"Extended-spectrum β-lactamase producing and Quinolone resistance *Salmonella spp.* in Dhaka city retail meat: an emerging public health concern of Bangladesh"

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DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "Extended-spectrum β-lactamase producing and Quinolone resistance *Salmonella* spp. in Dhaka city retail meat: an emerging public health concern of Bangladesh" has been carried out by the undersigned, Muhammad Sazzad Hossain, has been carried out under the joint supervision and guidance of Professor Dr. Naiyyum Choudhury, Chairman, Bangladesh Atomic Energy Regulatory Authority and Dr. Mohammed Abdus Samad, Senior Scientific Officer; Animal Health Research Division. BLRI, submitted to the Department of Mathematics and Natural Sciences, BRAC University in partial fulfillment of MS. in Biotechnology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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Dedication

To My Beloved Parents

Acknowledgment

ABSTRACT

Salmonella has represented as the primary cause of food poisoning in human. Immunocompromised people and infants are mainly vulnerable to salmonellosis; *Salmonella* can be found in many foods and food products including retail meat. Multi-drug resistance Salmonella has posed a robust challenge to food safety. Periodically, from March 2015 to December 2015,328 samples were collected from 10 live animal market (LAM) from Dhaka city in Bangladesh. In this study,15% (n=48) samples (13% chicken meat, 13% beef, 28% mutton, and 40% buffalo) were found to be *Salmonella* spp. positive. From (n=48) Salmonella isolates,58% nontyphoidal and 42% typhoidal *Salmonella spp*. were observed among the meat samples. A total of 13 antibiotics of β-lactam and Quinolone groups were tested to determine antibiotic resistance profile by using the Bauer method. Resistance to at least four antibiotic agents was detected in 100% (n=48) of isolates and the most extremely resistance were observed to Pefloxacin 100%, Enrofloxacin 78%, Nalidixic acid 74%, Ciprofloxacin 67% and ampiciline 58%. However, Ceftriaxone 4%, Cefixime 7%, Cefepime 9% and Imipenem 11% was shown relatively low antibiotic resistant. Presence of ESBLs (Extended-spectrum beta-lactamases) gene bla_{TEM} 89% was remarkable, another gene bla_{IMP} 29%, bla_{VIM} 29%, bla_{CTX-M} 27%, bla_{OXA} *20% and bla $_{KPC}$ 11%* was observed along or various combination. *GyrA* and *GyrB* was simultaneously observed in highly resistance quinolone antibiotics. Quinolone antibiotic resistance gene (*GyrA* 80%, *GyrB* 23% and *parc* 20%) was observed in phenotypic resistance isolate alone or concurrently. Resistant strains of *Salmonella* are common in retail meat, may be prudent use of antibiotics in livestock can mitigate this problem.

Key words: *Salmonella* **serovars, Retail meats, ESBLs, Quinolone**

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	Base Pair
pH	Negative logarithm of hydrogen ion concentration
ID	Identification
CDC	Centers for Disease Control
cfu	Colony Forming Unit
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside tri-phosphates
e. g.	Examplia gratia
et al	And others
kb	Kilo base
kbp	Kilo base pair
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standard
NCTC	National Collection of Type Cultures
PCR	Polymerase Chain Reaction
rpm	Rotation Per Minute
sec	Second
WHO	World Health Organization
XLD Agar	Xylose lysine deoxycholate Agar
SS Agar	Salmonella-Shigella Agar
NaCl	Sodium Chloride
aw	Water activity
NIH	National Institutes of Health
NIAID	National Institutes of Allergy and Infectious Disease
LPS	Lipopolysaccharides
IL	Interleukin
TNF	Tumor Necrosis Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
ISO	International Organization for Standardization
USFDA	United States Food and Drug Administration
RVS	Rappaport Vassiliadis Soy
MKTTn	Muller-Kauffmann Tetrathionate-Novobiocin
AOAC	Association of Analytical Communities
AFNOR	Association Française de Normalisation
EIA	Enzyme Imnunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
PFGE	Pulsed-Field Gel Electrophoresis
USDA	United States Department of Agriculture
FAO	Food and Agriculture Organization
WTO	World Trade Organization
PAHO	Pan American Health Organization
FSIS	Food Safety Inspection Service

HACCP	Hazard Analysis and Critical Control Points
KIA	Kligler's Iron Agar
H_2O_2	Hydrogen Peroxide
MDR	Multi Drug Resistant
PBS	Phosphate-Buffered Saline
EUCAST	European Committee on Antimicrobial Susceptibility Testing
qPCR	Quantitative Polymerase Chain Reaction
ICDDRB	International Center for Diarrhoeal Disease Research, Bangladesh
NST	National Science & Technology
CLSI	Clinical Laboratory Standard Institute

LIST OF SYMBOLS

%	Percentage
&	And
≤	Less than equal
≥	Greater than equal
°C	Degree Centigrade
°F	Degree Fahrenheit
μg	Microgram
μl	Microliter
g	Gram
M	Molar
ml	Milliliter
mM	Millimolar

Chapter
01
Introduction

CHAPTER 01: INTRODUCTION

1.1 Background:

Salmonella is gran-negative, rod-shape facultative anaerobe bacteria within the family Enterobacteriaceae, Salmonellosis is a result of *Salmonella* infection. (Acheson and Hohmann, 2001) Infection with *Salmonella* can result from the consumption of contaminated food and water. *Salmonella* can be the cause of nosocomial (hospital-linked) infections, and often, this strains found in hospitals is antibiotic resistant because of adaptations to widespread antibiotic use. (Davies and Davies, 2010) Many people are hospitalized each year after becoming infected, with some dying as a result.

Antimicrobial agents are currently used for three main reasons:(Swaminathan et al., 2006) to treat infections in humans, animals, and plants;(ANGULO et al., 2000) Prophylactically in humans, animals, and plants; and (Mathew et al., 2007) sub-therapeutically in food animals as growth promoters and for feed conversion (ANGULO et al., 2000). When antibiotic use became the norm in both human and animal medicine, selection pressure increased the bacterial advantage of maintaining and developing new resistance genes that could be shared among bacterial populations (Mathew et al., 2007).

The first suggestion that antibiotic use in livestock led to antibiotic resistant bacteria was in 1951. Starr and Reynolds reported streptomycin resistance in generic intestinal bacteria from turkeys that had been fed that antibiotic (Bauer-Garland et al., 2006). The use of antibiotics not only selects for antimicrobial resistant bacteria but may also increase the likelihood of disease transmission. In 2006, Bauer Garland et al. researched the transmission of multidrug resistant (MDR) *Salmonella typhimurium* in broiler chicks under selective pressure. An MDR *Salmonella typhimurium* strain had significantly increased transmission when chicks were treated with tetracycline, demonstrating that antimicrobial use influences transmission of antimicrobial resistant pathogens in poultry (Bauer-Garland et al., 2006).

The U.S. Centers for Disease Control and Prevention (CDC) estimates that antibiotic resistance is responsible for more than 2 million infections and 23,000 deaths each year in the United States, at a direct cost of $20 billion and additional productivity losses of $35 billion(CDC, 2013). In Europe, an estimated 25,000 deaths are attributable to antibiotic-resistant infections, costing €1.5 billion annually in direct and indirect costs (ECDC, 2009). Although reliable estimates of economic losses in the developing world are not available, it is estimated that 58,000 neonatal sepsis deaths are attributable to drug-resistant infections in India alone (Laxminarayan et al., 2013). Studies from Tanzania and Mozambique indicate that resistant infections result in increased mortality in neonates and children under five (Kayange et al., 2010)

1.2 Literature review

1.2.1 *Salmonella spp.:*

Salmonella is a genus of the rod-shaped gram-negative bacillus of the Enterobacteriaceae family. Which belonging in proteobacteria phylum and gammaproteobacteria class. The two species of *Salmonella*, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is the type species and is further divided into six sub species (Su and Chiu, 2007) that include over 2500 serovars. *S.enterica* sub species are found worldwide in all warm-blooded animal, and in the environment. S. *bongori* is restricted to cold-blooded animal particularly reptiles (Gerard J. Tortora, 2008).

In 1880 *Salmonella* was primarily observed by Karl Eberth and four years later Georg Theodor Gaffky was able to grow a pure culture of *Salmonella*. Finally, in 1885 scientist Theobald Smith discovered *Salmonella enterica* and named its head of the group Daniel Elmer Salmon in his honor (Eberth, 1880, Hardy, 1999). *Salmonella* cause for typhoid fever, paratyphoid fever, and food poisoning (salmonellosis) (Ryan KJ, 2004).

Genus	Species	Subspecies	Number
Salmonella	*enterica*	*enterica*	1547
		salamae	513
		arizonae	100
		diarizonae	341
		houtenae	73
		indica	13
	bongori		23

Table 1.1: Number of *Salmonella* species and subspecies (Guibourdenche et al., 2010)

1.2.2 *Salmonella* Serotypes

The first letter is a capital letter "*S*" followed by the serovar names of sub species *enterica* (e.g. *Typhimurium* or *Montevideo*). At the first citation of the serotype, the genus name is given followed by the word "serotype" or the abbreviation "ser." Followed by the serotype name. This project follows the abbreviated modern naming system, i.e. *S. Typhimurium* rather than a complete nomenclature *S. enterica,* subsp. *enterica* serovar *Typhimurium* (Brenner et al., 2000, Magistrali et al., 2008). The antigenic formulae are also used to name *Salmonella* serotypes. This designation

includes: (i) sub species designation (sub species I through VI), (ii) O (somatic) antigens followed by a colon, (iii) H (flagellar) antigens (phase 1) followed by a colon, and (iv) H antigens (phase 2, if present) i.e. *Salmonella* serotype IV 45:g,z51: (Cliver et al., 2011) . The nomenclature detailed above is internationally accepted based on recommendations of the WHO Collaborating Center (Cliver et al., 2011).

Serotype	Serogroup	Somatic antigen (O)	Flagella (H) antigens	
			Phase 1	Phase 2
S. Paratyphi A	A	1,2,12	A	(1,5)
S. Typhimurium	B	1,4, (5),12	I	1,2
S.Agona	B	4,12	f,g,s	-
S. Derby	B	1,4, (5),12	f,g	(1,2)
S. Typhi	D	9,12, (Vi)	C	1,2
S. Enteriditis	D	1,9,12	g,m	(1,7)

Table 1.2: Antigenic formulae of some *Salmonella* serotypes

1.2.3 *Salmonella*: A Foodborne Pathogen in Meat and Meat Product

Meat includes all the edible parts of slaughtered warm-blooded animals, fit for human consumption. Due to its chemical composition and to its intrinsic characteristics, fresh meat is a good substrate for microbial growth. For this reason, cooling after slaughter is a critical point because it determines the microbiological quality of the product and must occur as fast as possible (internal temperature = 7°C, within 24-30 hours following slaughter). The flesh of healthy and unstrained animals is devoid of microorganisms in depth; but due to stress before slaughter, disease, or weakness, microbial contamination can occur and is defined as endogenous: pathogens, in particular, starting from the intestine, spread into the blood due to the failing immune system, and reach the muscles, lymph nodes and internal organs. Among these microorganisms, there may also be *Salmonella*, if it is present in the intestinal content. On the other hand, the main microbial contamination occurs during the various stages of butchering and cutting, as well as in the following stages, such as the preparation one (minced meat, sausages,

kebabs, etc.) and processing (salami mixture), until the purchase and the preservation of meat products before the progress of zoonosis and foodborne diseases shows that the verification of *Salmonella* in intensive.(Giaccone et al., 2012)

1.2.4 *Salmonella* Infection

Salmonella enterica can cause four different clinical manifestations: gastroenteritis, bacteremia, enteric fever, and an asymptomatic carrier state (Ryan and Ray, 2004). It is more common in children under the age of 5, adults 20-30 years old, and patients 70 years or older (Ryan and Ray, 2004).

1.2.4.1 Gastroenteritis

Gastroenteritis or "food poisoning" is usually characterized by sudden nausea, vomiting, abdominal cramps, diarrhea, headache chills and fever up to 39 ºC(Ryan and Ray, 2004, Krauss, 2003, Brock, 2000). The symptoms can be mild to severe and may last between 57 days(Ryan and Ray, 2004, Krauss, 2003). The *Typhimurium* serotype is the most common cause of gastroenteritis and there are an estimated 1.3 billion cases and 3 million deaths annually (1.4 million cases and 600 deaths in the US alone) due to nontyphoidal *Salmonella* (Brock, 2000, Chimalizeni et al., 2010, Murray, 2007). In well-resourced countries with low levels of invasive complications, the mortality rate due to nontyphoidal *Salmonella* is lower than 1%(Murray, 2007); However, in developing countries, the mortality rate can be as high as 24% (Murray, 2007).

1.2.4.2 Bacteremia:

Bacteremia occurs in 3-10% of individuals infected with *Salmonella enterica* and certain serotypes (particularly serotype Choleraesuis) have higher mortality rates(Bronze, 2005, Woods et al., 2008). Immunosuppressed individuals and patients with comorbid medical conditions (e.g. HIV (AIDS), diabetes mellitus, malignancy, cirrhosis, chronic granulomatous disease, sickle cell disease, lymphoproliferative disease, or collagen vascular disease) have a higher risk of developing bacteremia due to a *Salmonella* infection(Ryan and Ray, 2004, Bronze, 2005). Bacteremia can cause septic shock; Endocarditis, especially in patients older than 50 or with heart conditions; Infection of the aorta, especially in patients with preexisting atherosclerotic disease; Liver, spleen, and biliary tract infections in patients with underlying structural abnormalities; Mesenteric lymphadenitis; Osteomyelitis in long bones and vertebrae; Urinary tract infection; pneumonia; Pulmonary abscess; Brain abscess; Subdural and

epidural empyema; meningitis; CNS infections (rarely) ; and death(Ryan and Ray, 2004, Bronze, 2005).

1.2.4.3 Enteric fever:

Also known as typhoid fever, this infection is caused by serotypes Typhi and Paratyphi (Ryan and Ray, 2004, Connor and Schwartz, 2005).Enteric fever: Also known as typhoid fever, this infection is caused by serotypes Typhi and Paratyphi(Ryan and Ray, 2004, Connor and Schwartz, 2005). Enteric fever is characterized by fever (rising within 72 hours after the onset of illness) and headache, bradycardia, faint rose-colored rash on the abdomen and chest, anorexia, abdominal pain, myalgias, malaise, diarrhea (more common in children) or constipation (more common in adults), hepatosplenomegaly, segmental ileus, meningismus, and neuropsychiatric manifestations (Ryan and Ray, 2004, Bronze, 2005). Less common symptoms are a sore throat, cough, and bloody diarrhea(Bronze, 2005). Complications include myocarditis, encephalopathy, intravascular coagulation, infections of the biliary tree and intestinal tract, urinary tract infection, and metastatic lesions in bone, joints, liver, and meninges(Ryan and Ray, 2004, Krauss, 2003). The most severe complication (occurs in about 3% of patients) is hemorrhage due to perforations of the terminal ileum of proximal colon walls(Ryan and Ray, 2004, Bronze, 2005). If untreated, the fever can last for weeks; However, with proper antimicrobial therapy, patients usually recover within 1014 days (Ryan and Ray, 2004). The disease is milder in children and, if treated, has a mortality rate of less than 1% (Krauss, 2003) Untreated cases can have a mortality rate greater than 10 % (Bronze, 2005, Murray, 2007).

1.2.4.4 Epidemiology:

Infections with *Salmonella enterica* occur worldwide; However, certain diseases are more prevalent in different regions. Nontyphoid salmonellosis is more common in industrialized countries whereas enteric fever is mostly found in developing countries (with the most cases in Asia)(Bronze, 2005, Connor and Schwartz, 2005). There are about 1.3 billion cases of nontyphoid salmonellosis worldwide each year and the WHO estimates that there are 17 million cases and over 500,000 deaths each year caused by typhoid fever(Murray, 2007, Bronze, 2005, Chimalizeni et al., 2010). There is a peak in disease in the summer and fall, and it is most common in children(Ryan and Ray, 2004, Brock, 2000, Chimalizeni et al., 2010). In the developing world, salmonellosis contributes to childhood diarrhea morbidity and mortality as bacteria are responsible

for about 20% of cases(Bronze, 2005). Epidemics of salmonellosis have been reported in institutions such as hospitals and nursing homes(Ryan and Ray, 2004).

1.2.4.5 Host Range:

For serotypes causing nontyphoidal salmonellosis, the primary hosts are domestic and wild animals such as cattle, swine, poultry, wild birds, and pets (particularly reptiles) as well as flies (Krauss, 2003, Greenberg et al., 1970).Humans are usually the final host (Krauss, 2003). For *Salmonella typhi*, humans are the only known host (Ryan and Ray, 2004, Krauss, 2003).

1.2.4.6 Infectious Dose:

The infectious dose varies with the serotype. For nontyphoidal salmonellosis, the infectious dose is approximately 10^3 bacilli (Ryan and Ray, 2004, Bronze, 2005). For enteric fever, the infectious dose is about 10^5 bacilli by ingestion (Ryan and Ray, 2004, Bronze, 2005) Patients with achlorhydria, depressed cell-mediated immunity, or who are elderly may become infected with at a lower infectious dose (Ryan and Ray, 2004, Bronze, 2005). The infectious dose may also be dependent on the level of acidity in the patient's stomach (Bronze, 2005).

1.2.4.7 Mode of Transmission:

Human infection usually occurs when consuming contaminated foods and water, contact with infected feces, as well as contact with infective animals, animal feed, or humans (Ryan and Ray, 2004, Krauss, 2003, Murray, 2007, Bronze, 2005). Foods that pose a higher risk include meat, poultry, milk products, and egg products (Krauss, 2003, Brock, 2000).In hospitals, the bacteria have been spread by personnel in pediatric wards, either on their hands or on inadequately disinfected scopes (Garrity et al., 2005) (Block, 2001). Flies can infect foods which can also be a risk for transmission to humans (GREENBERG, 1964, Ostrolenk and Welch, 1942).

1.2.4.8 Incubation Period:

For nontyphoidal salmonellosis, the incubation period is variable, depends on the inoculum size, and usually ranges between 5 and 72 hours(Krauss, 2003). For typhoid fever, the incubation period can be between 3 and 60 days, although most infections occur 7-14 days after contamination(Bronze, 2005). The incubation period for typhoid fever is highly variable and depends on inoculum size, host susceptibility, and the bacterial strain(Murray, 2007, Bronze, 2005)

1.2.4.9 Communicability:

Humans can spread the disease for as long as they shed the bacterium in their feces. Certain carriers shed the bacteria for years and 5 % of patients recovering from nontyphoidal salmonellosis can shed the bacteria for 20 weeks(Ryan and Ray, 2004). Animals can have a latent or carrier state where they excrete the organism briefly, intermittently or persistently(Bronze, 2005).

1.2.5 *Salmonella* identification

1.2.5.1 Characteristics of *Salmonella*

1.2.5.1.1 Morphology and isolation

Salmonella is 0.2 -1.5 x 2-5μm in size, Gram-negative, facultatively anaerobic, small rod-shaped motile bacteria (Cliver et al., 2011, Bell et al., 2002) belonging to family *Enterobacteriaceae*. Members of this genus are motile by peritrichous flagella, except, *Salmonella Pullorum* and *Salmonella Gallinarum*. *Salmonella* is chemoorganotrophic, with an ability to metabolize nutrients by both respiratory and fermentative pathways(Popoff and Le Minor, 2005). The temperature for growth ranges from 8°C to 45°C, strains can survive pH 4 to 9 and is able to grow at water activities above 0.94. *Salmonella* is heat labile so the organism can be inactivated at ordinary cooking temperatures (>70 °C) although the cooling time and values for temperature and time could change depending on the serotype and the food matrix. In addition, *Salmonella* has been shown to tolerate up to 20% salt concentration (Bell et al., 2002, Meneses, 2010). Under freezing conditions (from -23°C to -18°C) this microorganism is able to survive for as long as seven years (Bell et al., 2002).

Hydrogen sulfide is produced by most *Salmonella* but a few serovars like *Salmonella Paratyphi* A and *Salmonella Choleraesuis* do not produce H_2S. Most *Salmonella spp.* are aerogenic, however, *Salmonella Typhi* does not produce gas(Ziprin, 1994).

Most of the *Salmonella* do not ferment lactose and this property has been the basis for the development of numerous selective and differential media for the culture and presumptive identification of *Salmonella Spp.* (Rambach, 1990). Such media includes xylose lysine *deoxycholate* agar, *Salmonella-Shigella* agar, brilliant green agar, Hektoen enteric agar, MacConkey's agar, lysine iron agar and triple sugar iron agar (Anderson, 2001, Andrews et al., 2007).

Isolation of *Salmonella* from food and environmental samples with culture method utilizes the multiple steps of pre-enrichment and enrichment on the selective and differential media in order to increase the sensitivity of the detection assay (Andrews et al., 2007). Pre-enrichment is a process in which the sample is first cultured in a non-selective growth medium such as buffered peptone water or lactose broth with the intent of allowing the growth of any viable bacteria, and also useful in allowing recovery of injured cells. In the case of *Salmonella*, the next step of enrichment is usually achieved by culturing the pre-enriched samples in media containing inhibitors to restrict the growth of undesirable bacteria. Enrichment media commonly used to enrich *Salmonella* include the tetrathionate broth (Muller, 1923) and selenite cystine broth (Leifson, 1936).

More recently, selenite cystine broth has been replaced with Rappaport Vassiliadis broth(Andrews et al., 2007). The advantage of the Rappaport Vassiliadis medium is that it can be used as broth or semisolid medium. Following the enrichment period, the enriched cultures are spread onto a selective and differential agar plate, and then typical colonies for *Salmonella* has to be identified. Final confirmation of typical colonies is determined by a series of biochemical and serological tests. A total of 18 key biochemical reactions has been used in the identification and confirmation of *Salmonella* isolated from food or seafood (Andrews et al., 2007). A few *Salmonella* serovars do not exhibit the typical biochemical characteristics of the genus and these strains pose problem diagnostically because they may not easily be recovered on the commonly used differential media. About 1% of the *Salmonella* serovars submitted to the Centers for Disease Control (CDC) ferment lactose; hydrogen sulfide production too was quite variable(Ziprin, 1994). Most recently developed *Salmonella* chrom agar medium has been described very promising for detection of both lactose positive and lactose negative *Salmonella* isolates from food samples (Dick, 2005).

