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Prevalence of Multidrug-Resistant *Salmonella* in Raw Salad Vegetables in Dhaka Metropolitan Area

Palash Kumar Sarker[1*], Hafizur Rahman[2], Kulsum Ara[3], Md. Shahidul Kabir[4], Shishir Kanti Pramanik[5], and Maruf Hasan[6]

[1]Department of Science and Humanities, Military Institute of Science and Technology (MIST), Dhaka, Bangladesh
[2]Department of Environmental Science and Management, Independent University, Bangladesh (IUB), Dhaka, Bangladesh
[3]Institute of Public Health (IPH), Dhaka, Bangladesh
[4]Department of Microbiology, Stamford University Bangladesh, Dhaka, Bangladesh
[5]Department of Chemistry, Graduate School of Physical Sciences, Shahjalal University of Science and Technology, Sylhet, Bangladesh
[6]Department of Biomedical Engineering, Military Institute of Science and Technology (MIST), Dhaka, Bangladesh

emails: *[1]*sarker@sh.mist.ac.bd; [2]hrahman@iub.edu.bd; [3]arakulsum@yahoo.co.uk; [4]kabir@stamforduniversity.edu.bd; [5]shishir-che@sust.edu; and [6]maruf.hasan@bme.mist.ac.bd*

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ABSTRACT

Raw salad vegetables have become very popular among consumers due to their multiple health benefits. The bacteriological quality of these salad vegetables is of great public health concern. This study was conducted to determine the presence of potential pathogenic and multidrug-resistant (MDR) bacteria in raw salad vegetables served in different restaurants in Dhaka, the capital city of Bangladesh. A total of 50 samples, comprising different types of raw salad vegetables were collected from restaurants in Mirpur (n=10), Dhanmondi (n=10), Old Dhaka (n=10), Gulshan (n=10) and Bashundhara Residential Area (n=10). The highest counts for total coliforms and fecal coliforms were 4.02×10^3 cfu/ml and 1.3×10^3 cfu/ml, respectively. The isolates were *E. coli*, total coliform, fecal coliform and *Salmonella* spp. Among them, fecal coliform and *Salmonella* spp. were found to be more pathogenic. As potential pathogenic bacteria, *Salmonella* spp. were tested for antibiotic sensitivity and all of them showed resistance against amoxicillin, ampicillin, azithromycin, chloramphenicol, ciprofloxacin, erythromycin, kanamycin, streptomycin, tetracycline, norfloxacin, and trimethoprim. The findings assist to understand the level of contamination of pathogenic and MDR bacteria in raw salad vegetables, which will create awareness of food safety and public health.

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1. INTRODUCTION

Eating a diet rich in vegetables provides a lot of health-promoting benefits of the presence of different vitamins, minerals, fiber, and other bioactive agents in vegetables (Asaduzzaman and Asao, 2018; Liu, 2013; Samtiya *et al.*, 2021; Slavin and Lloyd, 2012). Many worldwide public health campaigns have also been engaged to promote the health benefits of vegetables. As a result, the consumption of vegetables is on the rise nowadays. Even though, in many countries, the intake of vegetables is below recommended levels (Frank *et al.*, 2019; Moura and Vialta, 2022). Again, research suggests that daily intake of raw vegetables as salad boosts up the health factor significantly as they have non-labile nutrients (Brookie *et al.*, 2018). However, the consumption of raw vegetables has been the cause of great public health concern as they may be contaminated with a wide variety of pathogenic microorganisms in so many ways (Amaechi *et al.*, 2016; Cardamone *et al.*, 2015; Chau *et al.*, 2014; IU *et al.*, 2015; Mohamed *et al.*, 2016; Tefera *et al.*, 2014). Several recent studies have reported the contamination of raw vegetables that harbor the pathogenic bacteria and parasites, e.g., *Salmonella, Shigella, Escherichia coli (E. coli), Clostridium, Staphylococcus, Campylobacter, Vibrio*, etc., which cause foodborne illness (Akoachere *et al.*, 2018; Cruz *et al.*, 2019; Luna-Guevara *et al.*, 2019).

