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Prevalence of Pathogenic Bacteria Isolated from Two Selected Salad Vegetables and Antibiogram Profile of *Klebsiella* spp.

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Abstract

The study was conducted to examine microbiological quality of raw salad vegetables of Jessore city of Bangladesh. Fourteen samples of two types of vegetables which are commonly used for salad eg. Tomato (*Lycopersicum esculentum*) and Cucumber (*Cucumis sativus*) were collected from different local markets of Jessore City and their bacteriological attributes were investigated using routine methods. All the vegetables were highly contaminated with coliforms and fecal coliforms. Range of Total Viable Count were $11x10^6$ CFU/g to $20x10^7$ CFU/g for tomato and $12x10^5$ CFU/g to $24x10^7$ CFU/g for cucumber, Total Coliform Count were $11x10^4$ CFU/g to 2 $x10^6$ CFU/g for tomato and $10.2x10^3$ CFU/g to $60x10^4$ CFU/g for cucumber and Total Staphyllococcal Count were $11x10^4$ CFU/g to $25x10^6$ CFU/g for tomato and $10x10^4$ CFU/g to $50x10^6$ CFU/g for cucumber. Total coliforms were identified as *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and *Shigella* spp. Antibiogram was conducted with twelve different antibiotics for some randomly selected pure colonies of *Klebsiella* spp. Multidrug resistance was observed in 90% isolates with resistance from two to six antibiotics. Hygienic practices should be followed in handling the vegetables in local markets and vegetables might have an important role as a source of antibiotic resistant bacteria.

Keywords: Salad vegetables, Coliforms, Microbiological quality, Antibiotic resistance, Multidrug resistance, Jessore city

1. Introduction

Salad is a food condiment and serves a major part of our food habit. Usually raw vegetables are used as salad items. Matter of facts, these raw vegetables harbor many pathogenic microorganisms. Many researchers claimed that vegetables could be associated with outbreaks of foodborne diseases. The outbreaks can differ in size from a few persons to many thousands (Halablab *et al*., 2011). Common pathogens found in vegetable (salad) include; *Staphylococcus aureus*, *Enterobacter* spp., *Klebsiella* spp., *Escherichia coli*, *Salmonella typhi*, *Serratia* spp., *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Aeromonas hydrophila* and *Shigella sonnei* (Poorna and Randhir, 2001). Contamination of vegetables may occur at all stages during pre and post-harvest techniques (Halablab *et al*., 2011). Use of untreated waste water and manure as fertilizers for the production of vegetables is a major contributing factor to contamination (Olayemi, 2007). Other possible sources of microorganisms include soil, feces (human and animal origin), animals (including insects and

birds), handling of the product, harvesting and processing equipment and transport (Johannessen *et al*., 2002). In developing countries, foodborne illnesses caused by contaminated vegetables are frequent and in some areas they cause a large proportion of illness. However, due to lack of foodborne disease investigation and surveillance, most outbreaks go unrevealed and the scientific literature reports only on very few outbreaks.

In recent decades, antimicrobial resistance and reduced sensitivity in pathogenic bacteria have become a major public health problem in many countries (Rabbi *et al*., 2011). Many studies have reported the presence of multidrug resistant bacteria in different types of food (Nipa *et al.*, 2011; Osibote *et al*., 2014). Drug resistance is spreading day by day mainly due to the overuse of antibiotics, incomplete and under use of medications and widespread practice of feeding livestock with low levels of antibiotics to promote growth (Sultana *et al*., 2014). In this case, raw salad vegetables could serve as vehicles of antimicrobial resistance to pathogenic bacteria from environments to humans and from one place to another. Considering these facts, the current study was undertaken to assess the microbiological quality of two vegetables commonly used as salad from different local markets of Jessore city by detecting the overall microbial load, identification of the pathogenic microorganisms and evaluation of the antimicrobial susceptibility test of isolated specific pathogen.

2. Materials and Methods

2.1. Collection of samples

A total of fourteen samples of tomato and cucumber were collected from different randomly selected local markets of Jessore city. The samples were collected during the period of January-June, 2014. All samples were collected in sterile polythene bag in an insulated box with ice to maintain a temperature ranging from 4 to 6 °C.

