Isolation of Antibiotic Resistant *Salmonella spp.* from Fresh Vegetables Collected from Local Wet Markets of Dhaka City

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing our undergraduate degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Approval

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Abstract

In Bangladesh, eating raw vegetables that have undergone less processing is becoming more and more popular. Consuming fresh vegetables contaminated with significant gastrointestinal pathogenic bacteria like *Salmonella* increases the risk of food poisoning. Salmonellosis is a food-borne illness that is brought on by a number of non-typhoidal *Salmonella enterica* (NTS) serovars, primarily serovars Enteritidis and Typhimurium. The aim of the study was to ascertain the prevalence of *Salmonella spp*. in fresh leafy vegetables from different areas of Dhaka city in two different seasons. A total of 39 of the 100 raw vegetable samples were positive for *Salmonella spp*. However, the highest positive samples for *Salmonella spp.* were mint (80%) and Green chili (70%), and the total percentage of positive samples was higher in summer (56.4%) than in winter (43.6%). Antibiotic susceptibility test of these isolates was performed using 14 antibiotics belonging to eleven groups which revealed that all the isolates (100%) were resistant to Penicillin, Clindamycin, and Metronidazole; followed by Erythromycin (95%), Tetracycline (39%), Amoxicillin (38%), and Streptomycin (12.5%). The study showed significant resistance of *Salmonella spp.* to third-generation antibiotics such as Amoxicillin, Ceftriaxone, and Levofloxacin which is a pure concern. However, commonly used antibiotics Gentamycin, Co–Trimoxazole, and Imipenem were reported to be very effective antibiotics against *Salmonella spp.* with sensitivity rates of 99%, 94%, and 88% respectively. In addition, the antibiotic susceptibility pattern of the bacterial isolates from the fresh vegetable samples revealed that all of the isolates were resistant to antibiotics of at least three different antibiotic groups with Multiple Antibiotic Resistant (MAR) index of 0.28 which denoted that all the isolates were multi-drug resistant and there was a significant risk of cross-contamination and uncontrol use of different antibiotics to the food products.

Keywords: *Salmonella spp.*; raw vegetables; resistance; sensitivity; MAR index

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List of Abbreviations

MR	Methyl Red
VP	Voges-Proskauer
TSI	Triple Sugar Iron
MIU	Motility Indole Urease
spp.	Species
µl	Microliter
LB	Luria – Bertani broth
XLD	Xylose Lysine Deoxycholate
SS agar	Salmonella Shigella Aga
H_2O_2	Hydrogen peroxide
MAR	Multiple Antibiotic Resistance
H2S	Hydrogen sulfide
^{O}C	Degree Celsius
%	Percentage
g/L	Grams per litre
mm	Millimetre
mg	Milligram
NA	Nutrient Agar
MHA	Muller Hinton Agar
WHO	World Health Organization
HGT	Horizontal Gene Transfer
h	hour

Chapter 1

Introduction

1.1 Background of the study

The bacterium *Salmonella*, which has a rod-like structure and is Gram-negative, is what causes salmonellosis in people. Salmonella was responsible for 1.2 million infections, 23,000 hospitalizations, and 450 fatalities in the United States per year (CDC, 2019). Most *Salmonella*-infected people experience diarrhea, fever, and cramps within 12 to 72 hours of infection. It can result in mild to moderate, self-limiting gastrointestinal illness; in a small percentage of cases, serious illness that results in death can happen. Traditional classifications of Salmonellae that cause human disease include a small number of invasive, human-restricted typhoidal serotypes (*S. enterica* var. Typhi and *S. enterica* var. Paratyphi A) and thousands of nontyphoidal *Salmonella* serotypes (NTS serotypes), which typically have a wide host range in vertebrates and cause a variety of presentations, most commonly diarrhoeal disease. When infected with *Salmonella*, vulnerable populations like children, the elderly, and those with compromised immune systems may suffer from acute dehydration that poses a life-threatening risk (WHO, 2018).

RTE salad vegetables are usually seen as being healthy to eat due to their vitamin, mineral, and antioxidant contents; as a result, their consumption in modern life has expanded both in quantity and diversity in recent years due to this rising demand from customers (de Giusti et al., 2010). The likelihood of exposure to foodborne pathogens through this food chain has increased along with the consumption of RTE salad vegetables. However, when they are consumed raw, poor processing hygiene can result in foodborne illnesses like salmonellosis because these foods are naturally contaminated by soil, water, and other poultry products. The two most prevalent foodborne pathogens in developed nations are *Salmonella enterica* serovars

Enteritidis and Typhimurium, two of the 2,659 serovars of *Salmonella* (Hendriksen et al., 2011). Since these bacteria have been known to live in the intestine of these animals, it is generally discovered that salmonella poisonings are linked to the ingestion of contaminated bovine or chicken products (Najwa et al., 2015). However, Salmonella poisonings have also been linked to the consumption of ready-to-eat (RTE) vegetables, including lettuce, tomatoes, carrots, alfalfa sprouts, bean sprouts, Asiatic pennyworts, water dropworts, cabbage, and cucumbers **(**Bordini et al., 2007; Centre For Food Safety, 2014; Najwa et al., 2015; Kuan et al., 2017). Following a *S. Typhimurium* epidemic in the US two years after the incident, which resulted in 183 people being ill after eating the contaminated tomatoes, a salmonellosis outbreak in the UK in 2004 that involved 350 people eating lettuce in fast food restaurants (Von Haaren, 2004; CDC, 2006). Similarly, 92 people in Australia who had eaten tainted pre-packaged lettuces were also diagnosed with *Salmonella* food poisoning (Suzanne, 2016). Therefore, it is important that people around the world take *Salmonella* in RTE vegetables seriously.

In Bangladesh, samples from poultry and poultry habitats both contained a comparable percentage (25%) of *Salmonella spp.* (Hassan et al., 2016). There have been reports of *Salmonella* being a prevalent microflora in chicken feeds, raw feeding materials, and animal feeds (Merchant & Packer, 1967). Salmonella contamination of fresh vegetables at local markets is frequently caused by careless and unclean handling practices. *Salmonella spp*. and other harmful bacteria spread from food handlers with poor personal hygiene to poultry and poultry products, including eggs, plastic-wrapped poultry meat, and ready-to-eat items, which may be found in many local and supermarkets. The incidence of *Salmonella spp*. in ready-to-food in touch with poultry products in Rajshahi and the neighboring districts was 45.1% according to a serological survey. Compared to children (16.7%), adults had the highest prevalence of *Salmonella* (37.6%). The biggest percentage was found in the season of summer

(30.4%), next came rainy (25.0%), winter (23.7%), and autumn (23.3%) (Hossain et al., 2010). In order to raise awareness about the safety and quality of RTE salad vegetables, additional prevalence research on *Salmonella* in vegetables is required in Bangladesh.