Dhaka city has been booming with restaurants and catering services for the past few years. According to a daily

newspaper "The Financial Express" published on 22nd February 2023, Dhaka city has over ten thousand restaurants and the numbers are increasing day by day. The variety of cuisines is diverse that covers many continents of the world. Furthermore, people are fond of going outside to eat at restaurants very often. While dining at a restaurant, most commonly salads are served as a side dish which usually contains a mixture of raw vegetables, e.g., cucumbers, onions, green chilis, lemons etc. (Rahman and Noor, 2012). However, good hygiene practice varies in different restaurants in different areas of the city. Restaurants in areas that contain high-class society maintain good hygiene practices, but such practice is rare in areas that contain a mixed class of people. In this area, the chances of contamination of raw salad vegetables with bacterial pathogens are high. Accordingly, an individual is more prone to be infected by different pathogens in the aforesaid type of unhygienic restaurants (Alam *et al.*, 2017; Nizame *et al.*, 2019).

Foodborne illnesses with microbial origins are very common in Bangladesh. Approximately, 30 million people suffer from foodborne illnesses such as diarrheal diseases, which are the most common food poisoning cases in Bangladesh, every year (Noor and Feroz, 2016). Pathogens such as *Escherichia coli, Salmonella, Klebsiella, Shigella*, etc. are transmitted by food such as raw meat and vegetables. These species are well known to originate foodborne diseases that can cause serious health issues such as diarrhea, stomach infection, kidney failure, and in some cases, death (Khairuzzaman *et al.*, 2014). Additionally, MDR microorganisms have made the food safety situation more vulnerable in public health in Bangladesh (Ali *et al.*, 2011). It might very well be that raw salad vegetables are also contaminated by MDR bacteria. A study has also reported that vegetables, crops etc. grown using manure as fertilizer, have shown the contamination of bacteria such as *Escherichia coli* O157:H7, *Salmonella, Klebsiella spp*., etc., which may act as very prominent MDR bacteria (Feroz *et al.*, 2013; Rahman and Noor, 2012).

In the present study, we aim to determine the pathogenic and MDR bacteria from raw salad vegetables served at different restaurants located in Mirpur, Dhanmondi, Old Dhaka, Gulshan, and Bashundhara Residential Area of Dhaka city.

2. MATERIALS AND METHODS

A. Sample Collection and Processing

Fifty different samples of common raw salad vegetables like cucumbers, carrots, radishes, cabbages, green chilis, and coriander leaves in cut or grated form were collected from different restaurants in Dhaka city. Each sample was placed in a sterile Ziplock bag and subsequently transported to the laboratory in a sampling box with ice packs maintaining a temperature of 4 to 6°C. Samples were then weighed while being sealed in the Ziplock bag. 50g of Ringer's solution was then added to 25g of the raw salad vegetables in the Ziplock bag (ratio of Ringer's solution and raw salad vegetable; weight/weight = 2:1) and mixed thoroughly by using a vortex mixer. Then, 50 µl of the sample was taken from the Ziplock bag and placed on a MacConkey agar plate to determine gram-negative and enteric bacteria. The rest of the sample in the Ziplock bag was passed through a membrane filter (pore size 0.2 µm) and subsequently the filter was put on an mFC agar plate for the detection and enumeration of fecal coliforms.

B. Microbiological Analyses

All media were prepared according to the manufacturer's protocol. Media used for bacterial growth, isolation, and maintenance were MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England), mFC agar (Sigma Aldrich, Germany), Salmonella-Shigella (SS) agar (Techno Pharmchem, India), Eosin-Methylene Blue (EMB) agar (Oxoid Ltd., Basingstoke, Hampshire, England). After incubation of the mFC agar plates at 37°C for 18-22 hours, it showed characteristic blue colonies which were counted as total coliform bacteria. Fecal coliforms were counted by incubating another set of inoculated mFC agar plates at 44.5 °C (Cappuccino and Sherman, 1996).

