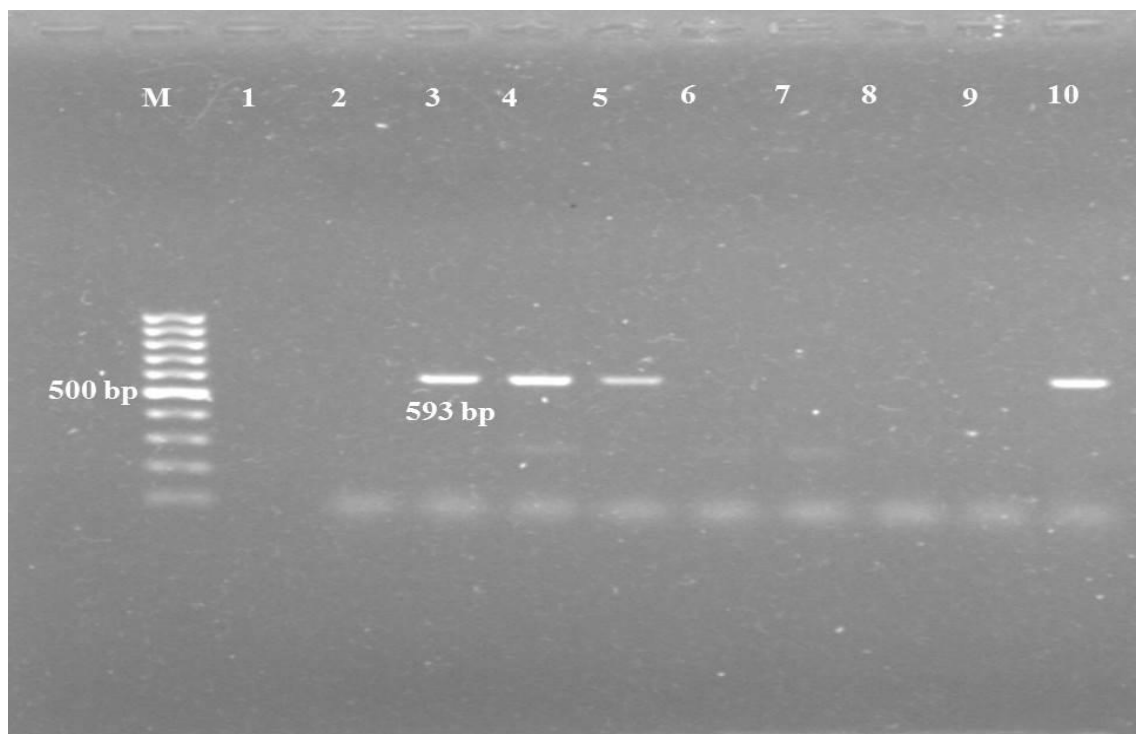


Fig. 30: PCR product of *bla*_{CTX-M} gene amplification, Lane M 100 bp DNA ladder, Lane 3 positive control, Lane 4, 5, 10 positive isolates

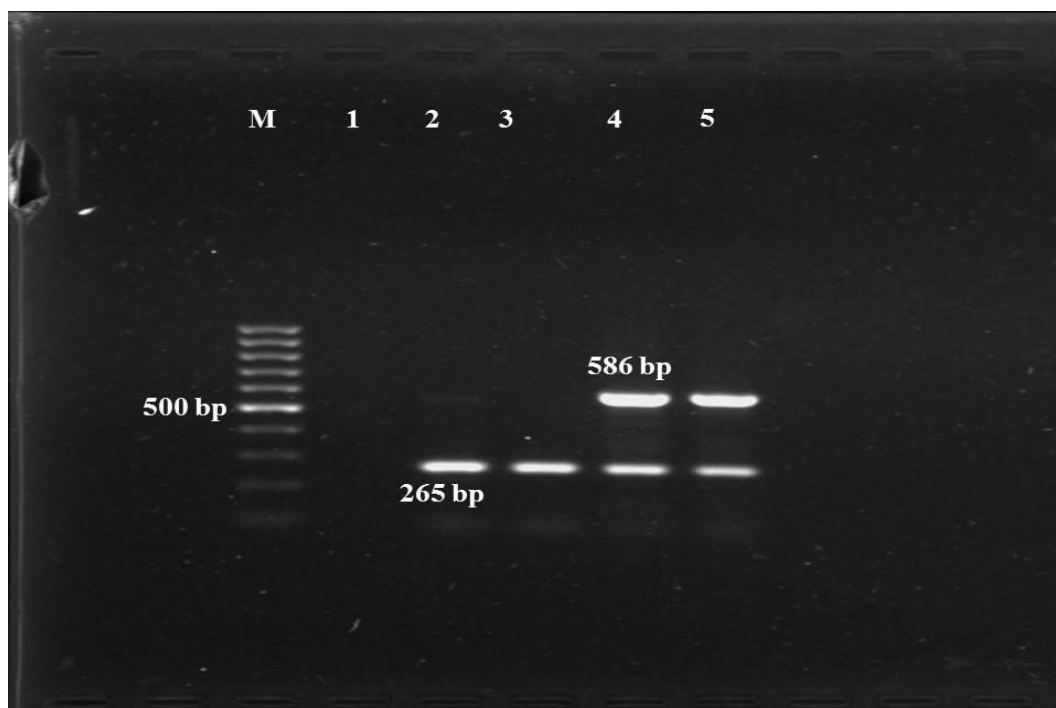


Fig. 31: PCR product of *parC* gene (265 bp) and *gyrA* gene (586 bp) amplification, Lane M 100 bp DNA ladder, Lane 2 and 3 positive for *parC* gene, Lane 4 and 5 positive for both *parC* gene and *gyrA* gene

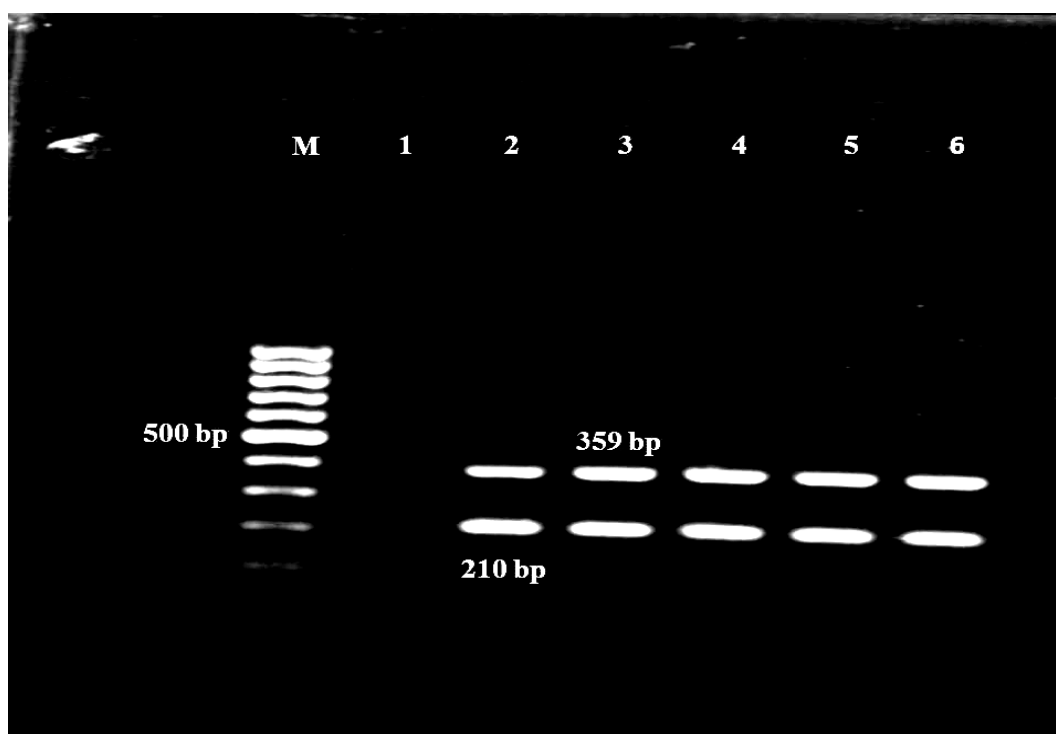


Fig. 32: PCR product of *tetA* gene (210 bp) and *tetB* gene (359 bp) amplification, Lane M 100 bp DNA ladder, Lane 2 to 6 amplification of *tetA* and *tetB* genes

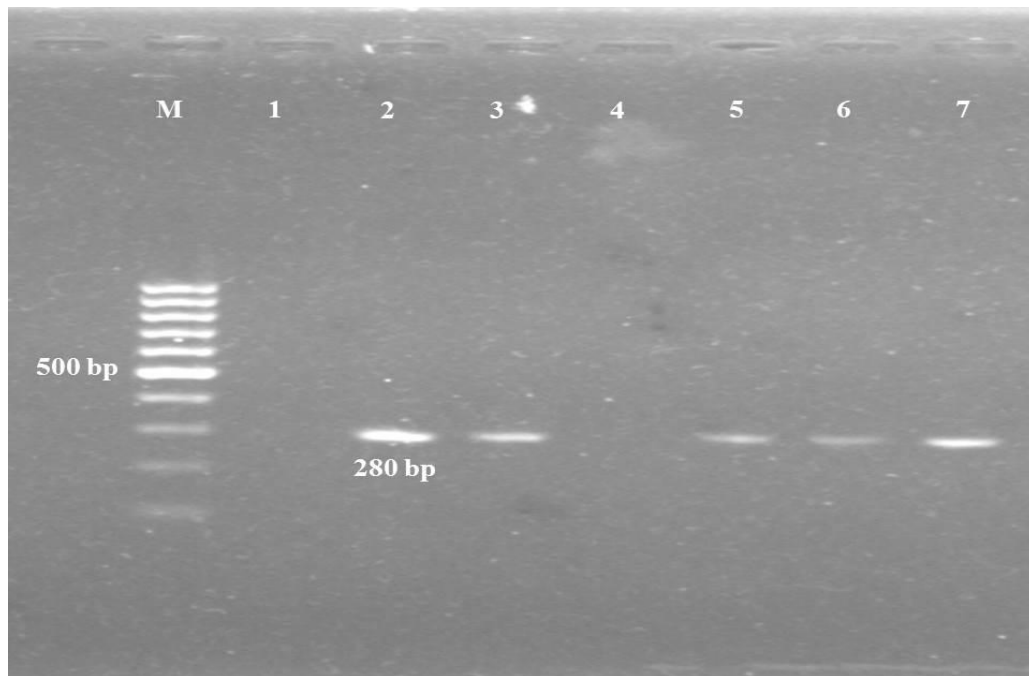


Fig. 33: PCR product of *Int1* gene (280 bp) amplification, Lane M 100 bp DNA ladder, Lane 2,3,5,6 and 7 show amplification of *Int1* genes

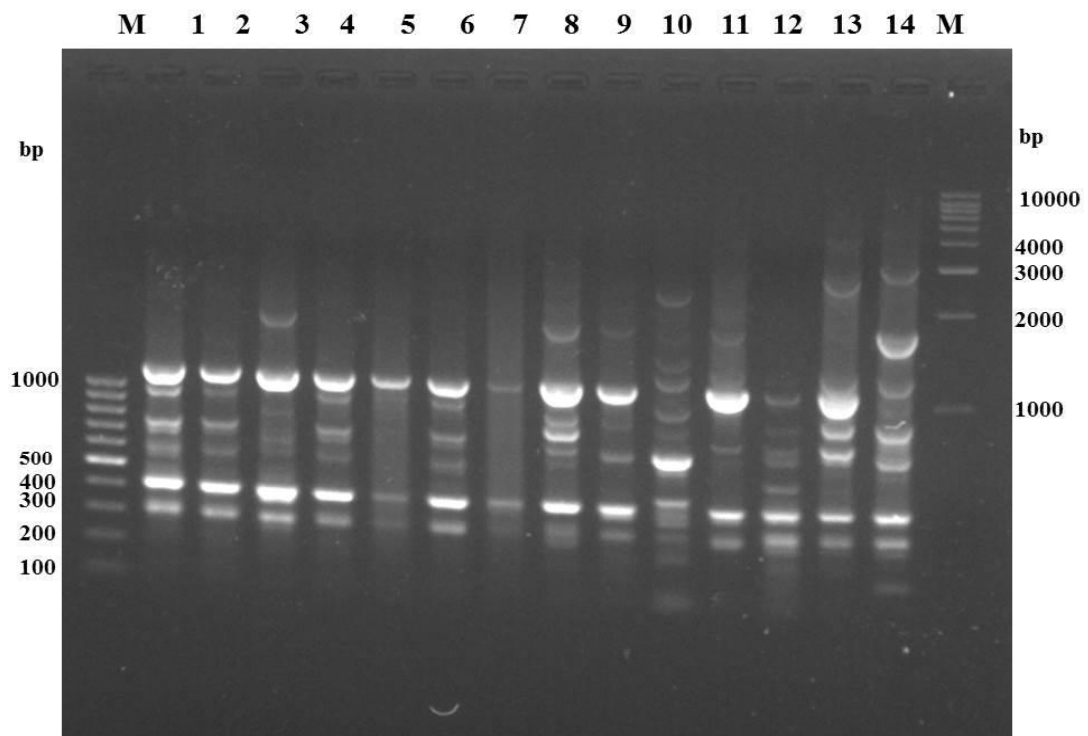


Fig. 34: Representative ERIC-PCR profile lanes 1-14, of different *E. coli* isolates. Lane M -100 bp and 10 kb DNA ladder

beta-lactamase gene conferring extended-spectrum-cephalosporin resistance among bacteria in the environment in Bangladesh (Toleman *et al.*, 2015). *bla*_{CTX-M} genes seem to be commonly detected from the environment in East Asia, Europe, and Australia (Amos *et al.*, 2014; Korzeniewska *et al.*, 2013).

