ANTIBIOTIC RESIDUES IN BROILER MEAT SOLD IN DHARAN

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A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfillment of the requirements for the degree of B. Tech. in Food Technology

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Approval Letter

This ***dissertation*** entitled ***Antibiotic Residues in Broiler Meat Sold in Dharan*** presented by **Santosh Thapa** has been accepted as the partial fulfillment of the requirement for the **B. Tech. degree in Food Technology**

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Abstract

Misuse of antibiotics in poultry production may lead to severe negative impacts among which occurrence of drug residues is a burning issue. Sunsari district, in which Dharan municipality is located, is one of the major poultry meat producer of Nepal. This study aims to study the status of antibiotic residues in broiler meat sold at Dharan. A semi-structured questionnaire survey was conducted among poultry farmers and veterinary shops to collect information regarding their education level, commonly used antibiotics, poultry diseases incurred and so on. Then samples of four types of broiler tissues, namely, liver, breast muscle, kidney and gizzard were collected and screening of antibiotic residues in them was performed by implying microbial inhibition technique. The samples found positive in this first stage of screening were subjected to thin layer chromatographic analysis in order to determine whether the positive samples contained residues of ciprofloxacin, doxycycline, enrofloxacin, gentamycin and tetracycline.

Most of the poultry farmers had an educational level upto school level and only a few of them had received training on poultry production. Not many of them had any idea regarding safety aspects of antibiotics and impacts of their misuse. The survey report showed maximum usage of tetracycline and doxycycline in poultry farms. Through microbial inhibition technique, 57% of chicken meat samples were found to contain residues among which the highest percentages was found in kidneys (72%) followed by liver (68%), gizzard (68%) and finally breast muscle (20%). Highest number of samples were positive towards β-lactams and/or tetracyclines (49%) followed by aminoglycosides (29%), sulfonamides (27%) and quinolones (17%). Residues of each groups of antibiotics were found in higher number of kidney samples in comparison to other tissues. Similarly, 36.84% of the positive samples contained a single group of antibiotics whereas the remaining 63.16% of positive samples contained multiple groups of antibiotics. Through thin layer chromatography, it was found that highest number of samples contained tetracyclines (21%) followed by doxycycline (17%), ciprofloxacin (9%), enrofloxacin (8%) and gentamycin (3%). Prevalence of antibiotics among different tissues was found to differ significantly.

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List of Abbreviations

Abbreviations	Full form
ADI	Acceptable Daily Intake
AFU	Agriculture and Forestry University, Chitwan
ATCC	American Type Culture Collection
BPKIHS	BP Koirala Institute of Health Science, Dharan
CAC	Codex Alimentarious Commission
CRD	Chronic Respiratory Disease
ELISA	Enzyme Linked Immuno-Sorbent Assay
EU	European Union
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
NA	Nutrient Agar
TLC	Thin Layer Chromatography
UV	Ultra Violet
VP	Voges-Proskauer
VTH	Veterinary Teaching Hospital, Chitwan

PART I

Introduction

1.1 General introduction

Poultry meat is one of the most consumed food product of animal origin. According to a 2013 report regarding global meat consumption, poultry meat is the second most consumed meat after pork meat (Ritchie and Roser, 2020). Chicken meat is considered to be healthier than red meat. It is also not associated with any major religious taboos such as pork and beef in Muslim and Hindu community respectively. In addition to this, poultry industry is growing tremendously because of its high productivity and thus has become available even to low income people. With the growing human population, income of people and urbanization, demand for animal derived foods is expected to rise at a very high rate (Alexandratos and Bruinsma, 2012). Among such foods, the demand for poultry meat is estimated to increase at the highest rate (Mottet and Tempio, 2017). A report given by FAOSTAT (2016) showed a tremendous increase in global per capita consumption of poultry meat from 2.88 kg to 14.13 kg between 1961 and 2010.

Poultry industry is reported to be the fastest growing meat industry showing more than 12 fold increase between 1961 and 2014 (Ritchie and Roser, 2020). Today, the world has over 23 billion poultry, about three birds per person on the planet (FAOSTAT, 2016). Several technological advancements have played a vital role to increase the productivity. Among them, usage of antibiotics has played a significant role in such tremendous growth of poultry industry (FAO, 2009).

After it was found that antibiotics can not only be used to prevent and treat infectious diseases but also to promote growth in animals in the 1940s, they were extensively used in animal husbandry and aquaculture (Gustafson and Bowen, 1997). Approximately upto 80% of all animals produced for food purposes receive medication for a part or for most of their lives (Lee *et al.*, 2001). Antibiotics have been found to be used for growth promotion, nutritive purpose as well as for therapeutic and prophylactic purposes in poultry production (Chowdhury *et al.*, 2009). Chickens were the first animals to receive antibiotics for disease prevention and as growth promoters in daily doses (McKenna, 2017). The use of antibiotics has facilitated the efficient production of poultry, allowing the consumer to purchase, at a

reasonable cost, high quality meat and eggs. Antibiotic usage has also enhanced the health and well-being of poultry by reducing the incidence of disease.

Initially, only the positive aspects of antibiotic usage in poultry industry were noticed and thus it was thought to be a great achievement in poultry sector (McKenna, 2017). Although these uses benefit all involved, unfortunately, consumer perceptions are that edible poultry tissues are contaminated with harmful concentrations of drug residues (Donoghue, 2003). According to Lee *et al.* (2001), such harmful concentrations of drug residues have resulted in various side effects such as acute allergic reactions, immune-depression, photo toxicity, aplastic anemia, thyroid adenoma and hyperplasia in humans. Some of them are carcinogenic and mutagenic as well. Misuse of antibiotics has produced several negative impacts on the poultry itself, on the consumers and on the environment. Development of antibiotic resistance in poultry and human gut bacteria has become a major threat. Incidences of occurrence of antibiotic resistance in bacteria isolated from poultry meat have been reported world-wide. The situation has been further worsened by the rapid increase in resistant microbes compounded by the lack of discovery of new highly effective antibiotics (Ventola, 2015). Occurrence of antibiotic residues in poultry meat have been reported throughout the world and this has resulted in several direct as well as indirect effects on the health of the consumers. So it is very important to perform routine analysis in order to monitor the levels of drug residues in poultry products so that the levels remain below the safe levels (Nirala *et al.*, 2018).

1.2 Statement of the problem

The use of antibiotics in livestock production has been successful in treatment of several zoonotic diseases. In addition to this, antibiotics make a valuable contribution in production enhancement by increasing weight gain, improving feed efficiency and modifying some production parameters (Gustafson and Bowen, 1997). But because of several negative impacts of overuse of antibiotics in poultry production, consumer awareness has aroused on this topic. Considering these negative impacts, several countries as well as the European Union have banned the prophylactic usage of antibiotics in poultry production. According to EC (2003) use of antibiotic growth promoters has been forbidden in the European Union since 2006. Similarly, FDA banned the use of several antibiotics including fluoroquinolones for poultry production in USA (Jones, 2005). Recently, India has also banned the use of colistin in poultry farms (Davies and Stockton, 2019). In context of Nepal, Department of

Health Services has targeted to develop and implement protocols for management of infectious diseases and to eliminate the non-therapeutic use of antibiotics for growth promotion in animals by 2020 A.D. Similarly it has targeted to stop selling antibiotics without prescription by 2025 A.D. (DoHS, 2016).

But a study regarding poultry production in Nepal by Osti *et al.* (2017) observed that the farmers used common antibiotics based on their individual judgement and analysis of disease and flock condition rather than consulting with veterinary doctors. 40% of the respondents who were asked about their knowledge on poultry vaccination stated that they had never been trained in poultry production and health management. A study in 2013 reported that 71 percent of veterinary drug sales were based on self-prescription by retailers (GARP-Nepal, 2015). Several workers have reported occurrence of antibiotic residues in poultry meat collected from different places (Gwachha, 2017; Maharjan *et al.*, 2020; Pandey *et al.*, 2009; Prajapati *et al.*, 2018; Raut *et al.*, 2017; Rawal and Manandhar; Sapkota *et al.*, 2019; Shrestha, 2017). Some workers have even reported residue levels to be above the maximum residue limits (Maharjan *et al.*, 2020; Raut *et al.*, 2017; Shrestha, 2017). Due to such an uncontrolled use of antibiotics, several side effects are seen both to human health and to the environment (Boxall *et al.*, 2002; Lee *et al.*, 2001). Development of antibiotic resistance in gut bacteria of human has emerged as a major health issue (Kirbis, 2007; Lee *et al.*, 2001).

A major reason for this problem is that the action taken by government to prevent antibiotic residue bearing meat is not satisfactory. Similarly, another reason is the lack of special training and awareness to farmers. Provisions of such trainings can help farmers to prevent infections so that the use of antibiotics can be minimized (Raut *et al.*, 2017). Effective implementation of good agricultural practices should be done by the farmers and respective authorities should monitor the situation. In addition to this, government should monitor the sales of such antibiotics in veterinary very effectively. This research aims to study the prevalence of antibiotic residues in poultry meat and organs, namely liver, kidney and gizzard sold at Dharan Municipality.

1.3 Objectives

1.3.1 General objective

1. To study the prevalence of antibiotic residues in poultry meat sold at Dharan municipality.

1.3.2 Specific objectives

1. To conduct a survey among farmers and veterinary shops regarding antibiotic usage in poultry industry in Dharan Municipality
2. To isolate *Bacillus subtilis* from soil and its identification for microbial screening of antibiotics
3. Determination of antibiotic succeptibility and minimum inhibitory concentration of antibiotics for test organisms
4. To screen antibiotic positive meat samples (liver, kidney, breast muscle and gizzard) by three plate test (for β-Lactams and/or tetracyclines, aminoglycosides and sulfonamides) and one plate test (for fluoroquinolones)
5. To determine the suitable solvent system for detection of antibiotic residue in poultry tissue
6. To determine antibiotic residue in meat tissue using thin layer chromatography

1.4 Significance of study

Due to an indiscriminate use of antibiotics for treatment and protection as well as to enhance production of livestocks, food stuffs of animal origin may contain antibiotics residues in amounts higher than the Maximum Residual Limits. So it is important to test the foods from animal origin for the welfare of consumers (Rawal and Manandhar). Nepal, being a member of the World Trade Organization (WTO) and World Organization for Animal Health (OIE), has an obligation to follow the standards and rules regarding Maximum Residue Limit (MRL) for different antibiotics set by the World Health Organization and the Codex Alimentarius Commission (Raut *et al.*, 2017). But researches regarding antibiotic residues in meat samples collected from western regions of Nepal has shown the prevalence of several antibiotics such as tetracycline, penicillin, aminoglycosides, sulphonamides, aminoglycosides and fluoroquinolones (Gwachha, 2017; Maharjan *et al.*, 2020; Pandey *et al.*, 2009; Prajapati *et al.*, 2018; Raut *et al.*, 2017; Rawal and Manandhar; Sapkota *et al.*, 2019; Shrestha, 2017). Till this date, no such studies have been done in Eastern Nepal regarding antibiotic residues in animal products. So an immediate need of a study on this topic is of prime importance. This research is expected to give information regarding safety of meat sold at Dharan and to point out the need of immediate action to be taken in order to regulate antibiotics residue levels in meat.

1.5 Limitations and delimitations

1. Quantification of antibiotic residues present in meat samples couldn't be performed.
2. Only five of the antibiotic standards were taken for thin layer chromatography.
3. Screening of macrolide group of antibiotics wasn't done due to lack of *Micrococcus luteus* culture.

PART II

Literature review

2.1 Growing demand for poultry meat

Food is a basic human requirement. A balanced diet is required for a healthy body and mind as it is an important factor in growth, function, maintenance and repair of all the cells of our body. Both macro and micronutrients should be supplied in required amounts. These nutrients are supplied by a number of food stuffs including meat, cereal grains, milk, fruits and vegetables. Among these food items, meat holds a significant role in fulfilling most of the protein requirements of humans. Meat is one of the most significant, nutritious and energy-rich natural food product utilized by humans to fulfill their body requirements (Ahmad *et al.*, 2018). Although few epidemiological studies have also pointed a possible relationship between its consumption and the elevated risks of having cardiovascular diseases, various forms of cancers and metabolic disorders but still its role in the human species evolution, specifically in its brain and intellectual development cannot be ignored (Pereira and Vicente, 2013).

According to an estimate given by Population division of Department of Economic and Social Affairs of the United Nations, there are 7.7 billion people worldwide in 2019 and the projection indicates that it could grow to around 8.5 billion in 2030, 9.7 billion in 2050 and 10.9 billion in 2100 (UN, 2019). Similarly, it is estimated that about 70% people will be living in urban areas and income of people could increase by 2% a year by 2050 (Mottet and Tempio, 2017). With this growing population, income and urbanization, the demand for animal derived foods is estimated to grow by 70% between 2005 and 2050 (Alexandratos and Bruinsma, 2012). Among such foods, demand for poultry meat is expected to increase at the highest rate, by 121% (Mottet and Tempio, 2017). In addition to this, chicken meat can be taken as a healthy meat when compared against red meat. It contains relatively lesser amounts of fat than other red meats. Breast meat contains less than 3 g fat/100 g. About half of this fat is made up of the desirable monounsaturated fats, and only one-third of the less healthy saturated fats. Other red meats contain much higher amounts of saturated fats. Unlike beef and lamb meat, it contains no trans-fats that contributes to coronary heart disease. In addition to this, poultry meat is an important source of essential polyunsaturated fatty acids (PUFAs), especially the omega-3 fatty acids. Not only this, poultry meat can be easily

enriched with several other important nutrients such as vitamins and minerals. Similarly, cheap inputs, technological change and scale efficiency in recent decades have resulted in declining prices for livestock products making them available to low income people as well (FAO, 2009). All these improvements in production and productivity has converted the chicken meat that was once a scarce and expensive Sunday treat to the meat that is growing the fastest in consumption all around the world (McKenna, 2017). A report given by FAOSTAT (2016) showed a huge increase in global per capita consumption of poultry meat from 2.88 kg to 14.13 kg between 1961 and 2010.

2.2 Status of poultry industry around the Globe

Poultry industry is growing rapidly throughout the world. This increase in poultry production has occurred in two ways, both in terms of increase in number as well as increased output per animal (FAO, 2009). According to a report given by FAOSTAT (2016), the average carcass weight has increased by 30%, from 1.3 kg in 1961 to 1.7 kg in 2013 which shows a remarkable increase in productivity. Several technological advancements such as advanced feeding technology, genetic advancements, health improvements by increased use of vaccines and antibiotics, development of better breeds, etc. has played a vital role to improve the productivity (FAO, 2009). Globally, chickens are the most commercialized variety of the avian species. Similarly, they are also the most transformed animals because of the post-w mission to feed the world at any cost (McKenna, 2017). Such transformations have made chicken able to convert many feed types, such as residuals from agricultural activities, households and food processing industries, into protein food more efficiently than other animal species (Nkukwana, 2018).

Today, the world has over 23 billion poultry, about three birds per person on the planet. The biggest poultry meat producers are the United States, with almost 20 million tons a year, followed by China, with 18 million tons, the EU and Brazil with about 13 million tons (FAOSTAT, 2016).

2.3 Scenario of poultry industry in Nepal

Nepal is one of the best place of poultry rearing due to its rich biodiversity (Dhakal *et al.*, 2019). According to CBS (2015), commercial poultry farming started in Nepal after 2031 B.S. but the industry flourished only after 2061 B.S. Nepal lies at 112th position for chicken meat production of the world (FAO, 2014). According to Nepal commercial poultry survey

report by CBS (2015), there are a total of 21,956 commercial poultry farms/ farmers across the country out of which 93.29% of them are broiler farms, 6.09% are layers farms, 0.58% are hatcheries and 0.04% are Giriraj/ Kroiler farms. More than 55,871 permanent and 103,035 temporary employees are involved in poultry sector. A total of 60,826,880 birds and 114,058 metric ton chicken meat was produced in the year 2015. Commercial poultry farming is practiced in 64 districts of the nation among which Chitwan, Kavrepalanchowk, Dhading, Kathmandu and Kaski are the major broiler meat producers that produce 5,362,591 (10%), 4,141,428 (8%), 3,294,347 (6%), 2,488,013 (5%) and 2,102,427 (4%) broilers (number) respectively (CBS, 2015).

Poultry industry has become a major attraction to Nepalese farmers. It may be because of a higher success rate and better profitability than other sectors. More than 75% of the poultry farms in Nepal are in profit (CBS, 2015). Thus, poultry industry has been growing at a rapid rate of around 17-18% (FAO, 2014). According to MOALD (2074/75), production of chicken has been found to be increased from 16,662 metric tons to 60,122 metric tons within a decade from fiscal year 2008/09 to fiscal year 2017/18.

Poultry farms in the country include broiler farms, layer farms, hatcheries and Giriraj/ Kroiler farms. But the most common ones are broiler farms. 93.29% of the poultry farms are broiler farms. Out of 60,826,880 birds produced in the year 2015, 87% were broilers. Similarly, out of 114,058 metric ton chicken meat produced, 97.05% of the meat was broiler meat (CBS, 2015). This shows that broilers are the most common birds reared in Nepalese poultry farms.

2.3.1 Status of poultry industry in Sunsari

Sunsari is a district in Province no. 1 of Nepal. This province has a total of 3,561 (16.2%) commercial poultry farms/ farmers out of which 992 (27.8%) are located in Sunsari district. Sunsari is ranked the 4th district of Nepal for having a very large number of poultry farms with 992 farms (4.5%) after Chitwan, Kavrepalanchowk and Dang with 1920 (8.7%), 1078 (4.9%) and 1056 (4.8%) poultry farms respectively. Broiler farms are more common in this district too. There are 952 broiler farms, 38 layers farms and 2 hatcheries. 96% of the farms are meat producers. Most of the poultry farms rear upto 1000 birds. 561 broiler farms have a capacity upto 1000 birds, 397 between 1,001-5,000 and 2 farms with capacity 5,001-10,000. In the year 2015, 229,931 birds were produced out of which 77.9% were broilers

and 22.1% were layers. 247124kg broiler meat and 35,214 kg layers meat was produced (CBS, 2015).

2.4 Antibiotics

Antibiotics are natural products of a micro-organism, identical synthetic products or similar semi-synthetic products that inhibit the growth of or destroy microorganisms (Kirbis, 2007). Antibiotics are probably one of the most successful forms of chemotherapy in the history of medicine. They have saved millions of lives and have controlled the majority of infectitious diseases that plagued human history for many centuries (Aminov, 2009). Antibiotics fall into five classes: penicillins, tetracyclines, macrolides, aminoglycosides and amphenicols. Although included under the topic antibiotics, sulfonamides, nitroimidazoles, nitrofurans and quinolones are not antibiotics as they are synthetic (Kennedy *et al.*, 1998).

2.4.1 Classification of antibiotics

A. Based on the nature of antimicrobial action

1. Bacteriostatic antibiotics: These drugs suppress the growth and multiplication of bacteria. Example: Sulphonamides, tetracyclines, chloramphenicol, erythromycin

2. Bactericidal antibiotics: These drugs cause death of bacteria. Example: penicillin, streptomycin, colistin, bacitracin, kanamycin, etc.

B. Based on antimicrobial spectrum

1. Narrow spectrum: These antimicrobials are effective against a small group of bacteria.

a. Against gram positive: penicillin, erythromycin, lincomycin

b. Against gram negative: streptomycin, gentamycin

2. Broad spectrum: These antibiotics are effective against both gram positive and gram negative bacteria. Example: Tetracycline, chloramphenicol, amoxicillin, cephalosporin.

C. Based on mechanism of action

Peach *et al.* (2013) have classified antibiotics into several classes based on their mode of action which are shown in Table 2.1.

Table 2.1 Classification of antibiotics based on their mode of action

S.N.	Antibiotic Class	Subclass	Antibiotic Name	Primary Target
1.	Cell wall synthesis inhibitors/ disruptors	β- Lactams	Penicillins (piperacillin, carbenicillin, ampicillin, penicillin G, coxacillin), cephalosporins (cefadroxil, cefaclor, ceftazidime)	Penicillin- binding proteins
		Lipopeptides	Polymyxin B	disruption of inner and outer membranes through binding to lipopolysaccharides in the outer membrane
		Others	Alafosfalin	Peptidoglycan units terminal D- Ala- D- Ala dipeptide
			Bacitracin	C_{55}- isoprenyl pyrophosphate
			D- cycloserine	D- alanine ligase and alanine racemase
			Fosfomycin	UDP- N-acetylglucosamine- 3-enolpyruvyltransferase
2.	DNA synthesis inhibitors	Fluoroquinolones	Nalixidic acid, Ciprofloxacin, Levofloxacin, Sparofloxacin, Norfloxacin	Topoisomerase II, topoisomerase IV
		Sulfonamides	Sulfamethazine, Sulfapyridine, Sulfamethoxazole, Sulfadiazine, Sulfamerazine	Competitive inhibitor for DHPS involved in folate synthesis
		Others	Novobiocin	DNA gyrase
3.	RNA synthesis inhibitor	Rifamycins	Rifampicin, Rifabutin, Rifaximin	DNA- dependent RNA-polymerase
4.		Tetracyclines	Oxytetracyclines, Doxycyclines,	30S ribosomes (inhibit tRNA binding to ribosome)

			Tetracyclines, Demeclocyclines, Minocycline	
		Aminoglycosides	Tobramycin, Gentamycin, Amikacin, Streptomycin, Spectinomycin	30S ribosome (mistranslation by tRNA mismatching)
	Protein synthesis inhibitor	Macrolides	Erythromycin, Clarithromycin, Midecamycin, Roxithromycin, Spiramycin, Azithromycin	50S ribosome (stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation)
		Amphenicols	Chloramphenicol, Thiamphenicol, Florfenicol	50S ribosome (inhibit elongation step)
		Lincosamides	Clindamycin, Lincomycin	50S ribosome (stimulate dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation)
		Pleuromutilins	Tiamulin	50S ribosome (prevent correct positioning of the CCA ends of tRNA for peptide transferase)
5.	DNA replication (Intercalators)	Anthracyclines	Doxorubicin, Epirubicin, Idarubicin	Intercalate DNA/ RNA strand and topoisomerase II
		Others	Actinomycin D	Intercalates G-C base pairs and minor groove DNA at the transcription initiation complex
			Mithramycin	Intercalates GC- rich DNA strands
			Tetracenomycin	Intercalates DNA
6.	Anaerobic DNA inhibitors	Nitrofurans	Furazolidone, Nitrofurantion	Highly reactive reduced form (by nitrofuran reductase)
		Nitro- imidazole	Ornidazole	Damages bacterial DNA

Source: Peach *et al.* (2013)

2.4.2 History of use of antibiotics in livestock production

A large scale production of Penicillin was implemented for the first time during the Second World War for the treatment of casualties in the war. During the latter stages of the war, lyophilized penicillin preparations were made available to veterinarians and were used for treatment of bovine mastitis. This treatment was proved to be far more effective than the previous treatments for dairy animals (Gustafson and Bowen, 1997). Antibiotics were first added to animal feeds in the late 1940s (McKenna, 2017). Moore *et al.* (1946) reported that antibiotic streptomycin, when added to the diet of chicks, could improve their growth. Stokstad and Jukes (1950) observed an improvement in weight gain of chickens and reduction in feed required to bring broilers to market weight which was a result of chlorotetracycline. The same broad spectrum antibiotics that promoted growth and feed efficiency at low levels was also shown to control endemic diseases in large groups of animals and poultry. As the cost of antibiotics came down, these uses became practical. Confinement rearing enhanced the transmission of infectious agents and provided opportunities to mass treat large number of animals at risk, particularly young, newly for weaned animals or those experiencing other types of stress. A few animals appearing sick in a herd or flock often provided diagnostic information that allowed successful prophylactic medication of the whole herd for a brief period, a practice which was both efficient and effective in maintaining herd health (Gustafson and Bowen, 1997).

