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BACTERIA ASSOCIATED WITH THE LEAFY SALAD VEGETABLES OF OLD DHAKA CITY AND THEIR MULTIPLE ANTIBIOTIC RESISTANCE (MAR) INDEX

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Key words: Bacteria, Antibiotics, Resistant, MAR index.

Abstract

The present work aimed to evaluate the bacteria associated with the most popular leafy salad vegetables like Coriander, Mint, and Lettuce and their antibiotic resistance pattern. Samples were collected from Rayshaheb Bazar, Nazira Bazar, and Shyam Bazar during June-August 2019. The maximum bacterial load was found in Coriander and the minimum bacterial load was in Mint. A total of 149 colonies were isolated and 35 isolates were selected for Culture and Sensitivity test against 15 common antibiotics like Amoxicillin (Ax), Cefixime (CFM), Levofloxacin (Lev), Clarithromycin (CLR), Ceftriaxone (CRO), Neomycin (N), Kanamycin (K), Ciprofloxacin (Cip), Erythromycin (E), Doxycycline (Do), Vancomycin (VA), Cefuroxime (CXM), Chloramphenicol (C), Rifampicin (RA), and Gentamycin (CN). Sixteen antibiotic resistant bacterial isolates were selected for their identification and drug resistance pattern. Among these 15 antibiotic resistant bacterial isolates, only 2 were Gram positive and were identified as *Enterococcus faecalis* and the Gram negative bacteria belonged to the genus *Enterobacter* (9), *Serratia* (3), *Klebsiella* (2).The drug resistance pattern showed that most of the isolated bacteria were resistant against Amoxicillin and susceptible to Chloramphenicol. The (multiple antibiotic resistance (MAR) inedx of the isolated bacteria ranged between 33.33% and 86.67% which is very alarming.

Introduction

Salad has received much attention due to health-related beneficial properties. Among various salad ingredients raw leafy vegetables are used frequently as salad ingredient. These are readily available, cheaper, nutritionally balanced and a good source of income for the vendors(1). Most of the time salad vegetables are consumed without any heat treatment, sometimes without washing , thus, has the possibility of causing food borne growing in the field, during harvesting, post-harvest handling, transporting, marketing or even at home. Several studies have shown that vegetables that are improper handling by vendors and sold at very dirty surroundings that make them prone to contamination and frequently cause diarrheal diseases. The great majority of people in developing

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countries obtain food from informal or "Wet Market" but these are often neglected by food safety authority[2].

Microorganisms that adhere to the surface of the vegetables may survive even after washing and sanitizing steps due to the formation of biofilms on the surface of the vegetables or from protection by the cuticle of the vegetables[3]. Common pathogens found in salad vegetables include: *Staphylococcus aureus, Enterobacter* spp*., Klebsiella spp., Escherichia coli, Salmonella typhi, Serratia* spp*., Pseudomonas aeruginosa, Yersinia enterolitica, Listeria monocytogenes, Aeromonas hydrophila, Shigella sonnei*[4]. The discovery of antibiotics was one of the greatest achievements of the 20th century. The subsequent introduction of broad spectrum bacteriostatic antibiotics, bactericidal antibiotics, synthetic chemicals and highly specific narrow spectrum antibiotics to clinical medicine transformed the treatment of bacterial disease[5]. However, due to excessive and inappropriate use of antibiotics there has been gradual emergence of antibiotic resistant bacteria, which pose a global health problem[6].

The decreasing effectiveness of antibiotics in treating common infections has been quickened in recent years. The main causes of resistances include increased global availability, uncontrolled sale in many low or middle-income countries where they can be obtained over the counter without physician prescription and use in livestock feed in low doses for growth promotion leads to increased levels of resistance. Now a days the release of large quantities of antibiotics into the environment by the pharmaceutical industries through inadequate waste water treatment. All these practices results in the development and spread of antibiotic-resistant bacteria. Considering all these facts and situations, the present study was undertaken to evaluate the bacterial load and multi-drug resistance pattern of leafy salad vegetables of old Dhaka city.