Another issue with regard to public health is the rise of microorganisms that are resistant to antibiotics in food. The World Health Organization (WHO) and the Centers for Disease and Prevention (CDC), among other organizations, have emphasized the need of preventing the spread of this resistance (Angulo et al., 2000). The uncontrol antimicrobial use in fruits and vegetables, as well as geographic differences, have an impact on the degrees of resistance on pathogens, which are variable.

1.2 Risk Factors

Salmonella infections can be fatal, especially for young children, expectant mothers, and the unborn child (Bryan, 2011). However, it can be avoided by cooking food at an average temperature of 68–72°C (145–160°F) and boiling liquids like soups or gravies. *Salmonella* is killed by freezing, but it cannot be completely eradicated to the point where it is no longer contagious. Salmonella is normally heat-sensitive, but in high-fat conditions like peanut butter, it does develop heat resistance. And a matter of concern that typhoid fever vaccinations are available, but non-typhoidal salmonellosis vaccines are still not (Saddler et al., 2019). In general, oral and intravenous antibiotic medication replaces fluid loss during salmonellosis therapies. Antibiotics are necessary for the treatment of typhoid fever and enteric fevers.

1.3 Causes of Salmonellosis

The main source of salmonellosis is raw meat. *Salmonella* can be found in the stomachs of humans, animals, and even birds. Human contamination is typically brought on by consuming food or drinking water that has been tainted with feces. During the slaughtering process, it's normal to find uncooked meat, fish, and poultry filth (Nordqvist, 2017). The shell of an uncooked egg could seem to be a significant barrier to contamination. Before the shell has formed, some unsanitary birds may lay eggs that are contaminated with *Salmonella*. We consume fruits and fresh vegetables, and there is a considerably increased risk of infection if they are washed or watered in filthy contaminated water. Some *Salmonella* outbreaks have been linked to contamination in spices, according to the US Food and Drug Administration (Nordqvist, 2017). Unhygienic kitchen surfaces that aren't cleaned after using the restroom or changing a baby's diaper, as well as those that don't get cleaned following those activities, are major sources of contamination and infection. Domestic amphibians and reptiles have the potential to carry *Salmonella* in their guts without getting sick. They contaminate anything they come into contact with, including cages, toys, clothes, furniture, and domestic surfaces, with the germs in their droppings, which can swiftly spread through their skin.

1.4 Antibiotic Resistance

Antibiotic resistance is most likely to emerge due to genetic diversity given the increased use of antibiotics in modern society, often against medical recommendations. Over time, there has been a significant increase in the number of countries where people or animals abuse or overdose on antibiotics. When germs are exposed to medications regularly, they learn how they work and might modify their defenses against the antibiotic. This is known as antibiotic resistance. When a microbe develops multiple antibiotic resistance, it is referred to as a "superbug". Because of this, some antibiotics lose their effectiveness, reducing the body's ability to fight off infections, and causing returning infections. Weakening medical control, ineffective infection control, poor sanitary conditions, and incorrect food handling management can all contribute to the development of antibiotic-resistant bacteria in the environment. We are most certainly moving towards a "post-antibiotic" period as a result of the rise of antibiotic resistance, in which ordinary infections and/or small wounds could once more result in death (WHO, 2018).

1.5 Safety from cross-contamination

Cross-contamination of *Salmonella spp.* in food must be prevented. Fresh meats and eggs contaminated with feces should not be stored next to cooked food, or food that is ready to consume. After handling raw foods, all utensils must be completely cleaned. Before handling different food products and handling food, hands should be thoroughly cleaned. People who have salmonellosis should refrain from handling food or water until their illness has cleared up (Brands et al., 2005b). People who have touched animal waste should wash their hands. All people should immediately wash their hands after handling reptiles since they are particularly susceptible to having *Salmonella*-contaminated skin. To prevent the spread of *Salmonella* from poultry to vegetables, fresh vegetables that are frequently consumed raw, such as cucumber, tomato, lettuce, carrot, green chili, capsicum, and coriander should be kept away from poultry products.

1.6 Literature Review

According to research by Sivapalasingam et al. (2004), eating raw or barely processed fruits and vegetables has been linked to an increase in the frequency of outbreaks of certain food-borne infections. 103 (54%) of the overall foodborne outbreaks in the United States from 1973 to 1997 were brought on by enteric agents (virus, protozoan, nematode, or bacterium), mostly through eating lettuce, melon, different salads, and drinking fruit and vegetable juices. Of these 103 epidemics, 62 were brought on by pathogenic bacteria, with *Salmonella* being responsible for 30 of these 62 outbreaks. Fresh produce-related foodborne outbreaks have become more prevalent in the United States, both in terms of absolute numbers and as a percentage of all foodborne outbreaks that have been documented. A healthy diet should mostly consist of fruits and vegetables, yet there are risks associated with consuming raw, fresh produce.

Tunung et al. (2007) investigated that there have not been many efforts made to address the issue, therefore it's unclear how many cases of foodborne diseases linked to the eating of fresh produce actually occur in Malaysia. A salmonellosis outbreak involving 171 people and fatalities following the eating of RTE vegetables, however, was documented in Kelantan, Malaysia in 2005. That incident made a lot of impact on further research about the correlation between the consumption of RTE vegetables and Salmonellosis. According to Nurul et al. (2012), due to their increased consumption of vegetables, particularly carrot, celery, spinach, cabbage, cauliflower, tomato, cucumber, and long beans, Malaysians people have a higher risk of contracting salmonellosis. In order to raise awareness about the safety and quality of vegetables, additional prevalence research on *Salmonella* in vegetables is required.

According to Ryan et al. (2004) and Sandler (2015), In Bangladesh, diarrhea is the leading cause of child mortality; however, due to the lack of a national health database, the real number of casualties may be underestimated. 501 cases of diarrhea are reported daily during monsoon seasons, according to incomplete statistics on food-borne infection. It is difficult to evaluate the effects of food-borne infections on public health since there is no consistent surveillance. Foods that are sold ready to eat are frequently eaten there, where they can be eaten hot, cold, cooked, or raw without any additional cooking technique.

1.7 Objective of the study

The specific aims and objectives of the study are as follows:

- To isolate and characterize *Salmonella spp.* from fresh vegetable samples collected from local wet markets in Dhaka city.
- To compare the percentage and availability of *Salmonella spp.* positive samples in two different seasons (winter and summer).
- To study the antibiotic resistance and multi-drug resistance pattern of the *Salmonella spp.* collected from fresh vegetables by comparing their MAR index.