Since then, antibiotics have been used in animal husbandry for chemotherapeutic, prophylactic purposes and also as feed additives to promote growth, improve feed efficiency, and breeding performance, and enhance feed acceptability (Chowdhury *et al.*, 2009; Lee *et al.*, 2001).

2.4.3 Role of antibiotics in poultry industry

Antibiotics and other drugs are administered to livestock at therapeutic, prophylactic, or sub-therapeutic concentrations. Therapeutic administration (220-1100 ppm) is used for disease prevention where animals are administered with high doses of antibiotics for relatively shorter periods. Prophylactic doses (110-440 ppm), where the animals are subjected to moderate doses for longer time durations, are used to prevent infectious diseases caused by bacteria and protozoa, whereas sub-therapeutic administration, where animals are subjected to very less doses of antibiotics for a very long duration or throughout the entire lifespan of

animals, is often used to increase feed efficiency and growth promotion (Deshpande, 2002; Mund *et al.*, 2017).

The use of antibiotics combined with strict biosecurity and hygiene measures has helped the poultry industry to reach a height that had never been reached before (Bermudez, 2008). Several groups of antibiotics including quinolones and fluoroquinolones are frequently used in veterinary medicine for treatment and prevention of diseases, thereby reducing famers' losses (Omotoso and Omojola, 2015). Use of such drugs also improve the rate of weight gain and improve feed efficiency. Thus, use of veterinary drugs has played a vital role to meet the challenge of providing adequate amounts of food for the growing world population (Beyene, 2016).

Although the mechanisms of action by which veterinary drugs cause growth and feed enhancement are not well understood, it is likely that these drugs exert their effects on animals in four different ways:

2.4.3.1 Disease prevention

Modern approaches for efficient poultry production, such as intensive rearing conditions with high stock densities, have provided ideal conditions for manifestation and transmission of several parasitic and viral diseases. Incidences of occurring these diseases have become more frequent, pronounced, unmanageable as well as difficult to control (Piatkowska *et al.*, 2012). The most prevalent diseases in poultry are typhoid, mycotoxicosis*, E. coli* infections, coccidiosis, Salmonellosis, enteritis, ascites, Newcastle disease, Marek's disease, hydropericardium syndrome, and Gumboro disease (Yunus *et al.*, 2009). Occurences of such diseases not only influences poultry growth and production, but also to the economic losses due to high mortality (Bera *et al.*, 2010; Chapman and Jeffers, 2014). The use of antibiotics can prevent such diseases by controlling zoonotic pathogens like *Salmonella*, *Campylobacter*, *Escherichia coli* and *Enterococci* (Hughes and Heritage, 2004). The use of drugs suppress the microorganisms responsible for mild but unrecognized infections. Microbial production of growth depressing toxins is also reduced (Deshpande, 2002).

Antibiotics such as tetracycline, gentamicin, neomycin, tylosine, erythromycin, virginiamycin and bacitracin are mostly used to reduce and prevent respiratory diseases and necrotic enteritis infections (Mund *et al.*, 2017). Fluoroquinolones and/or quinolone compounds are used for treating gastroenteritis, skin or soft tissue infections (Sarkozy, 2001;

Soni, 2012). Sulfonamide compounds are administered as preventive and chemotherapeutic agents against coccidiosis, fowl typhoid, coryza, and pullorun diseases (Mund *et al.*, 2017). Similarly, piperazine, oxytetracycline, amoxicillin, amprolium, ciprofloxacillin, and sulfa drugs are used to treat coccidiosis (Beyene *et al.*, 2015).

2.4.3.2 Growth promotion

According to Marshall and Levy (2011), the use of antimicrobial agents as growth promoters began in the mid-1950s. Since then, antibiotics such as tetracyclines, chloramphenicol, penicillin, virginiamycin, avoparcin, tylosin, etc have been extensively used at sub-therapeutic doses as growth promoters (Chowdhury *et al.*, 2009; Mund *et al.*, 2017). Several drugs when used in low doses in animal feeds increases the protein deposition by decreasing the fat content in the carcass and increasing meat leanness. It allows better feed efficiency and leaner meat (Hughes and Heritage, 2004; Lone, 1997). Antibiotics, such as penicillins, inhibit the growth of many gram-positive organisms, a process that leads to development of increased numbers of *Escherichia coli* and other beneficial intestinal bacterial flora that play an important role in the synthesis of many essential vitamins and amino acids. Tylosin has been reported to reduce population of *Lactobacilli* species (produce bile hydrolase salts) in ileum of chickens that increases the relative abundance of conjugated bile salts, thus promoting lipid metabolism and increase in weight gain (Lin *et al.*, 2013). Providing antimicrobial drugs in feed on a continuous basis makes the intestinal wall structure thinner and more adaptive due to which the efficiency of absorption and utilization of nutrients is greatly enhanced (Deshpande, 2002; Nirala *et al.*, 2018). It also relieves the animal from the need to produce catabolic hormones responsible for the wastage of muscles in order to prevent infections (Chattopadhyay, 2014).

2.4.3.3 Metabolic effects

Use of veterinary drugs is also found to contribute to modification of metabolic reactions. Tetracycline, for example, has been shown to affect water and nitrogen excretion (Deshpande, 2002).

2.4.3.4 Nutrient-sparing effect

A huge number of microorganisms are present in intestinal tract of birds and they compete with host animal for essential nutrients. Use of antimicrobial drugs depresses these

microorganisms and thus increases the nutrient availability through chelation and/or increasing their absorption from the GI tract (Deshpande, 2002).

2.4.4 Commonly used antibiotics in poultry industry

In intensive poultry farming, especially in North America, antibiotics such as tetracycline, bacitracin, tylosin, salinomycin, virginiamycin and bambermycin are often used (Diarra and Malouin, 2014). Tetracyclines represent more than two-thirds of antimicrobials administered to animals in the United States (Ronquillo and Hernandez, 2017). Antimicrobial classes used as therapeutics in the poultry industry include: aminoglycosides, tetracyclines, beta-lactams, fluoroquinolones, macrolides, polypeptides, amphenicols, sulphonamides and trimethoprim (Stolker and Brinkman, 2005).

The broad-spectrum beta-lactams such as amoxicillin are more effective for Gram-negative infections such as *E. coli* airsacculitis. Ceftiofur is the only cephalosporin approved for use in poultry in the United States (Tazrin, 2014). Three aminoglycosides are used in poultry: gentamicin, neomycin and streptomycin. Neomycin is commonly used to treat enteric infections and is administrated either in feed or water. Gentamicin is the most widely used aminoglycoside and it is used subcutaneously in day-old chicken or turkey chicks. Streptomycin is partially absorbed from the intestine and therefore can be used to treat systematic *E. coli* infections. Spectinomycin is highly effective for *E. coli* infections when combined with lincomycin (Smith *et al.*, 2007).

Quinolones are used in poultry against many gram-negative bacteria (Stolker and Brinkman, 2005). The fluoroquinolones are second generation quinolones that are highly effective against gram-positive, gram-negative and *Mycoplasma* infections. Enrofloxacin a fluoroquinolone with a good respiratory tract distribution, can eliminate *Mycoplasma gallisepticum* infection in laying hens (Smith *et al.*, 2007).

The tetracyclines are the most widely used antimicrobials in poultry. This is largely due to their affordability, a wide margin of safety and broad-spectrum (*Mycoplasma*, gram-positive and gram-negative bacteria) and intracellular activity. The three tetracyclines most commonly used in poultry are chlortetracycline, oxytetracycline and doxycycline (Smith *et al.*, 2007).

Bacitracin is the only poultry approved polypeptide antimicrobial. Bacitracin is very effective for treatment of enteric infections caused by *Clostridium perfringens* (Hofacre *et al*., 1998). It is also used as a performance enhancer in broilers (Phillips, 1999).

Sulphonamides are bacteriostatics that are used as veterinary drugs for prophylactic and therapeutic purposes, they also act as growth-promoting substances and are commonly administrated in drinking water as coccidiostats. Trimethoprim is a potentiator when administered together with sulphonamides as both act on different enzymes in the folic acid metabolic pathway (Balizs and Hewitt, 2003). Erythromycin is most frequently used in poultry to treat *Staphyloccus aureus* infection. Tylosin and tiamulin are considered to be highly effective in the treatment of *Mycoplasma* infections in laying hens to restore egg production and reduce transovarian transmission. The only poultry approved lincosamide is lincomycin, it is primarily used to treat infectious coryza and infectious synovitis. It is commonly used to treat *Clostridium perfringens* induced necrotic enteritis and also to enhance poultry performance (Smith *et al.*, 2007).

Awogbemi *et al.* (2018) concluded that penicillin (30%), amoxicillin (15%), tetracyclines (33.3%), oxytetracyclines (48.3%), doxycyclines (28.3%), streptomycin (16.7%), neomycin (66.7%), chloramphenicol (45%), cotrimoxazole (71.7%), gentamycin (5%) and erythromycin (28.3%) as majorly used antibiotics in poultry farms in Nigeria. Similarly, Wongsuvan *et al.* (2017) also reported timilcosin, doxycycline, amoxicillin, colistin and oxytetracyclines as major antibiotics being used in broiler production in Thailand. The most common antibiotics used in broiler production in Mymensingh district of Bangladesh were found to be ciprofloxacin (22.5%), followed by enrofloxacin (17.5%), amoxicillin (16.66%), oxytetracycline (10.83%), sulfa drugs (3.33%), and norfloxacin (1.66%) (Ferdous *et al.*, 2019).

2.4.5 Poultry diseases and antibiotic usage in Nepal

In Nepal, the major diseases affecting poultry are salmonellosis, fowl typhoid, colibacillosis and mycoplasmosis. Sulphonamides, neomycin, tetracyclines, amoxicillin and fluoroquinolones are used to treat salmonellosis. Similarly, tetracyclines and sulfa drugs are used to treat colibacillosis (GARP-Nepal, 2015). According to the report of CVL (2015), during the fiscal year 2071/72, the most common diseases identified on postmortem examination of poultry carcass are shown in Table 2.2. Discrete data regarding quantities of

antibiotics used in poultry industry in Nepal is not available. GARP-Nepal (2015) stated that in 2001, antibiotics accounted for 13% of total drug and feed supplement sales. The quantities of antibiotics consumed annually are shown in the Table 2.3. Antibiotics such as chlortetracycline, furazolidone, bacitracin methylene disalicylate, tylosine tartarate, lincomycin, neomycin, doxycycline, colistin sulphate, tetracycline and tiamutin are widely used in poultry feed as additives or growth promoters in Nepal (Acharya and Wilson, 2019). Table 2.4 shows usage of antibiotics in poultry feed in Nepal. A study in 2013 reported that 71% of veterinary drug sales were based on self-prescription by retailers (GARP-Nepal, 2015).

Table 2.2 Common poultry diseases identified during postmortem inspection of poultry carcass

S.N.	Laboratory	Common diseases
1	Central Veterinary Laboratory, Kathmandu	Colibacillosis, cCRD, Infectious Bursal Disease and Mycotoxicosis, coccidiosis, CRD
2	National Avian Disease Investigation Laboratory, Chitwan	Fowl cholera, CCRD, Salmonellosis, Collibacillosis, Mycotoxicosis
3	Regional Veterinary Laboratory, Biratnagar	IBD, CRD, Colibacillosis, mycotoxicosis, ND, mycoplasmosis, CCRD, salmonellosis
4	Regional Veterinary Laboratory, Janakpur	Colibacillosis, ascites, CRD, coccidiosis, Infectious Bursal Disease, salmonellosis, mycotoxicosis
5	Regional Veterinary Laboratory, Pokhara	Colibacillosis, mycotoxicosis, Infectious Bursal Disease, coccidiosis
6	Regional Veterinary Laboratory, Surkhet	IBD, colibacillosis, CRD
7	Regional Veterinary Laboratory, Dhangadhi	Coccidiosis, IBD, IBH, Colibacillosis, CRD, Mycotoxicosis, Asitis, Gout & Fatty liver syndrome

Source: CVL (2015)

2.4.6 Pharmacokinetics of antibiotics

The term pharmacokinetics refers to the movement of drug into, through and out of the body: the time course of its absorption, bioavailability, distribution, metabolism, and excretion. It is important in order to estimate how long an antibiotic takes to be depleted from the animal to safe levels and also to determine tissues in which the drug distribute or accumulate (Craigmill *et al.*, 1991).

Table 2.3 Antibiotics usage in animals in Nepal

S. N.	Antibiotics	Quantity (tons)
1	Tetracyclines	7,899
2	Enrofloxacins	529.1
3	Neomycin+doxycycline	229.47
4	Ampicillin	137
5	Tiamutin	109.46
6	Cephalexin	92
7	Ampicillin+coxacillin	90.02
8	Doxycycline+colistin sulphate	88.38
9	Gentamicin	75.53
10	Tylosin	71
11	Penicillin+streptomycin	48.97
12	Flumequin	16.96
13	Chloramphenicol	16.5

Source: GARP-Nepal (2015)

Drugs can be administered either intravenously, intramuscularly, subcutaneously, orally or by tropical routes (Craigmill *et al.*, 1991). The drugs are then absorbed from the site of administration into the bloodstream (Beyene, 2016). Antibiotics such as doxycycline are rapidly but partially absorbed from GI tract and because of their high lipid solubility, it is readily available for tissue distribution (Anadon *et al.*, 1994). They are then transported throughout the body via blood plasma (Mund *et al.*, 2017). Most of the antibiotics used in poultry production are administered in drinking water or incorporated in feed. It was reported that following administration these drugs are rapidly absorbed from the gastrointestinal tract of the chicken (Alhendi *et al.*, 2000). Distribution of these drugs to peripheral tissues is dependent upon physicochemical properties of the drug (pKa, lipid solubility and molecular

weight), the concentration gradient established between the blood and tissue, the ratio of blood flow to tissue mass and the affinity of the drug for tissue constituents. Lipid soluble drugs tend to distribute to adipose tissues because of their high lipid content. Most of the drugs are distributed rapidly to offal like liver and kidney because of higher blood flow to these organs (Aerts *et al.*, 1995; Craigmill *et al.*, 1991; Faten *et al.*, 2016; Islam *et al.*, 2016; Sahid *et al.*, 2007).

Table 2.4 Antibiotics used in poultry feed in Nepal

S.N	Antimicrobials	Mixing rate
1.	Bacitracin methylene	500g to 1 kg/ton
2.	Neomycin	500g to 1 kg/ton
3.	Doxycycline	500g to 1 kg/ton
4.	Chlorotetracycline	500g to 1 kg/ton
5.	Tylosin	500 g/ton
6.	Lincomycin	250-500 g/ton
7.	Colistin sulfate+ Doxycycline	500 g/ton
8.	Tetracycline+ Tiamutin	1-2 kg/ton
9.	Bacitracin+ Lincomycin+ Colistin sulfate	250-500 g/ton

Source: Ramdam (2015)

Finally, the drugs are eliminated from the body. Liver (hepatic mechanism) and kidneys (renal mechanism) are the most important organs involved in excretion of drugs. Drugs are excreted unchanged into bile or urine or are metabolized to more water-soluble compounds for subsequent excretion (Craigmill *et al.*, 1991). Most of the drugs such as sulfonamides and β-lactams are excreted unchanged and hence, there is a greater risk of dissemination of these excreted drugs to other untreated food animals through feed, water and environmental contamination (Herrera, 2010). Antibiotics like β- lactams, quinolones are rapidly absorbed from GI tract and are excreted by liver and kidneys (Dorrestein *et al.*, 1984; Goetting *et al.*, 2011). Aminoglycosides such as streptomycin are very slightly absorbed from the GI tract and thus excreted mostly in faeces (Dorrestein *et al.*, 1984).

Various antibiotics require different time periods to be eliminated from the body. This time period is identified as withdrawal period (WP) for the particular antibiotic. The length of WP depends on the dosage form, antibiotic type, and method of administration (Al-

mashhadany, 2019). Anadon *et al.* (1990) concluded that quinolones persist in chicken body for a relatively longer time period. Irrespective of the route or purpose of administration, antimicrobials can accumulate as residues in tissues, before they are completely metabolized or excreted from the body (Okocha *et al.*, 2018).

2.4.7 Safety aspects of antibiotics

2.4.7.1 Withdrawal period for Antibiotics

The withdrawal periods is the time which passes between the last dose given to the animal and the time when the level of residues in the tissues (muscle, liver, kidney, skin/fat) or products (milk, eggs and egg) is lower than or equal to the MRL/safe level (Khatun *et al.*, 2018). It depends upon drug, dose, formulation, route of administration, species, target tissues and diseases or management factors. These factors influence the way the drug moves in the animal body and how soon it will be eliminated (Tazrin, 2014). Antibiotics such as tetracyclines undergo extensive intrahepatic circulation due to which they stay in the body for a longer period of time after the secession of drug administration (Al-Bahry *et al.*, 2013; Hsiao *et al.*, 2016; Papich and Riviere, 2017). Withdrawal periods for some commonly used antibiotics as suggested by different workers is shown in Table 2.5.

2.4.7.2 Extra-label drug use (ELU)

When drugs are used in the approved manner in approved species, these legal withdrawal

Table 2.5 Withrawal periods for commonly used antibiotics in poultry

Antibiotics	Withdrawal period	Reference
Oxytetracycline	7 days	(Khatun *et al.*, 2018)
Sulphadimidine	5 days	(Alhendi *et al.*, 2000)
Ampicillin	6 days	(Alhendi *et al.*, 2000)
Enrofloxacin	10 days	(Khatun *et al.*, 2018)
Ciprofloxacin	10 days	(Khatun *et al.*, 2018)
Sulfonamide	4 days	(Khatun *et al.*, 2018)
Chloramphenicol	14 days	(Khatun *et al.*, 2018)
Gentamycin	14 days	(Khatun *et al.*, 2018)
Doxycycline	9 days	(Mestorino *et al.*, 2018)
Norfloxacin	12 days	(Interchemie, 2019)
Amoxicillin	7 days	(Khattab *et al.*, 2010)

times are generally sufficient. However, occasionally drugs must be used at extra-label doses, in non-approved species, or are used inadvertently at excessive dose levels (Craigmill *et al.*, 1991). ELU refers to the use of an approved drug in a manner that is not in accordance with the approved label directions. ELU occurs when a drug approved for one species of animal is used in another, when a drug is used to treat a condition for which it was not approved, or the use of drugs at levels in excess of recommended dosages (Beyene, 2016).

2.4.7.3 Maximum residue limits

The Maximum Residue Limit (MRL) is the maximum allowable level or concentration of a chemical in feed or food at a specified time of slaughter or harvesting, processing, storage and marketing up to the time of consumption by animal or human (Lee *et al.*, 2001). The standards on MRL for antibiotic residues in meat as given by Veterinary Standards and Drugs Administration Office, Nepal are given in Table 2.6. Similarly, the MRLs for antibiotics used in poultry allocated by the Codex Alimentarius Commission are shown in Table 2.7.

The MRLs is based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the ADI, or on the basis of a temporary ADI that utilizes an additional safety factor. In calculating an MRL, the ADI, the residue depletion patterns of a compound in the edible tissues of a particular food-producing animal and the theoretical food intakes are taken into account. Possible persistence of residues in organs or at the injection site is also considered (Fitzpatrick *et al.*, 1995).

2.4.8 Impacts of misuse of antibiotics

2.4.8.1 Occurrence of antibiotic residues in meat products

Veterinary medicines and chemicals used according to the label directions should not result in residues at slaughter. However, possible reasons for such residues include: Not following recommended label directions or dosage (extra- label usage); not adhering to recommended withdrawal times; administering too large volumes of drugs at a time; use of drug contaminated equipment, or failure to clean properly the equipment used in mixing or administering drugs; dosing, measuring or mixing errors; allowing animals to access medicated feeds; animal effects such as age, illness, allergies, etc. (Beyene, 2016). Antibiotic residues in foods of animal origin (eggs, meat and milk) that are meant for human consumption are one of the sources of concern among public and medical health

professionals(Shareef *et al.*, 2009). This is because man is the ultimate consumer of these toxic antibiotic residues in these products (Dipeolu and Alonge, 2002; Donoghue, 2003; Shareef *et al.*, 2009).

Table 2.6 The standards on MRL for antibiotic residues in meat in Nepal

Drugs	Drugs concentration (µg/kg/L)			
	Muscle	Liver	Kidney	Fat
Albendazole	100	5000	5000	100
Benzyl Penicillin/ Procaine Penicillin	50	50	50	-
Ceftiofur	1000	2000	6000	2000
Colistin	150	150	200	150
Chloramphenicol	100	100	100	100
Cypermethrin	50	50	50	1000
Deltamethrin	30	50	50	500
Dexamethasone	1	2	1	-
Erythromycin	100	100	100	100
Fenbendazole/ Oxfendazole	100	500	100	100
Gentamycin	100	2000	5000	100
Ivermectin	-	15	-	20
Levamisole	10	100	10	10
Spectinomycin	500	2000	5000	2000
Spiramycin	200	600	800	300
Sulfadimidine	100	100	100	100
Tetracycline	200	600	1200	-
Thiabendazole	100	100	100	100
Triclabendazole	200	300	200	100
Tylosin	100	100	100	100
Streptomycin	-	-	-	-
Neomycin	-	-	-	-
Ampicillin	-	-	-	-

Source: VSDAO (2017)

Table 2.7 The MRLs for antibiotics used in poultry allocated by the Codex Alimentarius Commission

Drugs	Drugs concentration (μg/kg)			
	Chicken Muscle	Chicken Liver	Chicken Kidney	Chicken Fat
Albendazole	100	5000	5000	100
Ampicillin	200	300	200	200
Benzylpenicillin	50	50	50	-
Chlorotetracycline/ Oxytetracycline/ Tetracycline	200	600	1200	-
Colistin	150	150	200	150
Danofloxacin	200	400	400	100
Streptomycin	600	600	1000	600
Erythromycin	100	100	100	100
Flubendazole	200	500	-	-
Flumequine	500	500	3000	1000
Levamisole	10	100	10	10
Lincomycin	200	500	500	100
Monensin	10	10	10	100
Narasin	15	50	15	50
Neomycin	500	500	10000	500
Nicarbazin	200	200	200	200
Sarafloxacin	10	80	80	20
Spectinomycin	500	2000	5000	2000
Spiramycin	200	600	800	300
Sulfadimidine	100	100	100	100
Tilmicosin	150	2400	600	250
Tylosin	100	100	100	100

Source: CAC (2018)

The main reason reported for drug residues occurrence is failure to observe the withdrawal times and early slaughter of animals. Impaired liver or renal function can result

in incomplete elimination of the parent compound or its active metabolites from the animal's system. Administration of a number of drugs in a short time period can affect drug elimination from the body, due to inhibition of hepatic enzymes essential for drug metabolism. Improper injection can result in deposition of the antibiotic, and the rate of elimination from the body is reduced. Also, in intensive farming systems where antibiotics are administered in drinking water or medicated feed, carry-over can result in the presence of residues in the finishing feed (Grane, 2000).

