CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Enumeration of Pathogenic Bacteria from Chicken Slaughter Containers in Twin Cities: A Source of Antibiotic Resistance

by

Sayed Shah Ali

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

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Dedicated to my parents and my teachers

CERTIFICATE OF APPROVAL

Enumeration of Pathogenic Bacteria From Chicken Slaughter Containers in Twin Cities; A Source of Antibiotic Resistance

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Abstract

Chicken meat is one of the widely used meats in the world due to its nutritious values, easy digestibility and low price. My current research work was on the enumeration of pathogenic bacteria found in the chicken slaughter houses (slaughter container, equipment and surfaces) which may contaminate chicken and cause disease in the consumer. As there are multiple sources of chicken meat contamination during the processing of chicken meat i.e. slaughter containers, surface of chicken slaughter house, equipment used for slaughtering and processing of chicken meat, hands of slaughterers and internal microbiota of chicken itself. Chicken meat gets contaminated from all these sources due to lack of awareness of the slaughterers and also the consumers. When enumeration of common pathogens found in the chicken meat was performed it was found that S.typhi was the most common contaminant found in the slaughter house and also in the chicken meat. S.aureus was the second most common pathogen while *C. jejuni, E. coli* and *C. perfringens* were at 3rd, 4th and 5th respectively. Different antibiotics are used in the chicken farming industry to enhance growth of chicken and to protect them from various diseases. This continuous exposure to antibiotics leads to the development of resistance in the pathogenic bacteria found in the poultry. These antibiotic resistant bacteria may cause severe infections in the humans due to consumption of such contaminated meat containing antibiotic resistant bacteria. These kinds of infections may be very difficult to treat.

Keywords: Slaughter houses; Slaughter containers; Surface of chicken slaughter house; Equipment used for slaughtering and processing of chicken meat.

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Abbreviations

AMK	Amikacin
AMP	Ampicillin
AMR	Antimicrobia Resistance
CFM	Cefixime
CFU	Colony Forming Unit
CFZ	Cefazolin
CIP	Ciprofloxacin
cm	Centimeter
CRO	Ceftriaxone
ES.	Extensive system
FOX	Cefoxitin
GEN	Gentamicin
GI	Gestro Intestinal
GLC	Global Livestock Counts
IPM.	Imipenem
Kg	Kilogram
LVO	Levofloxacin
MDR	Multidrug Resistance
mm	Millimeter
MOX	Moxifloxacin
NAL	Nalidixic Acid
SAM	Ampicillin-Sulbactam
spp	Species
SXT	Trimethoprim-Sulfamethoxazole

TBC	Total Bacterial Count
TET	Tetracycline
TSA	Tryptic Soya Agar
TSS	Toxic Shock Syndrome
TVC	Total Viable Count
UN-FAO	United Nations Food and Agriculture Organization
ZOI	Zone of Inhibition

Chapter 1

Introduction

1.1 Background

Animal based foods i.e. chicken; meat, beef etc. are a potential source of many microbial diseases. Microorganisms may be found on the feathers, feet, flesh or in the blood. Consumption of such kind of contaminated food containing pathogenic microorganisms may be a potential source of many food borne illnesses. Microorganisms may also contaminate flesh during or after slaughtering as they may be present on the hands of slaughterer, slaughtering knife or in the slaughtering containers. So consumption of such kind of food may lead to the disturbance of gut flora and food borne illness i.e. food poisoning. Normally flesh of healthy animals do not contain any microorganism and sterilized but surface of flesh may get contaminated during preparation for consumption. This contamination depends upon the method of slaughtering, slaughtering environment, processing and storage conditions. Contamination may be reduced by using proper hygienic conditions [1].

1.2 Nutritious Value of Chicken Meat

As far as nutritious value and price is concerned chicken meat is nutritious and cheap to consume. Chicken meat is a healthy food contains high level of protein

and fat in very low level. This is why chicken meat is one of the most consumed meats all around the world.

1.3 Problems Associated with Chicken Meat Consumption

Chicken is also used to produce sausages, salami, chicken meatballs and burgers etc. Food borne infections and intoxication caused by consumption of contaminated chicken meat is still a problem even in the developed countries of the world i.e. UK, Canada, Australia etc. Situation is worse in the developing countries. Chicken borne food poisoning ranks among first or second cause of food borne diseases in the Wales and at third place in the US. Epidemiological studies showed that 95% of food borne illnesses caused by chicken meat consumption [2].

Chickens are normally reared in close proximity to humans in many countries of the world so they may serve as a potential source of spread of many pathogenic microorganisms directly by their excreta or handling and indirectly through consumption of contaminated eggs and chicken meat. Chickens are also kept together for longer periods of time so they can transmit microorganisms to each other and so cross contaminate each other and as a result spread to humans [3].

1.4 Sources of Contamination of Chicken Meat

Contamination of chicken carcasses stored in cool air containers is much lowered as compared with stored in the ordinary conditions. Surface of chicken meat which comes into contact with the surrounding environment is more contaminated as compared with internal parts. Contamination may also arise from knife during cutting the chicken into smaller pieces [4]. Microorganisms may already be present on the surface of chicken as part of normal flora or may contaminate later during slaughtering, plucking and chopping or cutting [5]. Normally the number

of microorganisms on the surface of chicken slaughtered, processed and stored in well hygienic conditions is between 100–1000 microbes/cm^2 but the number of microorganisms may be 100 times more than this if slaughtered, processed and stored in unhygienic conditions.

The most common microorganisms found on the surface of poultry are Pseudomonas, Acinetobacter species, Salmonella species, Escherichia species and Flavo bacterium species [6]. Chicken meat is normally sterilized but it may get contaminated by a number of viruses, bacteria, fungi and their toxins during slaughtering (especially contaminated slaughter containers) and processing in unhygienic conditions. General sources of contamination are soil, water, animal feed, human hands, knives and other tools used for slaughtering and processing of flesh, storage area and packaging material etc. Soil is also a source of contamination as soil is the habitat of most of the microorganisms. So we can reduce contamination of chicken meat by keeping in view these sources of contamination and applying hygienic conditions [7].

To wash the poultry carcasses, slaughtering and processing tools and slaughter facilities water is used from water tanks. Microorganisms may also infect these tanks so this water from contaminated tank may also be a source of contamination. Although most of the microorganisms can be killed by boiling the water but thermophiles may survive [8]. Human hands, sweat, hairs and breath may also contaminate chicken meat. Various studies showed that almost 60% of food handlers/processors do not wash their hands properly and almost 25%–40% of diseases occur by consuming such food. Equipment and containers must be health friendly, easy and fast to be cleaned, able to be disinfected easily and should not support contamination of meat. Containers must be washed and disinfected adequately whenever necessary. Floor must be made up of water proof, easy to wash, easy to disinfect and non-slippery material. This should be washed and disinfected time to time. Water should not accumulate on the floor which may promote the growth of microorganisms. Doors should also be made up of smooth, stain free, water proof and easy to wash material. Doors should also be self-closing so to avoid contamination from outside [9].

1.5 Role of Disinfectants to Reduce Contamination of Chicken Meat

Packaging material should also be free of any sort of contaminants. Care must also be taken during packaging, storage and distribution. Proper hygiene and cleaning are very important in the chicken industry. We can reduce the rate of contamination, food borne infections and toxicities by proper hygiene and disinfection of the surrounding environment. The choice of disinfectant depends upon the type and number of microorganisms. So the type and number of microorganisms should be checked time to time so suitable disinfectant can be applied [10].

1.6 Chicken Meat Contamination During Processing

Processing may reduce the number of microorganisms but studies showed that the chicken may get contamination from slaughter containers. As slaughter containers are very unhygienic and contain a large number of microorganisms especially Salmonella species. Furthermore they can also get contaminated during the plucking or washing process. As water from a contaminated container contains enormous number of microbes. After processing chicken meat is cut off into pieces and packaged. So packaging material may also contain microbes, these microbes can contaminate the chicken meat. Various studies showed that the main phases of contamination are boiling, cutting, plucking, splitting giblets and packaging [11].

1.7 Bacterial Pathogens Associated with Chicken Meat Contamination

Staphylococcus aureus is a part of normal flora of chicken. The normal level of this microorganism on the skin of poultry is about 10g G^1 but his level may reach 10^3g

G[1] after slaughtering the chicken. Staphylococcus is a common cause of chicken borne intoxication mediated by enterotoxin released by this organism in the world. The most common cause behind this intoxication is contaminated hands and contaminated chicken containers. The toxin of *Staphylococcus aureus* is so powerful that it cannot be fully deactivated or destroyed by heat or pasteurization [12]. *Staphylococcus aureus* can cause direct damage i.e. invasion and bacteremia as a result or toxin mediated damage i.e. Toxic shock syndrome (TSS) or Staphylococcal scalded skin syndrome (SSSS). The main problem of Staphylococcal infections is resistance to antibiotics mediated by either plasmid or transposons. This resistance to antimicrobials is associated with increase morbidity and mortality. Also increase cost of management i.e. thorough susceptibility testing and long courses of antimicrobials are required [13]. *Escherichia coli* is also a part of normal gut microbial flora of chicken. Consumption of food contaminated by fecal content of chicken may cause diarrhea and vomiting. The most common serotype of *Escherichia coli* causes food poisoning is verotoxin O157:H7 *E. coli* [14].

According to a study conducted in the Belgium chicken meat samples were obtained from some retail stores and were examined for the contamination by pathogenic bacteria i.e. *Salmonella spp.*, *Campylobacter coli*, *Campylobacter jejuni* and *Listeria monocytogenes* showed that there was Salmonella spp. 36.5%, *Campylobacter coli* and *Campylobacter jejuni* 28.5% and *Listeria monocytogenes* 38.2% [15]. Among these bacteria, Campylobacter and Salmonella make a large and majority of the reports. These human pathogens can be present at high loads in the gastrointestinal tract of birds but, after contamination of poultry meat, it is important to detect their presence even at a very low level. *Salmonella Spp.* are found in the gut of chickens as normal microbial flora. So they can contaminate chicken meat while plucking, splitting giblets and processing. *Salmonella* can cause typhoid fever and septicemia [16]. Now a day's food borne infectious diseases are a major threat to mankind. These bacterial diseases range from mild illnesses to very severe life threatening infections. Chicken meat is an important source of many bacterial infections. Theses bacterial diseases can be prevented by applying safe and hygienic conditions while slaughtering and processing chicken meat. So this

study will be carried out to find what kind of bacterial contaminants are found in the/on the chicken meat and their role in the emergence of antibiotic resistance by analyzing their antibiotic susceptibility pattern. By understanding bacterial contaminants associated with chicken meat we can apply appropriate measures to prevent these infections.

1.8 Problem Statement

Slaughtering of chicken in the unhygienic environment is associated with contamination of chicken meat and infection in the consumer. Chicken meat consumption is associated with the emergence of antibiotic resistance as multiple antibiotics are used in the chicken farming industry.

1.9 Aims and Objectives

The aims and objectives of my research will be:

- To isolate and identify pathogenic bacterial strains from chicken slaughter containers from different practice, so to assess the health related issues due to the consumption of chicken meat contaminated by unhygienic processing
- To check the antibiotic susceptibility pattern of bacterial strains isolated from contaminated chicken so to assess the role of chicken meat in the emergence of antibiotic resistance in the environment both broiler and organic birds slaughter centers and meats will be used as source of possible pathogens

Chapter 2

Literature Review

Chicken meat is one of the widely used meats all around the world due to its nutritious value, easy availability and less ethical and social restrictions in the different societies.

2.1 Poultry Farming System, Annual Consumption and Nutritive Value of Chicken Meat

2.1.1 Brief History of Poultry Industry

Humans are raising poultry as a food source from thousands of years. According to the archeologists Chinese are raising chicken from last 8000 years as one of the important sources of food, subsequently chicken spread to the rest of the world especially to the Western Europe by sea and land routes from China.

The appearance of chicken in the Africa can be traced back many centuries ago and now chicken has established itself as a major source of food and as a part of daily African life [20].

2.1.2 Nutritive Value of Chicken Meat

Poultry meat is considered as a balanced diet for all age groups i.e. before conception and during pregnancy in females, during growth and development of children and also in the old age. It is also a suitable source of energy for those who need an increased amount of calories and protein. Poultry meat is a good source of easily digestible proteins, Vitamin B complex, unsaturated lipids and minerals. It is evident from various epidemiological studies that poultry meat is a balanced diet for all age groups and poultry meat consumption is associated with good health. Chicken meat consumption is associated with decrease risk of obesity, type 2 diabetes mellitus and cardiovascular diseases. Chicken meat consumption also decreases the risk of cancer to some extent. It has been established by United Nations Food and Agriculture Organization (UN-FAO) that because chicken meat is widely available and inexpensive and contain all the necessary nutrients so in the developing countries it can meet the shortage of food [21]. In the recent era chicken is considered as one of the major source of food and so chicken is among the highly consumed meat in both the developing and the developed countries of the world. According to a report by Global Livestock Counts (GLC) the total number of chickens in the world is almost 19 billion, so this number is enough to understand the importance of chicken as one of the food source. Chicken is the most commonly reared specie of the birds. According to an estimate consumption of chicken in the Europe is 2.5 kg per capita each year, whereas the consumption of chicken in the Africa is very high which is 6 kg per capita each year [22].

2.1.3 Annual Chicken Meat Consumption

The annual consumption of broiler meat in the Zambia is 4.8 kg per capita per year and the average consumption of chicken meat in the Zambia is approximately 62.9 million Kgs. An estimated annual production of chicken in the Zambia is 81.4 million Kgs. In most of countries of the world chicken is considered as one of the easily raised specie of the birds and an affordable source of food. Chicken can

easily be raised and slaughtered at home to get quality and nutritious food. Above all chicken meat has very few religious restrictions as a food source as compared to other animals reared for meat [23].

2.1.4 Poultry and Chicken

Poultry or Chicken is domesticated avian species raised worldwide to get meat, eggs and feathers (used for production of different items). Poultry is widely used term which includes a variety of avian species that are raised for food i.e. chickens, guinea fowls, geese, turkeys, ducks etc. The term poultry also includes other avian species that are often reared for game/games i.e. pigeons, quails, pheasants etc. Chickens constitute a major part (approximately 90%) of poultry and most commonly raised avian specie across the globe [24].

2.1.5 Poultry Farming System

Till the 20th century chickens were generally reared under an extensive system or ES. Extensive system is a system to raise poultry where they are raised in large numbers freely and depends upon scavenging and some supplementation is provided by the scavenger as a food source where the scavenging food does not provide them enough food to sustain their life. After the Second World War (II WW) the industry of chicken has been revolutionized and the rearing and production of poultry and poultry products i.e. eggs has been increased tremendously due to large domestication and rapid intensive poultry growth. Due to the newer technological advancements and biological discoveries the selection of high yield poultry and layer breeds has become a trend [25].

2.1.6 Advancements in Poultry Industry

Due to the recent biological advancements a range of new technologies and approaches are being employed to enhance poultry production and yield like chicken

FIGURE 2.1: Poultry Farming System in Pakistan

quantity and quality enhancement and production and nutritive value of eggs. Projects to raise small flocks of poultry on small scale have yield mixed results in the urban and peri-urban areas. Due to the unimproved extensive poultry raising system the mortality rate of poultry is very high so eggs and poultry is rarely consumed by people especially the children and women (who need eggs as a necessary part of their diet for proper growth and development) as these eggs are used for hatching to replace dead birds and to maintain their number. Newer technologies are now being incorporated in the Family Poultry Development manual. This manual toolkit is designed to improve and facilitate poultry production projects through an improved and newer decision making process. So mortality rate of chicken can be reduced and in return yield of meat and eggs can be enhanced [26].