Chapter 2

Materials & Methods

2.1 Study place

For this study, the vegetable samples were collected from different wet markets of Dhaka city.

2.2 Samples collection: Source, Seasons, and Number of isolates

From January 2022 to August 2022 a total 100 vegetable samples were collected in the winter and summer seasons from several wet vegetable markets of the city of Dhaka. Every sample was packed in sterile zipper bags with the appropriate labels, insulated in ice packs to maintain cold temperature, and transferred to the laboratory to analysis. At each step, while handling the samples, caution was exercised and hand gloves were worn to prevent contamination.

Samples were: cabbages (n = 10), lettuces (n = 10), mint (n = 10), coriander (n = 10), capsicums (n = 10), tomatoes (n = 40), carrots (n = 40), cucumbers (n = 40), green chilies (n = 10) and spring onions (n = 10).

The total number of samples, their source and seasons were shown in table 1.

Areas	Sample number	Winter	Summer
1. Uttara Azampur Kacha Bazar	20	10	10
2. Mirpur-11 Kacha Bazar	20	10	10
3. Tongi Bazar	20	10	10
4.Gulshan-1 DCC	20	10	10
5. Farmgate Bazar	20	10	10
Total	**100**	**50**	**50**

Table 1: Samples collection: Source, Seasons, and Number of isolates

2.3. Sample processing and inoculation to growth media

Each of the samples was cut into small pieces within laminar airflow using sterile knives. For 10 samples 500µl LB broth (Luria-Bertani Broth) was prepared, which is a nutritionally rich medium that promotes the growth of bacteria. For exclusive enrichment of *Salmonella*, the samples were also homogenized on Selenite broth. AT first, each of the excised vegetable samples (5g) was transferred to conical flasks containing 50µl of LB broth and incubated in a shaker incubator at 37 ºC for 24 h. After incubation, 10^{-2} serial dilution was performed by transferring 200µl of overnight grown LB broth to a test tube containing 15 ml of distilled water.

Figure 1: Vegetable samples inoculation in cultural broth

2.3.1. Growth on XLD media

After 10^{-2} serial dilutions, 30µl broth culture was poured into XLD agar plates (HiMedia, India), and using a sterile glass rod the plate was swirled with fingers while moving the glass rod back and forth in the media. When the sample became sticky, the plate was incubated at 37 ºC for 24 h in an inverted position. *Salmonella* breaks down thiosulfate that is present in the media to create hydrogen sulfide, which results in the development of red colonies with black centers in the media (Cappuccino & Sherman, 2005). Few presumptive colonies were selected for further biochemical and antimicrobial tests.

2.3.2. Growth on Salmonella - Shigella (SS agar)

Salmonella - Shigella (SS) agar was next streaked with presumptive colonies of 24-hour bacterial culture from the XLD agar and incubated for 24 hours at 37 ºC. As its name implies, SS Agar is yet another highly suggested selective medium for the identification of *Salmonella* from different food samples where *Salmonella* appears to be a colorless colony with a black center due to the production of H2S (Cappuccino & Sherman, 2005).

2.4. Biochemical Tests

A series of biochemical assays were used to further examine the 80 isolates that were thought to be *Salmonella spp*. in order to confirm. 24-hour bacterial cultures were employed and based on the variations in the metabolic activity of various bacteria, biochemical tests are performed. They are presumptive identification tests that enable the identification of a substance's existence in a sample and the decisions are typically made following a chemical reaction that results in the production of a certain color.

Biochemical identification of isolates was as described by Cappuccino & Sherman (2005). All isolates that had *Salmonella*-like reactions in the tests and substrates were thought to be *Salmonella*. *Salmonella* reactions are typically methyl red positive, citrate positive, lysine decarboxylase positive, Voges Proskauer negative, indole negative, oxidase negative, motile in motility media, urease negative, generate H_2S, ferments glucose but fails to ferment lactose and sucrose.

According to Cappuccino & Sherman (2005), the biochemical test results of the following table (table 2) represent the typical biochemical reactions of all the non-typhoidal *Salmonella enterica* (NTS) serovars, other than serovar Typhi and Paratyphi.

Catalase	Oxidase	Citrate (+/-)	Voges–Proskauer (VP)	Methyl Red (MR)	Triple Sugar Iron (TSI)				MIU		
					Slant	Butt	Gas	H2S	Motility	Indole	Urease
+	-	+	-	+	R/Y	Black	✓	+	+	-	-

Table 2: The desired biochemical reactions of all the non-typhoidal Salmonella enterica (NTS) serovars.

2.5. Antibiotic Susceptibility Test

The most used technique for determining antibiotic resistance or susceptibility of any bacteria including *Salmonella* is the Kirby-Bauer disk diffusion test. Special Mueller Hinton Agar (MHA) is used in antibiotic susceptibility testing because it has a higher diffusion rate than conventional growth media and contains starch as an energy source that absorbs toxins generated by bacteria so that they do not interfere with medications. Microorganisms are divided into Resistant (R), intermediate (I), and susceptible (S) based on their zone of diameter (mm) values in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2021). For the microbiological sensitivity testing, 14 antibiotics from 11 different groups were employed (Table 3).

According to CLSI's Antimicrobial Disk Susceptibility Test Performance Standards (CLSI'2021, 31st edition).

S. No:	Antibiotic classes/ groups	Antibiotics	Disc Content (µg)	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
1.	Beta-Lactam	Penicillin (PEN)	10 µg	≤ 19	20-27	≥ 28
2.		Amoxicillin (AMX/AML)	10 µg	≤ 13	14-17	≥ 18
3.	Aminoglycosides	Streptomycin (S)	10 µg	≤ 11	12-14	≥ 15
4.		Gentamycin (GEN)	10 µg	≤ 12	13-14	≥ 15

5.	Quinolones	Levofloxacin (LE)	5 µg	≤ 18	19-27	≥ 28
6.		Ciprofloxacin (CIP)	5 µg	≤ 20	21-30	≥ 31
7.	Macrolides	Erythromycin (E)	15 µg	≤ 13	14-22	≥ 23
8.	Tetracycline	Tetracycline (TE)	30 µg	≤ 11	12-14	≥ 15
9.	Phenicols	Chloramphenicol (C)	30 µg	≤ 12	13-17	≥ 18
10.	Cephalosporin	Ceftriaxone (CTR)	10 µg	≤ 19	20-22	≥ 23
11.	Carbapenem	Imipenem (IPM)	10 µg	≤ 19	20-22	≥ 23
12.	Lincosamides	Clindamycin (CD)	2 µg	≤ 14	15-20	≥ 21
13.	Sulfonamides	Co – Trimoxazole (COT)	1.25/23.75 µg	≤ 10	11-15	≥ 16
14.	Nitroimidazole	Metronidazole (MT)	5µg	≤ 15	16-18	≥ 20

Table 3: Antibiotic disks, their concentration, and Zone of Inhibition size (mm) for Enterobacteriaceae

2.5.1. Preparation of inoculum and compare with the McFarland Solution

Using an inoculating loop, one or two colonies of the bacterial isolates were taken up from sub-cultured nutrient agar plates and suspended in 5ml saline solution. After proper vortexing, the turbidity of the inoculums was compared to the standard McFarland 0.5, which provides the number of bacteria within a given range to standardize microbial testing (approximate cell count density: 1.5x10^8 CFU/ml).