2.4.8.1.1 Status of antibiotic residues in poultry meat in Nepal

Several workers have attempted to determine the antibiotic residues in poultry meat samples collected from different places of Nepal. Maharjan *et al.* (2020) performed antibiotic screening test in marketed broiler meat of Kathmandu valley using rapid test kits. Out of a total of 300 samples tested, 74 (24.66%) were found to be positive. 40% of liver samples and 10.66% of muscle samples were positive to antibiotics. Tetracycline group of antibiotics were present in 5.33% muscle samples and 22.66% of liver samples. Similarly, macrolides, aminoglycosides and sulphonamide group of antibiotics were present in 16% muscle samples and 54.66% liver samples. Among the positive samples, 71.62% samples exceeded the MRLs.

Another study regarding screening of antibiotic residues in poultry in Kathmandu valley was performed using disc assay method taking *E. coli* and *Staphylococcus aureus* as test organisms. 13% of the samples were reported to be positive. Among the samples, 16.67% of muscles and 10% of liver samples were reported as positive (Sapkota *et al.*, 2019).

Shrestha (2017) used ELISA technique to screen quinolone residues in poultry meat of Kathmandu valley and found 88.33% of the samples to be positive. On performing HPLC analysis, 3 samples were found to contain enrofloxacin above MRLs and one of the sample was found to contain ciprofloxacin residues above MRLs.

Using rapid test kits, Gwachha (2017) screened antibiotics in poultry meat samples from Kathmandu valley and found that 50.48%, 21.9% and 18.1% of the samples contained tetracycline, sulfonamide and penicillin residues respectively.

Raut *et al.* (2017) made a study on antibiotic residues in marketed meat of Kailali and Kavre of Nepal. A total of 55 samples (41 muscle samples and 14 liver samples) were

collected from different retail shops of Kavre and Kailali. Antibiotics penicillin, tetracycline and aminoglycosides, macrolides and sulfonamides were tested using test kit obtained from G9 Co. Ltd. It was found that 22% of samples were found positive for atleast one of the antibiotic tested. Tetracycline was detected in a maximum of 16 samples followed by macrolides, sulphonamides and aminoglycosides in 13 samples and finally penicillin in a minimum of 7 samples. It was also found that out of 41 muscle samples and 14 liver samples, 16 (39%) muscle samples and 10 (71%) were found positive for antibiotic residues. It showed the prevalence of antibiotic residues to be higher in liver than in muscles.

Similarly, another research on antibiotic residues in marketed broiler meat of Gorkha, Parsa, Chitwan and Kathmandu districts by (Rawal and Manandhar). A total of 80 samples, 40 muscle samples and 40 liver samples, were tested for the antibiotics penicillin, tetracycline, aminoglycosides and quinolones by using rapid test kits protocols. It was observed that 35%, 17.5%, 40% and 0% samples were positive for tetracycline, aminoglycosides, penicillin and quinolones respectively.

Another study was conducted by Pandey *et al.* (2009) to detect the antimicrobial drug residues in liver, kidney and breast meat samples collected from Chitwan and Kathmandu by using modified EU four plate test. Among them, 18.91% were found positive. The antimicrobial residues in liver, kidney and breast meat were 17.12%, 26% and 13.62% respectively. This study detected the residues of tetracyclines, β-lactams, sulfonamides, aminoglycosides, macrolides and fluoroquinolones that were found to be 33.95%, 26.45%, 20.41%, 7.91% and 5.83% respectively.

Prajapati *et al.* (2018) performed a screening test of poultry meat samples collected from Kathmandu, Kaski and Chitwan for antibiotics using ELISA technique. Out of 92 samples, 57 (62%) samples were found positive for antibiotics residue of which 38% samples were positive for strepromycin residue, 15.2% for ciprofloxacin and 8.7% for enrofloxacin residues.

Another study was performed by Pantha *et al.* (2019) to screening antibiotic residues in broiler meat sold in Kathmandu valley using test kits. 30.81% of total meat samples collected were found to contain antibiotic residues. The residues of tetracycline, macrolide/aminoglycoside/sulfonamide and penicillin were found 33.33%, 41.67% and 17.42% respectively in marketed broiler meat.

2.4.8.2 Development of drug resistance

During the growth of microorganisms they adapt to their environment. If some antimicrobial stops them from growing and spreading they evolve new mechanisms to resist the antimicrobials by changing their genetic structure. Bacterial resistance to antimicrobial agent may be due to inability of antimicrobial to reach the target or the target site may be altered so that the antimicrobial agent cannot bind to it. The failure of the drug to reach the target site may be due to impermeability of the bacterial cell membrane that will prevent influx of the drug. Hydrophilic antibiotics are transported across the cell membrane via aqueous channels or pores made up of specific proteins called porins. Some bacteria are deficient in these channels and hence resistant to these antimicrobial agents (Tazrin, 2014). Microbes may acquire resistance mainly by four methods- mutation, conjugation, transduction and transformation (McGowan, 2001). It can be spontaneously through mutation. Also, horizontal gene transfer (HGT) can allow the transfer of antibiotic resistance among different species of bacteria by transfer of mobile genetic elements such as plasmids (Read and Woods, 2014).

Disease organisms have been developing defenses against the antibiotics meant to kill them for as long as antibiotics have existed. Penicillin arrived in the 1940s, and resistance to it swept the world in the 1950s. Tetracycline arrived in 1948, and resistance was nibbling at its effectiveness before the 1950s ended. Erythromycin was discovered in 1952, and erythromycin resistance arrived in 1955. Methicillin, a lab-synthesized relative of penicillin, was developed in 1960 specifically to counter penicillin resistance, yet within a year, staph bacteria developed defenses against it as well, earning the bug the name MRSA, methicillin-resistant *Staphylococcus aureus*. After MRSA, there were the ESBLs, extended-spectrum beta-lactamases, which defeated not only penicillin and its relatives but also a large family of antibiotics called cephalosporins. And after cephalosporins were undermined, new antibiotics were achieved and lost in turn. Each time pharmaceutical chemistry produced a new class of antibiotics, with a new molecular shape and a new mode of action, bacteria adapted (McKenna, 2017).

What slows the emergence of resistance is using an antibiotic conservatively: at the right dose, for the right length of time, for an organism that will be vulnerable to the drug, and not for any other reason. Most antibiotic use in agriculture violates those rules. Resistant bacteria are the result (Grane, 2000).

Several workers have demonstrated antibiotic resistance in microbes isolated from poultry. Khanal *et al.* (2017) reported that *E. coli* isolated from poultry in VTH and AFU of Nepal were resistant most substantially towards cephalexin (81.2%) and amoxycillin (81.2%) followed by tetracycline (78.8%), colistin sulphate (62.5%). chloramphenicol (61.2%), ciprofloxacin (55.0%), enrofloxacin (53.8%) and levofloxacin (28.8%).

A study on antibiotic resistance of bacterial isolates of poultry collected from postmortem unit of National Avian Disease Investigation Laboratory, Chitwan, Nepal showed that 100%, 80%, 79.4%, 75.6%, 57.1%, 54.3%, 45.7%, 23.1%, 19.4%, 12.5%, 6.7% and 4.3% of the isolates are resistant to bacitracin, gentamycin, cotrimoxazole, cephalosporins, tetracyclines, neomycin, doxycyclines, azithromycin, ciprofloxacin, chloramphenicol, levofloxacin and amikacin respectively (CVL, 2012).

Another study of poultry meat samples from retail shops in Kathmandu in 2007 investigated the prevalence of multi-drug resistant *Salmonella*. Isolates showed resistance to tetracycline and nalidixic acid. Multi-drug resistance was observed in only 4% of *Salmonella* isolates (GARP-Nepal, 2015).

Similarly, Bantawa *et al.* (2019) studied the antibiotic resistance patterns of bacterial isolates from chicken, pork, buffalo and goat meat in eastern Nepal. It was found that 100%, 24%, 11% and 11% of *Salmonella* isolates were resistant to amoxicillin, tetracycline, chloramphenicol and nalidixic acid respectively. 100%, 80%, 60% and 20% of *E. coli* isolates were resistant to amoxicillin, tetracyclines, nalidixic acid and cefotaxime respectively. 100%, 63%, 17% and 13% *Staphylococcus aureus* isolates were resistant against amoxicillin, tetracyclines, nalidixic acid and cefotaxime respectively. The study showed very high resistance of isolates against amoxicillin. Resistance to tetracyclines was also found to be quite high.

Another study regarding antibiotic resistance in gram negative bacterial isolates from chicken meat in Bharatpur, Chitwan. It was found that 100%, 84.6% and 84% of *Salmonella* isolates were resistant to ampicillin, nitrofurantoin and doxycycline hydrochloride respectively. 32.6%, 19.5%, 19.5% *Citrobacter* isolates were resistant against imipenam, cefotaxime and ciprofloxacin respectively. *Proteus* isolates revealed 29.4% and 11.7% resistivity to imipenam and ciprofloxacin respectively. Similarly, *E. coli* isolates showed

100%, 80% and 80% resistivity to ampicillin, colistin and polymyxin B respectively (Shrestha *et al.*, 2017).

Dhakal et al. (2016) reported that the *Salmonella* isolates from livestock and poultry meat of Pokhara valley were resistant to erythromycin (76.92%), oxytetracycline (73.07%), cotrimoxazole (26.92%), gentamicin (11.54%), chloramphenicol (7.69%) and ceftriaxone (3.84%).

Similarly, Shrestha *et al.* (2010) reported 97.4%, 97.4%, 97.4%, 94.9%, 92.3%, 69.2%, 10.3%, 7.9%, 5.1% and % of *Salmonella* isolates from poultry in Nepal to be resistant to ampicillin, amoxicillin, nalidixic acid, cephalothin, tetracycline, cotrimoxazole, norfloxacin, ciprofloxacin, streptomycin and gentamycin respectively.

2.4.8.3 Impacts on consumers

The antibiotics used in livestock are ingested by humans when they consume food (Golkar *et al.*, 2014). All drugs have side effects when they are exposed to humans and animals with higher dose or prolonged time than recommended (Lee *et al.*, 2001). On one hand, they may lead to immunological effects (Nisha, 2008), imbalance of intestinal micro-flora (Javadi *et al.*, 2009; Kirbis, 2006; Olatoye and Ehinmowo, 2010), carcinogenicity (Sulfonamides, Arsenicals, oxytetracycline, Furazolidone) (Javadi *et al.*, 2009; Nisha, 2008; Olatoye and Ehinmowo, 2010), mutagenicity, loss of hearing, nepropathy (Gentamycin, Neomycin) and hepatotoxicity (Nisha, 2008). Other effects include reproductive disorders(Lawal *et al.*, 2015), bone marrow toxicity (Chloramphenicol), teratogenicity (Beyene, 2016) and anaphylactic reaction in individuals with known hypersensitivity to penicillin (Dipeolu, 2004; Shareef *et al.*, 2009).

On the other hand, consumption of tissues with toxic antibiotic can result in transfer of antibiotic resistant strain bacteria known to be food borne pathogen (e.g., *Salmonella* spp., *Escherichia coli* and *Campylobacter* spp.) to humans (Bartlett *et al.*, 2013; Boothe and Arnold, 2003; Hayes *et al.*, 2004; Nisha, 2008; Shareef *et al.*, 2009). These resistant microorganisms can cause infections in humans that may lead to adverse health consequences (CDC, 2013). Similarly, prolonged consumption of meat containing antibiotic residues can also lead to the development of antibiotic resistance in gut bacteria of humans (Kemper, 2008). Shrestha *et al.* (2011) found the *E. coli* obtained from people of Chitwan, Nepal showed 100%, 100%, 100%, 80%, 80%, 80%, and 60% antibiotic resistance against

ampicillin, cephalexin, tetracycline, doxycycline, ciprofloxacin, enrofloxacin, gentamycin and nitrofurantoin respectively. Antibiotic resistant bacterias are responsible for a huge number of deaths world-wide. Beyond those deaths, bacteria that are resistant to antibiotics cause millions of illnesses and cost billions in health care spending, lost wages and lost national productivity. It is predicted that by 2050, antibiotic resistance will cost the world $100 trillion and will cause a staggering 10 million deaths per year. If the situation continues, then one day surgery could be too dangerous to attempt and ordinary health problems such as scrapes, tooth extractions and broken limbs, could pose a deadly risk (McKenna, 2017).

2.4.8.4 Impacts on environment

With an increase in global consumption and production of animal products, large quantities of antibiotics are released to the environment. 30% to up 90% of the dose consumed by the animals is found in the urine and feces as parent compounds and/or metabolite compounds (Carvalho and Santos, 2016). According to Furtula *et al.* (2010), residues of bacitracin, salinomycin, penicillin and virginiamycin were detected in chicken litter at concentrations ranging from 0.07 to 66 mg/L. In agricultural countries like Nepal, manure from poultry farms are widely used in fields. According to the commercial poultry survey report by CBS (2015), a total of 49,47,548 sacks of poultry manure was produced and NRs 45,37,22,444 worth of poultry manure was sold in the year 2015. This shows a tremendous disposal of antibiotics to the environment through poultry feces. Vegetables may also be contaminated from feces (Phillips *et al.*, 2004). After farming process, resistant microorganisms may be transferred into rivers and other water sources through the waste disposal system or rainwater. Therefore, antibiotics may also enter water courses from farm waste, allowing the possibility for further selection of resistant organisms (Willis, 2000).

Antimicrobials have qualitative and quantitative effects on the microbial community residing in sediments, which in turn can affect the degradation of organic matter (Kummerer, 2009). The residues contribute strongly to the development of resistance in sensitive bacterial populations (Mehdi *et al.*, 2018). Bio-resistant bacteria (*Staphylococcus xylosus*) have also been reported in air in broiler farms (Vela *et al.*, 2012). According to Chen et al. (2015), the spatial distribution of antibiotics in the marine environment is significantly correlated with environmental variables such as chemical oxygen demand (COD) and nitrates (Chen *et al.*, 2015). Liguoro *et al.* (2003) mentioned that the biotransformation and biodegradation of antibiotics on agricultural sites can take up to 150 days. But those by-

products in the environment remain bioactive and can be potentially more toxic, stable and mobile than their parent compounds (Carvalho and Santos, 2016).

2.4.9 Methods for detection of antibiotic residues

2.4.9.1 Microbial inhibition techniques

Microbial inhibition assays (MIAs) are routinely used screening techniques offering the advantage of detecting the total biological activity associated with unknown residues. The MIAs are sensitive to compounds that inhibit or disturb the growth of a test microorganism. Agar diffusion methods based on determining inhibition zones of a standard test organism seeded in agar plates is perhaps the most widely used screening technology. The liquid sample diffuses from a carrier into the agar medium during incubation. If antimicrobial compounds are present above a certain concentration, the microorganism will be inhibited (as a result of microbial death and/or inhibition of growth) and clear zones are visible on the agar plates. Multiple plates with different indicator microorganisms can be employed for detecting a broader spectrum of antibiotics, e.g. gram-positive microbial inhibitors by *Bacillus* strains and gram-negative microbial inhibitors such as quinolones by *E. coli* strain (Stead and Stark, 2012). The test organisms commonly used include *Bacillus stearothermophilus* (Bielecka et al., 1981; Nonga *et al.,* 2009), *Bacillus subtilis* (Alla *et al.*, 2011; Jabbar and Rehman, 2013; Nonga *et al.,* 2009), *Bacillus cereus*, *Micrococcus luteus* (Ghasemi *et al.*, 2014), *Escherichia coli* (Andrews, 2001; Omotoso and Omojola, 2015; Sophila *et al.,* 2018) and lactic acid bacteria such as *Streptococcus thermophilus*.

Several variations of MIAs such as One Plate Test (Alla *et al.*, 2011; Omotoso and Omojola, 2015), Three plate test (Ezenduka, 2019), Four Plate test (Ghasemi *et al.*, 2014), Modified EU Four Plate test (Pandey *et al.*, 2009), STAR method (Watkins and Kozarova, 2019), etc. are used for routine screening purposes. Similarly, commercial MIAs for meat, eggs and honey based foods such as Premi Test, the Explorer test and the Kidney Inhibition Swab (KIS test) are available for convenient screening of antibiotics (Stead and Stark, 2012).

Unlike other tests, these tests are based on the combination of pH conditions, which consequently promote or inhibit the activity of antibiotics. The medium pH affects the activity of certain antimicrobial substances. For example, activity of tetracyclines and β-

lactams increase in acidic pH, while that of macrolides, quinolones and aminoglycosides in alkaline pH (Ferrini *et al.*, 2006; Hakem *et al.*, 2013).

2.4.9.2 Immunological techniques

These methods are based on the antigen-antibody interaction which is very specific for a particular residue. The most usual technique is the Enzyme Linked Immunosorbent Assay (ELISA) and the detection system is usually based on enzyme- labeled reagents. There are different techniques for antigen quantification such as double antibody or sandwich ELISA tests and direct competitive ELISA tests. Radioimmunoassay (RIA) is based on the measurement of the radioactivity of the immunological complex. Other assays use chemiluminescent compounds and use luminescence detection techniques (Reig and Toldra, 2008).

Development and application of ELISAs for analysis of antibiotics and drugs used therapeutically and sub-therapeutically in food producing animals have increased in the last decade. These immunochemical methods are capable of detecting low levels of residues in tissues as well as biological fluids (urine, blood, milk). These assays are rapid, sensitive, cost effective, require little sample clean-up and lend themselves to routine testing of large numbers of samples. They can be used for qualitative screening or quantitative analysis (E. and Dixon-Holland, 1992). Commercial ELISA test kits are available for specific antibiotics or a group of antibiotics. They have been used successfully for detection of antibiotics in meat like chloramphenicol (Murilla *et al.*, 2010); ciprofloxacin, streptomycin, sulphanilamide and tetracycline (Ramatla *et al.*, 2017); penicillins (Lee *et al.*, 2000), quinolones (Mashak *et al.*, 2017)etc.

Prajapati *et al.* (2018) performed antibiotic residue analysis to detect enrofloxacin, ciprofloxacin, streptomycin and chloramphenicol using ELISA in breast meat samples collected from Kathmandu, Kaski and Chitwan district. It was found that 38%, 15.2% 8.7% and 0% breast meat samples were positive against streptomycin, ciprofloxacin, enrofloxacin and chloramphenicol residues respectively.

2.4.9.3 Biosensors

Different types of biosensors have been developed as an alternative approach to screen veterinary drugs in meat. Generally, these sensors usually contain an antibody as a recognition element that interacts with the analyte. The resulting biochemical signal is

measured optically or converted into an electronic signal that is further processed in appropriate equipments. They are able to detect multiple residues in a sample at a time and thus allow the analysis of large number of samples (Reig and Toldra, 2008).

2.4.9.4 Chromatographic techniques

Chromatography is an important biophysical technique that enables the separation, identification and purification of the components of a mixture for qualitative and quantitative analysis. The mixture is applied onto the surface of a solid stationary phase and the components of the mixture are separated from each other while moving with the aid of a mobile phase. The factors affective for this separation process include molecular characteristics related to adsorption, partition and affinity or differences among their molecular weights. Because of these differences, some components of the mixture stay longer in the stationary phase and move slowly in the chromatography system, while others pass rapidly into mobile phase and leave the system faster. There are different types of chromatography such as column chromatography, ion-exchange chromatography, gel-permeation chromatography, affinity chromatography, paper chromatography, thin-layer chromatography (TLC), gas chromatography, high performance liquid chromatography (HPLC) and so on (Coskun, 2016). Among them few of them have been used for screening as well as quantification of antibiotic residues in animal tissues.

2.4.9.4.1 Thin layer chromatography (TLC)

TLC is a separation technique in which the separation process occurs in a uniform planar layer of sorbent placed on a glass or aluminum plate or plastic sheet. The sorbent is called the stationary phase. During analysis, the plate is immersed in the mobile phase, generally a mixture of two to four solvents, and developed vertically or horizontally. The separation process occurs due to various mechanisms such as adsorption (e.g., hydrogen bond interactions), partitioning between the stationary and mobile phases, or ion exchange, depending on the nature of the sorbent. After development, compounds can be detected (visualized) and identified by their natural color or fluorescence, quenching of fluorescence on a layer containing a fluorescent indicator, or by creating colored spots after treating the plate with a chromogenic detection reagent by spraying, dipping, or exposure to vapors (Komsta *et al.*, 2014).

Several works have been done regarding detecting antibiotic residues in biological samples by using TLC. Tajick and Shohreh (2006) performed TLC for detection of antibiotics residue in chicken meat in Iran. Out of a total of 50 chicken samples collected, more than 50% of the samples had noticeable antibiotics residue.

Similarly, Sattar *et al.* (2014) did a research on antibiotics residues in broiler and layer meat of Bangladesh. A total of 200 samples were collected. TLC method was used for screening detection of tetracycline, amoxicillin, ciprofloxacin and enrofloxacin residues. The residues of tetracycline were 48% in livers, 24% in kidneys, 20% in thigh muscles, and 24% in breast muscles. Ciprofloxacin residues were found 44% in liver, 42% in kidneys, 34% in thigh muscles and 30% in breast muscles. Enrofloxacin residues were found 40% in liver, 34% in kidneys, 22% in thigh muscles, and 18% in breast muscles. Amoxicillin residues were found 42% in liver, 30% in kidneys, 26% in thigh muscles and 22% in breast muscles. Another study by Hossain (2010) reported in 40.5% of poultry meat samples collected from Bangladesh.

