Distribution of *Aeromonas hydrophila* in Salad Vegetables and Treatment of Salad Vegetables

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Introduction

In Bangladesh and in many other developing countries, millions of people die every year due to diarrhoeal diseases. Recently, motile *Aeromonas* was recognized as a potential mediator of food associated gastroenteritis outbreaks. *Aeromonas* spp. have been reported to cause acute and/or chronic gastroenteritis in infants compromised by exposure to organisms from water or other sources followed by antibiotic therapy with a drug to which the colonizing aeromonads are resistant[1]. *Aeromonas* bacteria are increasingly being recognized as pathogens from environmental sources that may cause soft wound infection, septicaemia and meningitisin human beings[2-5]. Most studies involving the ecology of *a. hydrophila* gastroenteritis have concentrated on its transmission in contaminated water supplies [6, 7]. However, Buchanan and Palumbo[8] implicated *Aeromonas* sp. as potential food poisoning agents. *A. hydrophila* is psychotropic and has been associated with spoilage of refrigerated (5°C) animal products, including chicken, beef, pork, lamb, fish, oysters, crab, milk and grocery store products [9-14].

The purpose of this study was toisolate *Aeromonas* spp. from salad vegetables, survival pattern of *A. hydrophila* on salad vegetables at 5°C, nature of contamination, the storage conditionof salad vegetables and effect of temperature treatment on waterborne microor microorganisms.

Materials and methods

Salad vegetables investigated: Lettuce, cucumber and carrot were purchased from a retail grocer from

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local market in Dhaka, Bangladesh. All these vegetables were previously watered with dirty and cloudy water by the grocer. The purchased vegetables were kept at 2-5°C within 30 min of purchase and the experiments were started within 2 h.

Preparation of inocula: Three strains of *A. hydrophila* were taken for this study. Environmental strain k-5 was obtained from the Department of Microbiology, University of Dhaka, strain AG-15415 and AG-16315 were clinical isolates obtained from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). Stock cultures were maintained at -20°c in glycerol broth (15%). Strains were subcultured on Tryptic Soy Agar (TSA, pH 7.2, Difco) at 37°C for 24 h. A single colony was inoculated into Tryptic Soy Broth (TSB, pH 7.2, Dico) and incubated at 37°C for 24 h. The cells were harvested from the TSB by centrifugation (6000 × *g*) and washed twice with phosphatebufered saline (PBS, pH 7.2) and suspended in PBS at a concentration of 10^8 CFU/ml (adjusted spectrophotometrically).

Microbiological assay of vegetables : Microbiological analysis of the vegetables were done according to APHA15 and suspended in sterile PBS. Total count of aerobic and anaerobic organisms were measured by spreading and incubating 100 ml of the suspension onto TSA plate, one at 23°C for 7 days and another at 37°C for 24 h. Organisms were identified by biochemical test and by cultural characteristics (APHA)[15]. Each vegetable was washed with tap-water and sterile water, and microbial count of the vegetables was also recorded.

Procedures for inoculating vegetables : Each vegetable was first washed thrice with sterile PBS then heated at 60°C for 1 h, another set was washed with 70% alcohol and then washed with sterile PBS. Each vegetables were uncut. Each group of vegetables was inoculated by each strain of *Aeromonas* (106 CFU/g) and packed into sterile container and incubated at 5°C and at 25°C.

Procedure for enumeration of *A. hydrophila:* Population of A. hydrophila was measured at different time intervals. Fifty grams of vegetables were ground in sterile PBS and 10-fold serial dilution was made in 1% peptone (pH 7.2) and platedon starch-ampicillin agar according to Palumbo *et al.* 12. Ater incubation at 37°C, starch-ampicillin agar plates were floooded with approximately 5 ml of lugol iodine solution[16] and amylase positive colonies were scored as presumptive Aeromonas

spp[12]. On A hydrophila medium, the colony characteristic was taken as indicator or *A. hydrophila* organisms.

pH measurement: The pH of the primary diluent in which vegetable samples pummeled was measured at every step of microbiological analysis with a digital pH meter.

Temperature treatment of salad vegetables: Each vegetable was treated to various temperature for different time period. After heating, total count and *A. hydrophila* count were taken. Salad vegetables inoculated with *A. hydrophila* (5 ¥ 10^5 CFU/g) were also treated to various temperature for different time period and the microbial count was taken. For every individual analysis, the taste and odor of the salad vegetables were keenly observed by a group of volunteers (20 persons).