Fluoroquinolone resistance is mainly mediated by the accumulation of point mutations in the chromosomal genes encoding DNA gyrase and/or DNA topoisomerase IV (Hopkins *et al.*, 2005). Most of the fluoroquinolone-resistant isolates should have these point mutations because wild-type *E. coli* isolates are highly susceptible to this antibiotic (Hopkins *et al.*, 2005). Plasmid-mediated quinolone resistance genes, including *aac(6=)-Ib-cr*, *qepA*, *qnrA1*, *qnrB*, *qnrB10*, and *qnrS1*, that contribute to the low level of resistance to fluoroquinolones (Strahilevitz *et al.*, 2009). To some extent, these genes contribute to the fluoroquinolone resistance of these isolates. As many of the resistance genes are inserted as GCs in different types of integrons in resistance plasmids (R plasmids) (Grape *et al.*, 2005). Therefore presence of integrons is responsible for multidrug resistance because it works as a genetic cassette which harbors many other resistant genes.

With respect to the higher prevalence of *tetB* and *Int1* in the present study similar findings were reported by Su *et al.* (2011) but they reported higher prevalence of *tetA* gene in integrated pond than the monoculture pond.

Prevalence of beta-lactamase genes, *tetA* and *tetB* genes from water and fecal samples were reported in the present study which was comparable to the findings of

Zhang *et al.* (2013) and reported higher prevalence of *tetA*.

In the present study high prevalence of integrons and *bla*_{CTX-M} genes were found in healthy rural population which are utilizing community pond water. Results of study are in line with the reports of Dureja *et al.* (2014) and Purohit *et al.* (2017). On contrary Antony *et al.* (2018) reported higher prevalence of ESBL genes in MDR *E. coli* isolated from south Indian rural population.

Healthy cattle and buffalo *E. coli* isolates in present study showed prevalence of studied resistant genes. On contrary, no resistant gene prevalence was detected by Mahanti *et al.* (2013) from resistant fecal *E. coli* isolates from buffalo.

The prevalence of *tetB* (66.6%) and *bla*_{CTX-M} (16.66%) in fecal *E. coli* isolates of goats were found in the present study. Results of present study were found almost in line with the reports of Ndegwa *et al.* (2019).

The higher prevalence of different resistance genes in community pond and integrated pond than the conventional fish pond, obtained in the present study were comparable to those reported by Xu *et al.* (2015) and Subbiah *et al.* (2020).

4.2.4 Genetic diversity analysis

4.2.4.1 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

The ERIC-PCR method has been extensively employed to identify the genotypic diversity in *E. coli* (El-Badawy *et al.*, 2017; Pusparini *et al.*, 2018; Xie *et al.*, 2018; Khare *et al.*, 2020). This method is useful to differentiate clonal strains and hence, could be applied in epidemiological investigations (Dorneles *et al.*, 2014). Several genotyping methods have been explored for the characterization of *E. coli*.

Some techniques are relatively difficult, laborious and expensive to perform but have high resolution, while other easy, rapid and cheap techniques may have limited reproducibility and discriminatory powers. The ERIC-PCR is generally considered to be both reliable as well as sensitive and is much simpler procedure than Pulsed-Field Gel Electrophoresis (Kosek *et al*, 2012; Dorneles *et al.*, 2014).

The electrophoretic profiles of the ERIC-PCR products were determined for the *E. coli* isolated from different pond and fecal sample of different animal species. The data matrix based on the DNA fragments and the dendrogram using the PAST program (Hammer *et al.*, 2001) software were constructed, grouping all the *E. coli* isolates obtained in this study into different clusters or branches based on the ERIC-PCR pattern similarity. Furthermore, the ERIC DNA fragments from the isolates obtained from the same environment were amplified in one PCR reaction and loaded on the gel to obtain different gel electrophoresis pattern.

The ERIC-PCR fingerprinting patterns of different *E. coli* isolates (pond and fecal sample) were investigated using a single amplification protocol. ERIC-PCR profiles were discriminated by the number and position of the amplified fragments. Visual and software comparison of the banding patterns revealed multiple DNA fragments ranging in size between 100 and 4000 bp (Fig. 34). ERIC-PCR primer sets produced different type of profiles.

4.2.4.2 ERIC-PCR fingerprinting of community pond and related human and animal fecal *E. coli* isolates

The community pond, human fecal sample and cattle fecal sample *E. coli* dendrogram are shown in Fig. 35. The dendrogram analysis of the isolates (n = 20)

showed six clusters and one separate human isolate. Three human isolates were located in the cluster I, cluster II comprises two human, two pond and one cattle isolates, cluster III have only four cattle isolates followed by cluster IV which have only two isolates, one of cattle and other one is pond isolate, cluster V contain three pond isolates only and cluster VI contain human and pond isolates. The Jaccard coefficient showed around 67% of similarity between human and pond isolates in the cluster II, the similarity between cattle isolates, human and pond isolates was 58%. There was also 63% similarity between cattle and pond isolates in cluster IV. The cluster VI, composed of human and pond isolates showed 40% similarity which is lower as compared to other clusters.

4.2.4.3 ERIC-PCR fingerprinting of *E. coli* isolates of traditional fish culture pond

For traditional fish culture pond (Fig. 36), the 6 multidrug resistant *E. coli* isolates were divided into three clusters and each cluster has two isolates. Cluster I showed more than 55% similarity between two isolates whereas cluster II isolates showed higher similarity of about 80% and cluster III showed 78% similarity between the grouped isolates.

4.2.4.4 ERIC-PCR fingerprinting of *E. coli* isolates of integrated fish culture pond, duck and goat fecal samples

The dendrogram pattern of isolates from integrated fish culture duck and goat farm are distributed in four clusters and one single isolate (fig.37). Cluster-I contain two goat isolates with similarity of 66%. Cluster –II is the largest cluster, comprises

of nine isolates, is again divided in two major clusters which showed the grouping of duck and pond isolates separately with similarity of 50%. The other cluster III and IV the similarity between cluster III isolates was 53% where as 31% in cluster IV, they contain only two goat isolates in each cluster and two duck isolates are not grouped in any cluster and showed as single isolates.

In the *E. coli* genome, there are 30 to 50 copies of the ERIC sequence which contains potential stem-loop structure. The ERIC unit tends to occur singly, locating in non-coding regions (Hulton *et al.*, 1991; Martin *et al.*, 1992). The palindrome applied for the identification of bacterial species, types or isolates. The ERIC-PCR is an accurate and reliable molecular identification technology for the study on bacteria aetiology and molecular epidemiology (Hulton *et al.*, 1991; Ibenyassine *et al.*, 2006; Jurkovič *et al.*, 2007). It has been reported that the ERIC-PCR method is a faster, resolution-higher, and more cost-effective than pulsed-field gel electrophoresis (PFGE) or multilocus sequencing for generating information about the genetic similarity of bacterial strains (Jurkovič *et al.*, 2007). Duan *et al.* (2008) successfully identified genetic similarity of the airborne *E. coli* from five chicken houses.

As shown in Figs. 35 and 37, larger proportion of *E. coli* isolates are grouped according to their isolation source eg. in community pond human isolates grouped together like wise pond and cattle isolates are also grouped but some ERIC PCR cluster contain human, animal and community pond *E. coli* isolates with the similarity level of 59-67%. Integrated pond also reflect same type of clustering pattern where integrated pond isolates, duck isolates and goats isolates grouped separately but one major cluster contain both duck and pond isolates in same cluster

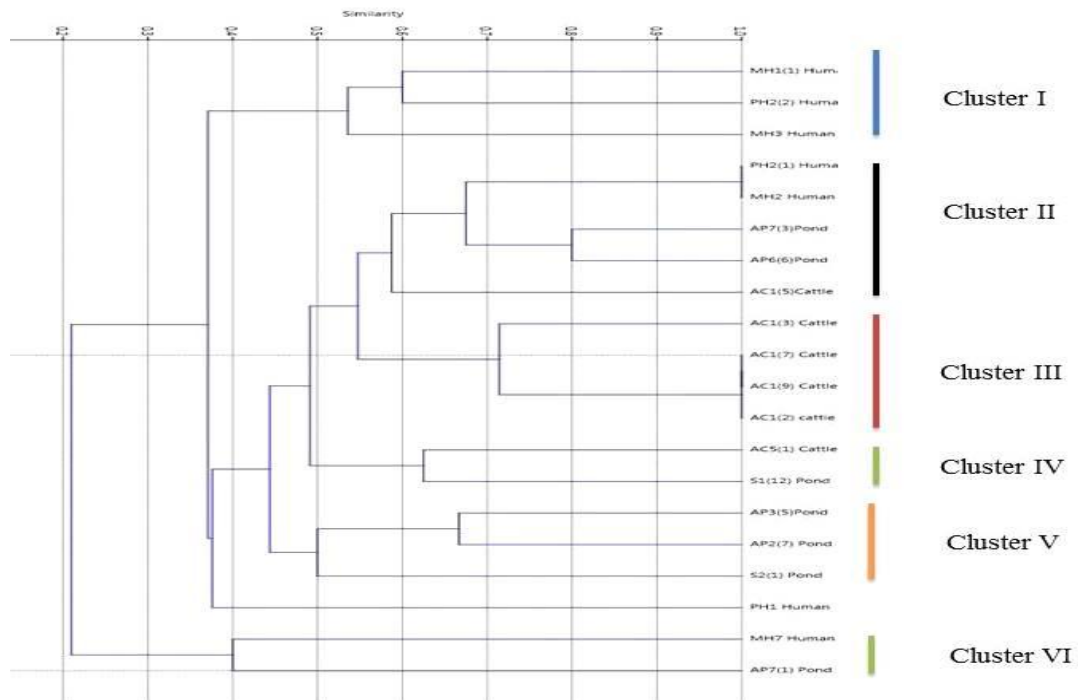


Fig. 35: ERIC-PCR analysis of *E. coli* isolated from feces (human and animal) and community pond water

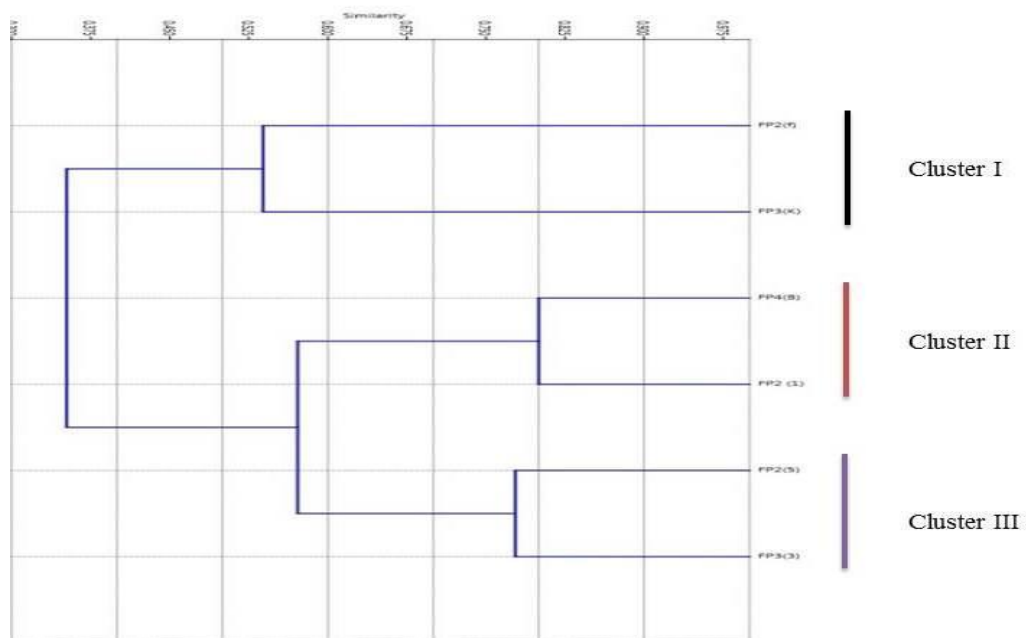


Fig. 36: ERIC-PCR analysis of *E. coli* isolates from conventional fish pond water

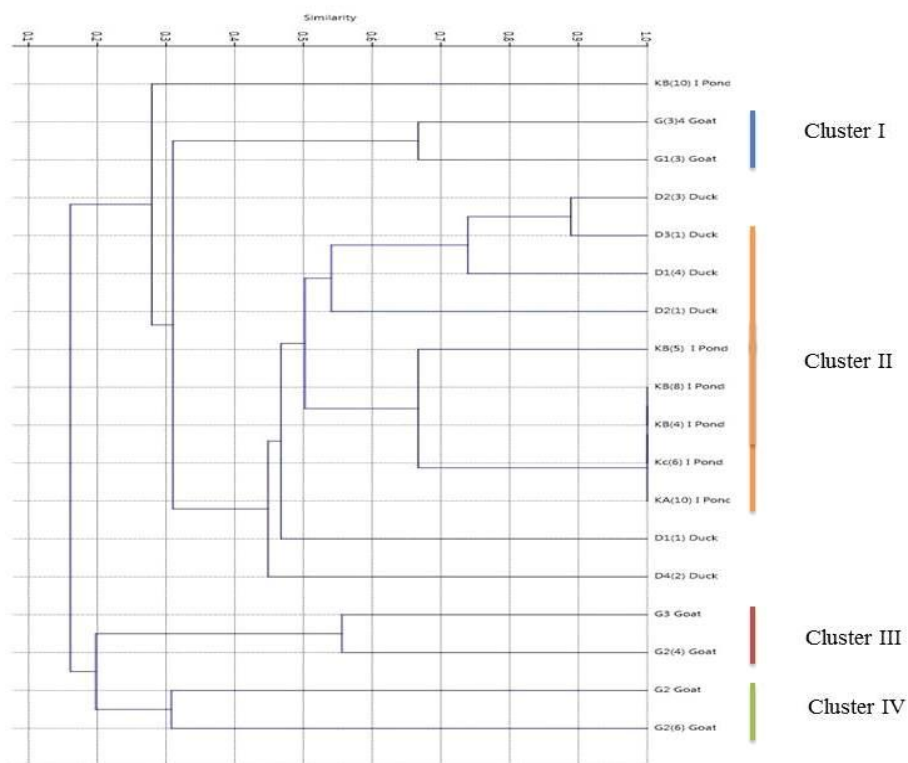


Fig. 37: ERIC-PCR analysis of *E. coli* isolated from feces (duck and goat) and integrated pond water

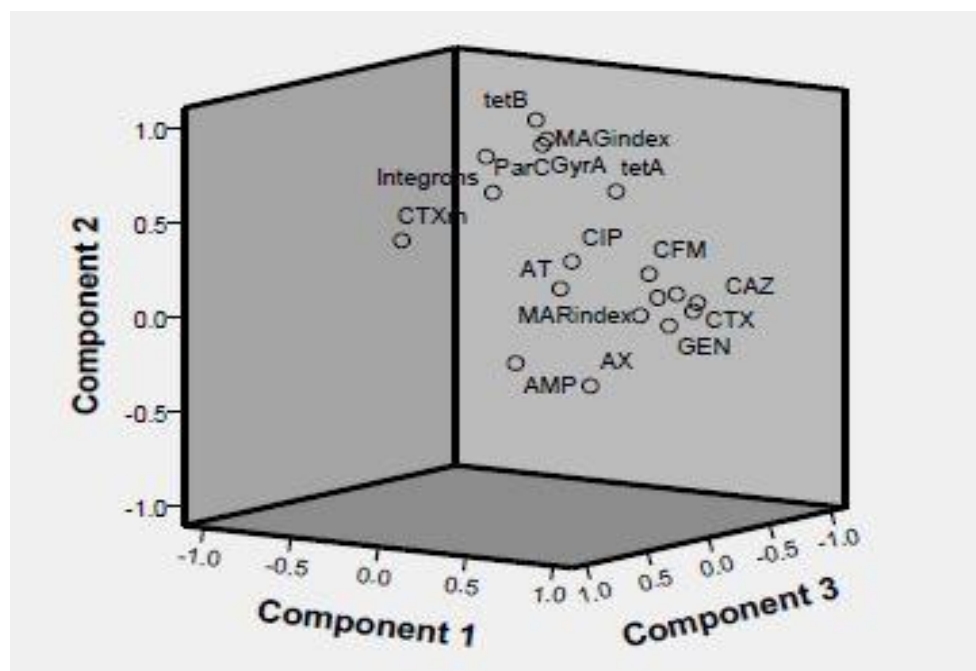


Fig. 38: A graphical representation for factor loading of principal component using resistant phenotype, antibiotic resistance genes, MAR index, MAG index and class 1 integrons

with 50% similarity. This represents the mixing of resistant *E. coli* from different compartments.

