Prevalence and Multidrug Resistance Pattern of *Salmonella* Isolated from Stray Dogs

A CLINICAL REPORT SUBMITTED
BY

Intern ID: D-39

Roll No: 08/105

Registration No: 367

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Chittagong Veterinary and Animal Sciences University

Khulshi, Chittagong-4225

January, 2014

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS USED

Abbreviation and symbol	Elaboration
BGA	Brilliant Green Agar
CDDEP	Center for Disease Dynamics, Economics & Policy
CLSI	*Clinical and Laboratory Standards Institute*
CS	Culture Sensitivity
^{0}C	*Degree Celsius*
DNA	Deoxyribonucleic Acid
EMB	Eosin Methylene Blue
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
hrs	Hours
I	Intermediately Sensitive
ICMSF	International Commission on Microbiological Specifications for Food
Ltd.	Limited
mcg	microgram
µg	Microgram
mg	milligram
ml	milliliter
mm	millimeter
NARMS	National Antimicrobial Resistance Monitoring System
NIAD	National Institute of Allergy and Infectious Diseases
n	Number
OM	Outer Membrane
/	per
%	Percent
±	plus-minus
PRTC	Poultry Research and Training Center
P.	*Pseudomonas*
R	Resistant
S.	*Salmonella*
SS	*Salmonella* Shigella
S	Sensitive
SL	Serial
TSI	Triple Sugar Iron
WHO	World Health Organization
www	World Wide Web

PLAGIARISM CERTIFICATE

Myself Tofazzal Md. Rakib strongly assures that I have performed all works furnished here in this report. The Information's have been collected from books, national and international journals, websites and other references. All references have been acknowledged duly.

Therefore, I hold entire responsibility of collection, compilation, preservation and publication of all data accumulated here in this report.

The Author

January, 2015

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The author,

January, 2015

ABSTRACT

Salmonellosis is one of the most common and widely distributed global public health issues. During the last decade, multi-resistance of *Salmonella* spp. has increased a great deal, especially in developing countries with an increased and indiscriminate use of antibiotics in veterinary practice. Therefore, a cross sectional study was conducted to investigate the prevalence and antimicrobial resistance pattern in *Salmonella* isolated from rectal swab of stray dogs in the randomly selected 9 areas of Chittagong City Corporation to assess the risk of possible transmission of *Salmonella* from dogs to humans. Rectal swabs were collected for isolating *Salmonella* spp. (bacteriological culture methods) followed by antimicrobial susceptibility testing (disk diffusion method) against *Salmonella* isolates during the period April to July, 2014. Inv A gene specific PCR was done to detect *Salmonella* Typhimurium from isolated *Salmonella* spp. to evaluate the public health crises. Out of the 108 samples, the highest prevalence of *Salmonella* spp. was found in Ambagan and New Market (100%) and lowest (20%) in Pahartoli area and variation in prevalence among the sites differs significantly ($p<0.05$). On the other hand, prevalence was highest (66.67%) in samples of females than in males (58.93%) but the difference was not varied significantly ($p>0.05$). Among the 67 isolates, 10.45% Inv A gene was indicating presence of *Salmonella* Typhimurium. Isolated *Salmonella* was tested for resistance to twelve different antimicrobial agents, using disc diffusion method. Among twelve antimicrobial tested, 100% resistance were found to Amoxicillin followed by Azithromycin (91.67-100%), Cefixime (90-100%), Ampicillin (83.33-100%), Pefloxacin (83.33-100%)%), Potentiated Sulfonamide (66.67-100%), Tetracycline (50-100%), Colistin (50-100%), Gentamycin (0-100%) and Ceftriaxone (0-70%). Ceftriaxone remained sensitive in 29.85%, Gentamycin and Colistin appeared to be 22.39% and 7.46%, respectively. *Salmonella* isolates were multidrug resistance up to nine of the twelve antimicrobials tested. In conclusion, it can be said that the rational use of antibiotics need to be adopt in veterinary and human practice of Bangladesh to prevent the emergence of multi-drug resistance *Salmonella.* In addition, appropriate measures should be taken to prevent occurrence of zoonotic *Salmonella* spp. in human.

Keywords: Antimicrobial, prevalence, resistance, *Salmonella* typhimurium, stray dog, PCR

CHAPTER - I

INTRODUCTION

The discovery and development of the main groups of antibiotics, mankind appeared to gain the upper hand against the legion of bacteria responsible for much morbidity and mortality among humans and their animals, but the advantage was illusory, because the bacteria were quietly and efficiently evolving and acquiring resistance genes and resistance gene arrays that provided them with protection against the pharmacopoeia of antibiotics deployed to contain them. Bacteria constantly surprise us and no more so than within the arena of antibiotic resistance. Bacteria have access, in principle, to a large selection of resistance genes scattered throughout the bacterial kingdom and mechanisms have evolved to re-assort these genes, moving them genetically from one DNA molecule to another and physically from one bacterial cell to another. Indeed, bacteria can be considered to have access to a comprehensive genetic engineering tool kit that provides the potential to remodel and to mix and match resistance genes according to requirements. Our experience over the last 20–30 years indicates that this tool kit is in frequent use (Bennett, 1999). Antimicrobial resistance (AMR) is an emerging problem in companion animals, because of difficult-to-treat infections, possible pressure to use antimicrobials that are essential in human medicine, and potential zoonotic transmission (Scott Weese, 2008).In recent years, antimicrobial resistance in bacteria of animal origin, including food-producing animals, pet and companion animals, fish and other aquatic animals as well as wild animals, has gained particular attention (Schwarz *et al.*, 2010).

Antimicrobial resistance (AMR) amongst companion animals, particularly household pets and horses, is a complex area that is of increasing importance because of both patient factors and public health issues. In this context, a unique and critical aspect of AMR in companion animals is their close contact with humans. This creates opportunities for interspecies transmission of bacteria, including multidrug resistant (MDR) bacteria, so that it is possible and likely that the increasing rates of MDR bacterial infection and colonization in humans may be reflected in their close contacts, including companion animals (Scott Weese, 2008).

Prior to the 1990s, the problem of antimicrobial resistance was never taken to be such a threat to the management of infectious diseases. But gradually treatment failures were increasingly being seen in health care settings against first-line drugs and second-line drugs or more.

Microorganisms were increasingly becoming resistant to ensure their survival against the arsenal of antimicrobial agents to which they were being bombarded. They achieved this through different means but primarily based on the chemical structure of the antimicrobial agent and the mechanisms through which the agents acted. The resistance mechanisms therefore depend on which specific pathways are inhibited by the drugs and the alternative ways available for those pathways that the organisms can modify to get a way around in order to survive (Sosa *et al.*, 2009).

Veterinarians and public health officials have recognized shedding *Salmonella*e by dogs as a possible source of *Salmonella* infection for dog owners and their communities (Sanchez *et al.*, 2002; Kahrs *et al.*, 1978; Kozak *et al.*, 2003). The prevalence of *Salmonella*e in dogs in the community is not well established, as dogs can be asymptomatic carriers, capable of shedding the organism without exhibiting signs of illness. The prevalence of *Salmonella* isolation from clinically healthy and hospitalized dogs has been estimated to be between 1% and 35% (Galton *et al.*, 1969; Greene *et al.*, 1998). Clinical salmonellosis is rare in dogs, but clinical signs include fever (40°C-41.1°C), anorexia, diarrhea, bloody diarrhea, abdominal pain, and abortion (Marks *et al.*, 2003). Asymptomatic dogs can shed *Salmonella*e for 6 wk or more, con- tinuously during the 1st week, and then intermittently (Marks *et al.*, 2003).

Salmonella is the aetiological agent of both human and animal salmonellosis, a very common and widely spread enteric disease. It is a significant cause of acute and chronic diarrhoea and death in numerous animal species and in human beings (McGavin *et al.*, 2001). Salmonellosis is therefore of significant importance both in animal production and in public health. Although there are *Salmonella* serovars that are strictly host-restricted (such as *Salmonella* Typhi in humans*, S.* Gallinarum and *S.* Pullorum in poultry*,* and *S.* Dublin in cattle), the majority of other *Salmonella* serovars can infect a wide host range. However, faeces of nearly all animal species may be a potential source of *Salmonella*; therefore, the zoonotic transmission of *Salmonella* is not limited to food animals alone. Pets, especially dogs that have close interaction with humans, may be responsible for *Salmonella* transmission (Ojo and Adetosoye, 2009).

To our knowledge, however, there has been no previous study on antimicrobial resistance in fecal indicator bacteria from stray dogs in Bangladesh. *Salmonella* Spp. is an inhabitant of normal flora of the gastrointestinal tract of humans and animals, and is believed to facilitate food digestion through enzyme synthesis; however, few of them are potentially pathogenic

and known to be a very good indicator for selection pressure by antimicrobial use and for resistance problems to be expected in pathogens. There is paucity of information on the role of dogs as a potential source of *Salmonella* infection to humans despite an increase in dog-keeping among the elite living in the metropolitan cities. Considering the above facts present study was undertaken to fulfill the following aims and objectives:

1. To assess the prevalence of *Salmonella* Spp. in fecal isolates recovered from rectal swab samples of stray dogs in Chittagong City Corporation, Bangladesh.
2. To detect the prevalence of *Salmonella* Typhimurium in isolated *Salmonella* organisms from rectal swabs of stray dogs.
3. To determine the antimicrobial resistance patterns of *Salmonella* spp. isolates to twelve antimicrobial agents, to verify their multidrug resistance patterns, and to assess their significance as sources of infection.

CHAPTER - II

REVIEW OF LITERATURE

2.1 Characteristics, taxonomy and nomenclature of *Salmonella*

The large family Enterobacteriaceae includes gram-negative bacteria along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella, Escherichia coli, Yersinia pestis, Klebsiella* and *Shigella*. Other disease-causing bacteria in this family include *Proteus, Enterobacter, Serratia* and *Citrobacter*. This family is the only representative in the order Enterobacteriales of the class Gammaproteobacteria in the phylum Proteobacteria (George, 2005). *Salmonella* have been known to cause illnesses for more than 100 years when it was discovered by Dr. Daniel Salmon (Steve Yan *et al.*, 2004)**.** *Salmonella* are Gram-negative bacilli belonging to the Family *Enterobacteriaceae*. *Salmonella*, like most *Enterobacteriaceae*, are motile, non-spore forming, and facultative anaerobes that reduce nitrates to nitrites, ferment glucose, and are oxidase negative (Steve Yan *et al.*, 2004)**.** The genus *Salmonella* consists of only two species (Grimont and Weill, 2007), *Salmonella bongori and Salmonella enterica,* with the latter being divided into six subspecies (I-VI); *Salmonella enterica* subsp. *enterica* (I), *Salmonella enterica* subsp. *salamae* (II), *Salmonella enterica* subsp. *arizonae* (IIIa), *Salmonella enterica* subsp. *diarizonae* (IIIb), *Salmonella enterica* subsp. *houtenae* (IV), *and Salmonella enterica* subsp. *indica* (VI) (Popoff *et al.*, 2003; Tindall *et al.*, 2005). All *Salmonella* strains are serologically classified using Kauffmann-Whitescheme, and at the present the genus contains more than 2,500 serotypes (Popoff *et al.*, 2003; Tindall *et al.*, 2005). The majority of the *Salmonella* serotypes belong to *Salmonella enterica* subsp, *enterica* (about 60%), followed by subspecies *salamae* (20%), *diarizonae* (13 %), *arizonae* (3.8 %), *houtenae* (2.8%) and *indica* (0.45%). Only (0.8%) belong to the second species *Salmonella bongori* (Pignato *et al.*, 1998). Strains that belong to *Salmonella enterica subsp*.I (*Salmonella enterica* subsp. *entericae*), are frequently pathogenic to humans and mammals while those belonging to subspecies II, IIIa, IIIb, IV, VI and *Salmonella bongori* are usually isolated from reptiles and other cold blooded animals (Brenner *et al.*, 2000). The genus consists of two species: (1) *S. enterica* which is divided into six subspecies: *S. enterica* subsp. enterica, *S. enterica* subsp. salamae, *S. enterica* subsp. arizonae, *S. enterica* subsp. diarizonae, *S. enterica* subsp. houtenae and *S. enterica* subsp. indica; and (2) *S. bongori* (formerly called *S.* enterica subsp. bongori). The subspecies name

does not need to be indicated, as only serovars of subspecies enterica has a name. Therefore we write e.g. *Salmonella* Typhimurium (Hendriksen *et al.*, 2009).

2.2 Salmonellosis

Salmonellosis is primarily a food-poisoning syndrome, which occurs when ingesting pathogenic *Salmonella* serotypes. The cause of food-borne Salmonellosis is the penetration and passage of *Salmonella* organism from the gut lumen into the epithelium of the small intestine where inflammation occurs. There is also evidence that the pathogenesis may involve two toxins; an enterotoxin and a cytotoxin (Jay, 2000).

Salmonellosis is an infectious disease in both humans and animals. Human infections are usually associated with animal contact and the consumption of contaminated food products such as poultry, meat and other dairy products (Uyttendaele *et al.*, 1998). Salmonellosis is usually considered as self-limiting illness, but it can also become invasive and fatal, especially for patients who are young or immune-compromized (Wilson *et al.*, 2003). Non-typhoidal *Salmonella* strains are imperative causes of infections in both humans and animals. This disease is caused by *Salmonella* serotypes other than *Salmonella typhi* and *Salmonella paratyphi*. It is a major food-borne infection with worldwide distribution. The majority of cases are self-limiting gastroenteritis (Kariuki *et al.*, 2002). The clinical symptoms usually appear 8 to 72h after contact with the pathogen. The typical symptoms are usually nausea, vomiting, abdominal pain and diarrhea with or without fever. Few (<5%) of the patients develop invasive *Salmonella* infections or bacteremia and about 10% of those with invasive disease develop localized infections (Steve Yan *et al.*, 2004). During the past decade, there had been a significant world-wide increase of non-typhoidal Salmonellosis especially in industrialized countries including, the United Kingdom, Germany, France, Austria, Denmark, and the United States of America. In the US, 1.3 million illnesses and 400 to 600 deaths were occurred in each year (Mead *et al.*, 1999). The most common serotypes responsible for the disease are *Salmonella* Enteritidis and *Salmonella* Typhimurium (Herikstad *et al.*, 2002).

2.2.1 Salmonellosis in Companion Animals

Infectious enteric pathogens have long been recognized as a significant problem owing to their pathogenicity potential to animals and their zoonotic risk to humans. Among them, two gastrointestinal bacterial pathogens, *Salmonella*e and campylobacters have been considered to be important food-borne pathogens causing human enteritis worldwide and leading to serious public health concern (Ethelberg *et al.*, 2004). *Escherichia coli* and *Salmonella*e are

important pathogens, causing gastrointestinal infections and septicaemia in humans and animals, and a range of secondary conditions including respiratory tract infections in animals. *Salmonella* gastroenteritis is usually only treated with fluoroquinolones when the patient is elderly or immuno-compromised, but antimicrobials are also used for treatment of patients with enteric fever, bacteraemia, metastatic infections or long-term *Salmonella* carriage (Hopkins *et al.*, 2005). In addition to causing enteritis, these organisms have also been reported in association with bacteraemia, reactive arthritis, and meningitis (Goldberg and Rubin, 1988; Peterson, 1994). Most dogs are asymptomatic when they act as reservoirs shedding *Salmonella*e or campylobacters in their faeces. Pathogens in their faeces may ultimately infect other animals by contaminating the environment (Morse and Duncan, 1975; Hald and Madsen, 1997). Recently, among immuno-compromised populations, i.e. those using immunosuppressive drugs having acquired immunodeficiency syndrome and the elderly these bacteria have become a great pathogenic risk (Robinson and Pugh, 2002). The prevalence of *Salmonella* spp. in stray dogs in New Zealand (Timbs *et al.*, 1975) and Sudan (Khan, 1970) has been reported to be 5.5% and 23.5%, respectively. However, Ojo (1994) failed to detect *Salmonella*e in the intestinal contents of stray dogs in Trinidad, West Indies. In Japan, 5.9% of stray or unwanted apparently healthy dogs were positive for the presence of *Salmonella*e in their intestinal contents and various serovars except S. Corvallis were identified (Fukushima *et al.*, 1985). In the present study, *Salmonella*e were isolated from 11% of stray dogs examined. This isolation rate is between the lowest 0.0% (Ojo, 1994) and the highest 23.5% (Khan, 1970) prevalence values of *Salmonella*e in stray dogs mentioned above. The differences in the sample sizes of dogs, year of sampling, type of faecal sample, geographical properties, and sampling strategies and isolation methods performed in the various countries may all affect the prevalence (Seepersadsingh *et al.*, 2004).

2.3 Public Health Significance

The presence of *Salmonella* in pet dogs makes them a potential source of infection to their human companions. Cases of dog to human transmission of *Salmonella* resulting in severe infection in the latter have been reported (Morse and Duncan, 1975). Dog-keeping households should be aware of this fact. Dogs might acquire the infection from their food sources and subsequently pass the infection on to their human companions. Contaminations of the immediate, shared-environment and household utensils as well as direct transmission through handling are some of the ways humans can acquire *Salmonella* from dogs. Close intimacy between dogs and human could facilitate easy transmission of *Salmonella* between

them (Ojo and Adetosoye, 2009). The current global scenario shows an increased incidence of antimicrobial resistance in *Salmonella* spp. from humans and animals. This creates a major public health concern that *Salmonella* spp. could become resistant to antibiotics used in human medicine, thus reducing therapeutic options and threatening the lives of infected individuals (Murray, 1986; Lee *et al.*, 1994). *Salmonella enterica*, Gram-negative, non-sporing, catalase-positive, oxidase-negative facultative anaerobic bacilli is a significant cause of morbidity and mortality in humans and animals, with multidrug-resistant *S. enterica* serovar Typhimurium being an emerging problem (Abouzeed *et al.*, 2000; Hendriksen *et al.*, 2004; Steve Yan *et al.*, 2004). *Salmonella* Typhimurium is a well-known zoonotic pathogen causing diarrhoea, pyrexia, and septicaemia in animals and humans. Non-typhoid *Salmonella serovars* remain a potential threat to human health, and beef cattle and broiler chickens are possible sources of these organisms in the environment. Although non-typhoidal salmonellosis in humans is usually a self-limiting disease confined to the intestinal tract, when infections spread beyond the intestine, or when immunocompromised persons are affected, it may have serious consequences requiring appropriate antimicrobial treatment. In animals, such symptoms can be lethal; so, prompt treatment with appropriate antimicrobial agents remains economically important (Abouzeed *et al.*, 2000).