Another research on screening of antibiotic residues in chicken meat in Bangladesh was done by Sarker *et al.* (2018) by TLC. A total of 160 samples (breast, thigh muscle and liver) were collected For comparison the standard antibiotics; Ciprofloxacin (CIP), Enrofloxacin (ENR), Oxytetracycline (OTC), Amoxicillin (AMOX) and Doxycycline (DOX) were prepared by dissolving in methanol. In breast muscle highest antibiotic was CIP (39%) followed by DOX (26%), AMOX (24%), and OTC (23%) and the lowest was ENR (21%). In thigh muscle, 42, 29, 28, 27 and 24% sample was positive for CIP, OTC, DOX, AMOX and ENR, respectively. Highest number of liver samples were shown positive result for all screened antibiotics (CIP-52%, OTC-46%, DOX-43%, AMOX-42% and ENR-36%).

Similarly, the residue of ampicillin, ciprofloxacin and enrofloxacin in liver and meat of broilers were evaluated by Khan *et al.* (2018) using thin layer chromatography (TLC). The highest percentages of antibiotic residues were detected in the birds of Kachijhuli bazar (26.67%) and lowest in Shankipara bazar (13.33%). The highest percentages of antibiotics used in poultry feed was enrofloxacin (46.67%) followed by ciprofloxacin (30.00%) and amoxicillin (23.33%). In addition, amoxicillin plus ciprofloxacin (30%) and ciprofloxacin plus enrofloxacin (43.33%) were commonly found in the liver of broilers. 100% of the

broiler livers contained antibiotic residues and 20% of the breast meat samples contained antibiotic residues.

Ramatla *et al.* (2017) evaluated antibiotic residues in raw meat samples in Mafikeng, South Africa using different analytical techniques including TLC. Meat extracts were spotted on pre-coated Silica gel plates and separation was done using acetone/methanol (1:1) as mobile phase. Visualization of spots was done under 254 nm UV light. It was found that 92.5%, 29.4%, 21.4% and 14.6% of the samples contained sulphanilamide, streptomycin, ciprofloxacin and tetracycline residues respectively.

Similarly, another study regarding detection of antibiotic residues in Sonali chicken tissues from Chittagong district, Bangladesh was performed by Tazrin (2014). Commercial silica TLC plates were used and acetone:methanol (1:1) as mobile phase. Out of a total of 120 samples (40 liver, 40 breast muscle and 40 thigh muscles), 3%, 2.5%, 8% and 0% samples contained ciprofloxacin, enrofloxacin, sulfachloropyradizine and tylosin respectively. Among the tissues, 12.5%, 0% and 15% of liver, thigh muscles and breast muscle samples contained antibiotic residues respectively.

Detection of antibiotic residues in stored poultry products in Mosul city, Iraq was performed by Shareef *et al.* (2009) using silica gel TLC plates and acetone: methanol (1:1) as mobile phase. 52% of the meat samples were found to be positive. A total of 75 samples stored poultry products; liver, breast and thigh muscle samples, were tested for the presence of four antibiotics residue; oxytetracycline, sulfadiazine, neomycin, and gentamycin using thin layer chromatography. The results revealed 39 (52%) positive samples. From 25 samples of each of liver, breast and thigh muscle samples tested, 7 (28%) of liver and breast muscle were positive for sulfadiazine and oxytetracycline while 7 (28%) of thigh muscle were positive for oxytetracycline and 4 (16%) samples were positive for sulfadiazine. No neomycin or gentamycin residues were detected on TLC plates in all samples tested. Oxytetracycline was the most predominant antibiotic detected (28%), among the four studied antibiotics and followed by Sulfadiazine (24%). Liver and breast muscle had the highest percentage of antibiotic detected (56%), followed by for thigh muscle (44%).

A study was conducted in Nigeria by Geidam *et al.* (2009) to detect oxytetracycline and penicillin residues in slaughtered cattle tissues. Oxytetracycline was detected in 32.6% muscles, 5% liver and 8.3% kidney samples. Similarly, penicillin residues were detected in

15.7% muscles, 13% liver and 8.3% kidneys. Sample extraction was performed by taking ethanol as solvent and TLC was performed using acetone: methanol (1:1) as mobile phase.

In addition to TLC, high performance thin-layer chromatography (HPTLC) has been applied successfully for the qualitative and quantitative detection of multi-residues in food samples. The plates are sprayed with an appropriate chromogenic reagent or viewed under UV light for visualisation of compounds. Detection by fluorescence is also applied. Quantitation is achieved by measuring the relative intensity of the spot vs that of the internal standard by scanning densitometry. Modern HPTLC has been automatized at a high level (Reig and Toldra, 2008). Use of HPTLC has been reported by several workers to detect sulfonamides (Bukanski *et al.*, 1988; Haagsma *et al.*, 1984; Poucke *et al.*, 1991), tetracyclines (Kodimalar *et al.*, 2014; Singh *et al.*, 2016), macrolides (Loya and Hamrapurkar, 2011), aminoglycosides (Bhogte *et al.*, 1997), ionophores (Bertini *et al.*, 2003)

Similarly, TLC- Bioautography has also been practiced in which combination of thin layer chromatography has been done with microbiological detection directly on the plates resulting in enhanced sensitivity (Toldra and Reig, 2006). This technique has been implied for several antibiotics such as quinolones (Choma, 2006a, 2006b; Choma *et al.*, 2004; Choma and Komaniecka, 2005), chloramphenicol (Hamburger and Cordel, 1987), macrolides (Ahmed et al., 2013), β-lactams (Kaya and Filazi, 2010) and ionophores (Vanderkop and MacNeil, 1989).

2.4.9.4.2 High performance liquid chromatography

HPLC expanded its use in the 1990s due to the availability of columns, good performance, variety of available detectors and possibility of automation. HPLC is a separation technique and its ability to detect compounds depends on the type of detector used. The choice of the detection system is very important for selectivity and sensitivity. Some analytes not detected by absorbance, refractive index or fluorescence may require chemical modifications to render chromophore, fluorescent or UV-absorbing compounds. Typical detections of multi-residues in meat samples are relatively simple and rapid, requiring a preliminary clean-up through solid-phase extraction followed by filtration before injection into a reverse-phase HPLC with diode array detection (Reig and Toldra, 2008).

Liquid chromatography techniques are getting expanded use in control laboratories due to the possibility of automation (injection, elution, washing of column, detection), computer-

controlled use and data manipulation and the relatively short time needed per sample. Recent developments in new systems and columns allow high speed and reduced analysis time. Even though the cost of the instrument is high, when a large number of samples are analyzed the costs are reduced and are more competitive (Reig and Toldra, 2008). This technique has been applied to meat for detection of antibiotics like quinolones (Kirbis *et al.*, 2005), sulphonamides (Machado *et al.*, 2013; Pecorelli *et al.*, 2003), β-lactams and macrolides (Nagata *et al.*, 2004), and tetracyclines (Aman *et al.*, 2017; Shalaby *et al.*, 2011).

2.4.10 Remedies to occurrence of antibiotic residues in foods from animal origin

In today's world, antibiotics have become an unavoidable part of both humans and animals as they are some of the most effective treatments for diseases. But the misuse of these antibiotics may result in the aforementioned hazards on poultry, consumers as well as on the environment. So, it is very important to control such indiscriminate use of antibiotics in poultry industry. According to Nirala *et al.* (2018), antibiotic residues in food animals of animal origin can be reduced as:

a. Reduce antibiotics use in food animal rearing.
b. Rapid screening methods should be developed for detecting and segregating samples contains above MRL levels of antibiotics.
c. Appropriate MRLs need to be set by regulatory bodies and should enforce it.
d. Appropriate withdrawal periods should be strictly followed. High usage of antibiotics leads to the occurrence of residues in blood and other tissues of the animals. However, since the antibiotics can be rapidly eliminated, the antibiotics disappear both from blood and tissues within a few days after the animals are placed on non-medicated feed (Nisha, 2008).
e. Improve the individual and organizational awareness by enhancing proper knowledge dissemination.
f. Follow best hygiene practices during animal rearing and avoid unwanted use of antibiotics.
g. Alternates to antibiotics like bio control measures and ethno-veterinary practices should be developed and followed.
h. Organic poultry farming may be encouraged by providing appropriate incentives to the farmers in form of subsidies.

i. Use of proper processing techniques to inactivate the antibiotic residue, e.g. refrigeration causes inactivation of penicillin. Javadi *et al.* (2009) reported that roasting meat at enough roasting temperature and time can have a great effect on antibiotic residue losses and provides an additional margin of safety for consumers.
j. Use of activated charcoal, resins and UV irradiation to inactivate residues.

Application of several processing techniques such as sufficient heating temperature and time can reduce nearly fifty percent of some antibiotics residues but it does not generally provide an additional margin of safety for consumers. So, veterinary officers should ensure the judicious use of antibiotics in combating bacterial infection. Furthermore, the observance of the pre-slaughter withdrawal periods after antibiotic usage should be emphasized (Hussein *et al.*, 2016).

PART III

Materials and methods

3.1 Materials used

All the chemicals, glasswares and equipments required were used from Central Campus of Technology, Hattisar laboratory. The major apparatus, chemicals and equipments used are listed in Appendix A.

3.2 Conducting Survey

A questionnaire survey was conducted to collect information regarding education level of farmers, common diseases incurred, common antibiotics given, technical knowledge of farmer regarding the usage of antibiotics, and so on from poultry farms situated within Dharan Municipality. Appendix G shows the questionnaire survey used in this study.

3.3 Collection of Sample

A total of 100 samples (25 samples of each tissue variety) were collected. The tissue samples (liver, breast muscle, kidney and gizzard) were collected from randomly selected poultry meat shops within Dharan municipality. Each sample was kept separately in sterile and clean plastic bag with proper labeling. All the collected samples were kept in an ice box and carried to the Research Laboratory of Central Campus of Technology, Hattisar, Dharan. These samples were stored in refrigerator at -20°C until further analysis (Sarker *et al.*, 2018).

3.4 Microbial inhibition test

A Three Plate test was performed as done by Ezenduka (2019) for detection of β-Lactams, tetracyclines, aminoglycosides and sulfonamides. Similarly, One Plate Test as performed by Omotoso and Omojola (2015) was used for the detection of fluoroquinolones. Among the required microorganisms, *Bacillus subtilis* was isolated and identified in the lab whereas *Escherichia coli* (ATCC 25922) was obtained from BPKIHS, Dharan.

3.4.1 Isolation and identification of *Bacillus subtilis*

According to Tamang (2003), *kinema* is a rich source of *Bacillus subtilis*. So, *kinema* was used as a source of this bacterium. 10 g of *kinema* was weighed and mixed well with sterile distilled water by magnetic stirrer for an hour. The suspension was heated at 90°C for an hour to encourage the formation of spores as well as for eliminating other unwanted

microorganisms (Ezenduka, 2019). Then 0.1 ml of this mix was plated on nutrient agar medium by using streak method of inoculation. The plates were then incubated at 30°C for 24 h. Colonies with different morphological appearance were sub-cultured onto fresh nutrient agar for identification purpose (Al-Humam, 2016).

3.4.1.1 Gram's staining

Gram's staining was performed as described in the protocol given by Smith and Hussey (2005). A smear of 24 h old bacterial culture was prepared on a glass slab. It was air dried and heat fixed. Then the smear was flooded with crystal violet staining reagent for 1 min. The slides were gently washed with indirect stream of tap water for 2 s. The slide was again flooded with Gram's iodine for 1 min. The slide was then washed with tap water for 2 s followed by flooding with decolorizing agent for 15 s. Finally, the slide was flooded with the safranin and after 30 s to 1 min, it was washed with gentle and indirect stream of tap water until no color appeared in the effluent. The slide was blot dried with blotting paper and was observed under oil immersion using a bright field microscope. The culture was classified as gram positive if the bacteria were stained blue/purple and as gram negative if stained pink/red.

3.4.1.2 Catalase test

This test was performed as described by Reiner (2010). 18 to 24 h old well isolated colony was placed onto microscopic slide. Then a drop of 3% H_2O_2 was placed onto the organism using a pipette. Observations were made for the formation of bubbles against a dark background. Organism showing bubbles formation are to be categorized as catalase positive.

3.4.1.3 Starch hydrolysis test

The starch hydrolysis test was performed as described in the protocol by Lal and Cheeptham (2012). Starch agar medium was prepared by taking beef extract (3 g/L), soluble starch (10 g/L) and agar (12 g/L). The media was then sterilized and poured onto petri plates. A single colony of fresh (16 to 18 h) pure culture was taken and inoculated onto the starch agar plate as a single streak. The plate was then incubated at 37°C for 3-5 days. After proper incubation, the plate surface was flooded with Gram's iodine solution. Appearance of a clear zone surrounding the bacterial growth would indicate positive starch hydrolysis.

3.4.1.4 Gelatin hydrolysis test

This test was performed as per the protocol described by Cruz and Torres (2012). Nutrient gelatin medium was prepared by taking peptone (5 g/L), beef extract (3 g/L) and gelatin (120 g/L) in 250 ml distilled water and making the final pH to 6.8. The media filled into test tubes and autoclaved. These tubes were allowed to cool in an upright position prior to use.

A heavy inoculum of an 18-24 h old test organism was stab inoculated into the tube containing nutrient gelatin. The inoculated tube and an un-inoculated control tube were incubated at 25°C for upto a week. The tubes were then placed in an ice bath for 15-30 min prior to observation. If the tube inoculated with the microorganism showed liquefied medium, then the organism is considered as positive to gelatin hydrolysis test.

3.4.1.5 Indole production test

This test was performed as per the procedure given by Aneja (2018). 1% tryptone broth was inoculated with nutrient broth culture of the test organism. The tubes were incubated for 48 h at 35°C. After incubation, 1 ml of Kovac's reagent was added to the tubes. The tubes were shaken gently after intervals for 10-15 min. Finally, the tubes were allowed to stand for some time to permit the reagent to come to the top.

Development of a deep red color on the top layer of the tube indicates positive results whereas absence of red coloration indicates indole negative.

3.4.1.6 Methyl red and Voges-Proskauer test

These tests were performed as per the protocol given by McDevitt (2009). MR-VP broth was prepared and filled in tubes. It was then sterilized. After the broth cooled down to room temperature, a light inoculum from 18-24 h old culture of the test organism was transferred to the MR-VP broth tube. It was incubated at 35°C for 48 h.

For methyl red test, about 2.5 ml of the culture was transferred to a new sterile culture tube. 5 drops of methyl red reagent was added to it. Formation of a red color in the tube indicated MR positive organism whereas presence of yellow coloration indicated MR negative organism.

For Voges-Proskauer test, the remaining 2.5 ml of culture was taken and 12 drops of VP reagent 1 was added followed by addition of 4 drops of VP reagent 2. The tube was shaken

carefully for 1 min to expose the medium to atmospheric oxygen. The tubes were allowed to stand for at least 30 min. If pink or red color is observed in the tube, it is an indicative of positive whereas no change in color is a negative test.

3.4.1.7 Citrate utilization test

This test was performed as per the protocol given by MacWilliams (2009). Simmons citrate agar medium was prepared and filled in test tubes. The tubes were then sterilized and slants were prepared. A 16-18 h old culture was taken as inoculum source. A single well isolated colony was taken and lightly streaked onto the surface of the slant. The tubes were then incubated at 37°C for 18-48 h. The microorganisms in the tube which developed intense Prussian blue color were taken as positive.

3.4.2 Preparation of 0.5 McFarland standard

0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (% w/v) with constant stirring until a uniform suspension is obtained (Gautam *et al.*, 2017).

3.4.3 Antibiotic susceptibility test and determination of minimum inhibitory concentration

Sensitivity of the test microorganisms towards antibiotics was performed as per the Kirby-Bauer disk diffusion susceptibility test protocol given by Hudzicki (2009). Mueller-Hinton agar media was prepared in distilled water the pH of which was adjusted to 7.2-7.4. The media was autoclaved at 121°C/15 psi pressure for 15 min and cooled to a temperature of about 40-50°C. Microbial culture suspensions of test microorganisms was prepared in sterile saline by taking four or five isolated colonies and suspending them in 2 ml sterile saline. The turbidity of this suspension was adjusted to 0.5 McFarland by visual inspection. Thus prepared standard microbial suspensions were swabbed uniformly on the surface of MHA plates by using sterile cotton swabs. Then standard antibiotic discs were applied to these inoculated plates by using sterile forceps. These plates were incubated at 35°C for 18-24 h and the diameter of inhibition zone was measured using a Vernier caliper.

Minimum inhibitory concentration for antibiotics were determined as described by Currie *et al.* (1998). Nutrient agar (pH 6) seeded with *B. subtilis*, nutrient agar (pH 8) seeded with *B. subtilis* and nutrient agar (pH 8) seeded with *E. coli* were prepared. Nutrient agar (pH 6) seeded with B. subtilis was used to determine minimum inhibition concentrations for

tetracycline and doxycycline, nutrient agar (pH 8) seeded with *B. subtilis* for gentamycin and nutrient agar (pH 8) seeded with *E. coli* for ciprofloxacin and enrofloxacin. 8 mm wells were bored in nutrient agar plates swabbed with test microorganisms. Stock solutions of tetracycline, ciprofloxacin and gentamycin of concentration 1 mg/ml were prepared in methanol, dilute acetic acid and water respectively. These solutions were diluted to concentration as low as 0.005 µg/ml using distilled water. Then 100 µL of each standard solution was added to the wells and the plates were incubated at 37°C for 24 h at upright position. The dilution after which no microbial inhibition occurred was taken as the minimum inhibitory concentration of the antibiotic for that microorganism.

3.4.4 Sample extraction

For each organ, 4 g piece was weighed and macerated with equal volume of phosphate buffer (pH 6.5). The mixture was centrifuged at 5,000 rpm for 10 min and the supernatant was filtered. The filtrate was used in microbial screening for antibiotics (Ezenduka, 2019).

3.4.5 Preparation of test plates

Nutrient agar media was prepared in distilled water according to manufacturer's instructions and adjusted to pH 6, 7.2 and 8 with NaOH and HCl. These media were autoclaved at 121°C/15 psi for 15 min and poured onto sterile petri plates. Petri plates with media of pH 6, 7.2 and 8 were swabbed with active culture suspension of *Bacillus subtilis* plus petri plates with media of pH 8 were swabbed with active culture suspension of *Escherichia coli* (Ezenduka, 2019). Each culture suspension was adjusted to 0.5 McFarland concentrations prior to swabbing. Test plates were prepared as shown in Table 3.1.

Five wells were dug into each petri plate by using a sterile 8mm cork-borer. About 100 µL of the organ extracts were then inoculated in 4 wells, each well representing an organ. The remaining well was inoculated with 100 µL of phosphate buffer as negative control (Ezenduka, 2019). Similarly, standard antibiotic discs of tetracycline, cotrimoxazole, gentamycin and ciprofloxacin were applied to the test plates I, II, III and IV respectively by the use of sterile forceps as positive control. Plates I, II and III were incubated at 30°C for 24 h whereas plates IV were incubated at 37°C for 24 h (Pandey *et al.*, 2009).

3.4.6 Interpretation of results

After 24 h, annular zones of inhibition were measured by using a Vernier caliper. The samples with 2 mm inhibition zones or more were considered as positive to indicate the presence of antibiotic residues while the sample with 1-2 mm inhibition zones were considered as suspects and the samples with less than 1 mm inhibition zones were considered as negative (Ghasemi *et al.*, 2014).

Table 3.1 Microbial culture and pH of media adjusted for screening of different groups of antibiotics

Plate	pH of media	Microbial culture	Antibiotic detected
I	6	*Bacillus subtilis*	β-Lactams and Tetracyclines
II	7.2	*Bacillus subtilis*	Sulfonamides
III	8	*Bacillus subtilis*	Aminoglycosides
IV	8	*Escherichia coli*	Fluoroquinolones

3.5 Thin layer chromatography

Samples found to be positive in the Three Plate Test and One Plate Test were subjected to thin layer chromatography.

3.5.1 Antibiotic standards preparation

0.05 g of ciprofloxacin, doxycycline, enrofloxacin, gentamycin and tetracycline were dissolved in 5 ml of dilute acetic acid, methanol, dilute acetic acid, distilled water and methanol respectively. Working concentration for each antibiotic was determined by hit and trial experiments such that a discrete spot without any tailing was obtained after chromatography.

3.5.2 Preliminary trials

A variety of solvent systems and TLC plate pretreatments as used by different authors were tested which are shown in Table 3.2.

3.5.3 Preparation of TLC plates

TLC plates were first pre-developed with methanol, dried at 100°C for 20 min and cut into suitable sizes. The TLC plates were again developed with an aqueous EDTA solution (100

Table 3.2 List of preliminary trials for Thin Layer Chromatography

Trial	TLC plate predevelopment	Mobile phase	Reference
A	No	Methanol: acetone (1:1)	(Sarker *et al.*, 2018)
B	No	Water: methanol: dichloromethane (6:35:59)	(Becic *et al.*, 2014)
C	No	n-butanol: oxalic acid: water (100 ml: 5 g: 100 ml)	(Kapadia and Rao, 1964)
D	No	Methanol: acetone: 1% aq. ammonia (4:4:1)	-
E	No	Water: methanol: dichloromethane: 1% ammonia (6:35:59:5)	-
F	No	Chloroform: methanol: conc. NH_4OH: H_2O (1:4:2:1)	(Cues and Vanderhaeghe, 1982)
G	Pretreatment by saturated EDTA solution	Chloroform: methanol: 5% aq. Na_2EDTA (65:20:5), lower layer	(Oka and Uno, 1983)
H	Pretreatment by saturated EDTA solution	Chloroform: methanol: acetone: 1% aq. NH_4OH (10:22:50:18)	(Xie *et al.*, 1997)
I	Predevelopment in aq. Na_2EDTA solution (100 g/l conc. and pH 8) and dried at 120°C/1 h	Methanol: acetone (1:1)	-
J	Predevelopment in aq. Na_2EDTA solution (100 g/l conc. and pH 8) and dried at 120°C/1 h	Water: methanol: dichloromethane (6:35:59)	-
K	Plates washed with methanol and predevelopment in aq. Na_2EDTA solution (100 g/l conc. and pH 8) and dried at 120°C/1 h	Chloroform: methanol: 25% NH_4OH (60:35:5)	(Chen and Schwack, 2013)

g/L adjusted to pH 8.0 by 20% NaOH). After EDTA modification, the plates were dried at 120°C for 1 h. The plates were then stored in a dessicator (Chen and Schwack, 2013).

