Middle-East Journal of Scientific Research 10 (1): 70-77, 2011
ISSN 1990-9233

Prevalence of Multi Drug Resistant Bacteria on Raw Salad Vegetables Sold in Major Markets of Chittagong City, Bangladesh

[1]Meher Nigad Nipa, [1]Reaz Mohammad Mazumdar, [2]Md. Mahmudul Hasan,
[1]Md. Fakruddin, [1]Saiful Islam, Habibur R. Bhuiyan and [2]Asif Iqbal

[1]Industrial Microbiology Research Division, BCSIR laboratories Chittagong, Chittagong, Bangladesh
[2]Department of Genetic Engineering and Biotechnology,
Shahjalal University of Science and Technology, Sylhet, Bangladesh

Abstract: The study was conducted to examine microbiological quality of raw salad vegetables and their role as a source of antibiotic resistant bacteria. Eight types of vegetables which are commonly used for salad i.e. Tomato, Cucumber, Carrot, Green chilli, Lemon, coriander leaf, Pepper mint, Beet root were collected from two Open markets and two Super shops of Chittagong City. All the vegetables were highly contaminated with Coliform and fecal Coliform (> 1100 CFU/100ml). Range of microbial count of Tomato was 9.0×10^4 CFU/ml to 3.8×10^5 CFU/g, Cucumber was 5.5×10^4 CFU/g to 1.9×10^6 CFU/g, Carrot was 1.2×10^4 to 2.6×10^6 CFU/g, Green chilli was 1.0×10^4 to 4.0×10^5 CFU/g, Lemon was 1.5×10^5 to 1.2×10^6 CFU/g, Coriander leaf was 5.87×10^5 to 1.8×10^6 CFU/g, Peppermint was 2.2×10^5 to 7.7×10^5 CFU/g and it was 5.0×10^3 to 5.4×10^5 CFU/g for Beet root. Yeast and mold was not detected in most of the vegetables. A total of 266 bacterial isolates of ten genera and three fungi *Rhizopus*, *Penicilium* and *Aspergillus* were identified. *Enterobacter* spp. (21.80%) was the most dominant followed by *Pseudomonas* spp. (19.17%), *Vibrio* spp. (16.92%), *Lactobacillus* spp. (15.04%), *Staphylococcus* spp. (10.15%), *Klebsiella* spp *(9.04%), E. coli* (4.89%), *Citrobacter* spp. (2.26%), *Serratia* spp. (0.37%) *and Salmonella* spp. (0.37%). Fifty-one selected isolates from Karnafully market were tested for antibiotic susceptibility. Multiple drug resistance was observed in 98.06% isolates with a resistance to two to seven antibiotics. These results suggest the necessity to follow the hygienic practices in handling the vegetables in open markets as well as the super shops and vegetables might have an important role as a source of multiple antibiotic resistant bacteria.

Key words: Salad vegetables · Microbiological quality · Multi-drug resistant bacteria · Chittagong city

INTRODUCTION

Vegetables have been associated with outbreaks of food borne disease in many countries. Organisms involved include bacteria, viruses and parasites [1]. Raw vegetables can harbour many microorganisms, which may be dispersed over the plant or appear as microcolonies embedded in the plant tissue [2]. The majority of microorganisms associated with raw vegetables are gram negative organisms tend to dominate the bacterial population. Vegetables are highly exposed to microbial contamination through contact with soil, dust and water and by handling at harvest or during postharvest processing. They therefore harbour a diverse range of microorganisms including plant and human pathogens [3-5]. Differences in microbial profiles of various vegetables result largely from unrelated factors such as resident microflora in the soil, application of nonresident micro flora via animal manures, sewage or irrigation water, transportation and handling by individual retailers [6,7]. Use of untreated waste water and manure as fertilizers for the production of fruits and vegetables is a major contributing factor to contamination [8,9]. Tomato, Cucumber, Carrot, Green chili, Lemon, Coriander leaf, Pepper mint and Beet root are some of the vegetables that are normally consumed raw in