1.2.5.1.2 Physiology and Biochemical Characteristics

The biochemical properties of *Salmonella Spp*. show that almost all *Salmonella* serovars do not produce indole, hydrolyze urea, and de-aminate phenylalanine or tryptophan. Most of the serovars readily reduce nitrate to nitrite and most ferment a variety of carbohydrates with the production of acid, and reported to be negative for Voges-Proskauer (VP) reaction(Popoff and Le Minor, 2005). The other prominent characteristics of *Salmonella* are that most serovars produce hydrogen sulfide (H_2S)

and decarboxylate lysine, arginine, and orinithine with few exceptions (e. g. *Salmonella enterica* subsp. *arizonae* and *Salmonella enterica* subsp. *diarizonae*). Most of the *Salmonella* utilize citrate with a few exceptions such as *Salmonella Typhi, Salmonella Paratyphi* A, and a few *Salmonella Choleraesuis* serovars. Dulcitol is generally utilized by all serovars except *Salmonella enterica* subsp. *arizonae* (Illa) and *Salmonella enterica* subsp. *diarizonae* (lllb), whereas, lactose will not be utilized by most of the *Salmonella* serovars (Popoff and Le Minor, 2005). Though lactose may not be utilized by most of the *Salmonella* serovars, it has been reported that less than 1 % of all *Salmonella* ferment lactose (Ewing, 1986). Most commonly, lactose negative (lac$^-$) *Salmonella* serovars are isolated and identified from food including seafood, which is more prevalent in nature. Several factors are responsible for the lower detection of lactose positive (lac$^+$) *Salmonella* serovars in food or seafood. Lac$^+$ *Salmonella* serovars, which are sporadic in the presence and also tricky to identify as many of the Enterobacteriaceae look similar with Lac$^+$ *Salmonella* on selective media plates, hence escaped detection during analysis. Further, *Salmonella* isolation from different sources with routine selective and differential media utilizes non-lactose fermentation as a key biochemical property and most commonly used differential plating media for isolation of *Salmonella* contains lactose. Routine selective and differential media for *Salmonella* was not efficient enough to identify *Salmonella arizonae* (*Illa*) group (Littell, 1977). The natural habitat of the *Salmonella* sub species*; Salmonella enterica* subsp. *salamae* (II), subsp. *arizonae* (Illa), subsp. *diarizonae* (lllb), subsp. *houtenae* and subsp. *indica* (VI) is considered to be the cold-blooded animals and environments (Popoff and Le Minor, 2005) and a large number of *Salmonella* serovars in these sub species are lactose fermenting in nature. Thus, it is suspected that seafood being cold blooded animals may harbor naturally lac$^+$ *Salmonella* serovars and actual incidence of lac$^+$ *Salmonella* in seafood may be much higher than the reported incidences. Outbreaks of disease from lac+ *Salmonella* have been reported(Camara et al., 1988, Ruiz et al., 1995). In India, *Salmonella arizonae* (*Illa*) infection in infants and children(Mahajan et al., 2003). *Salmonella* are considered resilient microorganisms that readily adapt to extreme environmental conditions. *Salmonella* grows best at moderate temperature (35 -37°C), they can grow over a much wider temperature range, as low as 4°C (D'Aoust, 1991) and as high as 48 °C (Baird-Parker). Thermal stress mutants of *Salmonella Typhimurium* have been reported to grow at an elevated temperature of 54°C (Droffner

and Yamamoto, 1991) and some other serovars exhibited psychrotrophic properties by their ability to grow in foods stored at 2 to 4°C (D'Aoust, 1991). The physiological adaptability of *Salmonella Spp*. was demonstrated by their ability to proliferate at pH values ranging from 4.5 to 9.5 (CHUNG and Goepfert, 1970). Acid-adapted *Salmonella* survival is increased in fermented milk and refrigerated temperature (Leyer and Johnson, 1992). Further studies showed that brief exposure of *Salmonella Typhimurium* to mild acid environment of pH 5.5 to 6.0 followed by exposure of the adapted cells to pH 4.5 (acid shock) triggers a complex acid tolerance response (ATR) that potentiates the survival of the microorganism under extreme acid environment (Foster and Hall, 1991, Hickey and Hirshfield, 1990).

Another factor such as high salt concentration has long been recognized for their ability to extend the shelf-life of foods by inhibiting the growth of inherent microflora (Hickey and Hirshfield, 1990). Although *Salmonella Spp*. are generally inhibited in the presence of 3 to 4 % NaCl, bacterial salt tolerance increases with increasing temperature in the range of 10 to 30°C. The magnitude of this adaptive response was food and serovar specific(D'Aoust, 1989). A recent report on anaerobiosis and its potentiation of greater salt tolerance in *Salmonella* raises concerns regarding the safety of modified atmosphere and vacuum-packed foods that contain high levels of salts(Anon, 1986)

1.2.5.2 Serological Identification (Kauffman White scheme)

The scheme used worldwide for serological identification of *Salmonella* serovars was first proposed by White and expanded by Kauffman (Le Minor and Popoff, 1987). The list of 2,501 *Salmonella* serotypes is maintained and annually updated by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France(Helmuth, 2000). The Kauffman-White scheme (KW) is based on the antigenic structure of *Salmonella* serotypes (Helmuth, 2000). The antigenic properties and variations of the O (surface polysaccharide) and H (flagellar) antigens from each serovar are summarized and described in what is known as the antigenic formulae (Mortimer et al., 2004, Wattiau et al., 2008).

The structure of each microbial cell is dependent on a variety of antigenic molecules, which are at the time dependent of many determinant groups (chemical groups). Thus it is the chemical makeup and the arrangement of these determinant groups what assign the immunological specificity of the antigen(Guthrie, 1991). The cross absorption of

antisera is used to reveal the antigenic structure of *Salmonella (Helmuth, 2000)*. The composition and structure of polysaccharides, which constitute a part of the structure of the cell surface, allow for recognition and differentiation of O antigens(Guthrie, 1991). In the KW scheme O antigens are indicated in brackets when they are easily modified by mutation, otherwise, they are underlined when these factors are determined by bacteriophages or plasmids(Helmuth, 2000). H antigens are present in the flagella, they are 9 composed of protein subunits called flagella in that are typically biphasic and thought to help the bacteria to survive host immune responses (Helmuth, 2000). A capsular polysaccharide is found in some serovars (Typhi, Paratyphi C, and Dublin) is termed "The virulence (Vi) antigen". This factor first needs to be heated to 100 °C for 60 min to remove the capsule, otherwise, it would not be agglutinable with anti-O antiserum(Helmuth, 2000).

Serological typing of *Salmonella enterica* serovars requires, over 150 O and H antigens and more than 250 antisera (Cai et al., 2005, Wattiau et al., 2008). The problem with this conventional method is that it is laborious, time-consuming, and cannot differentiate within serovars (Nashwa et al., 2009). It also depends on the availability of hundreds of antisera, needs highly trained personnel, consumes high volumes of reagents, and a minimum of three days is required to identify a serotype (Alvarez et al., 2004, Cai et al., 2005, Yoshida et al., 2007).

1.2.5.3 Molecular identification methods

1.2.5.3.1 Conventional Polymerase chain reaction (PCR)

Nucleic acid (DNA or RNA) based methods have become very popular for rapid detection of foodborne pathogens. The first in-vitro amplification of mammalian genes using the Klenow fragment of Escherichia coli DNA polymerase was carried out by Kary Mullis. (Saiki et al., 1985, Mullis and Faloona, 1987) This assay is now popularly known as polymerase chain reaction (PCR). PCR assay has proven to be a most powerful molecular tool and revolutionized the entire molecular biology. PCR assay requires the target template DNA, primers, dNTPs, and Taq polymerase, and based on the repeated cycles of enzymatic amplification of small quantities of target DNA in a thermocycler provide more than billion copies(Tenover et al., 1997). The role of PCR is applied in the various field of food microbiology such as detection of microorganisms, detection of virulence genes and detection of genes responsible for

antimicrobials(Cohen et al., 1996, Malorny et al., 2003, Del Cerro et al., 2002). More recently, PCR methods are used in the typing of bacterial isolates in an epidemiological investigation. PCR based methods are more promising and found to be very sensitive for detection of foodborne pathogens including *Salmonella* in food(Del Cerro et al., 2002). Different PCR validation studies showed that PCR method is one of the most promising techniques for the rapid detection of *Salmonella Spp.* in food (MAKINO et al., 1999, Ferretti et al., 2001, Kumar et al., 2005). Several PCR based detection assays for rapid and specific detection of *Salmonella* in seafood has been developed and assays were compared with conventional method and reported PCR method was comparable to the culture method(Fach et al., 1999, Kumar et al., 2003, Vázquez-Novelle et al., 2005) demonstrated the samples positive by eight-hour PCR assay were also positive by standard microbiological method. However, PCR assay was reported to be far superior to the conventional culture methods for detection of *Salmonella* in meat samples (Fratamico, 2003, Oliveira et al., 2003) showed the 15 meat samples positive for *Salmonella* by culture method and 33 samples were found positive by PCR method, when a total of 87 field meat samples were analyzed for the presence of *Salmonella* by culture and PCR assay. The main disadvantage for the adoption of *Salmonella* PCR in naturally contaminated foods is difficulties in times of amplification of dead cell's DNA and the occasional inhibition for PCR assay by food matrix, thus, presenting a few false results in terms of sensitivity and specificity. More recently, RNA based techniques have been used in the detection of viable and nonculturable (VBNC) and live and dead cells. The amplification of mRNA by reverse transcription-PCR showed the ability to distinguish between living and dead Escherichia coli cells(Oliveira et al., 2003). Detection of *Salmonella Enteritidis* by RT-PCR was reported by Szabo and Mackey (1999).

1.2.5.3.2 Real-time/ quantitative Polymerase chain reaction (Q-PCR)

Quantitative microbial risk assessment (QMRA) is an important step for food safety in which risk factor that influences food safety are identified. This approach is very important when low numbers of foodborne bacterial cells are present in a food sample. Currently, nearly all quantitative data generated for *Salmonellas* were obtained from traditional bacteriological methods(Jensen et al., 2003, Blodgett, 2006). Quantitative culture based method is both cumbersome and time-consuming, thus limiting the usage in routine analysis. PCR based method has been standardized by ISO and now being

used for food testing (Malorny et al., 2003). More recently, a second generation PCR called real-time PCR is developed and it offered the possibility of estimating the number of bacteria in different samples. The quantitation in real-time PCR is not based on the end point signal but rather based on the exponential increase in the initial target DNA amount with the number of PCR cycles performed. In real-time PCR, serial dilution of a known number of target copies is used to set up a standard curve which is used to determine an unknown amount of DNA in a sample, hence, provides an absolute quantitative data of target sample(Fey et al., 2004). The specificity of the real-time PCR is confirmed by the melting temperature (Tm) analysis of the amplicon obtained, which shows the temperature at which 50% of DNA amplicon is denatured (Ririe et al., 1997).

The automation of DNA sample preparation method and availability of large real-time PCR formats are undoubtedly useful for generating a large amount of quantitative data at a high speed and low cost. Real-time PCR has been successfully used to detect *Salmonella* in clinical, food, and environmental samples (Levin, 2005, Josefsen et al., 2007). Apart from the quantitative detection, there are several advantages of real-time PCR over conventional PCR. Conventional PCR requires post-PCR gel electrophoresis analysis to confirm the presence of the target in the sample. In contrast, the real-time method is based on the increase in fluorescence, which indicates the presence of the target and is monitored during PCR assay, thus, no post PCR handling of the samples and reducing the risk of the false positive due to contamination in the laboratory. A rapid and quantitative real-time PCR for the detection of *Salmonella* in raw and ready-to-eat meat products and reported to detect 1 cfu/ml of food homogenate (Ellingson et al., 2004). More recently, several real-time PCR based assays have been developed and perfected for quantitative detection of *Salmonella* in meat or food (Hein et al., 2006, Josefsen et al., 2007).

1.2.5.3.3 *inv*A Gene-Based Detection

invA is the first gene of an operon containing three (*invA,B,C* and *D*) or possibly more genes arranged in the same transcriptional unit (Galan et al., 1992). The *invA*, *B*, and *C* genes are located in the same transcriptional unit, while the *invD* gene is located downstream in a different transcriptional unit; fragment of *invA*, a very conserved gene which presents almost all *Salmonella* serotypes(Boyd et al., 1996) Plasmids carrying *invA* were derived from pYA2220 (Galan and Curtiss, 1989)
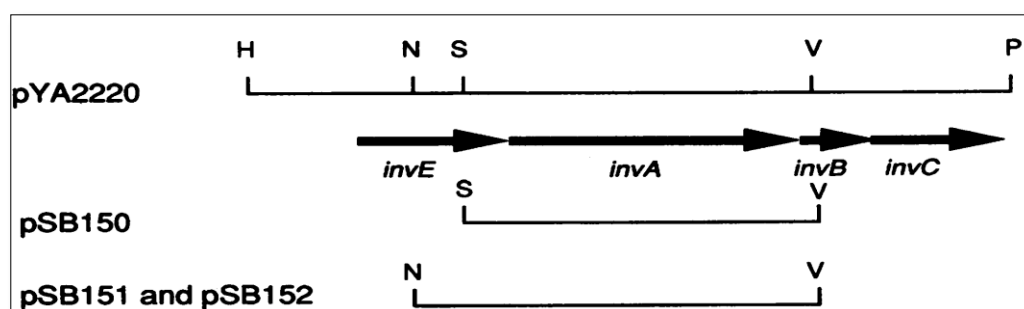
Figure 1.1: Restriction endonuclease map of the *inv* EABC region of *S. Typhimurium*. Positions of relevant restriction endonuclease sites are shown. The location and direction of transcription of the different inv genes are shown by the arrows. H, HindIII; N, NMuI; S, Sall; V, EcoRV; P, PstI.

Deletions for nucleotide sequencing were constructed in plasmid pSB002. This plasmid contains the HindIII-PstI fragment of pYA2220 cloned into the HindIII and PstI sites of pSKII. For expression of invA, plasmid pSB150 was constructed (Fig. 1). This plasmid carries a SalI-EcoRV fragment from pYA2220 containing invA plus 500 bp of nucleotide sequence upstream of the beginning of the invA open reading frame (ORF). This fragment was cloned into the SalI-EcoRV site of pSKII so that *invA* expression could be driven by the bacteriophage T7 promoter present in this plasmid vector.

1.2.6 *Salmonella*: Antimicrobial Resistance

Penicillin came to the light by Alexander Fleming in 1928 is a milestone in the history of medicine. As more antimicrobial compounds were discovered, it was predicted that infectious diseases would be eliminated through the use of these antimicrobials(Jorgensen and Turnidge, 2015). Unfortunately, the development of bacterial resistance to these antimicrobials quickly diminished this optimism and resulted in the need for physicians to request the microbiology lab to test a patient's

pathogen against various concentrations of a given antimicrobial to determine susceptibility or resistance to that drug. The original method of determining susceptibility to antimicrobials was based on broth dilution methods (Jorgensen and Turnidge, 2015, Kirby et al., 1955), which although still the gold standard today, is time-consuming to perform. This prompted the development of a disk diffusion procedure for the determination of susceptibility of bacteria to antimicrobials. By the early 1950s, most clinical microbiology laboratories in the United States had adopted the disk diffusion method for determining the susceptibility of bacteria to antimicrobials. Each lab modified the procedure to suit its own needs, which included using different types of media, inoculum concentration, incubation time, incubation temperature, and concentration of the antimicrobial compound. Interpretation of susceptibility and resistance was based only on the presence or absence of a zone of inhibition surrounding the disk (Bauer et al., 1959)

1.2.6.1 Mechanism of Antibiotic Resistance Process

Bacteria have become resistant to antimicrobials through a number of mechanisms (Mc Dermott et al., 2003):

- Permeability changes in the bacterial cell wall which restricts antimicrobial access to target sites
- Active efflux of the antibiotic from the microbial cell
- Enzymatic modification of the antibiotic
- Degradation of the antimicrobial agent
- Acquisition of alternative metabolic pathways to those inhibited by the drug
- Modification of antibiotic targets
- Overproduction of the target enzyme.

1.2.6.2 How Antibiotic Resistance Occur

1.2.6.2.1 Intrinsic Resistance

In some cases, a type of bacteria will survive antibiotic treatment and multiply because it is intrinsically resistant. For example, although many types of bacteria have cell walls, some don't. An antibiotic like penicillin that prevents cell-wall building can't harm a bacterium that doesn't build a cell wall in the first place. (Hawkey, 1998)

1.2.6.2.2 Acquired Resistance

Bacteria can also acquire resistance. This happens when a type of bacteria changes in a way that protects it from the antibiotic. Bacteria can acquire resistance in two ways: either through a new genetic change that helps the bacterium survive or by getting DNA from a bacterium that is already resistant. (Hawkey, 1998)

1.2.6.2.3 Genetic Change

DNA provides instructions to make proteins, so a change in DNA can cause a change in a protein. Sometimes this DNA change will affect the protein's shape. If this happens at the place on the protein where an antibiotic acts, the antibiotic may no longer be able to recognize where it needs to do its job. Changes like this can prevent an antibiotic from getting into the cell, or prevent the antibiotic from working once it's inside. Once a change occurs, it can spread in a population of bacteria through processes like reproduction or DNA transfer. (Blair et al., 2015)

1.2.6.2.4 DNA Transfer

Bacteria are very good at sharing genes, including genes for antibiotic resistance. They can share resistance genes that have been in the population, as well as new genetic changes that occur. Bacterium with an antibiotic resistance gene gives a copy of that gene to another bacterium. This process is called lateral gene transfer. (Blair et al., 2015). There are other ways bacteria can transfer DNA. Bacteria can get infected with a type of virus called a bacteriophage, as part of its life cycle, the bacteriophage packages DNA. When the bacterium dies, these packages of DNA (which sometimes include antibiotic resistance genes) are released and can be taken up and used by other bacteria. (Blair et al., 2015)

1.2.6.3 Spread of Antibiotic Resistance:

Figure: Overview Antibiotic resistance: Ecological relationships, selective pressures, main reservoirs, and routes of transmission. (Witte, 1998)
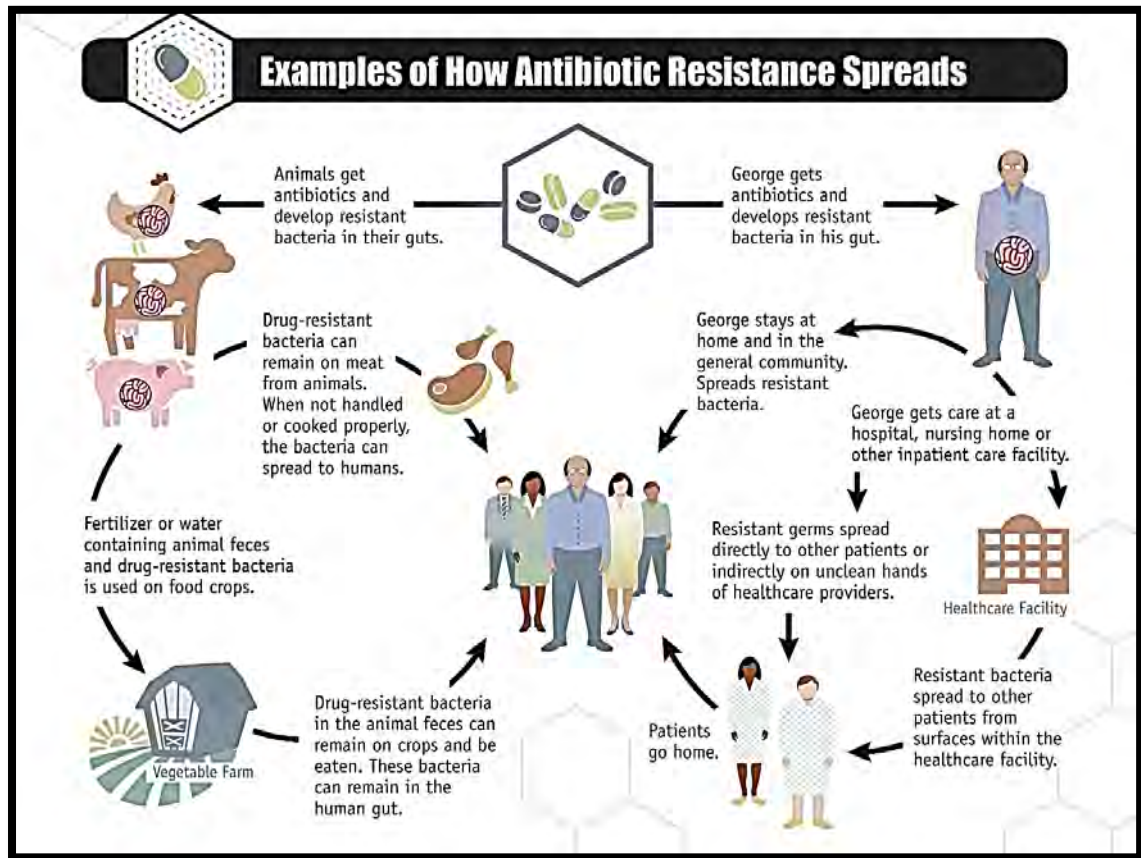
Figure 1.2: Possible antimicrobial resistance circulating process

1.2.6.4 Genotypic Resistance of Antibiotic

Antibiotic resistance gene was present in nature before the antibiotic invention (D'Costa et al., 2011). It was the first bacterium in which penicillin resistance was found in 1947, just four years after the drug started being mass-produced (Davies and Davies, 2010).

1.2.6.4.1 β-lactamase Mediated Resistance

The first plasmid-encoded β-lactamase that was able to destroy extended spectrum β-lactam antibiotics was described in Germany in 1983. It was related to the production of the variant of the *SHV-1* (sulfhydryl variable) enzyme, a broad-spectrum penicillinase found in *Klebsiella pneumonia (Cantón and Coque, 2006)*. *SHV-1* differed from *SHV-2* by replacement of a glycine with serine at the 238 positions.

This mutation alone accounted for the extended spectrum properties of *SHV*-2.*TEM*-1 was first reported in 1965 from a patient in Greece, named Temoneira. This report was followed by the description in France of variants of *TEM-1* and *TEM-2* enzymes with hydrolytic properties similar to *SHV-1* derivatives. *TEM-1* is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid. *TEM-2* has the same hydrolytic profile as *TEM-1*, but differs from *TEM-1* by having a more active native promoter and by a difference in isoelectric point (5.6 compared to 5.(Paterson and Bonomo, 2005). The *TEM* and *SHV* derivatives were named as an extended spectrum β-lactamases (ESBLs) in 1989 *(Cantón and Coque, 2006)*. According to the structural classification and the function scheme, these ESBLs are generally class A enzyme of the 2be group, arising as a result of a few amino acid substitutions, from the common *TEM* and *SHV-1* β-lactamases (Bonnet et al., 2000, Paterson and Bonomo, 2005). Changes at residue 164 are the most common changes observed in *TEM* variants. A reduction in the number of hydrogen bonds or the elimination of the electrostatic attraction weakens the linkage across the neck of the omega loop. This change allows more flexibility in the loop, which in turn opens more space for bulky β-lactam substituents, thus increasing resistance to these β-lactams (Knox, 1995).

More than 150 *TEM* and over 90 *SHV* enzymes have been documented. ESBLs hydrolyze oxyimino-cephalosporins but are inhibited by clavulanic acid, are inactive against cephamycins and are often encoded by large plasmids that carry resistance determinants to multiple antibiotics (Hopkins et al., 2008, Mhand et al., 1999).

There are two major concerns with pathogens producing ESBLs, i.e., their capacity to cause therapeutic failures with cephalosporins and aztreonam when the isolate is susceptible in vitro, and their capacity for undetected, widespread dissemination (Hanson et al., 2002). Although reports of ESBLs associated with *Salmonella Spp.* are not as many compared to those for other species in the family Enterobacteriaceae, the number of reported cases in this organism has been increasing (Mulvey et al., 2003).