2.5.2. Bacterial inoculation on MHA and measurement of Zone of Inhibition

Using a sterile cotton swab the entire surface of the MHA plates was streaked with the bacterial inoculum using the lawn technique and then antibiotic disks were placed using sterile forceps. After 24 hours of incubation at 37^0C, the zone of inhibition was measured following CLSI guidelines (Table 3). Isolates that showed resistance to at least one agent in three or more antibiotic categories were defined as multidrug-resistant strains (MDR) (Basak et al., 2016). And Multiple Antibiotic Resistance (MAR) Index of the isolates of more than 0.2 indicated the high-risk source of cross-contamination in the sample where antibiotics were commonly used (Rotchell & Paul, 2016).

2.6. Culture Preservation

For short-term preservation for 1-2 weeks, the 80 isolates of *Salmonella spp.* were streaked into nutrient agar (NA) media and stored in the refrigerator at 4 ºC.

For long-term preservation in 30% glycerol: A loop-full of a 24-hour bacterial culture inoculum was transferred into 5 ml of LB broth and incubated at 37°C for 24 hours. Then, 250µl of LB culture and 250µl of sterile 30% glycerol were transferred to a sterile eppendorf tube, and the mixture was mixed thoroughly by shaking the tube and preserved at a freezing temperature of –20°C.

Chapter 3

Results

3.1. Growth and appearance of 80 isolates on XLD Agar and SS Agar

All 80 isolates produced red colonies with black centers on XLD agar and colorless colonies with black centers on SS agar media after a 24-hour of incubation period at 37^0C which represent the desired growth appearance of *Salmonella spp*. on those particular growth media (Figure 2).

(a) (b)

Figure 2: Growth and appearance of Salmonella spp. on (a) XLD agar with characteristics of red colonies with black centers; and on (b) SS agar with distinctive colorless colonies with black centers.

3.2. Results of the biochemical tests of the 80 isolates

For the presumptive identification of *Salmonella spp.*, results of biochemical tests of 80 isolates were interpreted after observing all the growth media after 48 hours of incubation at 37^0C. Out of 80 isolates, 74 (92.5%) showed the typical biochemical test results of *Salmonella enterica*; the rest of 6 isolates were citrate-negative. The following figure (Figure 3) was the representation of the biochemical test results of the 80 isolates:

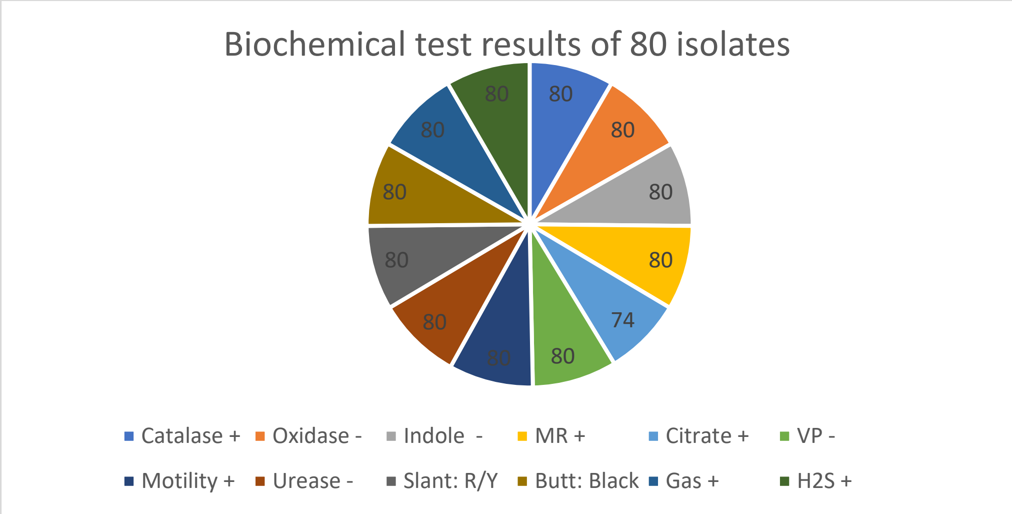

Figure 3: Biochemical Test Results of 80 isolates.

3.3. Distribution of *Salmonella spp.* on different vegetables

A total of 39 out of the 100 raw vegetable samples were positive for *Salmonella spp*. and 80 suspected bacteria were isolated from those 39 samples in two different seasons for further study. Among the 10 different types of vegetable samples, the highest positive rate for *Salmonella spp*. was found in mint (80%), followed by green chili (70%), coriander and cucumber (50%), capsicum (40%), cabbage, tomato and lettuce (30%), carrot (10%) and spring onion (0%) respectively.

Vegetables	Total number of samples	Number of *Salmonella spp.* positive samples (n)
Mint	10	8
Green chili	10	7
Coriander	10	5
Cucumber	10	5
Capsicum	10	4
Cabbage	10	3

