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Microbial load, antimicrobial sensitivity and plasmid profiles of *Vibrio cholerae* in fruit juice

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ABSTRACT

Vibrio cholerae are the causative agents of cholera, an acute dehydrating diarrhea. The research report was designed for isolation and identification of *Vibrio cholerae* from juices. A total of forty samples of different fruit juices such as orange, papaya, grape, malta, sugarcane juice, lemon juice, aloe vera and isupgul juice were collected from different areas of Dhaka city. The total viable count was varying from 7.73 $x10^4$-7.62 $x10^8$ cfu/ml where the highest count observed in sugarcane juice and lowest count in orange juice. In case of fungal count range was 2.7 $x10^2$ - 4.9 $x10^5$ cfu/ml. Total coliform count was between 11 - >2400/ 100 ml and *E. coli* count between <3 - 120/100 ml. *Vibrio cholerae* was present in 20% of samples where the 75% of *Vibrio cholerae* were detected from sugarcane juice which was also contaminated with high bacterial and fungal load. The occurrence of drug resistance was also determined in the 8 isolated *Vibrio cholerae*. All the isolates were resistant to polymixin B, and sensitive to gentamycin and ciprofloxacin. About 40% isolates were resistant to tetracycline and neomycin and 80% isolates were resistant to amikacin. Plasmid profiling of eight isolates also performed, where most of the strain harbor two plasmids. With all the investigation it is bearing on mind that *Vibrio cholerae* cause severe diarrhoea and occurance of *Vibrio cholerae* in juice may cause a serious health hazards.

Key words: Plasmid, *Vibrio cholera*, coliform, *E. coli*, fruit juice, Bangladesh

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INTRODUCTION

Juice is a liquid naturally contained in fruit or vegetable tissue. Juice is prepared by mechanically squeezing or macerating fresh fruits or vegetables without the application of heat or solvents. Many commercial juices are filtrated to remove fiber or pulp with addition of preservatives. Juice may be marketed in concentrated form, sometimes frozen, requiring the user to add water to reconstitute the liquid back to its "original state". However, concentrates generally have a noticeably different taste than their comparable "fresh-squeezed" versions (Fasoyiro et al., 2005). Freshly extracted juices considered as healthful drink may not always be safe (Kumari, 1995).

Commercial juice contains mainly water, sugar, food color, fruit pulp, and preservatives. Spoilage of juice may occur by the mechanical fracture of fruits; by autolysis of chemical and mineral contents, or by the microbial flora of fruit itself and along with the contaminating microbes which is the main concern to study. Environmental sources of contaminating organisms of juices are carefully considered as these microbes invade the drink preparation during processing, packaging, and handling (Jay, 1987) the major ingredient of juice such as water, sugar, fruit pulp may also carry some microbial contaminants.

Fruits are one of the important parts of human diet and food supplements over the years. Fruits in the

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daily diet have been strongly associated with reduced risk for some forms of cancer, heart disease, stroke, and other chronic diseases (Goldberg, 2003; Hyson, 2002; Prior and Cao, 2000; Tomas-Barberan and Espin, 2001; Wargovich, 2000). Fruits are the reservoir of vitamins (A, B1, B2, C, and D), proteins and minerals such as Ca, Mg, K, Zn, and Fe. Fruit consumption has been reported to be beneficial to health and to contribute to the prevention of degenerative processes, particularly lowering the incidence and mortality rate of cardio and cerebral-vascular diseases. Some components of fruits (phytochemicals) are strong antioxidants and function to modify the metabolic activation and detoxification/disposition of carcinogens, or even influence processes that alter the course of the tumor cell (Wargovich, 2000).

Fresh fruit and vegetables juice are recognized as an emerging cause of food borne illness (Parish et al., 1997). A major contributing factor in these raw agricultural commodities are contaminated by animal or human waste and consumption without processing steps that may killed or associated bacterial pathogens (Sandeep et al., 2001)

Fresh squeezed or pressed juices made from fruits and vegetables have a very high consumer preference both in terms of taste and health effects throughout the world, (Sandeep et al., 2001) however, in the current past, such juices, especially unpasteurized juices have been shown to be a potential source of bacterial pathogens notably, *Salmonella*, *E. coli* O 157:H7, (Ryu et al., 1998, Uljas et al., 1998, Zhuang et al., 1995). Disease outbreaks from enteropathogenic bacteria, such as *Salmonella*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Staphylococcus aureus* are common cases of food borne infection throughout the world (Chomvarin et al., 1993).