In present study community pond showed grouping of human animal and pond *E. coli* isolates in cluster II also integrated pond showed similar findings where duck and integrated pond isolates are grouped in same cluster. Similar findings were reported by Ahmed *et al.*(2020) where *E. coli* isolates assigning into a few clusters may indicate the spread of similar clonal groups. In Iraq, Merza and Jubrael (2015) mentioned similar patterns of dendrogram analysis where 98 *E. coli* isolates were divided into a few clusters (only two major cluster groups). In contrast, another study revealed different patterns of dendrogram analysis, Ramazanzadeh *et al.* (2013) found that ERIC-PCR differentiated *E. coli* isolates into a higher number of cluster groups (20 cluster groups) and suggested that these isolates were not clonally spread and resulted from individual infections.

4.2.4.5 Antibiotic resistance in *E. coli* isolates of different compartment and multivariate analysis

Bivariate statistical relation *i.e.* correlation matrix was not enough to delineate the relationship between traditional water quality parameters and emerging issue of antibiotic resistance. Graphical representation for factor loading of principal components (eigenvector > 1) along with their cumulative variance (%) is given in table 27. The raw data matrix was reduced to two or three principal component loadings which accounted for the majority of the variance. The first component loading explained most of the variance whereas each subsequent component explained progressively less. Therefore, multivariate statistical analyses like principle component analysis (PCA) and cluster analyses were performed and presented in

table 27 showing four principle components (PCs) explaining 92.1% of cumulative data variance. Several authors used X–Y plot of the first two PCA loading for visual interpretation; however, it is better to use 3D loading expressions for more robust visual interpretation (Fig.38).

Principal components analysis (PCA) with varimax rotation has been used in our study; four components were obtained (Table 27). The first principal component (PC1) had a variance of 37.5 % and was mainly represented high loading values of antibiotic resistance to CAZ, CFM, GEN and MAR index and PC1 is mainly influenced by antibiotic resistance score for different antibiotics which may be due to misuse of this antibiotic in human health, animal production and aquaculture activities (Gaude and Hattiholli, 2013; CDDEP, 2016; Malik and Bhattacharyya, 2019). Major loading on beta lactamase and GEN antibiotics indicate the use of this antibiotics increase in the studied area. PC2 is constituted by MAG index, *tetB*, *gyrA*, integrons, *parC* and *tetA* with a variance of 26.5 %, clearly representing the dominance of different antibiotic resistance genes and different studies also revealed that the presence of antibiotic resistance genes in bacterial isolates are responsible for the resistance of different antibiotics. Historical use of oxytetracyclin and gentamycin from long time in different production system is the possible cause of high loading values of these genes. Class 1 integrons is a genetic cassettes which harbor many resistance gene and also promote to their transfer between bacterial isolates by this way it influence the antimicrobial resistance (Zheng *et al.*, 2020). PC3 was comprised of *bla*_{CTX-M} gene, AMP and AT resistance with a variance of 16.0 %, potentially presenting the impact of use of these antibiotics and presence of resistance gene of

similar class. PC4 is constituted by CIP, O and *parC* with a variance of 12.0 %, indicating the use of fluoroquinolone antibiotic CIP and presence of *parC* gene is contributing factor for the resistance that's why the CIP and *parC* resistant gene show high loading.

Table 27 Principal component analysis of phenotypic antibiotic resistance and antibiotic resistant genes

Variables	PC1	PC2	PC3	PC4
AMP	0.51	-0.13	0.72	0.42
CTX	0.82	0.06	-0.32	-0.14
CAZ	0.98	0.07	-0.05	0.09
CFM	0.90	0.29	0.18	0.11
AX	0.73	-0.27	0.42	0.26
GEN	0.94	0.01	0.08	0.22
AT	0.68	0.25	0.60	0.09
CIP	0.42	0.31	0.14	0.83
O	0.71	0.10	-0.30	0.59
MAR index	0.92	0.09	0.28	0.08
MAG index	0.26	0.93	0.10	0.18
CTX-M	-0.06	0.47	0.82	-0.01
GyrA	0.22	0.90	0.09	0.08
ParC	-0.02	0.63	0.14	0.59
tetA	0.06	0.51	-0.74	0.12
tetB	0.05	0.99	-0.10	-0.04
Integrans	-0.27	0.75	-0.16	0.48

Eigen value	6.37	4.51	2.73	2.05
% of Variance	37.51	26.53	16.06	12.07
Cumulative %	37.51	64.05	80.11	92.18

4.2.4.6 Hierarchical cluster analysis

Hierarchical cluster analysis (HCA) was used in this study to detect the similarity and the differences between the different variables. To perform the HCA, Ward's method was used, with squared euclidean distance. Cluster analysis comprises a series of multivariate methods which are used to find true groups within the data. In clustering, the objects are grouped in such a way that similar objects form one cluster. Hierarchical clustering is the procedure which joins the most similar observations first, followed by the next most similar observations successively. The levels of similarity at which observations are merged are used to constructs a dendrogram (Kumar *et al.*, 2019).

The HCA revealed three clusters in the present study. The first cluster was formed by MAR index, MAG index, CIP, GEN, O, AT, CFM and CTX-M. The second cluster was formed by AMP, AX, CAZ and CFM. Third cluster was formed by *tetB*, interogrons, *parC*, *bla*_{CTX-M}, *gyrA* and *tetA*. It is clearly known fact that MAR index is similar to MAG index because higher resistance is a phenotype of multidrug resistant genes. HCA grouped similar variable into same cluster that's why antibiotic resistance phenotype to different antimicrobial agent was grouped together and this clustering also grouped the antibiotic in same cluster which have similar mode of action, likewise cluster 2 grouped different betalactamase antibiotic. Cluster

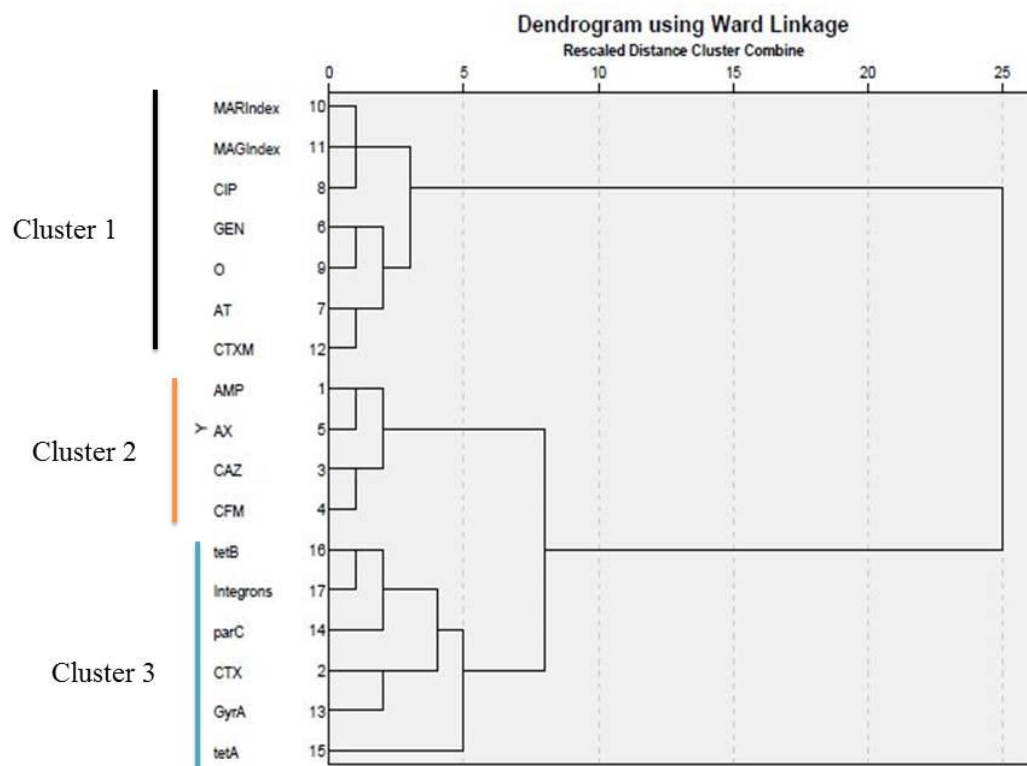


Fig. 39: Cluster analysis of antibiotic resistant phenotype, antibiotic resistance genes, MAR index, MAG index and class 1 integrons

3 grouped different resistance genes and integrons (Fig. 39). The figure indicated that each cluster has variable with same activity.

Antibiotic resistance has become a major challenge worldwide, especially in developing countries like India. Easy accessibility of antibiotics, self-prescription, incomplete treatment course of antibiotics combined with poor management of waste disposal, poor water quality and hygiene contribute to rapid distribution and occurrence of antibiotic resistance in pathogenic and environmental bacteria. The present study showed there is a high incidence rate of multiple antibiotic resistant *E. coli* in the pond water of three different ecosystem and fecal sample of human and animals and suggests that pond water with more animal species, may work as reservoir for antibiotic resistant *E. coli* and it may leads to sharing and transmission of resistant bacteria between human, animal and environment. The community may be at risk of contracting infection due to use of pond water for bathing and other household purposes. The high rate of antibiotic resistance occurrence among environmental bacteria could be a result of indiscriminate use of antibiotics in human, animal and agricultural sectors. The presence of MDR *E. coli* in the water bodies of the region warrants an instant commitment to the rational use of antibiotics as much as possible and discontinuing other practices like manuring of ponds and restrict the entry of organic waste of animal origin. However it has to be spotlighted that this study was conducted only in three different pond ecosystem involving human and animal. Thus the results may not completely reflect the overall prevalence of MDR *E. coli* in the region. The extent of the problem of this emergence of antibiotic resistance

can be managed by conducting the large scale surveillance. Rational use of antibiotics, use of the alternative bio-control agents, creating awareness in public etc.

4.3.1 Isolation of *E.coli* from flies sample

A total of 150 house fly (*Muska domestica*) samples including 73 from milk outlets and 77 from chevon meat shops, chicken meat shop and fish shop were collected and processed for the isolation of *E.coli*. Based on cultural characteristic and biochemical characterization, a total of 45 *E.coli* were isolated with overall prevalence rate of 30.00%. The higher prevalence rate was detected in samples collected from meat shops (37.06%), than the samples from the milk outlets (24.65%) (Table 28).

In house fly samples 30.00% prevalence of *E.coli* was recorded, which is higher than the findings of Collinet-Adler *et al.* (2015) who reported 6.7 % prevalence rate in house fly samples of Vellore, India.

In the present study 37.06% house fly infesting meat shops were found contaminated with *E. coli*. The level of contamination was significantly lower as compared to the findings of Songe *et al.* (2017) who recorded 69% prevalence in house fly samples collected from the fish market of Zambia.

Fly samples collected from milk shops/outlets showed 24.65% prevalence of *E.coli* during present study which is lower than the previously reported 70.67% prevalence rate by Sobur *et al.* (2019) in dairy farms reporting the prevalence of *E. coli* in flies (65.33%) of food centers, dustbins (64%) and area close to hospital (44%). Similarly higher prevalence of 51.4% was reported in fly samples collected

from seven different locations, including households, restaurants, the university canteen, veterinary teaching hospital, poultry farms, dairy farms, and Mymensingh Medical College Hospital of Bangladesh (Akter *et al.*,2020).

4.3.2 Biochemical and molecular characterization of isolates

The isolates of *E.coli* were further subjected to various biochemical test viz. Indole, Methylene blue, Voges Proskauer, and Citrate utilization. All the isolates of *E.coli* showed typical biochemical characteristics as typical IMViC +,+,-,-. Presumptive isolates were finally confirmed by polymerase chain reaction (PCR) using genus-specific oligonucleotide primers.

4.3.3 Antibigram of *E. coli* isolates

Antibiogram phenotype test confirmed the isolated *E. coli* as resistant to multiple antibiotics. The overall highest observed resistance rate was found against AMP (73.3%), followed by CTX and O (66.6% each), CFM (53.3), AT (48.8%), GEN (46.6), CIP (44.4%), AX,CAZ (42.2%) and lowest resistance was noted for the CN (37.7%). The *E.coli* isolates from milk shop/outlet's showed highest resistance against ampicillin (72.2%) followed by CFM (61.1%), CTX,CAZ,AT and O (55.5% each), AX,GEN,CIP (50% each), and lowest resistance was represented by CN (33.3%) (Table 29 and Fig. 43). However fly isolates from meat shops showed higher resistance to AMP, CTX and O (74%) followed by CFM (48.1%), GEN and AT (44.4%), CN (40.7%), AX(37%) and lowest resistance was denoted by CAZ (33.3%).