3.5.4 Sample preparation

Samples were drawn out from the freezer and further preparation was performed as cited by Tazrin (2014). The meat samples were grinded into a fine paste in mortar pestle. Then, 10 g of sample was taken in a centrifuge tube and 7 ml phosphate buffer (pH 6.5) was added to it. Then 3 ml aq. EDTA solution (0.1 mol/L and pH 8.0) was added to it (Chen and Schwack, 2013). They were mixed well using vortex mixture. It was followed by addition of 2 ml 30% trichloroacetic acid for protein precipitation. The mixture was centrifuged at 7000 rpm for 15 min. The supernatant was filtered and defatted with equal volume of diethyl ether. The upper oily layer was discarded and defatation of bottom layer was further done twice with equal volumes of diethyl ether. Tajick and Shohreh (2006) found that concentration of the extract made the detection easier while performing TLC. So the defatted extract was then concentrated to about 2 ml in a rotary vacuum evaporator at a temp of 50°C. The concentrate was collected in screw capped tubes and stored in refrigerator until TLC analysis.

3.5.5 Pointing, running and detection

A straight line was drawn on EDTA treated TLC plates using a pencil 1.5 cm above the lower end of TLC plate. The line was sufficiently high up the plate so that when it was placed in the solvent, the spotted samples remained above the level of solvent. Then each antibiotic standard solutions and concentrated sample extracts were spotted on the line 1 cm apart by using a micropipette. Proper care was taken to ensure that the spot was as small as possible and not greater than 2-3 mm in diameter. After spotting, the spots were left to dry properly.

Before placing the spotted TLC plates in TLC tank, 200 ml of mobile phase was poured into TLC tank lined with blotting paper and left for saturation for about an hour. The plates were then immersed carefully in the TLC tank. Before the mobile phase exceeds the upper end of TLC plate, the plates were taken out and solvent front was marked with a pencil. The plates were then left to dry for 30 min at room temperature.

The TLC plates were observed under UV light at 254 nm in a UV chamber. Dark or blue fluorescent spots seen against the green fluorescent background were circled and Rf values for the spots were calculated as

$$\text{Rf value} = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by mobile phase}}$$

3.6 Statistical analysis

Experimental data were introduced and well tabulated in Microsoft Excel 2016. Results were analyzed statistically for the test of significance using IBM SPSS Statistics 20. Tests were performed for descriptive statistics using Chi-Square test at 5% level of significance.

PART IV

Results and discussion

Several incidences regarding occurrence of antibiotic residues in poultry meat available in major cities of Nepal have been reported. So, in order to study status of antibiotic residues in poultry meat in Dharan, first of all, a survey was conducted among poultry farm owners regarding their education level, commonly used antibiotics, etc. Then different poultry meat samples, namely liver, breast muscle, kidney and gizzard, were collected and subjected to microbial screening. The microorganism *Bacillus subtilis* required for microbial screening was isolated and identified in the laboratory. Finally, the positive samples were further subjected to thin layer chromatography to identify the antibiotics present. Series of preliminary trials were also conducted in order to determine the most suitable mobile phase for TLC analysis.

4.1 Survey report

A total of 25 poultry farms situated in Dharan were surveyed and information regarding their education level, training level, antibiotics used in the farm, knowledge regarding occurrence of drug residues, maximum residue limits, withdrawal periods and antibiotic resistance were collected. Similarly, 10 veterinary shops located at Dharan Municipality were surveyed to find out the common diseases in poultry in Dharan.

4.1.1 Education level of poultry farm owners

The level of education of poultry farm owners is shown in Fig. 4. Most of the poultry farm owners reported to have obtained an education level upto S.L.C. (76%) and significantly high proportion ($p<0.05$) of the respondents fell under this group. According to CBS (2015), 84.7%, 10.4%, 3.5% and 1.4% of poultry farmers in Sunsari had an education level upto S.L.C., certificate level, bachelors level and masters level respectively. It was found that there's been a slight increase in level of education of owners.

Further survey informations on knowledge related to poultry farming and use of antibiotics are shown in Table 4.1.

CBS (2015) had reported that 23% of poultry farm owners in Sunsari district are trained. The present study revealed higher percentages of poultry farm owners to have received such

trainings at least once. Almost half of the respondents had a general concept regarding withdrawal period and reported to have stopped providing antibiotics to the birds for prophylactic purpose after they were above 30 days old. Almost none of the respondents had knowledge regarding antimicrobial resistance development in microorganisms.

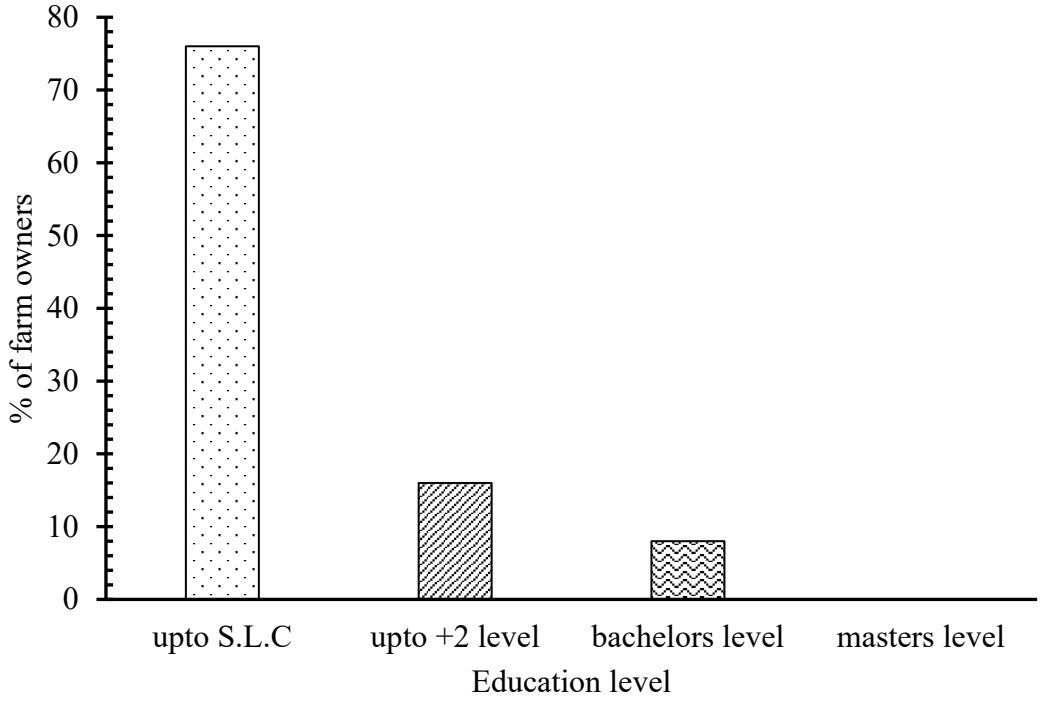

Fig. 4.1 Education level of broiler farm owners of Dharan Municipality

Table 4.1 Knowledge level of Poultry farm owners on several aspects related to poultry farming and antibiotic usage

S.N.	Particulars	Number of respondents (Percentages)
1.	Acquirement of trainings on poultry farming	11 (44)
2.	Knowledge regarding occurrence of antibiotic residues in meat	9 (36)
3.	Knowledge on withdrawal period of antibiotics	13 (52)
4.	Knowledge on antimicrobial resistance in microorganisms	1 (4)

4.1.2 Antibiotics usage in poultry farms

During the questionnaire survey, farmers were asked about the medicines and growth stimulants they provide to the broilers. The percentage of poultry farms that reported the use of different antibiotics is shown in Fig. 4.2. It was found that the most common groups of antibiotics to be used are tetracyclines (doxycycline, tetracycline), followed by quinolones (ciprofloxacin, enrofloxacin, levofloxacin), β-Lactams (amoxicillin), sulfonamides (sulfamethoxazole), aminoglycosides (neomycin) and macrolides (tylosin).

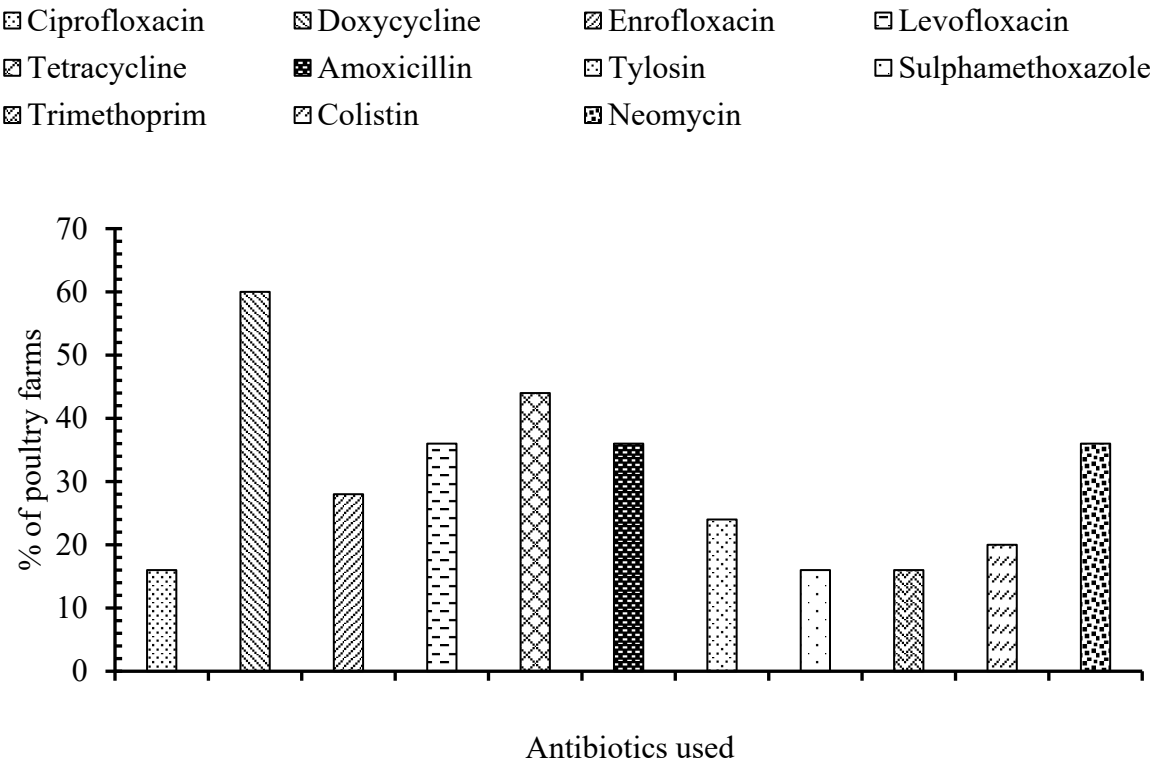

Fig. 4.2 Antibiotics usage in poultry farms as reported by poultry farmers

4.1.3 Common poultry diseases in Dharan

According to veterinary respondents of veterinary shops situated at Dharan, the common poultry diseases in Dharan are chronic respiratory disease (CRD), *E. coli* infections, coccidiosis and foulpox. They also mentioned that enrofloxacin, ciprofloxacin, levofloxacin and tylosin are prescribed in case of CRD, tetracycline groups such as chlorotetracycline against foulpox, amoxicillin, levofloxacin and colistin against *E. coli* infections and amoxicillin against coccidiosis. The findings are similar to a report by GARP-Nepal (2015) which mentioned colibacillosis, CRD and coccidiosis as some major poultry diseases

diagnosed at Regional Veterinary Laboratory, Biratnagar and Regional Veterinary Laboratory, Janakpur.

4.2 Isolation and identification of *Bacillus subtilis* from *kinema*

Pure culture isolate from *kinema* was subjected to several biochemical tests as suggested in the Bergey's Manual of Systematic Bacteriology (Vos *et al.*, 2009). The tests performed and the results are tabulated in the Table 4.2. Similar observations were reported by Jabbar and Rehman (2013) during isolation and identification of *Bacillus subtilis* from soil.

Table 4.2 Results of biochemical tests performed for identification of *Bacillus subtilis* strain isolated from *Kinema*

Characteristics	Result
Gram's staining	Positive
Shape	Rods
Growth on nutrient agar	Yellowish colonies with curly outlines
Catalase test	Positive
Starch hydrolysis test	Positive
Gelatin hydrolysis test	Positive
Indole production test	Negative
Methyl red test	Negative
Voges- Proskauer test	Positive
Citrate utilization test	Positive

4.3 Antibiotic susceptibility test for *Bacillus subtilis* and *E. coli*

The zones of inhibition formed by antibiotic discs on our test orgamisms is shown in Table 4.3. *Bacillus subtilis* was found to be more susceptible to β-Lactams (amoxicillin, penicillin, ampicillin), tetracyclines (doxycycline, tetracycline), sulfonamides (cotrimoxazole) and aminoglycosides (gentamycin, amikacin, kanamycin). Similarly, *E. coli* was found to be more susceptible to quinolones (ciprofloxacin, levofloxacin, norfloxacin, ofloxacin). *E. coli* was found to be resistant towards penicillin whereas both the test organisms were resistant towards nystatin.

Similar work was performed by Sophila *et al.* (2018) regarding antibiotic susceptibility testing of *E. coli* ATCC 25922 and *Bacillus subtilis*, but the zones of inhibition formed were slightly smaller in our case. According to Hudzicki (2009), the depth of MH agar in plates

should be 4 mm for the Kirby- Bauer disk diffusion susceptibility test. Since such precautions could not be followed precisely, the depth of media may have been higher due to which more of the antibiotic from discs diffused deeper down the media resulting in smaller zones of inhibition.

Table 4.3 Antibiotic susceptibility tests of test organisms

Antibiotics	Diameter of the Zone of Inhibition in mm	
	Bacillus subtilis	*E. coli*
Amikacin 30 µg	24.63±0.058	20.60±0.000
Amoxicillin 10 µg	32.60±0.100	10.57±0.058
Ampicillin 10 µg	41.20±0.000	18.83±0.058
Chloramphenicol 30 µg	29.47±0.058	22.60±0.000
Ciprofloxacin 30 µg	33.03±0.058	38.60±0.100
Cotrimoxazole 25 µg	31.07±0.115	27.43±0.058
Doxycycline 30 µg	27.80±0.000	21.03±0.058
Erythromycin 15 µg	31.37±0.058	20.63±0.058
Gentamycin 10 µg	22.60±0.000	20.07±0.115
Kanamycin 30 µg	24.57±0.058	21.80±0.000
Levofloxacin 5 µg	36.40±0.000	36.80±0.000
Nitrofurantoin 30 µg	21.47±0.058	18.43±0.058
Norfloxacin 10 µg	24.43±0.058	30.37±0.058
Nystatin 100 µg	6.03±0.058	5.93±0.115
Ofloxacin 5 µg	29.56±0.058	33.23±0.058
Penicillin 10 µg	37.63±0.058	6.03±0.058
Tetracycline 30µg	24.40±0.000	20.23±0.058
Trimethoprim 5 µg	32.40±0.100	26.83±0.058

*Values are the means of triplicate determinations ± Standard deviation

4.4 Minimum inhibitory concentration of antibiotics for test organisms

MIC of antibiotics for the test organisms is shown in Table 4.4. Since *Bacillus subtilis* was implied for detection of β-lactams and/or tetracyclines, aminoglycosides and sulfonamides but not for quinolones, MIC of quinolones for *Bacillus subtilis* were not evaluated. Similarly,

since *E. coli* was emplied for detection of quinolones only, the MIC of quinolones only was determined for *E coli*.

Table 4.4 MIC of antibiotics for test organisms

Antibiotics	Concentration of stock solution	*Bacillus subtilis*		*E. coli*	
		Dilution (fold)	MIC (µg)	Dilution (fold)	MIC (µg)
Ciprofloxacin	1 mg/ml	-	-	14	0.0061
Doxycycline	1 mg/ml	12	0.0244	-	-
Enrofloxacin	1 mg/ml	-	-	15	0.00305
Gentamycin	1 mg/ml	12	0.0244	-	-
Tetracycline	1 mg/ml	11	0.0488	-	-

Jabbar and Rehman (2013) had reported the MIC of gentamycin for a local strain of *Bacillus subtilis* isolated from soil to be 0.0625 µg which is higher than the MIC for *Bacillus subtilis* isolated during this work. Similarly, MIC of ciprofloxacin for *E. coli* (ATCC 25922) was reported to be 0.015 mg/L by Andrews (2001) which is equivalent to 0.0015 µg when 100 µL of the solution is used. The values for MIC were found to be lesser than that reported by other workers. This might be because of the use of primitive agar well diffusion technique. Since the antibiotic standard solutions applied in the agar wells diffused more into the agar medium and not all of the solution applied could act on the test organism present on the agar surface.

4.5 Microbial screening for detection of antibiotic residues in poultry meat samples

4.5.1 Overall occurrence of antibiotic residues

On performing the screening test of meat samples using microbial inhibition technique, 57% of the samples were found to be positive which indicates a high prevalence of antibiotic residues in poultry meat of Dharan. The occurrence was found to be lower than the findings of Prajapati *et al.* (2018) who had reported 62% of the samples collected from Kathmandu, Kaski and Chitwan to be positive. Similarly, the occurrence was found to be higher than the findings of Pantha *et al.* (2019), Sapkota *et al.* (2019) and Raut *et al.* (2017) who reported 30.81% chicken meat samples from Kathmandu, 13% chicken meat samples from Kathmandu valley and 22% broiler meat samples from Kavre and Kailali to be positive

respectively. Our findings showed higher prevalence than reported by Omotoso and Omojola (2015) and Ghasemi *et al.* (2014). This has made the safety of poultry meat available at Dharan questionable. Similar to other developing countries, a high occurrence of antibiotic residues have been recorded: 60% positive samples in Pakistan (Jabbar and Rehman, 2013); 52% in Iraq (Shareef *et al.*, 2009), and 70% in Tanzania (Nonga *et al.*, 2009). Such high occurrence may be due to not following recommended label directions or dosage (extra-label usage); not adhering to recommended withdrawal times; administering too large volumes of drugs at a time; dosing, measuring or mixing errors and allowing animals to access medicated feeds (Beyene, 2016).

4.5.2 Prevalence of antibiotic residues in different tissues

The percentages of tissue samples found to be positive for atleast one group of antibiotics tested by using combined three plate test and one plate test is shown in Fig. 4.3. Overall occurrence of residues among different tissue samples was found to differ significantly ($p<0.05$). The highest occurrence of residues was observed in kidneys (72%) followed by liver (68%), gizzard (68%) and breast muscles (20%). The % prevalence is higher for all tissues than in the findings of Pandey *et al.* (2009) who reported 17.12% liver, 26% kidney

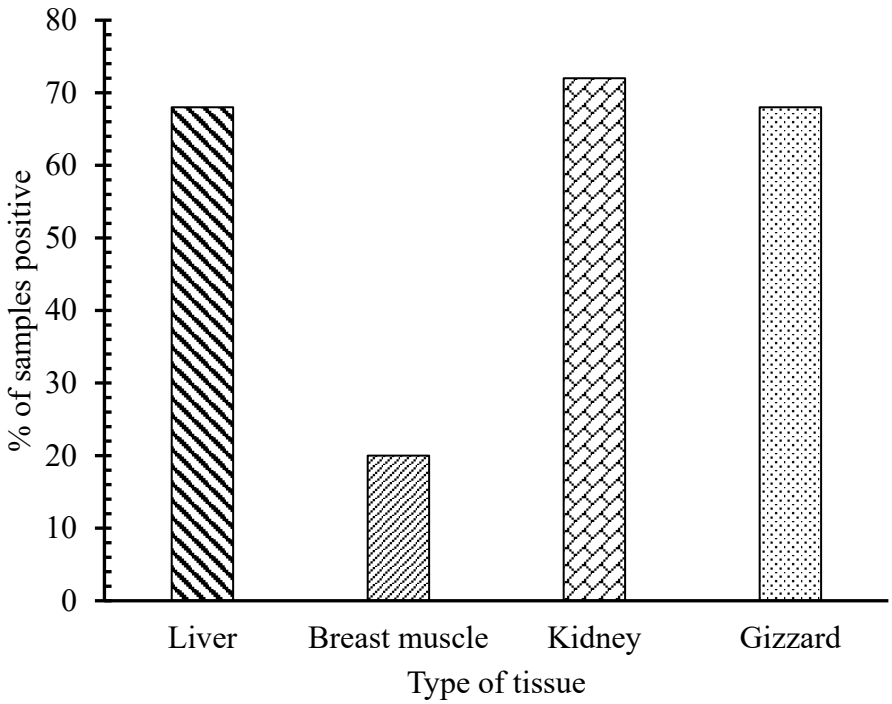

Fig. 4.3 Occurrence of antibiotic residues in different tissues by microbial inhibition technique

and 13.62% breast meat samples from poultry meat of Chitwan and Kathmandu to be positive. Raut *et al.* (2017) had reported 71% of chicken liver samples and 39% of muscle samples from Kavre and Kailali to be positive which are similar to our observed frequencies. Similar trends of occurrence of residues was observed by Ezenduka (2019) who found the highest prevalence of antibiotics in kidneys (60%) followed by liver (54%), gizzard (30%) and muscle (11%). Similarly, Jabbar and Rehman (2013) have reported 70% kidney, 60% liver and 50% muscle samples from Pakistan to be positive. Liver (hepatic mechanism) and kidneys (renal mechanism) are the most important organs involved in excretion of drugs. Most of the drugs are distributed rapidly to liver and kidney because of higher blood flow to these organs (Craigmill *et al.*, 1991). Thus, kidney and liver are generally found to contain elevated residue levels (Aerts *et al.*, 1995). Residues were detected in higher number of gizzard samples than reported by Morshdy *et al.* (2015) and Hussein *et al.* (2016) where 21.67% gizzard samples and 24% gizzard samples were found to be positive respectively. This indicates indiscriminate use of antibiotics in poultry farms of Dharan.

4.5.3 Occurrence of different groups of antibiotics

The overall percentage of samples found to be positive for different antibiotic groups is shown in Fig. 4.4. β-lactams and/or tetracycline, sulfonamides, aminoglycosides and quinolone residues were detected in 48%, 27%, 29% and 17% of chicken meat samples respectively. Similarly, 15%, 19%, 24% and 5% samples were suspected to contain β-lactams and/or tetracycline, sulfonamides, aminoglycosides and quinolone residues respectively. Overall occurrence of different groups of antibiotics was found to differ significantly ($p<0.05$) and occurrence of β-lactams and/or tetracycline was found to be the highest. The values obtained are found to be higher than in a similar study performed by Pandey *et al.* (2009) who reported 33.95%, 26.45% 20.41% and 5.83% of meat samples collected from Chitwan and Kathmandu to be positive towards β-lactams and/or tetracycline, sulfonamides, aminoglycosides and quinolones respectively. It indicates higher degree of misuse of antibiotics in poultry production in Dharan. Similarly, Hakem *et al.* (2013) reported β-lactams and/or tetracyclines, sulfonamides and aminoglycosides residues in 75.81%, 36.29%, 13.71% and 44.35% of chicken meat samples respectively.