2.1.7 Importance of Poultry Industry in Rural Life

Small scale poultry farmers commonly face shortage of resources and often employ such activities to achieve at least sustainable livelihood income. Under these tight conditions they are able to earn very little income to sustain their life. In this scenario poultry performs very important function for them from income generation

to home based quality food and also performs a function of social cohesion. So in such areas poultry industry performs a wide variety of very important function [27].

2.2 Bacterial Contamination Associated with Unhygienic Chicken Slaughtering Process

2.2.1 Sources of Poultry Meat Contamination During Slaughter Process

There are two main sources of poultry meat contamination during the slaughter process as; the surrounding environment of the slaughter (i.e. slaughtering equipment, live poultry or chicken and staff involved in the slaughtering process) and the second source of bacterial contamination during slaughter process is the microbes found in the digestive tract of the animal being slaughtered. A slaughter house is generally divided into two zones i.e. the dirty zone and the clean zone. This division of slaughter house helps to minimize bacterial contamination of the final product (poultry meat) and also ensures proper material flow. The clean area include water chilling, cutting, bone removal and final packaging area. This area should be neat and clean and contamination free as if meat gets contaminated in this area while processing this may lead to serious gastroenteritis to the user. The dirty area of chicken slaughter house generally includes area where poultry is shackled by feet and where poultry carcass is placed after electric immobilization [28]. During the processing of chicken meat leakage or breakage of gut may leads to the fecal contamination of the chicken carcasses so fecal Coliforms are commonly used as bacterial indicators of fecal contamination of chicken carcasses. Fecal coliforms are also used as an indicator for assessing the general hygienic condition of poultry slaughter houses. During slaughter process improper evisceration or breakage of gut may leads to the chicken carcass contamination from gut bacteria. These gut bacteria may also contaminate the slaughter house environment [29].

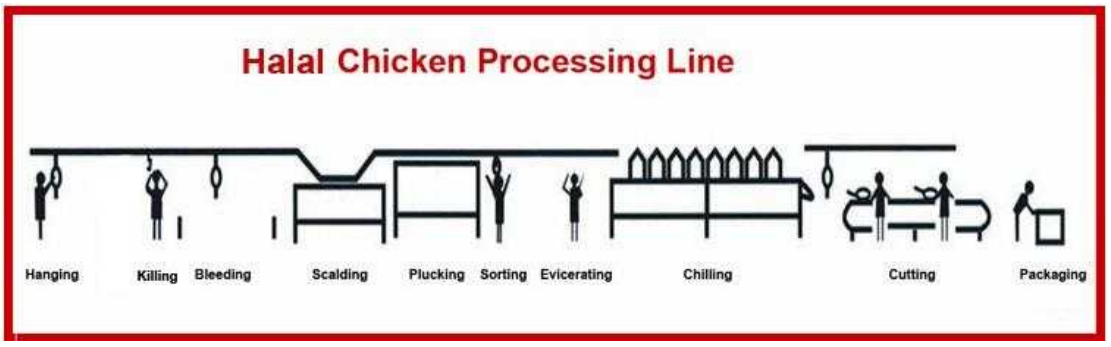

FIGURE 2.2: Chicken Meat Processing in Pakistan

FIGURE 2.3: Chicken Slaughtering Process in Pakistan

2.2.2 Contamination of Chicken Meat During Processing and Commonly Associated Pathogens

Poultry host a large number of disease causing organisms found on their skin, legs, feathers and also in their alimentary canal. These bacteria are eliminated during slaughter process, but contamination of chicken carcass or final product is possible at any stage of their processing i.e. during slaughter, plucking, washing, evisceration, cooling or packing. Poultry meat may be contaminated from either

poultry intestinal microbiota or from environment i.e. equipments and operators hands. Pathogenic microorganisms i.e. *Salmonella*, *E.coli*, *C.jejuni* or *S.aureus* may be present on neck, skin, breast, thighs and muscles in different frequencies. Table 1, showing the frequency of different pathogens isolated from different parts of poultry. *E.coli* was the found with highest frequency (100%) in both the fresh and frozen samples. The frequency of *S. aureus* (highest 46.6% and lowest 20%) was second to *E.coli*.

TABLE 2.1: Showing frequency of isolation of different pathogenic bacteria from different portions of chicken meat

Bacteria	**Meat**	**Breast**		**Thigh Muscle**		**Total**	
		No.	**%**	**No.**	**%**	**No.**	**%**
Salmonella	Fresh	4	26.6	2	13.3	3	20
	Frozen	2	13.3	2	13.3	1	6.6
E.coli	Fresh	15	100	15	100	15	100
	Frozen	15	100	13	86.6	15	100
C.jejuni	Fresh	12	80	11	73.3	10	66.6
	Frozen	5	33.3	7	46	8	53.3
S.aureus	Fresh	7	46.6	4	26.6	6	40
	Frozen	4	26.6	4	26.6	5	33.3
Bacteria	**Meat**	**Breast**		**Thigh Muscle**		**Total**	
		No.	**%**	**No.**	**%**	**No.**	**%**
Salmonella	Fresh	3	20	5	33.3	17	22.6
	Frozen	1	6.6	1	6.6	7	9.3
E.coli	Fresh	15	100	15	100	75	100
	Frozen	11	73.3	13	86.6	67	89.3
C.jejuni	Fresh	0	0	0	0	0	0
	Frozen	0	0	0	0	0	0
S.aureus	Fresh	3	20	4	26.6	24	32
	Frozen	3	20	4	26.6	20	26.6

The frequency of *Salmonella* was highest in the fresh samples of thigh muscles

(33.3%), and lowest in the frozen sample of breast (6.6%). *C.jejuni* was isolated from few samples [30]. Poultry meat is one of the commonly used meats in the world. Hence it is important to ensure microbial safety of chicken carcasses and final chicken products. There are many sources of consumption i.e. microbiota of poultry itself, slaughtering house environment and the equipment used for poultry slaughtering and processing. After contamination few of them survive during processing, storage and packing. These microbial species also include pathogenic microbes i.e. *Campylobacter* and *Salmonella*. These two pathogens are related with a high number of cases of food poisoning and gastroenteritis associated with contaminated meat consumption. Since last 15 years *Campylobacter* has been associated with most number of cases of gastroenteritis in the EU. The total number of cases reported in a single year (2015) caused by *Campylobacter* were 229,213 and in the similar time the number of confirmed cases of *Salmonella* were 94,625 [31].

2.2.3 Routes of Poultry Meat Contamination During Processing

The Figure below is showing the different steps involved in the slaughtering and poultry processing along with different sources of contamination. After slaughtering blood is let to flow freely and then chicken is scalded in hot water. Then feathers are abraded mechanically. Then carcasses are spray washed and evisceration is done (main stage of carcass contamination but its own gut flora). Then chilling and final processing is carried out. Bacterial contamination can occur at any stage as from equipment i.e. feather remover or carcass itself during evisceration [32].

2.2.4 Contamination from Surfaces of Slaughter House

As muscles are sterile in healthy chicken, while various microbes are found on the on the feathers, skin, lungs and especially in the GI tract of chicken. In the

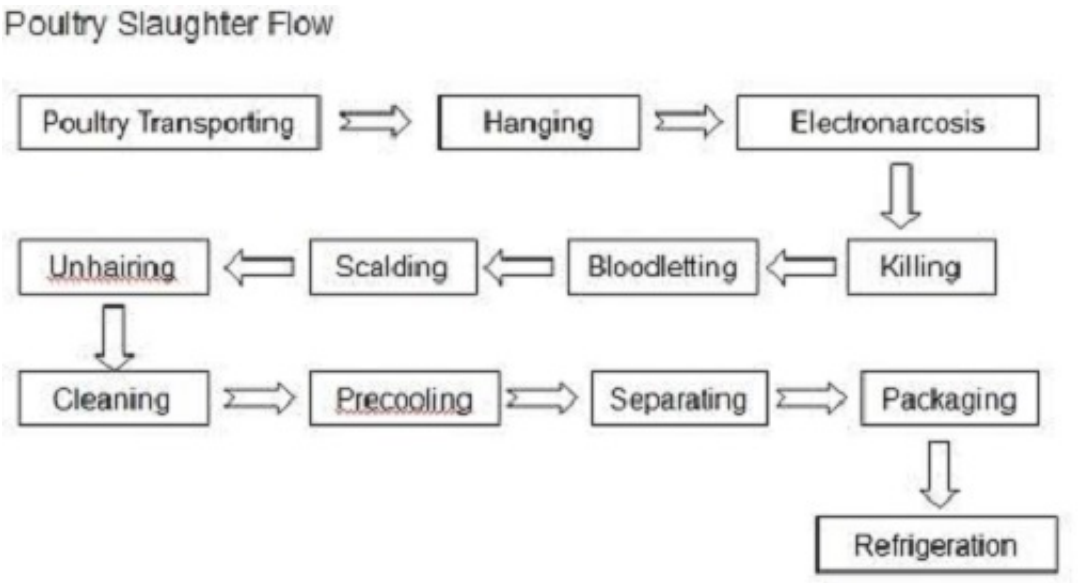

FIGURE 2.4: Sources of Poultry Contamination During Processing

slaughter houses surfaces encompass large number of bacteria. These bacteria may contaminate chicken meat during processing. Therefore surfaces should be cleaned properly with good quality disinfectants to reduce the risk of contamination [32].

FIGURE 2.5: Dirty Chicken Slaughter Container

2.2.5 Contamination from Air and Environment

After slaughtering contamination may occur from water and air. Bacterial pathogens found in the air can contaminate chicken meat during various steps. As skin and meat of chicken is directly in contact with air so pathogens found in air can easily contaminate the meat. In case of fresh meat bacteria only present on the surface rather than in the meat. However after processing these microbes may penetrate the skin and contaminate muscles [34].

2.2.6 Contamination from Equipment

Equipment also plays an important role in the contamination of chicken meat. There are many sources of contamination including rubber fingers used to remove feathers or belts that convey carcasses may be a source of contamination. Cross contamination may also occur between the carcasses during processing. Contamination may also occur during washing and cooling process. Equipment used to slaughter the chicken may also be a source of contamination i.e. knives. Packaging used for final packing of chicken meat may also be contaminated. Water bath used for processing can reduce the contamination because contain hot water but may increase cross contamination. Cold water as used for chilling may also increase cross contamination [35].

2.2.7 Contamination from Intestinal Flora

Meat may also get contaminated from carcass's own internal gut flora during evisceration process. As chicken contain high number of different pathogenic bacteria so improper handling may leads to the contamination of chicken meat. The most important human pathogens found in the gut of chicken are *Salmonella* and *Campylobacter*. Various researches showed that there is a relationship between numbers of *Campylobacter* found in the gut and number of bacterium found on the carcass. Microbiota found in the poultry GI tract is studied in detail and it is

found that poultry microbiota found in the GI tract correlates with feeding habits and health status of poultry [36]. The contamination level of chicken carcass decreases after the feather removal and evisceration process. This decrease in the number of contaminants is due to the effects of hot water found in the water bath and them immersion in the cold water during chilling. Number of contaminants again increases during storage and packaging. Some bacteria that survive during processing may persist storage and packaging step [37].

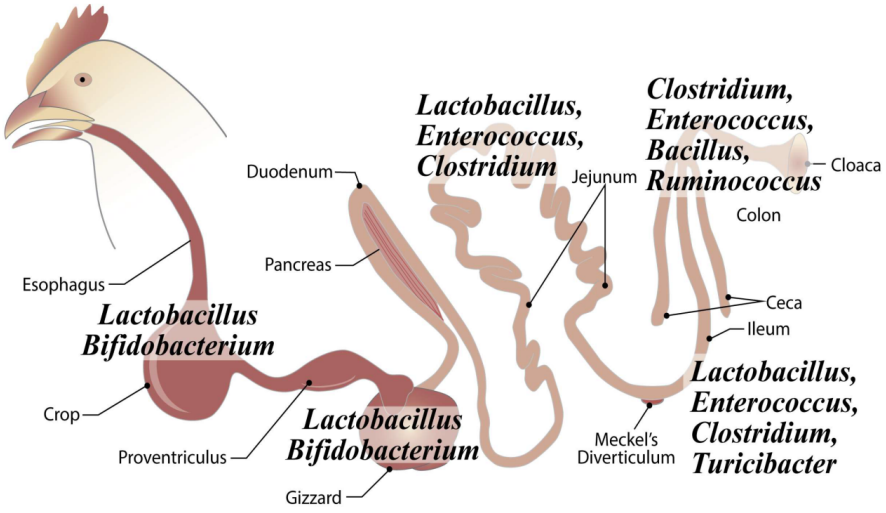

FIGURE 2.6: Contamination from Chicken Gut Microflora

2.2.8 Other Sources of Contamination

Contaminated chicken meat may contain *Salmonella* which can cause food poisoning and gastroenteritis in humans. *Salmonella* is found as a normal intestinal flora in most of the animal species. Contamination from intestinal contents of animal is a major route of transmission of *Salmonella* and other pathogenic microorganisms. Contamination may also occur during processing, preparation, transportation, storage and other services. At butcher's shop or slaughter house contamination may occur due to the other reasons i.e. by storage of meat in dirty and contaminated utensils, holding and storing meat at a temperature that may favor bacterial growth and multiplication, utilizing contaminated water for processing, using non-food grade quality and contaminated packaging material etc [38]. Other sources of contamination may include contaminated hands of butcher's and lack of

personal cleanliness and lack of proper awareness regarding safe handling of food. The primary responsibility of food safety is at the producer's end who produce, process and trade meat. So proper care should be taken while processing food so proper hygienic food should be supplied to the consumers [39].

FIGURE 2.7: Bacterial Contamination from Dirty Surfaces and Unhygienic Equipment

FIGURE 2.8: Cross Contamination of Chicken Meat During Processing

2.3 Bacterial Diseases Associated with Chicken Meat Consumption

2.3.1 Food Borne Bacterial Diseases

It is evident that chicken meat is one of the highly consumed animal based foods all around the world. In spite of technology improvement with the passage of time food borne diseases are as common as other diseases in all the developed and developing countries of the world. Though stringent hygienic practices are followed in all stages of meat production and processing but food borne infections is a continuous threat for humans. *Escherichia coli* and *Salmonella enterica* are major members of Enterobactericiae family involved in food borne infections. Development of antibiotic resistance is also a major problem in treating these infections [40]. As we know animal based food sources are involved in majority of microbial diseases in humans. The microbiom found in poultry is gaining attention because it is involved in most of the food borne bacterial infections in the US and worldwide. In the US food borne infections are as common as other diseases almost 47 million people develop food borne infections with an economic burden of $77 billion in a single year (2011) [41].