MacConkey agar plate was used to detect *Escherichia coli*. However, *Salmonella* could also be cultured on this media and detected through colourless and transparent colonies. A single colony from the MacConkey subculture plate is taken with the help of a sterile inoculation loop. The loop is then swabbed on the EMB agar plate and kept at 37 °C for 24 hours in an incubator. EMB agar plates were used here to identify *E. coli*. Colourless and transparent colonies grown on MacConkey agar plates were selected for sub-culture on SS agar, which was conducted and inoculated by using an inoculation loop. SS agar plates were used here to identify *Salmonella* spp.

C. Antibiotic Sensitivity Test

In order to determine the sensitivity of *Salmonella* spp., a panel of antibiotics with their respective concentrations was used (Jorgensen and Pfaller, 2015). A portion of the fresh culture of *Salmonella* isolates was used to prepare 0.5 Macferland standard concentration of cell suspension which was then spread onto Mueller Hinton agar (Himedia Ltd., India) and dried for 15 minutes. A total of 11 types of antibiotics amoxicillin (25 µg), ampicillin (10 µg), azithromycin (15 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), kanamycin (30 µg), norfloxacin (10 µg), streptomycin (10 µg), tetracycline (30 µg), and trimethoprim (5µg) discs were placed onto Mueller Hinton agar following standard procedure, plates were then incubated at 37 °C for 18-20 hours. The zone diameters were measured after incubation to determine their sensitivity and resistance against the tested antibiotics.

The inoculation loop used previously for the sub-culture of SS agar plates was streaked on the nutrient agar inside the vial tube and sealed to prevent any contamination of other species. The samples were then stored for biochemical and polymerase chain reaction (PCR) tests. Major biochemical tests such as Kligler's Iron Agar (KIA), Motility indole urease (MIU), Methyl-Red (MR), Voges-Proskauer (VP), citrate utilization and oxidase tests were carried out according to the standard methods (Alfrad, 2007; Cappuccino and Sherman, 1996).

D. Detection of Salmonella spp. by PCR

Isolated colonies of *Salmonella* spp. were used for molecular detection of the STM 3098 gene by PCR method (Kim *et al.*, 2006). A portion of a fresh colony of biochemically identified cells was dislodged in an Eppendorf tube in 1 ml sterile DNase and RNase-free water and boiled for 10 minutes. Samples were then centrifuged at 10000 rpm for 5 minutes and a DNA template was collected from the supernatant. A 50 µl PCR mixture contained 5 µl of DNA template, 1 µl (100 pmol) of each primer and a 25 µl of Taq PCR Master Mix polymerase containing 100 mM Tris-HCl, 500 mM KCl at pH 8.3 at 20 °C, 1.5 mM $MgCl_2$, 200 M each deoxyribonucleoside triphosphate and 0.025 U Taq polymerase (Qiagen, USA). Amplification of DNA was performed using Mastercycler® personal (Eppendorf, USA) PCR machine. Heat denaturation was performed at 95 °C for 5 minutes, followed by 35 cycles (90 s at 95 °C, 60 s at 62 °C, and 90 s at 72 °C) and an elongation step of 7 minutes at 72 °C. The primers used were STM 3098 F (5'-TTTGG CGGCG CAGGC GATTC-3') and STM R (5'-GCCTC CGCCT CATCA ATCCG-3'), which amplified a 423-bp fragment of *Salmonella* spp. specific genes, as shown in Figure 1.