ESBLs in *Salmonella* in Africa was first described in 1988 (Cardinale et al., 2001). *Salmonella* worldwide have been found to express a wide variety of ESBL- types including *TEM*, *SHV*, *CTX-M*, and *PER* enzymes. Additionally, *Salmonella* strains have been reported to produce plasmid-mediated *AmpC*-type β-lactamases, the *OXA*- type

class D (β-lactamase, and the plasmid-mediated Class A carbapenemase(Kruger et al., 2004, Miriagou et al., 2003). A nosocomial outbreak of *Salmonella* infection in pediatric patients caused by *Salmonella enterica* serovar Isangi producing ESBLs was first reported from the Chris Hani Baragwanath Hospital, Johannesburg South Africa, in 2006(Wadula et al., 2006). Fortunately, strains harboring metalloenzymes such as *VIM*-types or *IMP* types have not yet been reported for *Salmonella Spp.* In the early 1990s, nosocomial epidemics due to *TEM*-type ESBL-producing *Salmonella Spp.* Occurred in Algeria. During the period 1984 -1990 extended-spectrum cephalosporin resistant NTS producing *SHV*-type ESBLs were frequently isolated in pediatric units of Tunisian hospitals, while hospital outbreaks in Tunisia over the period 1995 -2001 were caused by *Salmonella* strains producing *SHV*-2a. *SHV*-12- producing isolates of a novel serotype was isolated from human and poultry specimens in Senegal. Production of *SHV*-and *TEM*-type ESBLs is evident in NTS strains isolated in various European countries. There have been sporadic isolations of *TEM*-3, *TEM*-25 and *SHV*-2 producing strains in French hospitals. In some of these cases, the index strains had probably been introduced by patients transferred from North African hospitals (Miriagou et al., 2004). An ESBL study of 160 *Salmonella Spp.* from 13 hospitals in South Africa conducted in 2004 reported that 15.6% of isolates produced *TEM* or *SHV* ESBLs (Kruger et al., 2004).

The *CTX*-M β-lactamases, a new family in class A ESBLs were characterized at the beginning of the 1990s with the first reports of the *CTXM*-1 enzyme from Germany (Bonnet et al., 2000). *CTX*-M enzymes share extensive sequence similarity with the chromosomal β-lactamases of *Klebsiella oxytoca*. They efficiently hydrolyze much newer broad-spectrum oxyimino- β-lactams including cefotaxime, ceftriaxone, and aztreonam and are readily inhibited by tazobactam and clavulanate (Tzouvelekis et al., 2000). There are over 70 *CTX*-M genes identified which are divided into five phylogenetic groups, (*CTX-M*-1, -2, -8, -9 and -25) based on their amino acid sequences. Ceftazidime hydrolyzing *CTX*-M-type β-lactamases such as *CTX-M-15*, *CTX-M-16*, and *CTX-M-19* was isolated in 2001 (Kimura et al., 2007). *CTX-M* type ESBLs display a level of resistance to cefotaxime and ceftriaxone significantly higher than to ceftazidime. The ceftazidime MICs for micro-organisms producing *CTX-M* type ESBLs are usually within the susceptible range. Therefore, the use of ceftazidime resistance as an indicator of ESBL production may miss ESBL producing bacteria in

the clinical microbiology laboratory(Rotimi et al., 2008). A number of *CTX-M* mutants with increased ceftazidimase activity have been described. The mutations in these variants occur in two of the structural elements that de-limit the β-lactam binding site, namely the terminal part of the B3 β-strand and the omega loop. The Asp 240 Gly substitution in the terminal part of the B3 β-strand is responsible for increased flexibility of the β-strand, rendering the active site more accessible to the bulkier ceftazidime molecule, while the substitutions in the omega loop (at position 167) apparently modify the mode of interaction of β-lactams with the binding site (Rossolini et al., 2008).

Most *CTX-M* β-lactamases reported in Africa (Kenya, Tanzania, Nigeria, Egypt) were from *K. pneumoniae* and *Escerichia coli* isolates. *CTX-M-3* was found in a *Salmonella* isolate from the military hospital in Tunisia in 2001 and *CTX-M-27* in isolates of S. *enterica* serotype Livingstone was the cause of a nosocomial outbreak in a neonatal ward in Tunisia in 2002 (Bouallègue-Godet et al., 2005). *CTX-M* enzymes are now endemic in many countries with both nosocomial and community emergence and some ESBL studies have identified *CTX-M* enzymes as the most prevalent ESBL. The epidemiology of *CTX-M*-producing strains is quite complex. Outbreaks of *CTX-M* clonal strains have been reported throughout the world(Touati et al., 2007).

PER-1, 2, and 3 comprise a highly clavulanate-sensitive family of ESBLs, with a different epidemiology from the *TEM* and *SHV* ESBLs. *PER*-1 was first identified in 1991 in a *Pseudomonas aeruginosa* isolated from a Turkish patient. *PER*-1 producing *Salmonella Typhimurium* strains were isolated from fatal nosocomial cases in 1992 at two hospitals in Istanbul (Vahaboglu et al., 1996). *PER*-1 has also been detected in France, Italy, Belgium, and Korea in *P. aeruginosa* and *Acinetobacter* spp. isolates (Paterson and Bonomo, 2005). *PER*-2 was first detected in *Salmonella Typhimurium* in Argentina and is now reported to be the second most prevalent ESBL in that country. Recently *PER*-3 was discovered in an isolate of *Aeromonas punctata* in France (Moland et al., 2008).

The presence of *AmpC* β-lactamases in pathogens known not to have chromosomal *ampC* genes (like *Salmonella)* eventually led to the discovery of plasmid-borne *AmpC* enzymes such as *ACT*, *ACC*, *DHA* and *CMY* (Babic et al., 2006). *CMY* (derived from *Citrobacter Freundii) DHA* (derived from *Morganella morganii)* and *ACC*-1 (derived

from *Hafnia alvei)* have been found in *Salmonella Spp.* (Miriagou et al., 2004). *CMY*-2 is the most prevalent of the plasmid-mediated

AmpC enzymes and the most widely distributed geographically. *CMY*-type β-lactamases found in nosocomial enterobacteria, particularly in *K. pneumoniae,* and *Salmonella Spp.* could have acquired the *ampC* gene from such microorganisms (Miriagou et al., 2002). *CMY*-2 confers resistance to various extended-spectrum cephalosporins, including ceftriaxone, which is the antibiotic of choice for invasive *Salmonella* infections in children. The movement of the *ampC* gene on to plasmids and transmission to other organisms is of major concern (Hanson et al., 2002). The expression of a plasmid-mediated *CMY*-2 β-lactamase has been responsible for most ceftriaxone resistance in *Salmonella Spp.* (Li et al., 2005). In Africa, the first report of the *CMY*-2 gene in *Salmonella* was from an Algerian clinical isolate of *S. enterica* serotype Senftenberg in 1997 (Koeck et al., 1997), and *CMY*-2 gene was reported in *Salmonella Typhimurium* and *S. enterica* serotype Schwarzengrund from South Africa (Kruger et al., 2004)

Many organisms producing class C β-lactamases may not be resistant to broad-spectrum cephalosporins when conventional Clinical laboratory standard institute breakpoints are used. Yet, adverse clinical outcomes in patients with infections caused by organisms producing plasmid-mediated class C β-lactamases have been reported when these patients were treated with cephalosporins. It is imperative that *Salmonella Spp.* producing plasmid-mediated class C β-lactamases are detected and reported so that appropriate antimicrobial therapy and infection control measures can be initiated (Doi and Paterson, 2007). *KPC*, *SME*, *NMC*-A, and *IMI* comprise a small group of class A β-lactamases (functional group 2f) with potent carbapenemase activities (Miriagou et al., 2003). *KPCs* are capable of hydrolyzing carbapenems, cephalosporins, and aztreonam, and they are inhibited by clavulanic acid and tazobactam (Cai et al., 2008). The only carbapenemase reported in *Salmonella Spp.* is of the *KPC* type. *KPC* producing *K. pneumoniae* strains have been found in hospitals in the USA and subsequently, *KPC*-2 was found in a *Salmonella* serotype *Cubana* isolate also in a hospital in the USA. The emergence of *Salmonella* and *K. pneumoniae* strains producing plasmid-mediated *KPC*-type β-lactamases in the USA further underlines the potential for exchange of resistance determinants between salmonellae and nosocomial enterobacteria (Miriagou et al., 2004). The *KPC*-type reportedly confers resistance to

all β-lactams with MICs of imipenem and meropenem reported as 16 and 8 mg/L respectively. These antibiotics are often the last therapeutic option used in cases of systemic infections in children due to ESBL-producing *Salmonella (Arlet et al., 2006)*. Other examples of non *TEM*, non *SHV* ESBLs such as *GES*, *BES*, *SFO*, *TLA*, *IBC*, and *VEB*-1 have been described(Paterson and Bonomo, 2005) but not reported in *Salmonella Spp.*

Historically the first characterized class D β-lactamases were also referred to as oxacillinases because they commonly hydrolyze the isoxazolyl penicillin, oxacillin much faster than classical penicillins, i.e. benzylpenicillin. The designation *OXA* of the class D (β-lactamases, thus, refers to their preferred penicillin substrate. Most *OXA*-type β-lactamases do not hydrolyze the extended-spectrum cephalosporins to a significant degree and are not regarded as ESBLs (Paterson and Bonomo, 2005). The first identified isolate expressing an *OXA*-type carbapenemase was the *OXA*-23 producing *A. baumannii*from Scotland. The isolate was recovered in 1985, before or at the time when imipenem was approved for general use. There have not been many reports of the *OXA* β-lactamase in *Salmonella Spp.* and the first, *OXA*-30, β-lactamase was reported from an Australian pediatric *Salmonella to* isolate in 2002. The substrate specificities of the *OXA*-type carbapenemases are diverse, but generally, the enzymes hydrolyze penicillins (benzylpenicillin, ampicillin, piperacillin, and ticarcillin) and the narrow spectrum cephalosporins, cephalothin and cephaloridine efficiently, while the extended-spectrum β-lactams, ceftazidime cefotaxime, and aztreonam are not or very poorly hydrolyzed. Most of the *OXA*-type carbapenemases have low hydrolytic activities against imipenem and especially against meropenem. Generally, class D (β-lactamases are inhibited less efficiently by clavulanate than the majority of the other group 2 (β-lactamases to which the class D enzymes belong. All *OXA*-type carbapenemases are inhibited more efficiently by tazobactam than by clavulanate. Most of the *OXA*-type carbapenemases confer only reduced susceptibility to the carbapenems, but unless secondary resistance mechanisms, such as altered permeability, reduced affinity of PBPs for carbapenems or increased influx are involved, the clinical detection of organisms producing these enzymes remains difficult. The chromosomal location of many of the *OXA*-type carbapenemase encoding genes has contributed to the slow spread of these genes(Walther-Rasmussen and Høiby, 2006).

1.2.6.4.2 Quinolone Resistance

The quinolones target bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, which play important roles in DNA replication, chromosome segregation, and DNA compaction. DNA gyrase is composed of two *GyrA* and two *GyrB* subunits and Topoisomerase IV are composed of two ParC and two ParE subunits (Okumura et al., 2008). *Qnr*-type plasmid-mediated quinolone resistance determinants belong to the pentapeptide-repeat family of proteins and protect DNA gyrase from quinolone inhibition. Three major groups of *Qnr* determinants, *Qnr* A, *Qnr* B, and *QnrS* have been identified worldwide in various members of the family Enterobacteriaceae(Wu et al., 2008). In *Salmonella Spp.* as in other Enterobacteriaceae, a single point mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene can mediate resistance to nalidixic acid and reduced susceptibility to fluoroquinolones such as ciprofloxacin. The most frequent point mutations in *Salmonella Spp.* associated with resistance to quinolones occur in the *gyrA* gene resulting in substitutions at the Ser-83 position, often to Tyr, Phe, or Ala, and Asp-87 substitutions to Asn, Gly or Tyr. Substitutions in ParC are not as frequent as those found in *GyrA*. Changes in *GyrB* and ParE are rarely found in *Salmonella Spp.* Although target gene mutations and efflux pumps are two mechanisms most commonly associated with fluoroquinolone resistance in bacteria, the additive or synergistic contribution of the two mechanisms in emerging fluoroquinolone resistance is not clear in *Salmonella Spp.* There is evidence of strains with no mutation in the QRDR, but with a lack of the OmpF porin, which showed decreased susceptibility to fluoroquinolones (Fierer and Guiney, 2001). Resistance to nalidixic acid has been suggested to be an indicator of low-level fluoroquinolone resistance (Rodriguez-Avial et al., 2005). Although resistance to fluoroquinolones remains rare in *Salmonella spp*, reduced susceptibility is increasing worldwide and it has been suggested that fluoroquinolone-susceptible strains that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extraintestinal salmonellosis (Cattoir et al., 2007, Wayne, 2007). Detection of *Salmonella Spp.* isolates showing decreased susceptibility to fluoroquinolones has become important as a result of the increasing prevalence of these strains and their association with treatment failure (Aznar et al., 2007). The increasing quinolone resistance in *Salmonella Spp.* may have serious clinical consequences. Although antimicrobial treatment is commonly not needed in

gastroenteritis caused by NTS, effective therapy is necessary for invasive infection. If such an infection is caused by a *Salmonella* strain with reduced fluoroquinolone susceptibility, treatment with a fluoroquinolone may not be a safe alternative (Hakanen et al., 2006). A better understanding of the biology and epidemiology of resistant *Salmonella* isolates is needed to combat the emergence and spread, and to determine appropriate empirical therapy of infections caused by these organisms (Hanson et al., 2002).

1.3 Objectives of the study

In the past few years, there has been a significant level of multidrug resistance *Salmonella spp*. infection increases, including invasive inflation throughout the world with significant impact on public health and remedial cost (Koshi, 1981). In Bangladesh, Non-typhoid *Salmonella* infections rate is higher than typhoidal *Salmonella* (Rahman et al., 2001). Contaminated retail meat is a major medium of *Salmonella* spp. infection (Antunes et al., 2016, Yang et al., 2010).Recent information on the prevalence of difference serogroups and predominant serotype of *Salmonella* species in retail meat sample is not available in Bangladesh. Besides, the emerging problem of antimicrobial resistance among *Salmonella* isolates in Bangladesh is not well documented

1.3.1 Specific objectives of this study are:

- To isolate *Salmonella spp*. from retail meat.
- Confirmation of isolated *Salmonella s pp*. up to genus level by PCR amplification.
- Determination of the prevalence of typhoidal and non-typhoidal *Salmonella* species in retail meat.
- Phenotypic and Genotypic antibiotic resentence in retail meat sample.
- Correlation between phenotypic and genotypic antimicrobial data.

Chapter
02
Method & Materials

CHAPTER 02: METHOD AND MATERIALS

2.1 Outline of the Research Work

The working strategy for isolation, identification and antibiotic resistance pattern of

Salmonella in meat samples from a retail shop in Dhaka city live animal markets is as

follows:

```
Collection of sample          Identify bacteria by          Identified typhoidal
and enclose into the          using conventional            and nontyphoidal
sterial plastic               PCR /RT PCR                   Salmonella by
container                                                   selective Primer

Enrichment by non-            Specify the isolated          Determine the drug
selective                     bacteria by various           resistance pattern
broth(BPW)                    biochemical test              by selective
                                                            Antibiotics

Selactive                     Isolation of                  Primer based
enrichment by                 salmonella by                 antibiotic resistance
selective broth (RV)          selective Agar                gene detection
                              media (XLD agar
                              ,SS agar)
```

Figure 2.1: Experimental design of the research work.

2.2 Sample collection area:

The meat samples were collected from 14 different live animal market in Dhaka city. According to population and socioeconomic aspect market was selected for sample collection. Source of sample was selected according to meat consumption by costumer and availability in market.
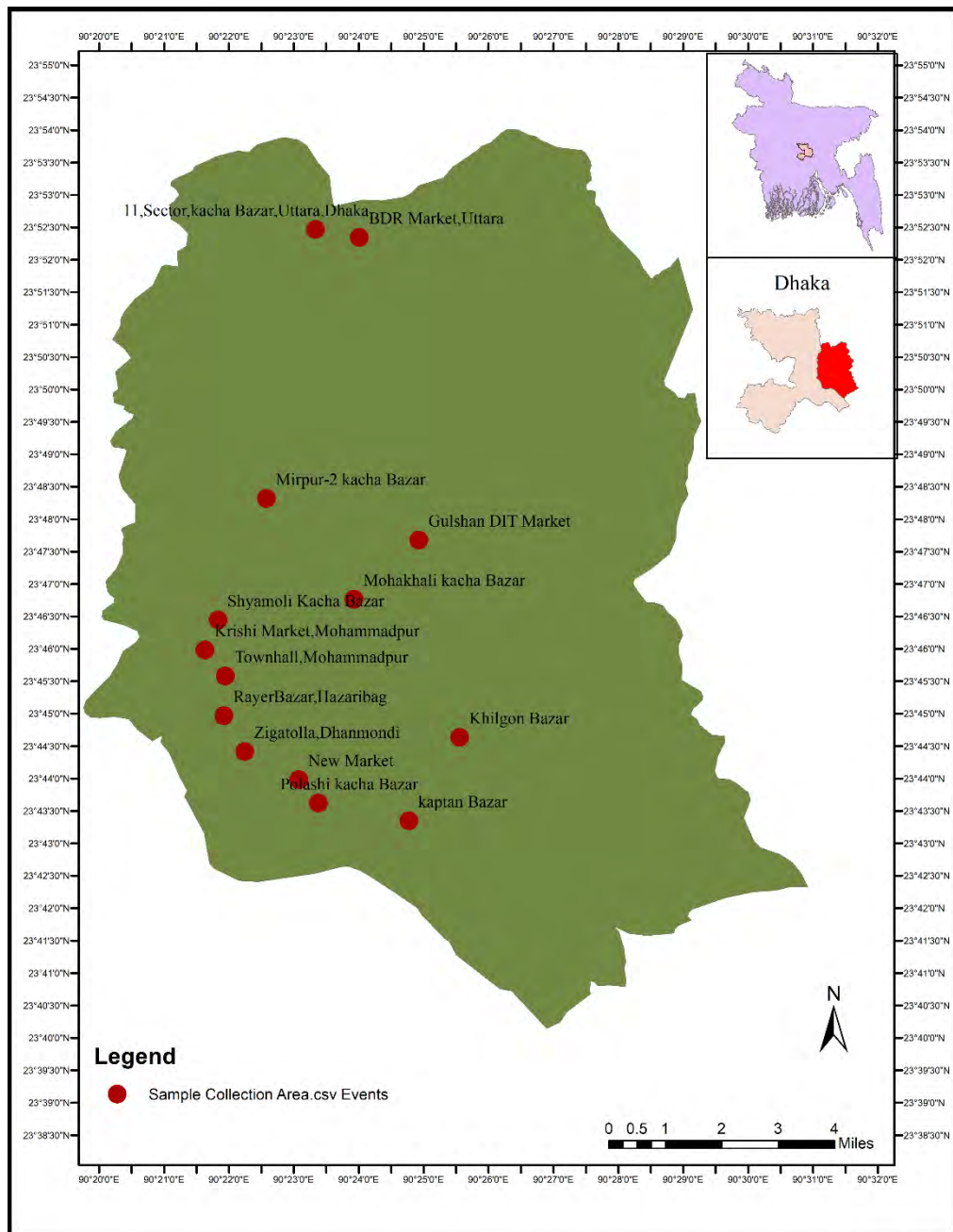
Figure 2.2: Map of sample collection area; This is Dhaka City map and red marks indicated the sample was collected from that place Live animal market (11-Sector, kacha Bazar,Uttara; BDR Market,Uttara;Mirpur-2 kacha bazar;mohakhali kacha bazar,shaymoli kacha bazar;krishi market,mohammadpur;townhall,mohammadpur;rayer bazar,hazaribag;zigatolla,dhanmondi; polashi bazar;new market;kaptan bazar;gulshan DIT market, khilgon bazar.

2.4 Collection of samples:

The source of the sample was selected from the Live Animal Market on basis of consumer demand. During slaughtering operation approximately (30 to 50) g meat sample was collected and put into a sterile container with Phosphate buffer saline

Figure 2.3: Live bird market sample collection picture

The sample was collected from a live Animal market at retail condition. Selection of sample and collection of the sample according to the questioner (Appendix I).

2.5 Sample transport

Meat samples were taken the different market from multiple places then put into a sterile plastic container in an ice box and maintain 2ºC to 4ºC temperature, within 2 to 4 hours' sample was taken in Bangladesh Livestock Research Institute (BLRI) at Food and Feed Safety Laboratory.

2.6 Detection of *Salmonella Spp.* (Presumptive)

2.6.1 Overview

Meat sample

Inoculam preparation

Culuter with nonselective enrichment media (1:10)
e.g:Buffered peptone water

Culture wit selective enrichment media
e.g: Rappaport Vassiliadis broth

Isolation of salmonella with the help of selective Agar Media
e.g:XLD,BSA,BG,SS Agar

Pick single colony and grow over night into nutrient broth;store into
20% Glyserol at -20ºC and -80ºC temperature

Biochemical test :indole test ,MR test,VP test,Motility test,Suger
ferantation test,oxidase test and Tripple Suger Iron Test is perform

Figure 2.4**:** Detection of *Salmonella* by nutrient media

2.6.2 Preparation of Samples (Inoculum)

Meat Sample was taken out from the plastic container and approximately 25 grams meat homogenized by sterilized mortar and pestle with Buffer Peptone Water (BPW). Finally, sample was inoculated into nonselective media (BPW) for overnight culture.

2.6.3 Isolation of *Salmonella spp.*:

2.6.3.1 Culture in Buffer Peptone Water (BPW)

Buffer peptone Water media were prepared by suspending 10 grams Buffer Peptone Water (BPW) powder in 500ml distilled water in a bicker and Sterilize by autoclaving at 121°C for 15 minutes.

9 ml Buffer peptone water was taken into a sterile test tube and sample inoculated into the media as 1: 10 ratio (1 ml homogenize sample + 9 ml BPW). Sample containing test tube was kept into test tube rack and placed in the incubator at 35±2ºC for 18 to 24 hours.

2.6.3.2 Selective pre-enrichment in *Rappaport Vassiliadis* (RV) Broth

Suspend 27.11 grams of dehydrated medium (*Rappaport Vassiliadis*) in 1000 ml purified/ distilled water in a round bottom flask and Sterilize by autoclaving at 121°C for 15 minutes.

4.5 ml Rappaport Vassiliadis was taken into a sterile test tube and 0.5 ml nonselective enrich sample inoculated into the media. Sample containing test tube was kept into test tube rack and placed to the incubator at 35±2ºC for 18 to 24 hours.

2.6.3.3 Inoculum preparation for plate

Before stick into pate selective inoculum is prepared with the help of serial dilution method. The samples were first cultured in the nonselective media such as nutrient Agar for the total bacterial count than these samples were subculture into the selective media for identification of the bacteria by their colony morphology. Again the samples were directly cultured to the selective media for enumeration of the total identified bacteria.

2.6.3.4 Culture in Nonselective Media: Nutrient Agar

Nutrient Agar media were prepared by suspending 14gm nutrient Agar in 500ml distilled water in a bicker and boiled to dissolve completely. The media and some Petridis were sterilized by autoclaving at 121ºC for 20 minutes at 15lbs.

15-20 ml Nutrient Agar media was poured into sterile 90 mm Petri plates and allowed to cool until usable. 20µl Selective bacterial inoculum (10^1-10^5) was poured into NA media and spread with the help of glass beads and placed in the incubator at 35±2ºC for 18 to 24 hours. Then the bacterial count was performed.