Tomato	10	3
Lettuce	10	3
Carrot	10	1
Spring onion	10	0
Total	100	39

Table 4: Distribution of Salmonella spp. on different vegetable samples

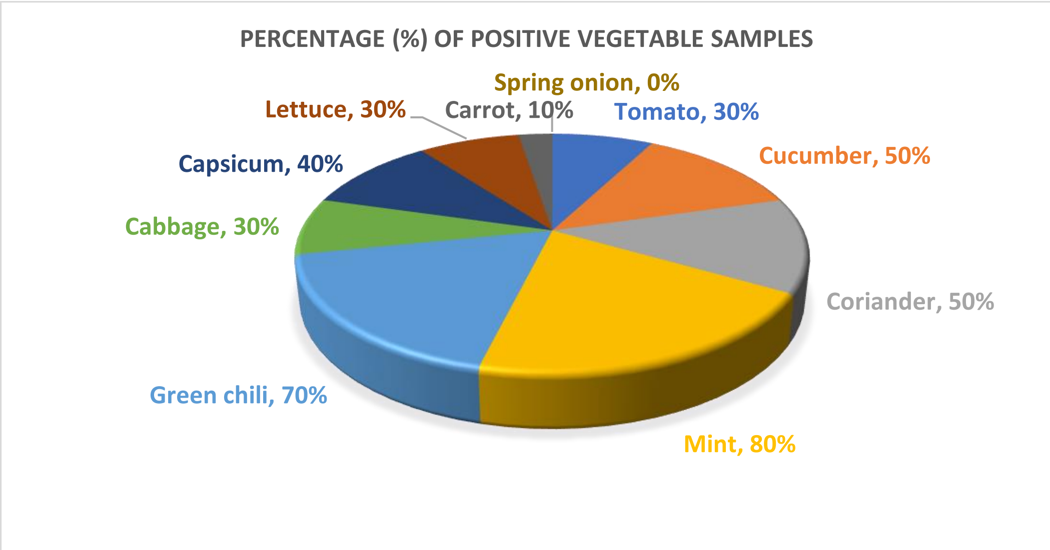

Figure 4: Percentages (%) of Salmonella spp. positive vegetable samples

3.3.1. Percentage of *Salmonella spp. positive vegetables* in winter vs summer

The percentage of *Salmonella spp.* positive samples in summer was 56.4%, which was higher than the percentage rate in winter (43.6%) in different areas of Dhaka City. The number of positive samples on two different seasons were shown in Table 5:

Areas	Total positive samples for *Salmonella spp.*	Winter	Summer
1. Uttara	6	3	3
2. Mirpur	14	7	7

3. Tongi (Gazipur)	6	2	4
4.Gulshan-1 DCC	3	1	2
5. Farmgate	10	4	6
Total	**39**	**17 (43.6%)**	**22 (56.4%)**

Table 5: Number of Salmonella spp. positive samples in two different seasons

The percentage of *Salmonella spp.* positive sample was highest in Mirpur (36%), followed by Farmgate (25.6%), Tongi Bazar & Uttara (15.4%), and DCC (7.7%) collectively in two seasons.

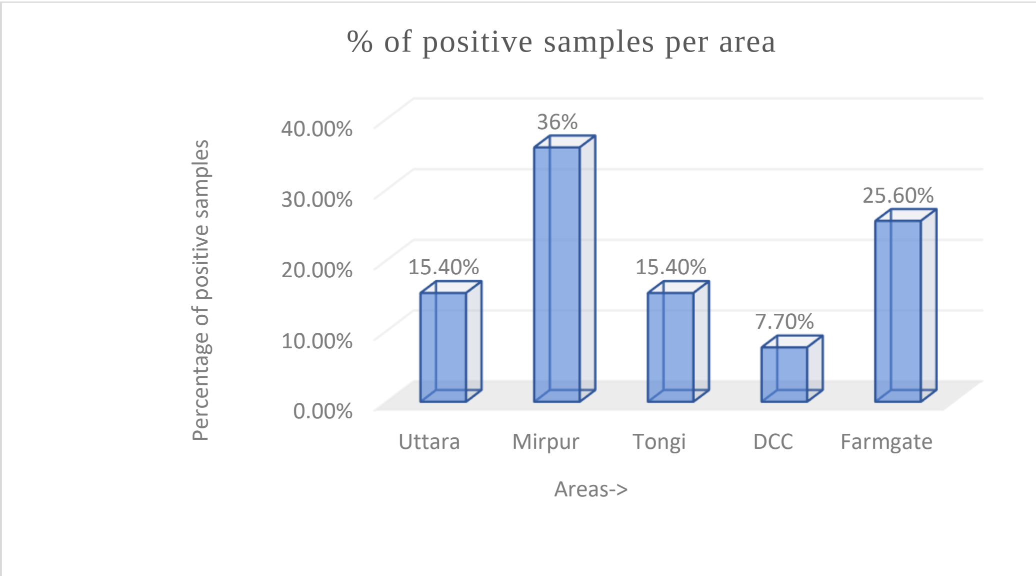

Figure 5: Percentage of Salmonella spp. positive vegetables in different areas of Dhaka city.

3.4. Antibiotic Susceptibility Test (AST)

3.4.1. Antibiotic Sensitivity Test of the 80 isolates and their resistance pattern

Antibiotic susceptibility test of these 80 isolates was performed using 14 antibiotics belonging to eleven groups: Aminoglycoside, Beta–Lactam, Lincosamide, Macrolide, Nitroimidazole, Quinolone, Sulfonamide, Phenicols, Carbapenem, Cephalosporin, and Tetracycline. The antibiotic susceptibility test reported that all the isolates (100%) were resistant to Penicillin, Clindamycin, and Metronidazole; Followed by Erythromycin (95%), Tetracycline (39%), Amoxicillin (38%), Streptomycin (12.5%), Chloramphenicol (11.25%), Ceftriaxone (7.5%), Co – Trimoxazole (6.25%), Levofloxacin (2.5%), and Gentamycin , Ciprofloxacin, and Imipenem (1.25%) respectively . However, Gentamycin, Co–Trimoxazole , and Imipenem were reported to be the most effective antibiotics against *Salmonella spp.* with susceptibility of 99%, 94%, and 88% respectively in this study.

The most antibiotic resistant isolate MT5-04 was found in fresh mint sample from DCC bazar and was resistant to 13 of the 14 antibiotics except Imipenem (Figure 6).

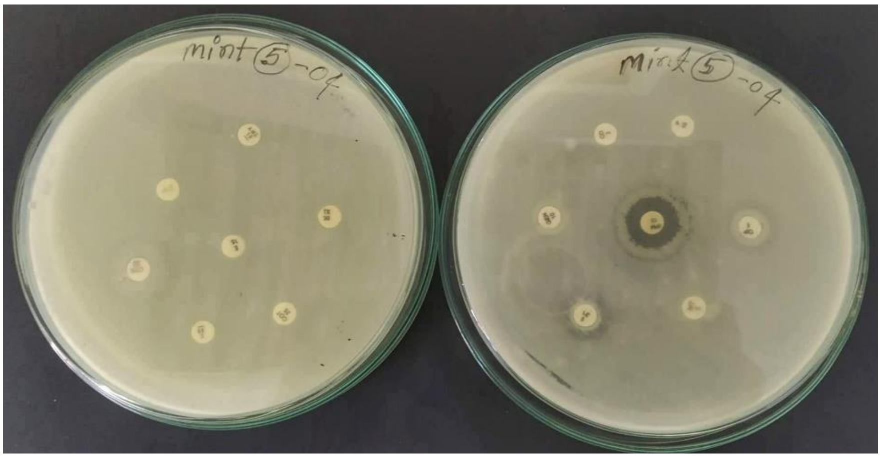

Figure 6: Antibiotic resistance of the most resistant isolate MT5-04 on MHA plate

According to CLSI (2021) guideline, the organisms were divided in three groups after measuring the zone of inhibition (mm): Resistant (R), Intermediate Resistant (I), and Sensitive (S) (Figure 7).