3. RESULTS

A. Incidence of Coliforms and Fecal Coliforms in the Study Area

The lowest and the highest total coliform counts were obtained from the samples of Dhanmondi (i.e., 4.0×10^1 cfu/ml) and Old Dhaka (i.e., 4.02×10^3 cfu/ml), respectively, as shown in Table 1. The highest fecal coliform count was also obtained from the samples of Old Dhaka (i.e., 1.2×10^3 cfu/ml), as shown in Table 1. In both cases, the highest average counts of organisms were found in Old Dhaka, as shown in Figure 2.

B. Presence of Salmonella spp. and E. coli in the Studied Samples

Salmonella spp. considered as very common food pathogens, were identified in the raw salad vegetable samples by plating them onto MacConkey agar. Additionally, *E. coli* and *Salmonella* spp. colonies were verified by sub-culturing on EMB and SS agar, respectively. However, the presence of *E. coli* and *Salmonella* colonies was found only in the samples collected from old Dhaka, Mirpur and Bashundhara R/A.

C. Identification and Further Confirmation of the Genera of the Selected Salmonella Species from the SS Plates

For the biochemical test, six isolates from the nine attributed *Salmonella* spp. based on their MDR test were selected for further characterization and identification. Among the selected six, one isolate was from Mirpur, four from Old Dhaka and one was from Bashundhara R/A. Biochemical test results of the samples shown in Table 2 suggested further confirmations of the genera of the selected *Salmonella* spp.

D. PCR Detection of Salmonella spp.

The result showed that out of five areas, three showed the presence of *Salmonella* spp. by culture technique which was further confirmed by PCR method. Figure 1 shows the PCR amplicons of the STM 3098 gene. *Salmonella* spp. were detected in samples collected from Mirpur (S-10), Old Dhaka (S-1, S-6, S-7, S-8) and Bashundhara R/A (S-3).

E. Antibiotic Resistance of Salmonella spp.

According to the morphological characteristics of the culture medium (SS plates), nine samples were attributed as *Salmonella* spp. and considered for antibiotics sensitivity test (Wayne, 2020). Among the nine isolates, three were from Mirpur, five from Old Dhaka and one from Bashundhara R/A. All *Salmonella* spp. were resistant to trimethoprim. Additionally, a few of the isolates were resistant to amoxicillin, ampicillin, erythromycin and chloramphenicol (Table 3).

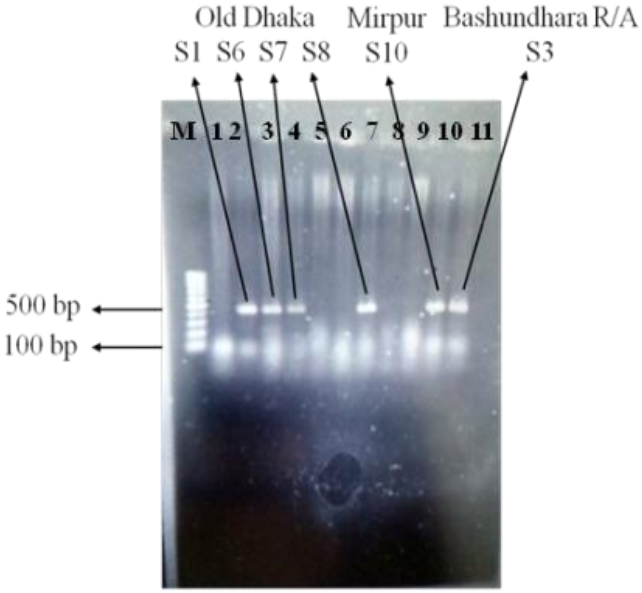

Figure 1: PCR detection of STM 3098 gene of *Salmonella* spp. after electrophoresis on 2% agarose gel. Lane M, 100bp DNA ladder, Lane 1, Negative control; 2, 3, 4 (S1, S6, S7), 7, 10, 11 (S8, S10, S3) show positive result for STM 3098 gene (423bp)

Table 1
Microbial load in raw salad vegetable samples collected from restaurants in different locations in Dhaka city