Vibrio cholerae serogroups O1 and O139 are the causes of epidemic cholera. This is predominantly a waterborne infection and high numbers of organisms are necessary to cause infection. Nevertheless, a significant number of fruit and vegetable borne outbreaks have been reported (Wachsmuth et al., 1994; Faruque et al., 1998; Anon, 2000). The characteristic profuse watery diarrhoea of cholera is due mainly to the effects of a heat labile enterotoxin elaborated by the organism in the intestine. Cholera is of rapid onset and can lead to severe dehydration and death within hours if left untreated. The illness usually lasts for 3-7 days (Anon, 2001).

The critical role of water in transmission of cholera has been recognized for more than a century. The classic demonstration of this event came in 1854, during the second cholera pandemic. In one illustrative study in Bangladesh, 44% of surface water sources in communities with cholera were culture positive for the organism; not unexpectedly, there was a significantly increased risk of infection associated with use of water from culture positive sources for cooking, bathing, or washing (but, interestingly, not with drinking) (Kaper et al., 1995).

In Bangladesh, especially in the metropolitan and other big cities a huge section of the population of all income and age groups consume fresh pressed and squeezed juices, most of these juices are sold by local market vendors. In Dhaka, the capital city of Bangladesh, approximately 94% of the population, including tourists comprising of all age and income groups prefer and consume freshly squeezed or pressed juices particularly, lemon, mango, orange, malta, papaya, apple, sugarcane etc. The present study was undertaken to investigate the microbial contamination in the commercially available juice in Dhaka city and determine microbial the load for risk assessment.

MATERIALS AND METHOD

Sample Collection

All the juice samples were collected from different area of Dhaka city, such as: New Market area, Science Laboratory, Farmgate, Gulistan, Motizhil, Saedabaad, Jatrabari, Sanirakhra etc. All samples were collected in sterile containers held at 4^0C and analyzed within 2 hours from procurement, 5ml portions of juice samples were removed aseptically for pH measurements.

A total of forty samples were collected which were categorized into five groups (Table 1)

Table 1
Collection of different types of juice from Dhaka city.

Groups	Number of samples	Juice category
Group-1	5	Packed juice of different commercial band.
Group-2	10	Freshly prepared vendor fruit juice collected from fast food shop.
Group-3	10	Freshly prepared Isubgul and Aloe Vera juice collected from road side.
Group-4	5	Lemon juice collected from road side.
Group-5	10	Freshly prepared sugarcane juice collected from road side.

Microbiological analysis of fruit juices

The samples were serially diluted (10 fold dilution) up to 10^6 by using Ringer solution. One ml from each diluted tube was transferred into sterile Petri dish. Then Plate Count Agar (PCA) media was poured into Petri dish (by means of pour plate technique) and plates were incubated into 37^0C for 24 hours for total bacterial count. Total fungal count was done in Potato Dextrose Agar (PDA) media by 0.2ml of samples from diluted tubes and spread by sterile glass rod (by means of spread plate technique) and incubated for 3-5 days at 25^0C- 30^0C.

Coliform count was done by MPN method (9 tubes method) in Lauryl Sulfate Broth (LST broth) & Brilliant Green 2% bile broth media. Gas production in the dirham tube means the positive test. Numbers of gas forming tubes were then recorded and comparing with the standard chart (APHA, 1971). Loopful inoculums from gas forming tubes of Brilliant Green 2% bile broth is streaked into Eosin Methylene Blue (EMB) agar plate. After 24 hours of incubation at 37^0C plates were observed. Colonies with green metallic sheen confirm the presence of indicator organism *E. coli*.