Materials and Methods

Three leafy vegetable samples *viz*. Lettuce, Mint, Coriander were collected from Rayshaheb Bazar, Nazira Bazar and Shyam Bazar for three times during June-August 2019. During sample collection, the samples were labeled properly and brought into the laboratory as soon as possible. Three different types of media viz. Nutrient agar, MacConkey agar, and Mannitol salt agar, were used to evaluate quantitative and qualitative nature of bacteria associated with the leafy salad vegetables. Serial dilution technique[7] was used for the isolation of bacteria. The inoculated plates were placed invertedly and incubated at 37 °C for 24 hours. After incubation, the plates having well discrete bacterial colonies were counted by a colony counter (Digital colony counter, DC-8 OSK 10086, Kayagaki, Japan). Well discrete colonies having distinctive morphology were primarily selected and isolated on NA slants. The selected isolates were purified through streak plate method.

The culture and sensitivity test was performed to reveal the drug resistance pattern of the isolated bacteria. For this purpose, the bacteria were grown on Muller-Hinton agar against common antibiotic impregnated discs. After 24 h the zone diameter around the discs were compared with the standard zones of inhibition for each antibiotic and the sensitivity, resistance or intermediary relationship of each bacterium was determined. Multiple antibiotic resistance (MAR) index % of the multi-drug resistant isolates was determined using the following formula:

$$\text{MAR index \%} = \frac{\text{No.of antiboitics to which pathogen showed resistance}}{\text{No.of antibiotics used}} \times 100$$

The isolates were tested against 15 common antibiotics, they are Amoxicillin (Ax), Cefixime (CFM), Levofloxacin (Lev), Clarithromycin (CLR), Ceftriaxone (CRO), Neomycin (N), Kanamycin (K), Ciprofloxacin (Cip), Erythromycin (E), Doxycycline (Do), Vancomycin (VA), Cefuroxime (CXM), Chloramphenicol (C), Rifampicin (RA), Gentamicin (CN). Finaly those isolates were selected which showed resistance against maximum number of antibiotics. As per standard protocol, morphological and Biochemical tests[(8)] were done and isolates were provisionally identified following Bergey's Manual of Systematic Bacteriology Vol. I[(9)] and Vol. II[(10)].

Results and Discussion

During this study, the bacteria were isolated from Lettuce, Mint and Coriander from Rayshaheb Bazar, Nazira Bazar and Shyam Bazar and was presented in Table 1. The bacterial load of the studied samples was ranged between $6.17 \pm 2.35 \times 10^7$ cfu/g and $18.21 \pm 5.87 \times 10^7$ cfu/g, $9.52 \pm 4.10 \times 10^6$ cfu/g and $6.11 \pm 4.13 \times 10^7$ cfu/g, and $3.02 \pm 0.45 \times 10^6$ cfu/g and $16.01 \pm 4.24 \times 10^6$ cfu/g on Nutrient agar, MacConkey agar and Mannitol salt agar, respectively. The Maximum bacterial load ($18.21 \pm 5.87 \times 10^7$, $6.35 \pm 3.8 \times 10^7$ and $16.01 \pm 4.24 \times 10^6$ cfu/ml on Nutrient agar, MacConkey agar and Mannitol salt agar respectively) was found in Lettuce and the minimum bacterial load ($6.17 \pm 2.35 \times 10^7$, $9.52 \pm 4.10 \times 10^6$ and $3.02 \pm 0.45 \times 10^6$ cfu/ml) on Nutrient agar, MacConkey agar and Mannitol salt agar, respectively was found in Mint leaf. This could be due to the presence of essential oils in the mint leaf to protect from bacterial harbor. In a study Khan *et al*.[(11)] mentioned the load of coliform and non-lactose fermenters were 6.0×10^6 and 1.0×10^7 cfu/g, respectively in fresh salad vegetables. On the other hand Ali *et al*.[(12)] observed enteric bacterial load as 6.1×10^3 to 1.22×10^6, 1.02×10^4 to 1.1×10^7, 22 to 870 and 1.3×10^3 to 1.96×10^7 cfu/ml on cucumber, carrot, green mangoes and wash water, respectively. Leafy green vegetables become more contaminated than other vegetables. The uneven surfaces of leafy greens facilitate microbial attachment. Abougraina *et al*.[(13)] reported the highest level of parasite contamination in Lettuce and the lowest in vegetables with smooth surfaces.