Sample ID	Sampling Locations									
	Mirpur (poor hygienie)		Dhanmondi (moderate hygiene)		Old Dhaka (poor hygiene)		Gulshan (Good hygiene)		Bashundhara R/A (Good hygiene)	
	TC	FC	TC	FC	TC	FC	TC	FC	TC	FC
	cfu/ml		cfu/ml		cfu/ml		cfu/ml		cfu/ml	
S-1	2.4×10^{2}	4.0×10^{1}	1.12×10^{3}	6.0×10^{1}	3.5×10^{3}	7.2×10^{2}	2.0×10^{2}	0	8.8×10^{2}	6.0×10^{1}
S-2	2.0×10^{2}	6.0×10^{1}	4.0×10^{1}	0	1.1×10^{3}	8.0×10^{1}	3.2×10^{2}	0	8.0×10^{2}	1.0×10^{2}
S-3	1.36×10^{3}	1.6×10^{2}	8.0×10^{1}	0	1.8×10^{2}	2.0×10^{1}	4.4×10^{2}	4.0×10^{1}	1.2×10^{3}	2.8×10^{2}
S-4	2.72×10^{3}	2.8×10^{2}	1.2×10^{2}	0	2.7×10^{3}	3.6×10^{2}	2.0×10^{2}	0	5.6×10^{2}	6.0×10^{1}
S-5	3.0×10^{3}	1.2×10^{2}	1.6×10^{3}	8.0×10^{1}	1.26×10^{3}	2.4×10^{2}	2.32×10^{3}	6.0×10^{1}	1.0×10^{3}	1.6×10^{2}
S-6	3.5×10^{3}	2.4×10^{2}	3.2×10^{3}	8.0×10^{1}	3.0×10^{3}	5.4×10^{2}	8.8×10^{2}	6.0×10^{1}	6.4×10^{2}	8.0×10^{1}
S-7	1.04×10^{3}	6.0×10^{1}	2.0×10^{2}	0	2.5×10^{3}	4.0×10^{2}	1.6×10^{2}	0	8.4×10^{2}	8.0×10^{1}
S-8	2.6×10^{3}	3.2×10^{2}	1.0×10^{3}	4.0×10^{1}	4.0×10^{3}	1.2×10^{3}	8.8×10^{2}	4.0×10^{1}	6.8×10^{2}	1.2×10^{2}
S-9	1.2×10^{3}	2.0×10^{2}	7.4×10^{2}	2.0×10^{1}	5.4×10^{2}	4.0×10^{1}	1.6×10^{2}	0	6.4×10^{2}	8.0×10^{1}
S-10	3.8×10^{3}	4.0×10^{2}	8.0×10^{2}	6.0×10^{1}	3.7×10^{3}	8.8×10^{2}	8.0×10^{2}	4.0×10^{1}	1.0×10^{3}	1.0×10^{2}