2.6.3.4 Culture in Selective media: *Xylose lysine deoxycholate (XLD) agar*

Suspend 53 grams XLD Agar powder in 1 liter of distilled water. Heat with frequent agitation until the medium boils. Overheat or Autoclave is forbidden by the manufacturer. Transfer immediately to a water bath at 50°C. 15-20 ml XLD Agar media was poured into sterile 90 mm Petri plates and allowed to cool until useable, then a loop full selective sample was taken and stick on Petri dish and placed to the incubator at 35±2ºC for 18 to 24 hours.

2.6.3.5 Culture in Selective media: Brilliant Green Agar (BGA)

Suspend 58.09 grams of dehydrated medium (Brilliant Green Agar) in 1000 ml purified/ distilled water in a round bottom flask and Sterilize by autoclaving at 121°C for 15 minutes, Cool to 45-50°C. 15-20 ml Brilliant Green Agar media was poured into sterile 90 mm Petri plates and allowed to cool until useable, then a loop full selective sample was taken and stick on Petri dish and placed to the incubator at 35±2ºC for 18 to 24 hours.

2.6.3.6 Culture in Selective media: *Salmonella-Shigella (SS) Agar*

Suspend 57.02 grams SS Agar powder in 1 liter of distilled water. Heat with frequent agitation until the medium boils. Overheat or Autoclave is forbidden by the manufacturer. Transfer immediately to a water bath at 50°C. 15-20 ml SS Agar media was poured into sterile 90 mm Petri plates and allowed to cool until useable, then a loop full selective sample was taken and stick on Petri dish and placed to the incubator at 35±2ºC for 18 to 24 hours

2.7 Screening for *Salmonella*

2.7.1 Gram Staining:

Gram stain was also carried out so as to confirm morphological character of *Salmonella* organism. This method was described by Merchant and Packer (1967).

- Smears containing thin and thick areas from the suspect's plates were prepared on clean glass slides, air-dried and fixed by quickly passing the slide three times through flame.
- Place slide with heat fixed smear on staining tray.
- Gently flood smear with crystal violet and let stand for 1 minute.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
- Gently flood the smear with Gram's iodine and let stand for 1 minute.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
- Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
- Immediately rinse with water.
- Gently flood with safranin to counter-stain and let stand for 45 seconds.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
- Blot dry the slide with bibulous paper.
- View the smear using a light-microscope under oil-immersion at 100x projection.

2.7.2 Biochemical Test:

2.7.2.1 Sugar Fermentation Test

Sugar media consisted of peptone water to which fermentable sugar was added to the proportion of 1 percent. Peptone water was prepared by adding one gram of peptone and 0.5 grams of sodium chloride in 100 ml of distilled water. The medium was boiled for 5 minutes, adjusted to pH 7.0, cooled and then filtered through filter paper. Phenol red, an indicator at the strength of 0.2% solution was added to peptone water and then dispensed in 5 ml amount into cotton plugged test tubes containing a Durham's

fermentation tubes, placed inversely. These were then sterilized in the autoclave at 121º C for 15 minutes maintaining a pressure of 15 lbs. per sq. inch (1kg/ cm2). The sugars used for fermentation were prepared separately as 10% solutions in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A gentle heat was necessary to dissolve the sugar completely and sterilized by stem sterilizer. Before use, the sterility of the sugar medium was judged by incubating the tubes overnight at 37ºC. The basic sugars dextrose, maltose, lactose, and dulcitol were used to prepare sugar medium. The carbohydrate fermentation test was performed by inoculating a loop full of thick test bacterial culture into the individual tubes containing sugars like dextrose, maltose, lactose, dulcitol and incubated at 37º C for 24 hours. Acid production was indicated by the change of media from pink to yellow color while gas production was indicated by the appearance of gas bubbles in the inverted Durham's fermentation tubes.

2.7.2.2 Methyl-Red and Voges-Proskauer (MR-VP) Test

A quantity of 3.4 gm of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the tubes were autoclaved at 121ºC for 15 minutes maintaining a pressure of 15 lbs per sq. inch (1 kg/ cm2). After autoclaving, the tubes containing medium were incubated at 37º C for overnight to check their sterility and then stored in a refrigerator for future use.

2.7.2.2.1 MR Test

The indicator methyl-red (MR) solution was prepared by adding 0.1gm of Methyl–red powder in 300 ml of 95% alcohol and diluting this to 500 ml with the adding of 200 ml of distilled water. The test was performed by inoculating a colony of the test organism in 5 ml sterile glucose phosphate broth. After overnight incubation at 37ºC, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of bright red color indicated acidity while a yellow or orange color was considered as negative.

2.7.2.2.2 VP Test

2.7.2.2.2.1 Preparation of Alpha-naphthol Solution

Alpha-naphthol solution was prepared by dissolving 5 grams of 1-naphthol in 100 ml of 95% ethyl alcohol.

2.7.2.2.2.2 Preparation of Potassium Hydroxide Solution

Potassium hydroxide (KOH) solution was prepared by dissolving 40 grams potassium hydroxide crystals in 100 ml of cold distilled water. All chemical and reagents were prepared according to the instruction of the manufacturer. (Hi-media, India)

2.7.2.2.2.3 Procedure

2ml of sterile glucose phosphate peptone broth was inoculated with a pure colony of test organisms and incubated at 37ºC for 24 hours. A very small amount (knife point) of creatine was added and mixed and 3 ml of sodium hydroxide were added and shaken well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink color for positive cases.

2.7.2.3 Indole test

Two ml of peptone water was inoculated with a pure colony of bacterial culture under observation and incubated at 37^0C for 24 hours after which 0.5 ml Kovac's reagent was added, shake well and examined after 1 minute. A red color in the reagent indicated a positive test.

2.7.2.4 Motility Test (Hanging Drop method)

Hanging drop preparation is a special type of wet mount (in which a drop of medium containing the organisms is placed on a microscope slide), in this method, a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and the petroleum jelly forms a seal that prevents evaporation. This preparation gives good views of microbial motility. At fast a clean glass slide, a cover slide, petroleum jelly, and bacterial culture were taken. Then a loop full bacterial culture was placed on a cover slide, 4 drops of petroleum were put on 4 edges of the cover slide. A glass slide was attached with cover slide carefully and flipped the slide which produces hanging drop of bacterial culture. The preparation was placed under the microscope and focus the edge of the drop carefully and look at each side of that line for very small objects that are the bacteria. The cells will look either like dark or slightly greenish, very small rods or spheres.

2.7.2.5 Triple Sugar Iron Agar (TSI) Test

65 grams of Triple Sugar Iron Agar were suspended in 1 liter of distilled water. Media was boiled to dissolve and 5 ml distributed into a test tube. After distribution, it was sterilized by autoclaving at 121°C for 15 minutes. A sterile straight inoculation was needle touched the top of a well-isolated colony, then Inoculated TSI Agar by first stabbing through the center of the medium to the bottom of the tube and then streaking

on the surface of the agar slant. Then a cotton plug was applied open of the test tube and incubated the tube at 35°C in ambient air for 18 to 24 hours.

2.7.2.6 Oxidase test

The oxidase test is designed to distinguish among groups of bacteria on the basis of cytochrome oxidases activity. Cytochrome oxidases catalyze the oxidation of a reduced cytochrome by molecular oxygen (O_2), resulting in the formation of H_2O, or H_2O_2. 24 hours fresh culture of a test organism on nutrient agar media, p-amino dimethylaniline oxalate, filter paper, inoculating loop, Bunsen burner, glassware marking pencil. One drop of oxidase test reagent was added onto a filter paper. Using an inoculating loop aseptically transfer a large mass of pure bacteria to the filter paper. The site of inoculation was observed for up to 10-30 seconds. If the area of inoculation turns pink to maroon to almost black, then the result is positive. If a color change does not occur within three minutes, the result is negative.

2.7.2.7 Catalase Test

The purpose of the test is to determine the ability of some microorganisms to degrade hydrogen peroxide (H_2O_2) by producing enzyme catalase. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. This reaction is evident by the rapid formation of bubbles.

24 hours fresh culture of a test organism on nutrient agar media, 3% hydrogen peroxide, microscopic slide, inoculating loop, bunsen burner, glassware marking pencil. A few drops of H_2O_2 was added onto a clean microscopic slide. Then a small amount of growth from bacterial culture was smeared into a drop of H_2O_2. If needed, the smear was mixed with a toothpick. A positive result is the rapid evolution of O_2 as evidenced by bubbling. A negative result is no bubbles or only a few scattered bubbles.

2.8 Preservation and Storage of Isolated *Salmonella*

2.8.1 Using Nutrient Broth:

The Nutrient Broth media were prepared by suspending 6.5 gm. the nutrient broth in 500ml distilled water. The media were heated to dissolve completely. The media were sterilized by autoclaving at 121ºC for 20 minutes at 15lbs pressure. 5ml broth poured into a serial test tube, a single colony was picked from selective medium and inoculated into a nutrient broth, then placed to the incubator at 35±2ºC for 18 to 24 hours.

The sample was stored at 20ºC and 80ºC by adding 20% of glycerol (800µl *Salmonella* overnight broth culture and 200µl glycerol) and mix properly with the help of a vortex machine.

2.9 DNA Extraction

Molecular techniques in bacteriology usually start with bacterial DNA extraction and purification. A large number of DNA extraction methods (performed manually or by automation) have been and are still being developed, each of which has its own advantages and disadvantages.

2.9.1 Procedure:

Salmonella Species DNA Extraction was performed manually by heat method

1. Biochemically positive pure bacterial samples were subcultured in Nutrient broth (NB) (Oxoid, England) overnight at 37°C.
2. The overnight grown culture was dispensed in 1 ml volumes in sterile vials,
3. The turbidity of bacterial suspension was adjusted approximately to 1McFarland which equal approximately to 3×10^8 CFU/ml.
4. The bacterial suspensions were centrifuged at 13,000rpm for 15 min,
5. The supernatants were discarded and the pellets were washed three times using phosphate buffered saline (PBS),
6. Suspension of the pellet into 150µl of Nuclease-free water (Qiagen GmbH, Hilden, Germany).
7. The bacterial suspension was subjected to boil at 100°C for 10 min.
8. Tubes were placed into ice immediately after boiling for another 10 min.
9. Tubes were centrifuged at 13,000rpm for 10 min.
10. The supernatant was collected in a new 1.5ml tubes carefully to avoid any debris or pellets and Extracted DNA was stored at -20ºC.

2.10 Molecular identification of *Salmonella Spp*. by *invA* gene PCR:

2.10.1 Real-time PCR based confirmation:

Real-time PCR, also known as kinetic PCR, qPCR, qRT-PCR, and RT-qPCR, is quantitative PCR method for the determination of copy number of PCR templates such as DNA or cDNA in a PCR reaction. There are two flavors of real-time PCR: Probe-based and intercalator-based. Both methods require a special thermocycler equipped with a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction. Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers as regular PCR does, an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. Intercalator-based method, also known as the SYBR Green method, requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and gives fluorescence. The TaqMan method is more accurate and reliable than SYBR green method, but also more expensive.

2.10.1.1 Reagents

- PCR Master Mix (2X) (Applied Biosystems, USA)
- Primer (Applied Biosystems, USA)
- Probe (Applied Biosystems, USA)
- DNase free water (Applied Biosystems, USA)

2.10.1.2 Master mix composition for the *invA* gene RT-PCR technique

Total DNA (5μl) was subjected to RT-PCR in a 25μl reaction mixture containing 1X PCR buffer (0.025U/μL Taq DNA polymerase, reaction buffer,2mM $MgCl_2$,0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP) and a variable concentration of specific prime and probe was added. (Table 2.1)

2.10.1.3 Procedure

- At fast, an iceless cold storage system for 96 well plates or ice cup and PCR tubes was taken, then Primer and the master mix was put into ice.
- Take PCR tube was labeled and put it in an iceless cold storage system for 96 well plates.

- Then 25μl reaction mixture per sample was prepared by adding H$_2$O, primer, probe, and master mix into the PCR tube according to the recipe.

2.10.1.4 The mechanical process of RT-PCR

- 5μl extracted DNA template was added to the master mix containing PCR tube and centrifuge at 1000 rpm for few second.
- Then PCR tube was placed into the thermal cycler.

Proceed to thermal cycling was programmed as given below:

2.10.1.5 Thermal Profile of RT-PCR *invA* gene

invA gene's RT-PCR amplification was carried out as follows: initial denaturation at 94°C for 15 min; 40 cycles of 94°C for 10 s, 60°C for 22 s and 66°C for 22 s.

PCR name	gene	Primer Name	Sequence (5′–3′)	Primer con. μM	A.T (°c)	Amplicon size (bp)	Reference
RT-PCR	*invA*	Sal-F	GCGTTCTGAACCTTTGGTAATAA	0.5	60	102 bp	(Ibrahim et al., 2014)
		Sal-R	CGTTCGGGCAATTCGTTA	0.5			
		Sal-TM (probe)	FAM-TGGCGGTGGGTTTTGTTGTCTTCTTAMRA	0.1			

Table 2.1: Real-time PCR primer composition of *Salmonella* identification/Virulence *invA* gene

2.10.2 Conventional PCR based confirmation:

2.10.2 .1 Reagents

- PCR master mix 2X (Taq 2X Master Mix-M0270L, New England BioLab)
- Primer (Forward and Reverse)
- DNase free water

2.10.2 .2 Master mix composition for invA gene Conventional PCR technique

Total DNA (5μl) was subjected to conventional PCR in a 25μl reaction mixture containing 1X PCR buffer (0.025U/μL Taq DNA polymerase, reaction buffer,1.5 mM MgCl$_2$,0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP),5% glycerol and a variable concentration of specific prime,0.5 mM MgCl$_2$ and 3% DMSO was added. (Table 2.2)

2.10.2 .3 Procedure

- At fast, an iceless cold storage system for 96 well plates or ice cup and PCR tubes was taken, then Primer and the master mix was put into ice.

- Take PCR tube was labeled and put it in an iceless cold storage system for 96 well plates.

- Then 25µl reaction mixture per sample was prepared by adding H_2O, primer, and master mix into the PCR tube according to the recipe.

2.10.2 .3 The mechanical process of conventional PCR of *invA* gene

- 5µl extracted DNA template was added to the master mix containing PCR tube and centrifuge at 1000 rpm for few second.

- Then PCR tube was placed into the thermal cycler.

Proceed to thermal cycling was programmed as given below:

2.10.2 .4 Thermal Profile of Conventional PCR *invA* gene

invA gene's RT-PCR amplification was carried out as follows: initial denaturation at 95°C for 15 min; 35 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 30 s and final elongation at 72°C for 5 min.

PCR name	gene	Primer Name	Sequence (5′–3′)	Primer con. µM	A.T (°c)	Amplicon size (bp)	Reference
Con. PCR	invA	139	GTGAAATTATCGCCACGTTCGGGCAA	0.4	64	284 bp	(Rahn et al., 1992)
		141	TCATCGCACCGTCAAAGGAACC	0.4			

Table 2.2: Conventional PCR primer composition of *Salmonella* identification /Virulence *invA* gene

2.10.2 .5 Gel electrophoresis and visualization:

According to table 2.6 and section 2.14 all method was performed

2.11 Serotyping of *Salmonella spp*.

Grossly, *Salmonella* Genus are divided into two species *Salmonella enterica* and *Salmonella bongori*. On the basis of O (surface polysaccharide) and H (flagellar) antigenic properties *Salmonella enterica* subspecies *enterica* distinguish

from other *Salmonella* species. Typing of O and H antigen denotes serogroup and Serotype accordingly. The Vi or Capsular protein are particular for *S.enterica* serover typhi typing. (Braden, 2006)

Salmonella Typhimurium and *Salmonella Typhi* are more common serover in *S. enterica*. Without consideration of plasmid, *Salmonella Typhimurium* and *Salmonella Typhi* cover 96.6% (4,338 genes) and 94.5% (4,348 genes) respectively in *S. enterica* genome (Porwollik et al., 2004). In elucidated open reading frames (ORFs) of *S. enterica* serovar Typhimurium LT2 supplemented with annotated chromosomal ORFs from the serovar Typhi CT18 strain that was >10% different from those of serovar Typhimurium. (Porwollik et al., 2003)

In this work, we describe a simple multiplex PCR method to serotype the 30 most common serovars of clinically relevant *S. enterica* subsp. *enterica*. This technique is based upon the PCR detection of genes present in specific serotypes but not others.

2.11.1 Reagents

- PCR Master Mix (2X) (Applied Biosystems, USA)
- Primer (Applied Biosystems, USA)
- DNase free water (Applied Biosystems, USA)

2.11.2 Master mix composition for serotyping gene PCR technique

Total DNA (5µl) was subjected to PCR in a 25µl reaction mixture containing 1X PCR buffer (0.025U/µL Taq DNA polymerase, reaction buffer,2mM $MgCl_2$,0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP) and a variable concentration of specific prime was added. (Table-2.3)

2.11.2 Procedure

- At fast, an iceless cold storage system for 96 well plates or ice cup and PCR tubes was taken, then Primer and the master mix was put into ice.
- Take PCR tube was labeled and put it in an iceless cold storage system for 96 well plates.
- Then 25µl reaction mixture per sample was prepared by adding H_2O, primer, and master mix into the PCR tube according to the recipe.

- S.Typhi and S. Enteritidis multiplex PCR was performed, but S. Paratyphi A and S. Paratyphi B PCR performed separately.

2.11.3 The mechanical process of RT-PCR

- 5μl extracted DNA template was added to the master mix containing PCR tube and centrifuge at 1000 rpm for few second.
- Then PCR tube was placed into the thermal cycler.

Proceed to thermal cycling was programmed as given below:

2.11.4 Thermal Profile of *Salmonella Spp.* serotyping

S.Typhi and *S. Enteritidis* multiplex PCR amplification was carried out as follows: initial denaturation at 95°C for 2 min; 30 cycles of 95°C for 1min, 57°C for 1 min and 72°C for 2min, and a final extension of 72°C for 5 min.

S. Paratyphi A PCR amplification was carried out as follows: initial denaturation at 95°C for 5 min; 40 cycles of 93°C for 30 s, 55°C for 30 s and 72°C for 40 s, and a final extension of 72°C for 5 min and *S. Paratyphi B* PCR amplification was carried out as follows: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s, and a final extension of 72°C for 10 min.

Assay	Primer	Primer sequence (5'- 3')	Final Primer Con. (μM)	A.T	Amp. size (bp)	Reference
S.PA	H_F	ACTCAGGCTTCCCGTAACGC	1	55		(Zhou et al., 2016)
	Ha_R1	TGCCGTCTTTATCGGTATATTCAG	1		880	
	Ha_R2	GACTTCGCTCTTCACATCATAT	1		372	
S. PB	pPB23_F	ACATAATGCTTTTCGTGCTCCTC	0.2	60	384	(Zhai et al., 2014)
	pPB23_R	GGCATAAATATCTTTCTCCCCTCC	0.2			
S.TY	TyphF	TTGTTCACTTTTTACCCCTGAA	0.1	57	401	(Olsen et al., 1995)
	TyphR	CCCTGACAGCCGTTAGATATT	0.1			
S.EN	ENTF	TGTGTTTTATCTGATGCAAGAGG	0.1	57	304	(Agron et al., 2001)
	ENTR	TGAACTACGTTCGTTCTTCTGG	0.1			

Table 2.3: Primer sequence and properties used in PCR based *Salmonella* serotype detection.

2.12 Phenotypic Antibiotic Tolerance test

There are a number of methods for antimicrobial susceptibility testing of bacteria, which can be categorized into (i) dilution method that generates MIC result and (ii) disk diffusion method that generates a zone diameter result. Susceptibility testing methods

can also be categorized as the genetic reference method, which is described by the standard-setting organization (e.g. The Clinical Laboratory Standard Institute [CLSI], The European Committee on Antimicrobial Susceptibility Testing [EUCAST] etc.)

2.12.1 Disk Diffusion Method:

The disk-diffusion agar method tests the effectiveness of antibiotics on a specific microorganism. An agar plate is first spread with bacteria, then paper disks of antibiotics are added. The bacteria are allowed to grow on the agar media and then observed.

2.12.1.1 Reagent and Media

- Muller Hinton Agar

- Antibiotic Disk

- Luria-Bertani (LB) broth

- 0.5 McFarland Standard

- 0.85% sodium chloride solution

2.12.1.2 Material

- Swab Stick

- 90mm plate

- Distill water
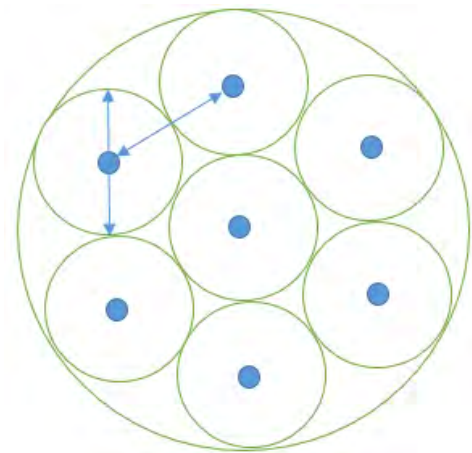
Figure 2.5: Antibiotic Disk Placement Design, 30mm distance from each Antibiotic Disk

2.12.1.3 McFarland Standard preparation

A McFarland Standard is a chemical solution of barium chloride and sulfuric acid; the reaction between these two chemicals result in the production of a fine precipitate, barium sulfate.

2.12.1.3.1 Required Regents:

- Barium Chloride ($Bacl_2 .2H_2O$)

- Sulfuric Acid (H_2SO_4)

2.12.1.3.2 Procedure:

- To produce a 0.5 McFarland turbidity standard, 0.05ml 1.175% $Bacl_2 .2H_2O$ and 9.95ml 1% H_2SO_4 was added which produce desired turbid solution.

- Reference data table is given below (McFarland, 1907)

Figure 2.6: Bacterial suspension prepared to match the turbidity of the 0.5 McFarland turbidity standard

- At fast 1.175 grams of Barium Chloride ($Bacl_2 .2H_2O$) was measured by electric balance, taken in a measuring cylinder and until 100ml Distil Water was added to produce 1.175% $Bacl_2 .2H_2O$.

- Another measuring cylinder was taken which contain 1ml 100% Sulfuric Acid (H_2SO_4) and until 100ml Distil Water was added to produce 1% H_2SO_4.

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density ($1x10^8$ CFU/mL)	1.5	3.0	6.0	9.0	12.0

Table 2.4: Composition of ingredients according to different McFarland standards

2.12.1.4 Procedure

- Before this test bacterium was cultured into Agar plate overnight.

- A single colony was taken into normal saline and adjusted the opacity to 0.5 McFarland Standard.

- Swab stick dip into the culture and spread into Muller Hinton Agar Plate for one time.

- Antibacterial Disk place into Muller Hinton Agar plate by Disk dispenser (Oxoid, UK).

- Antibiotic Disk place into MHA plate, each antibiotic Disk place 30mm far from each.

- Each Antibiotic Disk was taken from Antibiotic Cartage and put into MHA plate at the desired place in aseptic condition, then allowed to grow at 35ºC±2 for 18 to 24 hour.

- The zone of inhibition was measured by using a millimeter scale or slide calipers.

2.12.2 Minimum Inhibitory Concentration:

MIC was defined as the lowest concentration of antimicrobial agent that inhibited bacterial growth, as indicated by the absence of turbidity. MIC values were determined by the microtiter broth method (Amsterdam, 1996) in sterile flat-bottom 96-well polystyrene plates.