Table 1. Bacterial load of studied leafy vegetable samples of some local markets.

Sample type	Scientific name	Bacterial load (cfu/g) on		
		Nutrient agar	MacConkey agar	Mannitol salt agar
		Mean ± SD	Mean ± SD	Mean ± SD
Coriander	*Coriandrum sativum*	$9.25 \pm 6.01 \times 10^7$	$6.11 \pm 4.13 \times 10^7$	$7.52 \pm 3.98 \times 10^6$
Lettuce	*Lactuca sativa*	$18.21 \pm 5.87 \times 10^7$	$6.35 \pm 3.8 \times 10^7$	$16.01 \pm 4.24 \times 10^6$
Mint	*Mentha spicata*	$6.17 \pm 2.35 \times 10^7$	$9.52 \pm 4.10 \times 10^6$	$3.02 \pm 0.45 \times 10^6$

During this investigation, a total of 149 colonies were isolated from three leafy salad vegetable samples and then 35 were selected for their drug resistance nature. Finally, 16 antibiotic resistant bacterial isolates were selected for detailed study. Major biochemical characteristics of the isolates was shown in the Table 2. The Gram positive members were identified as *Enterococcus faecalis* while the Gram negative bacteria belonged to the genus, *Enterobacter, Serratia, Klebsiella* (Table 3). Among the Gram negative isolates, *Enterobacter* was the dominating genus found to be associated with leafy salad vegetables.

The culture and sensitivity test (Table 4) revealed that most of the bacterial isolates were resistant against Amoxicillin and Rifampicin and susceptible to Chloramphenicol (Fig. 1). About 65% isolates were found to be resistant and only 13% isolates showed susceptibility against amoxicillin. Only 9% isolates showed susceptibility against Rifampicin. Against Chloramphenicol, only 3% isolates showed resistance and 48% isolates showed susceptibility. The Gram negative isolates were found to be concern of interest against antibiotic resistance pattern. The MAR inedx of the isolates ranged between 33.33 and 86.67% (Table 5). Among isolated bacteria, the highest MAR index (86.67%) was showen by *Enterobacter intermedium, Klebsiella pneumoniae* and *Serratia ficaria*. In an earlier work Khan *et al.*(14) reported the MAR index in between 14.28 and 71.43% of the bacteria isolated from chatpoti and *Enterobacter* sp. showed the maximum MAR index. In the present study *E. intermedium* showed the maximum MAR index (86.67%) which is little bit higher than the previous report. Kim *et al.*(15) isolated a total of 132 *Klebsiella pneumoniae* isolates and all were found to be resistant against ampicillin, tetracycline, streptomycin, gentamycin and kanamycin. In the present study, *Klebsiella pneumoniae* was found to be resistant against amoxicillin, cefixime, clarithromycin, neomycin, kanamycin, erythromycin, gentamycin, rifampicin, vancomycin, doxycycline and cefotaxime. In another study *Klebsiella pneumoniae* was found to be resistant against

Table 2. Major biochemical characteristics of the bacterial isolates associated with the samples.