Legend: *TC – total coliform, FC – fecal coliform, R/A – residential area*

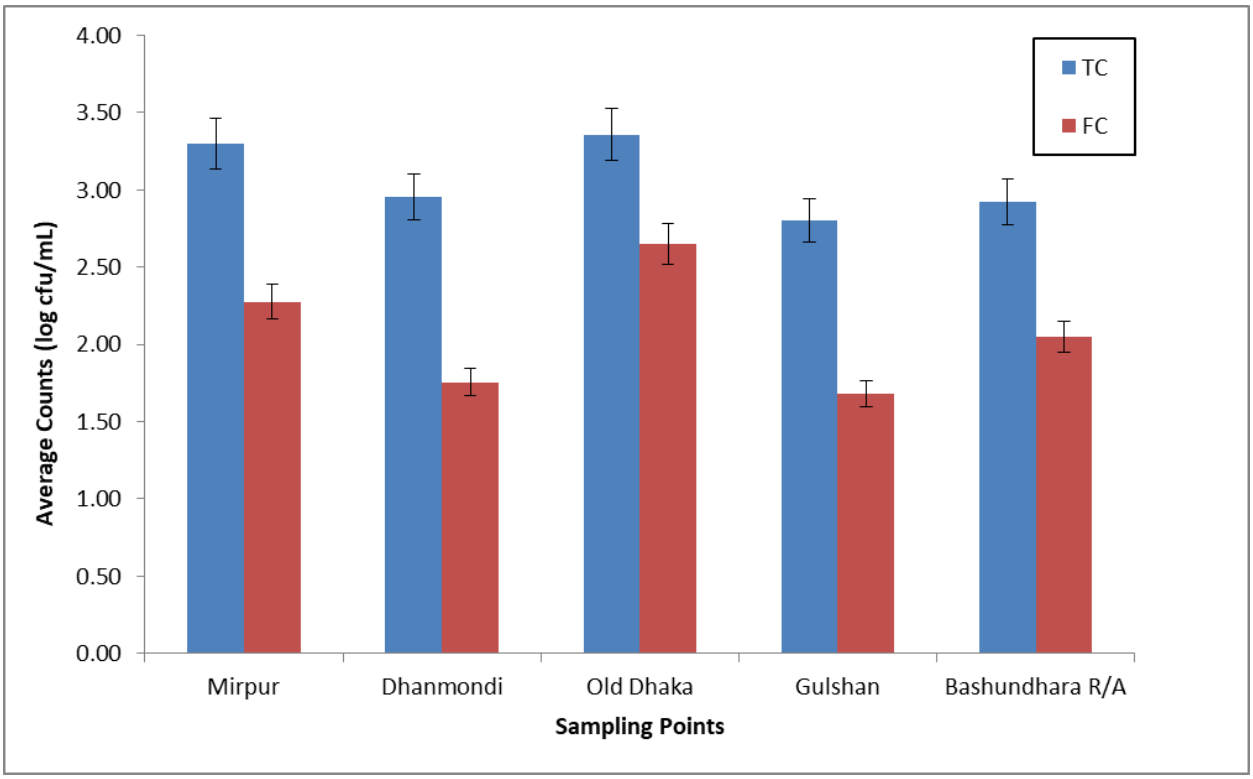

Figure 2: Total coliforms and fecal coliforms distribution in raw salad vegetables

Table 2
Results of biochemical tests of the pathogenic isolates

Sample ID	Locations	KIA				MIU			MR	VP	Citrate	Oxidase	Identified organisms
		Slant	Butt	Gas	H_2S	Motility	Indole	Urea					
S-10	**Mirpur**	K	A	-	+	+	-	-	+	-	V	-	*Salmonella* spp.
S-1	**Old Dhaka**	K	A	-	+	+	-	-	+/-	-	V	-	*Salmonella* spp.
S-6		K	A	+	+	+	-	-	+	-	-	-	*Salmonella* spp.
S-7		K	A	-	+	+	-	-	+	-	V	-	*Salmonella* spp.
S-8		K	A	-	+	+	-	-	+	-	V	-	*Salmonella* spp.
S-3	**Bashundhara R/A**	K	A	-	+	+	-	-	+	-	V	-	*Salmonella* spp.

Legend: V = 10-80% positive, K = alkaline, A = acidic, + = positive result, - = negative result, +/- = undefined result

Table 3
Antibiotic sensitivity pattern of the selected *Salmonella* spp.