Corresponding Author: Reaz Mohammad Mazumdar, Industrial Microbiology Research Division, BCSIR laboratories Chittagong, Chittagong, Bangladesh.
Tel: +8801819623623, Fax: +88031682505.

order to obtain their valuable nutrients in best form and their traditional use in best form and their traditional use in preparing salads is familiar throughout the world in the same manner. Increasing health awareness has increased the consumption of minimally processed foods in recent years [10]. Minimally processed foods or other raw vegetables have become popular since it suits the present necessity as need of elaborate preparations are not required. Fruits and vegetables carry microbial flora from the farm to the table even source of contamination however vary. The product is exposed to potential microbial contamination at every step including cultivation, harvesting, transporting, packaging, storage and selling to the final consumers. In developing coutries, food borne illnesses caused by contaminated fruits and vegetables are frequent and in some areas they cause a large proportion of illness. However, due to lack of food borne disease investigation and surveillance in most of these countries, most outbreaks go undetected and the scientific literature reports only on very few outbreaks.

In addition the increasing incidence of antimicrobial resistance (AMR) bacteria in humans, animals and the environment is a major concern in both human and veterinary medicine and is subject to increased monitoring [11,12]. However many studies have focused on pathogenic bacteria and indicator bacteria isolated from animals, humans and the environment [13,14]. Antimicrobial use has an impact on the distribution of AMR phenotypes [15] and resistance genes [16]. Use of antimicrobial agents in any environment creates selective pressures that favor the survival of antibiotic resistant pathogens. According to the Infectious Diseases Report released by the World Health Organization (WHO) in 2000, drug resistant organisms are prevalent world wide [12]. Since resistant genes may be in mobile genetic elements the use of specific antibiotic can also induce resistance to other antimicrobial agents and can be transferred to a wide variety of bacteria. Resistant plasmids may harbor a number of resistance genes and super plasmid encoding resistance to eight and more antimicrobials have been reported [17].

Due to the serious implications from the consumption of contaminated vegetables, this work aims at conducting market survey on vegetables sold in four major markets in Chittagong city to identify the associated antibiotic resistant microorganisms and their resistance pattern to create awareness among mass people and Physicians on indiscriminate use of antibiotics.

MATERIALS AND METHODS

A total of 32 samples of fresh vegetables each collected from two open markets (Karnafully market and Reazuddin bazaar) and two super shops (Meena bazaar and Khulshi mart) of Chittagong city. All samples were collected in sterile polythene bag in an insulated box with ice to maintain a temperature ranging from 4° to 6° and analyzed within one hour after procurement. Samples were rinsed with 100 ml distilled water and diluted 10 fold. 10 ml of the aqueous suspension obtained by washing the surface of each vegetable were inoculated to 90 ml Luria Bertani (LB) broth for overnight incubation at 37°C. This overnight culture in LB broth was used in streak plate technique on selective media for the isolation and identification of strains.

Bacterial Enumeration and Identification: Isolation and enumeration of bacteria were done by growing them on selective and non-selective media such as nutrient agar for total viable count (TVC), Potato Dextrose Agar for yeast and mold count (YC), MacConkey broth for total Coliform (TC) and fecal Coliform (FC) count. The pathogen from the surface of samples were enriched in LB broth and isolated on selective media by streak plate method. Sorbitol Macconkey for pathogenic *E. coli* and Thiosulfate Citrate Bile Sucrose Agar (TCBS Agar) for *Vibrios, Vibrio cholera* like organism (VCLO), Xylose Lysine Deoxycholate Agar (XLD agar) and Salmonella-Shigella Agar (S-S Agar) for *Salmonella*, Tomato Juice Agar for *Lactobacillus* spp. Cetrimide Agar for *Pseudomonas* spp. and Mannitol salt phenol-red Agar was used for S*taphylococcus* spp. All the selective media were obtained from Himedia Laboratories Limited, Mumbai, India. Plates were made in triplicates in appropriate selective media. For bacterial and fungal enumeration spread plate was used to determine the number of colony forming units (CFU). For computation average number per plate is divided by sample volume and expressed as CFU/g. Biochemical tests were performed using conventional methods. Identification of the enriched and isolated microbial isolates was done according to Bergey's manual of determinative Baceteriology [18].