Isolation and Identification of *Vibrio cholera*

Juice samples were transferred to Alkaline Peptone Water (APW) for 6-8 hours at 37^0C. *Vibrio cholerae* required 6-8 hours incubation where other *Vibrio* species requires 18-24 hours incubation. Following enrichment of samples into alkaline peptone water samples were inoculated into TCBS and *Vibrio* Agar plates and further incubated for 24 hours. After incubation typical colonies having the typical characteristics on TCBS and Vibrio Agar media was presumptively considered as *Vibrio cholerae* and the characteristics colony at TCBS agar and Vibrio agar is selected and then identified biochemically, serologically.

Biochemical identification

Several biochemical test were performed according to Microbiology a Laboratory Manual (4^{th} edition)" and Laboratory Methods in Food Microbiology, to identify the bacteria of interest such as oxidase test, Triple sugar iron agar test, Motility, Indole and H_2S production, MR-VP test, Citrate utilization test, Carbohydrate fermentation test, Salt tolerance test, Gelatinase test and growth in Gelatin Salt Agar, string test (CDC. 1994; Kay et al., 1994) (Table 3).

The eight isolates inoculated into blood agar plates to employ the hemolytic pattern, where a zone of inhibition around the inoculation was observed (Table 3) All the strains with typical biochemical behavior were subjected to serotyping for "O" antigen and "O139" antigen using O1 polyvalent antisera and polyvalent O139 antisera (Table 4) and Susceptibility of the isolates to different antimicrobial agents was determined '*in vitro'* by 'Kirby-Bauer Method (Bauer et al., 1966)

Agarose gel electrophoresis

Bacterial cells were enriched in Trypticase Soy Broth (TSB) and taken into eppendrof tube and passed through centrifugation. Then lysis buffer was added and tubes were incubated in water bath for 1 hour. Phenol:chloroform:isoamyl alcohol was added and vortex and centrifuged at 13000 rpm for 15. Plasmid was loaded in 1% agarose gel with tracking dye. 1% agarose gel was prepared and electrode was connected to gel tank to power pack. Gel was run at a constant voltage of 100V for 2.5-3 hours. Plasmid band was visualized because of tracking dye.

RESULTS AND DISCUSSION

Among the five groups of sample, Group 1 samples yield no viable count or fungal count. The reason may be the packed fruit juice carries high amount of preservatives. The most viable count was seen in sugarcane juice (7.62×10^8) and the lowest count observed in group 2 orange juice sample (7.73×10^4). The highest fungal count was observed in group 5 sugarcane sample (3.21×10^5) and lowest count was in group-4 lemon juices (1.52×10^2). There were three samples where highest number (>2400) of coliform was detected. The highest number (120) of *E. coli* was detected in group-2 papaya juice.

Antibiotic susceptibility pattern

All the strains were tested for their antibiotic susceptibility against 6 antibiotic discs belonging of different groups. Sensitivity to Ciprofloxacin and Gentamycin was 100% whereas resistance against Polymixin B antibiotic disc was observed 100%. In case of Tetracycline and Neomycin About 40% isolates were resistant and in case of Amikacin, 25% isolates were sensitive and the rest of 75% isolates were resistant.

Extensive biochemical tests were performed in order to measure the viability of biochemical behavior among the colonies. Detailed biochemical study assumed that the colonies had the biochemical behavior typical of *Vibrio cholerae*.

Agarose gel electrophoresis

The plasmids DNA was moves through the gel yielding a band into the gel which was visualize because of tracking dye was mixed with the plasmid suspension. The size of plasmid was measured by comparing the band with a ladder (V517) band. Ladder band was positioned in 35 MD (Mega Dalton), 30 MD, 25 MD, 20MD and lower. Most of the plasmid band was observed in between 25MD-35MD.

About 80% of the isolates yield two plasmid, 25% yield more than two plasmid. The size of plasmid band was between 25MD–35MD. The length of plasmid was detected by converting the size by multiplying as 1MD=1.56kbp (kilo base pair). The length of plasmid was varying from 3.9kbp to 4.9kbp.

Cholera transmission was associated with consumption of street-vended juice in Peru, Thailand (Swerdlow et al., 1992; Ries et al., 1992), Ecuador (Weber et al., 1994) and Guatemala (Koo et al., 1996). In West, Southern, and East Africa, water source contamination was the second most common risk factor reported, representing 32%, 30%, and 24% of the total, respectively. In Central Africa, water source contamination was the most common, accounting for 30% of the reported risk factors (Griffith et al., 2006). Thus the presence of these organisms in water and fruit juices is dangerous for human consumption (Salle, 2000).