Table 28: Prevalence of *E. coli* and antibiotic resistant genes in house fly collected from milk and meat shops.

Place	sample	Positive Samples (%)	Phenotypic Data (%)	Molecular data of ESBL resistant genes (%)			Tetracycline resistant genes (%)		Fluroquinolone resistant genes (%)		Class 1 integrons (%)
			ESBL and multiresistant	<i>bla</i> _{CTX-M}	<i>bla</i> _{TEM}	<i>bla</i> _{SHV}	<i>tetA</i>	<i>tetB</i>	<i>gyrA</i>	<i>parC</i>	<i>Int1</i>
Milk Outlets	73	18(24.65)	4(22.22)	3(16.66)	2(11.11)	0	4(22.22)	3(16.66)	4(22.22)	4 (22.22)	1(5.55)
Meat Shops	77	27(37.06)	13(48.14)	8(29.6)	3(11.11)	0	9(33.33)	9(33.33)	8(29.62)	10(37.03)	6(22.22)
Total	150	45(30.00)	17(37.77)	11(24.44)	5(11.11)	0	13(28.8)	12(26.66)	12(26.66)	14(31.11)	7(15.55)

E. coli=*Escherichia coli*, ESBL=Extended-spectrum beta-lactamase

Table 29: Antibiotic resistance frequency of *E. coli* isolates from house fly samples

Antibiotic	Milk shops (%)	Meat shops (%)	Overall (%)
Ampicillin (AMP)	72.2	74	73.3
Cefotaxime (CTX)	55.5	74	66.6
Ceftazime (CAZ)	55.5	33.3	42.2
Cefixime (CFM)	61.1	48.1	53.3
Ampicillin/ Cloxacillin (AX)	50	37	42.2
Gentamicin (GEN)	50	44.4	46.6
Aztreonam (AT)	55.5	44.4	48.8
Ciprofloxacin (CIP)	50	40.7	44.4
Oxytetracycline (O)	55.5	74	66.6
Cefalexin (CN)	33.3	40.7	37.7

Among 45 *E.coli* isolates, 44 isolates were resistant for two or more than two antibiotics. Highest MAR of 1.0 was recorded in 4 isolates, followed by 0.9 by 1 isolates, 0.8 by 1 isolates, 0.7 by 7 isolates, 0.6 by 5 isolates, 0.5 by 7 isolates, 0.4 by 9 isolates, 0.3 by 8 isolates and 0.2 by 2 isolates. Only one isolate representing 0.00 MAR index (Table 30).

The resistance against betalactam antibiotics observed during the present study is comparable to the findings of Songe *et al.* (2017) who reported that 100% *E.coli* isolates were resistance to ampicillin and cefotaxime, followed by tetracycline, norfloxacin and gentamycin (91.0% each). Similarly Sobur *et al.* (2019) also reported higher resistance for ampicillin and other beta-lactamase drugs, most of the isolates tested were resistant to ampicillin (61.75%), meropenem (78.69%), streptomycin



Fig. 40: *E. coli* isolates from house flies showing metallic sheen around the colonies on Eosin Methylene Blue agar (EMB) plate

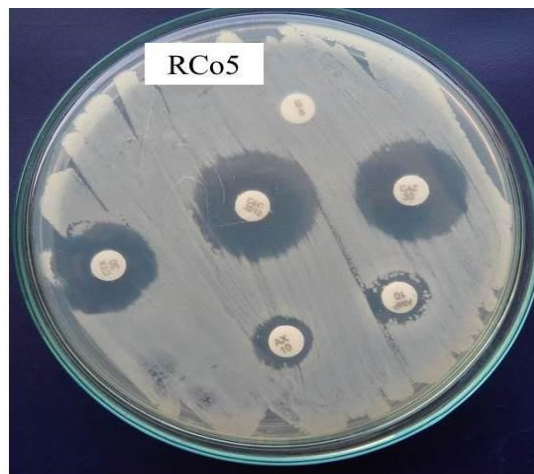


Fig. 41 A: *E. coli* isolate from house fly showing zone of inhibition against different antibiotics

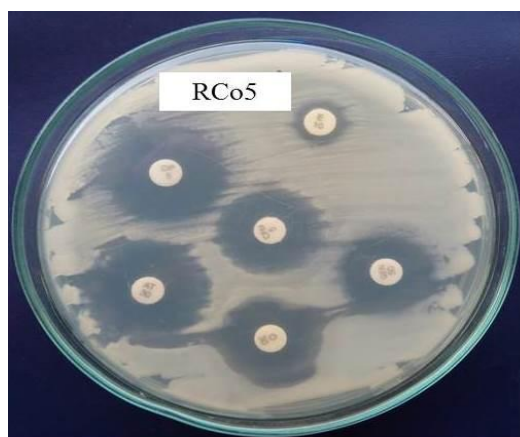


Fig. 41 B: *E. coli* isolate from house fly showing zone of inhibition against different antibiotics

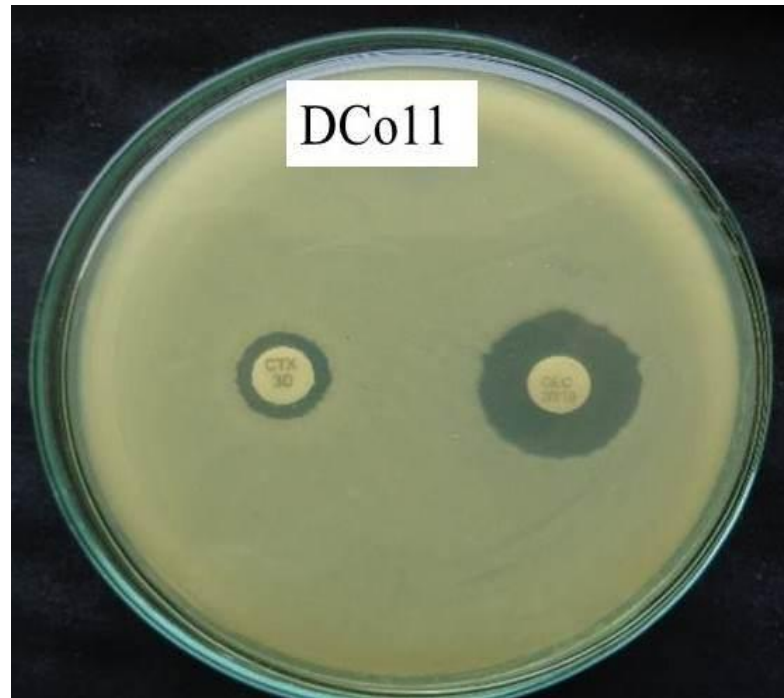


Fig. 42: *E. coli* isolate of fly sample showing phenotypic confirmatory test for ESBL production

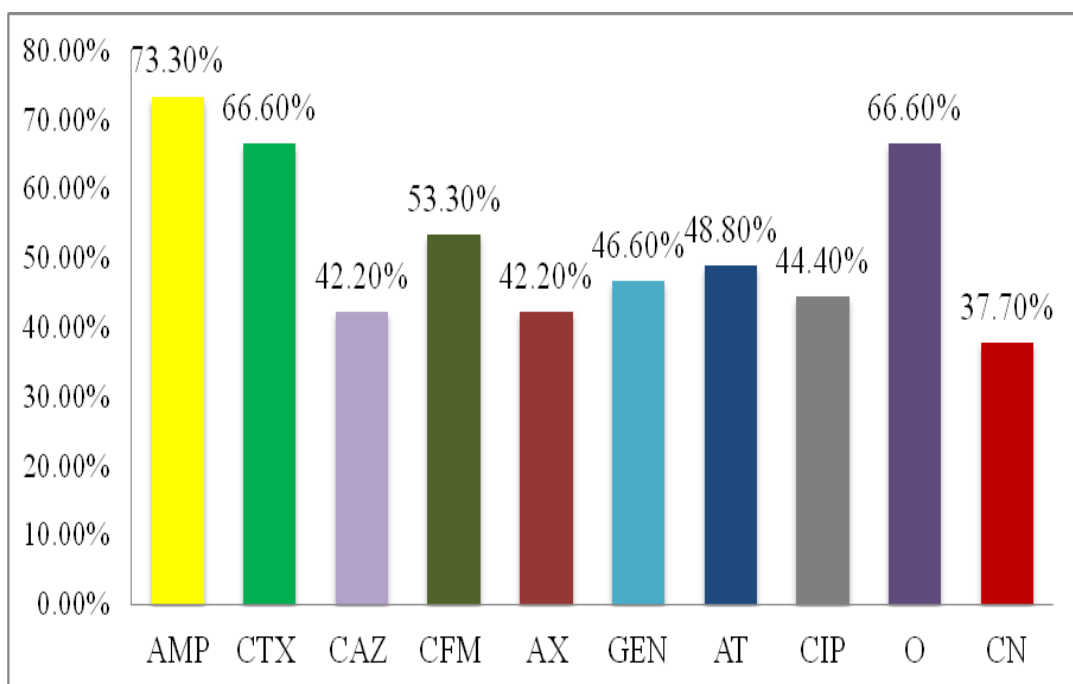


Fig. 43: Antibiogram pattern shown by *E. coli* isolated from house fly samples

(87.43%), erythromycin (91.8%) and tetracycline (93.35%) . Akter et al. (2020) in Bangladesh also reported that *E. coli* isolates of fly were 97% resistant to erythromycin and tetracycline, 88% to streptomycin, and 85% to amoxicillin. All these reports corroborate the findings of present study. MAR indexing is likely to provide a useful tool for better risk assessment by identifying contamination from high-risk environments (Krumperman, 1983). Present study showed that high MAR index between the *E. coli* isolates from fly samples and many author suggested that pathogens with higher indices of MAR in foods of animal origin may possibly be introduced from the environment. Therefore findings are indicating that house fly can be a potential source for dissemination of resistant *E. coli* from environment to animal originated foods.

Table 30:Detail of antibiogram pattern and MAR index for flies *E. coli* isolates

Milk shop fly isolates												
S.N.	Isolate No.	Antibiotic disc										MAR index
		AMP	CTX	CAZ	CFM	AX	GEN	AT	CIP	O	CN	
1	DM5	R	R	I	I	S	R	R	R	R	S	0.6
2	DM7	R	R	I	I	R	I	I	I	R	I	0.4
3	DM11	R	R	R	R	R	R	R	R	R	R	1
4	DM12	R	R	I	R	S	I	I	R	S	S	0.4
5	DM16	R	S	R	R	R	I	R	I	R	S	0.6
6	DM19	R	I	R	R	R	I	I	R	R	R	0.7
7	DM24	R	R	I	I	I	R	I	S	S	S	0.3
8	DM26	S	R	R	R	R	I	R	R	S	R	0.7
9	DM29	R	R	R	R	R	R	R	R	R	S	0.9
10	DM33	R	I	I	I	I	R	I	R	I	S	0.3
11	DM37	S	R	R	I	I	I	R	I	R	I	0.4
12	RM3	R	S	R	R	S	R	R	S	R	S	0.6
13	RM7	I	S	R	R	R	R	I	R	S	R	0.6
14	RM15	R	I	R	R	R	I	I	S	S	S	0.4

15	RM 20	R	R	R	R	R	R	R	R	R	R	1
16	RM25	S	S	I	S	S	S	S	S	S	S	0
17	RM29	R	R	S	R	I	R	R	I	S	S	0.5
18	RM32	S	S	I	S	I	I	R	I	R	R	0.3
Meat shop fly isolates												
S.N.	Isolate No.	Antibiotic disc										MAR index
		AMP	CTX	CAZ	CFM	AX	GEN	AT	CIP	O	CN	
1	DC ₀ 3	R	I	I	S	I	I	I	I	R	R	0.3
2	DC ₀ 7	R	R	I	S	S	S	S	R	I	I	0.3
3	DC ₀ 11	R	R	R	R	R	R	R	R	R	R	1
4	DC ₀ 12	R	I	I	R	I	S	R	R	S	R	0.5
5	DC ₀ 17	I	S	S	R	I	R	R	R	S	S	0.4
6	DC ₀ 19	R	R	I	R	R	R	I	R	R	S	0.7
7	DC ₀	I	R	R	R	R	I	I	R	S	S	0.5
8	DC ₀ 21	R	R	R	R	R	R	R	R	R	R	1
9	DC ₀ 23	R	R	I	I	I	I	S	R	R	S	0.4
10	DC ₀ 25	R	R	I	R	S	I	I	R	R	S	0.5
11	DC ₀ 26	R	R	I	I	R	I	I	I	R	I	0.4

12	DC ₀	R	R	I	I	I	R	I	I	R	S	0.4
13	DC ₀ 30	R	R	R	I	I	R	R	R	R	I	0.7
14	DC ₀ 32	R	R	R	R	I	R	R	I	R	S	0.7
15	DC ₀ 37	R	S	I	S	S	S	R	S	S	R	0.3
16	RC ₀ 5	S	R	I	I	S	S	R	S	R	S	0.3
17	RC ₀ 6	S	R	R	S	I	I	R	I	R	R	0.5
18	RC ₀ 7	R	R	R	R	I	R	R	I	S	R	0.7
19	RC ₀ 11	I	R	I	I	I	I	S	S	R	S	0.2
20	RC ₀ 12	I	R	R	R	R	I	R	I	R	R	0.7
21	RC ₀ 15	R	R	I	I	I	R	I	R	R	S	0.5
22	RC ₀ 18	R	S	S	R	R	R	I	S	R	R	0.6
23	RC ₀ 22	R	S	I	S	I	I	I	I	R	R	0.3
24	RC ₀ 25	R	R	I	I	R	I	I	I	R	I	0.4
25	RC ₀ 27	S	S	I	S	I	I	I	I	R	R	0.2
26	RC ₀ 30	R	R	R	R	R	R	R	I	R	S	0.8
27	RC ₀ 34	R	R	I	R	R	R	I	I	S	S	0.5

D= Durg, R= Raipur, M=Milk shops, C₀= Meat shops (Chicken, Fish and Chevon shops

4.3.4 Antibiotyping

Antibiotyping results revealed 40 patterns among the isolated *E. coli* (n = 45). Among the 40 phenotype patterns for the fly sample isolates, 38 patterns had a low frequency with only one *E. coli* isolates. Only two pattern of antibiotic resistance were denoted by two and more isolates. Pattern no. 16 (AMP.CTX.AX.O) and pattern no. 40 AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP.O.CN was found in 2 and 4 isolates respectively. There were 38 antibiotypes incorporating 42 (93.33%) isolates that were resistant to three or more antibiotics (Table 31). Sobur *et al.* (2019) reported 25 patterns among the isolated *Salmonella* spp (n = 34) from the flies. There were 23 antibiotypes incorporating 31 (91.2%) isolates that were classified as MDR. These findings are in accordance with the present study which represented the prevalence of high resistance among fly isolates.