*Salmonella*e are one of the most important food-borne pathogens. Owing to a high prevalence in livestock, they tend to spread through the food chain, exposing humans to the risk of salmonellosis (Kozoderović *et al.*, 2012). Non-typhoid *Salmonella* infection is one of the main zoonotic diseases in developed (Esaki *et al.*, 2004; Fisher and Threlfall, 2005) and developing countries (Bangtrakulnonth *et al.*, 2004). Multidrug-resistant (MDR) *Salmonella* isolates are a direct threat to human health when this multidrug resistance interferes with treatment and an indirect threat when resistance can be transferred to other human pathogens (FAO, 2003). Since 1981 the incidence of multiresistant strains isolated from man in England and Wales has doubled for *S.* Typhimurium and increased 50-fold for *S. virchow,* but remained constant for *S.* Enteritidis. For the remaining serotypes there has been a small overall increase in multi-resistant isolates (Ward *et al.*, 1990).

2.4 Mechanisms of Action of Antimicrobial Agents

Antimicrobial agents act selectively on vital microbial functions with minimal effects or without affecting host functions. Different antimicrobial agents act in different ways. The understanding of these mechanisms as well as the chemical nature of the antimicrobial agents is crucial in the understanding of the ways how resistance against them develops. Broadly,

antimicrobial agents may be described as either bacteriostatic or bactericidal. Bacteriostatic antimicrobial agents only inhibit the growth or multiplication of the bacteria giving the immune system of the host time to clear them from the system. Complete elimination of the bacteria in this case therefore is dependent on the competence of the immune system. Bactericidal agents kill the bacteria and therefore with or without a competent immune system of the host, the bacteria will be dead. However, the mechanism of action of antimicrobial agents can be categorized further based on the structure of the bacteria or the function that is affected by the agents (Sosa *et al.*, 2009).

Table 2.1: Summary of mechanisms of action of antimicrobial agents.

Group of antimicrobial agents	Effect on bacteria	Mode of action in general
Penicillins	Bactericidal	Inhibition of cell wall synthesis
Cephalosporins	Bactericidal	Inhibition of cell wall synthesis
Carbanepems	Bactericidal	Inhibition of cell wall synthesis
Polypeptide antibiotics	Bactericidal	Inhibition of cell wall synthesis
Quinolones	Bactericidal	Inhibits DNA synthesis
Metronidazole	Bactericidal	Inhibits DNA synthesis
Rifamycins	Bactericidal	Inhibitions of RNA transcription
Lincosamides	Bactericidal	Inhibition of protein synthesis
Aminoglycosides	Bactericidal	Inhibition of protein synthesis
Macrolides	Bacteriostatic	Inhibition of protein synthesis
Tetracyclines	Bacteriostatic	Inhibition of protein synthesis
Chloramphenicol	Bacteriostatic	Inhibition of protein synthesis
Sulfonamides	Bacteriostatic	Competitive inhibition

Courtesy by: **Sosa *et al.* (2009)**

2.5 Antibiotic Resistance

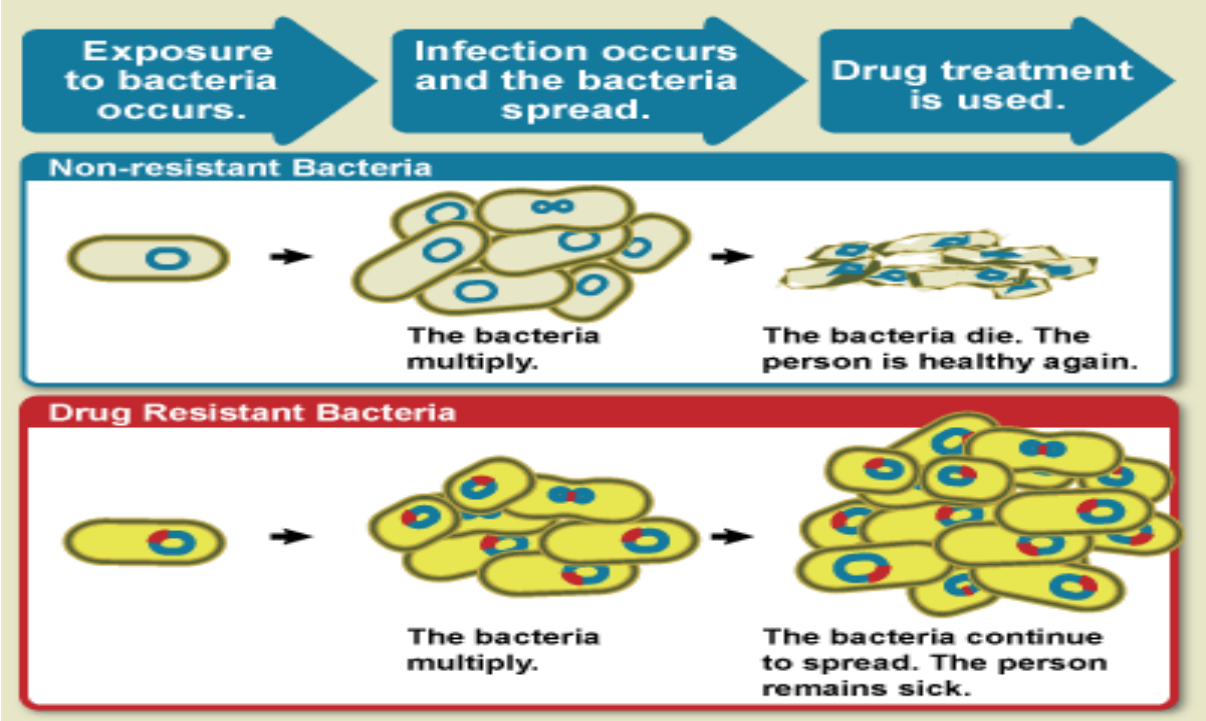
There is no doubt that antimicrobial agents have saved the human race from a lot of suffering due to infectious disease burden. Without antimicrobial agents, millions of people would have succumbed to infectious diseases. Man has survived the accidental wrath of microorganisms using antimicrobial agents and other mechanisms that keep them at bay. Hardly years after the discovery and use of the first antibiotics was observation made of organisms that still survived the effects of the antimicrobial agents (Sosa *et al.*, 2009).

Antibiotics play an important role in the treatment of bacterial infections. However, several reports indicate an increasing rate of bacterial resistance. Worldwide there is growing concern about the increased prevalence of antibiotic resistance. It is now generally accepted that the main risk factor for this increase in resistance in pathogenic bacteria is the increased use of antibiotics. This has inevitable lead to the emergence and dissemination of

resistant bacteria and resistance genes. This situation applies to antibiotic usage both in animals and in humans. In both populations antibiotics are used for therapy and prophylaxis of infectious diseases (Osoba *et al.*, 1984; Kesah *et al.*, 1999; van den Bogaard and Stobberingh, 2000). A bacterial strain can be defined resistant if it survives in the presence of higher antibiotic concentrations in comparison with phylogenetically related strains (Guardabassi *et al.*, 1998). Antibiotic resistance is not a bacterial property that can be determined by studying a single strain, but only by comparison under identical conditions of two or more strains belonging to the same species. The above mentioned definition of antibiotic resistance refers to *in vitro* conditions. Under *in vivo* conditions, antibiotic resistance is a context dependent term as it depends on the location of the bacterium and the bioavailability of the drug. Bacteria are less susceptible to antibiotics when assembled in compared with the same organisms living separately (Guardabassi *et al.*, 1998). In aquatic environments, binding of the antibiotic molecule with ions or substances present in sediment strongly reduces both the activity of the drug and its absorption in the intestine (Guardabassi, 2000). Antimicrobial resistance in *Salmonella* spp. is a major health problem in human and veterinary medicine worldwide (FAO, 2014). Many antimicrobial resistance genes are associated with genetic elements called integrons (Leverstein-van Hall *et al.*, 2003), which can be located on transposons and plasmids but also on the chromosome. They are able to integrate and express genes coding for antibiotic resistance (Stokes and Hall, 1989; Hall and Stokes, 1993). Uncontrolled use of antibiotics in farm animals and aquaculture system has contributed tremendously to the emergence and persistence of resistance strains (Novick, 1981; Young, 1994). There are three proven targets for the main antibacterial drugs: (i) bacterial cell-wall biosynthesis; (ii) bacterial protein synthesis and (iii) bacterial DNA replication and repair (Walsh, 2000).

2.6 Molecular Mechanism of Antimicrobial Resistance

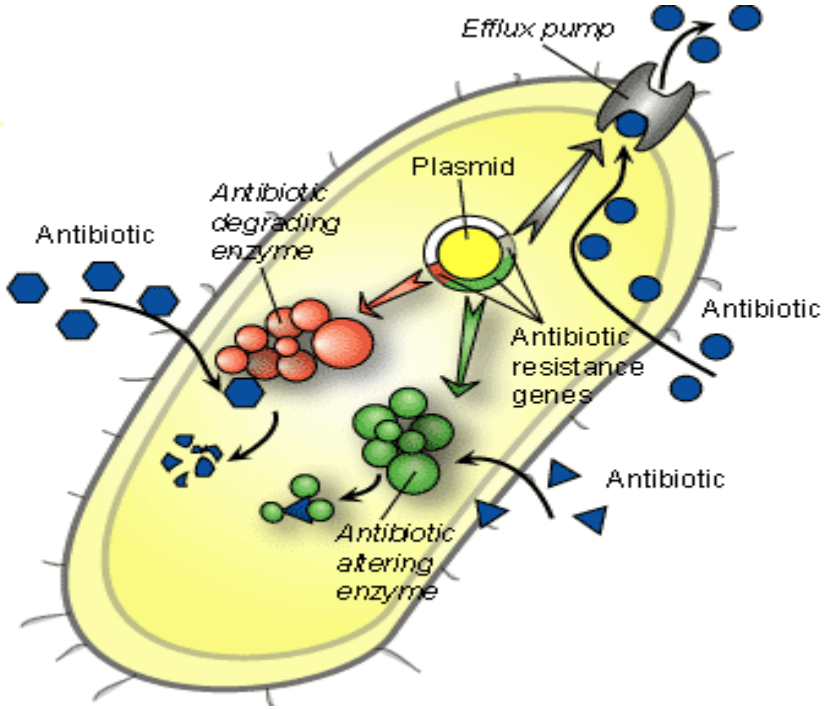
Resistance to antimicrobials can be acquired in two ways: mutations in pre-existing or previously acquired genes, and horizontal gene transfer (HGT), the acquisition of new genes from other bacteria. Depending on the antimicrobial, both mechanisms can play important roles in the development of the dramatic AMR situation that we face today (Boerlin and Reid-Smith, 2008). Biochemical and genetic aspects of antibiotic resistance mechanisms in bacteria are shown in **Figure 2.3**.



Courtesy by NIAID (2009)

Figure 2.1: Diagram showing the difference between non-resistant bacteria and drug resistant bacteria. Non-resistant bacteria multiply, and upon drug treatment, the bacteria die. Drug resistant bacteria multiply as well, but upon drug treatment, the bacteria continue to spread.

Most, but not all, resistance mechanisms are encoded by plasmids, which are potentially transmissible to other bacteria. Clockwise 12 o'clock: Efflux pumps are high-affinity reverse transport systems located in the membrane that transport the antibiotic out of the cell. This is the mechanism of resistance to tetracycline. 4 o'clock: A specific enzyme modifies the antibiotic in a way that it loses its activity. In the case of streptomycin, the antibiotic is chemically modified so that it will no longer bind to the ribosome to block protein synthesis. 9 o'clock: An enzyme is produced that degrades the antibiotic, thereby inactivating it. For example, the penicillinases are a group of beta-lactamase enzymes that cleave the beta lactam ring of the penicillin molecule (Todar, 2011)**(Figure 2.2)**.



Courtesy by Todar (2011)

Figure 2.2: Mechanisms of antibiotic resistance in bacteria

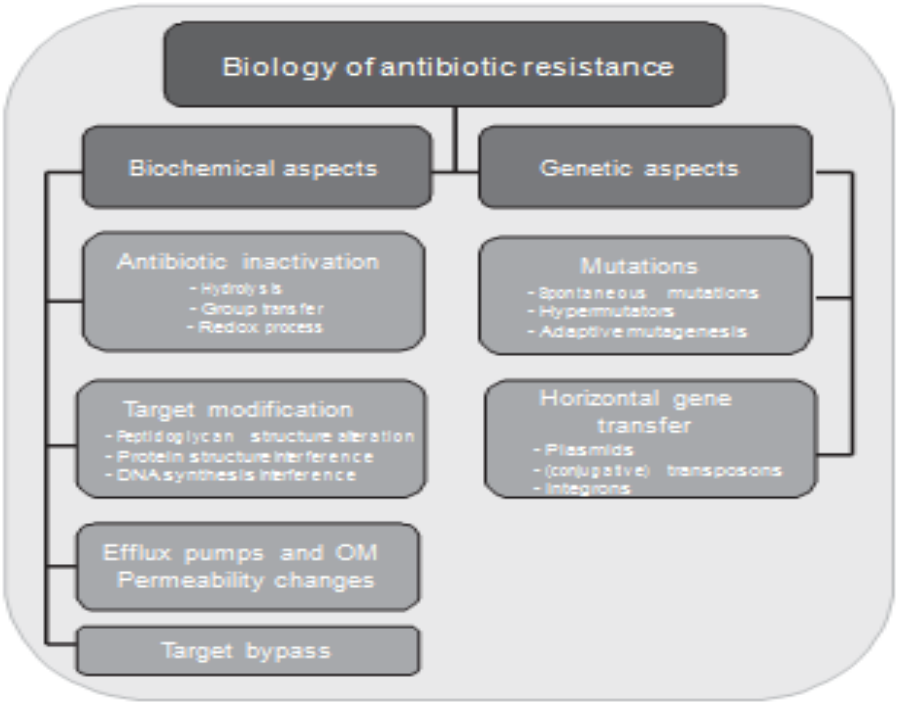


Figure 2.3: Biochemical and genetic aspects of antibiotic resistance mechanisms in bacteria (Džidić *et al.*, 2003).

2.7 Biochemical Aspects

The manner of acquisition of resistance may vary among bacterial species, resistance is created by only a few mechanisms: (i) Antibiotic inactivation– direct inactivation of the active antibiotic molecule (Wright, 2005); (ii) Target modification– alteration of the sensitivity to the antibiotic by modification of the target (Lambert, 2005); (iii) Efflux pumps and outer membrane (OM) permeability changes– reduction of the concentration of drug without modification of the compound itself (Kumar and Schweizer, 2005); or (iv) Target bypass– some bacteria become refractory to specific antibiotics by bypassing the inactivation of a given enzyme. This mode of resistance is observed in many trimethoprim-sulfonamide-resistant bacteria. The example is in bypassing inhibition of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes (involved in tetrahydrofolate biosynthesis). They are inhibited by trimethoprim and sulfonamides, respectively. In several trimethoprim- sulfonamide-resistant strains, a second enzyme that has low affinity for the inhibitors is produced (Mobashery and Azucena, 2002; Happi *et al.*, 2005).

There is an amazing diversity of antibiotic resistance mechanisms within each of these four categories and a single bacterial strain may possess several types of resistance mechanisms. Which of these mechanisms prevails depends on the nature of the antibiotic, its target site, the bacterial species and whether it is mediated by a resistance plasmid or by a chromosomal mutation (Džidić *et al.*, 2003).

2.7.1 Antibiotic inactivation

The defense mechanisms within the category of antibiotic inactivation include the production of enzymes that degrade or modify the drug itself. Biochemical strategies are hydrolysis, group transfer, and redox mechanisms (Džidić *et al.*, 2003).

2.7.1.1 Antibiotic inactivation by hydrolysis

Many antibiotics have hydrolytically susceptible chemical bonds (e.g. esters and amides). Several enzymes are known to destroy antibiotic activity by targeting and cleaving these bonds. These enzymes can often be excreted by the bacteria, inactivating antibiotics before they reach their target within the bacteria. The classical hydrolytic amidases are the b-lactamases that cleave the b-lactam ring of the penicillin and cephalosporin antibiotics. Many Gram-negative and Gram-positive bacteria produce such enzymes, and more than 200 different b-lactamases have been identified. b-Lactamases are classified into four groups on

the basis of functional characteristics, including preferred antibiotic substrate. Clinical isolates often produce b-lactamases belonging to different functional groups. They can be both chromosomal and plasmid-encoded b-lactamases transferred from different bacteria (Bush *et al.*, 1995; Bonnet, 2004; Poole, 2004).

Extended-spectrum b-lactamases (ESBLs) mediate resistance to all penicillins, third generation cephalosporins (e.g. ceftazidime, cefotaxime, ceftriaxone) and aztreonam, but not cephamycins (cefoxitin and cefotetan) and carbapenems. ESBLs are very diverse: more than 180 different ESBLs have been identified. They are most commonly detected in *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis*, but have also been found in other Enterobacteriaceae (Bradford, 2001). The website http://www.lahey.org/Studies/ was established to standardize the nomenclature for the growing number of b-lactamases and provide references to sources for nucleotide and amino acid sequence information. Other hydrolytic enzyme examples include esterases that have been linked to macrolide antibiotic resistance and ring-opening epoxidases causing resistance to fosfomycin (Nakamura *et al.*, 2000b; Kim *et al.*, 2002; Fillgrove *et al.*, 2003).