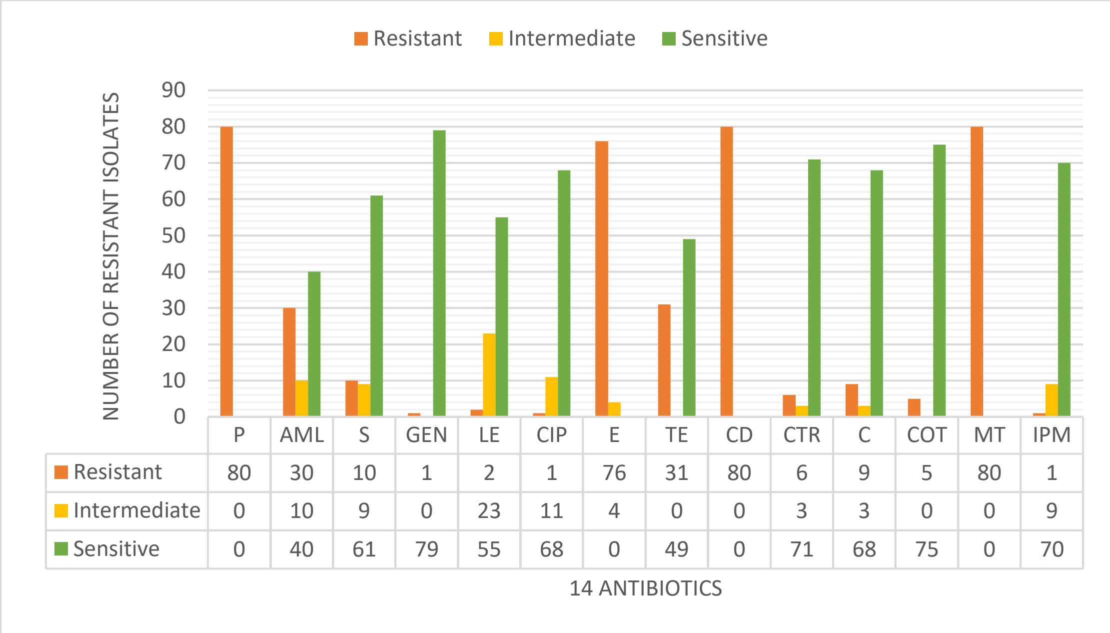

Figure 7: The number of Resistant, Intermediate, and Sensitive isolates for each of the 14 antibiotics.

3.4.2. Measuring of Multiple Antibiotic Resistance (MAR) Index of the isolates

The isolates' Multiple Antibiotic Resistance (MAR) Index revealed that all of them were resistant to at least one antibiotic of more than three different groups and had MAR Index of 0.28 and higher (Table 6).

Serial No	Samples of *Salmonella spp.*	Resistant Antibiotics (a)	Total Tested Antibiotics (b)	MAR(a/b)	Level of resistant
1	MT1-01	7	14	0.5	HIGH
2	MT2-01	8	14	0.57	HIGH
3	MT3-01	8	14	0.57	HIGH
4	MT5-01	8	14	0.57	HIGH
5	CR1-01	8	14	0.57	HIGH
6	CR2-01	5	14	0.35	HIGH
7	GC1-01	6	14	0.42	HIGH
8	LT1-02	6	14	0.42	HIGH
9	GC1-02	8	14	0.57	HIGH
10	TM1-02	5	14	0.35	HIGH
11	TM2-02	5	14	0.35	HIGH
12	MT1-02	4	14	0.28	HIGH
13	CP1-02	5	14	0.35	HIGH
14	CU1-02	5	14	0.35	HIGH

15	CR1-02	6	14	0.42	HIGH
16	MT1-03	6	14	0.42	HIGH
17	MT2-03	4	14	0.28	HIGH
18	MT3-03	5	14	0.5	HIGH
19	CB1-03	6	14	0.42	HIGH
20	CB2-03	5	14	0.5	HIGH
21	CB3-03	5	14	0.35	HIGH
22	CB4-03	6	14	0.42	HIGH
23	CU1-03	6	14	0.42	HIGH
24	CP1-03	6	14	0.42	HIGH
25	MT1-04	6	14	0.42	HIGH
26	MT3-04	5	14	0.35	HIGH
27	MT5-04	13	14	0.9	HIGH
28	GC4-04	6	14	0.42	HIGH
29	GC6-04	8	14	0.57	HIGH
30	GC8-04	5	14	0.35	HIGH
31	GC11-04	5	14	0.35	HIGH
32	CR1-05	4	14	0.28	HIGH
33	CR2-05	6	14	0.42	HIGH
34	GC2-05	6	14	0.42	HIGH
35	GC3-05	7	14	0.5	HIGH
36	GC5-05	5	14	0.35	HIGH
37	LT1-05	4	14	0.28	HIGH
38	CP1-05	5	14	0.35	HIGH
39	CP2-05	5	14	0.35	HIGH
40	CP3-05	5	14	0.35	HIGH
41	CB1-06	5	14	0.35	HIGH
42	CB2-06	5	14	0.35	HIGH
43	CR1-06	5	14	0.35	HIGH
44	CU1-06	5	14	0.35	HIGH
45	CP1-06	5	14	0.35	HIGH
46	CP2-06	5	14	0.35	HIGH
47	CP3-06	5	14	0.35	HIGH
48	MT4-06	5	14	0.35	HIGH
49	LT1-06	5	14	0.35	HIGH
50	GC4-06	5	14	0.35	HIGH
51	MT2-07	5	14	0.35	HIGH
52	MT3-07	5	14	0.35	HIGH
53	MT3-08	5	14	0.35	HIGH
54	CU7-08	4	14	0.28	HIGH
55	CAR1-08	4	14	0.28	HIGH
56	CAR2-08	4	14	0.28	HIGH
57	CB1-08	4	14	0.28	HIGH
58	CB2-08	4	14	0.28	HIGH
59	CR1-08	5	14	0.35	HIGH
60	GC4-08	4	14	0.28	HIGH
61	GC1-09	4	14	0.28	HIGH
62	GC2-09	4	14	0.28	HIGH

63	TM8-09	4	14	0.28	HIGH
64	TM9-09	4	14	0.28	HIGH
65	TM10-09	4	14	0.28	HIGH
66	TM11-09	4	14	0.28	HIGH
67	TM12-09	4	14	0.28	HIGH
68	MT2-09	4	14	0.28	HIGH
69	TM1-10	4	14	0.28	HIGH
70	TM2-10	4	14	0.28	HIGH
71	TM3-10	4	14	0.28	HIGH
72	TM4-10	4	14	0.28	HIGH
73	TM5-10	4	14	0.28	HIGH
74	TM6-10	4	14	0.28	HIGH
75	CU1-10	4	14	0.28	HIGH
76	CU2-10	4	14	0.28	HIGH
77	CU3-10	4	14	0.28	HIGH
78	CU4-10	4	14	0.28	HIGH
79	CU5-10	4	14	0.28	HIGH
80	CU6-10	4	14	0.28	HIGH

Table 6: Multiple Antibiotic Resistance (MAR) Index of the 80 isolates.