Antibiotics	Diameter of zone inhibition standard (mm)			Sensitivity pattern of isolates								
				Sample ID								
	Resistant ≤	Intermediate	Susceptible ≥	Mirpur			Old Dhaka					Bashundhara R/A
				S-4	S-8	S-10	S-1	S-6	S-7	S-8	S-10	S-3
Amoxicillin	13	14-17	18	I	R	R	R	R	R	R	R	R
Ampicillin	13	14-16	17	I	R	R	R	R	R	R	S	R
Azithromycin	13	14-17	18	R	S	R	R	S	I	S	R	S
Chloramphenicol	12	13-17	18	S	R	S	S	S	S	S	S	R
Ciprofloxacin	15	16-20	21	I	S	S	I	S	I	S	S	S
Erythromycin	13	14-22	23	R	I	R	R	R	R	S	I	S
Kanamycin	13	14-17	18	I	S	I	I	S	I	S	S	S
Streptomycin	11	12-14	15	S	S	S	R	S	S	S	S	S
Tetracycline	11	12-14	15	R	S	S	S	R	S	S	I	S
Norfloxacin	12	13-16	17	S	S	S	S	S	S	S	S	S
Trimethoprim	10	13-16	17	R	R	R	R	R	R	R	R	R

Legend: *S – susceptible, R – resistance, I – intermediate*

4. DISCUSSION

In the present study, total coliform counts were performed for all the samples and all of them showed the existence of coliform, while fecal coliforms were detected in most of them on mFC agar medium. But the salad samples collected from the Old Dhaka region were more contaminated with fecal coliforms and *Salmonella* spp. than that of other regions. The plausible cause might be the high population density and unhygienic practices in Old Dhaka. Too many people with overcrowded housing have generally steered the environment to an unhealthy state (Bangladesh Bureau of Statistics, 2012). Additionally, the staffs of the restaurants are often uneducated and they do not have adequate knowledge of safe food handling. Furthermore, unhygienic sanitation and contaminated drinking water supply made the situation more critical (Hasan *et al.*, 2019). Thus, dense population and improper management are the determinant factors for the highest count of bacteria in Old Dhaka. The second region is Mirpur where low- and middle-income groups live in, and the hygienic condition is also worse than in other study areas except for Old Dhaka (Akhie and Ahmed, 2018). The

third contaminated raw salad vegetable was found in Bashundhara R/A where three universities are located and more than thirty thousand students live in this area. Therefore, one of the significant factors influencing the quality of restaurants in this area is obviously to cater cheaper priced foods to students. Consequently, the restaurants are also more focused on their sales and profits rather than hygiene. On the other hand, Gulshan and Dhanmondi areas are fairly better as people of high income can afford to live there (Kamruzzaman & Ogura, 2007; Satu & Chiu, 2019). Hence, restaurants following different hygienic processes with different price ranges were explored in Dhaka city during this study. Only *Salmonella* spp. isolated from collected raw salad vegetables were considered for antibiotic sensitivity tests in our study as shown in Table 3. When isolates showed resistance against more than one antibiotic were considered to be multidrug-resistant (MDR) bacteria. In this study, MDR *Salmonella* spp. was more prevalent in Old Dhaka. Presently, it has been considered to be a menace to public health all over the world at an alarming rate (Rozario *et al.*, 2019; Tanwar *et al.*, 2014). Numerous studies also reported a significant number of MDR contaminating bacteria in raw salad vegetables, e.g., carrot, lettuce, cucumber, tomato, chili, onion, capsicum, and coriander (Alam *et al.*, 2015; Noor *et al.*, 2015; Rahman and Noor, 2012). The contamination and development of these MDR bacteria in these freshly produced salads have emerged due to the inappropriate use of antimicrobial drugs, maintaining poor hygiene, improper food handling etc.

5. CONCLUSIONS

The consumption of raw salad vegetables in restaurants with poor hygiene has emerged to the proliferation of food-borne pathogens. MDR in microbes is also spreading throughout Dhaka city. Dwellers are at a risk to be exposed to MDR pathogens through a variety of routes including food, food chain, contaminated water, unhygienic environment, etc. However, raw salad is an important route of possible MDR infection as raw salad intake is a common practice in restaurants in Dhaka city. Raw salad preparation, washing and poor hygiene knowledge of the restaurant workers have led to a potential threat to public health. Necessary steps should be taken to remove the pathogens from the raw salad preparation in restaurants. Food handlers should also be trained in food safety, which alternatively ensures the safety of the consumers.

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