2.3.2 Poultry Gut Microbiota

In the poultry production units chickens are commonly of single age cohort. The microbial load that they bear in their intestinal tract at the age of 6-8 weeks (market age) is up to 1011 bacterial cells per gram of their intestinal content. The microbiom found in the poultry gut differs vastly from mammals. In the poultry colonization of GI tract commonly occurs from the surrounding environment and as they grow in a same age cohort in a close proximity. Poultry may be colonize by pathogenic microbes from environment in the commercial poultry units this may have serious kind of concern if bacterial pathogens transferred from their environmental reservoirs to the humans via poultry [42].

2.3.3 Bacterial Contamination of Chicken Meat

Bacteria may contaminate chicken meat on various stages starting from growth of poultry till final processing and packaging. So this fact has lead to the development of Food Safety Modernization Act. This act states the importance of monitoring the entire food supply chain from growth to the consumption by the end user this is termed as farm to fork surrvillience. Detection of bacterial pathogens at every stage of poultry production is the matter of discussion from last few decades to ensure the safety of food. This is generally to focus on the common pathogens who are well known to be spread via poultry meat i.e. *C. jejuni*. Because the role of this organism is well establish with the consumption of chicken meat [43].

2.3.4 Chicken Meat Borne Bacterial Infections

Food borne infectious diseases are a major public health problem worldwide both in the developed and developing countries of the world. Each year thousands of millions of people get sick and die as a result of consuming unsafe food. Bacterial pathogens constitute a major portion of infectious diseases caused through consumption of contaminated food. Among these pathogenic bacteria *Salmonella* is the most common pathogen involved in human gastroenteritis. There are many factors involved that make *Salmonella* a major cause of human gastroenteritis i.e. easy adaptability of pathogen, changing trends and characteristics of the population, globalization and increase food trade among the different countries of the world and change in the structure of industries. All these factors contribute to make *Salmonella* a major threat to humans [44]. Chicken meat is popular among the people of every region of world because of its easy digestibility and acceptance although it could be a major cause of bacterial pathogens that may become a serious threat to consumers. The most common bacterial pathogens that can cause food borne illnesses in humans are Escherichia coli spp., *Salmonella spp.*, *Listeria spp., Campylobacter spp.* and *Staphylococcus aureus*. Chicken broilers may contain variety of food borne pathogens as a part of their gut microbial flora

i.e. *Salmonella spp.*, and Campylobacter spp., these pathogens may contaminate processing plant while processing and consumption of meat processed in such contaminated plant may cause human illness. Several epidemiological studies suggest that chicken broiler meat is still a major and primary cause of food bone bacterial diseases [45].

2.3.5 Clostridium Perfringens Associated with Chicken Borne Food Poisoning

According to a study approximately 4 million citizens of Canada suffer from food borne illnesses each year. *Clostridium perfringens* is the major pathogen involved in most of the cases of food poisoning. *Clostridium perfringens* also ranks second among the domestically acquired cases of food poisoning. The estimated number of cases of domestically acquired bacterial food poisoning caused by *Clostridium perfringens* in Canada is 544.5 cases per 100,000 inhabitants. Similarly the number of domestically interacted food poisoning cases caused by *Clostridium perfringens* is approximately 1 million/year. The most important transmission pathway which *Clostridium perfringens* follows is food and meat is the most common vehicle for the transmission [46].

2.3.6 Campylobacteriosis Associated with Contaminated Chicken Meat Consumption

There are 25 species of Campylobacter described to date but among these 25 species *C.jejuni* and *C.coli* are the major pathogens in the causation of gastroenteritis in humans. However some other species of Campylobacter may also cause gastrointestinal infections in humans i.e. *C.lari, C.concisus and C. upsaliensis.* Typically campylobacteriosis develops in humans 1-5 days post exposure to this organism. Campylobacteriosis is characterized by bloody diarrhoea, abdominal cramps, nausea, vomiting and fever. These symptoms last for 5-7 days. In most of

the cases campylobacteriosis is a self limiting infection and does not requires antibiotic treatment but in case of severe disease treatment is required. Campylobacter may also cause severe infections in immunocompromised patients so antibiotic treatment is required in these patients [47]. *Campylobacter jejuni* is mainly a food borne pathogen particularly transmit through the consumption of contaminated poultry. The role of *Campylobacter jejuni* as a food borne pathogen has been well established in the last 10 years in the US. This bacterial pathogen is the most common cause of bacterial gastroenteritis and most common pathogen involved in food poisoning in the US. The levels of coliforms and Mesophils, *E.coli*, *Staphylococcus aureus* and Psychrophils in the poultry meat can be routinely used to assess the safety and hygiene levels and proper processing. Because improper hygiene, safety, processing and storage may lead to the contamination and proliferation of pathogens and consumption of such contaminated meat may lead to the gastroenteritis [48].

The natural reservoirs of *Campylobacter jejuni* are poultry especially chicken and free living birds. *Campylobacter jejuni* is frequently isolated from numerous species of birds and also found frequently in the poultry production units. Prevalence rates of this bacterium may reach 100% at the slaughter age of poultry. *Campylobacter* is not harmful for poultry health but this organism is the leading cause of food borne infections worldwide. Contaminated meat of poultry is the main vehicle for the transmission of this organism to the humans. This organism is the most frequent cause of bacterial food poisoning worldwide. The total number of infections caused by *Campylobacter jejuni* exceeds the infections caused by *Salmonella*, *E.coli* and *Shigella* cause collectively [49].

2.3.7 Salmonellosis Associated with Contaminated Chicken Meat Consumption

Salmonella species especially *Salmonella enterica* is the major pathogen involved in most cases of food borne illnesses in the US. *Salmonella enterica* especially the Enteritidis serovar is one of the major cause of human salmonellosis in the

Europe and US. Contaminated poultry products especially eggs act as a main vehicle for human salmonellosis. Poultry especially chicken is the main reservoir for this pathogen where it is found on the feathers, skin and in the intestinal tract of live birds. Chicken meat is contaminated during slaughtering process and processing of chicken meat in the unhygienic conditions. *Salmonella* may disseminate throughout the chicken processing plant during processing, cooling, cutting and packaging.

According to the food standards presence of *Salmonella* in the chicken meat is a sign of contamination and this meat is not suitable for consumption. Typhoid fever is the most serious problem in humans caused by *Salmonella* characterized by diarrhea or dysentery, abdominal cramps, nausea, vomiting, fever and red rashes on the skin. According to an estimate Salmonellosis affects more than 90 million people each year worldwide [50].

2.4 Role of Chicken Meat in the Emergence of Antibiotic Resistance

In the recent years antimicrobial resistance is a global health problem. In the past few years it is well established that there is a relationship among the usage of antibacterial agents and resistance development against these agents. There is an excessive use of antimicrobials in the production of food animals. This use of antibiotics is also increasing with the passage of time due to increase risk of bacterial associated diseases in the poultry.

Now a day's much attention is diverted towards zoonotic infections associated with contaminated chicken meat consumption i.e. *E.coli*, *Enterococcus spp*; and *Staphylococcus aureus*. However with some exceptions, relatively there is a little knowledge about the prevalence of and mechanism of antimicrobial resistance in the bacteria associated with poultry production [51]. Chicken meat and meat

products are considered as an important vehicle for many bacterial diseases including *E.coli*, *Salmonella spp*, and *Klebsiella spp.* that are the main pathogens involved in human food poisoning.

Poultry meat also hosts antibiotic resistance strains of *E.coli* with high frequency than any other kind of meat. Extended spectrum β lactamases (ESBLs) enzymes found in bacteria especially *E.coli* are plasmid coded enzymes. These enzymes are found in the Gram negative class of bacteria that confer resistance against first three generations of Cephalosporin antibiotics and they are inhibited by clavulonic acid [52].

Gallibacterium is a member of *Pasteurellaceae* family and an important pathogenic organism associated with contaminated chicken meat consumption. To treat *Gallibacterium* effective antibiotic treatment is needed. Antibiotic resistance of *Gallibacterium* against commonly used antibacterial in the infected flocks is becoming a great problem. Currently very little information is available on the antibiotic susceptibility pattern of Gallibacterium, especially *G. anatis* which is one of the major human pathogen involved in the poultry meat borne bacterial infection. Emergence of resistance against a wide variety of antimicrobials has been observed among the members of *Pasteurellaceae* family.

High prevalence of antimicrobial resistance has been demonstrated in a remarkable number of organisms of *Pasteurellaceae* family [53]. Poultry is among the most widespread food industries across the globe, chicken is the most commonly farmed animal species across the globe and according to an estimate over 90 billion tons of chicken produced each year. The reason behind this huge production of chicken is the easy to raise and low costs required for this industry and less religious and cultural restrictions. To safeguard poultry against bacterial attacks a vast array of antimicrobials are used in most of the countries of the world. These antibiotics are mostly administered via oral route to prevent diseases and also to enhance growth and yield of meat. This excessive and improper usage of antimicrobial in the chicken industry leads to the emergence of antimicrobial resistance in the bacterial spp. that cause disease in the humans [54].

TABLE 2.2: Antibiotic susceptibility pattern of *E.coli* isolated from broiler chicken

Antibiotic	**Class**	**S**	**I**	**R**	**Resistance %age**
Penicillin	Beta lactam	0	1	55	98.2
Erythromycin	Macrolide	1	1	54	96.4
Rifampicin	Ansamycin	1	1	54	96.4
Trimethoprim	Diaminopyrimidine	19	1	36	64.3
Streptomycin	Aminoglycoside	12	14	30	53.6
Tetracyclin	Tetacyclin	24	4	28	50.0
Ceftazidime	Beta lactam	24	9	23	41.1
Amoxicillin	Beta lactam	29	12	15	26.8
Chloramphenicol	Non classified	41	2	13	23.2
Ciprofloxacin	Floroquinolone	38	6	12	21.6
Gentamicin	Aminoglycoside	38	7	11	19.4
Levofloxacin	Floroquinolone	35	13	8	14.3
Amikacin	Aminoglycoside	43	7	6	10.7
Imipenem	Beta lactam	48	7	1	1.8

Table2. shows the antibiotic susceptibility pattern of 56 *E.coli* isolates from broiler chicken meat. Antibiotic susceptibility testing was performed by broth microdilution test. This test is showing that most of the isolates were resistant to penicillin (55/56) only 1 isolates shown mild sensitivity. Imipenem showed greatest action against most of the *E.coli* isolates as 48/56 were sensitive, 7/56 were intermediate while only 1 isolate showed resistance [55]. Several antibiotics are used in the animal based food production i.e. in the poultry industry. This use of antibiotics in the animals has major public health implications.

Antibiotic routinely used in the chicken industry or poultry farms without any sort of precautionary measures leads to the pool of antibiotic resistant bacteria in the poultry. These poultry act as a reservoir for drug resistant bacteria which may cause serious infections in the consumers which are difficult to treat. These reservoirs may also lead to the emergence of antibiotic resistant bacteria in the

environment and spread of mobile antibiotic resistant genes to the other human pathogens. According to an estimate in 2015 almost 62% of the total 34.3 million pounds of antibiotics were consumed by the US food-animal production. This scenario shows the seriousness of antibiotic abuse in the food industry.

According to the US National antimicrobial resistance monitoring system it was well observed that antibiotic resistance was increasing in the *E.coli* isolated from livestock than human isolates. Despite these conditions the supply of antibiotics to the livestock and poultry industry increasing every year [56]. More recently colistin resistance mediated by plasmid containing genes is also being detected in the livestock industry and also in the human consumers. Colistin is the drug of choice to treat carbapenem resistant infections in humans. However Colistin is also used in the livestock industry to prevent disease and promote growth in some countries of the world. Antibiotic resistance of several isolates of *E.coli* from poultry industry was checked against different drugs i.e. Ampicillin, Cefazolin, Sulbactem, Ceftriaxone, Nalidixic acid, Ciprofloxacin etc. showed varying degree of resistance. Most of the isolates were resistant to Tetracyclin where as very low resistance was shown against Ciprofloxacin [57].

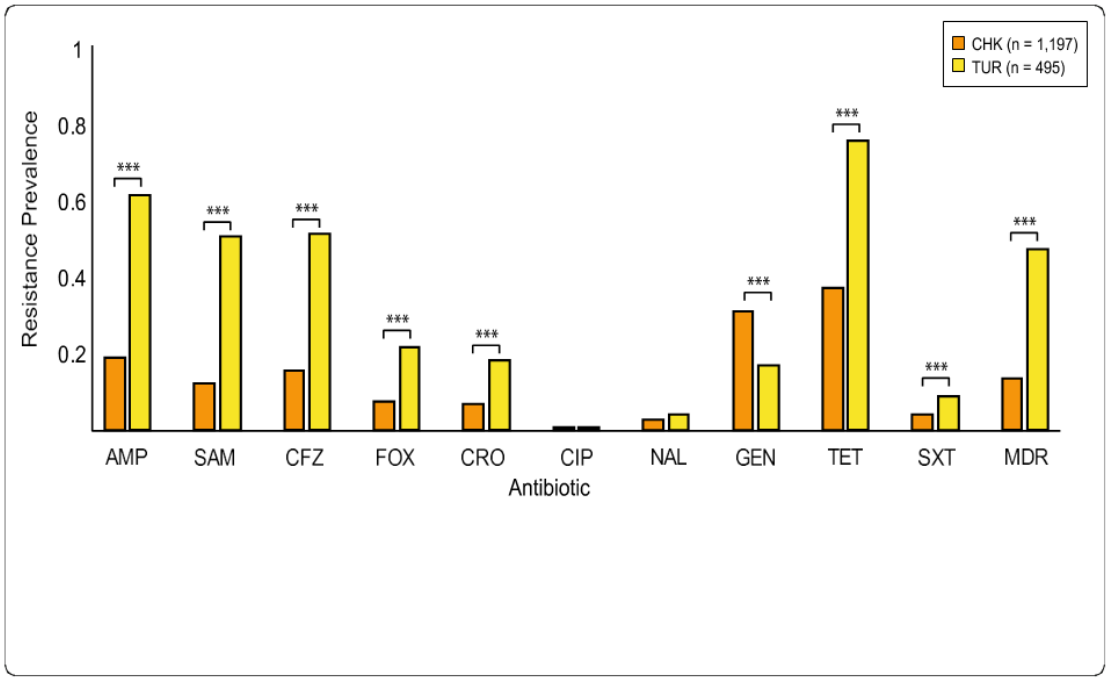

FIGURE 2.9: Prevalence of Antibiotic Resistance Among *E. coli* Isolates Contaminating Retail Chicken and Turkey.

(Ampicillin (AMP), ampicillin-sulbactam (SAM), cefazolin (CFZ), cefoxitin (FOX), ceftriaxone (CRO), ciprofloxacin (CIP), nalidixic acid (NAL), gentamicin (GEN), tetracycline (TET), trimethoprim-sulfamethoxazole (SXT), amikacin (AMK) and imipenem (IPM). Multidrug resistance (MDR) was defined as resistance to three or more classes of antibiotics). According to this study it is well illustrated that Penicillin was least effective against these isolates with a resistance percentage more than 98%, other drugs i.e. trimethoprim, streptomycin, tetracycline, ceftazidime and amoxicillin showed moderate action against these isolates.

While chloramphenicol, amikacin and Imipenem showed greatest activity against isolates with a sensitivity index of 76.8%, 89.3% and 98.2% respectively. The phenotypes of *Gallibacterium* and other members of *Pasteurellaceae* showed resistance against a wide array of antibiotics and so showed multidrug resistance. From last few years *Gallibacterium* has emerged as a major pathogen of poultry and other pet birds with high rates of mortality and multidrug resistance [58].