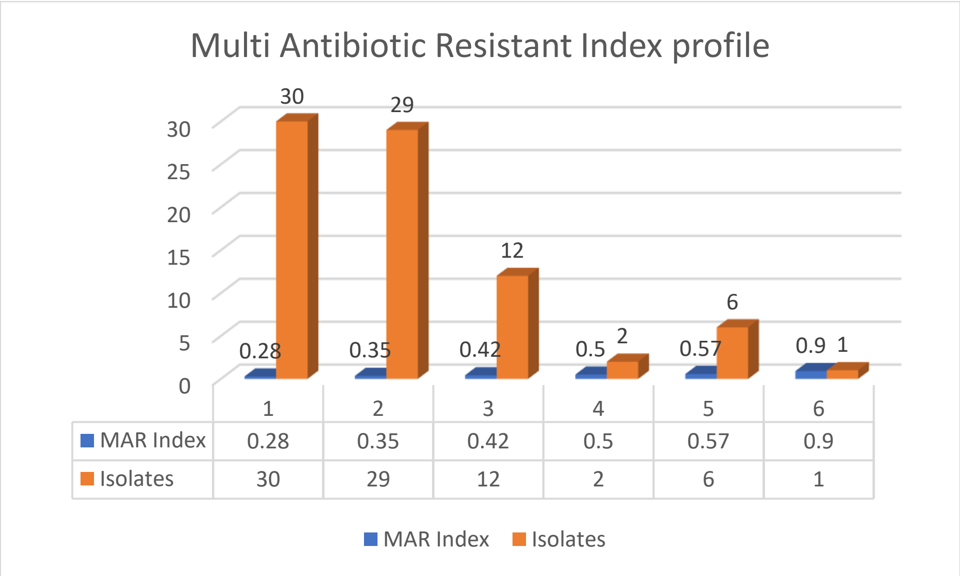

Figure 8: Multi Antibiotic Resistant (MAR) Index profile of the isolates for 14 antibiotics.

Chapter 4

Discussion

The goal of this research was to isolate and characterize antibiotic-resistant *Salmonella spp.* from fresh vegetables which is a serious foodborne pathogen. However, there are currently relatively few data available in Bangladesh for the identification of *Salmonella* from raw vegetables, which limits the potential risk assessment and establishment of safety standards. In Bangladesh, samples from poultry and poultry habitats both contained a comparable percentage (25%) of *Salmonella spp.* (Hassan et al., 2016). There have been reports of *Salmonella* being a prevalent microflora in chicken feeds, raw feeding materials, and animal feeds (Merchant & Packer, 1967). *Salmonella* contamination of fresh vegetables at local markets is frequently caused by careless and unclean handling practices and spread from food handlers with poor personal hygiene to poultry and poultry products, including eggs, plastic-wrapped poultry meat, and ready-to-eat items.

In this research, total 39 out of the 100 raw vegetable samples were positive for *Salmonella spp*. collected from local vegetable markets of five different areas of Dhaka city in two separate seasons (winter and summer). The isolates had been presumptively identified as *Salmonella* after observing the cultural and morphological characteristics of bacterial isolates in different selective and differential media and performing the conventional biochemical tests. In citrate test, out of 80 isolates, 74 were citrate positive and the rest of them were citrate negative. It is known that all the *Salmonella* serovars other than *S. enterica* serovar Typhi and Paratyphi are citrate-positive (Aryal, 2022). That means those 6 isolates could be typhoidal *Salmonella* serovars whereas the rest of them would be non-typhoidal serovars of the species *Salmonella enterica.* Presumptive tests, however, are not always conclusive, and additional confirmation testing might be required.

Among the 80 bacterial isolates selected from 39 positive samples in two different seasons of several areas of Dhaka city, the percentage of *Salmonella spp*. was highest in mint (80%); followed by green chili (70%), coriander and cucumber (50%), capsicum (40%), cabbage, tomato and lettuce (30%), carrot (10%) and spring onion (0%) respectively. So, there was the observation of very low and no presence of *Salmonella* in carrot and spring onion in this study. The percentage of *Salmonella spp.* positive samples in summer was 56.4%, which was higher than the percentage rate in winter (43.6%) collected from different wet markets of Dhaka City. That focused on the fact that *Salmonella* infections were more prevalent in the summer and developed better at higher temperatures, which results in a larger concentration of *Salmonella* in the food supply during the warmer months. Seasonal variations in salmonellosis cases are also caused by indirect seasonal impacts. Food consumption patterns, for example, fluctuate seasonally as leafy green vegetables, a growing cause of salmonellosis, are consumed in greater numbers during the warmer months (Liu et al., 2013).

Following the presumptive identification of *Salmonella spp.*, the antibiotic susceptibility patterns of the 80 bacteria isolated from fresh vegetables were tested against 14 antibiotics from 11 distinct groups : Aminoglycoside, Beta–Lactam, Lincosamide, Macrolide, Nitroimidazole, Quinolone, Sulfonamide, Phenicols, Carbapenem, Cephalosporin, and Tetracycline. The antibiotic susceptibility test reported that all the isolates (100%) were resistant to Penicillin (P), Clindamycin (CD), and Metronidazole (MT); followed by 95% of the isolates (n= 76) were resistant to Erythromycin (E), 39% of them (n=31) were resistant to Tetracycline (T), and 38% of them (N=30) were resistant to Amoxicillin (AML/AMX). The results also revealed that the isolates exhibited more susceptibility to the rest of the antibiotics; especially, only 1% of the isolates (n=1) were resistant to Gentamycin (GEN), Ciprofloxacin (CIP), and Imipenem (IPM); followed by 2.5% of the isolates (n=2) were resistant to Levofloxacin (LE), 6% of them (n=5) were resistant to Co–Trimoxazole (COT), 7.5% of them (n=6) were resistant to Ceftriaxone

(CTR), 11% of them (n=9) were resistant to Chloramphenicol (C), and 12.5% of the isolates (n=10) were resistant to Streptomycin respectively.