Isolate No.	Gram reaction	V.P. test	M.R. test	Deep glucose agar	Tyrosine degradation	Lecithinase production	Nitrate reduction	Utilization of propionate
105/C/N/MS	+	-	+	A	-	-	+	-
129/L/N/MS	+	-	+	A	-	-	+	-
19/C/R/NA	-	+	+	FA	-	-	+	-
50/L/R/NA	-	+	-	FA	+	+	+	-
49/L/R/NA	-	+	-	FA	+	-	+	-
127/L/S/MC	-	+	+	A	-	+	+	-
137/M/N/MC	-	-	+	A	+	-	-	+
138/M/N/MC	-	+	-	FA	-	-	+	-
95/C/S/MC	-	+	-	FA	-	-	+	-
77/L/N/MC	-	+	-	FA	-	-	+	-
60/M/N/MC	-	+	+	FA	+	-	+	-
122/L/R/MC	-	+	+	A	-	-	+	-
126/L/N/MC	-	+	+	A	-	+	+	-
22/C/R/MC	-	+	-	FA	-	-	+	-
28/M/R/MC	-	+	+	FA	-	-	+	-
103/C/N/MC	-	+	-	FA	-	-	+	-

Table 3. Provisional identification of the bacterial isolates associated with the studied samples.

Isolate No.	Identified bacteria	Isolate No.	Identified bacteria
103/C/N/MC 50/L/R/NA	*Enterobacter aerogenes*	129/L/N/MS 105/C/N/MS	*Enterococcus faecalis*
60/M/N/MC	*E. intermedium*	126/L/N/MC 137/M/N/MC	*Serratia ficaria*
49/L/R/NA 77/L/N/MC	*E. sakazakii*	95/C/S/MC	*S. rubidaea*
28/M/R/MC 19/C/R/NA	*E. gergoviae*	*127/L/S/MC* *122/L/R/MC*	*Klebsiella pneumoniae sub sp. rhinoscleromatis*
22/C/R/MC 138/M/N/MC	*E. cloacae*		

Table 4. Culture and sensitivity test of the selected isolates.

Isolate No.	Ax-10	CFM-5	Lev-5	CLR-15	CRO-30	N-30	K-30	Cip-5	E-15	Do-30	VA-30	CXM-30	C-30	RA-5	CN-10
E. gergoviae	R	I	I	R	I	R	R	R	R	R	R	R	I	R	R
Klebsiella pneumoniae	R	R	S	R	I	R	R	I	R	I	R	R	S	R	R
Enterococcus faecalis	R	R	R	I	I	R	R	R	I	S	I	I	I	I	I
Enterobacter aerogenes	R	R	R	R	I	R	R	R	R	R	R	I	I	R	I
E. gergoviae	R	I	I	R	I	R	R	R	R	R	R	I	S	R	I
E. cloacae	R	R	R	R	I	R	R	R	R	R	R	I	I	R	R
Serratia ficaria	R	R	S	R	R	R	R	R	R	R	R	R	I	R	R
E. cloacae	R	R	I	R	I	R	R	I	R	R	R	R	I	R	R
Enterobacter aerogenes	R	R	R	R	I	R	R	R	R	R	R	I	I	R	R
Serratia ficaria	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I
Klebsiella pneumoniae	R	R	R	R	R	R	R	R	R	I	R	R	I	R	R
E. intermedium	R	R	R	R	I	R	R	R	R	R	R	R	I	R	R
S. rubidaea	R	R	R	R	I	R	R	R	R	R	R	I	I	R	R
Enterococcus faecalis	I	R	I	R	I	R	I	R	R	S	R	I	I	I	S
E. sakazakii	R	R	R	R	I	R	R	I	R	I	R	R	I	R	R
E. sakazakii	R	R	R	R	R	R	I	S	R	S	R	I	S	R	I

Zone Diameter were studied in triplets. Ax=Amoxicillin, CFM=Cefixime, Lev=Levofloxacin, CLR=Clarithromycin, CRO=Ceftriaxone, N=Neomycin, K=Kanamycin, Cip=Ciprofloxacin, E=Erythromycin, VA=Vancomycin, DO=Doxycycline, CXM=Cefotaxime, C=Chloramphenicol, RA=Rifampicin, CN=Gentamycin.