4.3.5 Phenotypic detection of ESBL producing isolates

All the *E. coli* isolates were further tested for ESBL production. Among 45 isolates of *E. coli* only 17 (37.77%) isolates were found ESBL producer. The 4 (22.22%) isolates from milk outlets and 13(48.14%) isolates from meat shops were found as ESBL producer. Similar findings were also reported by Songe *et al.* (2017) who found 25.33% ESBL producing *E. coli* isolates from fly sample collected from fish and food market of Zambia. Tufa *et al.* (2020) also reported 50% *E. coli*, isolated from fly samples, collected from different clinical settings, as ESBL positive which is similar to the findings of present study (Table 28).

Table 31: Antibiotyping of *E. coli* isolates (n = 45) on the basis of resistant phenotype

Pattern No.	Antibiotic Resistance Pattern	No. of Antibiotics	Isolate No.
1	CTX.O	2	RC ₀ 11
2	O.CN	2	RC ₀ 27
3	AMP.CTX.GEN	3	DM24
4	AMP.GEN.CIP	3	DM33
5	AT.O.CN	3	RM32
6	AMP.O.CN	3	DC ₀ 3
7	AMP.CTX.CIP	3	DC ₀ 7
8	AMP.AT.CN	3	DC ₀ 37
9	CTX.AT.O	3	RC ₀ 5
10	AMP.O.CN	3	RC ₀ 22
11	AMP.CTX.AX.O	4	DM7
12	AMP.CTX.CFM.CIP	4	DM12
13	CTX.CAZ.AT.O	4	DM37
14	AMP.CAZ.CFM.AX	4	RM15
15	CFM.GEN.AT.CIP	4	DC ₀ 17
16	AMP.CTX.AX.O	4	DC ₀ 26, RC ₀ 25
17	AMP.CTX.CIP.O	4	DC ₀ 23
18	AMP.CTX.GEN.O		DC ₀ 28
19	AMP.CTX.CFM.GEN.AT	5	RM29
20	AMP.CFM.AT.CIP.CN	5	DC ₀ 12
21	AMP.CTX.CFM.CIP.O	5	DC ₀ 25
22	CTX.CAZ.CFM.AX.CIP	5	DC ₀ 20
23	CTX.CAZ.AT.O.CN	5	RC ₀ 6
24	AMP.CTX.GEN.CIP.O	5	RC ₀ 15
25	AMP.CTX.CFM.AX.GEN	5	RC ₀ 34
26	AMP.CTX.GEN.AT.CIP.O	6	DM5
27	AMP.CAZ.CFM.GEN.AT.O	6	RM3

28	CAZ.CFM.AX.GEN.CIP.CN	6	RM7
29	AMP.CFM.AX.GEN.O.CN	6	RC ₀ 18
30	AMP.CTX.CAZ.CFM.AX.AT.O	7	DM16
31	AMP.CAZ.CFM.AX.CIP.O.CN	7	DM19
32	CTX.CAZ.CFM.AX.AT.CIP.CN	7	DM26
33	AMP.CTX.CFM.AX.GEN.CIP.O	7	DC ₀ 19
34	AMP.CTX.CAZ.GEN.AT.CIP.O	7	DC ₀ 30
35	AMP.CTX.CAZ.CFM.GEN.AT.O	7	DC ₀ 32
36	AMP.CTX.CAZ.CFM.GEN.AT.CN	7	RC ₀ 7
37	CTX.CAZ.CFM.AX.AT.O.CN	7	RC ₀ 12
38	AMP.CTX.CAZ.CFM.AX.GEN.AT.O	8	RC ₀ 30
39	AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP.O	9	DM29
40	AMP.CTX.CAZ.CFM.AX.GEN.AT.CIP.O.CN	10	DM11, RM 20, DC ₀ 11, DC ₀ 21

4.3.6 Hierarchical clustering and heat map profile of antibiotic susceptibility

All the obtained *E. coli*, isolates and their antibiotic resistance profiles were used for cluster analyses using software package of HemI (Heatmap Illustrator, version 1.0). The results, presented as a heatmap, showed interesting associations between antibiotic resistance patterns of these isolates. We observed five major clusters of different *E. coli* isolated from flies collected from milk and meat shops and these cluster were grouped according to the similar antibiotic resistance pattern. Cluster I isolates, showed high resistance to ampicillin, cefotaxime and oxytetracycline. On the other hand, cluster I isolates were sensitive to cefalexin, and showed intermediate sensitivity to ceftazidime, aztreonam and cefixime. Cluster II and III isolates showed highest resistance to the antibiotics as compared to other

clusters during the study. It was, surprising to observe that some isolates (DM11, RM20, DC₀11 and DC₀21) from milk and meat shops showed resistance to all antibiotics tested. But some isolates of Cluster II were sensitive for cephalixin. Cluster III isolates were less resistant than cluster II isolates and they were sensitive for cephalixin and oxytetracycline. Cluster IV depicts four isolates of milk and meat shop and showed resistance for ceftazidime, aztreonam, cefixime, ciprofloxacin and cephalixin but sensitive for oxytetracycline. Cluster V isolates were mainly resistant for ampicillin, oxytetracycline and cephalixin, but this group contained less resistant isolates than the other clusters. Some isolates were not grouped in any cluster in present study (Fig. 44). The highest resistance shown by cluster II had *E. coli* isolates from milk, meat shops of Raipur and Durg, which indicated similar type of resistance pattern prevailing in the study area.

By cluster analysis, we could identify homogeneous potential clusters with predictor importance and it may help in reducing the antimicrobial resistant population of *E. coli* by adopting preventive measures based on predictor importance in the clusters. The cluster analysis may be used to detect the temporal and spatial distribution of resistance patterns and may also be used in statistical models to assess predictive factors for antibiotic resistance. Hence, cluster analysis may be a better alternate technique to assess epidemiological relationships between antibiotic resistances among bacterial isolates. The cluster analysis and heat map technique was used as an exploratory method that provided a clear and succinct summary tree to look for diversity of resistance phenotype with the approach taken by Eisen *et al.*(1998).

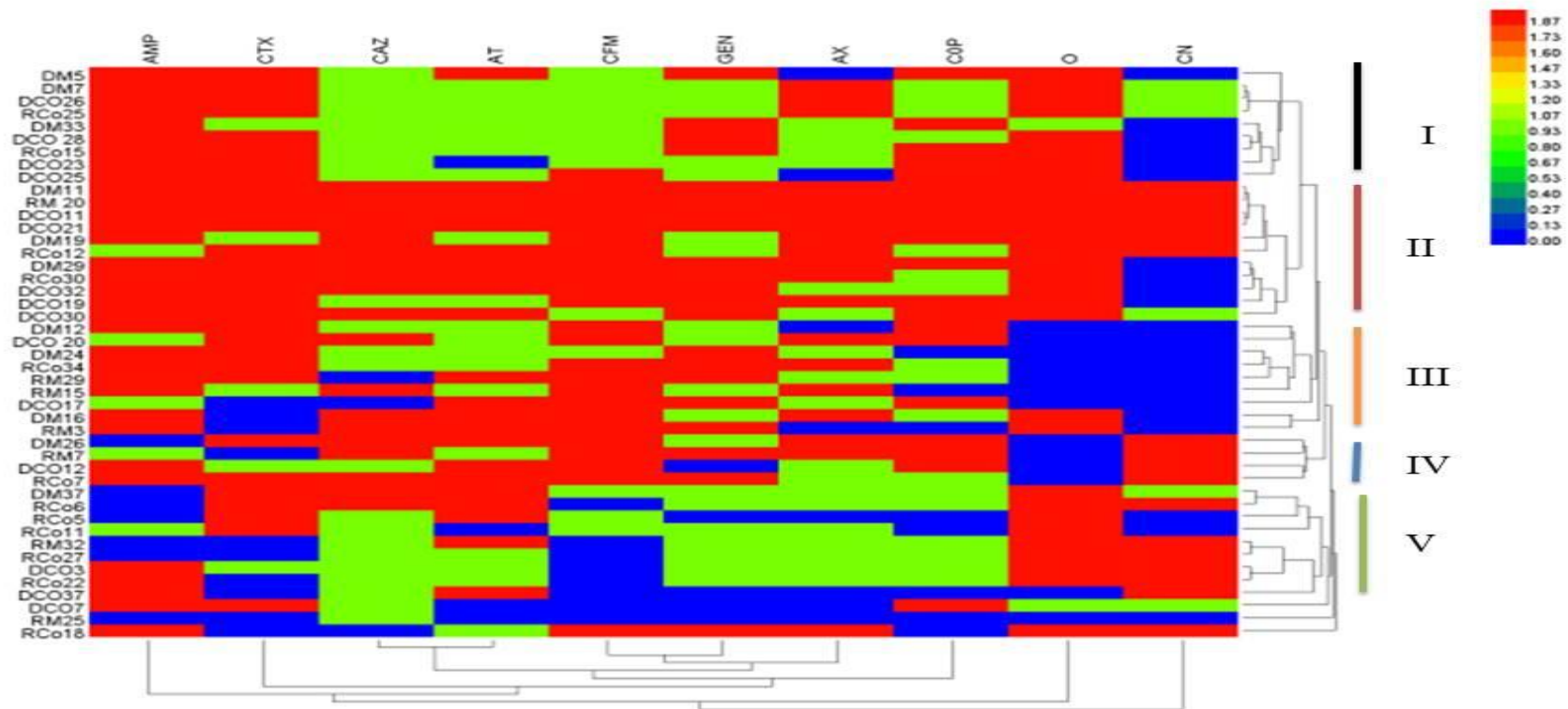


Fig. 44: Two-way hierarchical clustering with a heat map of antimicrobial susceptibility profiles to 10 antibiotics of *E. coli* isolated in this study. The heatmap indicates the categorical value of susceptibility from each antibiotic as indicated by a color bar (blue = sensitive, green = intermediate, and red = resistant)

4.3.7 Detection of antibiotic resistant genes and class 1 integron

Identification of resistance genes is significant, since AMR phenotypes are acquired by chromosomal DNA mutations and/or due to transfer and acquisition of new genetic material between bacteria of identical or distinct species or genera by horizontal transfer of resistance (Maiden, 1998; Ochman *et al.*, 2000). The resistance genes are located on plasmids which have the ability to transfer, to other bacteria. Plasmids in *E. coli* play an important role in the multiple antibiotic resistance genes transfer. Therefore in present study plasmid DNA was screened for the presence of *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, *tetA*, *tetB* and *Int1* while complete DNA was used for the detection of *gyrA* and *parC* for the fluroquinolone resistance. The presence of resistance gene thus indicates the possible development of resistance in the bacterial isolates.

E.coli isolates (n=17), phenotypically confirmed for ESBL production and multidrug resistant, were tested for the presence of antibiotic resistant genes. The isolates positive for *bla*_{CTX-M} and *bla*_{TEM} genes revealed expected product size of 593 and 445 bp respectively (Fig.46). All the beta-lactam resistant isolates were found negative for *bla*_{SHV} resistant gene. Overall prevalence of *bla*_{CTX-M} and *bla*_{TEM} genes were 24.44% and 11.11% respectively whereas, these resistant genes represent higher prevalence for the isolates recovered from the flies collected from meat shops. For the meat shop isolates *bla*_{CTX-M} and *bla*_{TEM} gene prevalence was 29.6% and 11.11 % respectively, whereas for milk shop fly isolates prevalence of *bla*_{CTX-M} and *bla*_{TEM} gene 16.66 and 11.11% respectively (Table 28 and Fig.45).

All the 17 MDR isolates were either oxytetracycline resistant or intermediate sensitive. *E. coli* isolates from fly samples of milk and meat shops revealed presence of *tetA* (210 bp) and *tetB* (359 bp) genes either single or in combination and interesting fact is that they are also present in the plasmid DNA. The prevalence of *tetA* and *tetB* was 28.80% and 26.66% respectively. Comparatively milk shop isolates represent more prevalence for *tetA* than the *tetB* but the meat shop isolates equal prevalence rate for both the resistant genes (Table 28 and Fig.32).

The isolates (n=17) resistant or intermediate sensitive to ciprofloxacin were found to have either *gyrA* (n=12, 26.66%) or *parC* (n=14, 31.11%) genes and showed the amplicon size of 586 bp and 265 bp (Fig.31) respectively. Interestingly *E. coli* isolated from fly samples from milk shops (n=4) were positive for both of the resistant genes with a prevalence rate of 22.22%. *E. coli* isolates from fly sample of meat shops represent higher prevalence for *parC* with the prevalence of 37.03% than the prevalence of *gyrA* 30.7%.

In the present study, class 1 integron of 280 bp size (Fig. 33) was detected only in 15.5% fly isolates. MDR *E. coli* isolates originating from milk and meat shops were showed presence of *Int1* genes with the prevalence rate of 5.55% and 22.22% respectively. A well-established relationship exists between *E.coli* MDR strains and the presence of integrons (Singh *et al.*, 2017).The most common integron types present in clinical isolates of the *Enterobacteriaceae* are class 1 integron, which along with other genomic elements like conjugative plasmids or transposons, play an important role in the carrying and disseminating antimicrobial resistance genes (Odetoyin *et al.*, 2017).