2.7.1.2 Antibiotic inactivation by group transfer

The most diverse family of resistant enzymes is the group of transferases. These enzymes inactivate antibiotics (aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin) by chemical substitution (adenylyl, phosphoryl or acetyl groups are added to the periphery of the antibiotic molecule). The modified antibiotics are affected in their binding to a target. Chemical strategies include O-acetylation and N-acetylation (Allignet and El Solh, 1995; Schwarz *et al.*, 2004; Vetting *et al.*, 2004), O-phosphorylation (Yazawa *et al.*, 1994; Nakamura *et al.*, 2000a; Matsuoka and Sasaki, 2004), O-nucleotidylation (Brisson-Noel *et al.*, 1988; Pedersen *et al.*, 1995), O-ribosylation (Houang *et al.*, 2003), O-glycosylation, and thiol transfer. These covalent modification strategies all require a co-substrate for their activity (ATP, acetyl-CoA, NAD+, UDP-glucose, or glutathione) and consequently these processes are restricted to the cytoplasm (Dzidic and Bedeković, 2003).

2.7.1.3 Antibiotic inactivation by redox process

The oxidation or reduction of antibiotics has been infrequently exploited by pathogenic bacteria. However, there are a few of examples of this strategy (Andersen *et al.*, 1997; Guengerich, 2001; Yang *et al.*, 2004). One is the oxidation of tetracycline antibiotics by the TetX enzyme. Streptomyces virginiae, producer of the type A streptogramin antibiotic

virginiamycin M1, protects itself from its own antibiotic by reducing a critical ketone group to an alcohol at position 16 (Džidić *et al.*, 2003).

2.7.2 Target modification

The second principal resistance mechanism is the modification of the antibiotic target site so that the antibiotic is unable to bind properly. Because of the vital cellular functions of the target sites, organisms cannot evade antimicrobial action by dispensing with them entirely. However, it is possible for mutational changes to occur in the target that reduce susceptibility to inhibition whilst retaining cellular function (Spratt, 1994).

In some cases, the modification in target structure needed to produce resistance requires other changes in the cell to compensate for the altered characteristics of the target. This is the case in the acquisition of the penicillin-binding protein 2a (PBP2a) transpeptidase in *Staphylococcus aureus* that results in resistance to methicillin (methicillin-resistant *S. aureus*, MRSA) and to most other b-lactam antibiotics. To save the efficiency of peptidoglycan biosynthesis, PBP2a needs alterations in the composition and structure of peptidoglycan, which involves functioning of a number of additional genes (Enright, 2003; Happi *et al.*, 2005; Łęski and Tomasz, 2005).

2.7.2.1 Peptidoglycan structure alteration

The peptidoglycan component of the bacterial cell wall provides an excellent selective target for the antibiotics. It is essential for the growth and survival of most bacteria. Consequently, enzymes involved in synthesis and assembly of the peptidoglycan component of the bacterial cell wall provide excellent targets for selective inhibition. The presence of mutations in the penicillin-binding domain of penicillin-binding proteins (PBPs) results in decreased affinity to b-lactam antibiotics. Alterations among PBPs result in ampicillin resistance among *Enterococcus faeciu*m, and penicillin resistance among *Streptococcus pneumoniae* (Dowson *et al.*, 1997; Nagai *et al.*, 2002; Kosowska *et al.*, 2004). Resistance to methicillin and oxacillin in *S. aureus* is associated with acquisition of a mobile genetic element called SCCmec, which contains the mecA resistance gene. The mecA determinant encodes PBP2a, a new penicillin-binding protein distinct from the PBPs normally found in *S. aureus*. PBP2a is highly resistant to inhibition by all clinically used b-lactams and remains active to maintain cell wall synthesis at normally lethal b-lactam concentrations (Tenover, 2006).

Glycopeptides such as vancomycin inhibit cell wall synthesis of Gram-positive bacteria by binding C-terminal acyl-D-alanyl-D-alanine (acyl-D-Ala-D-Ala)-containing residues in

peptidoglycan precursors. Resistance is achieved by altering the target site by changing the D-Ala-D-Ala to D-alanyl-D-lactate (D-Ala-D-Lac) or D-alanyl-D-serine (D-Ala-D-Ser) at the C-terminus, which inhibits the binding of vancomycin (Tenover *et al.*, 1998; Cooper *et al.*, 2000; Hiramatsu, 2001). As a consequence, the affinity of vancomycin for the new terminus is 1000 times lower than for the native peptidoglycan precursor in the case of D-Ala-D-Lac. Dissemination of glycopeptide resistance in Gram-positive cocci can occur at the level of the bacteria (clonal spread), replicons (plasmid epidemics) or of the genes (transposons). Glycopeptide (vancomycin) resistance can be intrinsic (VanC-type resistance) or acquired, present only in certain isolates belonging to the same species (VanA, B, D, C, E and G types of vancomycin resistance) (Courvalin, 2005).

2.7.2.2 Protein synthesis interference

A wide range of antibiotics interfere with protein synthesis on different levels of protein metabolism. The resistance to antibiotics that interfere with protein synthesis (aminoglycosides, tetracyclines, macrolides, chloramphenicol, fusidic acid, mupirocin, streptogramins, oxazolidinones) or transcription via RNA polymerase (the rifamycins) is achieved by modification of the specific target (Happi *et al.*, 2005).

The macrolide, lincosamide and streptogramin B group of antibiotics block protein synthesis in bacteria by binding to the 50S ribosomal subunit (Weisblum, 1998; Spigaglia and Mastrantonio, 2002; Ackermann *et al.*, 2003). Resistance to these antibiotics is referred to as MLS(B) type resistance and occurs in a wide range of Gram-positive bacteria. It results from a post-transcriptional modification of the 23S rRNA component of the 50S ribosomal subunit (Weisblum, 1995). Mutations in 23S rRNA close to the sites of methylation have also been associated with resistance to the macrolide group of antibiotics in a range of organisms. In addition to multiple mutations in the 23S rRNA, alterations in the L4 and L22 proteins of the 50S subunit have been reported in macrolide-resistant *S. pneumoniae* (Canu *et al.*, 2002). The mechanism of action of oxazolidinones (for example, linezolid) involves multiple stages in the protein synthesis (Bozdogan and Appelbaum, 2004). Although they bind to the 50S subunit, the effects include inhibition of formation of the initiation complex and interference with translocation of peptidyl-tRNA from the A site to the P site. Resistance has been reported in a number of organisms including enterococci and is linked to mutations in the 23S rRNA resulting in decreased affinity for binding (Wang and Taylor, 1998).

Mutations in the 16S rRNA gene confer resistance to the aminoglycosides (Suzuki *et al.*, 1998). Chromosomally acquired streptomycin resistance in *M. tuberculosi*s is frequently due to mutations in the rpsL gene encoding the ribosomal protein S12. Microorganisms that produce aminoglycosides have developed mechanism of high level antibiotic resistance by posttranscriptional methylation of 16S rRNA in the aminoglycoside binding site. This mechanism of resistance has recently been reported in human pathogens from nosocomial infections and animal isolates (Maravi *et al.*, 2010).

2.7.2.3 DNA synthesis interference

Fluoroquinolones interact with the DNA gyrase and topoisomerase IV enzymes and prevent DNA replication and transcription. Resistance is conferred by mutations in specific regions of the structural genes that sufficiently alter these enzymes preventing the binding of antibiotics (Khodursky *et al.*, 1995; Ince *et al.*, 2002). The most common mutations in this region cause resistance through decreased drug affinity for the altered gyrase–DNA complex (Willmott and Maxwell, 1993; Hooper, 1999; Eliopoulos, 2004).

2.7.3 Efflux pumps and outer membrane (OM) permeability

The efflux pumps are the membrane proteins that export the antibiotics out of the cell and keep its intracellular concentrations at low levels. Reduced outer membrane (OM) permeability results in reduced antibiotic uptake. The reduced uptake and active efflux induce low level resistance in many clinically important bacteria (Nikaido, 1994).

2.7.3.1 Efflux pumps

Efflux pumps affect all classes of antibiotics, especially the macrolides, tetracyclines, and fluoroquinolones because these antibiotics inhibit different aspects of protein and DNA biosynthesis and therefore must be intracellular to exert their effect. Efflux pumps vary in both their specificity and mechanism (Nikaido and Zgurskaya, 1999; Webber and Piddock, 2003). Although some are drug-specific, many efflux systems are multidrug transporters that are capable of expelling a wide spectrum of structurally unrelated drugs, thus contributing significantly to bacterial multidrug resistance (MDR) (Van Veen and Konings, 1997). Inducible multidrug efflux pumps are responsible for the intrinsic antibiotic resistance of many organisms, and mutation of the regulatory elements that control the production of efflux pumps can lead to an increase in antibiotic resistance. For example, the MexAB-OprM efflux pump in *Pseudomonas aeruginosa* is normally positively regulated by the presence of drugs, but mutations in its regulator (mexR) lead to the overexpression of MexAB-OprM,

which confers increased resistance to antibiotics such as b-lactams (Gotoh *et al.*, 1995; Köhler *et al.*, 1999; Poole, 2004). Both Gram-positive and Gram-negative bacteria can possess single-drug and/or multiple drug efflux pumps (Putman *et al.*, 2000; Langton *et al.*, 2005).

Bacterial drug efflux transporters are currently classified into five families (Pao *et al.*, 1998; Van Veen and Konings, 1998). The major facilitator superfamily (MFS) and the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily are very large and the other three are smaller families: the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) superfamily and the multidrug and toxic compound extrusion (MATE) family. Efflux transporters can be further classified into single or multicomponent pumps (Fath and Kolter, 1993; Okusu *et al.*, 1996; Ma and Chang, 2004). Single component pumps transport their substrates across the cytoplasmic membrane. Multicomponent pumps, found in Gram-negative organisms, function in association with a periplasmic membrane fusion protein (MFP) component and an outer membrane protein (OMP) component, and efflux substrates across the entire cell envelope (Džidić *et al.*, 2003).

Furthermore, the regulators of efflux systems may be attractive drug targets themselves. The regulators involved in efflux gene expression are either local or global regulators. Many

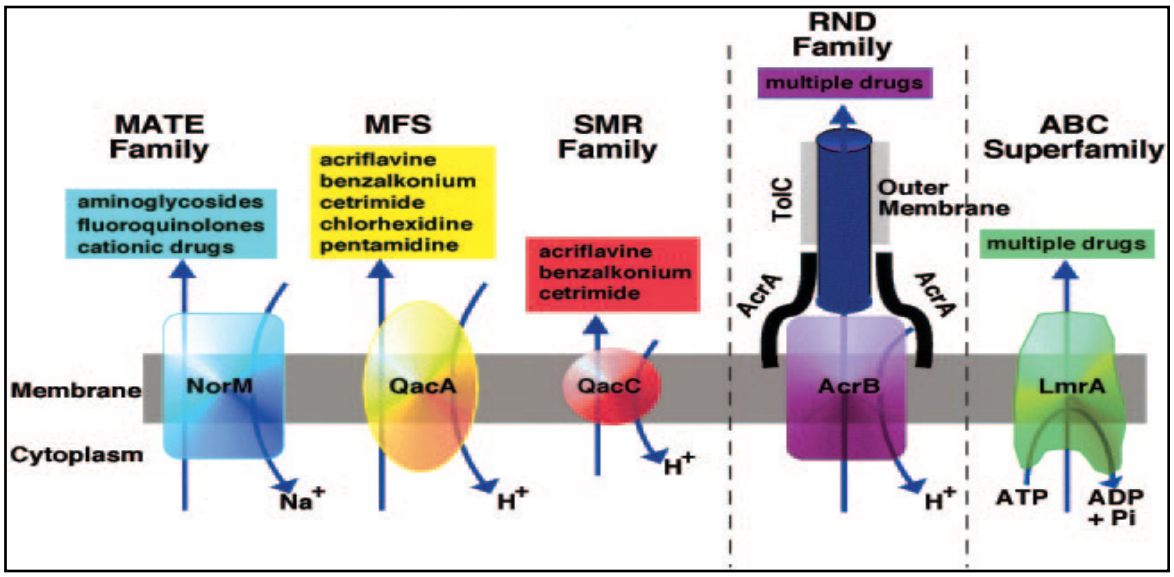


Figure 2.4: Diagrammatic comparison of the five families of efflux pumps. (Courtesy of Melissa Brown; reproduced by kind permission).

Pump component-encoding operons contain a physically linked regulatory gene. Some efflux pumps are known to be regulated by two-component systems. These systems mediate the adaptive responses of bacterial cells to their environment. Expression of various efflux pumps is also controlled by different global regulators. So far, several global transcriptional activators, including MarA, SoxS and Rob, have been shown to be involved in the regulation of expression of this system (Fath and Kolter, 1993; Okusu *et al.*, 1996; Ma and Chang, 2004).

2.7.3.2 Outer membrane (OM) permeability changes

Gram-negative bacteria possess an outer membrane consisting of an inner layer containing phospholipids and an outer layer containing the lipid A moiety of lipopolysaccharides (LPS). This composition of the outer membrane (OM) slows down drug penetration, and transport across the OM is achieved by porin proteins that form water-filled channels. Drug molecules can penetrate the OM employing one of the following modes: by diffusion through porins, by diffusion through the bilayer or by self-promoted uptake. The mode of entry employed by a drug molecule largely depends on its chemical composition. For example, hydrophilic compounds either enter the periplasm through porins (e.g. b-lactams) or self-promoted uptake (aminoglycosides). Antibiotics such as b-lactams, chloramphenicol and fluoroquinolones enter the Gram-negative outer membrane via porins. As such, changes in porin copy number, size or selectivity will alter the rate of diffusion of these antibiotics (Dé *et al.*, 2001; Denyer and Maillard, 2002; Hancock and Brinkman, 2002; Nikaido, 2003).

The role of LPS as a barrier to antibiotics is well documented. Mutations in LPS that result in antibiotic hypersusceptibility have been reported. Strains of E. coli and S. enterica serovar Typhimurium defective in LPS have been found to be at least 4-fold more susceptible to erythromycin, roxithromycin, clarithromycin and azithromycin than the wild-type strains (Vaara, 1993; Wiese *et al.*, 1999).

2.8 Genetics of Antibiotic Resistance

Studies of a wide variety of bacterial pathogens have identified numerous genetic loci associated with antibiotic resistance. For some types of resistance there is a large diversity of responsible genetic determinants.

Resistance can be an intrinsic property of the bacteria themselves or it can be acquired. Acquired bacterial antibiotic resistance can result from a mutation of cellular genes, the acquisition of foreign resistance genes or a combination of these two mechanisms. Thus,

there are two main ways of acquiring antibiotic resistance: i) through mutation in different chromosomal loci and ii) through horizontal gene transfer (i.e. acquisition of resistance genes from other microorganisms). This raises several questions about the evolution and ecology of antibiotic resistance genes. Phylogenetic insights into the evolution and diversity of several antibiotic resistance genes suggest that at least some of these genes have a long evolutionary history of diversification that began well before the antibiotic era (Aminov and Mackie, 2007).

2.8.1 Mutations

2.8.1.1 Spontaneous Mutations

Exploring the origins of resistant mutants began with the antibiotic era in 1940s, when researchers performed classical experiments proving that mutations conferring resistance to certain antibiotics arise prior to or in the absence of any selective pressure. These mutation events occur randomly as replication errors or an incorrect repair of a damaged DNA in actively dividing cells. They are called growth dependent mutations (spontaneous mutations) and present an important mode of generating antibiotic resistance (Krašovec and Jerman, 2003).

Antibiotic resistance occurs by nucleotide point mutations which are at the same time growth permissive and are able to produce a resistance phenotype (Woodford and Ellington, 2007). For instance, quinolone resistance phenotype in *Escherichia coli* is a result of changes in at least seven positions in the gyrA gene, but in only three positions in the parC gene (Nakamura *et al.*, 1989; Hooper, 1999). A variety of genes can be involved in antibiotic resistance either because there are several different targets, access, or protection pathways for the antibiotic in the bacterial cell or because each pathway requires the expression of several genes (Džidić *et al.*, 2003).

There is a substantial number of biochemical mechanisms of antibiotic resistance that are based on mutational events, like the mutations of the sequences of genes encoding the target of certain antibiotics (for instance, resistance to rifamicins and fluoroquinolones are caused by mutations in the genes encoding the targets of these two molecules, RpoB and DNA-topoisomerases, respectively) (Martinez and Baquero, 2000; Ruiz, 2003). The variation in the expression of antibiotic uptake or of the efflux systems may also be modified by mutation (for instance, the reduced expression or absence of the OprD porin of *P. aeruginosa* reduces the permeability of the cell wall to carbapenems) (Wolter *et al.*, 2004). Some of the

resistances associated with the uptake and efflux systems are caused by mutations in regulatory genes or their promoter regions (Piddock, 2006; Depardieu *et al.*, 2007). Also, the mutations leading to increased expression of the efflux systems, in general, confer resistance to multiple antibiotics (for example, mutations in the *Escherichia coli* mar gene affect the expression of about 60 different genes, including down-regulation of OmpF and up-regulation of AcrAB) (Barbosa and Levy, 2000). AcrAB is involved in the efflux of b-lactams, fluoroquinolones, chloramphenicol, and tetracycline. In *P. aeruginosa*, mutation in mexR up-regulates the mexA-mexB-oprM operon and raises resistance to most b-lactams, fluoroquinolones, tetracyclines, chloramphenicol and macrolides (Adewoye *et al.*, 2002). The overproduction of antibiotic-inactivating enzymes may also be achieved through mutational events. Many Gram-negative microorganisms produce chromosomal b-lactamases at low levels and mutations producing up-regulation of their expression may lead to the resistance to most cephalosporins (Džidić *et al.*, 2003).