2.2. Sample processing

From each sample, 20 g was aseptically weighed and ground to paste using mortar and pestle and later transferred into 100 ml conical flasks containing 80 ml sterile saline water under laminar air flow to prepare stock solution. Sterile serial dilution was made upto 10^{-6} using sterile distilled water as diluents.

2.3. Isolation and Enumeration of total bacteria

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), MacConkey agar for total coliform count (TCC) and Mannitol salt agar (MSA) for total staphylococcal count (TSC). For bacterial enumeration, spread plate method was used to determine the number of colony forming units (CFU/g).

2.4. Identification of coliforms

For presumptive identification of coliform bacteria isolates were randomly selected for major biochemical tests such as Triple Sugar Iron, Motility Indole Urease, Methyl Red, Voges-Proskaur, and Citrate Utilization test. The tests were performed using conventional methods. Isolates were then identified according to Bergey's manual of determinative bacteriology (Krieg and Holt, 1984).

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility of thirty isolates of *Klebsiella* spp. were performed by the disk diffusion technique using guidelines established by Bauer *et al*. (1966). A total of 12 antibiotic discs (Oxoid Ltd., Basingstoke, Hampshire, UK) comprising of amoxycillin (10µg), ceftriaxone (30 µg), imipenem (10 µg), ciprofloxacin (5µg), streptomycin (10µg), tetracycline (30µg), gentamicin (10µg), nalidixic acid (30 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (25µg), norfloxacin (10µg), and erythromycin (15µg) were used. Within 15 min of the application of the discs, the plates were inverted and incubated at 37°C. After incubation of 16-18 hours, the plates were examined, and the diameters of the zone of

inhibition were measured. The raw data were interpreted based on the available CLSI (Clinical and Laboratory Standards Institute) data and zone diameter interpretive standards.

3. Results and Discussion

3.1. Colony morphology, phenotypic and biochemical traits of the isolates

After 24 hours of incubation, typical pink, circular, convex colonies on MacConkey agar and light yellow colonies on Mannitol salt agar were primarily considered as coliforms and *Staphylococcus* spp., respectively. Isolates from MacConkey were observed as Gram negative, single, short rods which represent the typical properties of coliforms and isolates from MSA were Gram positive in a cluster arrangement which were typical for *Staphylococcus* spp. Based on the biochemical characteristics, isolates were confirmed as *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Shigella* spp. and *Staphylococcus* spp. (Table 1).

3.2. Bacterial load (CFU/g) of fresh salad vegetables

Total bacteria isolated from tomato and cucumber are listed in Table 2. The highest total viable count for tomato was $20x10^7$ CFU/g while the lowest was $11x10^6$ CFU/g. On the other hand, the highest total viable count for cucumber was $24x10^7$ CFU/g and the lowest was $12x10^5$ CFU/g. Unlike tomatoes, cucumbers are rarely contaminated by soil pathogens as they do not come in contact with the soil. So, contamination with pathogens could be due to poor hygienic practices by the handlers and sellers. The present results support the findings of Adebayo-Tayo *et al*. (2012) and Adeshina *et al*. (2012) who found that the total bacterial count in vegetable salads ranged from 1.28 x 10^6 to 3.20 x 10^6 CFU/g. Nawas *et al*. (2012) counted 9.0×10^4 CFU/g to 3.8×10^5 CFU/g total viable count from tomato and 5.5×10^4 CFU/g to 1.9×10^6 CFU/g from cucumber. These values are were comparatively lower than those of the present study.Results of our study partially supports the findings of Uzeh *et al*. (2009) who found 5.9×10^6 CFU/g from open markets and 2.6×10^4 CFU/g from fast food outlets.

Table 1. Results of biochemical tests of the bacterial isolates isolated from salad vegetables

Isolate No.	TSI test				CU test	MIU test					Probable organisms
	Butt	Slant	H_2S	Gas		Motility	Indole	Urease	MR	VP	
1,2,5,6,7,8,10,12,15, 17,18,19,22,23,25,27, 28,30,33,36,37,40,42, 43,45,46,52,55,56,57, 58,59,60.	A	A	-	+	+	-	-	+	+	-	*Klebsiella* spp.
3,11,21,24,26,39,47, 48.	A	A	-	+	+	+	-	-	-	+	*Enterobacter* spp.
14,29,41,44,49,50,51.	A	K	-	-	-	-	-	-	+	-	*Shigella* spp.
9,16,31,34,35.	A	K	-	+	+	+	-	+	+	-	*Citrobacte* spp.
14,29,41,44,49,50,51.	A	K	-	+	-	+	+	-	+	-	*E. coli*

In the present study, TVC were found higher compared to other studies, which might have resulted from relatively poor hygienic condition. However, our findings study differs from the findings of Osamwonyi *et al*. (2013), who isolated 1.46×10^4 to 2.80×10^4 CFU/g from vegetable salad and the microbial load was indicative of the fact that the microenvironments within these salads provided favorable conditions for the growth and proliferation of diverse groups of bacteria.