In case of group 1 sample which were the packed juice, had no microbial hazards. The main reason might be a high amount of preservatives were applied into juice, whereas the other groups of samples were examined had a large number of microbial contaminations. The total viable count was a high rate along with the total fungal count. Presence of coliform was also detected whereas the presence of *E. coli* was also detected. Presence of the indicator organisms (*E. coli*) indicated the presence of other pathogen in the fruit juice samples.

In the present study the isolated *Vibrio cholerae* was characterized. A total of eight (8) isolates were considered in this study randomly based on their primary identification by biochemical identification tests which were then further analyzed by serological and molecular biological techniques. Since the isolation and identification of *Vibrio cholerae* is very crucial for the characterization purpose that was aimed at, the colonies having typical cultural characteristics were selected as presumptive *Vibrio cholerae*, which was then subject to biochemical testes for confirmation. However all the strains showed the typical biochemical behavior characteristics of *Vibrio cholerae* as compared to the control strains (01N 16961, El tor strain). All the strains were positive for Indole, VP and Gelatinase production and all isolates were positive for oxidase and catalase test as well. All the strain could tolerate up to 6% of NaCl. In case of carbohydrate fermentation test, positive result was occurred from glucose sucrose and mannose but no fermentation was occurred in case of lactose. The organisms showed motility in SIM media and no H_2S formation was observed.

Table-2
Microbial load in different juice collected from Dhaka city

Groups of sample	Range of total viable count	Range of total fungal count	Range of total coliform count	Range of *E. coli* count
Group 2	7.73×10^4-6.52×10^7	2.7×10^2-2.32×10^4	11->2400	<3-120
Group 3	3.6×10^5-5.29×10^7	2.13×10^2-4.9×10^5	93->2400	3-43
Group 4	5.15×10^6-4.52×10^8	1.52×10^2-4.32×10^4	210-1100	11-21
Group 5	4.62×10^5-7.62×10^8	2.54×10^2-3.21×10^5	210->2400	<3-43

Table-3
Biochemical and carbohydrate fermentation test for identification of *Vibrio cholera*

Biochemical tests		Isolated colony characteristics								
		C	1	2	3	4	5	6	7	8
TSI test	slant	A	A	A	A	A	A	A	A	A
	butt	A	A	A	A	A	A	A	A	A
Oxidase		+	+	+	+	+	+	+	+	+
Catalase		+	+	+	+	+	+	+	+	+
Urease		-	-	-	-	-	-	-	-	-
SIM media	Motility	+	+	+	+	+	+	+	+	+
	Indole	+	+	+	+	+	+	+	+	+
	H_2S	-	-	-	-	-	-	-	-	-
MR-VP medium	MR	+	+	+	+	+	-	+	+	+
	VP	+	+	+	+	-	+	+	+	+
Carbohydrate fermentation test	Sucrose	+	+	+	-	+	+	+	+	+
	Glucose	+	+	+	+	+	+	+	+	+
	Lactose	-	-	-	-	-	-	-	-	-
	Mannose	+	+	-	+	-	+	+	+	+
Salt tolerance test	T1N0	+	+	+	+	+	+	+	+	+
	T1N1	+	+	+	+	+	+	+	+	+
	T1N3	+	+	+	+	+	+	+	+	+
	T1N6	+	+	+	+	-	+	+	+	+
	T1N8	-	-	-	-	-	-	-	-	-
	T1N10	-	-	-	-	-	-	-	-	-
Growth in	Gelatin	+	+	+	+	+	+	+	+	+
	GSA	+	+	+	+	+	+	+	+	+
String Test		+	+	+	+	+	+	+	+	+
Hemolysin test		β	γ	γ	γ	α	γ	β	α	β

Symbols: '+': positive result, '-': negative result. A: acidic, C: control strain,
TSI: Triple sugar iron testing, SIM: Hidrogen sulphide, motility indole test,
MR-VP: methyl red, vogas-proskauer test, T1N0- T1N10: tryptone broth with 0-10% salt concentration, GA: gelatin agar, GSA: gelatin salt agar.