In addition to these examples, there are some clinically relevant pathogens for which plasmid or transposon-mediated mechanisms of resistance have not been reported (*Mycobacterium tuberculosis* isolated in the infected patients lacks the horizontal transfer mechanisms and, consequently, can acquire antibiotic resistance by mutation exclusively) (Ramaswamy and Musser, 1998). *P. aeruginosa* from the lungs of patients with cystic fibrosis is almost impossible to eradicate, mainly because of the development of resistance to multiple antibiotics. In this particular environment resistance is achieved through chromosomal mutations that are able to produce resistance to all antibiotics used in clinical practice, without any acquisition of exogenous DNA (Oliver *et al.*, 2000).

2.8.1.2 Hypermutators

It has been widely accepted that mutation is the unavoidable consequence of errors produced in the DNA replication process or of the failure of the error-avoidance systems. Maintaining the stability of genetic information is vital for the perpetuation of species. Low spontaneous mutation rates are maintained by the activity of many molecular mechanisms that protect and repair DNA, as well as by the mechanisms that assure high-fidelity of DNA replication (Schaaper, 1993; Drake *et al.*, 1998). However, bacteria with an elevated mutation rate (hypermutable strains, or mutators) among natural and laboratory populations have been found. Experimental studies indicate that the frequency of mutators observed among natural and clinical bacterial isolates is much higher than expected, which suggests that there are situations in nature where being a mutator confers a selective advantage. According to

the currently most acceptable hypermutable state' model, during a prolonged non-lethal antibiotic selective pressure a small bacterial population enters a transient state of a high mutation rate. If a cell in this hypermutable state achieves a useful mutation, thus relieving the selective pressure, the cell begins to grow and reproduce, and at the same time exits the hypermutable state. It is still unclear what really triggers cells to enter the hypermutable state; however, it appears that a hypermutation is regulated by a special SOS-inducible mutator DNA polymerase (pol) IV (Krašovec and Jerman, 2003). Hypermutators have been found in populations of *E. coli*, *Salmonella enterica*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Helicobacter pylori*, *Streptococcus pneumoniae*, *P. aeruginosa* with frequencies ranging from 0.1 to above 60 % (LeClerc *et al.*, 1996; Denamur *et al.*, 2002).

Mutator phenotypes of different strengths could be created by inactivation of over 20 different *E. coli* genes (Horst *et al.*, 1999; Harfe and Jinks-Robertson, 2000). But the majority of strong mutators found in the laboratory and in nature have a defective mismatch repair (MMR) system due to the inactivation of mutS or mutL genes (Oliver *et al.*, 2002). This repair system controls the fidelity of DNA replication by eliminating biosynthetic errors. In addition, the mismatch repair system is involved in the maintenance of chromosomal structural integrity and in the control of horizontal gene transfer by preventing recombination between non-identical DNA sequences (Rayssiguier *et al.*, 1989). The results of various studies have shown that mutators play an important role in the evolution of antibiotic resistance (Martinez and Baquero, 2000; Giraud *et al.*, 2002; Chopra *et al.*, 2003; Eliopoulos and Blázquez, 2003). By increasing the possibility of mutations, they may accelerate the evolution of favourable mutations under certain conditions. During this process, mutators can be fixed in the population by getting along with the favourable mutations (e.g. resistance) they have created. Thus, the acquisition of a mutator phenotype may increase the chance of acquiring antibiotic resistance by mutational events. Hypermutators may also enable multiresistant phenotype (Maciá *et al.*, 2005).

2.8.1.3 Adaptive mutagenesis

The mutation process has classically been studied in actively dividing bacteria, as it was assumed that most mutations occur as the consequence of errors during the DNA replication process (spontaneous mutations). However, more recent experimental data have clearly shown that mutations arise also in non-dividing or slowly dividing cells and have some relation to the selective pressure used. These mutations, named adaptive mutations', arise only in the presence of non-lethal selective pressure that favours them. This is the main

feature that distinguishes them from the growth dependent, spontaneous mutations. The adaptive mutation process may be one of the main sources of the antibiotic resistant mutants under natural conditions (Taddei *et al.*, 1997; Bjedov *et al.*, 2003; Krašovec and Jerman, 2003). Analyses of several model systems have demonstrated that stress-enhanced bacterial mutagenesis is a regulated phenomenon (Sutton *et al.*, 2000). The main factors in this process are stress-responsive (as a part of finely regulated SOS response) error-prone DNA polymerases V (umuCD) and IV (dinB), which transiently increase the rate of mutation (Džidić *et al.*, 2003).

It has been demonstrated that some antibiotics (quinolones, for example) are able to induce the SOS mutagenic response and increase the rate of emergence of resistance in *E. coli* (Piddock and Wise, 1987). The emergence of multiresistant strains increases in *P. aeruginosa* under antibiotic challenge (Tenover *et al.*, 1998). *E. coli* exposed to antibiotic streptomycin displays a hypermutable phenotype (Ren *et al.*, 1999).

Some of adaptive mutations generated in mutator backgrounds (antibiotic resistance, for example) can be saved and fixed in a bacterial population either by horizontal transfer to a non-mutator background or by a reduction in the mutation rate of the adapted mutator strain before the load of deleterious mutations becomes too high. And then, the reduction of mutation rate might be achieved by several mechanisms: reversion of the mutator allele, acquisition of suppressor mutations or by reacquisition of a wild-type allele of mutator gene from non-mutator bacteria via horizontal gene exchange (Džidić *et al.*, 2003).

2.8.2 Horizontal gene transfer

A principal mechanism for the spread of antibiotic resistance is by horizontal transfer of genetic material. Antibiotic resistance genes may be transferred by different mechanisms of conjugation, transformation, or transduction. Resistance genes can be further incorporated into the recipient chromosome by recombination. These genes may contain single mutations or more severe sequence changes (Džidić *et al.*, 2003).

Tetracycline resistance in most bacteria is due to the acquisition of new genes often associated with mobile elements. These genes are usually associated with plasmids and/or transposons and are often conjugative. The website http://faculty.washington.edu/marilynr/, which is updated twice a year, was established to reflect the ongoing changes in information on acquired tetracycline resistance (tet) and oxytetracycline resistance (otr) genes, originally in antibiotic producing Streptomyces (Roberts, 2005; Roberts, 2006). Among Gram-negative

anaerobes and Gram-positive bacteria, conjugative transposons are recognized as important mediators of genetic exchange on a par with the large R-plasmids of enteric bacteria. These large (>25 kb) elements encode a fully functional conjugation apparatus and are capable of self-transfer to a wide variety of species. Conjugative transposons in the Bacterioides are referred to as Tcr-elements (tetracycline resistance elements) owing to the presence of tetracycline resistance genes (tetQ) and these elements are primarily responsible for more than 80 % of tetracycline resistance frequency among Bacteroides clinical isolates (Salyers *et al.*, 2007). High level resistance to gentamicin and all other related aminoglycosides with the exception of streptomycin was found in enterococci. The gene conferring this phenotype has been associated with both narrow and broad host range plasmids. The nature of these conjugative elements raises the possibility of the resistance gene spreading to other pathogenic bacteria (Simjee and Gill, 1997).

Horizontal transfer of resistance genes is a mechanism for the dissemination of multiple drug resistance because resistance genes can be found in clusters and transferred together to the recipient. This is enabled by the existence of specific DNA structures called integrons (Rowe-Magnus and Mazel, 1999; Ploy *et al.*, 2000). Integrons are DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination. These elements are located either on the bacterial chromosome or on broad host range plasmids. Integrons differ from transposons in two important characteristics: transposons have direct or indirect repeat sequences at their ends, but the regions surrounding the antibiotic resistance genes in the integrons are not repeats; and the integrons contain a site specific integrase gene of the same family as those found in phages but lack gene products associated with transposition. Integrons promote the capture of one or more gene cassettes within the same attachment site thereby forming clusters of antibiotic resistance genes (Džidić *et al.*, 2003).

Gene cassettes are the smallest mobile genetic entities that can carry resistance determinants. These can encode many types of resistance including to trimethoprim, chloramphenicol, b-lactams, aminoglycosides, fosfomycin and quinolones and for each of these antibiotic classes several distinct gene cassettes have been reported. Resistance gene cassettes have been found for the most classes of antibiotics, and the gene products are involved in various mechanisms of resistance, such as efflux, target bypass and drug inactivation. Over 40 gene cassettes and three distinct classes of integrons have been identified to date (Boucher *et al.*, 2007).

Integron movement allows transfer of the cassette-associated resistance genes from one DNA replicon to another. Horizontal transfer of the resistance genes can be achieved when an integron is incorporated into a broad host range plasmid. A plasmid with a pre-existing resistance gene cassette can acquire additional resistance gene cassettes from donor plasmids, thus spreading multiresistance. All resistance-encoding DNAs establish a resistance gene pool, which represents a potential source for the horizontal transfer between bacteria. There are many examples of horizontal gene transfer of resistance elements both within and between bacterial species (Spratt, 1994; Dzidic and Bedeković, 2003). Studies about horizontal gene transfer-emerging multidrug resistance in hospital bacteria have demonstrated that the transfer of antibiotic resistance genes can take place in the intestine between Gram-positive or Gram-negative bacilli (Dzidic and Bedeković, 2003).

Multidrug-resistance (MDR) in bacteria is often the result of the acquisition of mobile genetic elements that contain multiple resistance genes. Nucleotide sequence analysis of multiresistant integrons shows that the inserted resistance gene cassettes differ markedly in codon usage, indicating that the antibiotic resistance determinants are of diverse origins (Nirdnoy *et al.*, 2005). The fact that bacteria that have been separately evolving for up to 150 million years can exchange DNAs has strong implications with regard to the evolution of antibiotic resistance in bacterial pathogens (Vulić *et al.*, 1997; Normark and Normark, 2002; Džidić *et al.*, 2003).

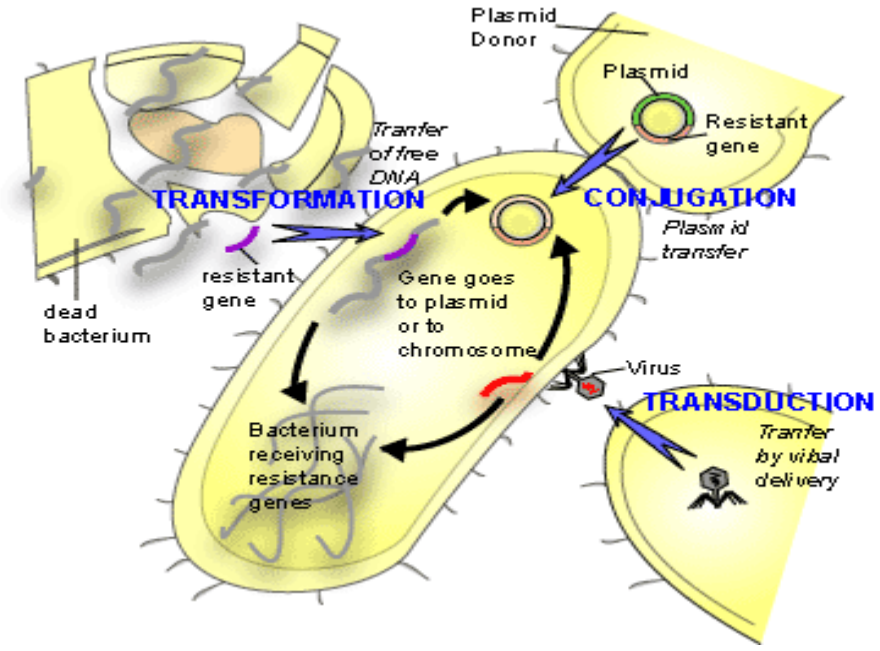


Figure 2.5: Mechanisms of horizontal gene transfer (HGT) in bacteria(Todar, 2011)

2.8.3 Vertical gene transfer

The spontaneous mutation frequency for antibiotic resistance is on the order of about of about 10^{-8}- 10^{-9}. This means that one in every 10^{8}- 10^{9} bacteria in an infection will develop resistance through the process of mutation. In *E. coli*, it has been estimated that streptomycin resistance is acquired at a rate of approximately 10^{-9} when exposed to high concentrations of streptomycin. Although mutation is a very rare event, the very fast growth rate of bacteria and the absolute number of cells attained means that it doesn't take long before resistance is developed in a population. Once the resistance genes have developed, they are transferred directly to all the bacteria's progeny during DNA replication. This is known as vertical gene transfer or vertical evolution. The process is strictly a matter of Darwinian evolution driven by principles of natural selection: a spontaneous mutation in the bacterial chromosome imparts resistance to a member of the bacterial population. In the selective environment of the antibiotic, the wild type (non mutants) are killed and the resistant mutant is allowed to grow and flourish (Todar, 2011).

2.9 Measurement of resistance in bacterial populations

The value of the term "measurement of antibiotic resistance" in environmental microbiology generally differs from that in clinical studies. The main concern for environmental microbiologists is to investigate the distribution of antibiotic resistance in bacterial populations rather than the level of resistance in individual strains. Unfortunately, culture methods are not efficient enough to determine the actual prevalence of antibiotic resistance in a bacterial population. In fact, only a small proportion of the aquatic bacterial flora (<1%) can be cultured on laboratory media (Pickup *et al.*, 1997). The method traditionally used for the measurement of antibiotic resistance at the population level consists in standard bacteriological counts on media containing specific concentrations of antibiotics. The main drawback of this method is the use of a single breakpoint for the determination of antibiotic resistance. In fact, the use of a single breakpoint, corresponding to the amount of antibiotic agent added to the medium, does not take into account the variability in the levels of antibiotic resistance existing among different bacterial species. Consequently, bacteria characterized by intermediate levels of resistance can be classified either as resistant or susceptible depending on the concentration of antibiotic added to the medium resistance (Cundliffe, 1989). An alternative approach is to use a group of phylogenetically related organisms as bacterial indicators of antibiotic resistance. This method is based on the principle that spatial and temporal differences observed in the levels of antibiotic resistance

of the bacterial indicator are indicative of the selective pressure to which the entire bacterial population is exposed. Thus, this method does not aim to determine the exact prevalence of antibiotic resistance in the bacterial population under study, but rather to detect the effect of potential sources of antibiotic resistance on the bacterial population (Cundliffe, 1989).

2.10 Antimcobial resistance pattern in *Salmonella* spp.

(Boonmar *et al.*, 1998) reported that a total of 1715 *Salmonella* strains, including 600 *S. Enteritidis*, 290 *S. derby*, 257 *S. weltevreden*, 122 *S.* 1,4,5,12:i:-, 235 *S. anatum*, and 211 *S. Typhimurium*, originating from 1308 human beings and 407 frozen chicken meat specimens collected in 1993 and 1994 were tested for antibiotic resistance. The disk diffusion method was used with nine disks of chloramphenicol, ceftriaxone, amikacin, kanamycin, ampicillin, sulfamethoxazole plus trimethoprim, nalidixic acid, gentamicin and ofloxacin. The resistance rates of human beings isolates in 1994 to ceftriaxone, amikacin and kanamycin were, respectively, 10.7%, 8.6%, 17.8% in *S. Enteritidis*; 23.1%, 17.3% 33.0% in *S. derby*; 30.9%, 40.2%. 60.4% in *S. weltevreden*; 16.1%, 17.7%, 70.9% in *S.* 1, 4, 5, 12:i:-; 25.7%, 21.6%, 24.7% in *S. anatum*; 18.9%, 15.7% 37.8% in *S. Typhimurium*, while those isolates in 1993 to the same three antibiotics were, respectively, 1.8%, 0.6%, 3.7% in *S. Enteritidis*; 0.8%, 0%, 9.1% in *S. derby*; 1.8%, 2.7%, 10.8% in *S. weltevreden*; 3.8%, 0%, 23.0% in *S.*1, 4, 5, 12:i:-; 2.2%, 2.2%, 6.7% in *S. anatum*; 4.5%, 1.5%, 10.6% in *S. Typhimurium*. It was shown that the resistance rates in 1994 were significantly higher than those in 1993. All isolates were susceptible to ofloxacin with the exception of one isolate.

(Arvanitidou *et al.*, 1997) reported that resistance to 20 antimicrobials was tested in 79 *Salmonella* strains isolated in northern Greece. Of the strains, 19 (24.1%) exhibited resistance to one or more of the antibiotics while single, double and multiple resistances were observed in 12.7%, 6.3% and 5.1% of the isolates, respectively. Streptomycin resistance was the most common and nine different antibiotic resistance patterns were recorded in total. All of the strains were susceptible to amoxycillin-clavulanate, cefuroxime, ciprofloxacin, colistin, amikacin and apramycin. Among the resistant *Salmonellas*, five (26.3%) were able to transfer R factors to the *Escherichia coli* recipient.

(Murti *et al.*, 1962) reported that resistance of *Salmonella typhi* to chloramphenicol has not been reported so far except in strains made resistant in the laboratory. While examining 52 smooth strains of *S. typhi* and three smooth strains of *S. paratyphi* A 10 strains of *S. typhi* were found to be resistant to 50 to 500µg chloramphenicol. Of these 10 strains, eight

appeared to be tolerant of the antibiotic, but the remaining two strains appeared to produce a substance that antagonizes or destroys chloramphenicol.

Rahman *et al.,* (2009) reported that 150 *Salmonella* isolates were 100% sensitive to Gentamycin followed by Amoxicillin (90%), Colistin (70%), Co-trimoxazole (60%) and Furazolidone (40%) but the isolates were highly resistant to Norfloxacin, Flumequine, Ciprofloxacin and Enrofoxacin. The study demonstrated that the *Salmonella gallinarum* were more sensitive to Gentamycin than Amoxycillin or Colistin.