Antimicrobial Susceptibility Testing: Fifty one strains were tested for antibiotic resistance by the standard agar disc diffusion technique [19] on Muller Hinton agar using commercial discs (Himedia, India). The following antibiotics with the disc strength in parentheses were used: Erythromycin (Ery, 10 μg), Gentamycin (Gen, 30 μg), Ampicillin (Amp, 10 μg), Ciprofloxacin (Cip, 5 μg), Cephalexin (ceph, 30 μg), Chloramphenicol (Chl, 30 μg) and Streptomycin (Strep, 10 μg).

Statistical Analysis: SPSS software (V12) was used for statistical analysis and T test was performed in the evaluation of the significance of the difference between the groups. The significance between the values was evaluated at 95% confidence ($p<0.05$) [20].

RESULTS AND DISCUSSION

Total Viable Count: Bacteriological findings of total bacteria are presented in Table 1. The retail markets and super shop vegetables showed no difference in term of Coliform and fecal coliform (more than 1100 cfu/100 ml). Range of microbial count of Tomato was 9.0×10^4 CFU/g to 3.8×10^5 CFU/g, Cucumber was 5.5×10^4 CFU/g to 1.9×10^6 CFU/g, Carrot was 1.2×10^4 to 2.6×10^6 CFU/g, Green chilli was 1.0×10^4 to 4.0×10^5 CFU/g, Lemon was 1.5×10^5 to 1.2×10^6 CFU/g, Coriander leaf was 5.87×10^5 to 1.8×10^6 CFU/g, Peppermint was 2.2×10^5 to 7.7×10^5 CFU/g and it was 5.0×10^3 to 5.4×10^5 CFU/g for Beet root. Mean TVC (Log CFU/g) of the Super shops and Open markets were 5.52 ±0.58 and 5.35±0.38 respectively. The significance of the average bacterial counts between the samples collected from open market and super shops was evaluated statistically. No significant difference ($p=0.51$) was observed in microbial load on the selected salad vegetables of Super shop and the Open market. Viswanathan and Kaur [21] reported total aerobic number as 10^5 to 10^{10} per gram and coliform number as 10^6 to 10^9 in raw vegetables used in salad mixture. The numbers of bacteria present will vary depending on seasonal and climatic variation and may range from 10^4 to 10^8 per gram. The inner tissues of fruits and vegetables are usually regarded as sterile [22]. However, bacteria can be present in low numbers as a result of the uptake of water through certain irrigation or washing procedures.

In open markets vegetables were seen displayed on open stalls in close proximity of fish display with flies swarming all over the places, mostly on bear rounds close to open gutter. The environments in the open market were generally unhygienic with open clogged gutters and refuse dumps with pieces of dirt littering virtually every available space. Water pools and pieces of rotten matters like fruits dotted the gutter and every other place. On the other hand, super shops vegetables were displayed and sold in a clean hygienic and satisfactory environment but the ultimate analysis for TVC, YMC, Coliform count, Fecal coliform counts indicate their contamination level was same as the open market products. Green vegetables are included in daily menus either raw or cooked, alone or together with either foodstuffs. Particularly freshly consumed vegetables or those which are used in salad mixture can be hazardous for health since they are not subjected to any thermal process.