Table-4
Agglutination of the isolates against *Vibrio cholerae* specific polyvalent antisera.

Slide agglutination test		Strain							
		1	2	3	4	5	6	7	8
Polyclonal antisera of	01	-	-	-	-	-	-	-	-
	0139	-	-	-	-	-	-	-	-

Symbols: '+': positive result, '-': negative result

Table-5
Antibiotic susceptibility pattern of *Vibrio cholerae* against various antibiotics

Strain ID	Antibiotic susceptibility					
	TE 30 µg	CIP 5 µg	CN 10 µg	AMC 30 µg	N 10 µg	PB 300 µg
1	S	S	S	R	S	R
2	S	S	S	S	R	R
3	S	S	S	R	S	R
4	R	S	S	R	S	R
5	S	S	S	S	S	R
6	R	S	S	R	R	R
7	S	S	S	R	R	R
8	R	S	S	R	S	R

Symbols: TE: Tetracycline, CIP: Ciprofloxacin, CN: Gentamycin, AMC: Amikacin,N: Neomycin, PB: Polymixin B.
S: sensitive, R: resistant.

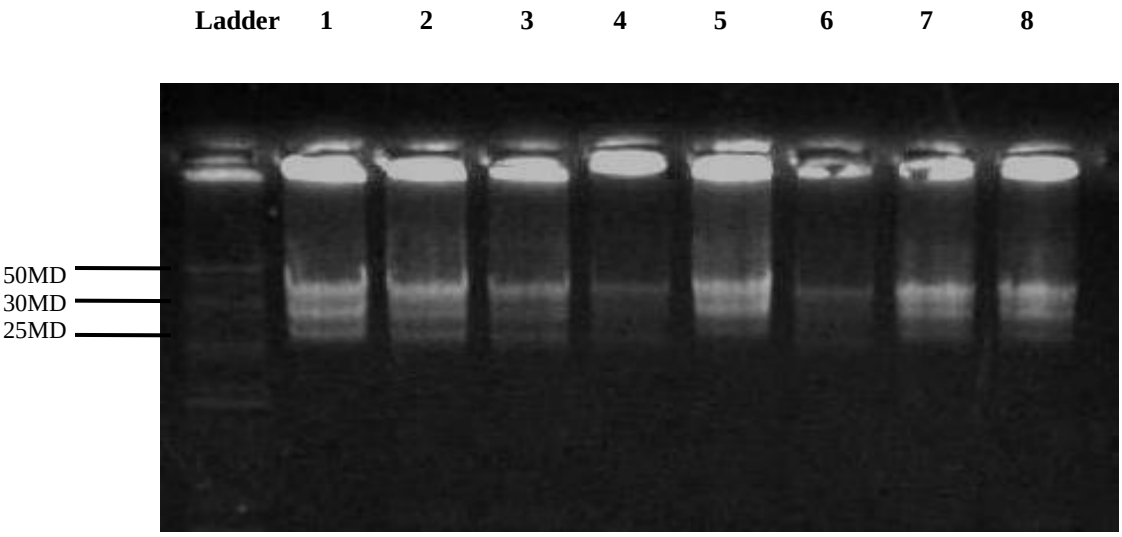

Figure 1
Agarose gel electrophoresis of eight isolates of *Vibrio cholera*. Lane 1-8 are samples

String test is one of the important biochemical testing for identification of *Vibrio choleare*. Not only the *Vibrio cholerae* yield yellow colony into TCBS, but also *Aeromonas* spp. also posses the same colony into TCBS agar media. On the other hand, to differentiate the *Aeromonas* spp from *Vibrio cholerae* may be very critical because the biochemical result of *Aeromonas* spp and *Vibrio cholerae* is relatively same. String test by 0.5% sodium deoxycholate differentiate *Aeromonas* spp from *Vibrio cholerae*.