(Lindgren *et al.*, 2009) reported that the fluoroquinolone susceptibility of 499 *Salmonella enterica* isolates collected from travelers returning to Finland during 2003–2007. Among isolates from travelers to Thailand and Malaysia, reduced fluoroquinolone susceptibility decreased from 65% to 22% (p =0.002). All isolates showing nonclassical quinolone resistance were from travelers to these 2 countries**.**

(Akoachere *et al.*, 2009) stated that cattle and pigs slaughtered in Buea as reservoirs of *Salmonella* Typhimurium and the susceptibility of isolates to antibiotics. In total, 230 specimens (comprising 50 each from the rectum, ileum, and gall bladder of cattle; and 10 each from same anatomical sites of pigs and 50 from abattoir drains) were analyzed for *Salmonella* using the standard microbiological, biochemical and serological techniques. Antibiotic susceptibility of the isolates was determined by the Kirby-Bauer disc-diffusion test. The most active drugs were ciprofloxacin (98.6%), ofloxacin (93.3%), amikacin (90.6%), and gentamicin (84%). All the isolates (100%) were resistant to tetracycline and ampicillin.

Ojo *et al.* (2009) reported that fecal *Salmonella* isolated have multiple antibiotic resistance Tetracycline (70.6%), Chloramphenicol (11.8%), Ampicillin (47.1%), Cefuroxime (52.9%), Amoxicillin (35.3), Erythromycin (100%) and Gentamicin (35.3%) in dog.

CHAPTER - III

MATERIALS AND METHODS

3.1 Description of study area

Chittagong City Corporation is located in the south-eastern part of Bangladesh, consists of 41 wards. There are lots of stray dogs without registration throughout the city. They have continuous access to abattoir, human food processing units and also contact with children and adults. A total of 108 samples were collected from 9 locations of metropolitan area, which were selected randomly.

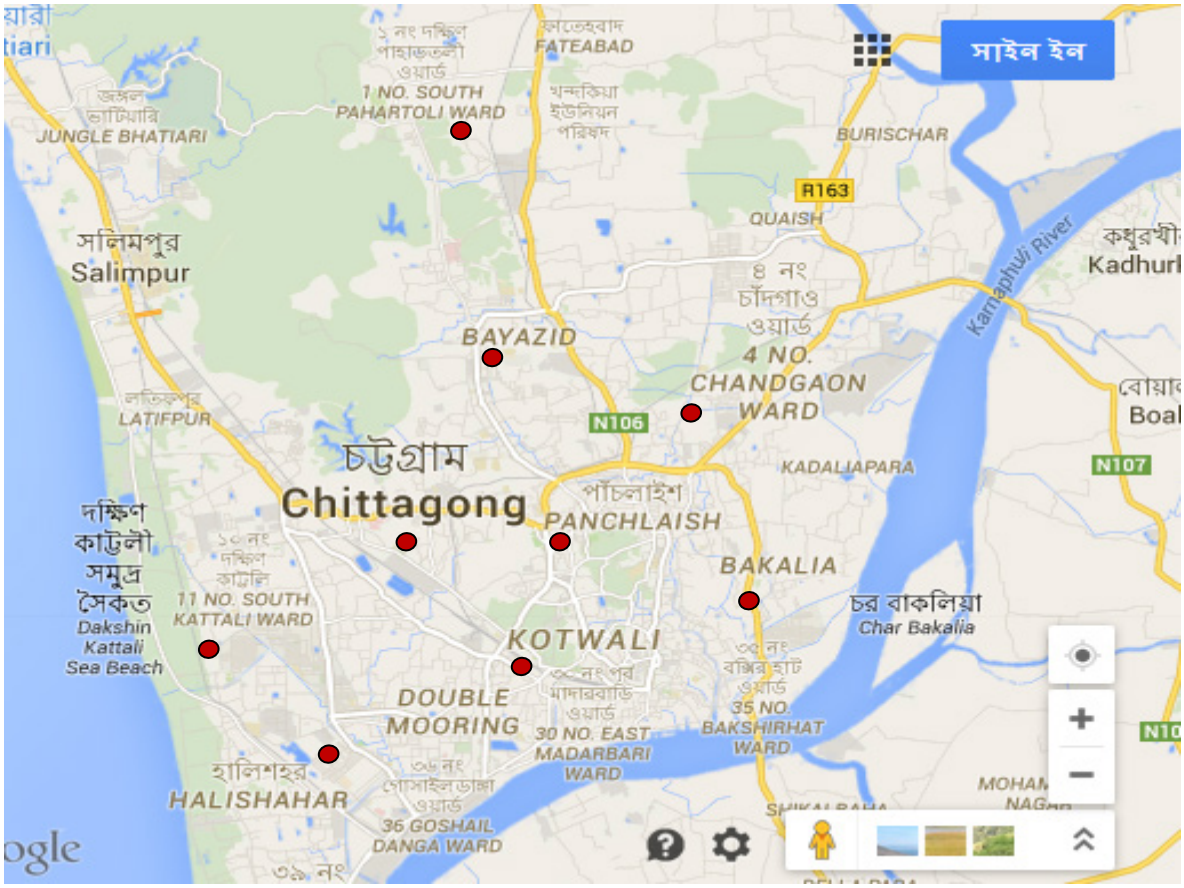


Figure 3.6: Map of study area

3.2 Study duration and sample collection

The study was conducted from April to November, 2014. The samples were collected as rectal swab with pre-sterilized cotton swab and immediately transferred into screw capped test tubes containing nutrient broth. Thermo flask containing ice was used to transport the

samples from the collection site to Poultry Research and Training Center (PRTC) laboratory for analysis.

Restraining Stray Dogs

Collection of Rectal Swab

Stray Dogs in Different Collection Sites

Figure 3.7: Status of stray dogs and collection of samples from different locations

3.3 Sample preservation

The collected samples were preserved in a refrigerator chiller at 4^0C during the research period at PRTC laboratory.

3.3 Media used

Some animals are infected with *Salmonella* without showing signs of the illness, i.e. they are subclinically infected. Faeces from these herds may contain *Salmonella* in low numbers (Handriksen, 2003).To diminish the risk of obtaining false negative results, a non-selective pre-enrichment of a large faeces sample and plating on two selective media were performed:

I. Primary enrichment media: nutrient broth (Oxoid Ltd., $P^{H:}$ 7.4±0.2)
II. Selective media: BGA agar (Merck, P^H: 6.9±0.2), SS Agar (Oxoid Ltd., P^H: 7.0±0.2), Blood Agar (Oxoid Ltd., P^H: 7.3±0.2) and TSI agar (Oxoid Ltd., P^H: 7.2±0.2)
III. Determination of antibiotic resistant bacteria (CS test): Mueller Hinton agar (Biotec, P^H: 7.3±0.1)

3.4 Isolation and identification of *Salmonella* spp.

3.4.1 Culture protocol for isolation and identification

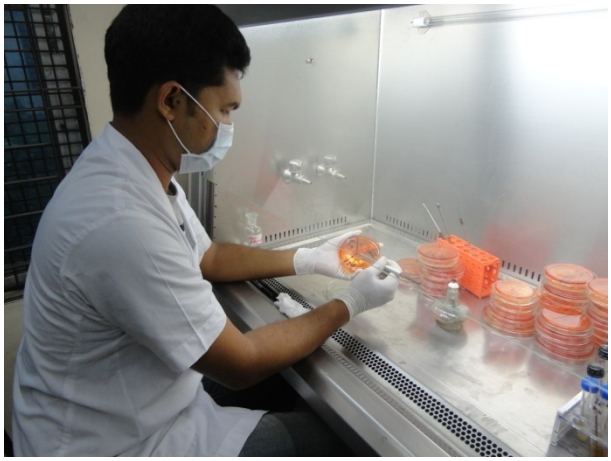
The collected samples were brought to the PRTC laboratory for isolation and identification of bacteria (*Salmonella* spp.). For the isolation of *Salmonella,* the cotton swab was inoculated in screw cap test tube containing nutrient broth (primary enrichment media) and incubated for 24 hours at 37^0C. After primary enrichment sample from buffer peptone was picked up and streaked on BGA, SS and Blood agar. The agar plates then were incubated at 37^0C for 24 hours. After development of characteristic colony the positives were selected for biochemical test (TSI stab) to confirm *Salmonella.*

3.4.2 Gram's staining

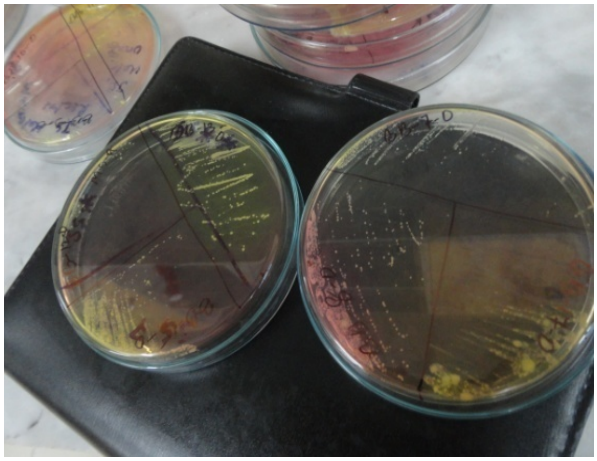
Gram's staining was performed as per procedures described by Merchant and Packer (1969) to determine the size, shape and arrangement of bacteria. Therefore**,** the suspected colonies were taken over a slid to make a thin smear that was done by sliding the edge of another glass slide across the glass slide containing the sample and then allowed it to air dry. The smear was then heat fixed by quickly passing it two to three times through a flame. After fixation the Gram's staining was done by follows: Crystal violet (primary stain) was used for two minutes, Gram's iodine (mordent) for 1 minute, Acetone (decoloriser) for 5-7 seconds and finally, Safranin (counter stain) for 1 minute. Gently rinsing was done with tape water after every step. The slide was then observed by microscope under 100X with emersion oil and characterization of bacteria was done.

3.4.3 Biochemical test

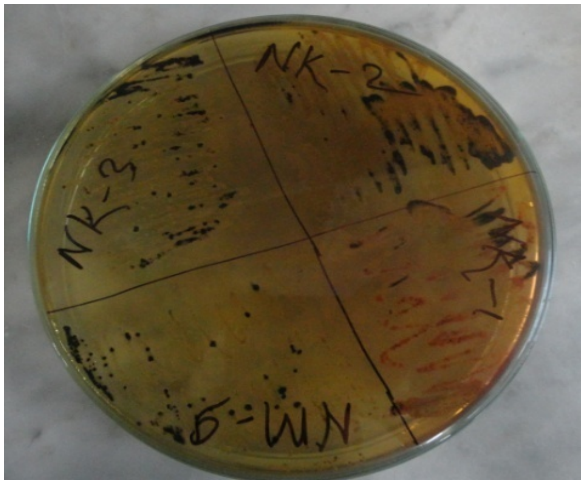
A straight inoculating needle was used to pick up isolated colony from culture of isolates. The TSI slant was inoculated by stabbing the butt down to the bottom, and then streaked over the surface of the slant (back and forth). The TSI slant was then incubated overnight at temperature of 37^0C. The positive result for *Salmonella* was detected based on the properties provided by (MacFaddin, 1985; Power and McCuen, 1988).



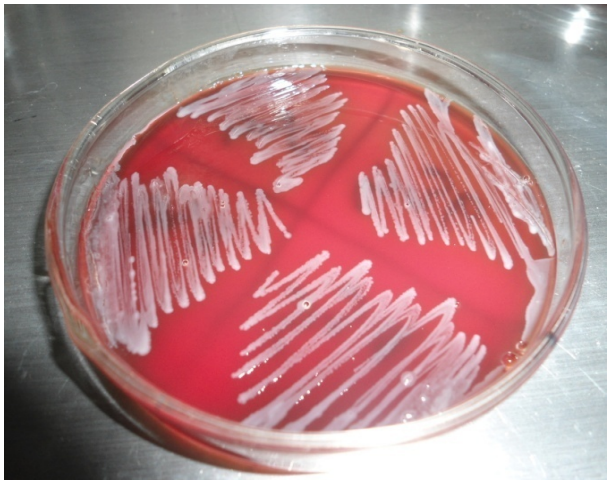
Streaking on sample Agar plate



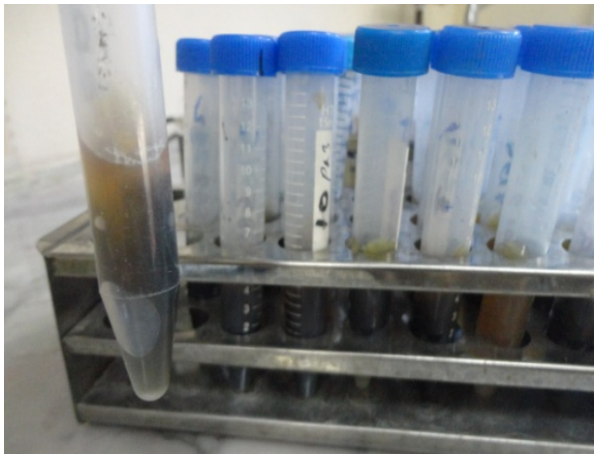
Pink color colony on BGA



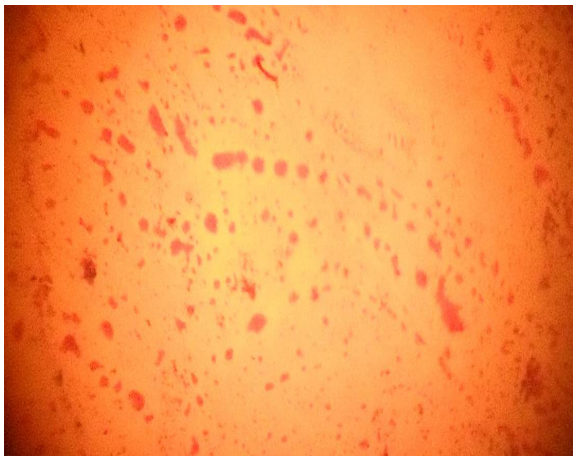
Black Color Colony on SS Agar



Pure colonies on Blood agar



Red slant, yellow butt and blackening



Gram-negative, pink colored, small rod shaped under microscope

Figure 3.8: Cultural properties of *Salmonella* spp. in different bacteriological media

3.5 Preservation of the culture

All positive isolates were inoculated into Tryptic Soy Broth (TSB) (Oxoid, England), incubated overnight at 37^{o}C and then preserved at -80^{o}C with 15% glycerol in 1.5 ml eppendorf tubes for future investigation.

3.6 DNA Extraction from bacterial culture for PCR test

The preserved isolates identified as *Salmonella* spp. using conventional bacteriological culture and biochemical characterization method were removed from the freezer and thawed at room temperature. Then each of the isolates were again streaked onto BGA agar plates as described under 3.4.1 incubated at 37^{o}C overnight. After incubation pink color colony for *Salmonella* was re-confirmed before initiating DNA extraction from the isolates. For DNA extraction boiling method was followed as per the steps given below:

i. Using a sterile inoculating loop, a loop full of fresh colonies (3-4) was picked up from each of the plates and transferred to 1.5 ml eppendorf tubes containing 100µl deionized water. The tubes were then vortexed to have a homogenous cell suspension.
ii. Using a sterile needle a ventilation hole was made on the lids of each of the eppendorf tubes. The tubes with the cell suspension were then boiled in a water bath at 100^{o}C for 10 minutes and immediately thereafter cooled by placing them into flaked ice for 10 minutes. This process of boiling and sharp cooling allowed the bacterial cell wall to break down thus releasing DNA.
iii. Then the eppendorf tubes with the suspension were centrifuged at 10000 rpm for 10 minutes and the supernatants containing DNA were then collected in the fresh eppendorf tubes and preserved at -20^{o}C until testing.

3.6.1 Identification of *Salmonella* Typhimurium (Inv A gene) by PCR

Table 3.2: Oligonucleotide primers used in PCR to detect *Salmonella*

Primers	Sequence (5´-3´)	G/C content (%)	Melting temp ^{o}C (Tm)	Annealing Temp (^{o}C)	Size of amplified product (bp)
ECO-1 (Forward)	GTG AAA TTA TCG CCA CGT TCG GGC AA	48	52	64ºC	284
ECO-2 (Reverse)	TCA TCG CAC CGT CAA AGG AAC C	63	50		

At first the stock solution (100 picomole concentration) of each primer was diluted with molecular grade water to make a 10 picomole concentration to be used for a PCR test. The reaction mixture, with the constituents and their amounts, prepared for detection of *Salmonella* Typhimurium using PCR test is shown in **Table 3.3**.

Adding reagents for PCR Placing Samples in PCR machine Filling Gel pores with samples

Figure 3.9: Different activities during PCR

Table 3.3: Contents of each reaction mixture of PCR used to detect *Salmonella*

CONTENT NAME	AMOUNT
Thermo Scientific dream Taq PCR Master Mix (2x) Ready to use	25 µl
Forward Primer	2µl
Reverse Primer	2µl
DNA template	2µl
Deionized water (Nuclease free)	19µl
Total	50µl

Amplification (PCR) was performed in a thermo cycler (Applied Biosystem, 2720 thermal cycler, Singapore). All reactions were carried out in a final volume of 50 µl. The cycling conditions used for PCR (Wang and Taylor, 1998) are shown in **Table 3.4**. A total of 35 cycles were run. We used negative control, i.e. samples with ddH_2O instead of genomic DNA

as template, to ascertain that the outcome of each PCR run was not affected by contamination.