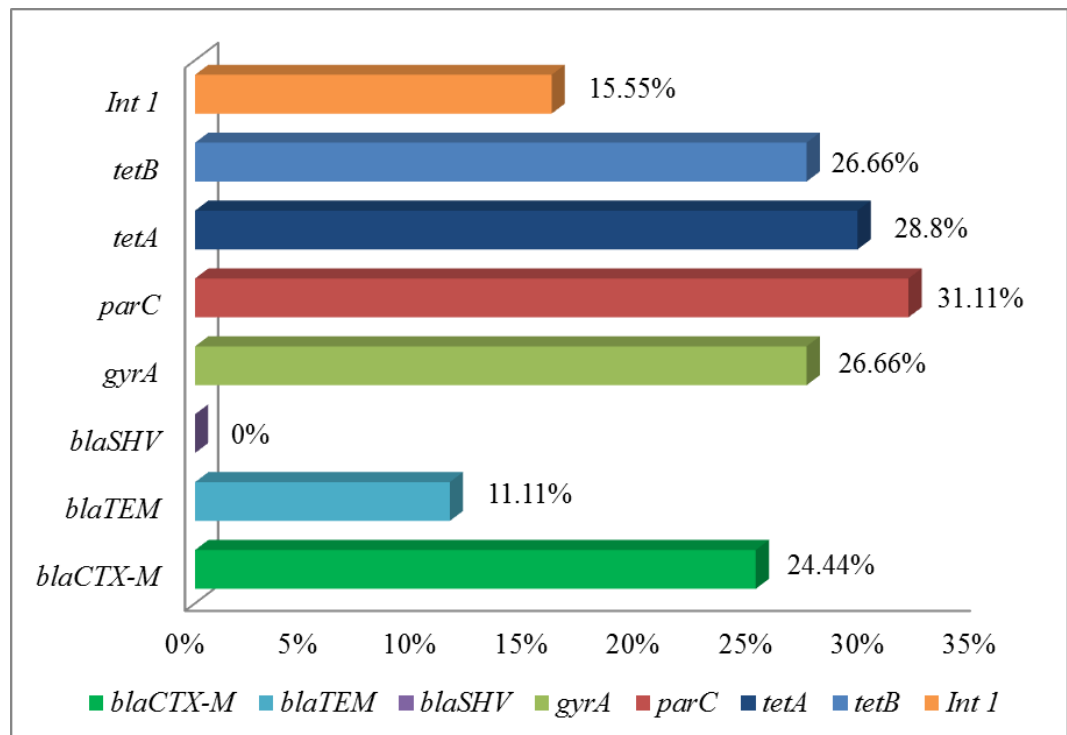


Fig. 45: Prevalence of resistant genes shown by *E. coli* isolates from house fly samples

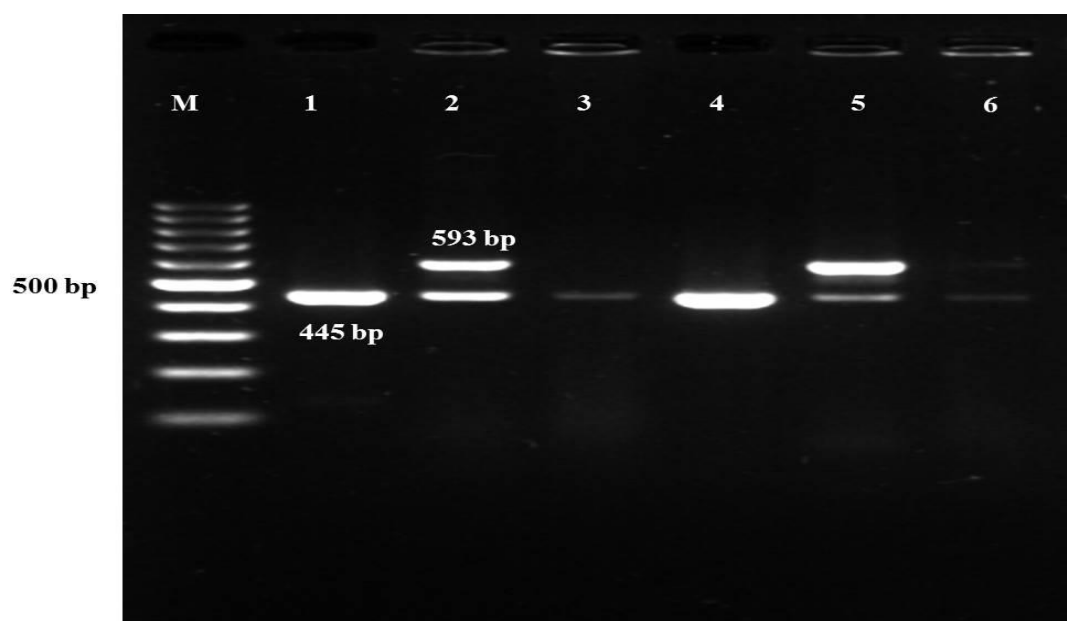


Fig. 46: PCR product of *bla*_{TEM} (445 bp) and *bla*_{CTX-M} (593 bp) gene amplification, in fly *E. coli* isolates. Lane M 100 bp DNA ladder

Sobur *et al.* (2019) collected house flies from various environmental settings and isolated *E. coli* showed the prevalence rates of *tetA* (88.75%) and *tetB* (48.52%), resistance determinants, the study revealed that higher prevalence of *tetA* gene which is in accordance to present findings. Similarly Akter *et al.* (2020) also reported higher prevalence of *tetA* gene than the *tetB* gene in *E. coli* isolated from fly samples of households, restaurants, the university canteen, veterinary teaching hospital, poultry farms, dairy farms, and Mymensingh Medical College Hospital of Bangladesh.

House flies are the most widely distributed vectors among all the arthropod insects. Flies have a great potential to contribute in the dissemination of bacteria both enteric pathogens and commensal microorganisms because of their remarkable ability to move freely among various habitats and overcome long distance flights up to 5–7 kilometers (Fotedar, 2001). Pathogens including bacteria, viruses, fungi and parasites may be transmitted passively through the body surface of the flies. Flies are thus involved in the spread of antimicrobial resistant pathogens between animals and humans (Onwugamba *et al.*, 2018). Flies are frequently found in close proximity to human activities including habitats, food corners, hospitals and animal farms. Several pathogens including MDR bacteria have been frequently isolated from the fly body surface (Khamesipour *et al.*, 2018). Flies can work as a mechanical and biological vector and also have potential capacity to transfer the MDR bacteria from environment to animal originated foods.

4.3.8 ERIC-PCR analysis

The electrophoretic profiles of the ERIC-PCR products were determined for the, MDR ESBL *E. coli* isolates (n=17) from fly samples collected from milk and

meat shops. All the *E. coli* isolates obtained in this study were grouped into different clusters or branches based on the ERIC-PCR pattern similarity. Furthermore, the ERIC DNA fragments from the isolates obtained from the fly samples were amplified in one PCR reaction and loaded on the gel electrophoresis for comparison. The data matrix based on the DNA fragments was then used to construct the dendrogram using the PAST program software (Hammer *et al.*, 2001).

The dendrogram analysis of the examined isolates (n = 17) showed four clusters and two unclustered meat shop isolates. Two milk shop isolates and two meat shop isolates grouped in cluster I. In this cluster milk shop fly isolates (DM19 and DM11) showed 100% similarity but meat shop isolates from Durg and Raipur showed low similarity. Cluster II comprises three isolates from the meat shops, out of them two fly isolates (DCo23 and DCo25) from meat shop of Durg showed 100% similarity but these isolates showed low similarity with meat shop fly isolate from Raipur. Cluster III contain three isolates from meat and milk shops. Meat and milk shop fly isolates (DCo32 and DM29) from Durg showed more than 90% similarity, whereas these isolates (RCo12) showed less than 75% similarity with the isolate from Raipur meat shop isolates. Cluster IV contain four meat shops and one milk shop fly isolates, out of them two isolates from Durg meat shop showed higher similarity of 88% whereas other isolates showed lower similarity. Two meat shop isolates of Durg and Raipur were not clustered in any cluster (Fig.47).

Findings of the ERIC PCR study revealed that the majority of flies from meat and milk shop were containing genotypically identical *E. coli* isolates. However non-identical one also found it indicated flies came from similar source as well as

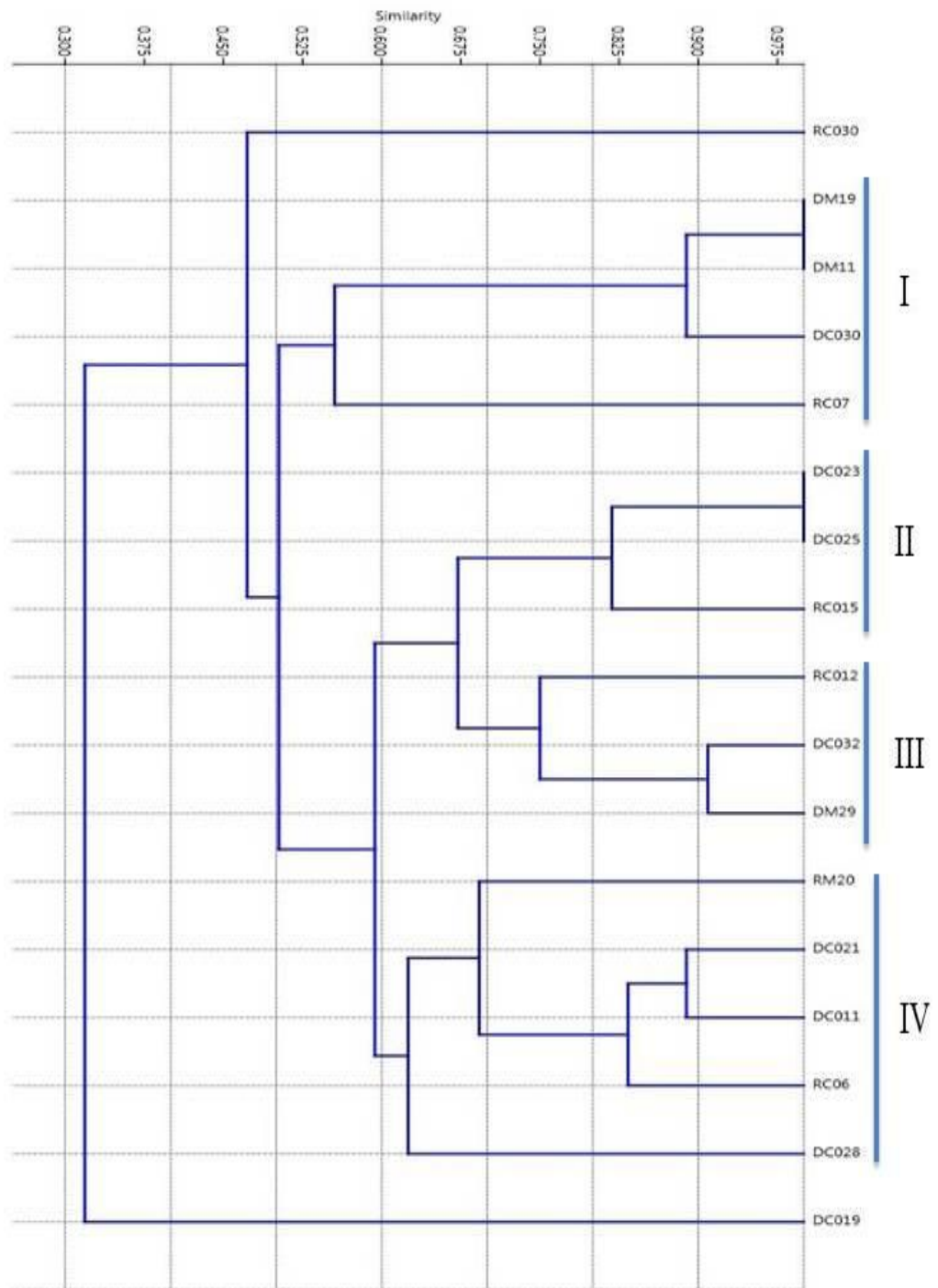


Fig. 47. ERIC-PCR analysis of *E. coli* isolated from fly samples collected from milk and meat shops

from diverse sources because they have great capacity to fly and their distribution is governed by various environmental and sociological factors. Studies proved that vectors such as flies might also play a role in spreading resistant bacteria. The flies might have come into contact with the resistant bacteria by interacting with the contaminated feces/environment (Von Salviati *et al.* 2015).

Earlier heatmap and hierarchical clustering analysis of *E. coli* isolates for antibiotic resistance pattern on the basis of their phenotypic character are similar to the clustering pattern of ERIC PCR analysis. Isolates like DM19 and DM11 who showed 100% similarity in ERIC PCR genotyping also showed similar resistance pattern and thus, by hierarchical clustering method grouped in the cluster II, likewise other isolates DCo23 and DCo25 which have 100% similarity and 80% similarity in between DCo21 and DCo11 by ERIC PCR are also grouped in Hierarchical cluster 1 and 2 respectively. Hierarchical clustering proved an easy and rapid tool able to group susceptibility testing results of a large series into clusters and to count them, according to the different specific resistance phenotype groups. So it is a easy method to identify the emergence of new resistant pattern among the isolates. Also use of ERIC PCR with the combination of Hierarchical clustering helpful for the detection of any genotypic variation. Several systems, including VITEK 2 and SIRWEB, incorporate expert systems to control the results of susceptibility testing by applying a series of predefined rules which detect frequent or infrequent phenotypes. However, such a system is unable to classify and count different antibiotic-resistance phenotypes and cannot survey the prevalence of unknown phenotypes that may spread locally in a environment (Joyanes *et al.*, 2001). In addition, laboratories in

low-income countries generally lack these systems because of the cost, so their data cannot be shared and compared with other laboratories and countries to provide an early warning of new or unusual outbreaks of drug-resistant bacteria (Bush *et al.*, 2011; WHO, 2011).

E. coli are found in the normal gut microflora and the gut parts that are left over after the animal were processed or cut, and these parts are easily accessible and exposed to the freely moving flies. Previously, the detection of *E. coli* in houseflies infesting fish in food markets was reported in Zambia (Songe *et al.*, 2017).