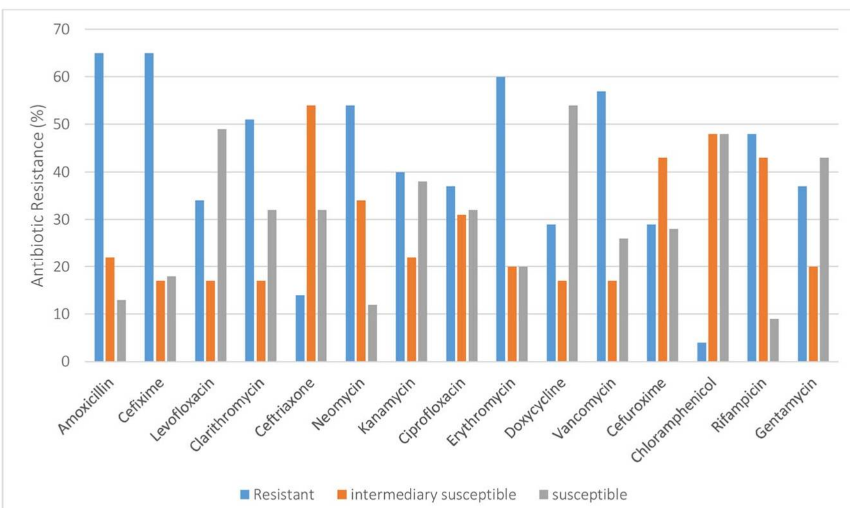

Fig. 1. Antibiotic resistance profile of the selected bacterial isolates.

Table 5. Multiple antibiotic resistance of the selected isolates.

Isolate No.	Name of the bacteria	Number of antibiotics against bacteria showed resistance	MAR value (%)
105/C/N/MS	*Enterococcus faecalis*	5	33.33
129/L/N/MS	*Enterococcus faecalis*	6	40.00
28/M/R/MC	*Enterobacter gergoviae*	11	73.33
127/L/S/MC	*Klebsiella pneumoniae* sub sp. *rhinoscleromatis*	10	66.67
50/L/R/NA	*Enterobacter aerogenes*	11	73.33
22/C/R/MC	*Enterobacter cloacae*	12	80.00
19/C/R/NA	*Enterobacter gergoviae*	9	60.00
126/L/N/MC	*Serratia ficaria*	13	86.67
138/M/N/MC	*Enterobacter cloacae*	11	73.33
103/C/N/MC	*Enterobacter aerogenes*	12	80.00
137/M/N/MC	*Serratia ficaria*	13	86.67
122/L/R/MC	*Klebsiella pneumoniae* sub sp. *rhinoscleromatis*	13	86.67
60/M/N/MC	*Enterobacter intermedium*	13	86.67
95/C/S/MC	*Serratia rubidaea*	12	80.00
77/L/N/MC	*Enterobacter sakazakii*	11	73.33
49/L/R/NA	*Enterobacter sakazakii*	9	60.00

ciprofloxacin and doxycycline(16). Adu-Gyamfi and Nketsia-Tabiri(17) reported *Enterobacter* spp. and *Klebsiella* spp. in food samples having leafy salad vegetables. The similar observation was found to be in our study where resistant *Enterobacter* spp. and *Klebsiella* associated with leafy salad vegetables. In a study Tabassum *et al.*(18) found multidrug resistant *Pseudomonas, Enterobacter cloacae, E. coli, Klebsiella* and *Yersinia enterocolitica* from velpuri, a very common street food. Similar trnd was noticed in our resent findings. Viswanathan and Kaur(19) showed the presence of *Enterobacter, Serratia, Salmonella, E. coli, Streptococcus aureus* in vegetables and fruits. The present study also showed the presence of *Enterobacter* and *Serratia* from studied leafy vegetables of the old Dhaka city.

In Bangladesh, people still are not aware of food hygiene, therefore, food borne disease become the most common phenomena. This study shows that there is an urgent need to sensitize people associated with vender and consumer for food hygiene with special attention to use of microbiologically safe water and proper washing leafy salad vegetables prior to sell and consumption.

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