Table 3.4: Cycling conditions used for PCR detection of *Salmonella.*

Serial No.	Steps	Temp. and Time
1	Initial incubation	94 ^{o}C for 60seconds
2	Final denaturation (35 cycles)	94 ^{o}C for 60 seconds
3	Annealing	64 ^{o}C for 30 seconds
4	Extension	72 ^{o}C for 30 seconds
5	Final extension	72 ^{o}C for 7 minutes
6	Final holding	4 ^{o}C

3.6.2 Visualization of PCR Product of *Salmonella* Spp. through agar gel electrophoresis

1.2% agarose gel w/v was made by using 0.5 g agarose powder and 50ml TAE buffer with ethidium bromide. The DNA amplicons were visualized using 4 µl of the final PCR product and 2 µl standard 100bp DNA markers (Invitrogen) at 120 V/ 90mA for 30 min. Gels were photographed using a gel documentation system Positive or negative amplifications were evaluated as presence or absence of visible orange colour bands on agarose gels under UV transilluminator (BDA digital, biometra GmbH, Germany).

Briefly the procedure was as follows:

3.6.2.1 Procedure of agar gel electrophoresis

1. For 1.2% agarose , 500 mg of agarose and 50 ml of 1 X TAE buffer was mixed thoroughly in a conical flask.
2. The mixture was heated in a microwave oven until agarose was completely dissolved.
3. The agarose-TAE buffer solution was then allowed to cool in room temperature.
4. Gel casting tray was prepared by sealing ends of gel chamber with tape or appropriate casting system and placed appropriate number of combs in gel tray.
5. 10 microlitre of ethidium bromide was added to agarose-TAE buffer mixture, shaked well and poured into gel tray.
6. The gel was then allowed to be cool (left for 15-30 minutes at room temperature).

7. The comb(s) were removed and the electrophoresis chamber was filled with 1x TAE buffer untill the casted gel is drowned completely.
8. 4 µl of DNA and 2 µl of 100bp marker (ladder) were loaded into gel.
9. The electrophoresis was run at 120 volt and 90 mA for 20 minutes.
10. Then the gel were taken to the UV transilluminator for image acquisition and analysis.

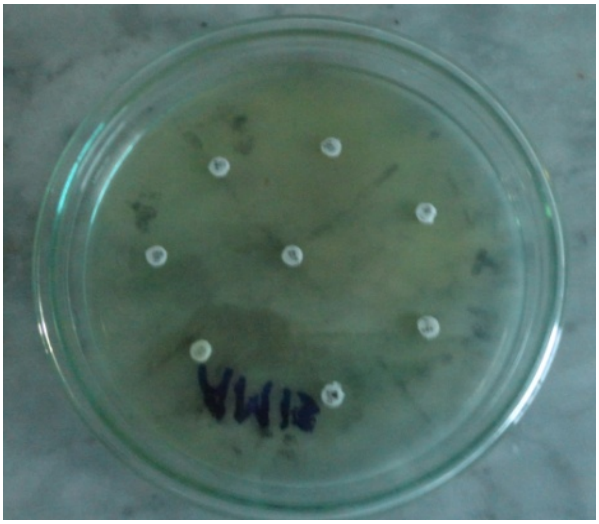
3.7 Cultural Sensitivity (CS) Test at Mueller Hinton Agar

After confirmation of isolates as *Salmonella* Typhimurium and *Salmonella* Entiritidis antimicrobial susceptibility of the isolates was determined by using the micro disc diffusion method, and the method was used according to guidelines established by *Clinical and Laboratory Standards Institute* (CLSI, 2007).

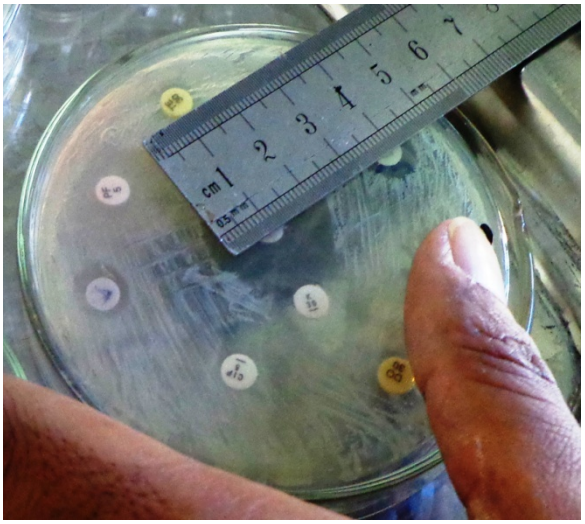
Antibiotics selected for susceptibility testing included a panel of antimicrobial agents of interest to the poultry industry and human (public) health authorities. From the range of antimicrobial drugs, 12 were selected on the basis of their range of activity against enterobacteria on their use in dog by veterinarians and human medicine. Veterinary antibiotics were chosen due to their use as therapeutic, prophylactic or growth promoting agents in livestock industry and human antibiotics were selected on the basis of their use and /or importance in human medicine.

The following antibiotics and disc potencies were used for *Salmonella*: CN: Gentamicin (10µg), ENR: Enrofloxacin (5µg), OT: Oxytetracycline (30µg), AML: Amoxicillin (10µg), AMP: Ampicillin (10µg), CT: Colistin sulfate (10µg), SXT: Sulfamethoxazole & Trimethoprim (25µg), CRO: Ceftriaxone (30µg), PEF: Pefloxacin (5 µg), CE: Cefixime (30 µg), AZN: Azithromycin (15 µg) and E: (15 µg). The antibiotic resistance pattern for the panel of antibiotics was determined considering the zone of inhibition sizes for each of the antibiotics as "resistant (R)", "intermediately resistant (I)", and “sensitive (S)” against the test isolates as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2010) shown in **Table 3.5**. A sterile swab was dipped into the inoculum prepared for antimicrobial sensitivity test and rotated against the side of the tube with firm pressure. Then after removing the excess fluid from the swab the dried surface of MH agar was inoculated by streaking the swab three times over the entire agar surface rotating the plates approximately at 60 degrees for each time to ensure an even distribution of the inoculums. The antimicrobial disks were then placed on the surface of the streaked agar. A separate forceps was always used to dispense each of the antimicrobial disks. The disks were placed carefully on the

surface of the agar with a gentle pressure to make a complete contact. After dispensing all the disks the agar plate was incubated at 37^{o}C for 16 to 18 hours. At the end of incubation the size of zone of inhibition around a micro-disk was measured with a digital slide calipers and the result was deduced according to CLSI, 2007. Details of the antibiotic discs used for the experiment is presented in **Table 3.5**.



Antibiotic Disc on Mueller Hinton Agar



Measuring Zone of Inhibition Diameter

Figure 3.10: Antimicrobial sensitivity test by Kirby-Bauer method.

Table 3.5: Diameter (zone of inhibition) standards for *Salmonella* Spp. (CLSI, 2007 & 2010).

Group of antimicrobials	Antimicrobial agents	Disk content	Zone of inhibition (diameter in mm)			Manufacturers
			R	I	S	
Penicillin	Ampicillin (AMP)	10µg	≤13	14-16	≥17	Oxoid, Ltd. Basingstoke, Hampshire, England
β-lactamase inhibitor	Amoxicillin (AML)	10µg	≤13	14-17	≥18	
Aminoglycosides	Gentamicin (CN)	10 µg	≤12	13-14	≥15	
Fluoroquinolones	Enrofloxacin (ENR)	5 µg	≤14	15-17	≥18	
	Pefloxacin (PEF)	5 µg	≤15	16-18	≥19	
Polypeptide antibiotics	Colistin sulphate (CT)	10µg	≤10	-	≥11	
Tetracycline	Oxytetracyclin (OT)	30 µg	≤11	12-14	≥15	
Potentiated Sulfur drugs	Sulfamethoxazole & Trimethoprim (SXT)	25 µg	≤10	11-15	≥18	
Macrolid	Azithromycin (AZN)	15 µg	≤13	14-17	≥18	
	Erythromycin (E)	15 µg	≤13	14-22	≥23	
Cephalosporine (3rd generation)	Ceftriaxone (CRO)	30 µg	≤13	14-20	≥21	
	Cefixime (CE)	30 µg	≤15	16-18	≥19	

CHAPTER - IV

RESULTS

4.1 Prevalence of *Salmonella* spp.

Salmonella spp. isolated from rectal swabs of nine (9) areas in Chittagong City Corporation, Bangladesh were evaluated for antimicrobial susceptibility to estimate the prevalence and pattern of antimicrobial resistance and sensitivity among *Salmonella* spp. isolates.

Table 4.6: Prevalence of *Salmonella* in different sex and sampling sites

Variables	Categories	No. of Sample	Prevalence % (n)	χ^2-value	P-value
Sex	Male	56	58.93 (33)	0.774	0.379
	Female	51	66.67(34)		
Sampling site	Muradpur	22	45.45 (10)	17.64	0.024
	Panchlaish	7	28.57 (2)		
	Chawkbazar	8	62.50 (5)		
	Alankar	15	66.67 (10)		
	Kattoli	16	75 (12)		
	Pahartoli	5	20 (1)		
	Ambagan	6	100 (6)		
	Bayezid Bostami	18	61.11 (11)		
	New Market	10	100 (10)		

Table 4.6 shows the prevalence of *Salmonella* spp. in both sexes of stray dogs and different sampling sites. The prevalence of *Salmonella* spp. was found higher in females (66.67%) than males (58.93%). There was found no statistically significant difference ($p>0.05$) between sexes for prevalence of *Salmonella* spp. On the other hand, prevalence was highest (100%) in samples of Ambagan and New Market and lowest (20%) in samples of Panchlaish. There was found statistically significant differences ($p<0.05$) among the sampling sites for the prevalence of Salmonaliosis.. The prevalence of *Salmonella* in stray dogs was represented in **Figure 4.11**.

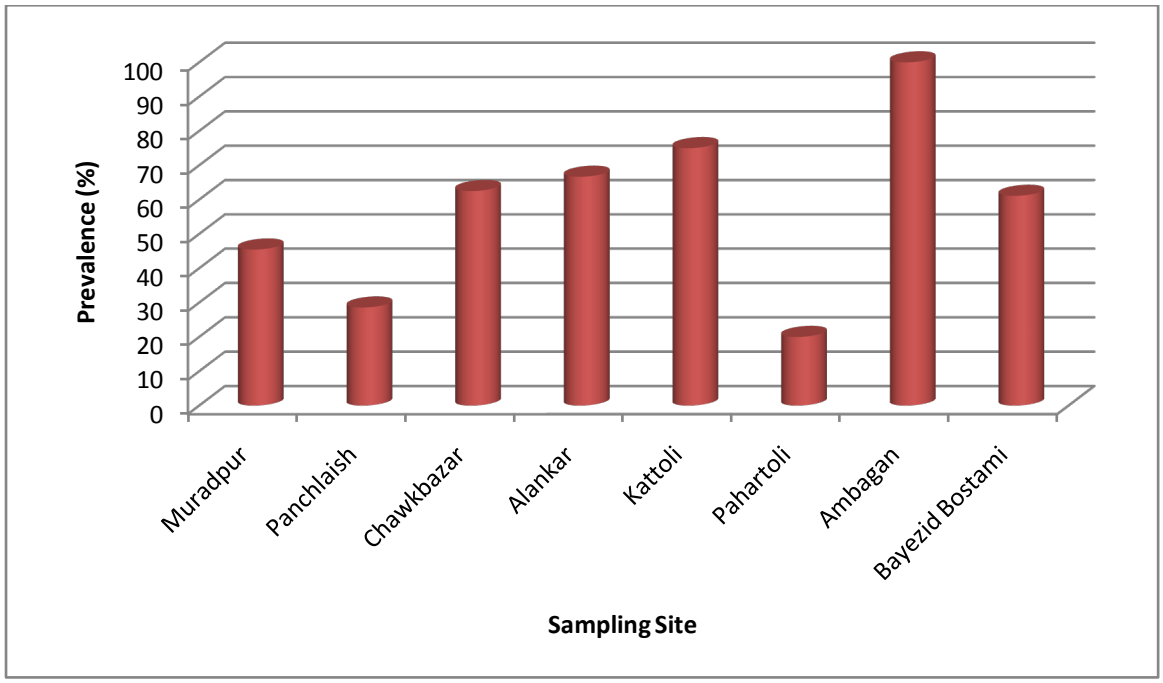


Figure 4.11: Prevalence of *Salmonella* in different sampling site

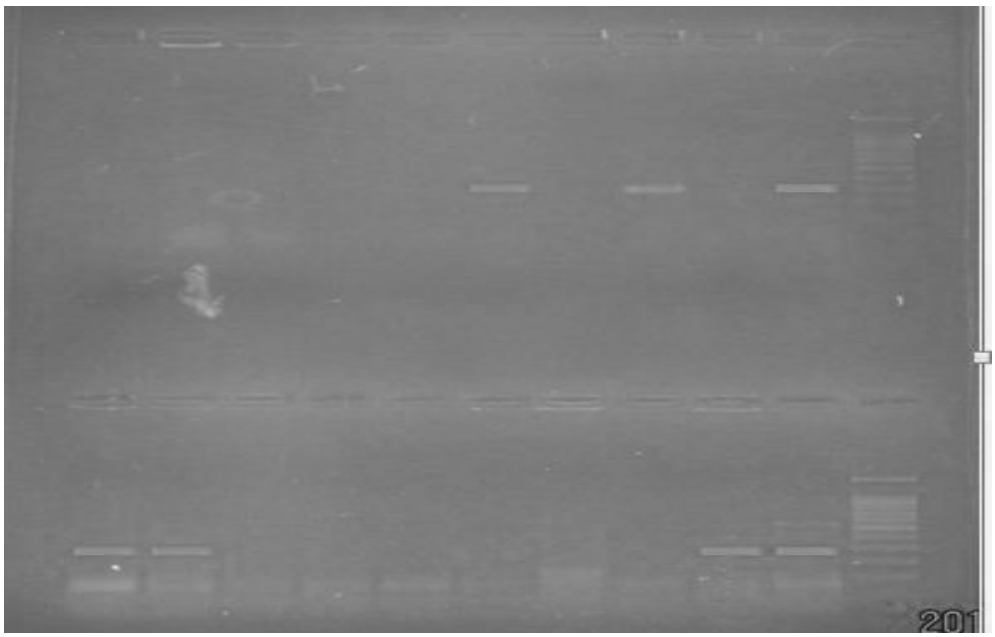


Figure 4.12: Positive band at 284bp site agar-gel

4.2 Prevalence of *Salmonella* Typhimurium

Among the 67 isolates of *Salmonella* Spp. of 7 isolates (10.45%) were found positive for *Salmonella* Typhimurium by PCR. That is prevalence of *Salmonella* Typhimurium was 6.49% in the study area.

4.3 Antimicrobial Resitance Pattern

Table 4.7: Antimicrobial resistance pattern of *Salmonella* isolates

Antibiotics	*Salmonella* positive isolates	Pattern		
		Resistant % (n)	Intermediate % (n)	Sensitive % (n)
Ampicillin	67	98.51 (66)	1.49 (1)	0 (0)
Amoxicillin	67	100 (67)	0 (0)	0 (0)
Gentamicin	67	64.18 (43)	13.43 (9)	22.39 (15)
Enrofloxacin	67	97.01 (65)	2.99 (2)	0 (0)
Pefloxacin	67	94.03 (63)	4.48 (3)	1.49 (1)
Colistin sulphate	67	92.54 (62)	-	7.46 (5)
Oxytetracyclin	67	97.01 (65)	1.49 (1)	1.49 (1)
Sulfamethoxazole & Trimethoprim	67	91.04 (61)	4.48 (3)	4.48 (3)
Azithromycin	67	98.51 (66)	1.49 (1)	0 (0)
Erythromycin	67	76.12 (51)	19.40 (13)	4.48 (3)
Ceftriaxone	67	43.28 (29)	26.87 (18)	29.85 (20)
Cefixime	67	95.52 (64)	4.48 (3)	0 (0)

The prevalence and pattern of antimicrobial resistance of *Salmonella* isolates has been outlined in **Table 4.7**.