In the current study, houseflies were collected from a fish market, where live birds were also slaughtered and goats, meat was sold together with fishes. Flies carrying *E. coli* therefore cross-contaminate the surrounding environment during slaughter, cutting, and further processing of fish, meat, or other food products (Usui *et al.*, 2015; Li *et al.*, 2016). There was a close proximity of milk shops with those of this meat market or otherwise the sale of raw milk was in the dairy farm itself, that's why flies in the farm and surrounding environment were the main contaminating agent. In addition, several studies showed that flies from the environment harboring human pathogens contaminate themselves spontaneously (Doza *et al.*, 2018) thus making the flies more likely to transmit pathogens and caused foodborne illnesses. Many resistance genes like tetracyclin and betalactamase can be easily transmitted among various bacterial species due to their location on mobile genetic elements such as plasmids, transposons, and integrons. Large conjugative resistance plasmids are capable of transferring several resistance genes. These plasmids have been detected in *E. coli* isolates in several countries and caused cross-resistance to tetracycline

(Hopkins *et al.*, 2005; Van *et al.*, 2008; Strahilevitz *et al.*, 2009; Rozwandowicz *et al.*, 2018). It is important to recognize that although this study focused on flies collected from animal originated food shops, this commodity is usually cooked before it is eaten, and so any Enterobacteriaceae will die during these procedures. Our concern, though, is cross contamination of other products such as fresh salad, fruit and ready-to-eat food products in close proximity, as well as the indication of high pathogen prevalence and antimicrobial resistance contamination of environment and foods.

E. coli isolates in the present study showed very high resistance, however it has to be spotlighted that this study was conducted only in some markets of Durg and Raipur and sample size taken for study was not very large; therefore, the results may not completely reflect the overall prevalence of MDR *E. coli* in the region. Further determination of the serotypes and sequence types, as well as the whole genome characterization of the detected *E. coli* isolates in this study, might provide important information about the circulating strains and their impact on food safety and human health. Additional hygienic measures, particularly for the appropriate disposal of inedible and unused parts of the meat markets as well as proper cleaning of dairy barn and use of fly repellent and other measure for hygienic sale of these product, should be undertaken to reduce the number of flies in selling areas to limit the dissemination of AMR bacteria, thus to ensure improved consumers' health and safety.



*SUMMARY, CONCLUSIONS
AND SUGGESTIONS FOR
FUTURE RESEARCH WORK*



CHAPTER-V

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH WORK

SUMMARY:

The intensification of farming in India corresponds with the increasing consumption of animal protein, particularly meat, fish, poultry and eggs. Urbanization, population growth and rising incomes contribute further to this demand for animal-based foods. Poultry production is considered a high risk for ABR (Anti Bacterial Resistance) emergence in low-income settings, particularly in smaller scale unregulated operations. Poultry commonly receives higher quantities of antibiotics than other animal livestock, and resistance is more likely to develop in conditions of overcrowding and poor sanitation. The most commonly used antimicrobial groups in dairy animals and poultry are tetracyclines, fluoroquinolones, sulphonamides, betalactams, aminoglycosides, macrolides etc. The extensive use of antibiotics in poultry and cattle and non-adherence to withdrawal periods results in the occurrence of their residues in animal originated foods. The consumption of foods containing residues may lead to various adverse effects to consumers such as allergic reactions, disorders of intestinal flora, pathological effects, carcinogenicity, mutagenicity, bone marrow toxicity etc. In addition to aforementioned effects, another most important effect of excessive use of antibiotics is the emergence of resistant bacteria

due to selection pressure. Antibiotic resistance has emerged as one of the major health related problem throughout the world with increased concern in developing countries like India. The infection with antibiotic resistant pathogens is a matter of immense concern as it may result in treatment failures in humans.

Chhattisgarh is a leading state for fish production and also have a rich pond tradition where three type of pond system exist. These include village ponds used for the fish culture as well as for bathing and other community work, ponds only used for fish culture and low resource setting integrated livestock and fish farm. The mixing of different animal species and manuring of pond by cattle and poultry fecal matter may lead to transmission of resistance bacteria to pond and may act as sink for different resistant gene and bacteria. As a commensal bacterium, *E. coli* colonizes the gut of humans and animals and ubiquitous in soil, plants, vegetables and water. For these reasons, *E. coli* is chosen as a sentinel organism for One Health studies of ABR.

The house fly, *Musca domestica* Linnaeus (Diptera: Muscidae), is the well-known and widely distributed species of muscid nonbiting filth flies on earth. *Musca domestica* is eusynanthropic and endophilic are cosmopolitan pest of medical and veterinary importance that lives nearly in association with human and it can complete its whole life cycle inside the residence of humans and animals. House flies are regularly found in huge numbers in regions of human activities such as food centers or eateries, butcher houses, hospitals,

markets, poultry and dairy farms, where they create an annoyance to people, poultry and domesticated farm animals. House flies are important medical and veterinary insects due to their potential for pathogen transmission from animal feces and leftover decaying organic matters. House flies can harbor and transfer pathogenic and MDR bacteria that pose serious threats to human and animal health.

Keeping in view the aforementioned facts, in the present study, 214 broilers and 35 backyard marketed chicken meat samples from three agro climatic zone of Chhattisgarh state were collected. The samples were analyzed to determine antibiotic residues and assess potential health risks to consumers. Additionally, to know the status of antibiotic resistance in different three types of pond ecosystem, water samples and fecal samples from pond associated cattle, human, human not using pond, duck and goat of integrated farm were collected for isolation of *E. coli* and assessment of resistance in them. House fly samples (n=150) from milk shops and meat shops located in Durg and Raipur district were also collected to assess the possible roles of these flies in dissemination of the antibiotic resistant *E. coli* from environment to animal originated foods.

In the present study, a confirmatory HPLC multi-residue method for the detection and extraction of oxytetracycline, tetracycline, chlortetracycline, sulphamethazine, ciprofloxacin and enrofloxacin from chicken meat was optimized and validated which can be applied for detection of antibiotic residues in chicken meat in the routine analysis.

The six antibiotics belonging to three different groups were detected in the present study. Each antibiotic was detected at its maximum wavelength; 360 nm for oxytetracycline, tetracycline, chlortetracycline and 280 nm for sulphamethazine and 280 nm for ciprofloxacin and enrofloxacin. The chromatographic separation was performed on ZORBAX Eclipse XDB column with isocratic elution of 0.01 M oxalic acid (pH 4.0), methanol and acetonitrile (70:10:20, v/v/v), for separation of tetracycline group and sulphamethazine antibiotics. Xbridge-C18 column with gradient elution was used for the separation of fluoroquinolone group antibiotics.

After standardization of HPLC conditions, the method for simultaneous extraction of oxytetracycline, tetracycline, chlortetracycline, sulphamethazine, ciprofloxacin and enrofloxacin from the meat matrix was optimized. The main experimental steps evaluated were: protein precipitation, extraction, clean-up using solid-phase cartridges and concentration of elute. The use of 20% trichloroacetic acid in acetonitrile as meat protein precipitant, sodium EDTA-McIlvaine buffer at pH 4 as extraction buffer and methanol as eluting solvent were found excellent.

The analytical methods for detection of all the studied antibiotics by HPLC-PDA as well as the sample preparation methods were optimized and validated. All the performance parameters viz. linearity, limit of detection, limit of quantification, accuracy, precision and selectivity

were found to be in compliance with the European Commission decision 2002/657/EC.

In this study, a total of 249 chicken meat samples (214 commercial broiler and 35 backyard) were collected from three agroclimatic zones of Chhattisgarh. The samples were processed for analysis of antibiotic residues using method optimized and validated in the present study.

A total of 104 meat samples collected from Plain agroclimatic zone of Chhattisgarh, out of these 44, (42.3%) samples were found positive for antibiotic residues. Among positive samples, 13(12.5%) were positive for oxytetracycline, 20 (19.2%) for tetracycline, 2(1.9%) for chlortetracycline, 5 (4.8%) for ciprofloxacin and 4 (3.8%) for enrofloxacin. None of the sample from plane zone was found positive for sulphamethazine residues. A total of 21 (20.1%) samples [5 (4.8%) for oxytetracycline, 9 (8.6%) for tetracyclin, 2(1.9%) for chortetracycline, 3(2.8%) for ciprofloxacin and 2(1.9%) for enrofloxacin] exceeded the established MRL.

Out of 55 meat samples collected from Northern hill agroclimatic zone 28 (50.9%) samples were found positive for antibiotic residues with mean $\pm$ SD of 148.9 $\pm$ 68.1 μ g/kg. Among positive samples, 5(9.0%) were positive for oxytetracycline, 7(12.7%) for tetracycline and 16(29.0%) for sulfamethazine. None of the samples from northern hill zone was found positive for chlortetracycline, ciprofloxacin and enrofloxacin residues. A total of 17 (30.9%) samples [1(1.8%) for oxytetracycline, and 16 (29.0%) for

sulfamethazine] exceeded the established MRL whereas none of the sample was found above MRL for tetracycline residues.

A total of 24 (46.6%) out of 55 meat samples collected from Bastar plateau were found positive for antibiotic residues. Out of 24 positive samples, 12 (21.8%) samples were positive for oxytetracycline, 5 (9.0%) for sulphamethazine, 6(10.9%) for ciprofloxacin and 1 (1.8%) for enrofloxacin. None of the sample was found positive for tetracycline and chlortetracycline residues. A total of 5 (9.0%) samples [1 (1.8%) oxytetracycline, 2 (3.6%) for sulphamethazine and 2 (3.6%) for ciprofloxacin] exceeded the established MRL whereas none of the sample was found above MRL for enrofloxacin residues.

The 35 backyard chicken meat samples were processed for analysis of antibiotic residues and 13 (37.1%) samples were found positive for antibiotic residues with mean $\pm$ SD of 86.3 $\pm$ 26.8 μ g/kg. 12 (34.2%) samples were positive for oxytetracycline residues while 1 (2.8%) sample was positive for ciprofloxacin residues.

The above findings highlighted that the percentage of positive samples, mean concentration of residues in positive samples as well as percentage of samples above MRL were higher in meat samples collected from plane region and northern hill region as compared to Bastar plateau . Also, the statistical analysis highlighted significant difference in mean concentration of antibiotic residues detected in Bastar plateau as compared to Northern hill and Plane agroclimatic zone ($p < 0.05$). Statistically significant

higher residual mean concentration detected in broiler chicken samples than the backyard chicken samples.

The risk assessment for meat samples from broiler chicken meat and backyard chicken meat was carried out to determine the hazards to consumers associated with consumption of meat containing antibiotic residues. Samples were analysed for determining any health risks to consumers. The HQ for antibiotic residues was detected less than 1 in meat samples from broiler chicken meat and the backyard chicken. Thus no significant adverse effect on the health of consumers due to consumption of the meat was found.

In the present study, *E. coli* isolates (n=223) of different ponds and fecal samples (human and animal) origin were studied to understand their antimicrobial resistance pattern and genetic diversity. A total of 87 *E. coli* were isolated from the pond water samples (W1, W2 and W3) and 136 from the fecal samples (M1, M2, M3, M4 and M5). The results showed that $53.2 \pm 20.7\%$ of the *E. coli* isolates from all samples were found to be resistant to AMP, $76.4 \pm 16.9\%$ to CTX, $55.8 \pm 27.0\%$ to CAZ, $38.3 \pm 24.7\%$ to CFM, and $47.9 \pm 16.6\%$ to AX. In contrast, a lower bacterial resistance incidence was found for the other antibiotics with their frequencies of $24.2 \pm 23.7\%$ to GEN, $20.2 \pm 20.1\%$ to O, and $11.3 \pm 7.4\%$ to CIP. Similar bacterial antibiotic resistance profiles for the water and fecal samples were found with a significantly higher resistance incidence to beta-lactamase group of antibiotics viz. AMP, CTX, CAZ, CFM, and AT than to CIP, GEN and O (One-wayANOVA, $p < 0.05$).

Analysis of the overall mean percentage of resistance showed variations based on the different pond and their related activity for *E. coli* isolates by ANOVA ($p \leq 0.05$). The low overall resistance for conventional pond, W2 (27.3 ± 28.9) and high resistant to community pond W1 (58.8 ± 26) to different antibiotics was detected.

A total of 502 resistance phenotype patterns of 9 antibiotics based on the combination theoretical analysis by using online software were calculated. Among the 223 *E. coli* strains, 14.3% were found to be not resistant to any antibiotics. Only 4.0% and 1.7% were resistant to eight or nine type of antibiotics, respectively and 52.4% showed resistance to three to seven types of antibiotics. The 49 phenotype patterns in the water samples and 54 phenotype patterns in the fecal samples were observed among all *E. coli* isolates from the three aquaculture systems and five types of fecal samples. The *E. coli* isolates exhibited 16 phenotype patterns for W1, 14 patterns for W2, 22 patterns for W3, 14 patterns for M1, 21 patterns for M2, 7 patterns for M3, 15 patterns for M4, and 23 patterns for M5. The MAR index ranged from 0.40 to 0.56 in the *E. coli* isolates from the water samples and from 0.25 to 0.53 in the fecal samples. The highest MAR index was found in W1 (0.56), followed by M4 (0.53), W3 (0.51), M5 (0.43), W2 (0.40) and M2 (0.35), M3 (0.28), and M1 (0.25).

A total of 44 multidrug resistant isolates were selected from pond and fecal sample groups for detection of antibiotic resistance genes. Seven

antibiotic resistance genes (beta-lactamase resistant genes: *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV} ; plasmid mediated tetracycline efflux genes: *tetA*, *tetB*; fluroquinolone resistant genes: *gyrA* and *parC*) and Integron class 1(*Int1*) gene were detected in these resistant *E. coli* isolates. But two target genes *bla*_{TEM} and *bla*_{SHV} were not detected in these isolates. The detection frequency of different resistant genes and integrons in water *E. coli* isolates had the following order: *gyrA*>*parC*>*tetB*> *Int1*>*bla*_{CTX-m} >*tetA*, for the fecal *E. coli* isolates the order of resistant gene detection frequencies was *gyrA*>*parC*> *Int1*>*tetA*>*tetB* and *bla*_{CTX-M} .

E. coli isolates were selected from the pond water samples (W1, W2 and W3), with the average resistance gene frequencies of 30.9% to *bla*_{CTX-M}, 95.2% to *gyrA*, 72.2% to *parC*, 16.6% to *tetA*, 63.4% to *tetB* and 46.8% to *Int1*. *E. coli* isolates six of each group were selected from the fecal samples of (M1, M2, M4 and M5), with the average resistance gene frequencies of 12.49 % to *bla*_{CTX-M}, 95.8% to *gyrA*, 74.9% to *parC*, 66.6% to *tetA*, 62.4% to *tetB* and 83.3% to *Int1*. Higher resistance gene frequencies to *gyrA*, *parC*, *tetA*, and *tetB* were detected in the *E. coli* isolated from the fecal samples.