2.12.2.1 Reagent, Media, and Machine

- Raw Antibiotic

- DMSO

- 100% Ethanol

- Luria-Bertani *(*LB) broth

- PowerWave-340 Microplate Spectrophotometer (BioTek)

2.12.2.2 Material

- 96 well plate
- Taste Tube
- Syringe, 0.2µm Syringe Filter
- Pipette, Multichannel Pipette
- Distill water

2.12.2.3 Stock Solution preparation (Andrews, 2001):

	Where,
$$w = \left(\frac{100}{P}\right) \times V \times C$$	P = potency given by the manufacturer (µg/mg), V = volume required (mL), C = final concentration of solution (multiples of 1000) (mg/L), and W = weight of antibiotic in mg to be dissolved in volume V (mL).

2.12.2.4 Antibiotic Dilution from stock solution:

	Where,
$$V2 = \frac{V1S1}{S2}$$	V_1=Volume for the desired solution S_1 =Desire strength V_2=Volume of stock solution is needed S_2=Strength of stock solution

2.12.2.5 Procedure:

- The sample was grown into an agar plate for 24 hours, colonies transfer aseptically in sterile saline (0.85%).

- Turbidity was adjusted spectrophotometrically to 0.08-0.12 at 625 nm according to 0.5 McFarland, the broth methods give a microbial suspension of (1-2) $\times 10^8$ CFU /ml for bacteria.

- After the drug solution was prepared at the desired concentration in sterile distilled water, Ethanol or DMSO dilution of 1/10 in sterile Luria-Bertani (LB) broth.

- Then 100µl media with drug solution was transferred into 1st row of a microtiter plate.

- 50µl LB broth media was filled from 2 to 12 well in a microtiter plate.

- Two-fold dilution was performed by transferring 50 µl from the 1st to the 10th well, 50µl media discard from 10th well after dilution.

- 11th and12th number row was free from the drug.11th number row was drug-free culture growth and 12th number column was free from drug and microbes, which was media control.

- Then the bacterial suspension was diluted 1/150 into LB broth, which contains 10^6 CFU /ml of bacteria.

- Then inoculum was given 50 µl from 1st to 11th row of a microtiter plate and incubate it 18 to 24 hours at 35ºC±
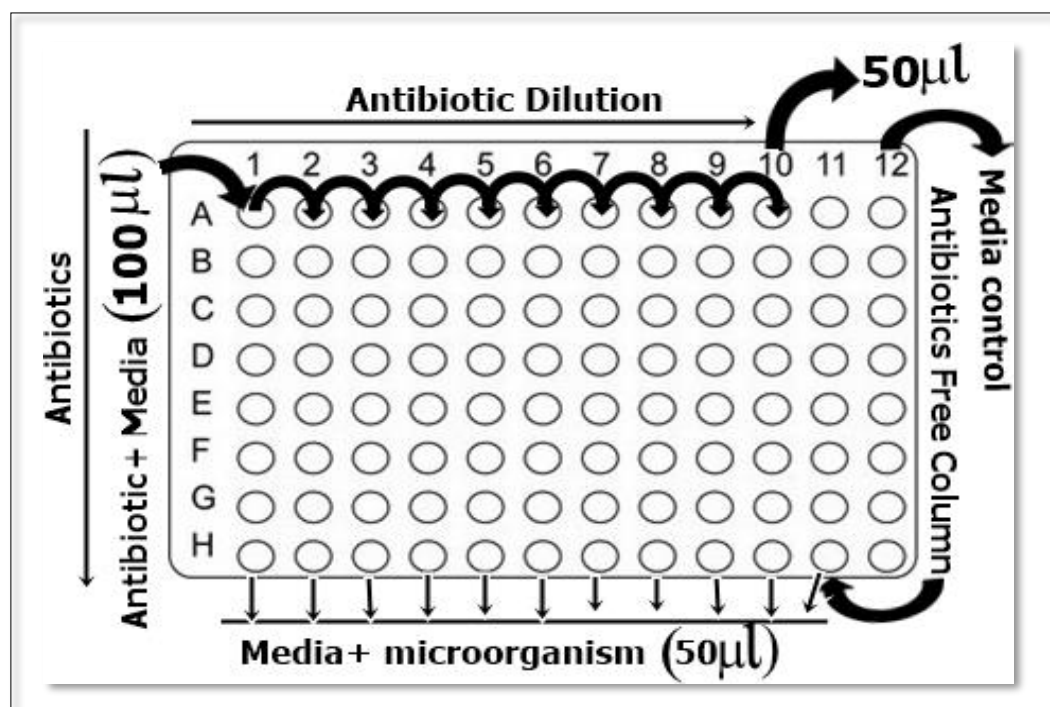
Figure 2.7: MIC plate design (1st to the 10th well antibiotic dilution (1024, 512, 256, 128, 64, 32, 16, 8, 4, 2) mg/l accordingly

2.13 Antibiotic Resistance Gene Identification using conventional PCR:

2.13.1 Reagents

- PCR Master Mix (2X) (Applied Biosystems, USA)

- Primer (Applied Biosystems, USA)

- DNase free water (Applied Biosystems, USA)

2.13.2 Procedure

- At fast, an iceless cold storage system for 96 well plates or ice cup and PCR tubes was taken

- Primer and master mix was put into ice cup.

- Take PCR tube was labeled and put it in an iceless cold storage system for 96 well plates.

- Then 25µl reaction mixture per sample was prepared by adding H_2O, primer, probe, and master mix into the PCR tube according to the following recipe

2.13.2.1 Master-mix composition

Total DNA (5µl) was subjected to each PCR /multiplex PCR in a 25µl reaction mixture containing 1X PCR buffer (0.025U/µL Taq DNA polymerase, reaction buffer,2mM $MgCl_2$,0.2 mM of each dNTP (dATP,dCTP,dGTP and dTTP) (Thermo Scientific PCR Master Mix 2X) and a variable concentration of specific prime /group primers. (Table 2.5)

2.13.2.2 The mechanical process of Conventional PCR

- 2µl extracted DNA template was added to the master mix containing PCR tube and centrifuge at 1000 rpm for few second.

- The PCR tube was placed into the thermal cycler.

2.13.2.3 Thermal profile used for Antimicrobial resistance gene:

2.13.2.3.1 Thermal Profile used to detect ESBLs resiatance gene

β-Lactam, Carbapenem, and Quinolone amplification was carried out as follows: initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 7 min. For the carbapenemase gene multiplex PCR assays, the annealing temperature was optimal at 55°C for amplification of bla_{VIM}, bla_{IMP}, and bla_{KPC} genes, and optimal at 57°C for amplification of bla GES and blaOXA-48 genes.

2.13.2.3.2 Thermal Profile used to detect Quinolone resistance gene

Quinolone gene (GyrA, GyrB, and parc) amplification was carried out as follow: initial denaturation at 94°C for 10 min; 35 cycles of 94°C for 30 s, 30 s of annealing at 50°and 72°C for 1 min; and a final elongation step at 72°C for 10 min. n.

Assay	T. gene	Primer	Sequence (5′–3′)	Primer (µM)	A. T.	Amp. (bp)	Ref.
Multiplex I TEM, SHV and OXA-1-lik	TEM variants including TEM-1 and	MultiTSO-T_for	CATTTCCGTGTCGCCCTTATTC	0.4	60	800	(Dallenne et al., 2010)
		MultiTSO-T_rev	CGTTCATCCATAGTTGCCTGAC	0.4			
	SHV variants including SHV-1	MultiTSO-S_for	AGCCGCTTGAGCAAATTAAAC	0.4		713	
		MultiTSO-S_rev	ACCCGCAGATAAATCACCAC	0.4			
	OXA-1, OXA-4 and OXA-30	MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	0.4		564	
		MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG	0.4			
Multiplex II CTX-M group 1, group 2 and group 9	variants of CTX-M group 1 including CTX-M-1, CTX-M-3 and CTX-M-15	MultiCTXMGp1_for	TTAGGAARTGTGCCGCTGYA	0.4	60	688	
		MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT	0.2			
	variants of CTX-M group 2 including CTX-M-2	MultiCTXMGp2_for	CGTTAACGGCACGATGAC	0.2		404	
		MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT	0.2			
	variants of CTX-M group 9 including CTX-M-9 and CTX-M-14	MultiCTXMGp9_for	TCAAGCCTGCCGATCTGGT	0.4		561	
		MultiCTXMGp9_rev	TGATTCTCGCCGCTGAAG	0.4			
CTX-M group 8/25	CTX-M-8, CTX-M-25, CTX-M-26, and CTX-M-39 to CTX-M-41	CTX-Mg8/25_for	AACRCRCAGACGCTCTAC	0.4		326	
		CTX-Mg8/25_rev	TCGAGCCGGAASGTGTYAT	0.4			
Multiplex VI IMP, VIM and KPC	IMP variants except IMP-9, IMP-16,IMP-18, IMP-22 and IMP-25	MultiIMP_for	TTGACACTCCATTTACDG	0.5	55	139	
		MultiIMP_rev	GATYGAGAATTAAGCCACYCT	0.5			
	VIM variants including VIM-1 and VIM-2	MultiVIM_forc	GATGGTGTTTGGTCGCATA	0.5		390	
		MultiVIM_revc	CGAATGCGCAGCACCAG	0.5			
	KPC-1 to KPC-5	MultiKPC_for	CATTCAAGGGCTTTCTTGCTGC	0.2		538	
		MultiKPC_rev	ACGACGGCATAGTCATTTGC	0.2			
Multiplex GyrA,GyrB and parc	GyrA	GyrA-F	CTGAAGCCGGTACACCGTCG	0.2	50	290	(Casin et al., 2003)
		GyrA-R	TCGGCCATCAGTTCGTGGGC	0.2			
	GyrB	GyrB-F	TTATCGATGCTGCGCGTGCC	0.2		1280	
		GyrB-R	TCGCCGCTTTCAGGGCGTTC	0.2			
	parc	parc-F	CGCCTACTTAAACTACTCCA	0.2		540	
		parc-R	ATCAGCGTAATCGCCGCTTT	0.2			

Table 2.5: List of PCR primers, properties and targets β- Lactam, Carbapenem and Quinolone resistance gene focused in this study; A.T: Annealing temperature, T. gene: Target gene.

2.14 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a standard method used to separate, identify, and purify cDNA fragments. When agarose (D-galactose and L-galactose) is melted and then allowed to harden, it forms a matrix, which serves a molecular sieve to separate DNA fragment of different sizes.

2.14.1 Agarose Gel Preparation Reagent

- Ultrapure Agarose.
- 1x TBE buffer
- Ethidium bromide.

		Amount Of Gel (ml)						Recommended	Resolution for Linear DNA
		30	50	80	110	130	150	% Agarose	Optimum
Percent of Gel (%)	0.5	0.15	0.25	0.4	0.55	0.65	0.75	0.5	1,000-30,000bp
	1	0.3	0.5	0.8	1.1	1.3	1.5	0.7	800-12,000bp
	1.2	0.36	0.6	0.96	1.32	1.56	1.8	1	500-10,000bp
	1.5	0.45	0.75	1.2	1.65	2.15	2.25	1.2	400-7,000bp
	2	0.6	1	1.6	2.2	2.6	3	1.5	200-3,000bp
Ethidium Bromide		Amount of agarose(gm)						2	50-2,000bp
		1.5	2.5	4	5.5	6.5	7.5		

Table 2.6: Recipe of Gel preparation

2.14.2 Procedure

- Thoroughly clean the appropriate gel apparatus by washing with detergent, completely removing the detergent mixture with tap water and rinsing with distilled water three to five times. Allows the apparatus to dry at room temperature.
- 0.9gm (1.8%) agarose powder (Thermo Fisher Scientific, MA, US) was added with 50ml 1x TBE buffer in a conical flask.
- The flask was heated for 2 min in a microwave oven.
- Heated gel was cool to 60-70°C.
- 2.5µl Ethidium Bromide from10mg/ml stock was added and mixed properly.
- Gel tray was equipped with 25 well comb (1.5mm).

- Slowly pour the gel into the assembled gel tray and wait for at least 15 minutes for solidification at room temperature.

- Proper safety precaution was taken during gel preparation due to the hazardous effect of Ethidium Bromide

2.14.3 Gel electrophoresis

- Electrophoresis tank was filled with 1x TAE buffer and fill up the maximum level.

- Gel was put into the desired place, DNA containing side put at the negative side.

- 1μl (5x) loading dye and 4μl DNA was added, then place into the gel pocket.

- Carefully loaded the positive control in the very left or the very right well of the gel.

- Ladder was used very left or very right well, opposite sight of positive control.

- Sample was loaded one by one, into the empty wells in the submerged gel.

- Electrophoresis was performed in Mupid®-One Electrophoresis System (Mupid Co., Ltd, Tokyo, Japan) for 30 min at 100V

- The gel was observed under UV light in gel-doc machine (Alphagram mini system, Protein Simple, CA, US) and imaged using Alpha View software

2.15 Data Analysis

- Sample collection and geographical Data managed by ArcGIS.

- First of all, primary Data was imputed into Microsoft office (Word and Excel), and Analysis by Excel and SPSS statistical software.

- Antimicrobial data was managed by WHONET-2017

Chapter
03
Result & Discussion

CHAPTER 03: RESULT AND DISCUSSION

3.1 RESULT

The present study was carried out on 328 samples were collected from different live animal market at 4 categories are chicken meat, Beef, Mutton and Buffalo.

This study has been delineated into four distinct phases:

- Sample collection and isolation

- Molecular identification

- Phonotypical antimicrobial resistance determination & observe minimum inhibitory concentration.

- Genotypic Drug resistance pattern determination.

All test was performed at Food and Feed Safety Laboratory in Bangladesh live Stock Research Institute (BLRI), Saver, Dhaka.

3.1.1 Enrichment Sample to isolation microorganism

After sample collection and processing that was enriched all sample into non selective broth (buffer peptone water), after satisfactory growth or after 18 to 24 hours all samples inoculated into selective broth media (Rappaport Vassiliadis) after 18 to 24 hours then stick into selective media (XLD Agar, SS Agar) and all sample are analyzed and separate according to their typical morphological colony characteristics.
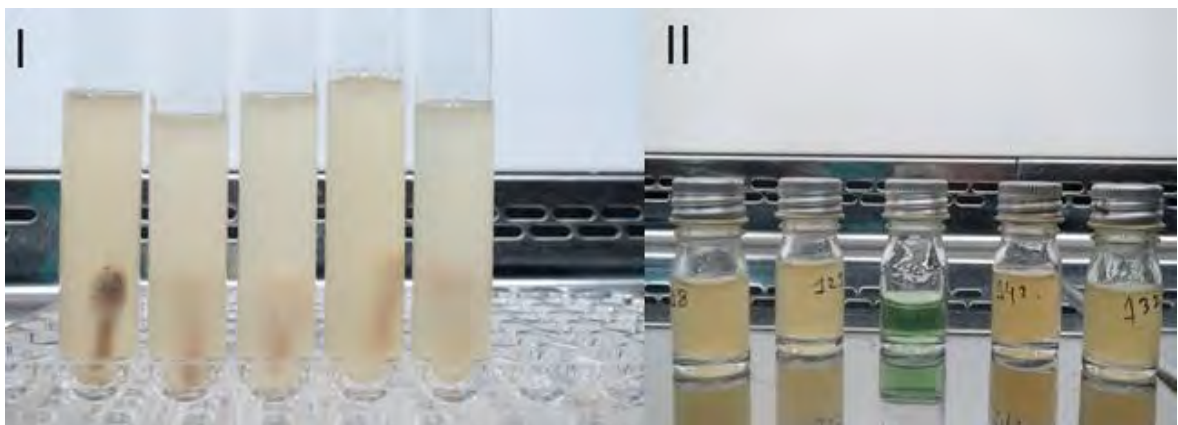
Figure 3.1: (I) Non selective media (Buffer Peptone Water) enrichment (II) Selective media Rappaport Vassiliadis broth enrichment

3.1.1.1 Isolation and identification *Salmonella Spp.* and other bacteria by XLD Agar Medium

Xylose Lysine Deoxycholate (XLD agar) is a selective growth medium used in the isolation of *Salmonella* and *Shigella* species from clinical samples and from food.(Zajc-Satler and Gragas, 1976, Nye et al., 2002) Generally its pH approximately 7.4, because of phenol red it's looks bright pink or red color. Sugar fermentation lowers the pH and the phenol red indicator registers this by changing to yellow. Most gut bacteria, including *Salmonella*, can ferment the sugar xylose to produce acid; *Shigella* colonies cannot do this and therefore remain red. After exhausting the xylose supply *Salmonella* colonies will decarboxylate lysine, increasing the pH once again to alkaline and mimicking the red *Shigella* colonies. *Salmonella* metabolize thiosulfate to produce hydrogen sulfide, which leads to the formation of colonies with black centers and allows them to be differentiated from the similarly colored *Shigella* colonies.(Park et al., 2012)

Other Enterobacteria such as *E. coli* will ferment the lactose and sucrose present in the medium to an extent that will prevent pH reversion by decarboxylation and acidify the medium turning it yellow.(Park et al., 2012)

Microorganism Name	Colony Characteristic
Salmonella Typhi	Red Colonies, Black Centers
Salmonella choleraesuis	Red Colonies
Shigella sonnei	Red Colonies
Shigella flexneri	Red Colonies
Escherichia coli	Large, Flat, Yellow Colonies; some strains may be inhibited
Proteus vulgaris	Yellow Colonies
Enterobacter/ Klebsiella	Mucoid, Yellow Colonies
Pseudomonas aeruginosa	Pink, Flat, Rough Colonies
Gram-positive bacteria	No growth to slight growth

Table 3.1 : Typical colonial morphology on XLD Agar are as follows (Aryal, July 15, 2015)

From Live Animal Market total (n=328) samples was collected, here chicken (n=259), beef (n=32), mutton, (n= 32) and buffalo (n=5) was taken.

In Live Animal Market chicken sample XLD agar media was shown 14%(n=35) red black center colony, 4%(n=10) red or pink colony, 39% (n=100) yellow colony, 5% (n=14) yellow black center colony and 19% (n=50) clear zone.

In Live Animal market beef sample XLD agar media was shown 19%(n=6) red black center colony, 3%(n=1) red or pink colony, 28%(n=9) yellow colony, 28% (n=9) yellow black center colony and 22% (n=7) clear zone.

In Live Animal Market mutton sample XLD agar media was shown 34%(n=11) red black center colony, 9%(n=3) red or pink colony, 19%(n=6) yellow black center colon,19%(n=6) yellow colony and 13%(n=4) clear zone.

In Live Animal market buffalo sample XLD agar media was shown 60% (n=3) red black center colony, 0%(n=0) red or pink colony, 0%(n=0) yellow colony, 0% (n=0) yellow black center colony and 9% (n=3) clear zone.
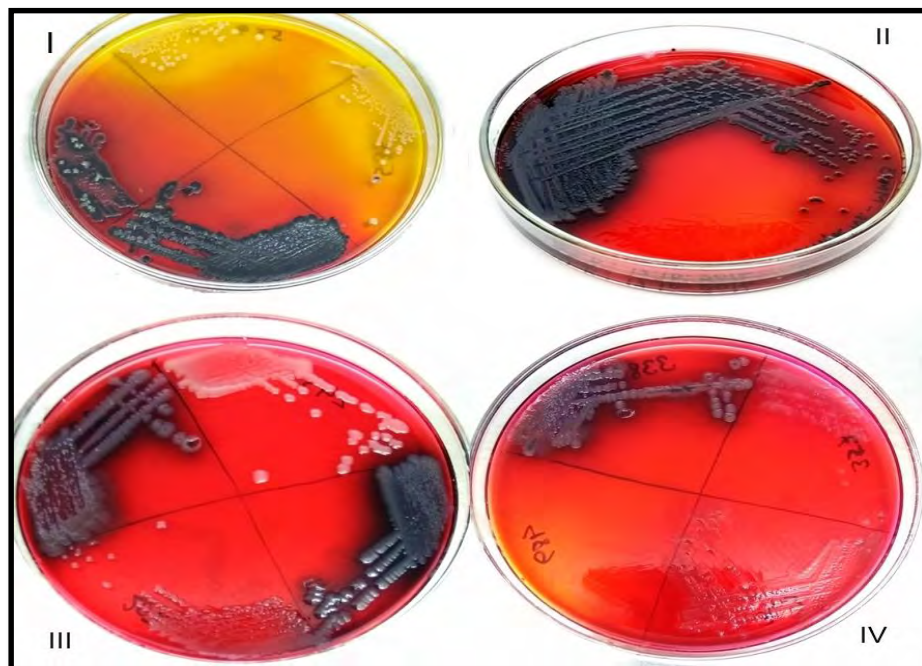
Figure 3.2: Typical colony characteristic on XLD Agar media was shown Yellow colony, red colony with black center, pink colony and inhibition according to I, II, II and IV.
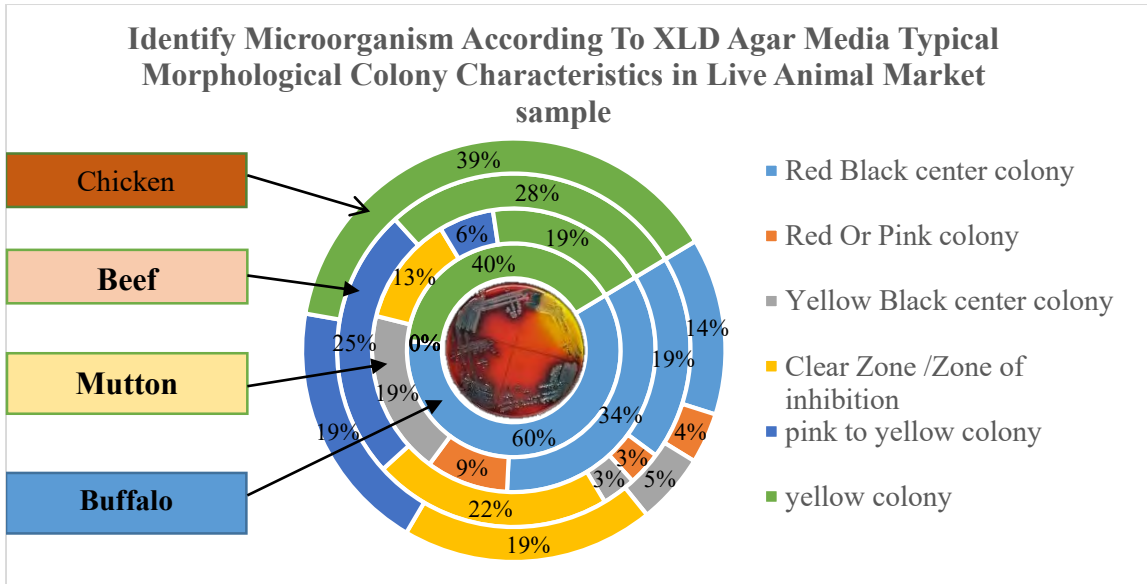
Figure 3.3: XLD Agar Media Typical Morphological Colony Characteristics in Live Animal market sample

3.1.1.2 Isolation and identification *Salmonella Spp.* and other bacteria by SS Agar Medium
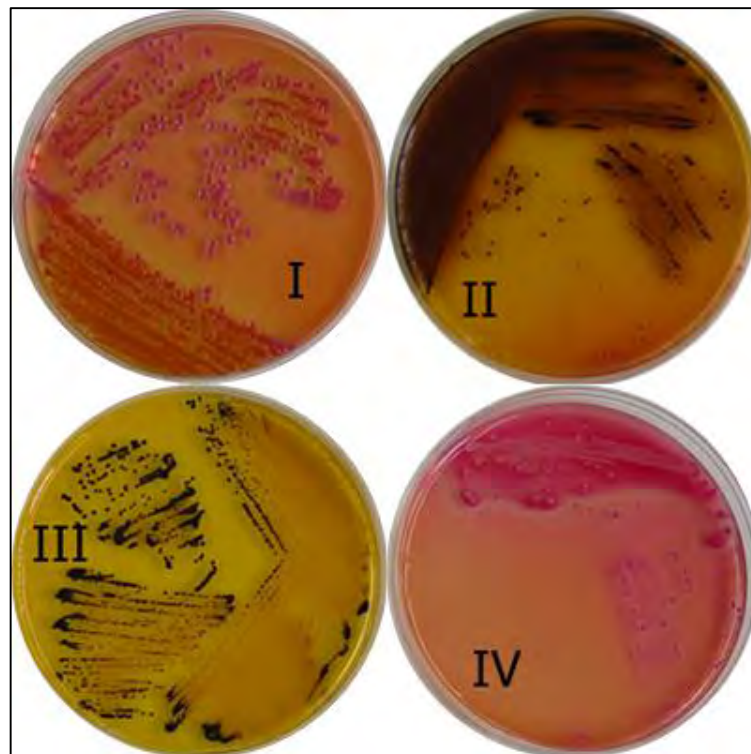
Figure 3.4: Typical colony character in SS Agar (I) *Klebsiella pneumonia*, (II) *Proteus mirabilis* (III) *Salmonella spp.* (IV) *Escherichia coli*.