According to Gwachha (2017), tetracycline and penicillin residues were detected in 50.48% and 18.1% of broiler meat samples from Kathmandu valley which is similar to our findings. Such a high prevalence of β-lactams and/or tetracyclines is probably due to higher

usage of this group of antibiotics. Our survey reports also shows doxycycline and tetracycline as the most commonly used antibiotics in poultry production by farmers. In addition to this, such high prevalence of β-Lactams and/or tetracyclines is attributed by high usage of these antibiotics in poultry feed. According to Ramdam (2015), doxycycline, chlorotetracycline and tetracycline are added each at the rate of 500 g to 1 kg per ton of feed during feed preparation. Tetracycline antibiotics have a higher tissue affinity compared to other tissues and some of them, like doxycycline, have a very slow elimination rate due to which they may have persisted longer in the tissues (Ferrini *et al.*, 2006).

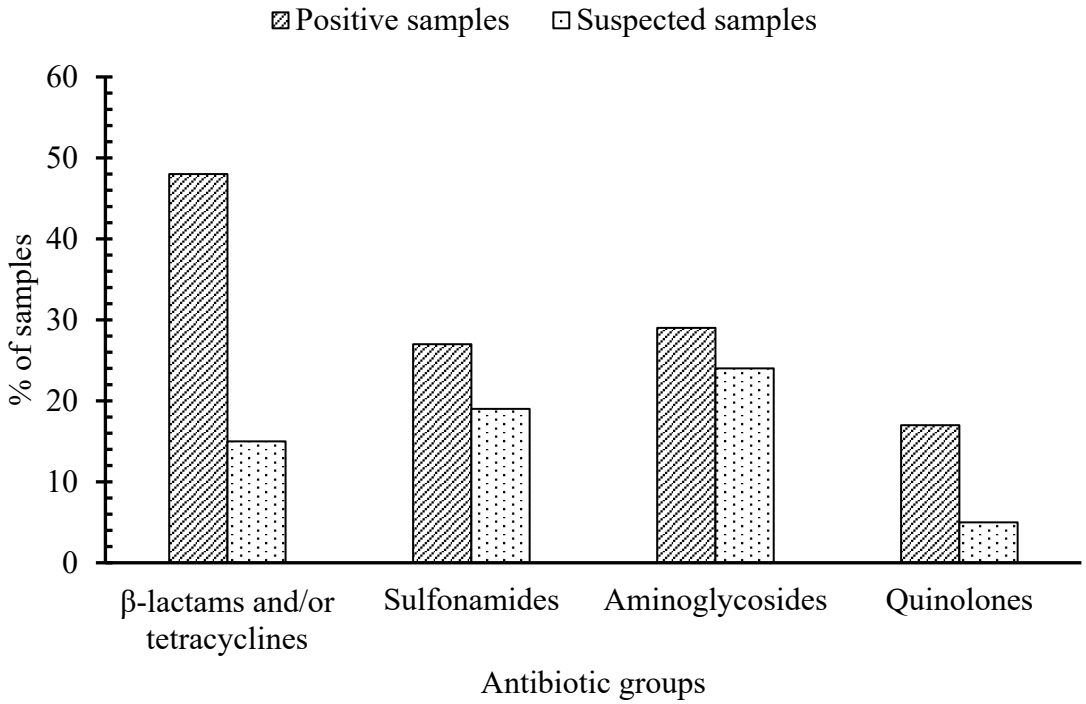

Fig. 4.4 Occurrence of different groups of antibiotics by microbial inhibition technique

Similarly, 27% of total samples screened were found to be contain sulfonamide residues. Slightly lower frequency of occurrence of sulfonamides (21.9%) in broiler meat of Kathmandu valley was reported by Gwachha (2017).

Quinolone residues were detected in an overall of 17% samples. In contrast to our findings, Shrestha (2017) reported 88.33% of chicken meat samples from Kathmandu valley to be positive towards quinolones when detected by ELISA where three samples were found to contain residues of enrofloxacin above MRLs while one sample contained residue levels

of ciprofloxacin above MRLs. Higher prevalence of quinolones was reported by Shrestha (2017) which might be due to better sensitivity of ELISA test kit.

4.5.4 Occurrence of single and multiple antibiotic residues

In the current study, four different plates optimized for detection of specific group of antibiotic were used. The detection of antimicrobials by more than one plate for the same sample indicated the possibility of presence of multi-residues in those samples. Table 4.5 shows that only 36.84% of the positive samples were found to contain a single group of antibiotics whereas the remaining were detected with multiple groups. The concurrent prevalence of different antimicrobials in tested samples has also been reported by several workers (Ezenduka, 2019; Shareef *et al.*, 2009).

Table 4.5 Occurrence of single and multiple antibiotic residues in different tissues

Residues	Plates	Number of samples positive				
		Liver	Breast muscle	Kidney	Gizzard	Total
Single residue	I	5	1	1	5	12
	II	0	0	1	2	3
	III	0	0	0	1	1
	IV	2	0	1	2	5
Multiple residues	I and II	1	0	2	1	4
	I and III	2	2	1	2	7
	I and IV	0	1	1	1	3
	II and III	0	0	0	0	0
	II and IV	0	1	0	0	1
	III and IV	0	0	0	0	0
	I, II and III	6	0	5	2	13
	I, II and IV	1	0	0	0	1
	I, III and IV	0	0	1	0	1
	II, III and IV	0	0	0	0	0
	All	0	0	5	1	6
Total		17	5	18	17	57

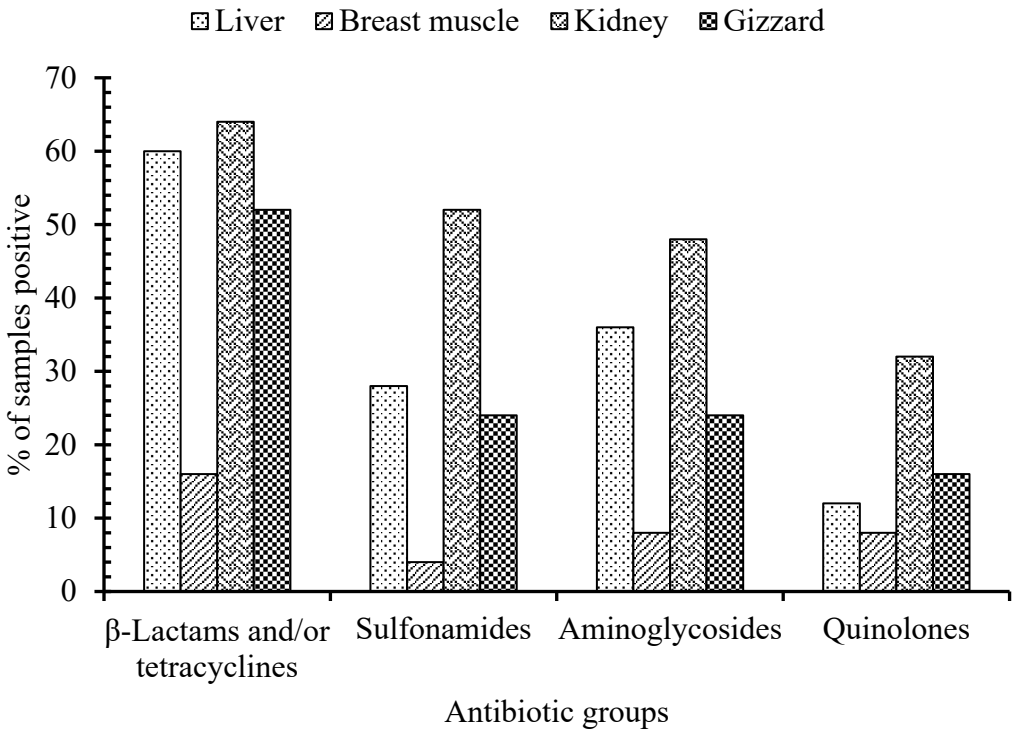

Fig. 4.5 Tissue wise occurrence of different groups of antibiotics by microbial inhibition test

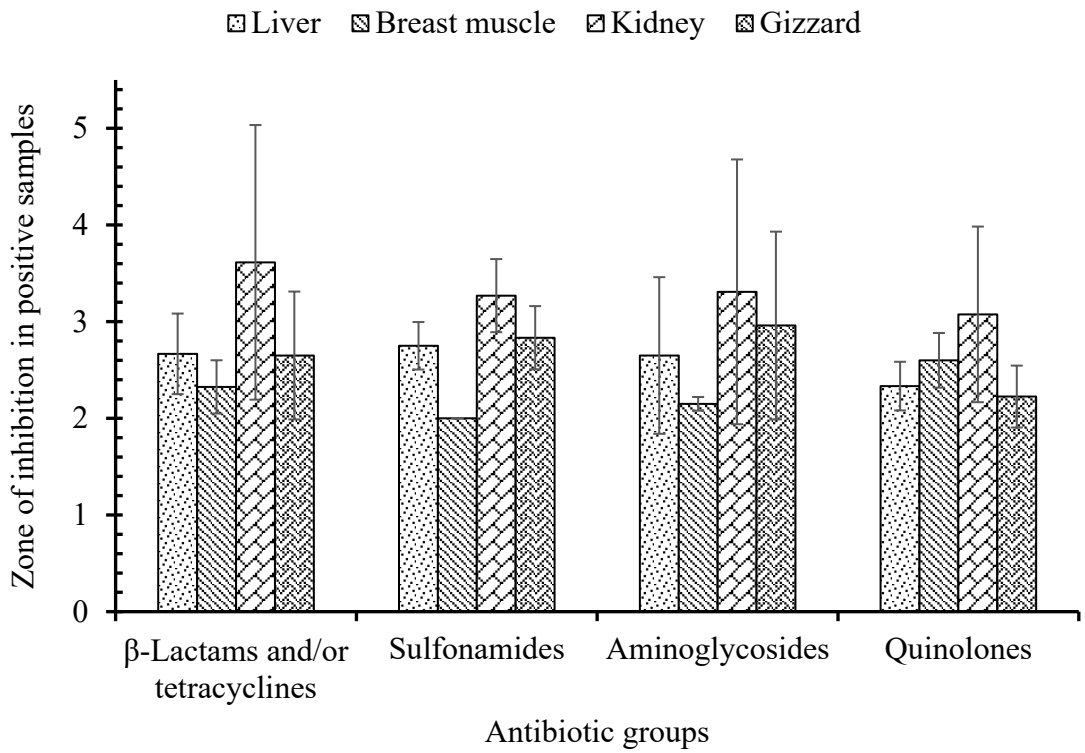

Fig. 4.6 Mean zones of inhibition shown by positive samples in different test plates. Vertical error bars represent ± standard deviation

4.5.5 Individual residues in different tissues

Percentage of individual tissue samples found to be positive to different groups of antibiotics are shown in Fig. 4.5. Significant difference was observed in occurrence of β-Lactams and/or tetracyclines, sulfonamides and aminoglycosides among different types of tissues ($p<0.05$). But occurrence of quinolone groups among different tissues was not found to differ significantly ($p>0.05$). It may be due to extensive distribution properties of this group of antibiotics owing to their physicochemical characteristics (Goetting *et al.*, 2011; Sarkozy, 2001).

Fig. 4.5 shows that the occurrence of all groups of antibiotics is the highest in kidneys followed by liver, gizzard and breast muscles. Similar findings was reported by Pandey *et al.* (2009) in which occurrence of β-Lactams and/or tetracyclines, sulfonamides as well as aminoglycosides was found to be the highest in kidney samples among kidney, liver and breast muscle samples. Kidney, being the most important organ for the excretion of antibiotics, obviously has relatively higher residues and is often used as sample matrix in many countries in which level of antibiotics in meat is to be assessed (Ezenduka, 2019). Similarly, each type of tissue screened showed highest prevalence of β-Lactams and/or tetracycline among the four groups of antibiotics tested.

4.5.6 Zone of inhibition of antibiotic residues in positive samples

The size of inhibition zones formed in the test plates is shown in Appendix A. Fig. 4.6 shows the largest zones of inhibition in each plate to be found for kidney samples. Zones of inhibition as large as 8.2 mm, 7.1 mm, 6.4 mm and 5.2 mm annular radii of inhibition zones were observed in plates I, II, III and IV respectively for kidney samples. According to Okerman *et al.* (1998), zone of inhibition shows linear relationship with the log concentration of the drug. This indicates each group of antibiotics are more concentrated in kidneys than other tissues. Such observation was also reported by Pandey *et al.* (2009) where largest zones of inhibition was found in kidneys followed by liver and breast muscle samples. Similarly, Shahid *et al.* (2007) reported significantly higher concentrations of oxytetracycline in chicken kidneys than in liver and muscles. Ciprofloxacin residues were also found in higher amounts in chicken kidneys than in liver and muscles (Faten *et al.*, 2016).

4.6 Thin layer chromatography

4.6.1 Preliminary trials

Almost all of the works regarding detection of antibiotic residues in meat tissues using TLC have been performed using acetone: methanol (1:1) as the mobile phase (Geidam *et al.*, 2009; Hossain, 2010; Khan *et al.*, 2018; Ramatla *et al.*, 2017; Sarker *et al.*, 2018; Sattar *et al.*, 2014; Shareef *et al.*, 2009; Tajick and Shohreh, 2006; Tazrin, 2014). But it couldn't be implied in this study due to very unsatisfactory results and thus a number of solvent systems were tested to determine the best one. Among different types of solvent systems and TLC plate pretreatments used, trial K was found to be the optimum solvent system for TLC analysis of the test antibiotics in this study. The photographs showing results of each trial are placed in the color plate P.1.

One of the major problems encountered during TLC was excessive tailing of doxycycline and tetracycyline antibiotics even though very small concentration of these antibiotic standards (as small as 0.1 mg/ml) were spotted on the plates. Such tailing was observed in trials A and B for all of the antibiotics tested. In order to minimize the problem, mobile phases added with a few amount of liquid ammonia was tried as in trials D, E and F. But no any significant improvement was observed. Oka and Uno (1983) described n-butanol as a suitable developing solvent for TLC of tetracycline and thus trial C containing greater fractions of n-butanol was tested. But very unsatisfactory results were obtained that covered the entire TLC plate with a dark patch when visualized under UV light. The reason for this could not be explained. The possibility is that some form of interaction might have taken place between the fluorescent material of the TLC plate and the component of solvent system used.

Chen and Schwack (2013) reported that the analytes (especially tetracyclines) displayed a strong tendency to form chelate complexes with alkaline earth and transition metal ions present in the silica plate, leading to serious tailing effects. So trials G, H, I and J were conducted which involved predevelopment of TLC plates in saturated Na_2EDTA solution prior to running the antibiotic standards. This technique was found to improve the results to some extent but still the results were not satisfactory as the antibiotic standards incurred similar retention factors. Finally, trial K was found to be the most satisfactory one with better resolution of the spots as well as minimum occurrence of tailing effect.

Another major problem encountered during TLC analysis was the occurrence of a dark band on the TLC plate just below the solvent front after the solvent was run through the plate. It was initially suspected to be because of the impurities that may have been present in the solvent. But even on running distilled water through the plate, the dark band was formed. It indicated that the band might have been formed due to impurities in the TLC plate itself. So to avoid the band, the plates were first pre-developed in methanol.

4.6.2 Overall detection of antibiotics

The samples found to be positive in microbiological screening test were subjected to TLC. The Rf values of spots located on the TLC plate for positive samples are tabulated in Appendix B. The percentages of samples detected with different antibiotics is shown in Fig. 4.7. Prevalence of different antibiotics in the meat samples was found to differ significantly ($p<0.05$). Tetracycline and doxycycline are found to be the most common antibiotics detected followed by ciprofloxacin and enrofloxacin. The results obtained are also justified by the survey report in which tetracycline and doxycycline are found to be the most commonly used antibiotics. Such a high incidence of tetracycline and doxycycline residues in meat can also be attributed to their usage in poultry feed. According to Ramdam (2015), doxycycline, chlorotetracycline and tetracycline are the major antibiotics added to the feed and they are each added at the rate of 500 g to 1 kg per ton of feed during feed preparation.

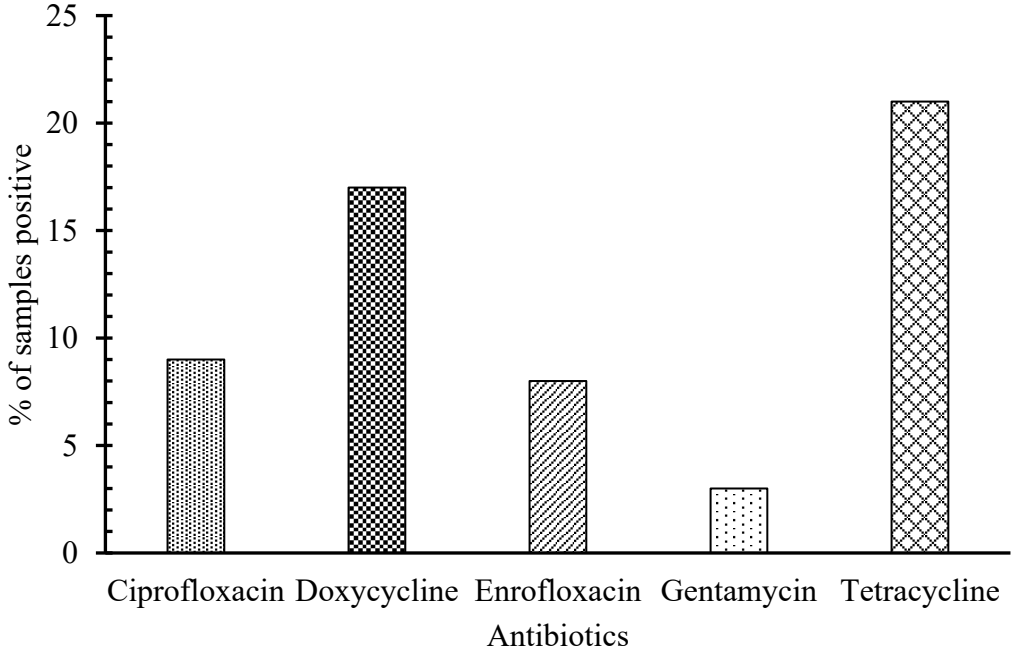

Fig. 4.7 Percentage of samples found to contain antibiotics on TLC analysis

4.6.3 Occurrence of residues in different tissues

4.6.3.1 Residues of ciprofloxacin

Residues of ciprofloxacin were detected in 9% of the samples which is lower than the findings of Prajapati *et al.* (2018) who reported ciprofloxacin in 15.21% broiler meat samples collected from Kathmandu, Kaski and Chitwan. Different workers have reported various percentages of ciprofloxacin in chicken meat. Residues of ciprofloxacin in as high as 40.7%, 44.37% 21.4% have been reported by Sattar *et al.* (2014), Sarker *et al.* (2018) and Ramatla *et al.* (2017) respectively. Similarly, residues of ciprofloxacin residues in as low as 3% chicken meat samples have been detected by Tazrin (2014).

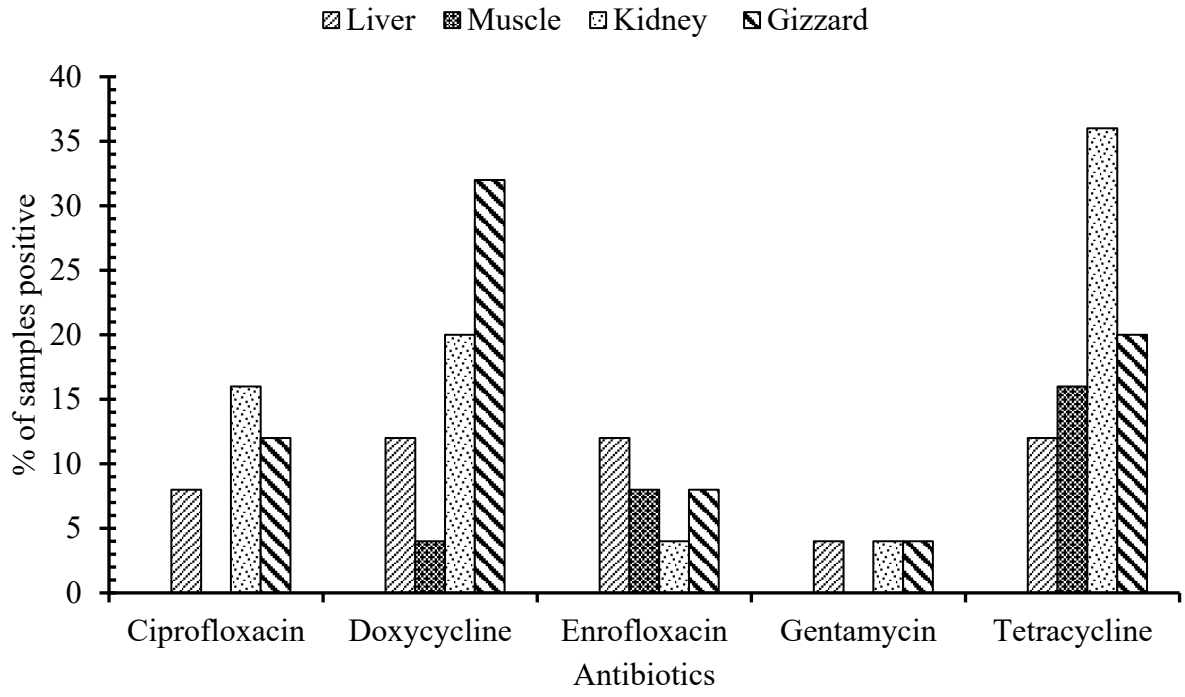

Fig. 4.8 Prevalence of antibiotics in different tissues on TLC analysis

Percentage of different types of tissues in which ciprofloxacin was detected is shown in Fig. 4.8. Although no significant difference in occurrence of ciprofloxacin among different tissues was observed, highest incidence was observed in kidneys (16%) followed by gizzard (12%) and liver (8%). No residues of ciprofloxacin was detected in breast muscle samples. Maximum occurrence of ciprofloxacin in broiler kidneys (48.57%) among different tissues was also reported by Sattar *et al.* (2014) followed by liver (42.85%) and breast muscles (31.42%). In contrast to the results in this study, Sattar *et al.* (2014) found higher prevalence of ciprofloxacin residues in liver (44%) than in kidneys (42%) and breast muscles (30%).

Similarly, Sarker *et al.* (2018) also reported 52% liver samples and 39% breast meat samples to be contain ciprofloxacin residues.

4.6.3.2 Residues of doxycycline

Fig. 4.7 shows that residues of doxycycline were detected in 17% of the samples. Prevalence of doxycycline as high as 32.3% was reported in chicken meat of Bangladesh (Sarker *et al.*, 2018).