The study also showed significant arising of resistance of *Salmonella spp.* to third generation Penicillin, such as Amoxicillin, third generation Cephalosporin, such as Ceftriaxone, and third-generation quinolone, such as Levofloxacin which is a pure concern as those are the major and supposedly the most effective antibiotics that are commonly used to treat *Salmonella* illness. This is very alarming, as the incidence of high resistance of the isolates to the Quinolone group of antibiotics is consistent with a study conducted in Vietnam, which revealed moderate to high resistance to the Quinolone group of antibiotics (Nhung et al., 2018). Furthermore, a study in Southern Brazil discovered a significant proportion of resistance to both Beta-lactam and Quinolone antibiotics (Giuriatti et al., 2017).

Resistance to third-generation cephalosporins in *S. enterica* is primarily owing to the formation of extended-spectrum-beta-lactamases (ESBLs) or AmpC-lactamases by some of the strains found in a study in Germany (Hasman et al., 2005). AmpC genes are frequently found on plasmids, allowing them to spread by horizontal gene transfer (HGT) between enterobacterial species and genera. In this study, though 7.5% of the isolates were resistant to the third generation Cephalosporin, Ceftriaxone (CTR), it was effective against 71 out of 80 isolates. So, CTR could an effective treatment alternative even though the horizontal transformation of the AmpC-lactamases gene is spreading within its serovars which is alarming.

Cephalosporins work in the same way as penicillins, inhibiting bacterial cell wall peptidoglycan formation by inhibiting penicillin-sensitive enzymes. These antibiotics, which inhibit the transpeptidase activity of penicillin-binding proteins, are used for patients who cannot tolerate sulfamethoxazole-trimethoprim. Third-generation cephalosporins in combination with ampicillin, or Clavulanic acid (a beta-lactamase inhibitor) in combination with amoxicillin is an effective combination for managing and treating bacterial infections, particularly those caused by beta-lactamase-producing bacteria. However, when used with amoxicillin, clavulanic acid can produce some moderate gastrointestinal side effects (Matho et al., 2018).

However, commonly used antibiotics Gentamycin (GEN), Co–Trimoxazole (COT), and Imipenem (IPM) were reported to be the most effective antibiotics against *Salmonella spp.* with sensitivity rates of 99%, 94%, and 88% respectively in this study. Gentamicin is a broad-spectrum antibiotic that belongs to the aminoglycoside class of medicine. Its method of action involves inhibiting bacterial protein synthesis by binding to 30S ribosomes. 79 out of 80 isolates were sensitive towards Gentamicin, which made it the most effective antibiotic out of all 14 antibiotics to treat *Salmonella* illness. Co-trimoxazole is a medication in the sulfur or sulfonamide class that combines two antibiotics, trimethoprim and sulfamethoxazole, that work together to treat bacterial infections. Both of these antibiotics inhibit enzymes in bacteria that are essential for the manufacture of tetrahydrofolic acid, a cofactor required for the synthesis of DNA nucleotide bases. With the rising occurrence of multidrug-resistant (MDR) bacterial pathogen infections in hospitals and communities, the discovery of novel antibacterial medicines is critical. However, because the development of newer antimicrobial drugs is slow, reintroduction of previously used antibiotics active against MDR pathogens is a viable option for antibiotic-resistant bacterium control. Cotrimoxazole is a miracle medicine with a broad antibacterial spectrum that makes it acceptable for use in a wide range of illnesses and good tissue penetration that makes it beneficial for the treatment of diseases involving numerous systems of the body (Batra et al., 2017).

Imipenem (Carbapenum) another very effective antibiotic with an 88% sensitivity rate is a beta-lactamase inhibitor, binds irreversibly to the beta-lactamases generated by many bacteria, inactivating the enzymes and rendering the microorganisms susceptible to beta-lactamase-sensitive antibiotics (Moellering,1991). Beta-lactamase inhibitors are frequently used in conjunction with beta-lactam antibiotics to broaden the spectrum of antibacterial action in clinical practice (Gilchrist et al., 2007). As a result, imipenem and other beta -lactamase inhibitors are the drugs of choice for treating serious *Salmonella spp.* infection or killing highly resistant *Salmonella spp.*

The isolates' Multiple Antibiotic Resistance (MAR) Index revealed that all of them were resistant to antibiotics of at least three different groups and had a MAR Index of 0.28 or higher. Which means, all of the isolates experienced a significant amount of antibiotic exposure, resulting in high antibiotic resistance. The MAR index is a reliable, valid, and cost-effective tool for tracing the origins of antibiotic-resistant microbes (Sandhu et al., 2016). The highest MAR Index was 0.9 for sample MT5-04 where the isolate were resistant to 13 of the 14 antibiotics (93%) of different groups except Imipenem, and the lowest MAR Index was 0.28 (>0.2) expressed by 34% (n=27) of the total 80 isolates. So, all the bacteria isolated from fresh vegetables were reported to be multi-drug resistant and had a significant risk of cross-contamination with *Salmonella* infected food products with a high level of antibiotic resistance.

As a result, *Salmonella spp.* infections are quite harmful, and even more so if these organisms turn out to be highly resistant. Horizontal Gene Transfer may result in cross-contamination (HGT). If HGT occurs, it may result in an increase in highly resistant bacteria in the surrounding environment. Since widespread antibiotic usage has resulted in the emergence of antibiotic-resistant bacteria, it would be prudent to explore whether antibiotic resistance is plasmid-mediated or chromosomally mediated in order to plan and create effective treatments accordingly. Antibiotic resistance can pose a major hazard to both humans and other animals. This trend of increasing antibiotic resistance may be driven by the uncontrolled use of antibiotics in animal treatment and their inclusion in animal feeds that can be transferred to fresh vegetables directly or indirectly from food handlers due to careless and unclean handling practices (Galland et al., 2001).

Chapter 5

Conclusion

Overall, *Salmonella spp.*, *S. enterica* serovar Enteritidis, and *S. enterica* serovar Typhimurium were not found in Spring onion. However, a low frequency of *Salmonella spp.* was found in carrots, tomato, cabbage and lettuce samples, whereas a high percentage of *Salmonella spp.* positivity was found in mint, green chili, cucumber, coriander, and capsicum respectively. The presence of *Salmonella spp.* in these frequent daily eating of fresh vegetables raises concerns about the hygiene and safety of consuming them, as well as other raw vegetables. Various infections can grow in food and endanger the consumer's health. As a result, vulnerable groups such as the elderly, immunocompromised patients, and pregnant women should avoid eating raw vegetables to limit the risk of bacterial infection. Because an increasing number of individuals in Bangladesh use raw or little processed vegetables on a regular basis, the findings of this study may be valuable as baseline information for risk assessment on the microbiological quality of vegetables.

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