Generally excessive and non-judicious use of antibiotics and other antimicrobials in the veterinary and poultry farming has been implicated in the emergence of antimicrobial resistance (AMR) in the pathogenic organisms. Excessive use of antibiotics in the humans is also the main reason in the emergence of multidrug resistant strains (MDR strains) in the environment. Multidrug resistance in the *Salmonella* is a major problem in all the countries of the world though varying extent.

The problem of multidrug resistant strains of *Salmonella* is a major threat in the developing countries of the world. These multidrug resistant strains of *Salmonella* are very difficult to treat with conventional antibiotics [59]. According to a study conducted by Garedew et al. on the antimicrobial susceptibility patterns of *Salmonella* against commonly used antibiotics, he found that; highest percentage (60%) of multidrug resistant *Salmonella* were isolated from meat samples followed by samples obtained from hands (26.7%). This study also found that more than 1 in 4 (28.3%) of *Salmonella* were multidrug resistant. There was high resistance of *Salmonella* against Ampicillin, Amoxicillin, Nitrofuranthoin, Sulfamethoxazole;

this resistance was due to the uncontrolled availability and non-judicious use of antimicrobials. These multidrug resistant strains exert selection pressure for the resistant strains and making them resistant to antimicrobials. This prevalence of antimicrobials resistance has very bad health effects for humans by causing infections in humans that are difficult to treat by conventional antibiotics [60].

Chapter 3

Material and Methods

All the lab work of this research project was done in the lab of department of Bioinformatics and Biosciences, Faculty of Health and Life Sciences, Capital University of Science and Technology, Islamabad. For isolation and identification by culturing on selective media was done in the department of microbiology Cure laboratories Islamabad. For further identification of isolated organisms biochemical tests were performed in the Islamabad Research lab Islamabad.

FIGURE 3.1: (A) Swab Cticks Used for Bacterial Sampling, (B) Sterile Distilled Water Used for Sample Preparation and Dilution, (C) Sample Preparation for Further Analysis

3.1 Culture Media That Were Used As

Table 3.1: Culture Media List

Name of culture media	**Used to isolate**	**Manufacturer**
Nutrient agar	Total bacterial count (CFU)	Conda
Mueller Hinton agar	For antibiotic sensitivity testing	Merck
MSA (Mannitol Salt agar)	*S.aureus*	Conda
XLD (Xylose Lysine Deoxycholate agar)	*S.typhi*	Oxid
VRB (Violet Red Bile agar)	*E.coli*	Conda
CVA agar	*C.jejuni*	Merck
TSA agar	*C.perfringens*	Merck

3.2 Antibiotics That Were Tested As

- OFX (Ofloxacin)
- LEV (Levofloxacin)
- MOX (Moxifloxacin)
- CFM (Cefixime)

3.3 List of Biochemical Tests Used

- Indole
- Catalase
- Oxidase
- H_2S production
- Citrate utilization test

3.4 Methodology

3.4.1 Sample Collection

Samples were collected from different chicken slaughter facilities in the twin cities (Islamabad and Rawalpindi) with the help of a sterile cotton swab. Total 24 samples were collected from different settings i.e. from local vendors and from supermarkets. Local vendors in the remote areas mostly slaughter and process chicken meat in the open environment, so there are most chances of contaminated from surrounding areas. There are also chances of chemical contamination from the open environment. On the other hand slaughtering and processing of chicken meat in the supermarkets was all done in the closed environment.

FIGURE 3.2: Sample Collection from Hack Used for Chicken Slaughtering and Processing

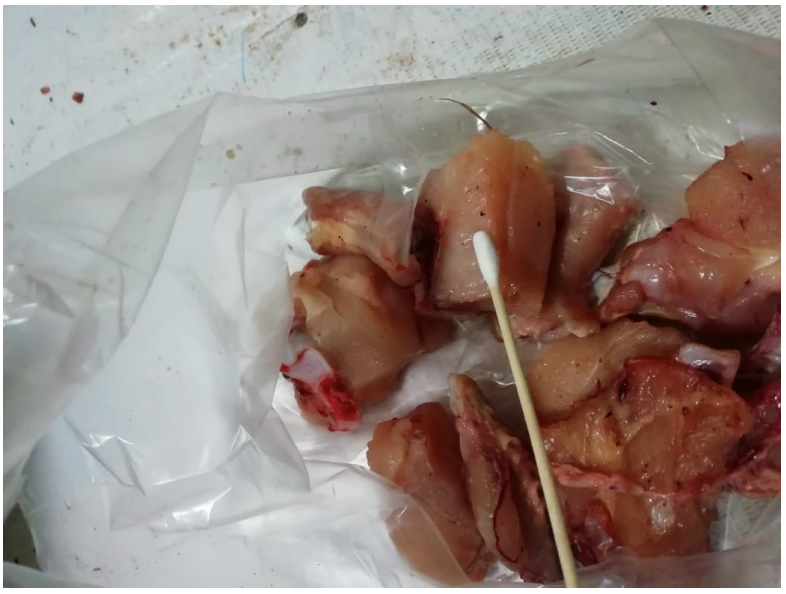

FIGURE 3.3: Swab Taken from Chicken Meat for Bacterial Analysis

3.4.2 Sample Preparation

Swab was placed in a sterile screw capped test tube and 10 ml sterile distilled water was added in the tube and mixed well for 2 minutes. So 1:10 dilution was prepared called as parental dilution.

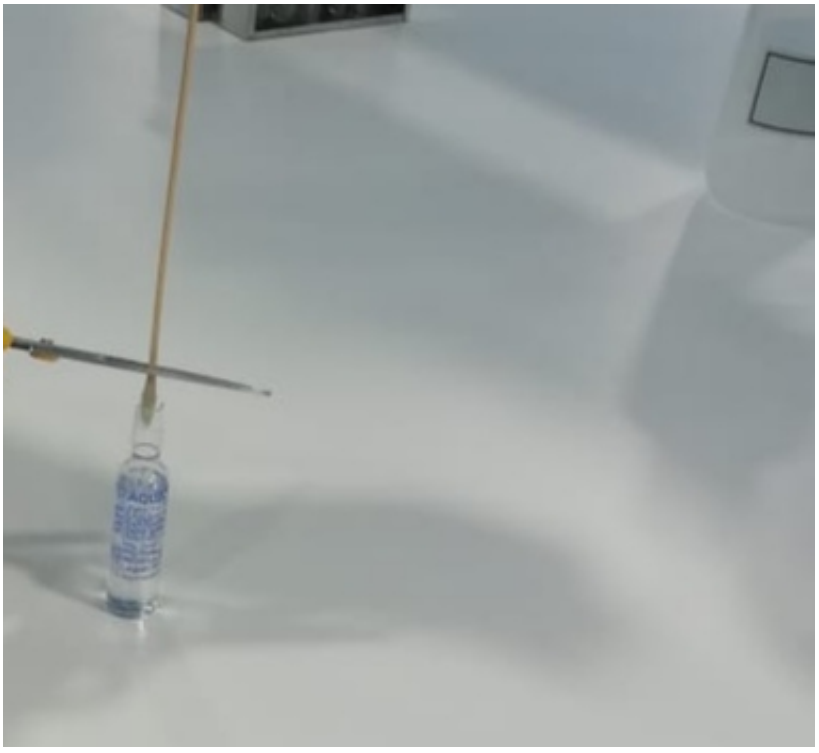

FIGURE 3.4: Sample Dilution in Sterile Distilled Water

3.4.3 Sample Processing

3.4.3.1 Bacterial Isolation and Identification

3.4.3.1.1 TBC (Total Bacterial Count)/CFU (Colony Forming Unit)

- Nutrient agar was prepared in sterile distilled water and autoclaved at 121℃.
- Media was allowed to cool at 42℃.
- At 42℃ 1 ml of parental dilution of sample was added in a sterile petri dish.
- 25 ml of already prepared and autoclaved media was then added in the petri dish (pour plate method).
- 4 of the samples were also streaked with the help of a sterile cotton swab (streak method)
- Plates were incubated at 35℃ for 24-48 hours.
- After 48 hours colonies were counted for TBC.
- If colonies were too numerous to count then further 1:10 dilution of parental dilution was made (total dilution 1:100) (ISO certified method)

3.4.3.1.2 Bacterial Identification

- Biochemical tests and growth characteristics on the selective media were used to identify bacteria.
- XLD media was used to isolate and identify *Salmonella spp.*
- For *S. aureus* MSA was used.
- For *E. coli* VRB media was used.
- CVA agar was used for the isolation and identification of *C. jejuni.*
- TSA agar was used for the isolation and identification of *C. perfringens.*

- Re-culturing on selective media was used to obtain purified colonies of bacteria.

3.4.3.1.3 Enumeration of Pathogenic Bacteria

- 1 ml of the sample dilution was added in the petri dish with the help of a sterile micropipette.
- Then 15 ml of already prepared media (temp. 44-47℃) was added into each petri dish.
- 2 plates were inoculated against each dilution.
- Medium was added in the petri dish containing inoculum within 10 minutes.
- Then inoculum was carefully mixed in the media and then allowed to solidify in the horizontal position.
- Then plates were incubated at temperature and time according to the type of isolate suspected to be present in the sample.
- Then after incubation colonies were observed and counted on each plate.
- Colonies were counted with the help of a digital colony counter.
- Then restreaking on the selective media was performed to obtain pure colonies of organisms for further processing.
- When pure colonies of bacteria under study were obtained and identified on the selective media further confirmation by biochemical tests was done.

3.4.3.1.4 These Biochemical Tests were Used

- Indole
- Catalase
- Oxidase

- H_2S production
- Citrate utilization test

3.4.3.1.4.1 Indole Test

Principle:

Indole test was used to identify bacteria which have the ability to convert amino acid tryptophan into indole. This test helped us to differentiate between members of Enterobactericiae family.

Procedure

- A small amount of pure culture of test organism was inoculated in the tryptone broth.
- Then it was incubated for 24-48 hours at 35℃.
- Then 5 drops of Kovacs reagent were added to the tube.
- Tube was observed for formation of pink to red color [61].

Indole Positive Organisms:

Escherichia coli is indole positive.

3.4.3.1.4.2 Catalase Test

Principle:

This test was used to detect the presence of catalase enzyme in the bacterial cell. Hydrogen peroxide is converted into oxygen and water by this enzyme.

Procedure:

- A microscope slide was placed in a petri dish.
- A colony (18-24 hours old) of test organism was placed on the slide.

- Few drops of 3% H_2O_2 were dropped on the colony and observed for any gas bubbles [62].

Catalase Positive Organisms:

*Staphylococcus aureu*s is a catalase positive organism.

3.4.3.1.4.3 Oxidase Test

Principe:

This test was done to check the presence of cytochrome oxidase enzyme in the bacterial cell. This enzyme oxidizes the reduced colorless Kovacs reagent into an oxidized colored product.

Procedure:

- A small filter paper was taken and dipped in the Kovacs oxidase reagent.
- A colony (18–24 hour old) of test organism was rubbed on the filter paper.
- Filter paper was observed for color change (deep purple) within 5-10 seconds [63].

Oxidase Positive Organisms:

P. aeruginosa

3.4.3.1.4.4 H_2S (Hydrogen Sulfide) Production Test

Principle:

This test was used to check the ability of bacteria to reduce sulfur containing compounds to hydrogen sulfide. This test was used for the identification of *enterobactericiae*.

Procedure:

- Test organism was inoculated on the media by stab inoculation.
- Then culture tubes were incubated 37℃ for 24-48 hours.
- After incubation tubes were observed for the formation of black precipitate [64].

H_2S Producing Organisms:

Salmonella, E. coli

3.4.3.1.4.5 Citrate Utilization Test

Principle:

This test was used to identify organisms that produce citrase enzyme i.e. *Salmonella*. These organisms can use citrate as a source of carbon for metabolism. This test was performed on Simmons Citrate agar.

Procedure:

- Slant of Simmons citrate agar was inoculated with 18-24 hours old colony of test organism.
- Tubes were incubated for up to 7 days at 37℃.
- After 7 days tubes were observed for any color change from green to blue [65].

Citrate Positive Organisms:

Salmonella

3.4.3.2 Antibiotic Susceptibility Testing

- Antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion method using Mueller Hinton agar.
- With the help of a sterile wire loop 4-5 colonies of test organisms were touched.
- Then these colonies were suspended in 2 ml sterile saline solution.
- This suspension was mixed with the help of a vortex.
- Turbidity of suspension was adjusted by comparing it with a 0.5 McFarland suspension.
- A sterile swab was dipped into the inoculum prepared and excess fluid was removed by pressing the swab along the wall of the tube.
- Dried surface of MH agar was inoculated by streaking the swab and excess fluid was removed.
- Plate was allowed to sit at room temperature for 3-5 minutes while lid was slightly aside.
- Then antimicrobial discs were applied (OFX, LEV, MOX, CFM) on the agar surface with the help of sterile forcep.
- After the application of antibiotic disks plate was incubated at 35℃ overnight.
- After incubation plates were observed for the zone of inhibition of growth from backside of the plate.
- Zone of inhibition was noted on the record sheet [66].

Chapter 4

Result and Discussion

24 samples were collected from 6 different chicken slaughter houses of twin cities (Islamabad and Rawalpindi). There were 4 samples were collected from each slaughter facility. 1st swab was taken from chicken slaughter containers (where chicken are placed after killing/slaughtering) from each facility. 2nd swab was taken from the different surfaces of chicken slaughter houses. 3rd Swab was taken from knives used for the slaughtering of chicken. 4th swab was taken from the processed chicken. All the samples were collected with the help of a sterile swab stick and transported to the microbiology lab for microbial analysis as soon as possible.

FIGURE 4.1: Sampling from Chicken Slaughter Container with the Help of Sterile Swab Stick

FIGURE 4.2: Sampling from the Surface of Chicken Slaughtering Equipment for Bacteriological Analysis

In the microbiology lab samples were analyzed for the presence of pathogenic bacteria. Antibiotic susceptibility pattern of isolates was also checked.

All 24 samples were analyzed for the isolation of bacteria and all the samples were found positive for bacterial growth. The average number of CFU isolated was 23.25×10^3 from all the samples. After CFU determination presence of *E. coli, C. jejuni, S. aureus, C. perfringens* and *S. typhi* was checked. Samples were analyzed for the isolation and identification of these pathogens. All the samples were analyzed and it was found that 17 samples out of 24 were positive for *S.typhi* (70%), 15 out of 24 samples were positive for *C. jejuni* (62.5%), 12 samples out of 24 were positive for *S. aureus* (50%), 11 samples out of 24 were positive for *E. coli* (45.8%) and 6 samples out of 24 were positive for *C. perfringens* (25%). While 2 samples showed no growth of any of these pathogens (8.3%). So 22 samples were positive for these pathogens, while 2 samples were negative for these

FIGURE 4.3: Sampling from Stump Used for Slaughtering and Processing of Chicken

pathogens. *Salmonella typhi* was the predominant pathogen in the samples while *C. perfringens* was the least isolated pathogen in the samples.