After the selective enrichment of the retail meat sample, at the same time sample was plated into XLD agar and SS agar. In live animal market total (n=57) was shown positive *Salmonella* characteristic in SS agar plate.

3.1.2 Gram reaction and microscopic observation

According to growth characteristics in selective culture media, all isolates were subjected to gram staining. Among them, all isolates showed gram negative criteria (Figure-3.5). They were all gram negative and found to be single, short rods in appearance, then all isolates were selected for further biochemical tests.
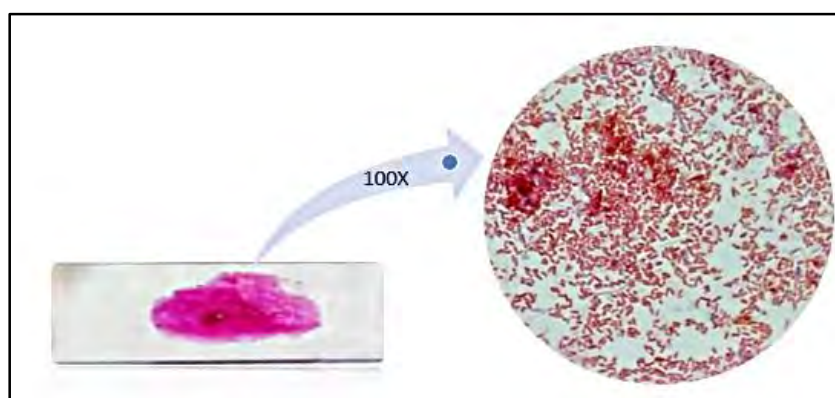
Figure 3.5**:** Microscopic view of *Salmonella Spp.* after gram staining. Magnification: 10*100x, Photo was taken using Olympus CX41, Olympus Corp., Tokyo, Japan.

3.1.3 Biochemical properties of isolated colonies

The various biochemical test has been performed on selected suspected meat sample, which is identified according to selective media according to morphological colony character.

TSI test, MR-VP test, Indole Test Motility Test, Oxidase Test and Catalase Test was performed and compared with *Salmonella Spp*. reference value

3.1.3.1 Catalase test result

All Gram-negative bacteria isolates were examined for catalase enzyme production using 3% H_2O_2.In Catalase Test all of the samples were shown the catalase positive result.

Figure 3.6: Catalase test positive for *Salmonella Spp.*

3.1.3.2 TSI (Triple Sugar Iron) Test result

TSI test was performed for Live animal market (n=57) suspected sample. TSI Result Data shown in the (Table 3.2), Alkaline (K) slant appear red color; Acid (A) slant appear yellow color. (+) and (-) sign denoted present and absent gas.

Test Appearance				Result (%)
Slant	Butt	Gas	H_2S	Live Animal Market (n=44)
K	A	(+)	(+)	87.72% (n=50)
K	A	(-)	(+)	7.02%(n=4)
A	A	(+)	(+)	NA
K	A	(-)	(-)	5.3%(n=3)
*NA= Not Appeared				

Table 3.2: TSI Test Result

Figure 3.7: TSI test result ;(I) Media Control. (II) & (III) sample, Slant-Alkaline; Butt-Acid; H_2S-pos (+); Gas-(II) negative but (III) positive. (IV) Positive control.

3.1.3.3 **MR** (Methyl Red) Test result

MR test was performed for Live bird market (n=57) suspected sample. Red color indicates the test is positive and yellow color indicates a negative result. In live animal market sample, 91.22% (n=52) was shown red color in the test, rest of the sample was shown yellow color. *Salmonella spp.* shown positive (red color) in MR test.
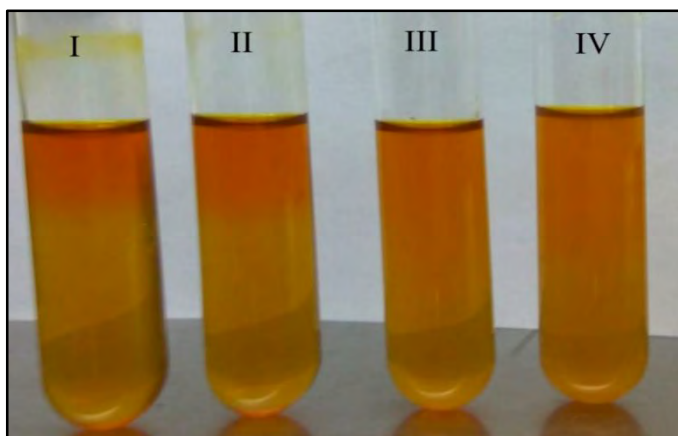
Figure 3.8: MR Test, (I) Positive control and (II), (III), (IV) test sample.

3.1.3.4 Voges-Proskauer (VP) Test result

VP test was performed for Live animal market (n=57) suspected sample. Red color indicates the test is positive and yellow color indicate a negative result. In live animal market sample,89.5%(n=51) was shown yellow color in the test, rest of the sample was shown red color. *Salmonella spp.* shown negative (yellow color) result in VP test .
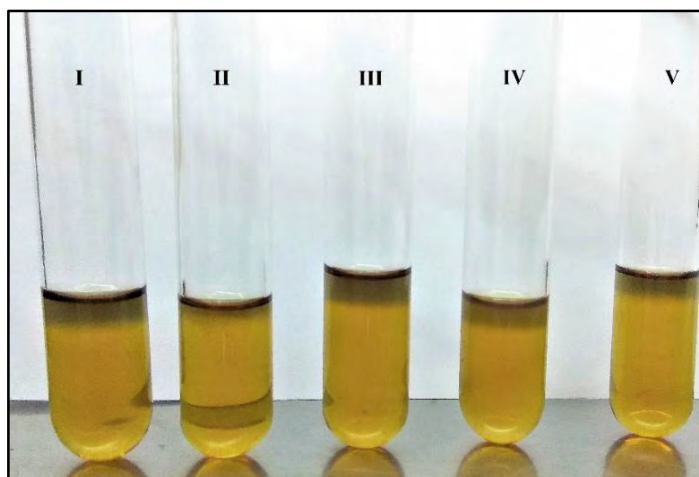
Figure 3.9: VP Test, (I) Positive control and (II), (III), (IV), (V) test sample

3.1.3.5 Indole Test result:

Indole test was performed for Live bird market (n=57) suspected sample. Red color indicates the test is positive and yellow color indicates a negative result. In live animal market sample, 87.72% (n=50) was shown yellow color in the test, rest of the sample was shown red color. *Salmonella spp.* shown negative (yellow color ring) in indole test.
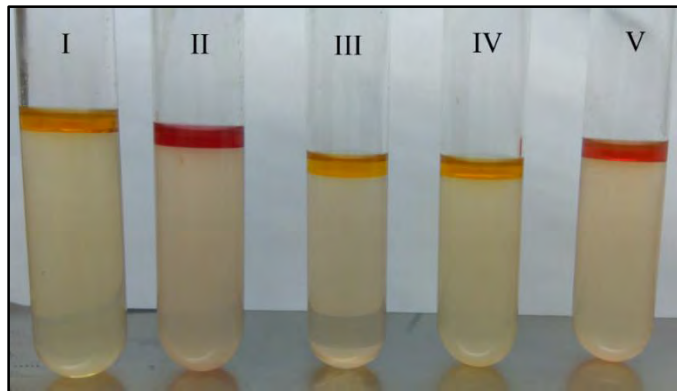
Figure 3.10: Indole Test, (I) Positive Control ;(II), (III), (IV) and (V) test sample, (II) & (V) positive   in test ;(III) & (IV) negative in test.

3.1.3.6 Motility Test result:

All of the suspected positive sample, Live animal market n= 57 was performed motility test by hanging drop method. *Salmonella Spp.* is motile by peritrichous flagella.(Andino and Hanning, 2015).Presence of flagella was observed by microscopic examination. In live animal market 98.25%(n=56) was shown motile positive.

3.1.3.7 Sugar Fermentation Test result:

Sugar fermentation test was performed for Live animal market (n=57) suspected sample. Sugar fermentation test result data shown in the (Table), Alkaline (K) appear red or orange color; Acid (A) appear yellow color and gas (G). (+) and (-) sign denoted present and absent. Glucose, Mannitol, Maltose Produce Acid/gas; *Salmonella typhi* do not produce gas; lactose, sucrose not fermented.

	Alkaline (K)	Acidic (A)	Gas (G)	Result (%)				
				Glucose	Mannitol	Maltose	Lactose	Sucrose
Live Bird Market	(-)	(+)	(+)	84.1% (n=31)	84.1% (n=31)	81.81% (n=47)	15.90% (n=9)	13.63% (n=8)
	(-)	(+)	(-)	2.27% (n=1)	2.27% (n=1)	0% (n=0)	2.27% (n=1)	4.55% (n=3)
	(+)	(-)	(+)	11.36% (n=6)	13.63% (n=8)	13.63% (n=8)	6.81% (n=4)	4.55% (n=3)
	(+)	(-)	(-)	15.91% (n=9)	13.64% (n=8)	13.64% (n=8)	0% (n=0)	0% (n=0)

Table 3.3: Sugar Fermentation test result
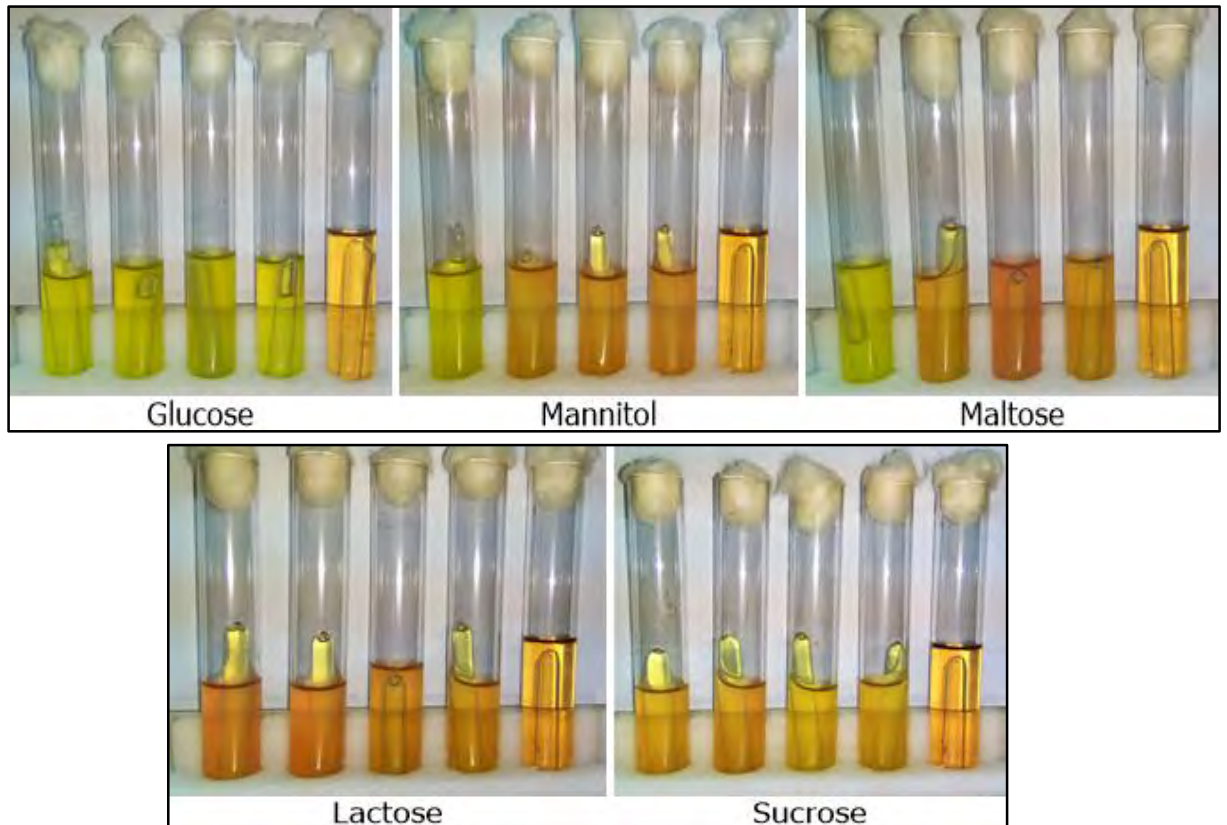
Figure 3.11: Sugar fermentation test result

3.1.4 Molecular Identification or Confirmatory test of suspected sample:

3.1.4.1 *Salmonella* identification conventional PCR result

Conventional PCR was performed with inva gene specific primers (Table 2.2) for all

biochemically positive live animal market chicken meat isolates. Among them, 13%

(n=33) had shown positive amplification in gel electrophoresis (Figure 3.12).
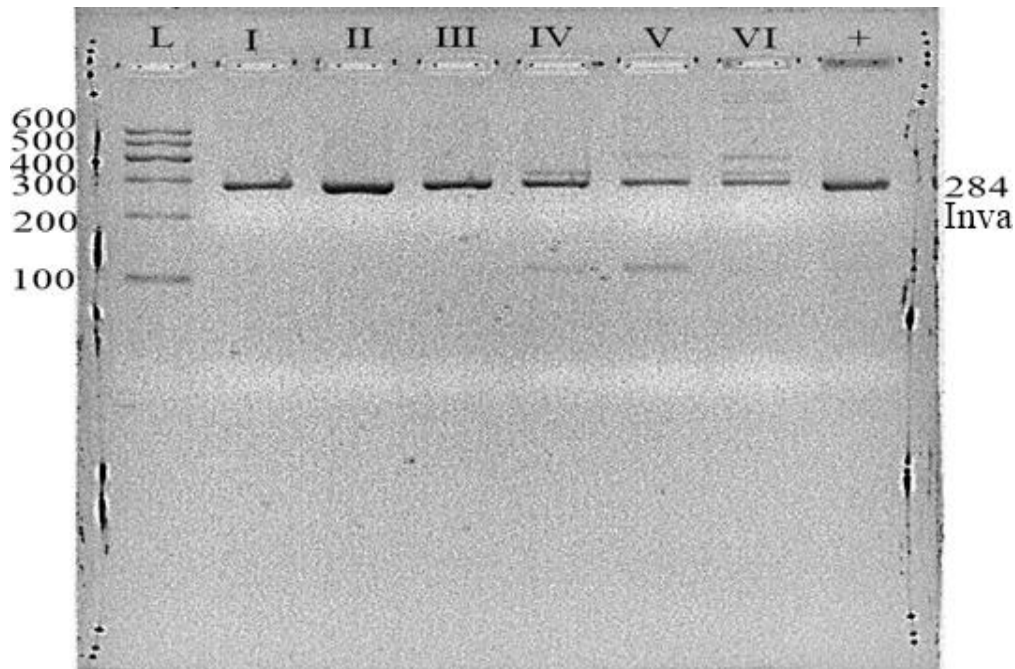
Figure 3.12: *invA* gene Conventional PCR gel electrophoresis image, PCR positive

isolates were observed as amplified DNA band near 284bp after the Gel electrophoresis

of PCR product. Image of Gel electrophoresis was taken using the gel-doc machine

(Alphagram mini system, ProteinSimple, CA, US) and Alpha View software V2.1;

3.1.4.2 *Salmonella* identification real-time PCR result

RT-PCR was performed with *invA* gene specific primers (Table 2.1) for all biochemically positive live animal market beef, mutton and buffalo isolates. Among them beef 12.5%(n=4), mutton 28.13%(n=9) and buffalo 40%(n=2) was shown positive ct value.
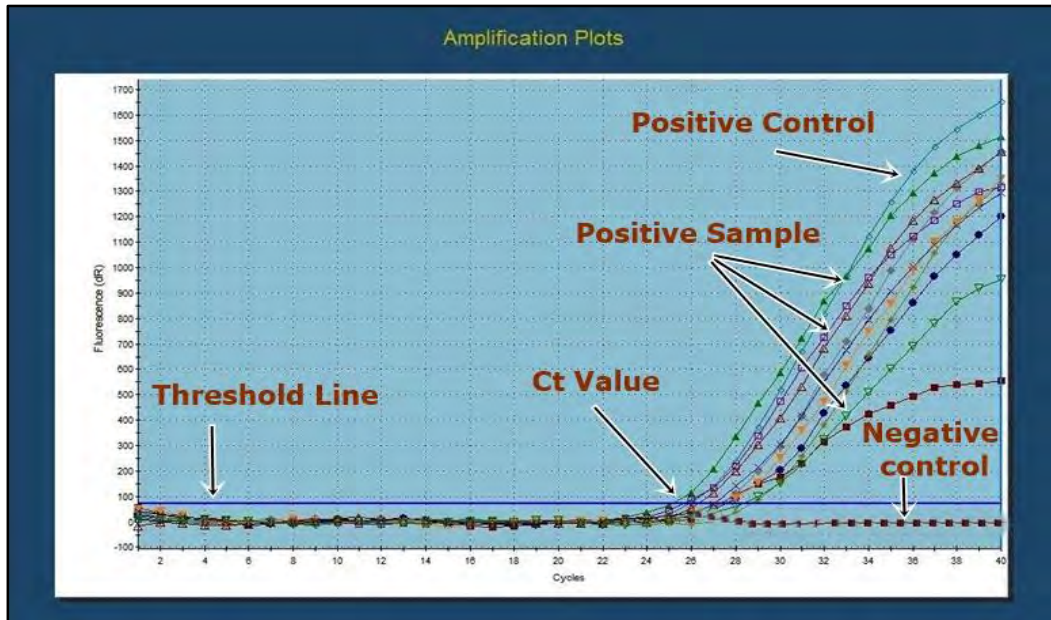
Figure 3.13: RT-PCR of *Salmonella spp. invA* gene amplification curve picture

3.1.5 Prevalence of *Salmonella Spp*. in retail Meat
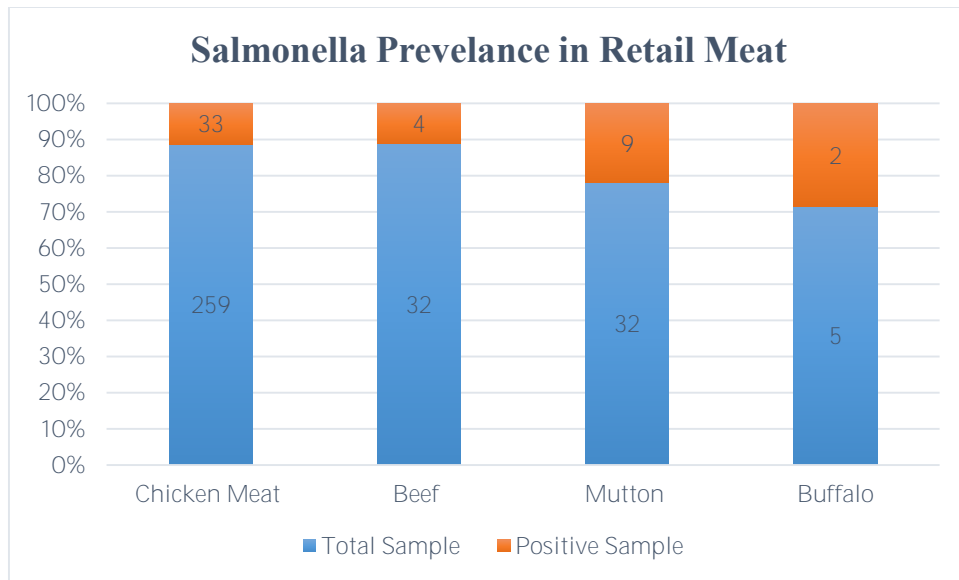
Figure 3.14: Prevalence of *Salmonella Spp*. according to Sample Source

Overall, *Salmonella* prevalence in retail meat was 14.63%(n=48) ,without considering type of sample *Salmonella* contamination was observed live animal market chicken 12.74% (n=33/259),beef 12.5% (n=4/32),mutton 28.13% (n=9/32) and buffalo 40%(n=2/5) individually

3.1.6 Differentiation of Typhoidal and non-typhoidal *Salmonella spp*.

According to this study protocol (Table 2.3) *Salmonella spp*, was differentiated into two main categories: Typhoidal and non-typhoidal *Salmonella*. Among the all positive *Salmonella* isolate 58% non-typhoidal and 42% typhoidal was observed.
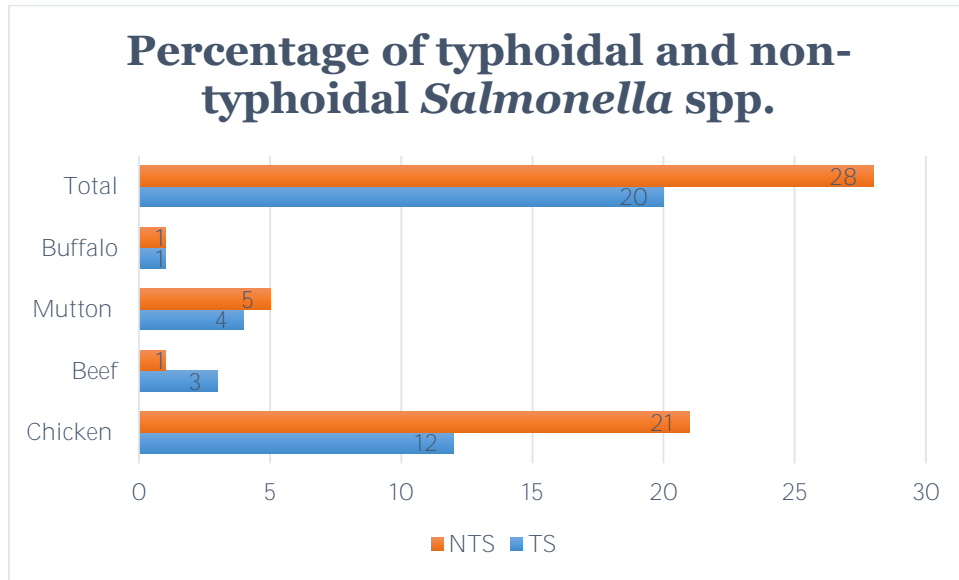
Figure 3.15: Seroprevalence of *Salmonella Spp.* in Dhaka city retail meat sample

In total 48 *Salmonella* isolate 25% chicken,6%beef,8% mutton and 2% buffalo was shown present of typhoidal *Salmonella*, another 44% chicken ,2% beef ,10% mutton and 2% buffalo was non-typhoidal *Salmonella*.