The coliform count was also high in all samples (Table 2). Range of total coliform count of Tomato was $11x10^4$ CFU/g to $27x10^6$ CFU/g and $10.2x10^3$ CFU/g to $60x10^4$ CFU/g for cucumber. Higher prevalence of coliform bacteria was reported by many workers. In Turkey, Hasan *et al*. (2006) showed the presence of *E. coli* (1-3.8 CFU/g) in raw eaten vegetables. In India, the salad vegetables were contaminated by coliforms (Rajvanshi, 2010). The findings of the present study is strongly supported by Adebayo-Tayo *et al*. (2012) who found the total coliform count in vegetable salads ranging from 2.35×10^6 to 3.28×10^6 CFU/g. The finding of the present study findings also supports with the findings of Rabbi *et al*. (2011), Osamwonyi *et al*. (2013) and Nipa *et al*. (2011) tested different samples of salad vegetables and observed that the differences between the mean bacterial counts were statistically insignificant ($P>0.01$). The presence of *E. coli* in raw salad vegetables analyzed is a common indicator of fecal contamination. *E. coli* are part of the normal flora of the human gastrointestinal tract. Some strains of *E. coli* have been related to diahorrea, gastro-enteritis and urinary tract infections (Hassan *et al*., 2006). The samples were also found to contain gram negative bacteria like, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and *Shigella* spp. which are potential pathogens and thus pose a serious threat to public health. *Klebsiella* spp. is second only to *E. coli* as a human urinary tract pathogen. It is familiar to the environment and can be cultured from soil, water and vegetables when consumed raw as in salads. The isolation of coliforms from salad vegetables was not unexpected since water used to irrigate the vegetable crops and the manure used as fertilizer are reported to contain coliforms and other enteric bacteria (Gagliardi and Karns, 2000).

Table 2. Bacterial load (CFU/g) of fresh salad vegetables

Sample no.	Types of Salad Vegetables.	Total Viable Count (TVC)	Total Coliform Count (TCC)	Total Staphyllococcal Count (TSC)
1.	Tomato	$10.2x10^6$	$12x10^4$	$50x10^4$
2.	Tomato	11×10^6	10×10^4	12×10^4
3.	Tomato	20×10^6	27×10^6	15×10^6
4.	Tomato	21×10^6	17×10^5	25×10^6
5.	Tomato	20×10^7	26×10^5	17×10^5
6.	Tomato	17×10^6	11×10^4	12×10^4
7.	Tomato	13×10^6	10×10^5	11×10^4
8.	Cucumber	26×10^5	17×10^4	15×10^6
9.	Cucumber	12×10^5	10.2×10^3	10×10^4
10.	Cucumber	25×10^5	10×10^4	25×10^4
11.	Cucumber	80×10^5	17×10^4	10×10^5
12.	Cucumber	25×10^5	30×10^4	50×10^6
13.	Cucumber	40×10^5	50×10^4	50×10^6
14.	Cucumber	24×10^7	60×10^4	11×10^4

Table 3. Antibiotic sensitivity of isolated *Klebsiella* spp. from salad vegetables