4.1 TVC (Total Viable Count) Isolated from Slaughter House

Samples were analyzed for the total viable count as CFU per sample. Samples to analyze TVC were cultured on the Nutrient agar. Samples from containers showed the highest number of isolates. The average number of TVC in the samples collected from chicken slaughter containers was 70.6×10^3 with a highest number shown by sample 6 which was 17.0×10^3. Samples from surfaces of chicken slaughter houses showed a total viable count less than containers. The average number of TVC from samples swabbed from surfaces of slaughter houses was 14.5×10^3.

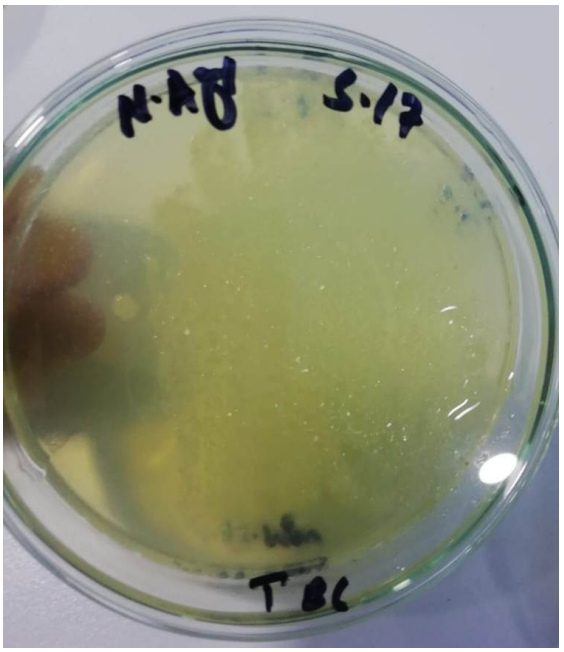

FIGURE 4.4: TVC Analysis on Nutrient Agar

Samples were also swabbed from knives used for slaughtering and cutting of chicken. Samples from equipment showed a TVC 11×10^3 colonies per sample.

This number was less than TVC isolated from chicken slaughter containers and surfaces of slaughter houses. Swabs were also taken from processed chicken meat. Samples from meat showed an average number of TVC of 1.1×10^3. This number was well below than the number of total viable bacteria isolated from slaughter houses. Table 4.1 shows the number of TVC isolated from chicken slaughter houses and chicken meat. Sample 006 showed a highest number of TVC (170×10^3) from all the sites and also from the chicken meat. This sample was collected from a chicken slaughter house with very low level of hygiene. Graph (Figure 4.1) below shows the average number of bacteria (TVC) isolated from different sites. Samples from chicken slaughter containers showed the highest number of bacterial isolates. While samples swabbed from the surfaces of chicken slaughter houses showed the second highest number of total viable count isolated from these samples. When equipment used for slaughtering and cutting of chicken were analyzed it was also contaminated from bacterial pathogens. The number of bacteria from equipment was much lower than from chicken slaughter containers and surfaces of slaughter houses. When samples swabbed from processed chicken before delivery to the

customer were analyzed they showed a very few number of bacteria as compared to samples from different areas of slaughter houses.

TABLE 4.1: TVC Isolated from the Samples of Chicken Slaughter Houses and Chicken Meat

Sample code	[1]**Container**	[2]**Surfaces**	[3]**Equipment**	[4]**Meat**
001	90.0×10^3	11.0×10^3	10.0×10^3	1×10^3
002	26.0×10^3	5.0×10^3	0.3×10^3	0.9×10^3
003	$71.0\times\ 10^3$	19.0×10^3	07.0×10^3	01.1×10^3
004	14.0×10^3	02.0×10^3	4.00×10^3	0.50×10^3
005	53.0×10^3	09.0×10^3	6.0×10^3	1.0×10^3
006	17.0×10^3	41.0×10^3	11.0×10^3	02.1×10^3
Average	70.6×10^3	14.5×10^3	6.8×10^3	1.1×10^3

TVC: Total Viable Count, 1. Container (Chicken slaughter container) 2. Surfaces (Surface of slaughter house) 3. Equipment (Equipment used in the slaughter process) 4. Meat (Processed chicken meat)

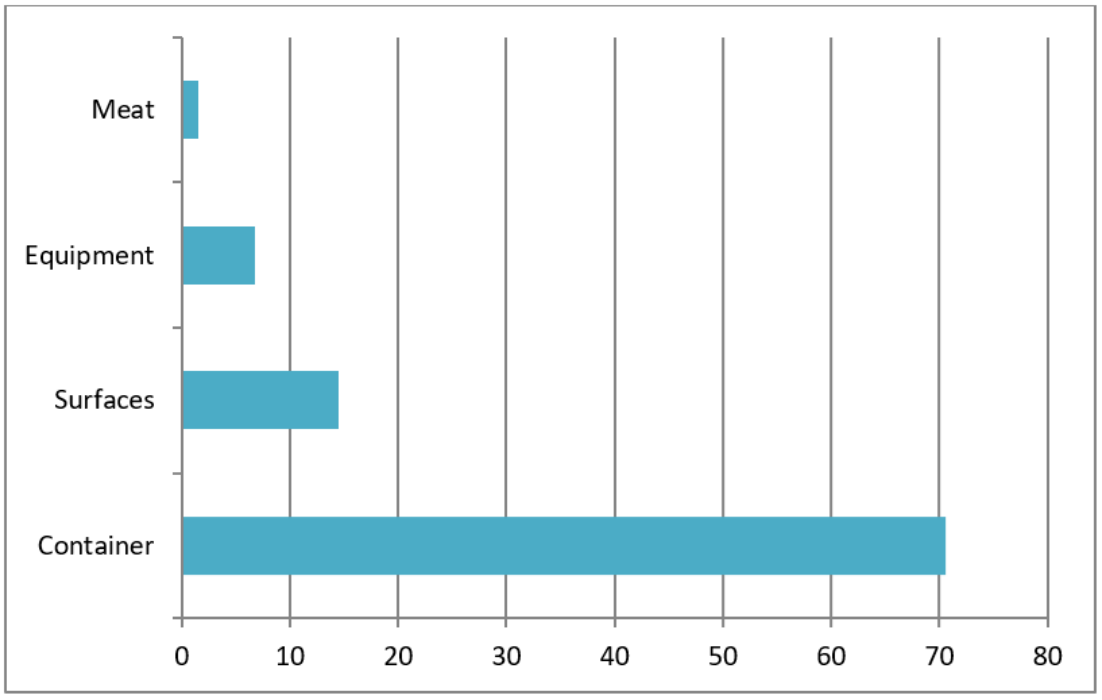

FIGURE 4.5: Avg. TVC Isolated from Samples of Chicken Slaughter Houses and Chicken Meat

Table 4.2 below is depicting the prevalence rate of these pathogens among the samples from poultry slaughter house facilities.

TABLE 4.2: Prevalence rate and frequency of prevalence in the samples from slaughter houses

Pathogen	**Prevalence rate**	**Frequency of pathogen (%)**
S. typhi	17	70
C. jejuni	15	62.5
S. aureus	12	50
E. coli	11	45.8
C. perfringens	6	25

S.typhi was the most predominant pathogen among the 5 pathogens under study in the samples from chicken slaughter facilities and chicken meat with a prevalence rate of 70%. *C. jejuni* was the second most prevalent pathogen in the samples with a frequency of 62.5%. *S. aureus, E. coli* and *C. perfringens* were present with a frequency of 50, 45.8 and 25% respectively. *C. perfringens* was the least isolated organism.

Graph (Figure 4.6) below is showing the frequency of different pathogens found in the samples of chicken slaughter houses and chicken meat.

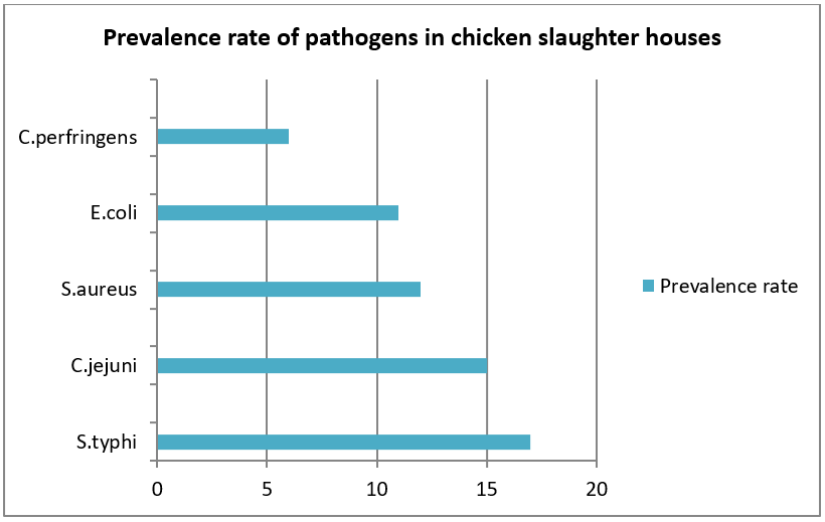

FIGURE 4.6: Prevalence Rate of Pathogens Isolated from Chicken Slaughter Houses and Chicken Meat

4.2 Samples from Chicken Slaughter Container

Samples were also analyzed to check which type of pathogen was more prevalent in these samples. When samples from chicken slaughter containers were analyzed it was found that was the most prevalent pathogen in the samples and *S. aureus* was the second most prevalent pathogen. *C. jejuni, E. coli* and *C. perfringen*s were at third, fourth and fifth. The average number of colonies of these organisms in the samples collected from chicken slaughter containers was 14×10^3, 8×10^3, 6.4×10^3, 2.2×10^3, and 1×10^3 for *S. typhi*, *S. aureus, C. jejuni, E. coli* and *C. perfringens* respectively. Table 4.3 shows the count of pathogens isolated from chicken slaughter containers.

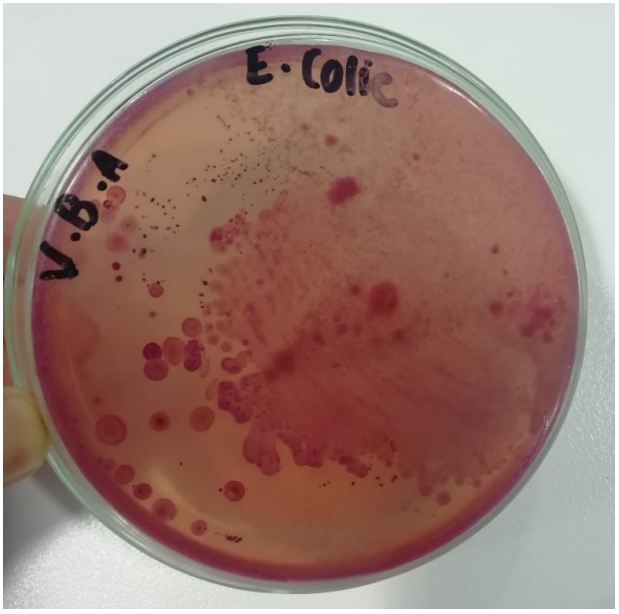

FIGURE 4.7: Colonies of *E. coli* on Selective Media

TABLE 4.3: Colonies of Pathogens Isolated from Chicken Slaughter Containers

Pathogen	**Isolated Colonies**
S.typhi	14×10^3
S. aureus	8×10^3
C. jejuni	6.4×10^3
E. coli	2.2×10^3
C. perfringens	1×10^3

FIGURE 4.8: Colonies of *S. aureus* on Selective Media

So in the samples collected from chicken slaughter containers *S. typhi* was the predominant pathogen while S. aureus was at second. Other pathogens were also found in low numbers as compared to these two pathogens.

Graph (Figure 4.9) below depicts the number of colonies of pathogens isolated from samples of chicken slaughter containers.

4.3 Samples from Surfaces of Chicken Slaughter House

When samples swabbed from surfaces of chicken slaughter houses it was found that S. aureus was the major contaminant of surfaces of slaughter houses. *S. typhi* was second to *S. aureus*. *C. jejuni* was at third and *C. perfringens* and *E. coli* at fourth

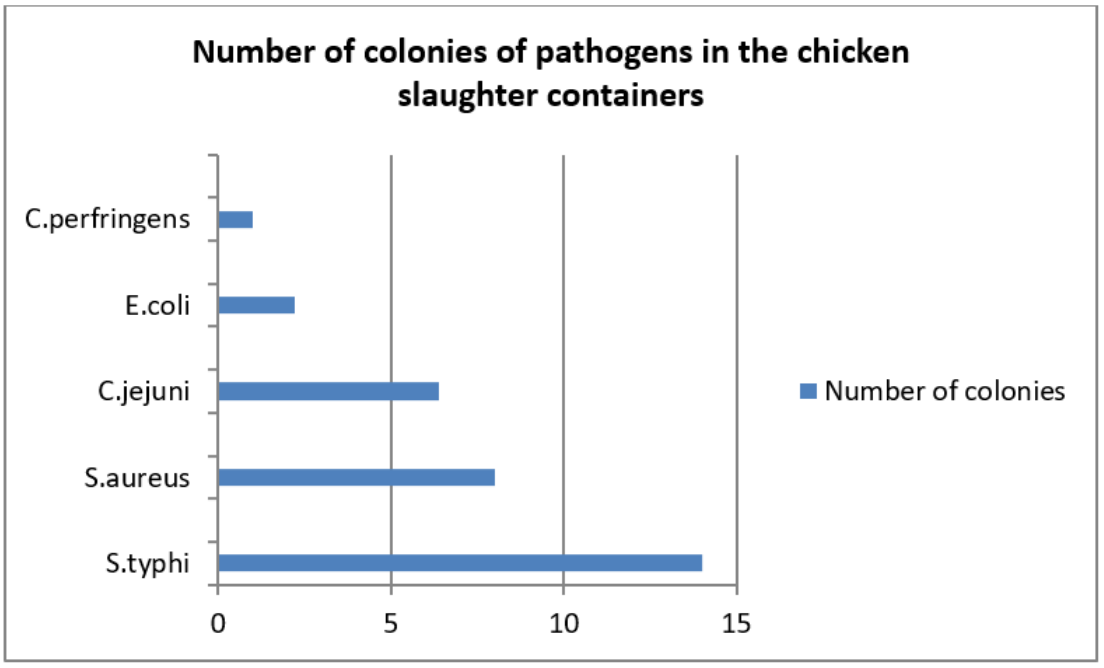

FIGURE 4.9: Number of Colonies of Pathogens ($\times 10^3$) Isolated from Chicken Slaughter Containers

and fifth respectively. The average number of these pathogens was calculated for each pathogen. It was found that the average number of these pathogens was; S. aureus 2.9×10^3, *S. typhi* 2.5×10^3, *C. jejuni* 1.6×10^3, *C. perfringens* 0.9×10^3 and *E. coli* 0.4×10^3. Table 4.4 show the number of pathogens isolated from surfaces of slaughter houses.

TABLE 4.4: Colonies Isolated from Surfaces of Slaughter House

Pathogen	**Number of colonies isolated**
S. typhi	2.5×10^3
S. aureus	2.9×10^3
C. jejuni	1.6×10^3
E. coli	0.4×10^3
C. perfringens	0.9×10^3

Graph in Figure 4.12 depicts the number of colonies isolated from samples swabbed from surfaces of chicken slaughter houses.