3.1.7 Phenotypically Antibiotic Resistance Profile:

3.1.7.1 Disk diffusion result:

Agar diffusion test (Kirby–Bauer**)** was performed PCR positive live animal market (n=48) positive sample. The result interpretation was carried out according to Clinical & Laboratory Standards Institute: CLSI Guidelines-2017

Salmonella Positive isolate in live animal market sample (n=48) are categorize into four type, Chicken (n=33), Beef (n=4), Mutton (n=9) and Buffalo (n=2).

Antibiotic disk (n=13) was used to determine antibiotic resistance profile for all sample.
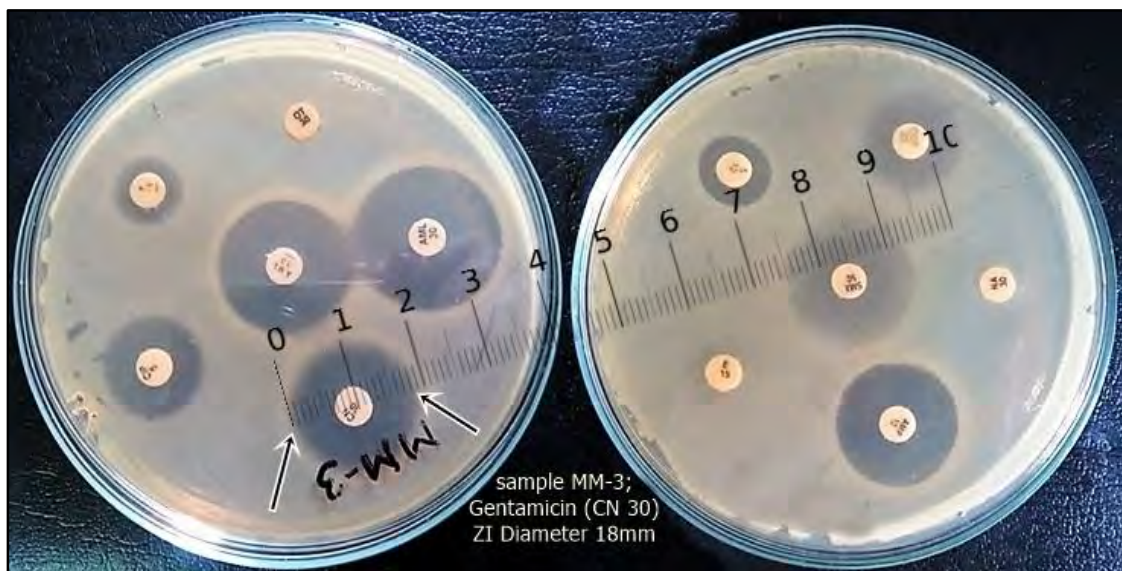
Figure 3.16: Disk diffusion method performed *Salmonella* positive sample

3.1.7.1 .1 Percentage of Antibiotic non-susceptible in Meet Sample

Ampicillin(AMP) is a bacteriolytic antibiotic. In this study, chicken meat 71% and beef 33% was shown resistance in ampicillin. Chicken meat 3%, beef 17%, mutton 50% and buffalo 100% was intermediate in Dhaka city meat sample.

Amoxicillin/Clavulanic acid (AMC) Amoxicillin is a bactericidal and clavulanic acid is a beta-lactamase inhibitor, a combination of amoxicillin and clavulanic acid was given synergistic effect by inactivation of amoxicillin. In this study, chicken meat 29%, beef 17% and mutton 25%was shown resistance in AMC. Chicken meat 15%, beef 17%, mutton 25% and buffalo 100% was intermediate.

Ceftriaxone(CRO) is a third-generation cephalosporin antibiotic which lysis the bacteria cell. In this study, chicken meat 6% was shown resistance in ceftriaxone. Chicken meat 3%, beef 17%, mutton 25% and buffalo 100% was intermediate.
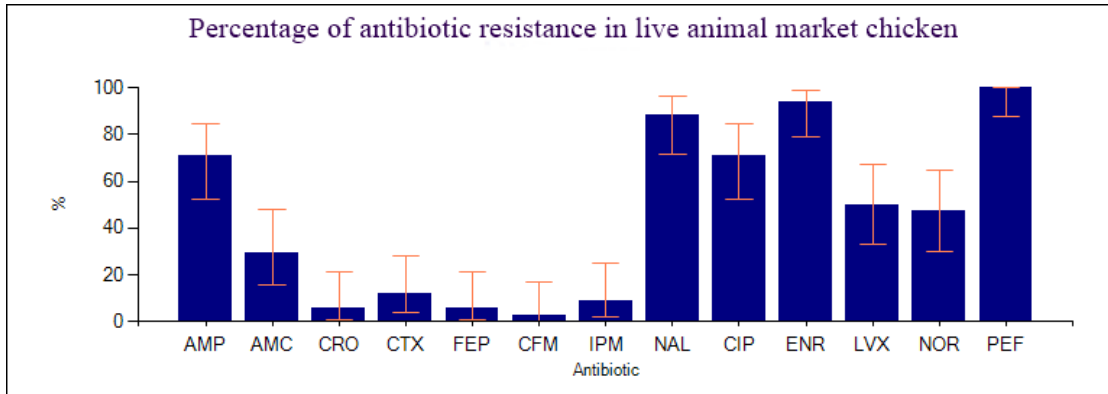
Figure 3.17: Histogram with confidence interval representing antibiotic resistance profile in live animal market chicken meat sample

Cefotaxime(CTX) is a third-generation cephalosporin antibiotic which was given bactericidal action through inhibition of bacterial cell wall synthesis. In this study, chicken meat 12%, beef 67% and mutton 75% was shown resistance in cefotaxime. Chicken meat 21%, mutton 25% and buffalo 50% was intermediate.

Cefepime(FEP) is a fourth-generation cephalosporin antibiotic which was given bactericidal action through inhibition of bacterial cell wall synthesis. In this study, chicken meat 6%, beef 17% and mutton 25% was shown resistance in cefotaxime. Chicken meat 12%, beef 17% mutton 50% and buffalo 50% was intermediate.
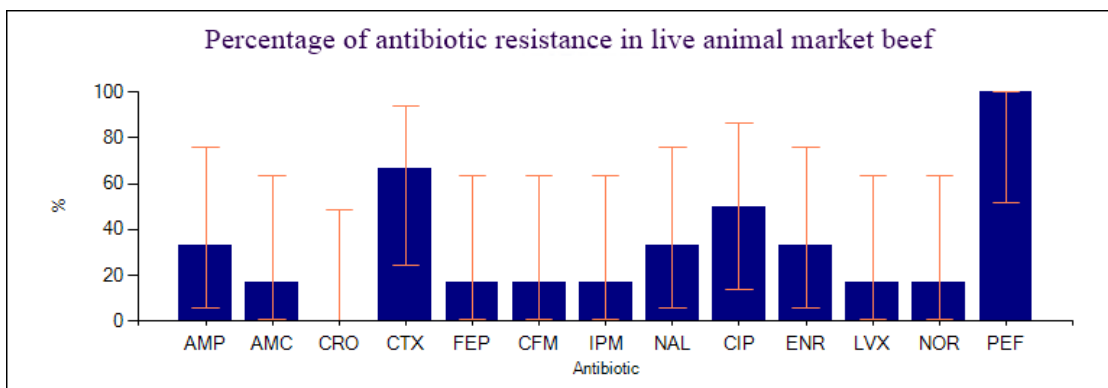
Figure 3.18: Histogram with confidence interval representing antibiotic resistance profile in live animal market beef sample

Cefixime(CFM) is a third-generation cephalosporin antibiotic which was given bactericidal action through inhibition of bacterial cell wall synthesis. In this study, chicken meat 3%, beef 17% and buffalo 100% was shown resistance in cefixime. Chicken meat 9%, beef 17% and mutton 50% was intermediate.
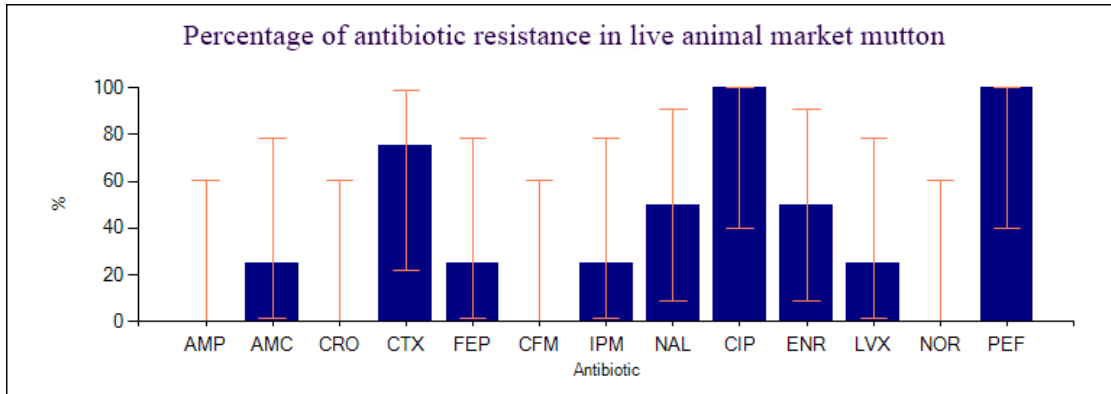
Figure 3.19: Histogram with confidence interval representing antibiotic resistance profile in live animal market mutton sample

Imipenem(IMP) has the ability to kill a wide variety of bacteria. Imipenem is the active antibiotic agent and works by interfering with their ability to form cell walls, so the bacterial cell wall lysis occur. In this study, chicken meat 9 %, beef 17% and mutton 25% was shown resistance imipenem. Chicken meat 41%, beef 33% and mutton 75% was intermediate

Nalidixic acid(NAL) is the first synthetic quinolone antibiotic which inhibit protein synthesis. In this study, chicken meat 88 %, beef 33% and mutton 50% was shown resistance nalidixic acid. Chicken meat 12%, beef 17% and mutton 25% was intermediate.

Ciprofloxacin(CIP) is broad spectrum fluoroquinolone antibiotic which given bacteriostatic effect. In this study, chicken meat 71%, beef 50% and mutton 100% was shown resistance ciprofloxacin. Chicken meat 29%, beef 50% and bufallo100% was intermediate.

Enrofloxacin(ENR) is a fluoroquinolone antibacterial drug, which inhibit bacterial DNA and RNA synthesis. In this study, chicken meat 94%, beef 33% and mutton 50% was shown resistance enrofloxacine. Chicken meat 6%, beef 67%, mutton 50% and buffalo 50% was intermediate.

Levofloxacin(LVX) is a synthetic fluoroquinolone (fluoroquinolones) antibacterial agent that inhibits the supercoiling activity of bacterial DNA gyrase, halting DNA replication. In this study, chicken meat 50%, beef 17% and mutton 25% was shown resistance Levofloxacin. Chicken meat 18% and beef 17% was intermediate

Norfloxacin(NOR) is a synthetic fluoroquinolone (fluoroquinolones) with broad-spectrum antibacterial activity against most gram-negative and gram-positive bacteria. Norfloxacin inhibits bacterial DNA gyrase. In this study, chicken meat 47%, beef 17% and mutton 25% was shown resistance norfloxacin. Chicken meat 12%, beef 33%, mutton 25% and buffalo 100% was intermediate.

Pefloxacin(PEF) is a synthetic broad-spectrum fluoroquinolone antibacterial agent active against most gram-negative and gram-positive bacteria. In this study, chicken meat 100%, beef 100%, mutton 100% and buffalo 100% shown resistance pefloxacin.
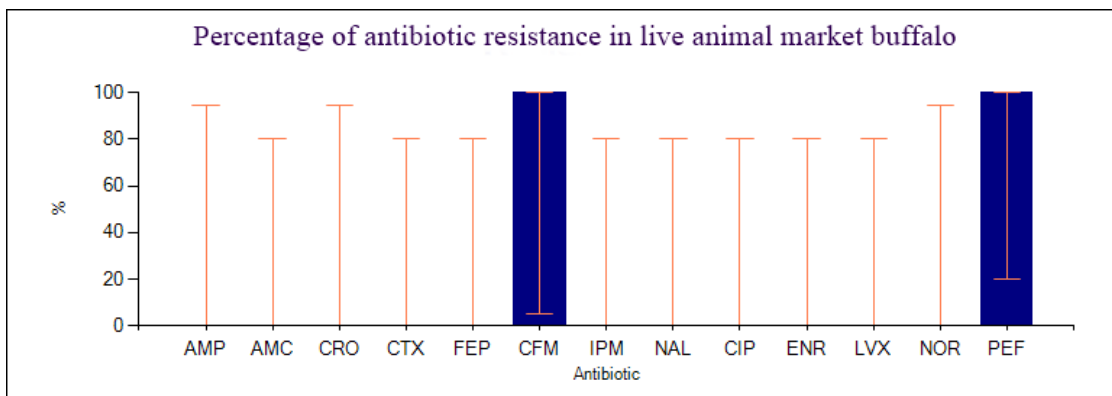
Figure 3.20: Histogram with confidence interval representing antibiotic resistance profile in live animal market buffalo sample

3.1.7.2 Minimum Inhibitory Concentration (MIC)

Broth dilution testing allows the option of providing both quantitative (MIC) and qualitative (category interpretation) results. MIC can be helpful in establishing the level of resistance of a particular bacterial strain and can substantially affect the decision to use certain antimicrobial agents.

Figure 3.21: Minimum Inhibitory Concentration (MIC) data analysis Picture; result was determining in Nanoplate with the help of GENE 5 2.09 software.11th column is free from antibiotic and 12th column free from antibiotic and bacterial inoculum.

3.1.7.2 .1 Antibiotic Tolerance Test in *Salmonella Spp*. Contaminated Meat Sample

Antibiotic Name	Minimum Inhibitory Concentration(MIC)μg/ml									
	Breakpoint CLSI-2016		Live Animal Market							
			Chicken		Beef		Mutton		Buffalo	
	S	R	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Amoxicillin	NA	NA	1024	>1024	1024	>1024	128	>1024	1024	>1024
Flucloxacillin	NA	NA	512	1024	512	1024	256	512	512	>1024
Cephradine	NA	NA	256	1024	256	512	32	64	512	>1024
Ceftriaxone	≤1	≥4	≤2	8	≤2	≤2	≤2	8	≤2	4
Cefixime	≤1	≥4	≤2	512	≤2	1024	16	>1024	≤2	512
Ciprofloxacin	≤1	≥4	4	32	≤2	8	≤2	32	≤2	8

Table 3.4: Result of Minimum Inhibitory Concentration (**MIC**);

MIC_{50}**:** Concentration of antibiotic (μg/ml) at which 50% of isolates were inhibited,

MIC_{90}**:** Concentration of antibiotic (μg/ml) at which 90% of isolates were inhibited.

Data were interpreted according to CLSI-2016 guideline; **NA**=No Define MIC value, MPM=Meat processing machine.

3.1.8 Phonotypic Multidrug Resistance(MDR) Profile

A total (n=48) meat sample was subjected to 13 antibiotics in β-lactam and quinolone. If a sample is resistant to three or more antimicrobial classes is called MDR (Multidrug Resistance)(Magiorakos et al., 2012).

Amoxicillin/Clavulanic acid (AMC), Ampicillin (AMP), Cefixime (CFM), Ceftriaxone (CRO), Cefotaxime(CTX), Cefepime(FEP), Imipenem (IPM), Levofloxacin(LVX), Nalidixic acid (NAL), Norfloxacin (NOR), Pefloxacin (PEF), Enrofloxacin (ENR) and Ciprofloxacin (CIP) all of the antibiotics was used at a time for positive isolates to determine antibiotic resistance profile.

All of the Sample are MDR (Multidrug Resistance) positive, ≥4 to12 ESBLs and Quinolone drug are resistance in meat sample.

In chicken sample 27 types of antibiotic resistance combination was occurred. (LVX, NAL, PEF, ENR, CIP)-2; (AMP LVX NAL NOR PEF ENR CIP)-2; (AMC, AMP, LVX, NAL, NOR, PEF, ENR and CIP)-2; (AMC, AMP, CTX, LVX NAL NOR PEF ENR and CIP)-2 and (AMP, IPM, LVX, NAL, NOR, PEF, ENR and CIP)-3 these type of combination had seen more than one time. But different type of resistance combination had seen in beef, mutton and buffalo sample.

3.1.9 Genotypic Drug resistance:

According to beta lactamase and quinolone phenotypically resistance sample were sorted out from all positive samples, then perform beta lactamase, quinolone and other antibiotic resistance encoded gene identification test by primer based PCR technique. ESBLs (n=10) and Quinolones (n=3) antibiotics encoded genes were examined in this study.

3.1.9 .1 ESBLs (Extended spectrum beta-lactamases) encoded resistance gene prevalence

3.1.9 .1.1 Multiplex PCR of *bla TEM*, *bla SHV* and *bla OXA*-1 like **genes result**

Phenotypically resistance beta lactam antibiotics (e.g. ampicillin) most commonly shown beta lactamase encoded gene *bla TEM* and *bla OXA-1.* *bla TEM* -type are the most prevalent beta-lactamases in enterobacteria; they hydrolyze the beta-lactam bond in susceptible beta-lactam antibiotics, thus conferring resistance to penicillins and cephalosporins.

Overall,89%retail meat samples (Chicken 24%, Beef 40%, Mutton 100% Buffalo 100%)was shown *bla TEM* (*bla TEM -1* and *bla TEM -2*) positive and 20%retail meat sample (Chicken 4%, Beef 20%, Mutton 100% Buffalo 100%).But *bla SHV* (*bla SHV* variants including *bla SHV-1*) were not present.
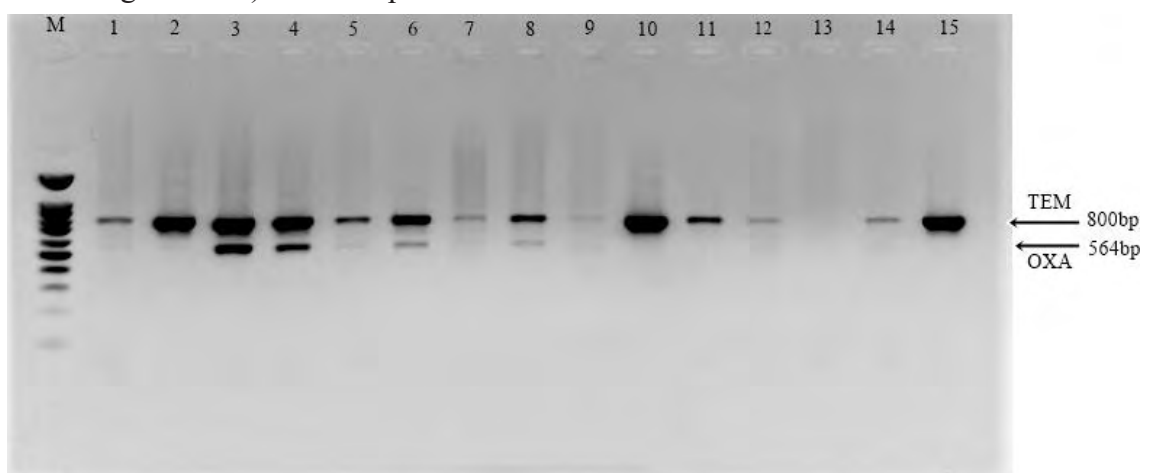
Figure 3.22: Multiplex PCR, gel electrophoresis picture of *bla TEM, bla SHV* and *bla OXA-1-like* β-lactamases encoded gene

3.1.9 .1.2 Multiplex PCR of bla CTX-M gens result

In many parts of the world and *CTX-M* type enzymes have become the most dominant ESBLs (Bonnet, 2004). Of particular concern is the emergence and dissemination of *CTX-M* family ESBLs among E. coli within the community (Pitout and Laupland, 2008). The *CTX-M* family is composed of more than 80 heterogeneous ESBLs and can be divided into five different groups (*CTX-M-1, M-2, M-8, M-9* and *M-25*) based on amino acid sequence similarities. Within each group, ESBLs share greater than 90% sequence identity(Bonnet, 2004).

In *bla CTX-M group 1*(*bla CTX-M-1, bla CTX-M-3* and *bla CTX-M-15*) overall 26 % retail meat samples (chicken 24%, beef 20% and mutton 50%) was shown positive, *bla CTX-M group-2* (*bla CTX-M-2*) 26% retail meat sample (chicken 12%, beef 40%, mutton 75% buffalo 100%) was shown positive, *bla CTX-M group-9* (*bla CTX-M-9* and *bla CTX-M-14*) 26% retail meat sample (chicken 8%, beef 40%, mutton 50% buffalo 100%) was shown positive and *bla CTX-M group-8/25* (*bla CTX-M-8* and *bla CTX-M-25, bla CTX-M-26* and *bla CTX-M-39* to *bla CTX-M-41*) 37% retail meat sample (chicken 32%, beef 60% and mutton 50%) was shown positive.
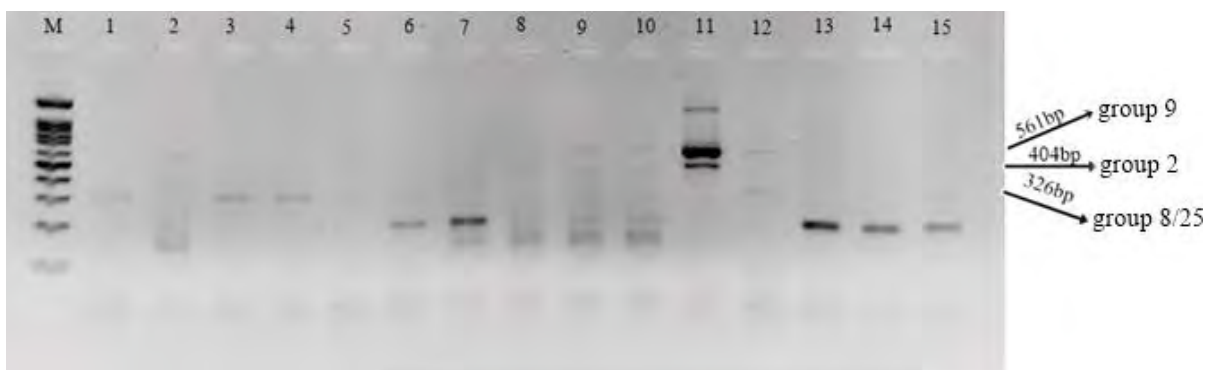
Figure 3.23: Multiplex PCR, gel electrophoresis picture of *bla CTX-M* group 1 (including *bla CTX-M-1, bla CTX-M-3* and *bla CTX-M-15*), variants of *bla CTX-M* group 2 (including *bla CTX-M-2*), variants of *bla CTX-M* group 9 (including *bla CTX-M-9* and *bla CTX-M-14*) *bla CTX-M group 8/25*(*bla CTX-M-8, bla CTX-M-25, bla CTX-M-26* and *bla CTX-M-39* to *bla CTX-M-41*) β-lactamases encoded gene.