Table1: Microbiological study of the proposed raw salad vegetables collected from various places

	Location	Sample							
	Code	Tomato	Cucumber	Carrot	Green Chili	Lemon	Coriander Leaf	Pepper Mint	Beet Root
Coliform (CFU/100 mL)	M1	+++	+++	+++	+++	+++	+++	+++	+++
	M2	+++	+++	+++	+++	+++	+++	+++	+++
	M3	+++	+++	+++	+++	+++	+++	+++	+++
	M4	+++	+++	+++	+++	+++	+++	+++	+++
Fecal coliform (CFU/100 mL)	M1	+++	+++	+++	+++	+++	+++	+++	+++
	M2	+++	+++	+++	+++	+++	+++	+++	+++
	M3	+++	+++	+++	+++	+++	+++	+++	+++
	M4	+++	+++	+++	+++	+++	+++	+++	+++
Viable Bacterial colony (CFU/g)	M1	$7.2x10^5$	$3.6x10^5$	$2.6x10^6$	$8.0x10^4$	$1.2x10^6$	$1.8x10^6$	$7.72x10^5$	$5.4x10^5$
	M2	$9.0x10^4$	$1.4x10^5$	$1.2x10^4$	$1.0x10^4$	$7.4x10^5$	$5.87x10^5$	$2.2x10^5$	$2.4x10^5$
	M3	$3.8x10^5$	$1.9x10^6$	$2.7x10^5$	$4.0x10^5$	$1.5x10^5$	$7.7x10^5$	$2.5x10^5$	$4.5x10^5$
	M4	$8.7x10^5$	$5.5x10^3$	$9.8x10^5$	$2.6x10^3$	$4.4x10^5$	$7.5x10^5$	$3.2x10^5$	$5.0x10^3$
Viable Yeast and Mold (CFU/g)	M1	$8x10^1$	ND	ND	ND	ND	ND	ND	$2.0x10^1$
	M2	$6.5x10^1$	ND	$5.0x10^1$	ND	ND	$1.8x10^1$	ND	$2.5x10^1$
	M3	ND	ND	ND	$1.2x10^1$	ND	ND	$2.0x10^1$	$5.0x10^1$
	M4	$4.7 x10^1$	ND	ND	ND	ND	$9.0x10^1$	ND	$1.8x10^2$

Super shop: M2=Meenabazar, M3=Khulshimart; Open market: M1=Karnafully market, M4=Reazuddin Bazar, +++ = >1100 CFU/100ml, ND= Not Detected

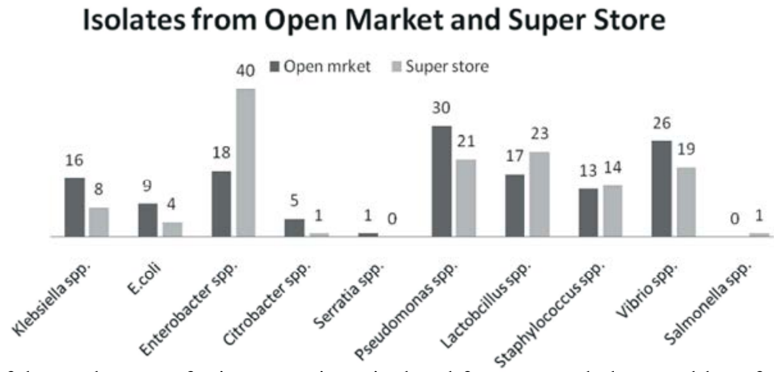

Fig.1: Determination of the total count of microorganisms isolated from raw salad vegetables of open markets and super stores.

Isolation of Bacteria: Of the vegetable samples a total of 266 isolates were identified up to the genus level as presented in the fig 1. The prevalent bacterial genera were *Klebsiella* spp. (9.04%), *E.coli* (4.89%), *Enterobacter spp.* (21.80%), *Citrobacter* spp. (2.26%), *Serratia* spp. (0.37%), *Pseudomonas* spp. (19.17%), *Lactobacillus* spp. (15.04%), *Staphylococcus* spp. (10.15%), *Vibrio* spp. (16.92%) *and Salmonella* spp. (0.37%). *Rhizopus, penicillium* and *Aspergillus* were the fungal isolates. The frequency of the associated microorganisms on vegetables as shown in Table 2 varies. *Pseudomonas* were present on all vegetables in all markets i.e. 100 % occurrence, followed by *Vibrio* spp. and *Lactobacillus* spp., 96.87 %; *Enterobacter* 84.37%: *Staphylococcus*, 81.25 %; *Klebsiella* 68.75%; *Escherichia coli*, 40.62 %; *Citrobacter* 12.5%; *Salmonella* and *Serratia*, 3.12 %. *Pseudomonas* were present on all vegetables of the four markets. *Vibrio* spp., was absent on peppermint of Reazuddin Bazar only. *Lactobacillus* spp., was not seen on carrot of the same market. *Enterobacter* spp. was present on all vegetables of the four markets except tomato, cucumber, carrot and green chili from Karnafully market. All vegetables from Meenabazar along with lemon and Coriender leaf from Karnafully