Results

Organisms isolated from salad vegetables: All the salad vegetables (carrot, cucumber and lettuce) contained a large number of microorganisms. The total bacterial count ranged from 10^5 to 10^7 CFU/g. Twenty-five samples of each vegetable contained Aeromonas sp (1 ¥ 10^2 to 8 ¥ 10^3 CFU/g). Most of the vegetables contained different types of fecal coliform organisms.The water samples that was used for watering the vegetables contained most of the fecal organisms whose total aerobic count ranged from 10^6 to 10^8 CFU/ml. The counts of Aeromonas spp. and total aerobic bacterial counts observed were summarized in Table-1.

Survival of A. hydrophilaon salad vegetables : One environmental isolate and two clinical isolates of *A. hydrophila* were found were found to grow at 5°C and 25°C at storage condition. At storage, the number of the colony were found to be increased for the first 7 days then it was found to become decreased (Table-II). It was found that both clinical and environmental isolates were able to grow at 5°C. It was also observed that pH of the vegetables changed with time (Table-IIII).

Temperature treatment of salad vegetables: The result of heat treatment of salad vegetables for different time periods summarized in table-IV. At 50°C, the salad vegetables remained more resh, and all the volunteers mentioned positive results, but the microbial load remained higher. At 60°C or acceptable result where there was a slight change in salad taste and the microbial count along with A. hydrophila were much low. At temperature 70 and 80°C, though the total count reduced much more

Table 1. Aeromonas count and total count of organisms on salad vegaytebles

Vegetable	Vegetable (CFU/ml)				Water used for washing the vegetables by salesman (CFU/ml)	
	Just after purchase		After washing with tap-water by salesman			
	Total count (Aearobic+ anaerobic)	Total count (Aearobic+ *Aeromonas*	Total count (Aearobic+ anaerobic)	Total count (Aearobic+ *Aeromonas*	Total count (Aearobic+ anaerobic)	Total count (Aearobic+ *Aeromonas*
Lettuce	2.6×10^6 to 3.3×10^8	2.3×10^2 to 4.0×10^3	6.0×10^5 to 5.0×10^7	1.6×10^2 to 4.4×10^2	3.0×10^6 to 5.2×10^8	1.0×10^2 to 1.0×10^4
Carrot	1.8×10^5 to 3.0×10^2	1.0×10^2 to 2.2×10^3	3.0×10^4 to 1.8×10^5	0.5×10^2 to 1.7×10^2		
Cucumber	3.2×10^4 to 4.3×10^6	1.0×10^2 to 2.0×10^3	2.3×10^4 to 2.0×10^6	1.2×19^2 to 1.3×10^2		

Table 2. Survival pattern of A. hydrophila on salad vegetables at 5°C (A) and 25°C (B)

Vegetable	Day->	Organism (log10 CFU/g)				
		0	3	5	7	10
Lettuce	A	5.2±0.2	5.4±0.2	5.9±0.1	6.2±0.1	6.2±0.1
	B	5.2±0.2	6.0±0.2	6.5±0.3	7.3±0.2	7.0±0.2
Carrot	A	5.2±0.2	5.5±0.2	6.1±0.2	6.4±0.3	6.4±0.1
	B	5.2±0.2	5.3±0.1	7.0±0.2	7.5±0.2	6.8±0.2
Cucumber	A	5.2±0.2	5.5±0.2	6.0±0.1	6.3±0.2	6.0±0.2
	B	5.2±0.2	6.2±0.2	6.8±0.2	7.2±0.1	6.5±0.1

Table 3. Changes in pH of salad vegetables during storage at 5°C (c) and 25°C (D)

Vegetable	Day->	pH				
		0	3	6	9	12
Lettuce	C	7.2±0.2	7.0±0.2	6.8±0.1	6.4±0.2	6.0±0.2
	D	7.2±0.2	6.8±0.2	5.2±0.2	5.0±0.2	4.8±0.2
Carrot	C	7.1±0.1	6.4±0.2	6.4±0.1	6.0±0.2	6.0±0.1
	D	7.1±0.1	5.0±0.1	55±0.2	4.4±0.2	4.4±0.2
Cucumber	C	7.3±0.2	6.4±0.2	6.3±0.1	6.3±0.2	4.6±0.2
	D	7.3±0.2	4.4±0.2	5.3±0.1	4.0±0.2	4.0±0.2