3.1.9 .1.3 Multiplex PCR of *bla* IMP, *bla* VIM and *bla* KPC

Carbapenemases are a member of molecular classes A, B and D β-lactamases, which could hydrolyze β-lactam antibiotics. Class B carbapenemases, metallo-B-lactamases (MBLs), are resistant to β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam, but sensitive to inhibition by metal ion chelators such as Ethylene Diamine Tetra-acetic Acid (EDTA), a chelator of Zn^{2+} or other divalent cations. Metallo-B-Lactamases are classified to two major groups, IMP and VIM. Metallo-β-lactamase of the IMP are clinically important and active against many β-lactam antibiotics such as carbapenems (Karlowsky et al., 2003, Giakkoupi et al., 2003).Another gene bla KPC, Ambler class A enzymes KPC-type enzymes in carbapenem-resistant (Ambler et al., 1991)

Overall 29% retail meat samples (Chicken 32%, Beef 20% and Mutton 25%) was shown *bla* IMP variant (except *bla* IMP-9, *bla* IMP-16, *bla* IMP-18, *bla* IMP-22 and *bla* IMP-25) positive, In *bla* VIP variant (including *bla* VIM-1 and *bla* VIM-2) 29% retail meat samples (Chicken 32%, Mutton 25% buffalo 100%) was shown positive and bla KPC (*bla* KPC-1 to *bla* KPC-5) 11% retail meat samples (Beef 20% and Mutton 75%) was shown positive.
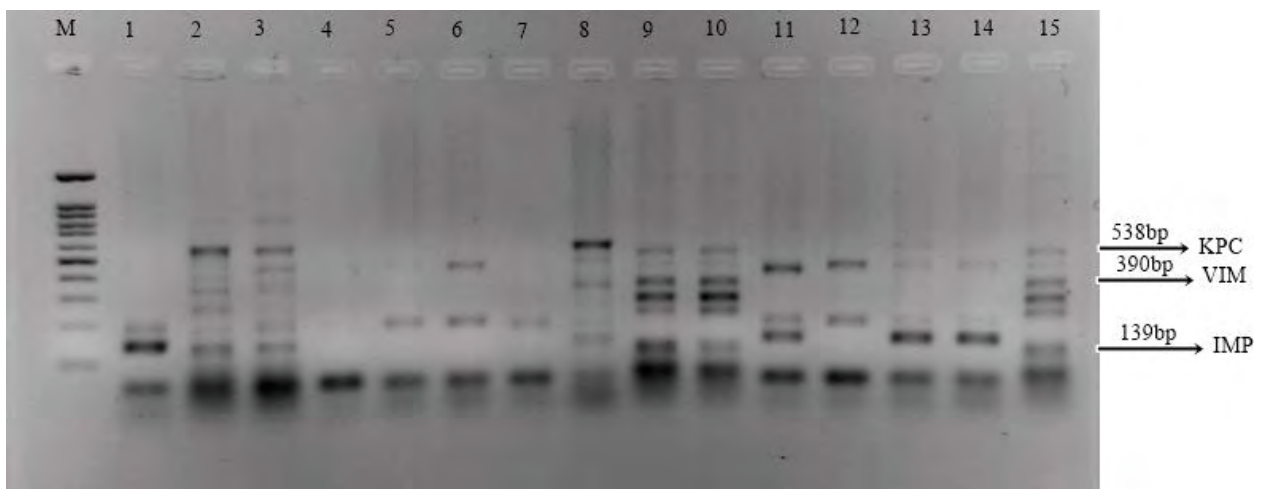
Figure 3.24: Multiplex PCR, gel electrophoresis picture of *bla* IMP (*bla* IMP variants except *bla* IMP-9, *bla* IMP-16, *bla* IMP-18, *bla* IMP-22 and *bla* IMP-25) *bla* VIM (*bla* VIM variants including *bla* VIM-1 and *bla* VIM-2) *bla* KPC (*bla* KPC-1 to *bla* KPC-5) β -lactamases encoded gene.

3.1.9 .2 Quinolone resistance gene prevalence

3.1.9 .2 .1 Multiplex PCR of *GyrA* and *GyrB* gene

Quinolonesare a large and widely used class of synthetic drugs. Expanded-spectrum quinolones, like ciprofloxacin are highly effective against Gram-negative bacteria. The major target for quinolones is DNA gyrase. This enzyme is composed of two subunits, GyrA and GyrB encoding by *gyrA* and *gyrB*, respectively. Mutations in either of these genes cause quinolone resistance. Mutations in quinolone resistance determining region (QRDR) section of *gyrA* are more common in quinolone resistant clinical isolates. However, a mutation outside of this region was also reported.

Overall,80% retail meat samples (chicken 72%, beef 100%, mutton 100% buffalo 3%); was shown GyrA positive and Only 32% chicken was shown GyrB positive.
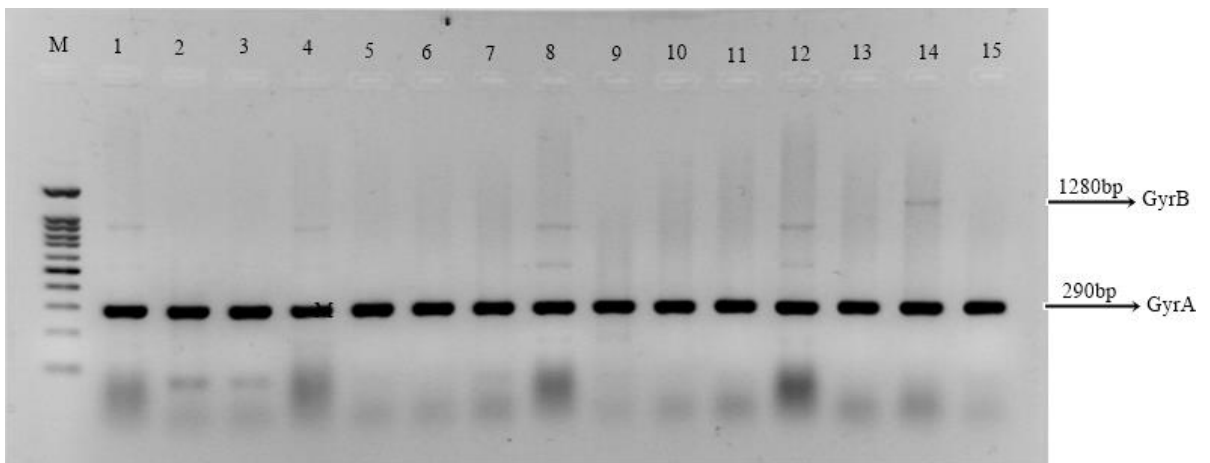
Figure 3.25: Multiplex PCR, gel electrophoresis picture of *GyrA* and *GyrB* gene

3.1.9 .2 .2 PCR of quinolone resistance *parc* gene

Overall,20% retail meat samples (Chicken 16% and mutton 75%) was shown *parc*

positive
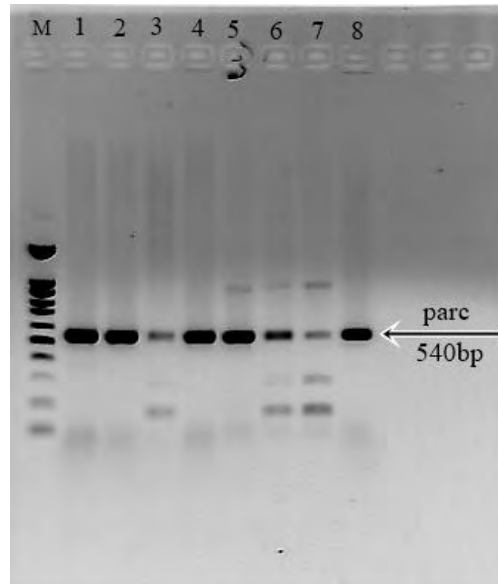
Figure 3.26: Gel electrophoresis picture of *parc* gene

3.1.10 Multiple Antibiotic Resistance Gene Profile:

According to phenotypic resistance of β-lactam and quinolone group antibiotics PCR was performed to identify the antibiotic resistance gene. In *Salmonella* meat isolate 80% sample was shown presence of multiple antibiotic resistance genes. Highly resistance quinolone isolates were shows *GyrA* and *GyrB* gene presence at a time. A total 27 combination of resistance gene was observed in retail meat sample.

3.2 Discussion

Salmonella is a notorious human pathogen and can lead to acute intestinal disease outbreaks in humans through consumption of contaminated foods (Kunwar et al., 2013). Salmonellosis is caused by *Salmonella* species; it is a socioeconomic problem for many countries. salmonellosis is a zoonotic disease which transmitted by food, especially by retail meat (Control et al., 2005). Plating on XLD, SS agar, and BS agar after pre-enrichment followed by selective enrichment in RV medium has been recommended for the isolation of *Salmonella* from foods by the U.S. FDA (Andrews, 1993). However, XLD has a high sensitivity and specificity, Proteus and Citrobacter produce colonies indistinguishable from those of *Salmonella* on this medium (Cooke et al., 1999, Rambach, 1990, Tate et al., 1990).

Retail meat sample was enriched and inoculated on *Salmonella*-selective media for their isolation. From selective XLD and SS agar media, we presumably isolated *Salmonella* like bacteria. Red, a black-centered colony on XLD and colorless, black centered on SS agar dictates us to select. Standard cultural methods for isolation of *Salmonella* from the sample were labor-intensive and time-consuming, requiring a minimum of 4-5 days to obtain presumptive evidence of *Salmonella.* Early studies showed that direct selective enrichment or direct plating were often unsuccessful for the detection of *Salmonella* because the bacterium may be in a stressed state, damaged by processing procedures such as freezing, chilling or heat. The isolation of *Salmonella* from meat can be difficult because the target bacterium may be present in low numbers, and they are often in the presence of high numbers of non-*Salmonella* (Prendergast et al., 2009). Pre-enrichment in a non-selective broth medium (buffered peptone water) provided growth and multiplication of indigenous bacterial flora as well as the accompanying micro-flora and resuscitation and proliferation of stressed or injured *Salmonella* to detectable levels.

In this study, Among the total sample, 17%(n=55) Live animal market sample were shown positive *Salmonella* characteristic in XLD media. At the same n= 57 live animal market SS agar plates, isolates showed *Salmonella* typical colony characteristics. It has been found that both Proteus and *Salmonella* shows the same type of colony characters on selective media XLD agar and SS agar. Some strains of Proteus and *Salmonella* were identified on non-selective nutrient agar media, as Proteus produce swarming colonies on it but *Salmonella* did not. But most of them could not be differentiated. So, further

characterizations of these isolates were needed through various biochemical tests and molecular identification.

All of the isolated both showing *Salmonella*-specific and non-specific colony characteristics were examined for biochemical characterizations. In this study, the bacterial isolates were considered as *Salmonella* by various biochemical tests such as TSI, MR, VP, Indole, Sugar (fermentation of glucose, lactose, sucrose, and mannitol) test Oxidase test and Catalase test. After performing all these biochemical tests, we have found around (n=50) isolates only show *Salmonella*-specific biochemical characteristics

invA gene-based molecular detection was shown overall 15% (Chicken 13%, Beef 12.5%, Mutton 28.3 %, and buffalo 40%) of 328 live animal market samples were *Salmonella* positive in this study. A previous study on poultry meat the prevalence of *Salmonella spp.* 31.66% at Gazipur and Mymensingh local market in Bangladesh (Al-Salauddin et al., 2015),which indicating present study prevalence is lower than previous study in chicken. The incidence of *Salmonella* has been studied in retail meat in many countries such as Greater Washington, D.C. Area (4.2% chicken,2.6% Turkey,3.3%Pork and Beef 1.9%) (Zhao et al., 2001),Northern China (26.7% pork,15.8% chicken,33.3% beef and 33.3% mutton)(Yan et al., 2010) China (54% chicken, 31% pork, 17% beef and 20% lamb)(Yang et al., 2010), Vietnam(69.9% pork , 48.6% beef , 21.0% chicken , 22.3% duck)(Phan et al., 2005), South Korea (2.0% beef, 8.9% pork, and 42.3% chicken meat)(Hyeon et al., 2011) Colombia (26% chicken meat)(Donado-Godoy et al., 2012). *Salmonella* prevalence in retail meat, compare this study showing moderately high, low or identical among the different country.

Salmonella spp. can be differentiated into two major groups typhoidal *salmonella* (TS) and non-typhoidal *Salmonella* (NTS) on the basis of diseases and distinct immune responses in humans (Gal-Mor et al., 2014). In this study, *Salmonella* were isolated where typhoidal 58% and nontyphoidal 42% *Salmonella* was present. Higher level of typhoidal *Salmonella* (TS) and non-typhoidal *Salmonella* (NTS) was prevalence observe than previous study reports (Faleke et al., 2017, FDA, 2016). Where the overall typhoidal *Salmonella* prevalence was quite low than other studies (Ren et al., 2017).

Antimicrobial resistance is a global issue, abuse of antibiotic in meat producing animals may be increasing AMR foodborne pathogen which transmitted to human by

contaminated food (White et al., 2002).A total 13 ESBLs and quinolone group antibiotics were used to carry out this study. Highly resistance β-lactam (Ampicillin 58%) and quinolone (Pefloxacin 100%, Enrofloxacin 78%, Nalidixic acid 74% and Ciprofloxacin 67%) antibiotic was observed in the retail meat. Whereas several other studies also showed high prevalence Ampicillin 22.9% resistance (FDA, 2016). Another antibiotics β-lactam (Amoxicillin/Clavulanic acid 26%, Cefotaxime 24%, Cefepime 9%, Cefixime 7% and Ceftriaxone 4%), Carbapenems (Imipenem 11%) and quinolone (Levofloxacin 41% and Norfloxacin 38%) was comparatively low in retail meat. In 2007-2008 study in china retail meat sample resistance to Nalidixic acid 35% and Ciprofloxacin 21%was lower than present study but high in ceftriaxone 16% resistance (Yang et al., 2010). Whereas similar level of resistance observed according to US. FDA National Antimicrobial Resistance Monitoring System (NARMS) database 2017 (FDA, 2017). This   study Confession that, Bangladesh is not behind the antibacterial resistance.

Almost all *Salmonella spp.* have showed multidrug resistance (MDR). In 13 antibiotics 35 type of drug resistance combination was pragmatic among *Salmonella* positive sample, which indicating higher prevalence of MDR in Bangladesh than other part of the world and increasing day by day (Zaki and Karande, 2011, Baltazar et al., 2015).

Genetic Antibiotic resistance is a result of intrinsic (mutation of normal cellular genes) or extrinsic (acquisition of foreign resistance genes) mechanism of microorganism genes, otherwise a combination of these two mechanisms (Harbottle et al., 2006). Almost 13 antibiotic resistance encoded genes (Beta-lactam, penems and Quinolone) with 27 different types of combination was observed in this study. *bla$_{TEM}$* 89% and *GyrA* 80% gene was observed ample amount in meat sample. In ESBLs encoded resistance gene, *bla$_{CTX-M\ Group-4}$(bla$_{CTX-M-8}$, bla$_{CTX-M-25}$, bla$_{CTX-M-26}$ and bla$_{CTX-M-39}$ to bla$_{CTX-M-41}$)* 37%, *bla$_{IMP}$* 29%, *bla$_{VIM}$* 29%, *bla$_{KPC}$* 11%, *bla$_{CTX-M-Group-1}$(variants of bla$_{CTX-M}$ group 1 including bla$_{CTX-M-1}$, bla$_{CTX-M-3}$ and bla$_{CTX-M-15}$)* 26%, *bla$_{CTX-M-Group-2}$(bla$_{CTX-M-2}$)*, *bla$_{CTX-M-Group-3}$(variants of bla$_{CTX-M}$ group 9 including bla$_{CTX-M-9}$ and bla$_{CTX-M-14}$)*20%, *bla$_{OXA}$* 20% was present in study sample. Among the phenotypically quinolone resistance antibiotic isolates were shown *GyrB* 23% and *parC* 20% quinolone resistance genes presence in Dhaka city meat sample. In highly resistance quinolone sample was carrying *GyrA* and *GyrB* gene. *bla$_{TEM}$*, gene was dominant among the resistance isolates according to the US. FDA database (FDA, 2016)

Chapter
04
Conclusion

CHAPTER 04: CONCLUSION

Presence of both typhoidal (42%) and Non-typhoidal (58%) in retail meat. Which can lead to food borne illness as well as enteric diseases. An alarming level of multidrug resistance (MDR) *Salmonella* (100%) observed in retail meat which embedding pathogenicity. More than 12 plasmid mediated β-lactam and quinolone antibiotic resistance gene were found in this pathogen which was highly associated with phenotypic resistance. It is obvious from this study that the high frequency of MDR *Salmonella* carrying mobile drug-resistance gene is an ultimate threat to public health specially in Dhaka city. This observation indicates possibly poor hygiene during slaughtering meat processing as well as imprudent use of antibiotic in livestock. So more extensive study is required to define the cause.

4.1 Recommendations for future work:

The present study was confined to retail shop distributed meat samples so that origin of *Salmonella* and genetic profile cannot be ascertained. Right from location of animals, slaughtering storage distribution storage at retail shops and distribution spot are all reservoirs of contamination and such environmental factors in addition to the antibiotic treatment may affect the extent of *Salmonella* contamination. Genetic analysis of meat samples at such different spot would give more clear picture of *Salmonella* origin and distribution. Future work in this regard may include:

I.   Larger sample size (present study was conducted with 328 samples of chicken, beef, mutton and buffalo).

II.   Distribution of *Salmonella* at different spots from the meat collected there and distribution of *Salmonella* serotype.

III.   Root case analysis (Specific antibiotic treatment with feed at the location of collection and correlating with *Salmonella* distribution, serotype, phenotypic and genetic analysis) in meat producing animal for antimicrobial resistance.

IV.   More detailed study with chicken samples on a larger scale to authenticate the present finding.

V.   Development of a model for proffer feed ingredients with minimum specific antibiotic for reducing the antibiotic resistance.

For the purpose of public health, use of antibiotics in feed ingredients may be drastically reduced or avoided as growth promoters for animals and apparently healthy animals may cause more harm to the consumers of meat. Recently formed Bangladesh Food Safety Commission may take necessary initiative in this regard in cooperation with veterinary officials.

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APPENDIX- I

Sample Collection form or Questioner

Bangladesh Live Stock Research Institute (BLRI), Saver Dhaka, Bangladesh

Food Safety Laboratory (Prevalence of Salmonella in chicken from live bird market on retail condition)

Sample Collection Date	Name of sample collection place:		
Sample Basic Information:			
Sample source	Local	Broiler	others
Sample type	Meat	Intestinal Swab	Environmental Swab
Sample treatment process	By worker hand		Dressing by hot water
Sample Details Information :			
Chicken Meat	• Leg • Chest • Liver	Intestinal Swab	• Upper part • middle part • Lower part	Environmental Swab	• Hand ,knives ,chopping board • waste been, Water, • Floor ,Case of bird ,Feed
Sample Background Information:			
Chicken supplier information	Name of supplier		Supplier Location
Chicken age			
Antibiotic Treatment for chicken	When Antibiotic was given		What types Antibiotic Use and Name
Sample Collector Name and Date			
Opinion			
Customer			
Shopkeeper			
Remark			

APPENDIX-II

Appendix-II (A): Microbiological Media

- A-01: Buffered peptone water (Oxoid, England)

Ingredients	Amount(g/L)
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5
pH 7.2 ± 0.2 @ 25°C	

- A-02: Rappaport-Vassiliadis medium (Oxoid, England)

Ingredients	Amount(g/L)
Soya peptone	5.0
Sodium chloride	8.0
Potassium dihydrogen phosphate	1.6
Magnesium chloride 6H_2O	40.0
Malachite green	0.04
pH 5.2 ± 0.2 @ 25°C	

- A-03: Xylose lysine deoxycolate (XLD) agar (Oxoid, England)

Ingredients	Amount(g/L)
Yeast extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium desoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5
pH 7.4 ± 0.2 @ 25°C	

- A-04: *Salmonella Shigella (SS)* agar (Oxoid, England)

Ingredients	Amount(g/L)
`Lab-Lemco' powder	5.0
Peptone	5.0
Lactose	10.0
Bile salts	8.5
Sodium citrate	10.0
Sodium thiosulphate	8.5

Ingredients	Amount(g/L)
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	15.0
pH 7.0 ± 0.2 @ 25°C	

- A-05: Peptone Water (Oxoid, England)

Ingredients	Amount(g/L)
Peptone	10.0
Sodium chloride	5.0
pH 7.2 ± 0.2	

- A-06: MRVP Medium (Oxoid, England)

Ingredients	Amount(g/L)
Peptone	7.0
Glucose	5.0
Phosphate buffer	5.0
pH 6.9 ± 0.2	

- A-07: Nutrient Agar (Oxoid, England)

Ingredients	Amount(g/L)
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
pH 7.4 ± 0.2 @ 25°C	

- A-08: Nutrient Broth (Oxoid, England)

Ingredients	Amount(g/L)
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH 7.4 ± 0.2 @ 25°C	

- A-09: Luria Bertani Broth, (Himedia)

Ingredients	Amount(g/L)
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Final pH (at 25°C) 7.5±0.2	

Appendix-II(B): Chemical & Reagent

- B-01: Phosphate buffered Saline (PBS)

Formula	Amount
KCl	0.2g
Na2HPO4	1.44g
NaCl	8.0g
KH2PO4	2.0g
Water	1L

- B-02: 10 x TBE

Formula	Amount
Tris-base	54.0g
Boric acid	27.5g
EDTA (0.5 M)	20ml
Water upto	500ml

- B-03: Gel loading buffer (10X)

Formula	Amount
Ficoll (20%)	800μl
EDTA (0.1M)	400μl
Bromophenol blue (0.25%)	10μl
SDS (1%)	200μl
Water	590ml

- B-04: Ethidium bromide solution (Sigma, USA)

Formula	Amount
Ethidium bromide	2.5g
Water	5ml

- B-05: McFarland Solution

Formula	Amount
BaCl2.2H2O (1.175%)	0.5ml
Water	99.5ml

Appendix-II(C): Instruments

Important instruments those were used in current study are given below

Instruments	Company
Autoclave, Model No: SA-300V	STURDY, Taiwan
Centrifuge, Model:5804	Eppendorf, Germany
Stomacher, Model: 80	Seward,USA
Class II Microbiological Safety Cabinet	Gelman Science, US
DNA Sequencer, Model: 3130 Genetic Analyzer	Applied Biosystems, US
Freezer (-80oc)	A ngelentoni, UK
Gel Electrophoresis, Model: Mupid®-One	Mupid Co., Ltd , Japan
Gel Documentation, Model: Alphagram mini	Protein Simple , U S
Incubator, Model: CB-150	Binder , Germany
Light microscope, Model: CX41	Olympus Corp., Japan
Metter balance, Model: HR-200	METLER, Switzerland
Micropipettes	Labsystem, Finland
Microplate Spectrophotometer, Model: Power Wave-xs	BioTek, US
Oven Drier, Model: ED-115	Binder, Germany
PH Meter, Model: Mp220	Hach, US
Refrigerator (-20oC), Model: 360LTR	Samsung, South Korea
Spectrophotometer, Model: NanoDrop 2000c	ThermoFisher Scientific, US
Real-Time qPCR System, Model: Mx3005p	Stratagene California, US
Water Bath, Model: Sub6	England