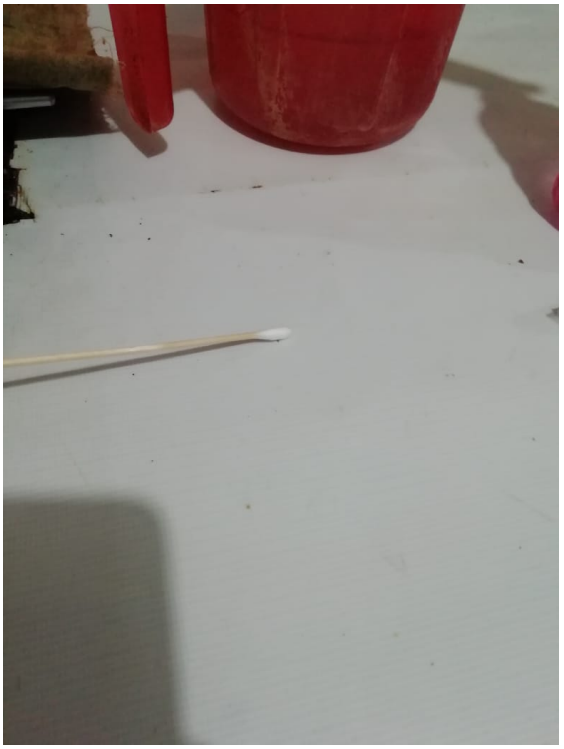

FIGURE 4.10: Swab Taken from Surface of Chicken Slaughter House

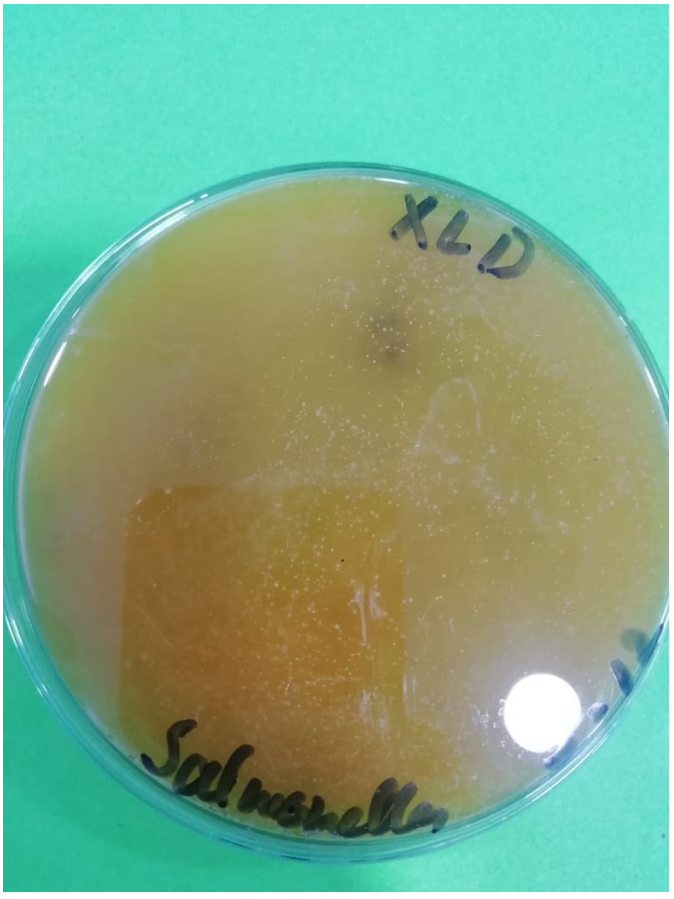

FIGURE 4.11: Colonies of *S.typhi* on Selective Media

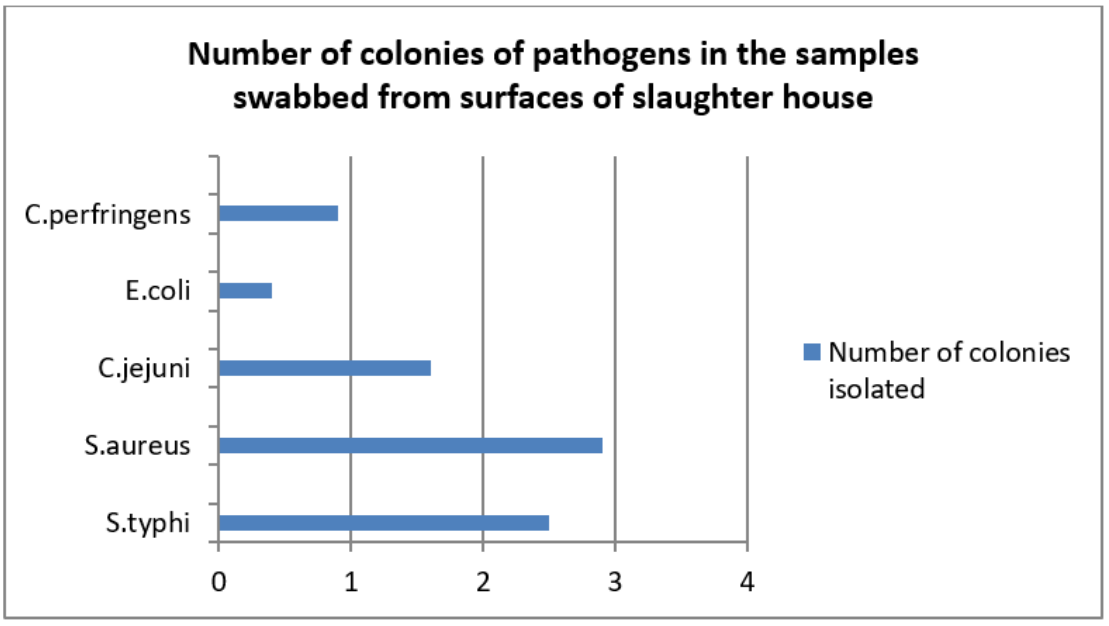

FIGURE 4.12: Number of Colonies of Pathogens ($\times 10^3$) Isolated from Surfaces of Chicken Slaughter Containers

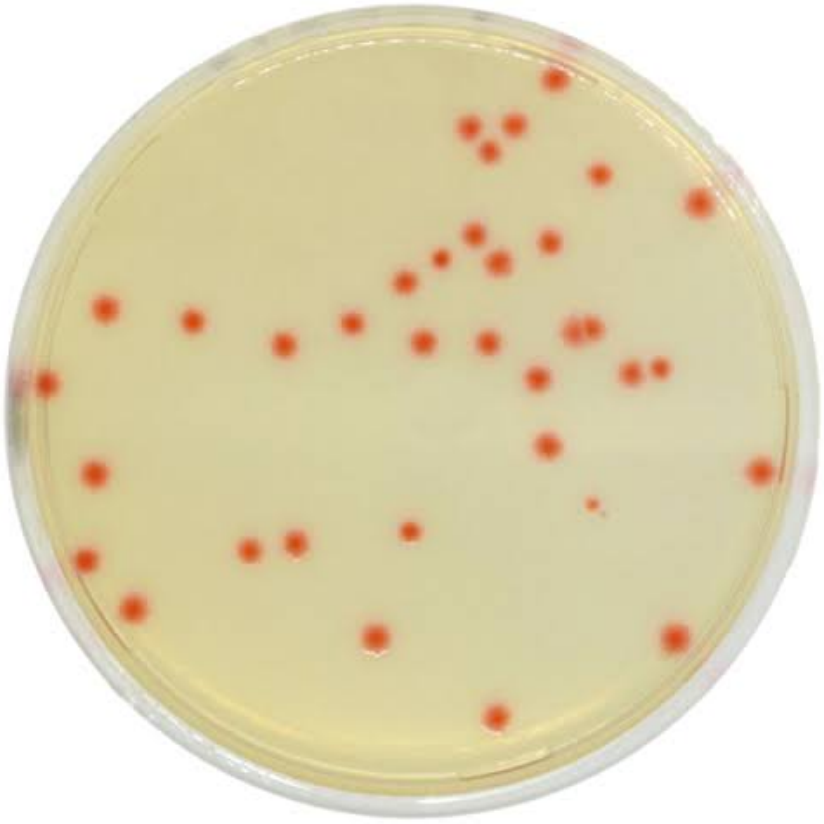

FIGURE 4.13: Colonies of *C. jejuni* on Chrome Agar

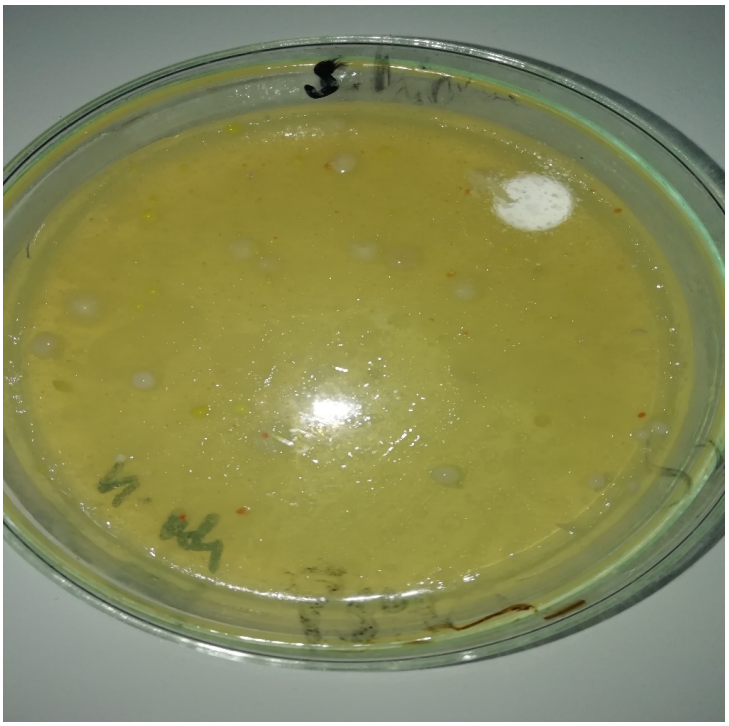

FIGURE 4.14: Colonies of *C. perfringens* on TSA Agar

4.4 Samples from Equipment Used for Slaughtering and Processing of Chicken

When samples swabbed from equipment were analyzed it was found that *S. typhi* was the major contaminant of slaughter equipments. Other four organisms were isolated in different numbers. The number of pathogens isolated was as; *S.typhi* 1.6×10^3, *S. aureus* 1.3×10^3, *E. coli* 0.8×10^3, *C. jejuni* 0.6 and *C. perfringens* 0.3×10^3. Table 4.5 show the number of colonies of pathogens isolated from equipment used in slaughter house.

S. Typhi was the most abundant contaminant isolated from chicken slaughter equipment while *S. aureus* was at second. *E. coli, C. jejuni* and *C. perfringens* were at third, fourth and fifth respectively according to number of colonies isolated. Graph in figure 4.16 shows the colonies of pathogens isolated from equipment used for slaughtering and cutting of chicken.

TABLE 4.5: Number of Colonies of Pathogens Isolated from Equipment

Pathogen	**Number of colonies**
S. typhi	1.6×10^3
S. aureus	1.3×10^3
C. jejuni	0.6×10^3
E. coli	0.8×10^3
C. perfringens	0.3×10^3

FIGURE 4.15: Swab from Hack Used for Slaughtering

Samples were also swabbed from chicken meat after processing. Contaminants were checked and it was found that *S.typhi* was found in higher numbers than other pathogens. The average number of this pathogen was 0.5×10^3. Other contaminants were *C. jejuni*, *C. perfringens* and *S. aureus*. *E. coli* was isolated from

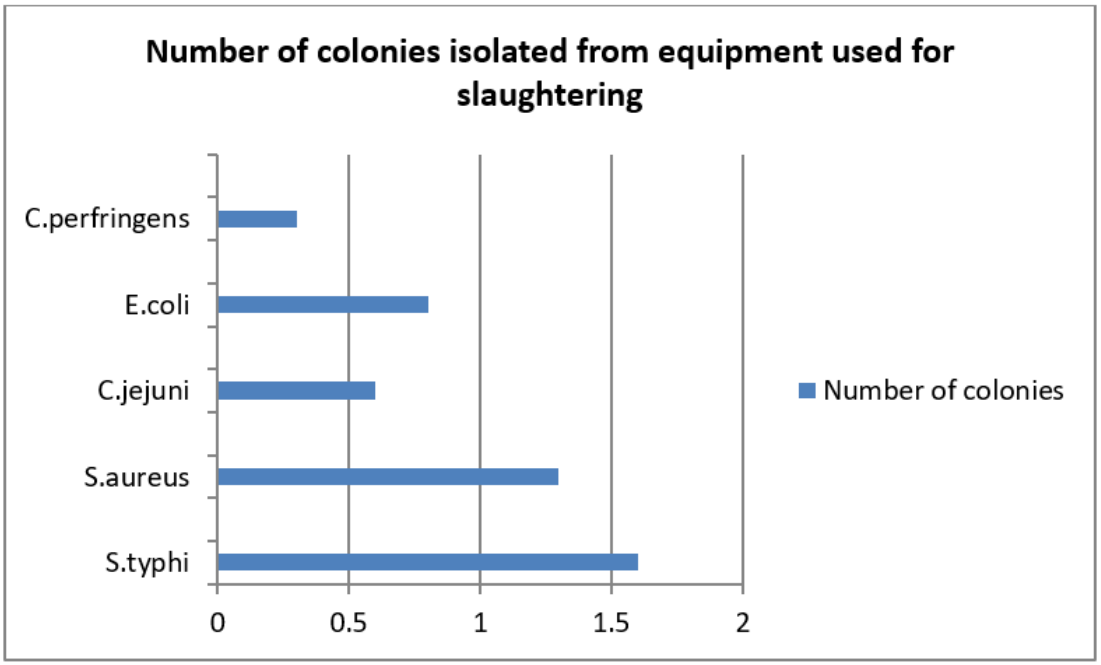

FIGURE 4.16: Number of Colonies ($\times 10^3$) of each Pathogen Isolated from Equipment

2 samples in very low numbers. So according to my finding *S. typhi* was the predominant pathogen found in the chicken slaughter houses and also in the processed chicken meat.

4.5 Samples from Processed Meat of the Chicken Slaughter House

Samples were also swabbed from chicken meat after processing. Contaminants were checked and it was found that *S. typhi* was found in higher numbers than other pathogens.

The average number of pathogens isolated was as; *S. typhi* 0.5×10^3, *S. aureus* 0.3×10^3, *E. coli* 0.4×10^3, *C. jejuni* 0.2 and *C. perfringens* 0.1×10^3.

So according to my finding *S. typhi* was the predominant pathogen found in the chicken slaughter houses and also in the processed chicken meat.

S. typhi was the predominant pathogen found in the chicken slaughter container, equipment and processed meat of chicken slaughter houses but *S. aureus* was the predominant pathogen in the surface of the chicken slaughter house.