Occurrence of doxycycline residues in gizzard and breast muscles was found to differ significantly ($p<0.05$). In contrast to other antibiotics, doxycycline was found to be the most prevalent in gizzard samples (32%) followed by kidney (20%), liver (12%) and breast muscles (4%) which is shown in the Fig. 4.8. A study regarding tissue depletion of doxycycline administered orally at high dosage to broiler chickens via drinking water was conducted by Hsiao *et al.* (2016) and found out doxycycline concentrations to be higher in gizzard followed by kidney, liver and breast muscles after the third day of administration while on the fifth day, the concentrations in gizzard depleted at a higher rate than in kidney and liver. It may be because of 5 to 10 times higher liphophilicity of doxycycline, resulting in higher tissue penetration, larger volume of distribution and longer elimination times (Papich and Riviere, 2017).

4.6.3.3 Residues of enrofloxacin

Residues of enrofloxacin were found in 8% of the collected chicken meat samples. This findings is much smaller than the findings in Bangladesh where 26.8% and 27.85% of the samples were detected with enrofloxacin residues by Sarker *et al.* (2018) and Sattar *et al.* (2014) respectively. Similarly, 46.7%, 2.5% of meat samples were detected with enrofloxacin residues by Khan *et al.* (2018) and Tazrin (2014) respectively.

Occurrence of enrofloxacin among different tissues was not found to differ significantly ($p>0.05$). Highest occurrence of enrofloxacin was found in liver (12%) followed by breast muscles (8%), gizzard (8%) and kidney (4%) which is shown in Fig. 4.8. Sattar *et al.* (2014) also reported highest occurrence of enrofloxacin in liver among liver, kidney and breast muscle samples in Bangladesh. Similar values of enrofloxacin residues in breast muscles was shown by Prajapati *et al.* (2018) who reported 8.7% of breast meat samples collected from Kathmandu, Kaski and Chitwan to be positive. In contrast to other antibiotics, least number of kidney samples were detected with enrofloxacin residues. Higher prevalence of

enrofloxacin residues in chicken liver than in kidney samples was also reported by Islam *et al.* (2016).

4.6.3.4 Residues of gentamycin

As shown in Fig. 4.7, gentamycin residues were detected in the least number of samples (3%). Shareef *et al.* (2009) reported none of the chicken meat samples to contain gentamycin residues in chicken meat of Iraq.

Occurrence of gentamycin residues in different tissues was not found to differ significantly ($p>0.05$). 4% of each of liver, kidney and gizzard samples were found to contain gentamycin residues whereas no residues of gentamycin was detected in breast meat samples which is shown in Fig. 4.8.

4.6.3.5 Residues of tetracycline

Tetracycline was found to be the most prevalent antibiotic in poultry meat of Dharan and was found in 21% of the meat samples. Tetracycline residues were also detected by Sattar *et al.* (2014), Hossain (2010) and Ramatla *et al.* (2017) in 30%, 11.83% and 14.6% of chicken meat samples respectively. According to Pantha *et al.* (2019), 33.33% of chicken meat samples from Kathmandu valley to be positive towards tetracycline by using rapid test kits. The test kit detected not only tetracycline but also other antibiotics of the tetracycline group like chlortetracycline and oxytetracycline due to which the obtained values might have been higher. Similarly, Raut *et al.* (2017) reported 29.09% of chicken meat samples from Kavre and Kailali to contain tetracycline residues.

As shown in Fig. 4.8, among different tissues, tetracycline residues were the most prevalent in kidney samples (36%), followed by gizzard (20%), breast muscles (16%) and finally liver (12%). Occurrence of tetracycline residues between liver and kidney was found to differ significantly ($p<0.05$). According to Sattar *et al.* (2014), residues of tetracycline were in 48% livers, 24% kidneys and 24% breast muscles. Although a large number of liver samples showed zone of inhibition in plate I during microbial inhibition test, only a few were found to contain tetracycline residues. It may probably be due to occurrence of other antibiotics of the tetracycline group such as cholorotetracycline and oxytetracycline or of the β-lactams group.

4.6.4 Unidentified spots during TLC

Due to the limited number of antibiotic standards available, all the spots separated on the TLC plate could not be identified. In addition to spots whose Rf values matched with that of the standard antibiotics, several samples showed spots which had Rf values different from that of the standards.

During the first run, two of the samples showed spots with an Rf value of 0.065. During microbial inhibition test, both these samples were found to be positive for β-Lactams and/or tetracyclines. So this spot can potentially be of a β-Lactam or tetracycline. Similarly, 13 of the samples showed an unknown spot with Rf value of 0.093. During microbial inhibition test, all of these samples were positive in Plate I, i.e., these spots could potentially belong to a β-Lactam or tetracycline. Likewise, 10 samples showed an unknown spot with Rf value of 0.315. All these samples were also found to form zone of inhibition in plate I during microbial inhibition test. So, this spots might belong to another β-lactam or tetracycline antibiotic.

During the second run, two samples showed a spot with Rf value of 0.05. During microbial inhibition test, these samples showed inhibition zones both in plate I and plate III. So, this spot might be of an antibiotic of the group β-lactam or tetracycline or aminoglycoside. Similarly, three samples showed a spot with Rf value of 0.081. All of these samples showed zones of inhibition in plate I and III during microbial inhibition test. So, this spot might belong to an antibiotic from β-lactam or tetracycline or aminoglycoside group. Likewise, 9 of the samples showed spot with Rf value of 0.11. All these samples formed zones of inhibition in plate I during microbial inhibition test. So, this spot could belong to β-lactam or tetracycline antibiotic. Another 3 samples also showed spots with Rf value of 0.343. Since all of these samples formed inhibition zones in plate I and II during microbial inhibition test, the spot can probably belong to a β-lactam or tetracycline or sulfonamide antibiotic.

During the third run, 13 samples showed a spot with Rf value of 0.08. On comparing with microbial inhibition test, all of these samples were found to form a zone of inhibition on plate I. Thus, the spot may probably belong to a β-lactam or tetracycline antibiotic. Similarly, 8 of the samples showed spot with Rf value of 0.283. All of these samples were also found to form a zone of inhibition on Plate I during microbial inhibition test. Thus, it can be

potentially the spot of a β-lactam or tetracycline antibiotic. Likewise, two of the samples in this run showed a spot with Rf value of 0.487. During the microbial inhibition test, both of these samples had formed zones of inhibition on plate I, II and III. Thus, the spot may belong to a β-lactam or tetracycline or sulfonamide or aminoglycoside antibiotic.

During the fourth run, 5 of the samples showed a spot with Rf value of 0.304. During the microbial inhibition test, all of these samples had formed zones of inhibition on plates I and III. So, this spot may potentially be of a β-lactam or tetracycline or aminoglycoside. Similarly, 6 of the samples showed an unknown spot with Rf value of 0.34. Since all of these samples had formed zones of inhibition on plate I and III during microbial inhibition test, the spot may probably belong to a β-lactam or tetracycline or aminoglycoside antibiotic.

PART V

Conclusions and recommendations

5.1 Conclusions

Based on the survey conducted among poultry farmers and veterinarians of Dharan Municipality as well as microbial screening of meat samples followed by thin layer chromatography of samples collected from Dharan market, following conclusions were drawn:

1. Significantly high proportion of poultry farm owners of Dharan have an education level upto S.L.C. The farmers have inadequate knowledge regarding occurrence of antibiotic residues, antibiotic resistance and withdrawal period of antibiotics. The common poultry diseases in Dharan are chronic respiratory disease (CRD), *E. coli* infections, coccidiosis and foulpox. Similarly, tetracyclines are the most commonly used antibiotics in poultry farms.
2. For the microbial screening of antibiotics, *Bacillus subtilis* was isolated and identified from *kinema.*
3. *Bacillus subtilis* is more susceptible to β-Lactams, tetracyclines, sulfonamides and aminoglycosides while *E. coli* is more susceptible to quinolones.
4. 57% of chicken meat samples sold at Dharan contain atleast one group of antibiotics tested. Among different tissues, kidneys (72%) have the highest incidences of antibiotics followed by liver (68%), gizzard (68%) and breast meat (20%). Highest percentages of samples contain β-lactams and/or tetracycline residues (49%) followed by aminoglycosides (29%), sulfonamides (27%) and quinolones (17%).
5. Use of silica gel TLC plates prewashed with methanol followed by predevelopment with aq. Na_2EDTA combined with the use of chloroform: methanol: 25% NH_4OH (60:35:5) as mobile phase gives the best separation of the five test antibiotics.
6. Highest number of samples contain tetracyclines (21%) followed by doxycycline (17%), ciprofloxacin (9%), enrofloxacin (8%) and gentamycin (3%). Occurrence of tetracycline and doxycycline among different tissues differs significantly in poultry meat of Dharan whereas occurrence of ciprofloxacin, enrofloxacin and gentamycin doesn't differ significantly among different tissues. Several samples showed spots with unknown Rf

values which may belong to other antibiotics that were not taken as references in this study.

5.2 Recommendations

1. As per the findings in this study, more than half of the broiler meat sold within Dharan municipality contains antibiotic residues. This indicates that antibiotics aren't used wisely by farmers. Such carelessness can lead to severe health hazards as well as development of antibiotic resistance in zoonotic pathogens that may erupt as a serious threat to public health.
2. So in order to stem such misuse of antibiotics in broiler production, Nepal Government should strictly implement a national action plan on antibiotics usage and it should include strategies and policies to promote good husbandry practices, nationwide antibiotic residue surveillance program and raising awareness among producers and consumers on this issue. Buying and usage of antibiotics should be strictly be done under the supervision of a veterinary professional. Similarly, proper insurance facilities should be provided to the farmers in order to prevent losses if incurred by death of the birds.

PART VI

Summary

Use of antibiotics for therapeutic and prophylactic purposes have made it possible for poultry industry to reach the heights it had never reached before. But with excessive use of such antibiotics, there is a risk of occurrence of antibiotic residues in the meat produced. This work is intended to study the prevalence of antibiotic residues in poultry meat sold at Dharan municipality. At first, a survey was conducted among poultry farmers and veterinary shops. Then samples of four varieties of poultry tissue, namely, liver, breast muscle, kidney and gizzard were collected and subjected to microbial inhibition test using combine three plate test using *Bacillus subtilis* as test organism and a one plate test using *E coli* as test organism. The samples found positive in this first stage of screening were further subjected to thin layer chromatographic analysis for identification of the antibiotics present.

Most of the poultry farmers have an educational level upto S.L.C and only a few of them have received training on poultry production. Not many of them have any idea regarding safety aspects of antibiotics and impacts of their misuse. The survey report shows maximum usage of tetracycline and doxycycline in poultry farms. Through microbial inhibition technique, 57% of chicken meat samples are found to be contain residues among which highest percentages of kidneys (72%) contain antibiotic residues followed by liver (68%), gizzard (68%) and finally breast muscle (20%). Highest number of samples are positive towards β-lactams and/or tetracyclines (49%) followed by aminoglycosides (29%), sulfonamides (27%) and quinolones (17%). Residues of each groups of antibiotics are found in higher number of kidney samples in comparison to other tissues. Similarly, 36.84% of the positive samples contain a single group of antibiotics whereas the remaining 63.16% of positive samples contain multiple groups of antibiotics. Through thin layer chromatography, it is found that highest number of samples contain tetracyclines (21%) followed by doxycycline (17%), ciprofloxacin (9%), enrofloxacin (8%) and gentamycin (3%). Prevalence of antibiotics among different tissues is found to differ significantly. Such a high prevalence of residues in poultry meat indicates excessive usage and misuse of antibiotics. So, in order to control these levels, strict regulatory measures should be implemented by the municipality.

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Appendices

Appendix A

Materials used

Chemical reagents:

1. Acetic acid (Labort, Minimum assay 99.7%)
2. Acetone (SRL Chem, Minimum assay 99%)
3. Agar agar
4. Ammonium hydroxide (Qualigens)
5. Antibiotic standard powers of ciprofloxacin, doxycycline, enrofloxacin, gentamycin and tetracycline
6. Antibiotic susceptibility discs (Himedia)
7. Barium Chloride (Qualigens)
8. Beef extract (Himedia)
9. Chloroform (SRL chem, Minimum assay 99%)
10. Crystal violet
11. Dichloromethane (Qualigens)
12. Diethyl ether (Qualigens, Assay 98%)
13. Disodium Hydrogen Phosphate (Merck, Minimum Assay 98%)
14. Gram's Iodine
15. Hydrochloric acid (Qualigens, Minimum assay 35-37%)
16. Hydrogen peroxide
17. Kovac's reagent
18. Methanol (Qualigens, Assay 99%)
19. Methyl red
20. MRVP broth
21. Mueller Hinton Agar (Himedia)
22. Na_2EDTA (Qualigens, Minimum assay 98%)
23. NaOH (Labort, Minimum assay 96%)
24. n-Butanol (Qualigens, Minimum assay 98%)
25. Nutrient Agar (Himedia)
26. Nutrient Broth (Himedia)
27. Oxalic acid (Qualigens, Assay 99.5%)

28. Safranin
29. Simmons Citrate agar (Himedia)
30. Sodium Dihydrogen Phosphate (Merck, Minimum Assay 98%)
31. soluble starch
32. Sulphuric acid (Qualigens, Minimum assay 97%)
33. Trichloroacetic acid (Qualigens, Assay 98%)
34. VP reagents

Equipments:

1. 8mm cork borers
2. Autoclave
3. Bacteriological incubator
4. Brightfield microscope
5. Centrifuge
6. Heating arrangements
7. Hot air oven
8. Ice box
9. Inoculating loop
10. Magnetic stirrer
11. pH meter (HANNA HI 96017, Sensitivity ± 0.01)
12. Reclosable plastic pouches
13. Rotary vacuum evaporator (IKA® RV 10 Model-HB 10 D S96- 2425W)
14. Sterile cotton swabs (Himedia)
15. TLC development tank
16. TLC Silica Gel 60 F_{254} aluminium plates (Merck, Germany)
17. UV Visualization chamber (CAMAG)
18. Vernier caliper
19. Weighing balance

Glasswares:

1. Beakers
2. Conical flasks
3. Funnel
4. Glass rods

5. Measuring cylinders
6. Mortar and Pestle
7. Petri plates
8. Pipettes and micro pipettes
9. Separating funnel
10. Test tubes
11. Volumetric flasks

Appendix B

Table B.1 Zones of Inhibition shown by different sample tissues on test plates

Samples	Annular radii of zones of inhibition (mm)			
	Plate I (β-Lactams and/or tetracyclines)	Plate II (Sulfonamides)	Plate III (Aminoglycosides)	Plate IV (Quinolones)
L1	2.5	2.8	3.1	1.4
L2	1.3	1.8	-	2.1
L3	2.3	1.3	1.2	1.3
L4	1.3	-	1.3	-
L5	2.4	1.9	1.5	-
L6	2.5	-	2.1	1.8
L7	3.1	3	2.1	1.6
L8	1.7	-	1.5	2.6
L9	-	-	-	-
L10	3.3	1.5	1.7	1.2
L11	2.8	-	3.6	2.3
L12	2.9	2.3	1.5	-
L13	2.3	-	-	-
L14	2.7	-	2.2	-
L15	1.9	1.9	-	-
L16	3.5	4.3	4.4	-
L17	1.9	-	1.1	-
L18	2.3	2.2	2.1	-
L19	1.4	1.3	0.9	-
L20	2.2	1.1	1.4	1.3
L21	2.2	2.3	2.2	-
L22	3	2.8	2.7	-
L23	-	-	-	-
L24	-	-	-	-
L25	-	-	-	-
M1	-	-	-	1.8
M2	1.2	2.4	-	2.8
M3	2	-	1.2	-
M4	-	-	-	-
M5	1.8	-	1.6	1.2
M6	-	-	-	-
M7	-	-	-	-
M8	-	-	-	-
M9	2.2	1.5	-	2.4

M10	2.6	1.6	2.1	-
M11	2.5	-	2.2	-
M12	-	0.9	0.9	-
M13	-	0.5	0.5	-
M14	1.5	1.8	1.9	-
M15	-	-	-	-
M16	-	-	-	-
M17	-	-	1.3	-
M18	-	-	-	-
M19	-	-	-	-
M20	0.5	-	0.5	-
M21	-	1.2	1.5	-
M22	-	-	-	-
M23	-	-	-	-
M24	-	-	-	-
M25	-	-	-	-
K1	8.2	7.1	6.4	5.2
K2	1.4	-	-	2.5
K3	-	-	-	-
K4	-	-	1.2	-
K5	3	-	2.2	-
K6	2.8	2.2	2.1	-
K7	-	-	-	-
K8	3.4	2.6	2	2.9
K9	4.4	1.3	1.6	2.4
K10	3.1	2.3	1.7	-
K11	3.7	-	4.3	2.9
K12	4.1	4.2	3.7	3.3
K13	4.1	3.1	3.6	2.9
K14	3.7	3.6	2.3	-
K15	2.6	3.7	4	-
K16	3.2	4.1	4.6	2.5
K17	0.5	0.8	0.6	-
K18	2.3	-	0.9	-
K19	2.3	2.7	2.4	1.7
K20	3.3	2.1	1.3	1.6
K21	2.3	2.5	2.1	1.8
K22	-	-	-	-
K23	-	-	-	-
K24	-	2.3	-	-
K25	-	-	-	-
G1	2.9	4.4	4.2	2
G2	1.2	-	-	2.7

G3	-	-	-	-
G4	2	1.5	1.5	-
G5	2.3	2.3	1.2	-
G6	-	-	-	-
G7	2.5	1.7	1.9	-
G8	2.5	-	1.5	2.1
G9	3.5	1.5	-	-
G10	4.2	-	2.2	-
G11	1.4	-	0.9	-
G12	1.5	2.4	0.9	-
G13	-	-	0.9	-
G14	1.7	2.8	1.6	-
G15	3	2.3	2.1	-
G16	2	2.8	3.8	1.3
G17	2.1	1.1	2.5	-
G18	2.1	0.9	1.9	1.5
G19	0.9	-	1.3	2.1
G20	2.1	1.1	0.9	1.5
G21	1.1	1.1	2	-
G22	2.6	1.1	1.5	-
G23	-	-	-	-
G24	-	-	-	-
G25	-	-	-	-

*L, M, K and G represent liver, muscle, kidney and gizzard sample respectively.

Appendix C

Table C.1 R_f value of spots seen on performing thin layer chromatography of positive samples

Sample	Retention factors									
	First run									
Ciprofloxacin							0.36			
Doxycycline			0.17							
Enrofloxacin										0.50
Gentamycin								0.42		
Tetracycline					0.24					
L1	0.06					0.31				
K1		0.09			0.24	0.31				
G1		0.09			0.24	0.31				0.51
L2		0.09				0.31				0.50
M2		0.09			0.24					0.50
K2		0.10			0.24	0.31				0.50
G2		0.09			0.24					0.50
L3	0.06	0.10				0.31				0.51
M3		0.10			0.24					
G4		0.09				0.31				
L5		0.09				0.31				
M5		0.09			0.23					0.50
K5		0.09			0.23	0.31				
G22		0.09	0.17			0.31				
	Second run									
Ciprofloxacin								0.54		
Doxycycline				0.22						
Enrofloxacin										0.62
Gentamycin							0.49			
Tetracycline					0.29					
L6	0.04	0.08		0.22	0.28		0.49			
L7	0.05		0.10	0.22		0.34				
G7			0.10		0.28					
L8			0.11					0.54		
K8					0.28					
G8			0.11	0.22						
M9			0.11							
K9			0.12		0.29					
G9			0.11	0.22		0.34				
L10		0.08								
M10		0.08	0.11	0.23		0.35				
G10			0.11	0.22						

Third run							
Ciprofloxacin					0.47		
Doxycycline		0.16					
Enrofloxacin							0.51
Gentamycin				0.41			
Tetracycline		0.20					
L11	0.07		0.27				
M11	0.07	0.20					
K11	0.07	0.17	0.27	0.41			
L12	0.07	0.20	0.28				
K12	0.07	0.17	0.28				
G12	0.08		0.27				
L13	0.08	0.17	0.28				
K13	0.07	0.20	0.28				0.48
L14	0.07	0.20	0.28				
K14	0.08	0.20					
G14	0.08	0.21					
K15		0.21					0.49
G15	0.08	0.21					
Fourth run							
Ciprofloxacin						0.50	
Doxycycline	0.22						
Enrofloxacin							0.57
Gentamycin					0.45		
Tetracycline		0.26					
L16			0.30				
K16	0.22			0.33		0.50	
G16	0.23		0.31			0.50	
G17	0.23						
L18				0.34			
K18	0.23			0.34			
G18	0.23		0.30		0.45	0.50	
K19						0.50	
L20				0.34		0.50	
K20	0.23			0.33		0.50	
G20	0.23		0.30			0.50	
L21			0.31				
K21		0.25		0.33		0.50	
L22							0.58

Appendix D

Table D.1 Test of significance between education levels of poultry farm owners

Chi-Square	20.720[a]
Df	2
Asymp. Sig.	.000

a. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 8.3.

Table D.2 Test of significance between frequencies of usage of different antibiotics by poultry farmers

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	22.917[a]	10	.011
Likelihood Ratio	22.686	10	.012
Linear-by-Linear Association	2.615	1	.106
N of Valid Cases	275		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 7.55.

Appendix E

Table E.1 Test of significance of overall occurrence of different groups of antibiotics

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	23.828[a]	3	.000
Likelihood Ratio	23.645	3	.000
Linear-by-Linear Association	19.575	1	.000
N of Valid Cases	400		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 30.25.

Table E.2 Test of significance of occurrence of overall antibiotic residues between different tissue samples in microbiological screening test

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	18.727a	3	.000
Likelihood Ratio	19.308	3	.000
Linear-by-Linear Association	1.365	1	.243
N of Valid Cases	100		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 10.75.

Table E.3 Test of Significance for overall occurrence of different groups of antibiotic residues

Chi-Square	16.620[a]
df	3
Asymp. Sig.	.001

a. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 30.3.

Table E.4 Significance test for occurrence of β-Lactams and/or tetracyclines between tissue samples

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	14.423[a]	3	.002
Likelihood Ratio	15.547	3	.001
Linear-by-Linear Association	.286	1	.593
N of Valid Cases	100		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 12.00.

Table E.5 Significance test for occurrence of sulfonamides between tissue samples

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	14.764[a]	3	.002
Likelihood Ratio	16.436	3	.001
Linear-by-Linear Association	.814	1	.367
N of Valid Cases	100		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.75.

Table E.6 Significance test for occurrence of aminoglycosides between tissue samples

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	13.952[a]	3	.003
Likelihood Ratio	15.946	3	.001
Linear-by-Linear Association	.010	1	.920
N of Valid Cases	100		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.75.

Table E.7 Significance test for occurrence of quinolones between tissue samples

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.691[a]	3	.128
Likelihood Ratio	5.630	3	.131
Linear-by-Linear Association	1.932	1	.165
N of Valid Cases	100		

a. 4 cells (50.0%) have expected count less than 5. The minimum expected count is 4.50.