Antibiotic disc diffusion method demonstrated the sensitivity and resistance against the drug applied. Most of the environmental isolates are now become resistant which was sensitive in previous time, which makes the main health hazards. Mainly the tetracycline, fluoroquinolones were used to treat cholera. But now a day's most of the strain becomes resistant to tetracycline, so that this antibiotic may not show activity against *Vibrio cholerae*. If these criteria of antibiotic resistance develop with time, it may cause a serious health problem. Resistance of antimicrobial agent is acquired by the transmission of drug resistant gene from other isolates. These genes are carried by the plasmid which may transmit from cell to cell. The plasmid carrying the drug resistant gene is called the Resistant (R) plasmid.

Traditional identification system is based on the cultural characteristics and the metabolic characteristics of an organism. Although it has a good potential for the characterization of microorganisms, but still it is not up to the mark. Rather the recent developments in molecular biological techniques are found to be more effective in diagnostic microbiology as well as for research purpose. Extremely sensitive methods based on molecular biology principles are available for detecting pathogens and to demonstrate their toxigenicity.

Several researchers contributed similar type of investigations in different places with different street vended fruit and vegetable juices. Tambekar et al (2009) reported the food borne illness associated with the consumption of road side freshly squeezed fruit juices at public places in Amaravati city, India and samples were also analyzed for the presence of dominant enteric bacterial pathogens were *Escherichia coli* (40%), followed by *Pseudomonas aeruginosa* (25%), *Salmonella* spp (16%), *Proteus* spp (9%), *Staphylococcus aureus* (6%), *Klebsiella* spp (3%) and *Enterobacter* spp (1%). Sandeep et al., (2004) have detected total *Staphylococcus* counts or coliform counts in three samples of carrot juices and Kinnow-mandarin juices obtained from two different areas of the Patiala city. Moushumi et al., (2004) explained the presence of faecal coliforms in fresh squeezed carrot juices and explained the possible entry points of bacterial pathogens in carrot at several points during course in the distribution chain and hence into carrot juice. Overall the results of the present study indicate that, majority of the street vended fresh fruit juices in many parts of the city showed contamination with faecal coliforms.

In Bangladesh vendor juices are prepared in open area. As a result there remains a huge chance of contamination. Most of the contamination occurs by the handlers because of lack of knowledge about hygiene and sanitation. In this study, a number of packaged and vendor fruit juices were handled to examine the microbiological quality and isolate the *Vibrio cholerae* from those fruit juice samples.

Fruit juices are well recognized for their nutritive value, mineral and vitamin content. In many tropical countries they are common man's beverages and are sold at all public places and roadside shops. However in view of their ready consumption, quick methods of cleaning, handling and extraction they could often prove to be a public health threat. There are reports of food borne illness associated with the consumption of fruit juices at several places in India and elsewhere (Parish, 1997; Sandeep et al., 2001). Sources of contamination however vary. Most fruits contain bacterial counts up to 1.0×10^5 CFU/cm2 on their surface (Harrigan 1998). Improper washing of fruits add these bacteria to extracts leading to contamination. In addition, use of unhygienic water for dilution, dressing with ice, prolonged preservation without refrigeration, unhygienic surroundings often with swarming houseflies and fruit flies and airborne dust can also act as sources of contamination.

In a study in Malysis *Vibrio cholerae* in fruit juices 36.7% and the overall, the prevalence of *Vibrio cholerae* in all the drink samples was 28.3% (Ubong, 2011). In another study in Dhaka city total bacterial load, coliforms and staphylococci were observed in juice samples in

the range between 10^2 -10^7 cfu/ml. Fecal coliforms were found in vendor fruit juice samples (102 cfu/ml), while in the industrially packed samples, they were completely absent. Drug resistance among the isolates was found against ampicillin, ciprofloxacin, amoxicillin, erythromycin, chloramphenicol, ceftriaxone, piperaciline, trimethoprime-sulfomethoxazole, nalidixic acid and vancomycin (Rashed et al., 2012). In this study microbial load of fungus, coliform, *E. coli* and *V. cholera* was observed which were found sensitive to ciprofloxacin and gentamycin, amikacin whereas resistance against polymixin B, tetracycline and neomycin. Overall, the study demonstrates that the quality fresh juices were unsatisfactory and hence the products need for the improvement of the hygiene and sanitation practice in preparing the fruit juice.

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