TABLE 4.6: Number of Colonies of Pathogens Isolated from Processed Chicken Meat

Pathogen	**Number of colonies**
S. typhi	0.5×10^3
S. aureus	0.3×10^3
C. jejuni	0.2×10^3
E. coli	0.4×10^3
C. perfringens	0.1×10^3

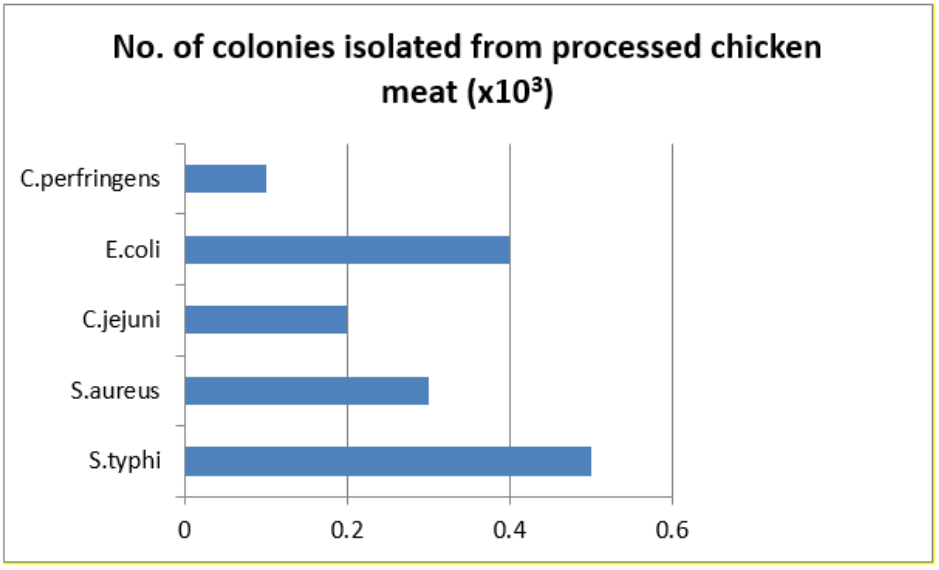

FIGURE 4.17: Shows the Colonies of Pathogens Isolated from Processed Chicken Meat.

4.6 Morphological Characteristics of Isolates

When morphological characteristics of isolates were analyzed it was observed that some isolates were cocci while others were rod shaped and *C. jejuni* was helical

in shape. Some were Gram positive while others were Gram negative. There was also difference in the size of the organisms.

When oxygen requirement of isolates was checked some were aerobes while others were facultative anaerobe. *C. perfringens* was anaerobe according to its metabolic requirements. Motility of isolates was also analyzed and some isolates were motile while others were non motile. Colony morphology on selective media was used for identification of the isolates. Table 4.7 show the morphological characteristics of isolates.

4.7 Antibiotic Susceptibility Testing of Isolates

After isolation antibiotic susceptibility testing of isolates was also checked. Susceptibility of isolates was checked against four antibiotics which are commonly used to cure bacterial infections in humans. These antibiotics were Ofloxacin, Levofloxacin, Moxifloxacin and Cefixime.

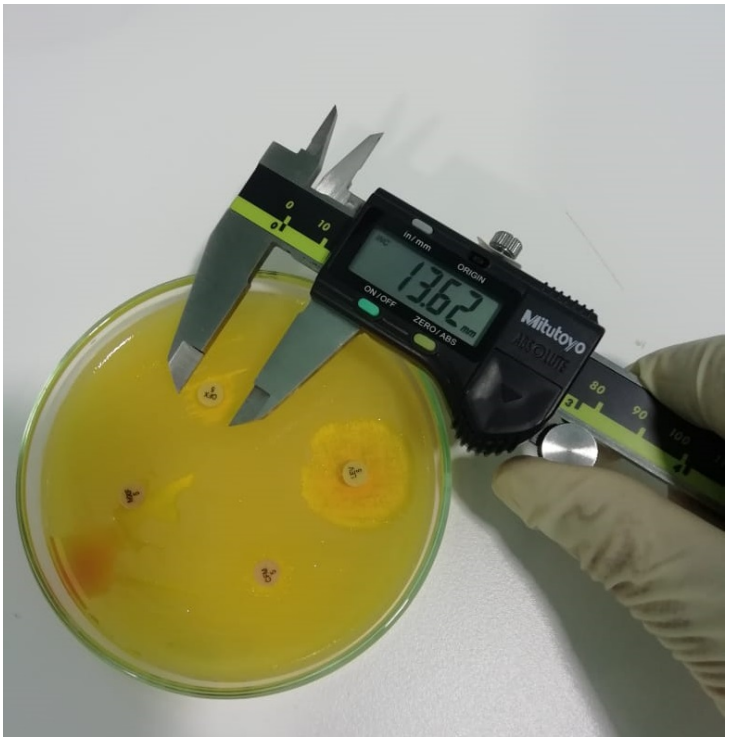

FIGURE 4.18: Measurement of ZOI Against Selected Antibiotics

Table 4.7: Morphological Characteristics of Isolates

Characteristics	***S. typhi***	***S. aureus***	***C. jejuni***	***E. coli***	***C. perfringens***
Shape	Rod	Cocci	Helical	Rod	Rod
Size (Micron)	2 - 5 x 0.5 – 1.5	1 – 1.5 x 0.1 – 0.25	0.5 – 5.0 x 0.2 – 0.5	1- 2 x 0.5	4 – 8 x 0.8 – 1.5
Gram reaction	Gram negative	Gram positive	Gram negative	Gram negative	Gram positive
Oxygen requirement	*F. anaerobe*	*F. anaerobe*	Micro-aerophilic	*F. anaerobe*	Anaerobe
Selective media	XLD agar	MSA	Charcoal based selective media	MacConkey's agar	Roberts-on's cooked meat broth
Colony	Red colored colonies on selective media	Yellow colonies on MSA	Black colonies on selective media	Pink colonies on selective media	
Motility	Motile	Non motile	Motile	Motile	Non motile

TABLE 4.8: Antibiotic Susceptibility Pattern of Isolated Organisms Against Selected Antibiotics

Organism	**OFX (mm)**	**LEV (mm)**	**MOX (mm)**	**CFM (mm)**
S. typhi	25.76	12.11	22.61	21.21
S. aureus	26.41	23.14	24.79	19.61
C. jejuni	19.15	18.71	13.71	12.11
E. coli	25.33	20.51	22.61	19.71
C. perfringens	22.60	21.82	18.66	21.22

OFX (Ofloxacin), LEV (Levofloxacin), MOX (Moxifloxacin), CFM (Cefixime).

TABLE 4.9: Interpretation of Zone Diameter (mm)

Antibiotic	**R $\leq$**	**I**	**S $\geq$**
Ofloxacin	11	12-14	15
Levofloxacin	13	14-16	17
Moxifloxacin	16	17-19	20
Cefixime	19		20

These four antibiotics were checked against these isolates and it was found that Levofloxacin was least effective against *S. typhi* and it showed a 12.11 mm zone of inhibition of growth on Mueller Hinton agar. So *S. typhi* was resistant to Levofloxacin. OFX, MOX and CFM showed a zone of inhibition of 25.71, 22.61 and 21.21 respectively against this isolate. So over all OFX, MOX and CFM were effective against this organism. While LEV was found to be less effective against this isolate. Graph in figure 4.20 shows zone of inhibition of *S. typhi* against antibiotics tested.

FIGURE 4.19: Measurement of ZOI of *S. typhi* Against CFM

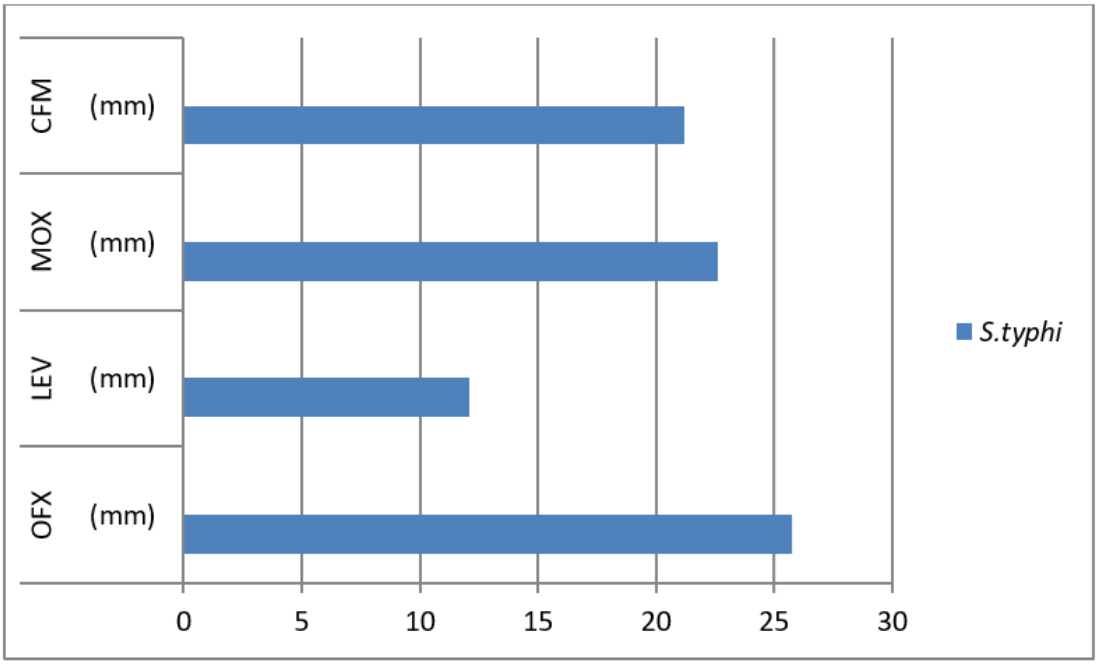

FIGURE 4.20: Zone of Inhibition (mm) of *S. typhi* Against OFX, LEV, MOX and CEF.

S. aureus isolated from these samples was also tested against these four antibiotics. *S. aureus* showed a zone of inhibition of 26.41 against OFX, 23.14 against LEV, 24.79 against MOX and 19.61 against CEF. So this organism showed almost similar pattern of susceptibility against these all antibiotics. OFX showed a wide area of inhibition while MOX showed a least area of inhibition. Graph in figure 4.21 shows a zone of inhibition of *S. aureus* against four antibiotics tested.

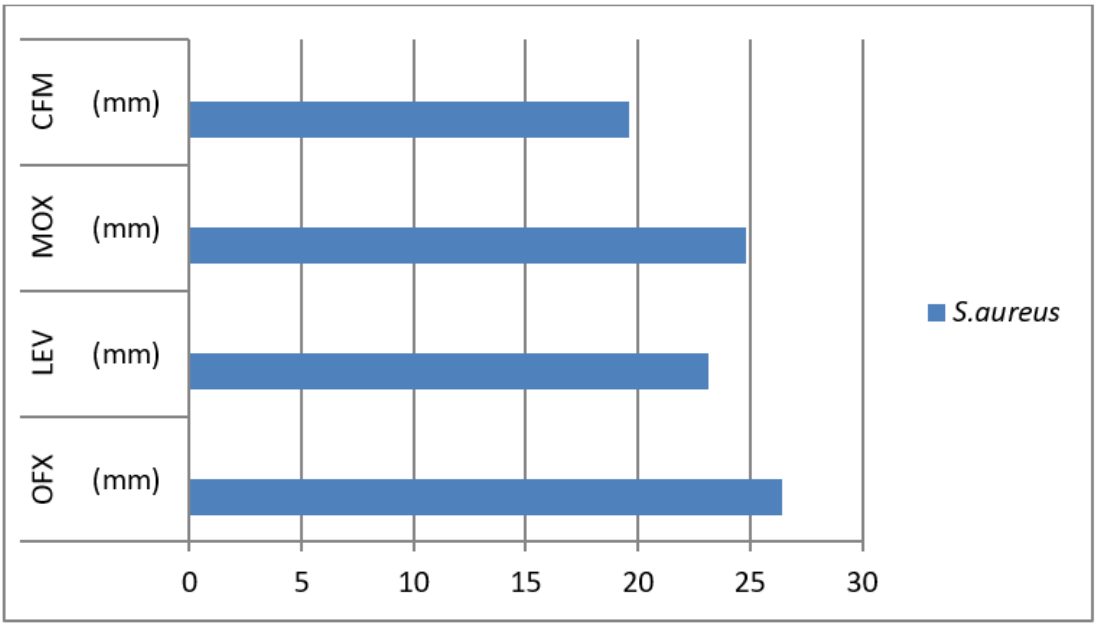

FIGURE 4.21: Zone of Inhibition (mm) of *S. aureus* Against OFX, LEV, MOX and CEF.

When susceptibility pattern of *C. jejuni* isolated from chicken slaughter houses and chicken meat was checked against these antibiotics they showed a variable zone of inhibition. The zone of inhibition was 19.15 against OFX, 18.71 against LEV, 13.71 against MOX and 12.11 against CFM. This organism was resistant against MOX and CEF. While two other antibiotics were effective against this isolate. This organism was sensitive against LEV and OFX [Figure 4.22].

Susceptibility pattern of *E. coli* was also tested against OFX, LEV, MOX and CFM. OFX showed a greater zone of inhibition against *E. coli* (25.33 mm). While CFM showed a smaller zone of inhibition (19.71 mm) against this isolate. The zone of inhibition of this organism against LEV and MOX was 20.51 and 22.61

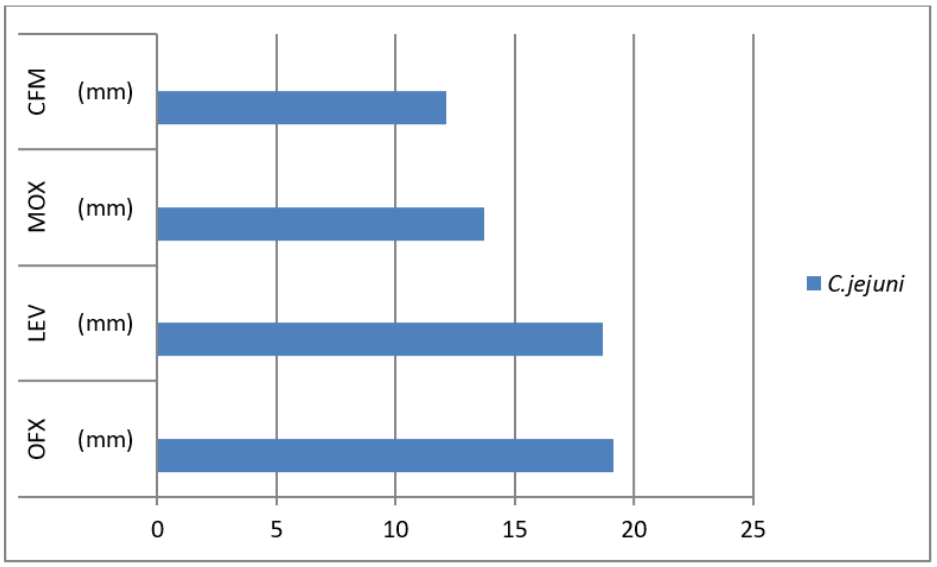

FIGURE 4.22: Zone of Inhibition (mm) of *C. jejuni* Against OFX, LEV, MOX and CEF.

respectively. Graph in figure 4.24 shows the results of antibiotic susceptibility pattern of *E. coli*.

Antibiotic susceptibility pattern of *C. perfringens* against four antibiotics tested was almost similar ranging from 18.66 mm against MOX to 22.60 mm against OFX. The two other antibiotics LEV and CEF showed 21.82 mm and 21.22 mm respectively. OFX showed wider zone of inhibition while MOX showed smallest zone of inhibition. Graph below shows zone of inhibition of *C. perfringens* against OFX, LEV, MOX and CFM. So *S. typhi* was the most prevalent pathogen found in the chicken slaughter houses and also on the processed chicken.