Table 2: Occurrence of microorganisms on vegetables

	Location Code	*Citrobacter* spp.	*Enterobacter* spp.	*E. coli*	*Klebsiella* spp.	*Lactobacillus* spp.	*Pseudomonas* spp.	*Salmonella* spp.	*Serratia* spp.	*Staphylococcus* spp.	*Vibrio* spp.	*Aspergillus* spp.	Rhizpous spp.	*Penicillium* spp.
Tomato	M1	-	-	-	+	+	+	-	-	+	+	+	-	-
	M2	-	+	-	-	+	+	-	-	+	+	+	-	-
	M3	+	+	-	+	+	+	-	-	+	+	-	-	-
	M4	+	+	-	+	+	+	-	-	+	+	-	+	+
Cucumber	M1	-	-	+	+	+	+	-	-	+	+	-	-	-
	M2	-	+	-	-	+	+	-	-	+	+	-	-	-
	M3	-	+	-	+	+	+	-	-	+	+	-	-	-
	M4	-	+	-	+	+	+	-	-	+	+	-	-	-
Carrot	M1	-	-	-	+	+	+	-	-	+	+	-	-	-
	M2	-	-	+	-	+	+	+	-	+	+	-	+	-
	M3	-	-	-	+	+	+	-	-	-	+	-	-	-
	M4	-	+	-	+	-	+	-	-	+	+	-	-	-
Green Chilli	M1	-	-	+	+	+	+	-	-	+	+	-	-	-
	M2	-	+	+	-	+	+	-	-	+	+	-	-	-
	M3	-	+	-	+	+	+	-	-	+	+	-	+	-
	M4	-	+	+	+	+	+	-	-	+	+	-	-	-
Lemon	M1	-	+	+	-	+	+	-	-	+	+	-	+	+
	M2	-	+	-	-	+	+	-	-	+	+	-	-	-
	M3	-	+	-	+	+	+	-	-	+	+	-	-	-
	M4	+	+	-	+	+	+	-	-	-	+	-	-	-
Coriender leaf	M1	+	+	+	-	+	+	-	+	-	+	-	-	+
	M2	-	+	+	-	+	+	-	-	-	+	-	-	+
	M3	-	+	-	+	+	+	-	-	-	+	-	-	-
	M4	-	+	+	+	+	+	-	-	-	+	+	-	-
Peppermint	M1	-	+	+	+	+	+	-	-	+	+	-	-	-
	M2	-	+	-	-	+	+	-	-	+	+	-	-	-
	M3	-	+	-	+	+	+	-	-	+	+	+	+	-
	M4	-	+	-	+	+	+	-	-	+	-	-	+	-
Beet root	M1	-	+	+	+	+	+	-	-	+	+	-	+	-
	M2	-	+	+	-	+	+	-	-	+	+	-	-	+
	M3	-	+	-	+	+	+	-	-	+	+	-	-	-
	M4	-	+	+	+	+	+	-	-	+	+	+	-	-

market lacks *Klebsiella* spp.. Less frequent bacteria on the selected vegetables of the considering markets were *Citrobacter* spp., *Salmonella* spp. and *Serratia* spp. Vishawnathan and Kaur [21] also reported presence of *Salmonella* spp., *Serratia* spp., *Enterobacter* spp., *Staphylococcus aureus*, fecal *E.coli, Pseudomonas aeroginosa* in vegetables. Salmonella was found only on carrot may be due to the fact it comes in direct contact with soil. Saddik *et al*. [23] reported about the presence of salmonella on vegetables in Egypt.