Appendix F

Table F.1 Significance test for overall occurrence of different antibiotics detected by TLC

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	20.596[a]	4	.000
Likelihood Ratio	21.700	4	.000
Linear-by-Linear Association	.973	1	.324
N of Valid Cases	500		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 11.60.

Table F.2 Significance test for occurrence of ciprofloxacin among different tissues

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.274[a]	3	.233
Likelihood Ratio	6.239	3	.101
Linear-by-Linear Association	1.185	1	.276
N of Valid Cases	100		

a. 4 cells (50.0%) have expected count less than 5. The minimum expected count is 2.25.

Table F.3 Significance test for occurrence of doxycycline among different tissues

Pearson Chi-Square Tests

		Doxycycline
Tissue	Chi-square	7.583
	df	3
	Sig.	.055[a]

Results are based on nonempty rows and columns in each innermost subtable.

a. More than 20% of cells in this subtable have expected cell counts less than 5. Chi-square results may be invalid.

Table F.4 Significance test for occurrence of doxycycline between liver and gizzard

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.030[a]	1	.082		
Continuity Correction[b]	1.705	1	.192		
Likelihood Ratio	3.275	1	.070		
Fisher's Exact Test				.189	.095
Linear-by-Linear Association	2.970	1	.085		
N of Valid Cases	50				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.00.

b. Computed only for a 2x2 table

Table F.5 Significance test for occurrence of enrofloxacin among different tissues

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.087[a]	3	.780
Likelihood Ratio	1.133	3	.769
Linear-by-Linear Association	.430	1	.512
N of Valid Cases	100		

a. 4 cells (50.0%) have expected count less than 5. The minimum expected count is 2.00.

Table F.6 Significance test for occurrence of gentamycin among different tissues

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.031[a]	3	.794
Likelihood Ratio	1.757	3	.624
Linear-by-Linear Association	.068	1	.794
N of Valid Cases	100		

a. 4 cells (50.0%) have expected count less than 5. The minimum expected count is .75.

Table F.7 Significance test for occurrence of tetracycline among different tissues

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.003[a]	3	.172
Likelihood Ratio	4.771	3	.189
Linear-by-Linear Association	1.444	1	.229
N of Valid Cases	100		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.25.

Table F.8 Significance test for occurrence of tetracycline residues between liver and kidney

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.947[a]	1	.047		
Continuity Correction[b]	2.741	1	.098		
Likelihood Ratio	4.091	1	.043		
Fisher's Exact Test				.095	.048
Linear-by-Linear Association	3.868	1	.049		
N of Valid Cases	50				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.00.

b. Computed only for a 2x2 table

Appendix G

Poultry Farms Survey

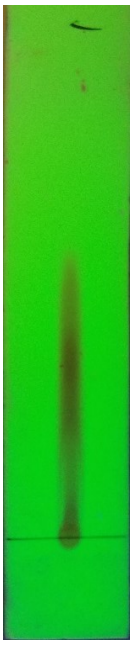
Dharan Municipality

Date: _____

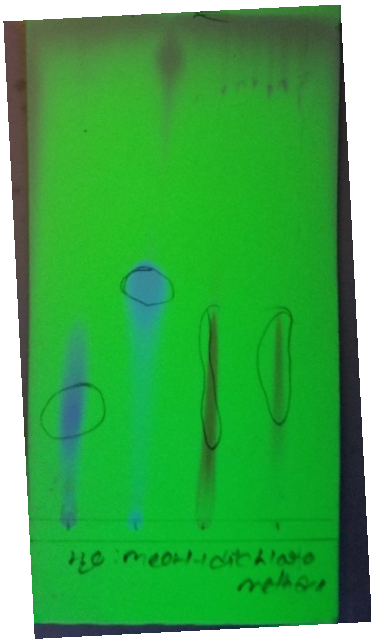
1. Name of Poultry Farm Owner ☐
2. Maximum capacity of the poultry farm ☐
3. Education level of the owner ☐
4. Training acquired on poultry farming ☐ Yes ☐ No
5. Knowledge regarding occurrence of antibiotic residues in meat ☐ Yes ☐ No
6. Knowledge regarding withdrawal period of antibiotics ☐ Yes ☐ No
7. Knowledge regarding occurrence of antibiotic resistance in microorganisms ☐ Yes ☐ No
8. Commonly used antibiotics in poultry farms

Plates

Plate P.1 Results of preliminary trials of TLC using different solvent systems

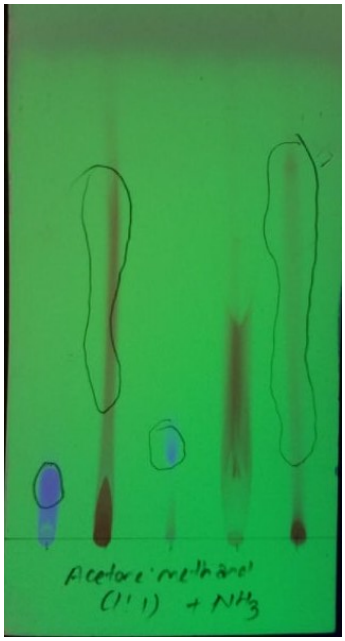


Trial A



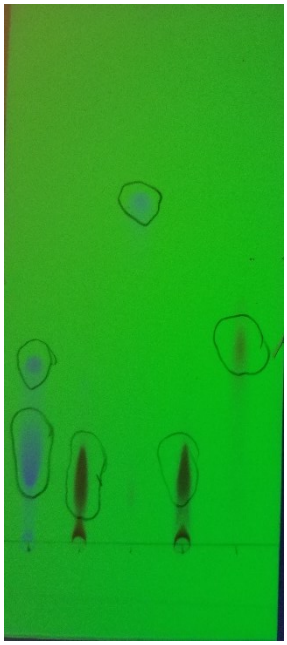
Trial B

Trial C

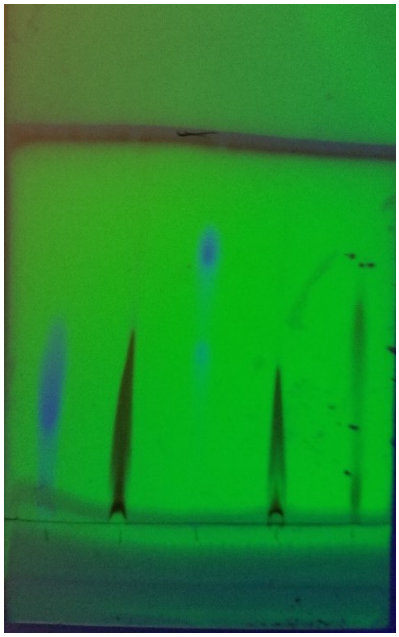


Trial D

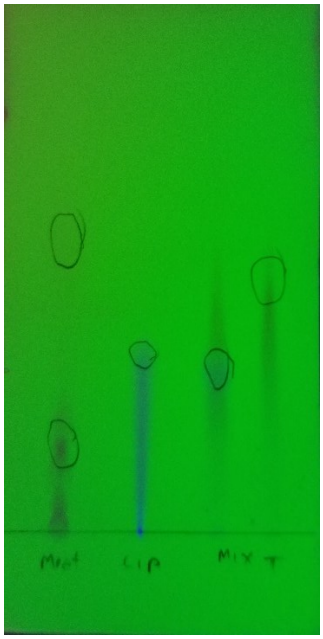
Trial E



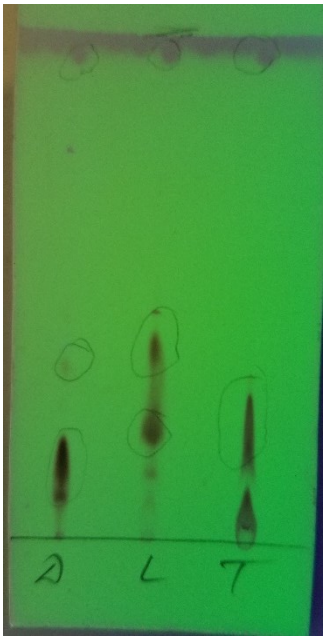
Trial F



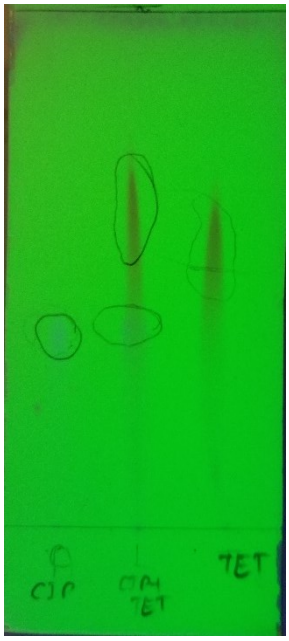
Trial G



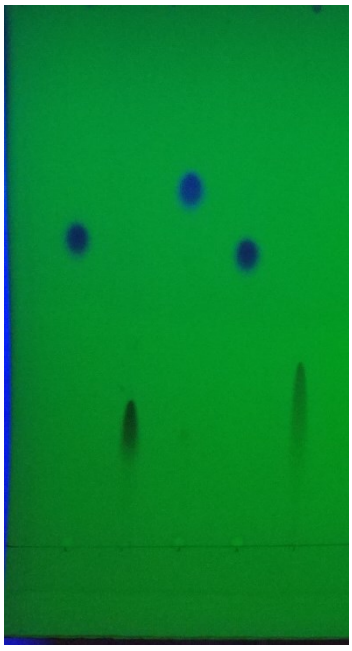
Trial H



Trial I



Trial J



Trial K

Plate P.2 Conducting survey

Plate P.3 *Bacillus subtilis* isolated from *Kinema*

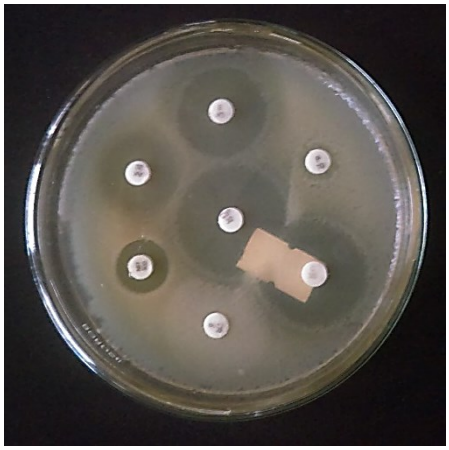


Plate P.4 Antibiotic susceptibility testing of test organisms

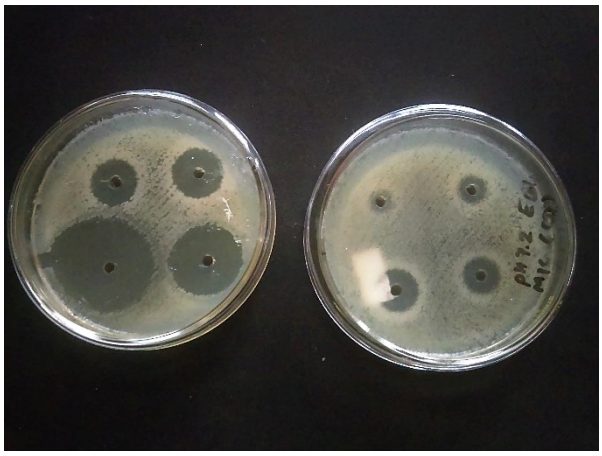


Plate P.5 Determination of MIC of antibiotics for test organisms

Plate P.6 Microbial screening for presence of antibiotics in broiler meat

Plate P.7 Observation of petri plates after incubation for determination of inhibition zones

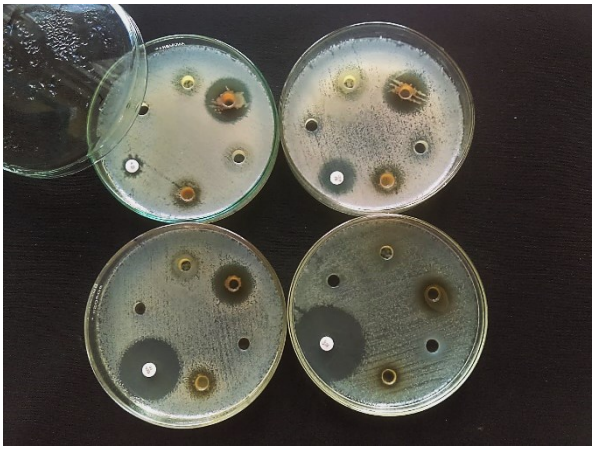
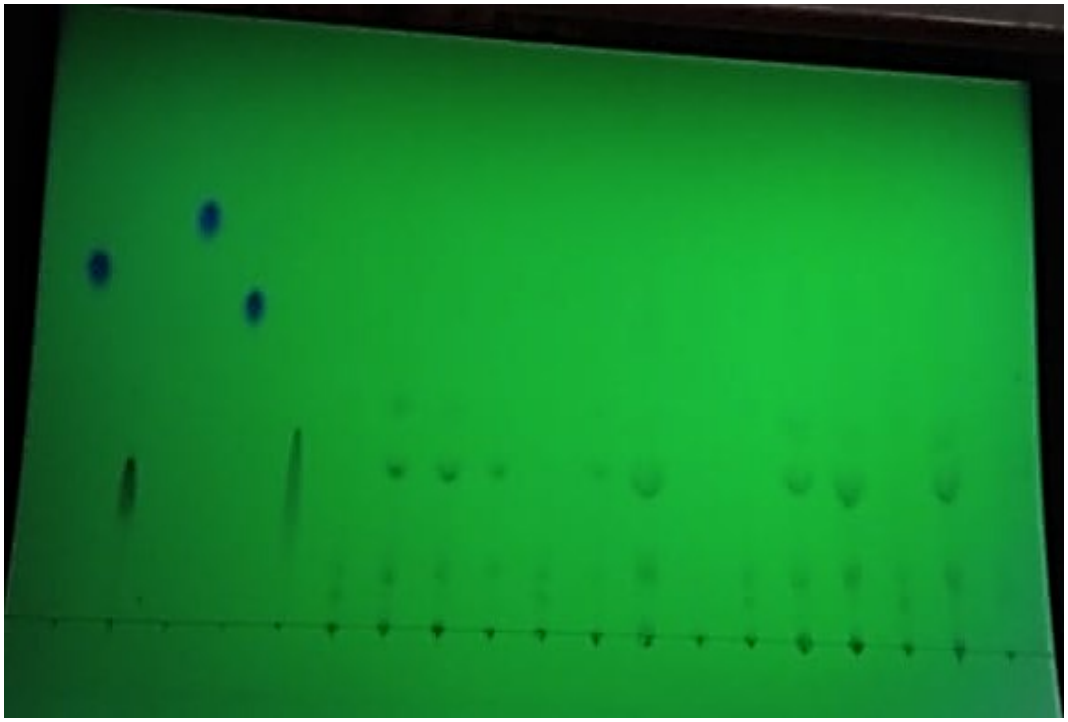


Plate P.8 Occurrence of zones of inhibition in antibiotic positive samples

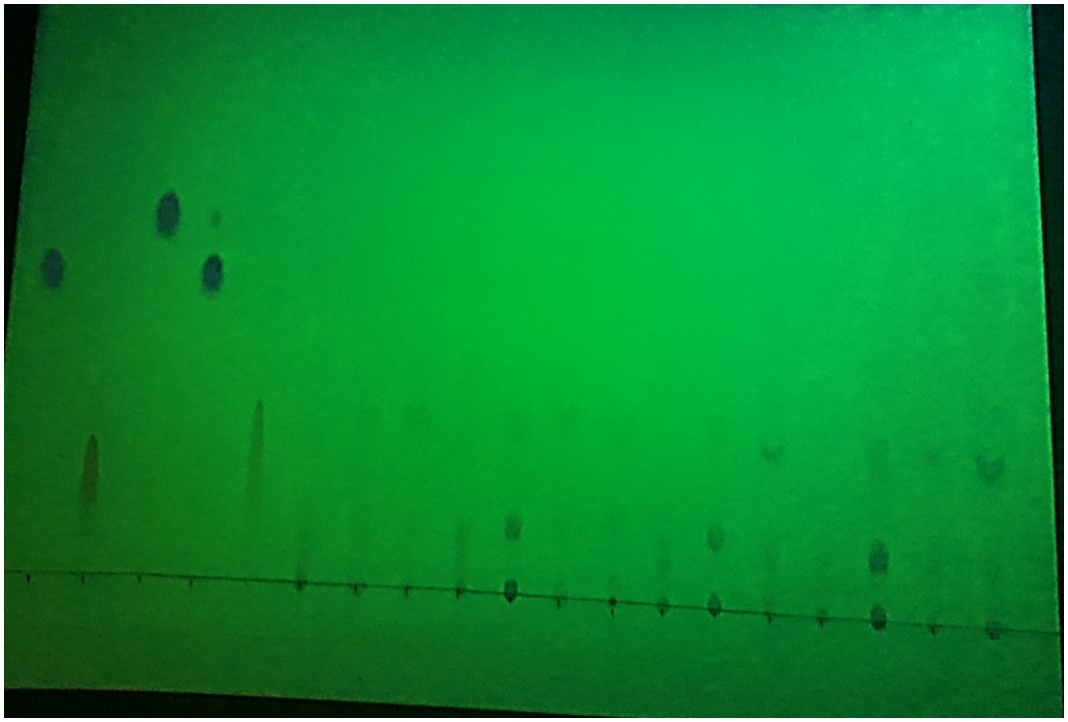
Plate P.9 Spotting of sample extract on TLC plate

Plate P.10 Running of spotted samples using suitable solvent

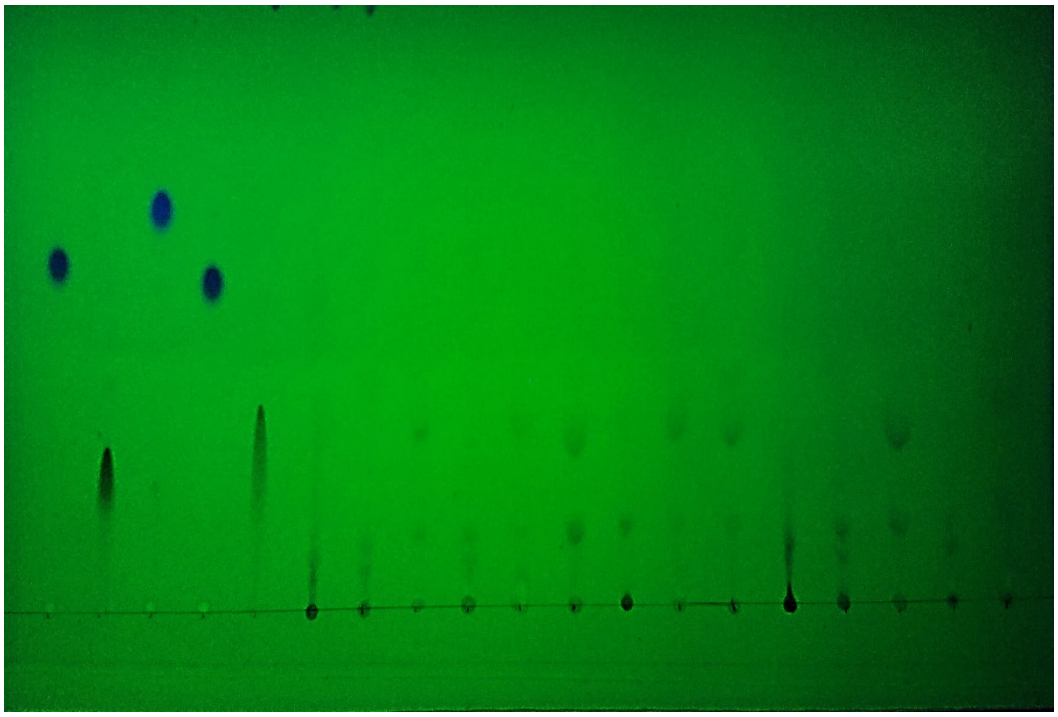
Plate P.11 Identification of antibiotics present in positive samples using TLC



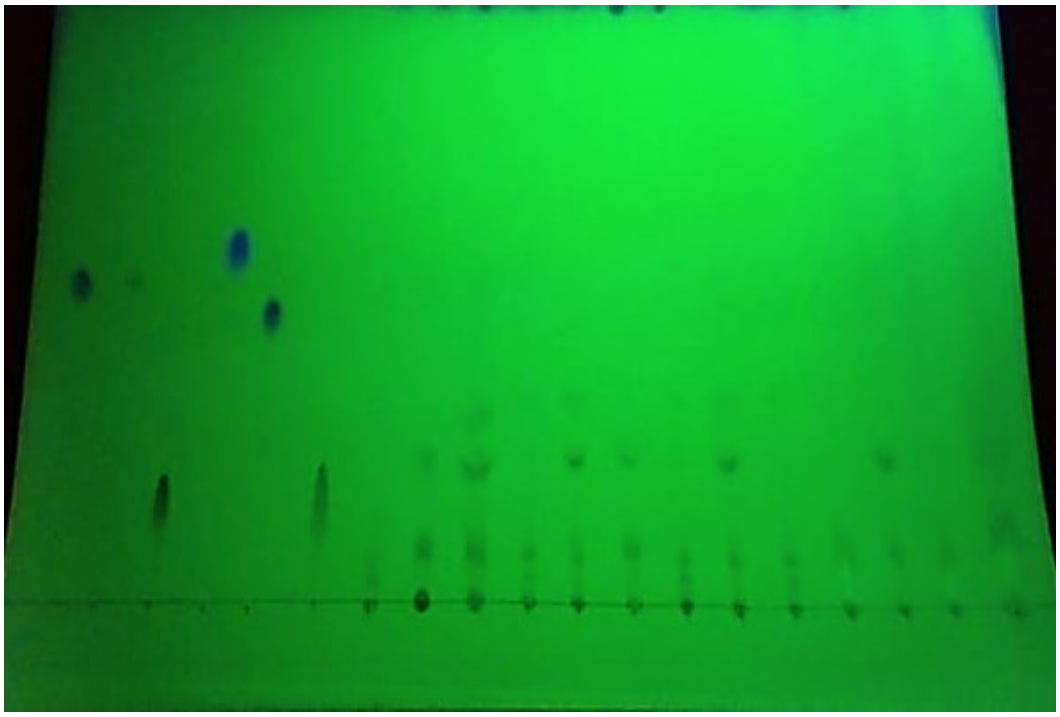
First run of TLC (first five spots from left are of ciprofloxacin, doxycycline, enrofloxacin, gentamycin and tetracycline respectively)



Second run of TLC (first five spots from left are of ciprofloxacin, doxycycline, enrofloxacin, gentamycin and tetracycline respectively)



Third run of TLC (first five spots from left are of ciprofloxacin, doxycycline, enrofloxacin, gentamycin and tetracycline respectively)



Fourth run of TLC (first five spots from left are of ciprofloxacin, doxycycline, enrofloxacin, gentamycin and tetracycline respectively)