C. jejuni was the second most prevalent pathogen isolated from samples. *S. aureus* was the third among the isolates of chicken slaughter houses and chicken meat. *E. coli* and *C. perfringens* were at fourth and fifth respectively. These findings were same as found in another study done in the Gondar town of Ethiopia where they found that *Salmonella* was the major pathogen isolated from raw meat with an isolation rate of 35.6 % [67]. This study was also in accordance with the findings of another study which concluded that *S. enterica* serovar Enteritidis was the most predominant pathogen in the frozen poultry meat samples imported from Brazil to Canary Island and Spain [68].

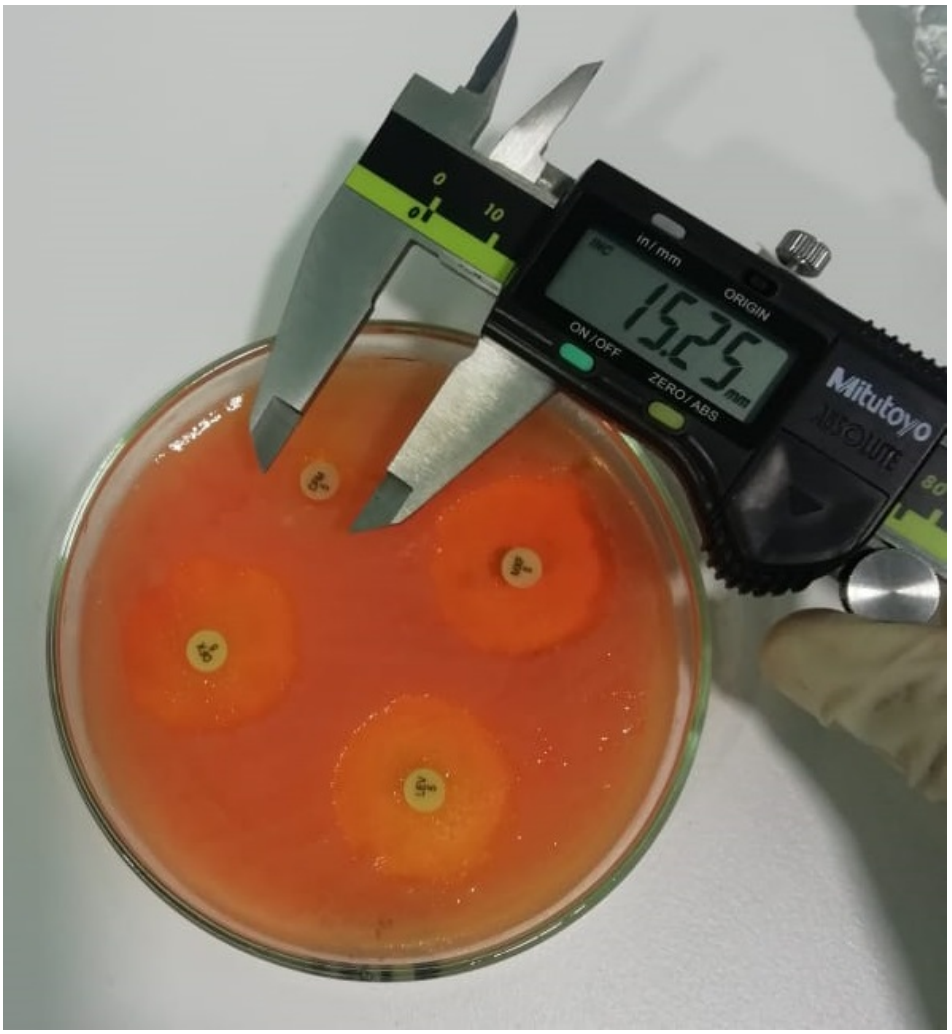

FIGURE 4.23: Measurement of ZOI of *C. perfringens* Against Selective antibiotics

In another study done in Zambia it was found that the prevalence rate of *S. typhi* was 20.5% which was much lower than the findings of my study (70% prevalence rate). So this study was in contrast with the findings of my study which showed a very high prevalence of *S. typhi* [69]. This difference may be attributed due to multiple factors i.e. difference in the size of sample, difference in the processing environment and difference in the hygienic practices of the slaughter houses. According to a study the high prevalence of *Salmonella* in the chicken was found to be associated with poor farming practice and unhygienic conditions in the slaughter houses. By improving these conditions Salmonella and other pathogens can be reduced in a larger extent.

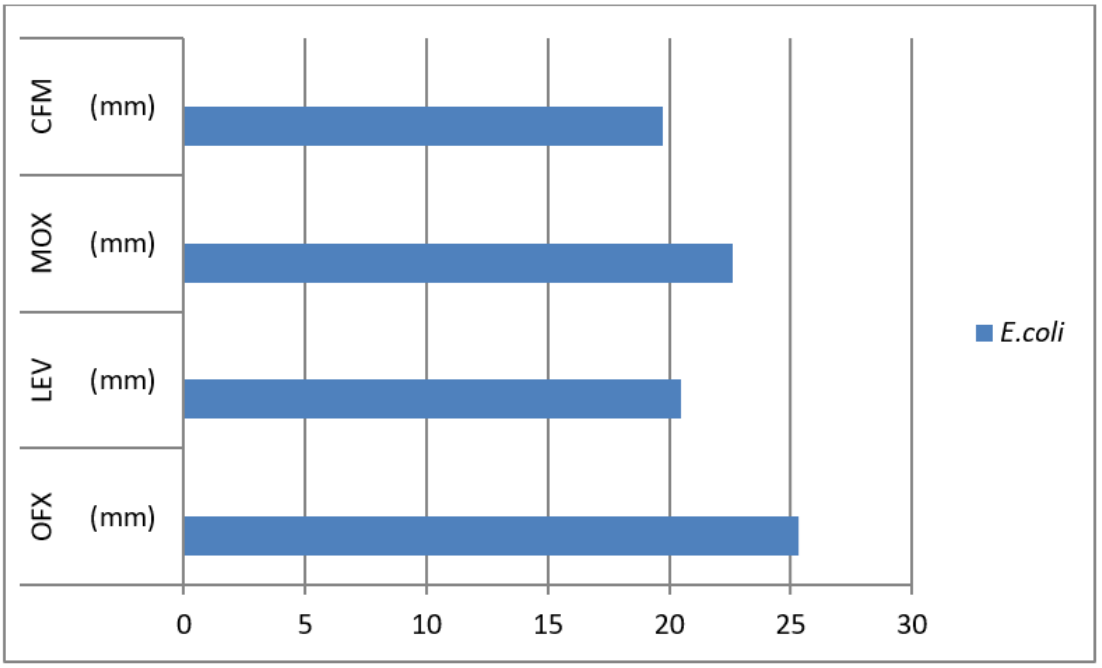

FIGURE 4.24: Zone of Inhibition (mm) of *E. coli* Against OFX, LEV, MOX and CEF.

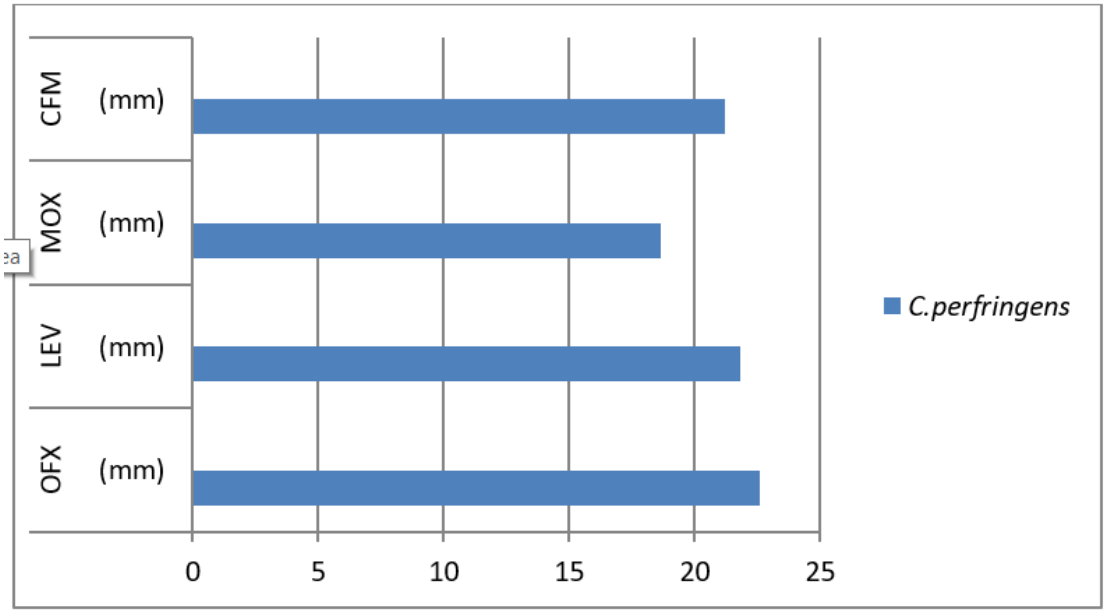

FIGURE 4.25: Zone of Inhibition (mm) of *C. perfringens* Against OFX, LEV, MOX and CEF.

It was also found that there were some other sources of Salmonellosis i.e. consumption of untreated raw milk and untreated water but consumption of contaminated poultry products was the major risk factor [70]. *Salmonella* was the major pathogenic bacteria found in the samples of chicken slaughter houses. This may

be due to the fact that Salmonella is found as normal bacterial flora in the gut of chicken. Chicken meat may get contaminated during evisceration. As offal is wasted in the slaughter container so slaughter container gets contaminated with Salmonella found in the GIT of chicken. Environmental conditions may also play a role in the excessive growth and survival of this organism. *Salmonella* also resist bad environmental conditions. The other reason may be the contamination of chicken meat from the hands of chicken slaughterers while processing of chicken meat.

S. typhi is also found in the drinking water so water which is used to drink by chicken may also spread this organism to the chicken. Due to all these factors *S. typhi* was the most prevalent bacteria in the samples taken from chicken slaughter houses and chicken meat. *E. coli* was also found in the variable numbers in the samples of slaughter containers and slaughter houses but the number of this pathogen was much lower than the *S. typhi*. This finding was same found in another study by Sharma KP. shown that *E. coli* was found in lower numbers as compared to *S. typhi* in the chicken meat. The prevalence of this pathogen was observed by various authors in different numbers as there was 34.5% prevalence of this pathogen was found in India [71]. Where as in another study conducted in Sudan the prevalence of this pathogen was 57.8% was observed [72].

There was a huge difference among the number of pathogens isolated from different sites of even a single slaughter house. The highest number of pathogens (TVC) was isolated from chicken slaughter containers followed by surfaces of slaughter house and equipment used for the slaughtering and processing of chicken meat. Least number of pathogens was isolated from final processed chicken. This difference in the number of isolates may be attributed due to the level of hygiene of these sites. As chicken slaughter containers are not cleaned and disinfected properly after every slaughter process but equipments are cleaned after every slaughtering. Chicken meat is also cleaned and washed properly to remove pathogenic bacteria and most of the pathogens removed by washing as a final step of chicken processing. Four antibiotics were tested against the isolates it was found that *S.typhi* was resistant against Levofloxacin. This antibiotic is commonly used in the chicken farming to

enhance growth of chicken and also to protect chicken against bacterial diseases. So this was the major reason of resistance of *S. typhi* against this antibiotic. This is a serious problem which should be considered. Resistant genes may be transfer to the offspring of chicken. Antibiotics are used in the chicken feed and also used as injectables to protect chicken from diseases. Consumption of such chicken meat may transfer resistance against this organism to humans.

C. jejuni showed resistance against Moxifloxacin and Cefixime. This resistance may be acquired by different ways i.e. by use of these antibiotics in the chicken feed, from environment and may also transfer from humans to the chicken by close contact of humans to chicks. This is very alarming condition because there is a chance of transfer of resistant genes to the humans against this organism as well as to the other organisms. So there should be judicious use of antibiotics in the chicken farming. This emergence of resistance is very alarming especially for the developing countries like Pakistan where health infrastructure is very weak. In the recent past XDR *S.typhi* is reported in the few districts of Sindh and Punjab. Chicken is consumed widely in our country so spread of such resistant bacteria is very easy. So spread of such infection would be drastic. *S. typhi* is a gram negative rod shaped facultative anaerobe motile organism. This organism belongs to the enterobactericiae family. This organism invades gastrointestinal tract of humans and cause Salmonellosis or typhoid fever. Symptoms may resolve without antibiotics in mild cases. In severe cases a course of antibiotics is needed if left untreated may cause shock and death. This pathogen spread via faeco-oral route by consumption of contaminated water, food and chicken. *S. typhi* causes gastrointestinal diseases in humans. *S. typhi* ingested in food or water survives acid barrier in the stomach reaches the small intestine and invades it and produces toxins which leads to gastrointestinal symptoms [73].

C. jejuni is a Gram negative helical shaped bacterium which is motile by polar flagellum. *C. jejuni* is commonly found in the gut of chicken. By reaching in the intestinal tract via food it causes bloody diarrhea, fever, abdominal cramps, nausea and vomiting [74].

S. aureus is a round shaped Gram positive bacterium mostly found on the skin and respiratory tract of humans. This organism produces toxins and enzymes which cause abscesses, respiratory tract infection, sinusitis, pneumonia, otitis and various infections in humans.

E. coli is a rod shaped Gram negative bacteria. In humans *E. coli* found as normal resident flora in the lower intestine. *E. coli* is harmless most of the time but sometimes cause serious food poisoning, urinary tract infection and meningitis which are caused due to the production of toxins by this organism. *C. perfringens* is a Gram positive spore forming bacterium. This organism is found in the intestinal tract of humans. Also found on poultry and meat. *C. perfringens* causes food poisoning, diarrhea, myonecrosis and gastroenteritis etc [75].

Chapter 5

Conclusions and Recommendations

Chicken meat is used across the globe as a common source of food. Chicken meat is a protein rich nutritious food. But slaughtering of chicken in unhygienic environment is putting human health at stake. Slaughtering and processing of chicken meat in unhygienic conditions is associated with the spread of many pathogenic bacterial pathogens. In our country slaughtering of chicken in open environment is very common practice which leads to the contamination of chicken meat and as a result disease in the consumer. Chicken slaughter container is the main source of contamination of chicken meat. Most of the slaughterers are uneducated and they don't even know the hazards and diseases associated with the consumption of contaminated chicken meat. Non judicious use of antibiotics in the poultry industry is associated with the emergence of drug resistance in the environment. Antibiotics which are used to protect poultry from diseases and to promote growth are now becoming a major source of emergence of resistance against the commonly used antibiotics. This is very alarming condition. We recently have faced emergence of XDR *S.typhi* in some districts of Sindh province of Pakistan. These kinds of infections are very difficult to treat and require multiple drugs to treat them. As many diseases associated with the consumption of contaminated poultry meat especially bacterial infections so there should be proper SOPs (Standard Operating

Procedures) for the slaughter houses. Poultry should be slaughtered and poultry meat should be processed in close environment. There should be proper training of the slaughterers about the slaughtering and cleaning process. Slaughtering and processing of chicken meat in open environment should be strictly banned. There should be a strict surrvillience for the use of antibiotics in the poultry farming. There should be time to time evaluation of the effects of use of antibiotics in the poultry industry.

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