The young, the old, the pregnant and the immune compromised consumers potentially have a higher risk of bacterial infection than other groups. This factor is important in risk assessment and risk management relating to the consumption of vegetables. A particular concern relates to infection of young children with *E. coli* O157:H7 and the potential for these infections to progress to Hemolytic Uremic Syndrome (HUS) [24]. Older patients may be at increased risk of *Salmonella* infection because of achlorhydria, decreased intestinal motility associated with medications, gastrointestinal diseases prevalent in the elderly and more frequent use of antibiotics [25-27]. Vegetables can become contaminated with pathogenic microorganisms during harvesting through fecal material, human handling, harvesting equipment, transport containers, wild and domestic animals, air, transport vehicles, ice or water [28]. The majority of bacteria found on the surface of plants is usually Gram-negative and belong either to the *Pseudomonas* group or to the *Enterobacteriaceae* [22]. Many of these organisms are normally non-pathogenic for humans.

Antimicrobial Resistance: Table 3 presents the distribution of isolates resistant to specific antibiotic. Isolates resistant to individual antibiotic Erythromycin, Gentamycin, Ampicillin, Cephalexin or Streptomycin were found more than 78%. 96.07% (49) of isolates were resistant to Ampicillin. The antibiotic to which maximum isolates found sensitive were Ciprofloxacin and only 3.92% were resistant to it. However, 27.45% isolates were resistant to Chloromphenicol. 17 different resistance patterns were observed when analyzed with the number of antibiotics (Table 4). Only one isolates (*Pseudomonas* spp.) were resistant to all antibiotics tested and eight isolates were resistant to six antibiotics. Highest 49.01% (25) of isolates showed resistance with any five antibiotics and 84% (21) of them were resistant to Erythromycin, Gentamycin, Ampicillin, Cephalexin, Streptomycin. 3.92%, 9.88% and 17.64% of the isolates were resistant to any 2, 3 and 4 antibiotics respectively. Such antimicrobial resistance pattern clearly indicates that isolated bacteria were more resistant to easily available and most frequently used antibiotics.

A high percentage (98.06%) of the isolates was multiple drugs resistant. The emergence of drug resistance is one of the most serious health problems in developing countries, particularly in Bangladesh. This happens, for instance, when antibiotics are misused or overused [29]. Frequent use of antibiotics in medicine and in food of animal origin production has resulted in an increase in the prevalence of bacterial strains resistant to these antimicrobial agents [30-32]. The low affectivity of antibiotics results in infections that are more difficult to treat [33]. In the medical community, the need for prudent use of antibiotics is accepted worldwide. Furthermore, the European Union has banned the use of several antibiotics as growth promoters (avoparcin, bacitracin, spiramycin, tylosin and virginiamycin) in the animal industry and there are proposals to withdraw more antibiotics [34]. In contrast, in the United States, antimicrobial agents are used widely as food additives to improve growth and feed conversion in many types of

Table 3: Distribution of antibiotic resistant isolates

	Antibiotic						
	Erythromycin	Gentamycin	Ampicillin	Ciprofloxacin	Cephalexin	Chloromphenicol	Streptomycin
Pseudomonas spp. *(7)*	7	6	7	1	7	3	6
Vibrio spp. *(10)*	8	8	10	0	10	4	9
Lactobacillus spp. *(9)*	4	8	8	0	7	1	6
Klebsiella spp. *(6)*	5	4	6	0	6	4	5
Enterobacter spp. *(7)*	7	5	7	1	7	1	5
E.coli spp. *(4)*	3	4	4	0	3	1	4
Citrobactor spp. *(3)*	3	3	3	0	3	0	3
Staphylococcus spp. *(2)*	1	2	2	0	3	0	3
Salmonella spp. *(1)*	1	1	1	0	1	0	1
Serratia spp. *(1)*	1	1	1	0	1	1	0
% isolates	78.43	84.31	96.07	3.92	94.11	27.45	82.35