Klebsiella spp.(Isolate no.)	Zones of inhibition (mm)											
	G-I (Cell wall synthesis inhibitor)			G-II (Protein synthesis inhibitor)					G-III (Nucleic acid synthesis inhibitor)			
	AML	CRO	IPM	CIP	S	TE	CN	E	NA	C	SXT	NOR
1.	16	32	21	34	13	17	16	R	22	22	21	32
5.	16	30	21	35	13	15	17	R	21	24	21	35
6.	R	25	20	31	13	16	17	R	20	20	22	30
10.	R	R	21	32	14	16	17	R	20	21	25	32
12.	R	29	24	28	15	17	18	R	20	23	21	25
15.	R	27	22	27	16	18	17	R	18	24	21	25
17.	12	R	22	22	17	16	20	17	15	R	20	17
18.	17	28	21	26	15	17	16	R	R	17	25	13
19.	17	28	22	33	15	17	17	R	R	22	22	31
22.	16	R	30	23	17	18	20	R	16	25	23	18
23.	R	R	23	22	16	18	20	R	17	26	21	21
25.	29	25	23	25	16	20	18	R	15	23	12	21
27.	R	R	19	27	15	R	17	15	16	18	21	25
28.	R	R	21	26	15	R	16	R	20	19	21	25
30.	R	R	19	25	R	R	15	R	18	18	17	25
33.	R	R	21	30	13	R	20	R	R	13	R	31
36.	R	R	35	26	16	21	20	R	15	R	20	26
37.	15	R	26	27	16	R	20	R	22	15	R	25
40.	R	R	28	23	20	20	20	R	16	22	R	21
42.	12	R	20	30	12	16	20	R	18	21	20	20
43.	15	R	30	24	16	24	21	R	16	22	25	25
45.	R	R	19	30	15	16	18	R	18	20	16	30
46.	R	R	24	21	16	R	19	R	R	20	R	20
52.	R	R	29	23	16	16	18	R	16	20	19	17
55.	12	R	30	26	23	19	26	26	17	22	R	24
56.	R	R	21	22	17	R	21	19	R	20	R	21
57.	14	R	32	25	20	15	23	R	20	22	R	23
58.	R	R	18	23	16	17	18	R	17	20	20	21
59.	R	R	28	25	17	17	22	R	15	20	R	24
60.	R	R	30	22	15	R	18	R	15	20	16	25

AML=Amoxycillin (10µg), CRO=Ceftriaxone (30 µg), IPM=Imipenem (10 µg), CIP=Ciprofloxacin (5µg), S=Streptomycin (10µg), TE=Tetracycline (30µg), CN=Gentamicin (10µg), NA=Nalidixic acid (30 µg), C=Chloramphenicol (30 µg), SXT=Trimethoprim-sulfamethoxazole (25µg), NOR=Norfloxacin (10µg), E=Erythromycin (15µg), R=Resistant.

In this study, the total staphylococcal count ranged from $10x10^4$ CFU/g to $50x10^6$ CFU/g (Table 2). The highest staphylococcal count may results from poor hygienic condition of cultivated areas or contamination from respiratory system and body surface of food handlers or by infected workers. *Staphylococcus aureus* has been reported to remain the most prominent aetiological agent of pyogenic infections and that staphylococcal infection leads

to a worsening of some already existing superficial infections (Adegoke and Komolafe, 2009). This findings are in agreement with that report Rabbi *et al*. (2011) who found all food samples including vegetables were contaminated by *Staphylococcus* spp. The presence of *Staphyllococcus* spp. (2×10^5 to 5.95×10^7 CFU/g) has been reported to contaminate some salad vegetables such as carrots, cucumber, tomato and lettuce (Rahman and Noor, 2012).

3.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility of 30 isolates of *Klebsiella* spp. from salad vegetables are presented in Table 3. Figure 1 summarizes the antimicrobial resistance in *Klebsiella* spp. isolated from salad vegetables.

In this study, the highest (86.66%) resistance among *Klebsiella* spp. was observed to erythromycin. In case of Ceftriaxone and Amoxycillin, 73.33 and 60% isolates were detected as resistant, respectively. 26.66% *Klebsiella* spp. were observed to be resistant and 56.66% isolates were intermediate to tetracycline. It was observed that, 76.66% isolates were streptomycin-intermediate and 33.33% isolates were gentamicin-intermediate. On the other hand, imipenem, ciprofloxacin and norfloxacin were found to be completely sensitive against the *Klebsiella* isolates tested. The lowest resistance value was figured out as 6.66% for chloramphenicol, 16.66% for nalidixic acid and 26.66% for trimethoprim-sulfamethoxazole.

Antibiotic resistance patterns and multidrug resistance pattern for *Klebsiella* species from salad vegetables are presented in Table 4. Among 30 isolates of the total *Klebsiella* spp., 90% isolates displayed multidrug resistance (MDR). Highest number of resistant antibiotics (AML-CRO-TE-E-NA-SXT) was observed to 6.66% isolates. Multidrug resistance was observed in 98.06% isolates with resistance to two to seven antibiotics (Nipa *et al.,* 2011).