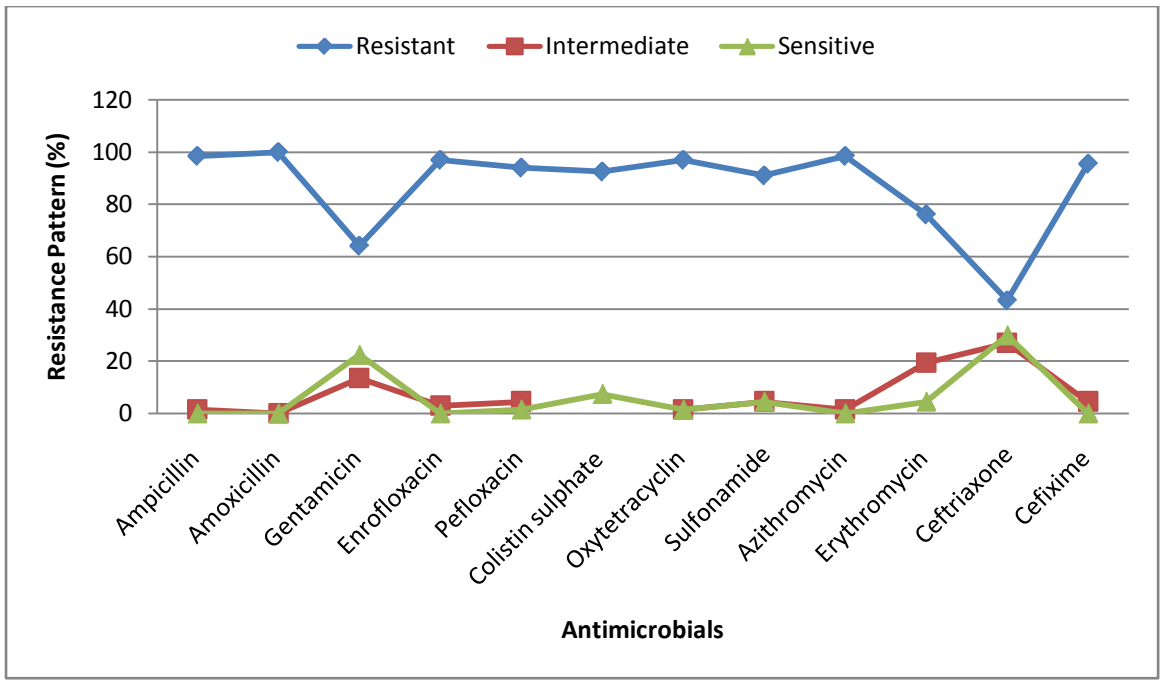


Figure 4.13: Antimicrobial resistance pattern of *Salmonella* isolates

Resistance patterns of *Salmonella* were highest in Amoxicillin (100%) followed by Ampicillin (98.51%), Azithromycin (98.51%), Enrofloxacin (97.01%), Tetracycline (97.01%), Cefixime (95.52%), Pefloxacin (94.03%), Colistin (92.54%), Potentiated Sulfonamide (91.04%), Erythromycin (76.12%), Gentamicin (64.18%) and Ceftriaxone (43.28%). It was revealed that no isolates were found sensitive to Ampicillin, Amoxicillin, Azithromycin, Enrofloxacin and Cefixime. Ceftriaxone showed highest level of sensitivity (29.85%) followed by Gentamicin (22.39%), Colistin (7.46%), Erythromycin (4.48%), Potentiated Sulfonamide (4.48%), Pefloxacin (1.49%) and Tetracycline (1.49%). In current research, all the isolates of *Salmonella* showed multiple antimicrobial resistances. **Figure 4.13** shows the graphical presentation of resistance pattern of *Salmonella* isolates of stray dog.

Table 4.8: Patterns of multidrug resistance in isolates of *Salmonella* in different sexes

Antimicrobial	Disc conc. (µg)	Male (n=33) (%)			Female (n=34) (%)		
		R	I	S	R	I	S
Ampicillin	10	100	0	0	97.06	2.94	0
Amoxicillin	10	100	0	0	100	0	0
Gentamicin	10	66.67	9.09	24.24	61.76	17.65	20.59
Enrofloxacin	5	96.97	3.03	0	97.06	2.94	0
Pefloxacin	5	93.94	3.03	3.03	94.12	5.88	0
Colistin sulphate	10	96.97	-	3.03	88.24	-	11.76
Oxytetracyclin	30	96.97	3.03	0	97.06	0	2.94
Sulfamethoxazole & Trimethoprim	25	93.94	3.03	3.03	88.24	5.88	5.88
Azithromycin	15	96.97	3.03	0	100	0	0
Erythromycin	15	78.79	15.15	6.06	73.53	23.53	2.94
Ceftriaxone	30	39.39	24.24	36.36	47.06	29.41	23.53
Cefixime	30	93.94	6.06	0	97.06	2.94	0

Resistance pattern of *Salmonella* isolates among the twelve tested antimicrobials, Amoxicillin and Ampicillin turned out as the highest level of resistance (100%) followed by Enrofloxacin (96.97%), Colistin (96.97%), Azithromycin (96.97%), Tetracycline (96.97%), Sulfonamide (93.94%), Pefloxacin (93.94%), Cefixime (93.94%), Erythromycin (78.79%), Gentamicin (66.67%), and Ceftriaxone (39.39%) in males while in female Amoxicillin and Azithromycin showed 100% resistance followed by Ampicillin (97.06%), Cefixime (97.06%), Oxytetracycline (97.06%), Pefloxacin (94.12%), Colistin (88.24%), Potentiated Sulfonamide (88.24%), Erythromycin (73.53%), Gentamicin (61.76%) and Ceftriaxone (47.06%). In both sexes of stray dogs, Ceftriaxone showed highest sensitivity (36.36 & 23.53%) against *Salmonella* isolates from different sampling sites and no isolates are found to be sensitive towards Amoxicillin, Ampicillin, Enrofloxacin, Cefixime and Azithromycin. Besides those antibiotics, Gentamicin showed 24.24% sensitivity followed by Erythromycin (6.06%), Pefloxacin (3.03%), Colistin (3.03%), Potentiated Sulfonamide (3.03%) and others (0%) in male and Gentamicin (20.59%), Colistin (11.76%), Potentiated Sulfonamide (5.88%), Oxytetracycline (2.94%), Erythromycin (2.94%) and others (0%). Patterns of multidrug resistance isolates of *Salmonella* in both sexes presented graphically in **Figure 4.14.**

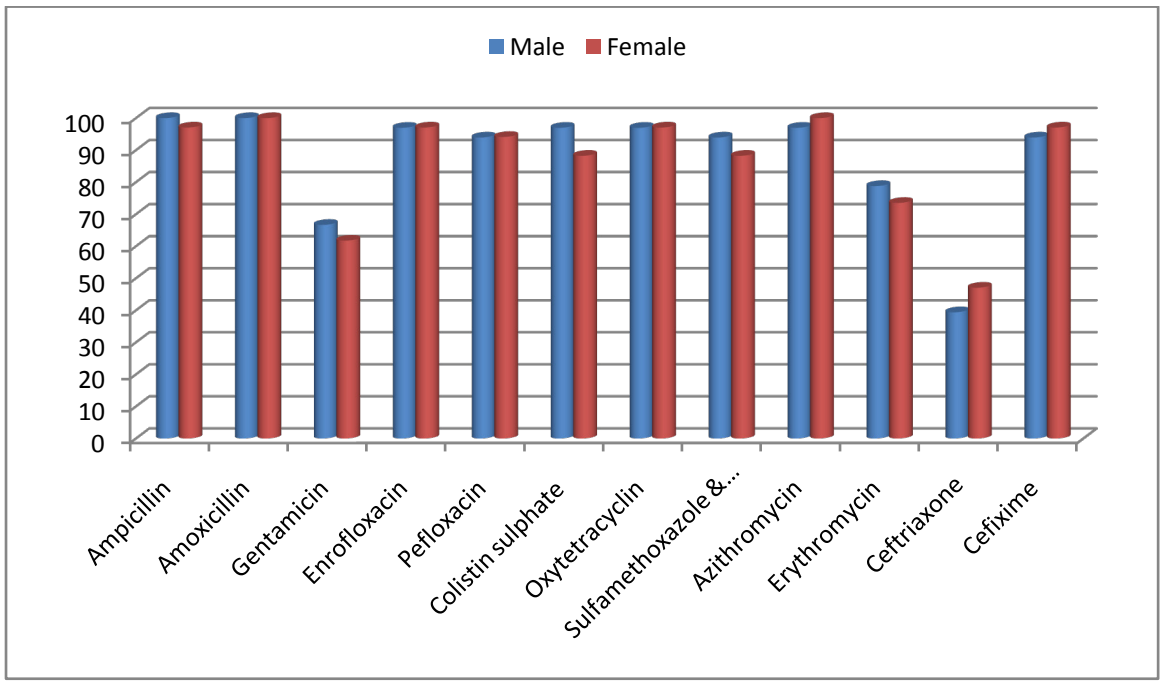


Figure 4.14: Patterns of multidrug resistance isolates of *Salmonella* in both sexes

Table 4.9: Patterns of multidrug resistance in isolates of *Salmonella* in different sampling sites

Antimicrobial		Muradpur n=10 (%)	Panchlaish n=2 (%)	ChawkBazar n=5 (%)	Alankar n=10 (%)	Kattoli n=12 (%)	Pahartoli n=1 (%)	Ambagan n=6 (%)	Bayezid n=11 (%)	New Market n=10 (%)	Range
Ampicillin	**R**	100	100	100	100	100	100	83.33	100	100	83.33-100
	I	0	0	0	0	0	0	16.67	0	0	0-16.67
	S	0	0	0	0	0	0	0	0	0	0
Amoxicillin	**R**	100	100	100	100	100	100	100	100	100	100
	I	0	0	0	0	0	0	0	0	0	0
	S	0	0	0	0	0	0	0	0	0	0
Gentamicin	**R**	90	100	60	70	66.67	0	16.67	54.55	70	0-100
	I	10	0	0	10	16.67	0	50	18.18	0	0-50
	S	0	0	40	20	16.67	100	33.33	27.27	30	0-100
Enrofloxacin	**R**	100	100	100	100	91.67	100	83.33	100	100	83.33-100
	I	0	0	0	0	8.33	0	16.67	0	0	0-16.67
	S	0	0	0	0	0	0	0	0	0	0
Pefloxacin	**R**	100	100	100	100	91.67	100	83.33	90.91	90	83.33-100
	I	0	0	0	0	8.33	0	16.67	9.09	0	0-16.67
	S	0	0	0	0	0	0	0	0	10	0-10
Colistin	**R**	90	50	100	90	83.33	100	100	100	100	50-100
	S	10	50	0	10	16.67	0	0	0	0	0-50
Oxytetracyclin	**R**	100	50	100	100	100	0	100	100	100	50-100
	I	0	0	0	0	0	100	0	0	0	0-100
	S	o	50	0	0	0	0	0	0	0	0-50
Sulfamethoxazole & Trimethoprim	**R**	90	100	80	100	100	100	66.67	90.91	90	66.67-100
	I	10	0	0	0	0	0	16.67	0	10	0-16.67
	S	0	0	20	0	0	0	16.67	9.09	0	0-20
Azithromycin	**R**	100	100	100	100	91.67	100	100	100	100	91.67-100
	I	0	0	0	0	8.33	0	0	0	0	0-8.33
	S	0	0	0	0	0	0	0	0	0	0
Erythromycin	**R**	70	50	60	80	75	100	83.33	81.82	80	50-100
	I	30	50	20	20	16.67	0	0	18.18	20	0-50
	S	0	0	20	0	8.33	0	16.67	0	0	0-20
Ceftriaxone	**R**	70	0	40	60	41.67	0	33.33	45.45	20	0-70
	I	20	50	20	20	25	0	16.67	36.36	40	0-50
	S	10	50	40	20	33.33	100	50	18.18	40	10-100
Cefixime	**R**	100	100	100	90	100	100	100	90.91	90	90-100
	I	0	0	0	10	0	0	0	9.09	10	0-10
	S	0	0	0	0	0	0	0	0	0	0

Table 4.9 stated that among the twelve tested antimicrobials, resistance pattern of *Salmonella* isolates Amoxicillin turned out as the highest level of resistance ranged at (100%) followed by Azithromycin (91.67-100%), Cefixime (90-100%), Ampicillin (83.33-100%), Enrofloxacin (83.33-100%), Pefloxacin (83.33-100%), Potentiated Sulfonamide (66.67-100%), Tetracycline (50-100%), Colistin (50-100%), Gentamicin (0-100%), and Ceftriaxone (0-70%) across the study sites. The rate of sensitivity to individual antibiotics against *Salmonella* isolates from different sampling sites was higher in Ceftriaxone ranged (10-100%) followed by Gentamicin (0-100%), Colistin (0-50%), Oxytetracycline (0-50%), Erythromycin (0-20%), Potentiated Sulfonamide (0-20%) and Pefloxacin (0-10%). There were no isolates are found to sensitive against Amoxicillin, Ampicillin, Azithromycin and Oxytetracycline in all sampling sites. In case of Enrofloxacin, 100% of *Salmonella* isolates were resistance to Enrofloxacin in Muradpur, Panchlaish, Chawk Bazar, Alankar, Pahartoli, Bayezid Bostami, New Market and lowest resistance against Enrofloxacin (83.33%) in Ambagan. All *Salmonella* isolates were found resistant against Amoxicillin in all sampling sites. The resistance level against Colistin was higher (100%) in Chawk Bazar, Pahartoli, Ambagan, Bayezid Bostami, New Market and lowest in Panchlaish (50%). In this study, there was no variation of resistance level in case of Enrofloxacin.100% resistance against Erythromycin was found in Pahartoli, New Market and lowest (50%) in Panchlaish. No isolates of *Salmonella* was resistance to Ceftriaxone (0%) in Pahartoli and Panchlaish and 70% was resistance in Muradpur. The sensitivity of Colistin is better in Panchlaish (50%) than of any other site (0-20%). In case of Gentamicin, the higest level of sensitivity (100%) was found in Pahartoli and lowest (0%) in Muradpur and Panchlaish. Besides Ceftriaxone and Gentamicin, Potentiated Sulfinamide was sensitive 20% in Chawk Bazar and lowest (0%) in Muradpur, Panchlaish, Alankar, Kattoli, Pahartoli and New Market. Patterns of multidrug resistance isolates of *Salmonella* in different sites presented graphically in **Figure 4.15.**

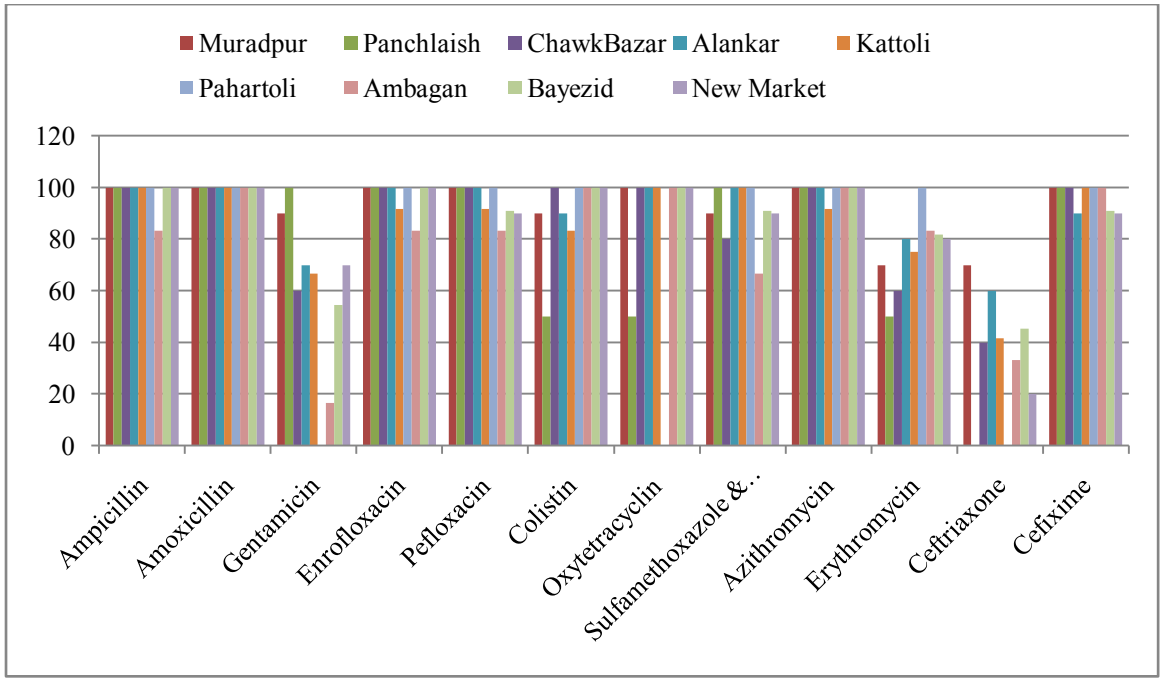


Figure 4.15: Patterns of multidrug resistance *Salmonella* isolates in sampling sites

CHAPTER - V

DISCUSSION

Stray dogs are common in Bangladesh and they feed on household wastage in dustbins, roadside restaurants, abattoir etc., and drink on sewage and drain water which is frequently contaminated with microorganisms. So they get infections frequently. On the other hand, stray dogs have close attachment with people and children. Children often love to play with a dog nearby his house and also offer food. There is high chance of cross infection to human. The aim of this study was to determine the occurrence of zoonotic *Salmonella* spp. in fecal material along with estimating the prevalence of antimicrobial resistance against *Salmonella* spp. in randomly selected areas in Chittagong City Corporation of Bangladesh. To the best of my knowledge, this is the first study in Chittagong, Bangladesh to tackle this issue.

5.1 Prevalence of *Salmonella* spp. in rectal swab

Salmonella Heidelberg was also the most common serotype isolated from the feces of dogs. Results of a retail surveillance conducted in Canada in 2003, showed *S.* Heidelberg to be the most prevalent serovar (73%) in 16% of chickens purchased from retail stores and markets, followed by *S.* Kentucky (11%). In Canada, *S.* Heidelberg was the most common cause of human salmonellosis, accounting for 26% of all *Salmonella* isolates obtained from human cases through enhanced passive surveillance in 2003. *Salmonella* Typhimurium was the second most common serotype (25%) associated with human salmonellosis, followed by *S.* Enteritidis (15%) (Finley *et al.*, 2007). All the *Salmonella* isolates were identified as *Salmonella* Typhimurium in the study of Ojo and Adetosoye (2009). Morse and Duncan (1975) isolated 53 *Salmonella* serovars from dogs while Seepersadsingh *et al.* (2004) reported 28 different serovars of *Salmonella* in dogs. *Salmonella* Typhimurium is the serotype most commonly isolated from dogs (Morse and Duncan, 1975). Human cases of Salmonellosis caused by *Salmonella* Typhimurium have been linked with likely contact with the faeces of infected dogs (WHO, 2001).