The electrophoretic profiles of the ERIC-PCR products were determined for the *E. coli* isolates separated from different pond and fecal samples from different animal species. The data matrix based on the DNA fragments and the dendrogram using the PAST statistical software were constructed, grouping all the *E. coli* isolates obtained in this study into

different clusters or branches based on the ERIC-PCR pattern similarity.

The dendrogram analysis of the examined isolates ($n = 20$) showed six clusters and one separate human isolate. The similarity between human and pond isolates of the cluster II was calculated by Jaccard coefficient and showed around 67% of similarity. The similarity between cattle, human and pond isolates was detected as 58%.

For traditional aquaculture culture pond, the 6 multidrug resistant bacterial isolates were divided into three clusters and each cluster has two isolates. Cluster I show more than 55% similarity between two isolates whereas cluster- II isolates show higher similarity about 80% and last cluster showed 78% similarity between the grouped isolates.

The dendrogram pattern of isolates from integrated fish culture pond and related duck and goat isolates are distributed in four clusters and one single isolate. Cluster-I contain two goat isolates with similarity of 66%. Cluster –II is the largest cluster which comprises of nine isolates and this big cluster is again divided in two major clusters which showed the grouping of duck and pond isolates separately and showed 50% similarity between pond and duck isolates.

Graphical representation for factor loading of principal components (eigenvector > 1) along with their cumulative variance (%) has showed that data matrix was reduced to two or three principal component loadings and

accounted for the majority of the variance.

The first principal component (PC1) had a variance of 37.5 % and was mainly represented by high loading values of antibiotic resistance to CAZ, CFM, GEN and MAR index. PC2 is constituted by MAG index, *tetB*, *gyrA*, integrons, *ParC* and *tetA* with a variance of 26.5 %, clearly representing the dominance of different antibiotic resistance genes. PC3 was comprised of CTX-M gene, AMP and AT resistance with a variance of 16.0 %, potentially presenting the impact of use of these antibiotics and presence of resistance gene of similar class. PC4 is constituted by CIP, O and *ParC* with a variance of 12.0 %.

Hierarchical cluster analysis (HCA) was used in this study to detect the similarity and the differences between the different variables. To perform the HCA, Ward's method was used, with squared Euclidean distance. Cluster analysis comprises a series of multivariate methods which was used to find true groups within the data. The first cluster was formed by MAR index, MAG index, CIP, GEN, O, AT, CFM and CTX-M. The second cluster was formed by AMP, AX, CAZ and CFM. Third cluster was formed by *tetB*, integrons, *parC*, CTX-M, *GyrA* and *tetB*.

A total of 150 house fly (*Musca domestica*) samples, 73 from milk shops and 77 from meat shops were processed for the isolation of *E. coli*. Based on cultural characteristic and biochemical characterization, a total of 45 *E. coli* isolates were obtained with overall prevalence rate of 30.00%. Higher

prevalence rate was detected in samples collected from meat shops (37.06%), than the samples from the milk outlets (24.65%).

Antibiogram phenotype test confirmed that the fly isolated *E. coli* were resistant to multiple antibiotics. The overall highest observed resistance rate was found against AMP (73.3%), followed by CTX and O (66.6% each), CFM (53.3), AT (48.8%), GEN (46.6), CIP (44.4%), AX (42.2%) and lowest resistance was noted for the CN (37.7%). Antibiotyping results revealed 40 patterns among the isolated *E. coli*. and out of them 38 patterns had a low frequency with only one *E. coli* isolate.

The heatmap and hierarchical clustering, showed interesting associations between antibiotic resistances pattern of these isolates. *E. coli* isolated from flies were grouped into five major clusters on the basis of similar antibiotic resistance responses.

Fly isolates of *E. coli* (n=17) were phenotypically confirmed for ESBL production and multidrug resistant and these isolates were tested for the presence of antibiotic resistant genes and class 1 integron. Among the ESBL and MDR *E. coli* isolates, *bla*_{CTX-M} (24.44%), *bla*_{TEM} (11.11%), *tetA* (28.8 %), *tetB* (26.66%), *gyrA* (26.66%), *parC* (31.11%) and *Int1* genes (15.5%) were detected and none of isolates was found positive for *bla*_{SHV} genes.

The dendrogram analysis of the examined isolates showed four clusters and two separate meat shop isolate. Two milk shop isolates and two

meat shop isolates grouped in cluster I. In this cluster milk shop fly isolates showed 100% similarity. Cluster II comprised of three isolates from the milk and meat shops, and two fly isolates from meat shop showed 100% similarity. Cluster III contain three isolates from meat and milk shops. Meat and milk shop fly isolates from Durg showed more than 90% similarity, whereas these isolates showed less than 75% similarity with the isolate from Raipur meat shop. Cluster IV mainly contain meat shops fly isolates and a single isolate from milk shop fly isolate two isolates from Durg meat shop showed higher similarity of 88% whereas other isolates showed lower similarity.

CONCLUSIONS:

1. The multi-residue method for the determination of antibiotics in chicken meat was validated according to international guidelines and found to be simple, rapid, reliable, safe and easily adoptable.
2. The application of present method to chicken meat samples collected from three agroclimatic zones of Chhattisgarh revealed the presence of oxytetracycline, tetracycline, sulphamethazine and ciprofloxacin residues. This suggested that these antibiotics are most commonly used in poultry farming in the state.
3. The risk assessment based on per capita consumption of meat in humans revealed that the current levels of antibiotic residues in meat pose no significant adverse effects on the health of consumers.

4. Community pond and integrated pond *E. coli* isolates have higher antibiotic resistance than conventional fish pond which may be due to multiple species involvement thus increasing the chances of dissemination of resistant bacteria.
5. Pond and fecal isolates showed different resistant genes *bla*_{CTX-M}, *tetA*, *tetB*, *gyrA*, *parC* and class 1 integrons. Some genes were present on plasmid thus responsible for resistance transmission between the bacteria.
6. ERIC PCR dendrogram showed similarity between pond, human and cattle isolates which is due to the spread of similar clonal groups between the different compartments of ecosystem.
7. Prevalence of *E. coli* in fly samples was 30.00%. Higher prevalence rate was detected in samples collected from meat shops (37.06%), than the samples from the milk outlets (24.65%).
8. Except only one *E. coli* isolate, all fly isolates were resistant. The results of this study suggest that flies are important vectors in transmitting resistant *E. coli* from contaminated environment to the animal originated food shops.

SUGRESSIONS FOR FUTURE RESEARCH WORK:

1. A comprehensive study with more number of antibiotics and samples from different compartment of ecosystem (livestock products, water from different source, soil sample) can be undertaken for ascertaining the status of antibiotic residues and their role in AMR.
2. Detection and molecular characterization of various transposons and integron genes which facilitate the transmission of different beta-lactamase genes from one bacterium to another.
3. Proteomics analysis of antimicrobial resistant bacteria for further insights into the ideology of antimicrobial resistance.



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APPENDIX

Media for isolation of bacteria (HiMedia, Pvt. Ltd., Mumbai)

1. Nutrient broth

Ingredients

Peptic digest of animal tissue	5.0 g
Sodium chloride	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Distilled water	to make 1000 ml
Final pH (at 25 ⁰ C)	7.4 ± 0.2

Suspended 13.0 g in 1000 ml of distilled water. Heated to boiling to dissolve the medium completely. The medium was used after autoclaving at 121⁰C, 15 lb for 15 min.

2. Nutrient agar

Composition

Peptic digest of animal tissue	5.0 g
Sodium chloride	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Agar	15.0 g
Distilled water	to make 1000 ml
Final pH (at 25 ⁰ C)	7.4 ± 0.2

Suspended 2.80 g in 1000 ml of distilled water. Heated to boiling to dissolve the medium completely. The medium was used after autoclaving at 121⁰C, 15 lb for 15 min.

3. M-TEC Agar

Composition

Proteose peptone	5.0 g
Yeast extract	3.0 g
Lactose	10.0 g
Sodium chloride	7.5 g
Potassium dihydrogen phosphate	1.0 g
Dipotassium hydrogen phosphate	3.3 g
Sodium lauryl sulphate	0.2 g
Sodium deoxycholate	0.1g
Sodium deoxycholate	0.08g
Bromphenol red	0.08g
Agar	15g
Distilled water	to make 1000 ml
Final pH (at 25 ⁰ C)	7.3±0.2

Suspended 45.2 g in a clean glass flask containing 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45°C and pour into sterile Petri plates.

4. Eosin Methylene Blue (EMB) agar

Composition

Peptic digest of animal tissue	10.0 g
Dipotassium phosphate	2.0 g
Lactose	5.0 g
Sucrose	5.0 g
Eosin-Y	0.40 g
Methylene blue	0.065 g

Agar	13.50 g
Distilled water	to make 1000 ml
Final pH (at 25 ⁰ C)	7.2 ± 0.2

Suspended 36.0 g in a clean glass flask containing 1000 ml distilled water. After autoclaving at 121⁰C, 15 lb for 15 min, the medium was poured into sterile plates, allowed to cool and then preserved at 4⁰C till use.

5. Mueller-Hinton agar

Composition

Beef	300.0 g
Casein acid hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	to make 1000 ml
Final pH (at 25 ⁰ C)	7.3 ± 0.1

Suspended 38.0 g in 1000 ml of distilled water. Heated to boiling to dissolve the medium completely. The medium was used after autoclaving at 121⁰C, 15 lb for 15 min.

6. Luria-Bertani broth

Composition

Casein enzymatic hydrolysate	15.0 g
Yeast extracts	5.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml
Final pH (at 25 ⁰ C)	7.2 ± 0.2

Suspended 24.0 g in 1000 ml of distilled water. Heated to boiling to dissolve the medium completely. The medium was used after autoclaving at 121⁰C, 15 lb for 15 min.

THESIS ABSTRACT

Title of thesis: Antibiotic residues in chicken meat and status of antibiotic resistance in *E.coli* isolated from different ecosystems of Chhattisgarh

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The present study was carried out with the objectives to determine the occurrence of antibiotic residues in chicken meat samples, collected from three agroclimatic zones (Plain, Northern hill and Bastar plateau) of Chhattisgarh and assessment of antibiotic resistance in *E. coli*, isolated from house flies and water and fecal samples of animals and human from different ecosystems.

A total of 249 chicken meat (214 commercial broiler and 35 backyard chicken) samples were analyzed for antibiotic residues using, optimized and validated HPLC method during the present study. Results revealed that 42.3% of chicken meat samples found positive for antibiotics while 20.1% samples had concentration above EU MRL in Plain region. In Northern hill and Bastar plateau 50.9% and 43.6% samples were found contaminated with antibiotic residues while, 30.9% and 9% samples showed concentration higher than the EU MRL level in this region. Backyard chicken meat samples were found contaminated with oxytetracycline (34.2%) and ciprofloxacin (2.8%) residues. Oxytetracycline was the leading antibiotic detected in the meat samples (38%) followed by tetracycline (25%) and sulfamethazine (19%). Human health risk assessment suggested that people of Chhattisgarh at the current levels of antibiotic residues in chicken meat pose no significant adverse health effects.

A total of 223 *E. coli* isolated from the water and fecal samples were evaluated for resistance against nine antibiotics. The results showed that 53.2±20.7% of the *E. coli* isolates were resistant to AMP, 76.4±16.9% to CTX, 55.8±27.0% to CAZ, 38.3±24.7% to CFM, 47.9±16.6 to AX. Antibiotic

resistance profiles for the water and fecal samples *E. coli* showed significantly higher resistance incidence to beta lactamase group of antibiotics AMP, CTX, CAZ, CFM, and AT than to CIP, GEN and O (One-way ANOVA, $P < 0.05$). Analysis of the overall mean percentage of resistance in *E. coli* showed variations based on the different pond and their related activity. The community pond involving human, animal and aquaculture showed highest resistance to different tested antibiotics. A total of 49 and 54 resistance phenotype patterns in *E. coli* isolated from water and fecal samples, respectively were observed.

The resistance genes and integrons were detected in water *E. coli* isolates with frequency order: *gyrA* > *parC* > *tetB* > *Int1* > *bla*_{CTX-M} > *tetA*, where as fecal origin *E. coli* isolates was *gyrA* > *parC* > *Int1* > *tetA* > *tetB* and *bla*_{CTX-M}. The ERIC-PCR fingerprinting patterns of pond and fecal sample *E. coli* isolates of different ecosystem revealed that isolates of human, animal and water of community pond are grouped in a same cluster indicating the similar resistant clonal group responsible for resistance in the ecosystem.

PCA results suggest that among all the resistance indicators tested, the MAR index, resistance to CAZ and GEN antibiotics represented the antibiotic resistance profile comprehensively and hierarchical cluster analysis grouped betalactamase antibiotic and multiple resistant genes in different groups.

A total of 45 *E. coli* isolates were obtained from fly samples with overall prevalence rate of 29.33%. Higher prevalence rate was detected in samples collected from meat shops (33.33%), than the samples from the milk outlets (24.65%). Antibigram test confirmed that fly *E. coli* isolates were resistant to multiple antibiotics.

Among 45 isolates of *E. coli* only 17 (37.77%) isolates were found ESBL producer. Among the ESBL and MDR *E. coli* isolates, *bla*_{CTX-M} (24.44%), *bla*_{TEM} (11.11%), *tetA* (28.8 %), *tetB* (26.66%), *gyrA* (26.66%), *parC* (31.11%) and *Int1* genes (15.5%) were detected and none of isolates was found positive for *bla*_{SHV}

genes. Heatmap and hierarchical clustering analysis of *E. coli* isolates for antibiotic resistance pattern on the basis of their phenotypic character are similar to the clustering pattern of ERIC PCR genotyping analysis, because similar isolates were grouped in same cluster by both the methods.

This study highlighted a need to generate awareness among poultry farmers towards judicious antibiotic usage to prevent occurrence of antibiotic residues in chicken meat and to prevent an unacceptable health risks to consumers. Mixing of animal species leads to higher resistance in *E. coli* isolated from water bodies may responsible for resistance dissemination.

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