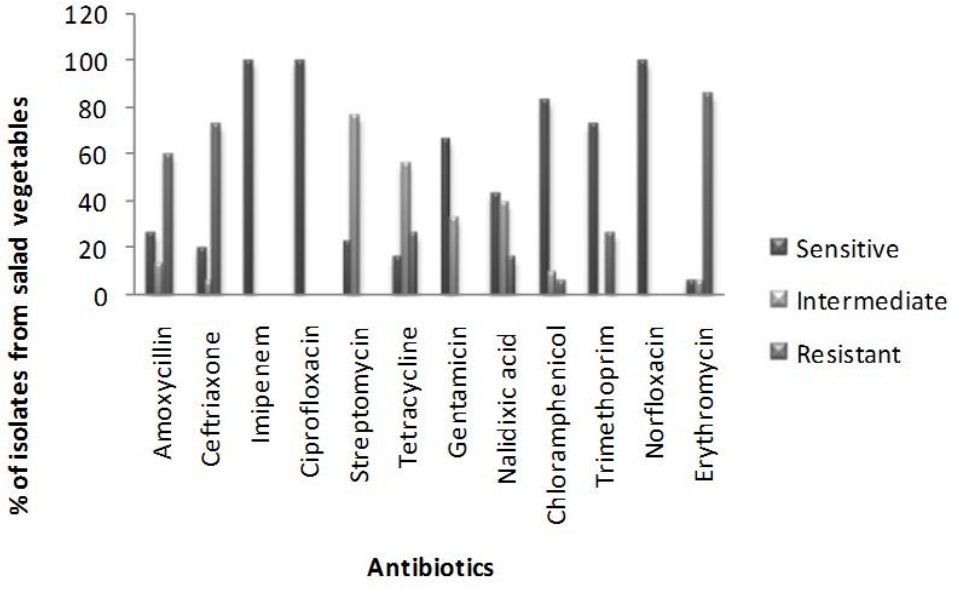

Figure 1: Antibiotic sensitivity pattern of *Klebsiella* spp. isolated from salad vegetables

Table 4. Antibiotic resistance profiles for *Klebsiella* species isolated from salad vegetable samples

No. of resistant antibiotics	No. of isolates	Antimicrobial resistance profile	%	Resistance classification
6	2	AML-CRO-TE-E-NA-SXT	6.66	MDR
5	1	AML-CRO-TE-NA-SXT	3.33	MDR
4	5	AML-CRO-E-TE/SXT	16.66	MDR
3	9	AML-CRO-E/TE	30	MDR
2	10	AML-E, CRO-C/E/SXT, E-NA	33.33	MDR
1	3	E	10	NMDR

*MDR - Multidrug resistance, NMDR - Non multidrug resistance.

Oluyege *et al*. (2009) showed 85% of the resistant isolates were multidrug resistant where highest (89.1%) resistance was to the amoxicillin. Adeshina *et al*. (2012) found some sensitive isolates to amoxicillin, tetracycline and cotrimoxazole; conversely, some were also resistant to gentamicin, nalidixic acid as well as ofloxacin. The highest number of pathogenic bacteria isolated from ready-to-eat fresh vegetables were resistant to penicillin and vancomycin where, the isolates were sensitive to streptomycin and gentamycin (Ali *et al*., 2011). In general, bacteria can develop resistance for antimicrobial agents, which share similar genetic structures (Angela *et al*., 2006). Van de Boogard and Stobberingh (2000) reported that due to indiscriminate use of antibiotics, such high incidence of multidrug resistance may apparently be occurred which may ultimately replace the drug sensitive microorganisms from antibiotic saturated environment. Sometimes abuse of antibiotics as well as poor sanitation systems could also play potential roles.

4. Conclusions

The high bacterial load and presence of multidrug resistant organisms in the salad vegetable samples could serve as an indicator for the necessity to promote consciousness about the possible health hazards due to poor handling of these vegetables. There is therefore, the necessary for the regulatory authorizations to ensure that the microbiological standards are established and practiced by the farmers and sellers for handling and distribution of salad vegetables.

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