5.2 Prevalence of *Salmonella* Typhimurium in rectal swab

Salmonella Typhimurium is the serotype most commonly isolated from dogs (Morse and Duncan, 1975). Human cases of Salmonellosis caused by *Salmonella* Typhimurium have been linked with likely contact with the faeces of infected dogs (Anonymous). *Salmonella enterica serovar* Typhimurium and *Salmonella enterica serovar* Enteritidis are the most

frequently isolated serovar from food borne outbreaks throughout the world (Herikstad *et al.,* 2002). Established conventional methods to detect and identify *Salmonella* are time consuming and include selective enrichment and plating followed by biochemical tests (Bennasar *et al.,* 2000). In vitro amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics (Malorny *et al.,* 2003). Several genes have been used to detect *Salmonella* in natural environ-mental samples as well as food and faecal samples. Virulence chromosomal genes including; invA, invE, himA phoP are target genes for PCR amplification of *Salmonella* species. (Jamshidi *et al.,* 2009). *Salmonella* specific PCR with primers for invA is rapid, sensitive, and specific for detection of *Salmonella* in many clinical samples (Lampel *et al.,* 2000). Ojo and Adetosoye (2009) reported prevalence of *Salmonella* typhimurium in non-diarrhaeic and diarrhoeic feces were 4.0 and 3.7%, respectively which agree with present study (6.49%).

5.3 Antimicrobial resistance

Approaches to prevent and control Salmonellosis in the food animal industry by various means such as improved bio-security, vaccination, use of competitive exclusion products, and the introduction of novel immuno-potentiators with limited success has necessitated the use of antimicrobial chemotherapy in the treatment and control of Salmonellosis (Zhao *et al.,* 2007). The use of antimicrobials in food animals has resulted in the development of antimicrobial resistance (White *et al.,* 2001), through mutation and acquisition of resistance encoding genes (Fluit, 2005). The situation in developing countries like Bangladesh may be exaggerated by easy accessibility of antimicrobials at a cheaper price and their extensive use in poultry production system (Prakash *et al.,* 2005). Another major setback might be the quality and potency of locally produced antimicrobial drugs; for example, there are over 80 different brands of the Fluoroquinolones (Ciprofloxacin) in Bangladesh. Thus there is widespread availability and uncontrolled use of antibiotics poses the antimicrobial resistance in food animals and their products which is the actual threat of public health. The current study recorded multiple antimicrobial resistances against *Salmonella* spp. (up to seven) and in most cases, estimated 100% resistance for category 2-4 antimicrobials across the study sites. These threatening correspond to the many non-epidemiological and opportunistic earlier studies in Bangladesh (Kohinur *et al.*, 2010; Ahmed *et al.*, 2011), India (Suresh *et al.*, 2006), Nepal (Pokharel *et al.,* 2006; Dahal, 2007), Bhutan (Sing *et al.,* 1992; Silva *et al.,* 2000; Dahal, 2007).

5.4 Level of antimicrobial resistance of *Salmonella*

5.4.1 Amoxicillin

The results of antimicrobial resistance of *Salmonella* isolated revealed that all the isolates were resistance to Amoxicillin. Earlier studies conducted at CVASU, Bangladesh (Chowdhury, 2012) and other European countries detected amoxicillin residues in eggs (Reidiker and Stadler, 2001; Luboslava *et al.,* 2006) and other poultry and livestock products (Hossain, 2012; Mahmud, 2013). Amoxicillin commonly used in breeder, commercial farms and animals. Therefore, residues of these antibiotics could have been passed through eggs followed by day old chicks from the breeder farms and directly through farm effluents. The complete resistance of Amoxicillin in rectal swabs of present study might be due to indiscriminate use of this antibiotic in the breeder, commercial farm and other veterinary practices.

5.4.2 Ampicillin

Resistance to Ampicillin in this study was higher. Almost similar (87-100%) resistance of Ampicillin was reported earlier in Bangladesh (Kohinur *et al.*, 2010; Ahmed *et al.*, 2011) and India (Suresh *et al.*, 2006). Ampicillin resistant isolated from eggs was in higher proportion than from the broiler chickens and environmental samples from India (Suresh *et al.,* 2000). Amoxicillin and Ampicillin antibiotics might have been used as growth promoters or prophylactics or therapeutics in breeder farms from where farmers purchased day old birds or pullets. Ojo *et al.* (2009) reported 47.2% resistance to Amphicillin in *Salmonella* isolated rectal swab of pet dogs. Cross antimicrobial resistance cannot be ignored as it is evident in many earlier studies (Goldstein *et al.,* 1983, Gupta *et al.,* 1990 and Rowe *et al.,* 1992) and causes higher resistance to Ampicillin and Amoxicillin in food and companion animals.

5.4.3 Erythromycin

In this study Erythromycin was resistance against *Salmonella* spp. in Chittagng that are higher than the resistance of Erythromycin (62.5%) reported in Dhaka city (Ahmed *et al.*, 2011). This result are consistent with many other previous studies in developing countries including Bangladesh (Verma *et al.,* 1993; Anjanappa *et al.,* 1994; Rahman *et al.,* 2004 ; Akter *et al.,* 2007). Ojo *et al.* (2009) stated 100% resistance against Erythromycin in *Salmonella* Typhimurium isolated from rectal swab of dogs in Nigeria. It could be happened due to heavily use of Erythromycin against different infectious diseases including Salmonellosis without proper diagnosis of diseases. Resistance of Erythromycin was

evidenced as a serious problem in the study sites at it might be due to Subnormal doses and incomplete treatment course of Erythromycin.

5.4.4 Tetracycline

Tetracycline showed higher resistance in present study that are alarming for the layer farms and consumers of Bangladesh and the results agreed with the earlier researcher of Bangladesh (Islam *et al.,* 2006; Akter *et al.,* 2007) and India (Sing *et al.,* 1992; Harsha *et al.,* 2007; Sivkumar *et al.,* 2012). Tetracycline has been used to treat animals and poultry, which might have resulted in the emergence of tetracycline resistant *Salmonella* in animals and human (Ekperigin *et al.,* 1983). Earlier study in Bangladesh Tetracycline reported 32% resistance from egg and its environment (Kohinur *et al.*, 2011). Contrarily 100% of cases were reported to be resistance to Tetracycline against *Salmonella* spp. in India (Suresh *et al.*, 2006), 88% were Italy (Ludovico *et al.,* 2000), Spain (Hernandez *et al.,* 2002) and England (Jones *et al.,* 2003). The pattern of tetracycline resistance against *Salmonella* spp. in Bangladesh is one of the commonest antimicrobials. Tetracycline widely used in veterinary practice in Bangladesh and other developing countries for prophylaxis, therapeutic and feed additive purposes (William, 1984; Harsha *et al.,* 2011; Donado-Godoy, 2012; Sivkumar *et al.,* 2012). In addition, sometimes farmers select drugs by their own or rely on neighbor experienced farmers or non veterinarians and these mal-practices may not always ensure proper drugs doses, frequency of drug administration and complete course of drug treatment. The above factors might have influenced on Tetracycline to be resistance against *Salmonella* spp.

5.4.5 Enrofloxacin

In present study we observed higher resistant of Enrofloxacin against the *Salmonella* isolates. The result was not agreed with Sing, (2012) because no isolates were found to resistant to Enrofloxacin in north India. In Several investigations resistant to Enrofloxacin were found 14% (EFSA and ECDC, 2012), 7% (FSA, 2003) in UK, 0.6% - 2% (Cheng *et al.*, 2012) in Australia that were comparatively lower than the current investigation. Higher resistant to Enrofloxacin in the study site due to indiscriminate usage of Enrofloxacin against bacterial disease causes higher resistant to Enrofloxacin in Bangladesh.

5.4.6 Pefloxacin

Resistance to Pefloxacin was recorded relatively at lower proportions in current study. Pefloxacin is a Fluoroquinolones antimicrobial that is increasingly and successfully used for the treatment of Salmonellosis in humans and animals (Brown *et al.*, 1994; Griggs *et al.*, 1994). Among Fluoroquinolones, resistance to Pefloxacin was found comparatively lower in

the present study as compared to 24% resistance in USA (Cui *et al.,* 2005b), 9 -14% in Germany (Malorny *et al.,* 2003). The lower resistance of this antibiotic might be due to newly introduced in poultry industries in Bangladesh and nature of relatively less resistance.

5.4.7 Ceftriaxone

Resistance to Ceftriaxone was recorded relatively lower proportions in study area which is consistent with the findings of Ojo *et al.* (0%) in Nigeria. The lower resistance was may be due to selective use of this antibiotic in veterinary practice. But the resistance is increasing day by day due to lower dose and incomplete dosing for relatively higher price than other antibiotics in developing countries like Bangladesh.

5.4.8 Colistin

The resistance pattern of Colistin to *Salmonella* was high in the isolates of the present study. Although, resistances to Colistin among rectal isolates are reported from Senegal (Bada-Alambedji *et al.,* 2006), Mexico (Zaidi *et al.,* 2006) and USA (Zhao *et al.,* 2006). Colistin resistance was comparatively increasing might be due to increasing use and recent availability in Bangladesh.

5.4.9 Cefixime

The resistance pattern against Cefixime in our study was higher which is consistent with Ojo *et al.* (2009) that reported 52.5% resistance from fecal isolates in dogs. Cefixime is not usually indicated in Salmonellosis in animals. We used this antibiotic to know the present response against *Salmonella*. There was found considerable intermediately sensitive isolates during this study.

5.4.10 Azithromycin

Present study showed that there was 100% resistance against Azithromycin in fecal isolates of *Salmonella*. The multiple antibiotic resistances of enterobactericeae demonstrated in the study accord with those found in our study. In recent years, testing of *Salmonella* isolates from different environments has shown an increasing proportion of multidrug resistant *Salmonella* spp. (Cheng *et al.,* 2004). The resistance might be due to genetic and biochemical mechanism alteration by microbes.

5.4.11 Gentamicin

Gentamicin presented higher resistance in the present study in stray dogs. Ojo *et al.* (2009) reported 35.3% resistance in fecal isolates of *Salmonella* Spp. in dog which is consistent with our findings. The increasing resistance might be due to improper dosing and frequent underdosing of Gentamicin in veterinary practice in Bangladesh.

5.4.12 Potentiated Sulfonamide

Our study demonstrated above 90% resistance against *Salmonella* Spp. from rectal swab of stray dogs. This resistance might be due to frequent use sulfonamides in animal and avian medicine in recent years in Bangladesh.

5.5 Resistance pattern

Overall, twelve antimicrobial resistance pattern were observed among *Salmonella* isolated . *Salmonella* spp. was resistant to five of the twelve antimicrobials tested with simultaneous multi-drug resistance to antimicrobials. A similar study in Turkey showed higher multi-drug resistance in *Salmonella* spp. isolates as compared to human (Icgen *et al*, 2002). *Salmonella* spp. was also found to be comparatively resistant to as many as 5 drugs tested in United States (Berrang *et al.,* 2006). The increasing rates of resistance to Ampicillin, Amoxicillin, Tetracycline and Erythromycin among the isolates might be attributed to the emergence of multi resistance *Salmonella* spp. Multidrug resistance, with rates of resistance to Ampicillin, Chloramphenicol, and Trimethoprim-Sulfamethoxazole of more than 50%, has been reported in many areas of the world. Extended-spectrum Cephalosporins and Fluoroquinolones have been suggested as alternative agents in the treatment of infections caused by multidrug resistant *Salmonella* serotypes (Cheng *et al.,* 2004), these data correspond to results obtained in this study showing that the serotype isolated *S. typhimurium* is resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole/trimethoprim and tetracycline. Resistance to antimicrobial agents in bacteria is mediated by several mechanisms including changes in bacterial cell wall permeability, energy dependant removal of antimicrobial agents via membrane-bound efflux pumps, modification of the site of drug action, and destruction or inactivation of antimicrobial agents (Cheng *et al.,* 2004).

5.6 Level of antimicrobial sensitivity

Ceftriaxone, Colistin and Gentamicin appeared to be sensitive against *Salmonella* spp. This is because these drugs are newly introduced and not commonly used against canine diseases in Bangladesh as well as in the study areas that's why these drugs may remain sensitive against *Salmonella* spp. The *Salmonella* strains having similar level of resistance and resistance pattern indicates their origin from a common source. The logical interpretation of the results of the multiple antibiotic resistances (MAR) index is that all *Salmonella* isolated in the study showed that the strains might have originated from environments where antimicrobials are often used. Dogs, one of the major reservoirs of *Salmonella* spp. are considered to be a high-risk source. Isolates resistant to three or more antimicrobials were classified as multi-drug-

resistant (MDR). Multidrug-resistant (MDR) strains of *Salmonella* are now encountered frequently worldwide and the rates of multidrug-resistance have increased considerably in recent years. Even worse, some variants of *Salmonella* have developed multidrug-resistance as an integral part of the genetic material of the organism, and are therefore likely to retain their drug-resistant genes even when antimicrobial drugs are no longer used, a situation where other resistant strains would typically lose their resistance (WHO, 2005). *Salmonella* isolate and its environment showed resistance to 10 antimicrobials in the US (NARMS, 2004), the isolates of *Salmonella* in this study were found resistant to Ampicillin, Amoxicillin, Erythromycin, and Tetracycline. Multi-drug resistant *Salmonella* Typhimurium was reported in the past few decades and is frequently reported from the Indian subcontinent (Rahman *et al.,* 2004). A higher proportion of antibiotic resistance in *Salmonella* Enteritidis has been reported from southern Brazil (Oliveira *et al.,* 2005).

CHAPTER - VI

CONCLUSION

Salmonellosis is a leading food-borne and zoonotic disease worldwide. A wide range of foods and companion animals has been implicated in such disease. However, close living with animal, especially pet animals and food animal products, have been consistently implicated in sporadic cases and outbreaks of human Salmonellosis. The results of the present study indicate that *Salmonella* contaminated dog feces are common in the environment of Chittagong, Bangladesh. The poor sanitation and handling of sewage and slaughter house products in the city area could be a source of contamination. In the current study the prevalence of *Salmonella* spp. was higher in the rectal swab of stray dogs. *Salmonella* and antibiotic resistance was a big problem in Bangladesh. In the current study, *Salmonella* isolates displayed resistance to antimicrobial and displayed a larger number of multiple resistances. From the findings of the study, it is avowed that a larger number of resistant isolates of *Salmonella* for Ampicillin, Amoxicillin, Tetracycline, Enrofloxacin and Erythromycin. The excess utilization of antibiotics in the dwells and veterinary practice, which might be the cause of increased resistance to Potentiated sulfur drugs, Pefloxacin and Colistin identified as sensitive drugs previously. Ceftriaxone should be used to treat Salmonellosis carefully as it showed sensitivity to *Salmonella* Spp. Cares should be taken to maintain the sensitivity of the drugs selecting the correct drugs, proper doses and complete course of treatment. This multiple antimicrobial-resistant nature of the organism adds to the gravity of the problem. The level of resistance of *Salmonella* to antibiotics should be alarming to veterinary treatment, the food processing, distribution and handling of food product. The prevalence of *Salmonella* Typhimurium in the rectal swabs of stray dogs indicates greater public health concern should be undertaken to prevent human salmonellosis. Therefore, it is necessary to inform people involved in the dwelling and veterinarians as well as distributors to take care in handling of pets, stray dogs and the food products. The present situation underlines the increased public and governmental interest in eliminating sub-therapeutic use of antibiotics in poultry and livestock, particularly those that are also used to treat humans. There is need for more rational use of antibiotics in animal production and more prudent use in humans.

CHAPTER - VII

RECOMMENDATIONS

The current study reveals presence of *Salmonella* spp. in the rectal swab of stray dogs of Chittagong City Corporation of Bangladesh. Isolation of antimicrobial resistant *Salmonella* spp. including multi-drug resistance poses a concern to public health authorities and the general Bangladeshi people. In view of this research finding, there is a need to develop comprehensive policies to ensure stray dog health maintenance and prevent multi-drug resistant. The following recommendations should prove useful to prevent the contamination of environment and to ensure the judicious uses of antibiotics.

To prevent the multidrug resistant *Salmonella* spp. the practicing veterinarians should follow the following recommendation

1. Infection by such multi-drug resistant *Salmonella* may no longer be treated by conventional therapeutic agents
2. Sensitive drugs should be identified and choice to treat Salmonellosis
3. Care should be taken to use the sensitive drugs judiciously in terms of dose, frequency of administration and course of drug treatment

To prevent occurrence in *Salmonella* spp. in dog feces and its environment-

1. In order to control *Salmonella* infection of dogs in Bangladesh detailed epidemiological investigation and strain identification are prerequisites
2. As the results from this single investigation are not sufficient for formulating standards by the regulatory agencies, more large-scale studies are required to explore prevalence of *Salmonella* in stray dogs and their environment and antibiogram against *Salmonella* spp. isolates
3. All stray dogs should be registered and their health status should be evaluated routinely
4. Further studies on the national level to identify *Salmonella* serotypes and specific antimicrobial resistant gene of *Salmonella* isolates should be needed.

CHAPTER - VIII

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CHAPTER – IX

APPENDIX

9.1 Peptone Water

Composition	gm/liter
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5

9.2 Nutrient broth

Composition	gm/liter
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

9.3 BGA (Brilliant Green Agar)

Composition	gm/liter
Proteose peptone	10.0
Yeast extract	3.0
Lactose	10.0
Sucrose	10.0
Sodium chloride	5.0
Phenol red	0.08
Brilliant green	0.0125
Agar	12.0

9.4 SS (*Salmonella* Shigella) Agar

Composition	gm/liter
'Lab-Lecmo' powder	5.0
Peptone	5.0
Lactose	10.0
Bile Salts	8.5
Sodium Citrate	10.0
Sodium Thiosulphate	8.5
Ferric Citrate	1.0
Brilliant Green	0.00033
Neutral Red	0.025
Agar	15.0

9.5 Blood Agar Base

Composition	gm/liter
Tryptone	14.0
Peptone Neutralised	4.5
Yeast Extract	4.5
Sodium Chloride	5.0
Agar	12.0

9.6 TSI (Triple Sugar Iron) Agar

Composition	gm/liter
`Lab-Lemco' powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

9.7 Mueller Hinton Agar

Composition	gm / liter
Beef, infusion from	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0