Table 4. Effect of tempe rature on salad vegetable inoculated with *A. hydrophila* (5×10^5 CFU/g)

Tempera-ture (°C)	Organisms (CU/g)							
	5 min		10min		20min		30 min	
	Total count	*A. hydrophila*	Total count	*A. hydrophila*	Total count	*A. hydrophila* count	Total count	*A. hydrophila*
50	7.0×10^4	4.2×10^3	4.2×10^4	4.0×10^3	2.0×10^3	2.0×10^3	2.0×10^3	$2.0\times10^2$60
	2.0×10^3	2.0×10^2	10^2	1.0×10^1	2.0×10^2	0	1.5×10^1	0
70	4.0×10^2	1.0×10^1	2.2×10^1	0	10.0	0	10	0
80	2.0×10^2	0	0	0	0	0	0	0

but the taste of salad vegetables had changed to a considerable extent where the vegetables could not be used as salad.

Discussion

All the salad vegetables (carrot, cucumber and lettuce) studied contained a large number of aerobic organisms. The presence of significant population of *Aeromonas* spp. in observed samples quantitatively virtually established the organisms as ubiquitous in salad vegetables. All the water samples that were used for watering the vegetables alsocontained *Aeromonas* spp. in large scale, so it might be possible that salad vegetables were contaminated through water. The number of *Aeromonas* spp. ater 7 days of incubation at 5°C, confirmed the indings of Palumbo *et al.*[12], Callister

and Agger14 that Aeromonas spp. are psychrotrophic organisms.

The growth of *A. hydrophila* in salad vegetables refrigerated (5°C) implicated *Aeromonas* spp. as potential pathogens and through salad vegetables, it might cause disease. It is well established that diarrhoea in the developing countries is a major sufferings of human. *A. hydrophila* contains different types of toxins, cytolytic enterotoxin, haemolysins, cytotoxins[17-20] and can cause different types o diseases, like diarrhoea, septicemia, wound infection, etc. In Bangladesh, the normal tradition of washing salad vegetables is to wash it under tap-water or normal water used for washing, which also contain these organisms. Besides these, in the market, the grocers use contaminated water to wash these vegetables. As a result, organisms are introduced in a large number. these vegetables in storage at room temperature or in the refrigerator may help to increase th number of *A. hydrophila* and its different types of toxin. These toxins in fact can cause considerable damage to human body.

The number of recovered A. hydrophila represented only small portion of the bacterial flora of the samples, and this load would be sufficient to cause disease. Changes in pH of the salad vegetables depend on temperature and time. in practice, it was found that salad vegetables preserved at 25°C were spoiled more than when stored at 5°C.

Salad vegetables which were washed with tap-water also gave a sufficient number of total bacterial population. It is because all these tap or normal water containcd a large number of aerobic organisms and this washing did not make any difference. Every year, diarrhoea due to Aeromonas spp. occur in Bangladesh. It could be said that contaminated salad vegetables may play one of the major part of the distribution of these organisms.

In this investigation, it was observed that heat treatment play an effective role to lower the microbial load (such as coliform and *A. hydrophila*) from salad vegetables. The volunteers who tasted the salad vegetables after heat treatment, reported that the taste and odour of the salad vegetables remained unaltered after heating them at 60°C for 10 and 20 min. Though high temperature (70-80°C) can kill 100% of the microbial population from the salad vegetables, but that temperature completely changed the texzture of the salad vegetables. As a result, consumers do not allow that vegetables.

This study gives the idea about salad organisms, its source of contamination, its public awareness and is finally suggested that the heat treatment (60°C for 10 and 20 min) is the best way to overcome the problem of *A. hydrophila* contamination.

Summary

Aeromonas spp. were isolated from salad vegetables (cucumber, lettuce and carrot) collectedfrom local market. The number of these organisms in the vegetables ranged from 102 to 4¥103 CFU/g and after washing with tap-water, the number remained between 0.5 ¥ 10^2 CFU/g. These salad vegetables were contaminated mainly by water used for watering the vegetables. *Aeromonas hydrophila* could survive in vegetable for long time in large number at 5 and 25°C. The pH of the vegetables changed with time. At 60°C for 20 minutes, most of the organisms were killed with sligh change in taste of these salad vegetables.

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