Table 4: Antibiotic resistance pattern of isolates from Karnafully Market

Antibiotic	Isolates (51)
Erythromycin	*Lactobacillus* spp.(1)
Ampicilli, Cephalexin	*Klebsiella* spp. (1)
Cephalexin, Streptomycin	*Staphylococcus* spp. (1)
Erythromycin, Ampicilli, Cephalexin	*Enterobactor* spp. (1)
Gentamycin, Ampicilli, Cephalexin	*Enterobactor* spp. (2), *E.coli* (1)
Gentamycin, Ampicilli, Streptomycin	*E.coli* (1)
Erythromycin, Gentamycin, Ampicillin, Cephalexin	*Vibrio* spp. (1)
Erythromycin, Ampicillin, Cephalexin, Chloromphenicol	*Pseudomonas* spp. (1)
Erythromycin, Ampicillin, Cephalexin, Streptomycin	*Vibrio spp. (* 1) *Enterobactor* spp. (1)
Gentamycin, Ampicillin, Cephalexin, Streptomycin	*Vibrio* spp. (1) *Lactobacillus* spp. (3) *Staphylococcus* spp. (1)
Erythromycin, Gentamycin, Ampicillin, Cephalexin, Chloromphenicol	*Enterobactor* spp. (1), *Serratia* spp. (1)
Erythromycin, Gentamycin, Ampicillin, Cephalexin, Streptomycin	*Vibrio* spp. (4), *Pseudomonas* spp. (4), *Lacto bacillus* spp. (2), *Enterobactor* spp. (3), *Klebsiella* spp.(1), *E.coli* (2), *Staphylococcus* spp.(1), *Citrobactor* spp.(3), *Salmonella* spp.(1)
Erythromycin, Ampicillin, Cephalexin, Chloromphenicol, Streptomycin	*Vibrio* spp (1)
Gentamycin, Ampicillin, Cephalexin, Chloromphenicol, Streptomycin	*Vibrio* spp (1)
Erythromycin, Gentamycin, Ampicillin, Cephalexin, Chloromphenicol, Streptomycin	*Vibrio* spp. (1), *Pseudomonas* spp. (1), *Lacto bacillus* spp. (1), *Klebsiella spp.* (3), *E.coli* (1)
Erythromycin, Gentamycin, Ampicillin, Ciprofloxacin, Cephalexin, Streptomycin	*Enterobactor* spp. (1)
Erythromycin, Gentamycin, Ampicillin, Ciprofloxacin, Cephalexin, Chloromphenicol, Streptomycin	*Pseudomonas* spp. (1)

animal operations, including poultry, swine and cattle operations. As a result, antibiotic resistance in the bacterial communities in the intestinal tracts of domestic animals has become common [35-37]. In that case the natural strain disappears and is replaced by the drug resistant strain. Also an efficient treatment of the natural strain may give an edge to the drug resistant strain. These pathogens are responsible for potentially severe infections in the community and have a great capacity for acquisition of resistance to antibacterial agents [38].

CONCLUSION

The study demonstrated the occurrence of multiple antibiotic resistances among bacterial isolates on salad vegetables sold on markets in Chittagong, Bangladesh. Thus, intensive surveillance of isolates to detect emerging antimicrobial resistance phenotypes especially in the developing world is needed. As preparation of salads does not require further heat treatment, it is important to thoroughly wash vegetables and dip them in food grade antibacterial chemicals for a good time to eliminate pathogens and significantly reduce the microbial load.

ACKNOWLEDGEMENT

We thank Mr. Suman Das, Scientific Officer, Bangladesh Council of Scientific and Industrial Research (BCSIR), for his assistance. We would like to thank BCSIR for having supported the project entitled “Microbiological quality of raw and processed fruits and vegetables of Chittagong city”.

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