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Assessment of microbiological proliferation and *in vitro* demonstration of the antimicrobial activity of the commonly available salad vegetables within Dhaka Metropolis, Bangladesh

Tasnia Ahmed[1], Nusrat Jahan Urmi[1], Md. Sakil Munna[1], Kamal Kanta Das[1], Mrityunjoy Acharjee[1], M Majibur Rahman[2], Rashed Noor[1, *]

[1]Department of Microbiology, Stamford University Bangladesh, Dhaka, Bangladesh
[2]Department of Microbiology, University of Dhaka, Dhaka, Bangladesh

Email address:
noor.rashed@yahoo.com (R. Noor)

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Abstract: Present study mapped a complete pathogenic profile of the salad vegetables in Dhaka Metropolis, Bangladesh. In addition to a huge bacterial load found previously in lettuce, tomato, cucumber and carrot, current study further detected microbial contamination in chili, onion, capsicum and coriander samples. While *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. fecal coliform and *Eshcherichia coli* were found to be absent within these vegetable samples; a colossal burden of *Aeromonas* spp. (>10^6 cfu/g) was observed in chili, capsicum, coriander, whereas *Staphylococcus aureus* (1.2×10^8 cfu/g) and *Klebsiella Pneumoniae* (10^4 cfu/g) were detected in onion. Fungal growth was also observed in all samples. Most of the pathogens from all 8 samples were resistant against erythromycin (15 µg), amoxicillin (30 µg) and ampicillin (10 µg) while susceptible against ciprofloxacin (5 µg), kanamycin (30 µg) and gentamicin (10 µg). Interestingly, lettuce and cucumber samples were found to exhibit the anti-bacterial activity against *Staphylococcus aureus* and *Aeromonas* spp.

Keywords: Salad Vegetables, Pathogens, Drug-Resistance, Antibacterial Activity, Food Safety

1. Introduction

Vegetables may undergo microbial spoilage by a huge array of pathogenic bacteria, fungi, viruses and parasites [1-2]. Raw vegetables may be bruised during processing and distribution resulting in the release of plant nutrients which may serve as the potential organic and inorganic substrates for microbial growth [3]. A variety of pathogenic bacteria including *Salmonella* spp., *Escherichia coli* O157:H7, *Bacillus anthracis*, *Mycobacterium* spp., *Brucella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium perfringens*, and *Klebsiella* spp. can be introduced into vegetables from the manure used to promote their growth [4]. Moreover, pathogens existing in contaminated foods may harbor virulence genes and toxins/ enzymes, which further evoke the pathogenesis [5-7]. As a consequence, outbreaks of food borne diseases associated with vegetables in many countries are not unusual [4, 8-10].

Besides such spoilage threat, a major consideration in the medication of diseases has to be brought into the development of drug-resistance of the pathogens against commonly used antibiotics [11]. Such a situation drives the current interest on natural antimicrobial molecules in hope that they may be considered as anti-infective drug candidates. Many antimicrobial agents have previously been isolated from plant including secondary metabolites such as essential oil and terenoides, i. e., xanthones, benzophenones, coumarins and flavonoids [12]. These chemical substances can be even used as templates to produce more effective drugs through semi-synthetic and total synthetic procedures [13]. A new interest has occurred in the last decade in search of phytochemicals of native plants for pharmaceutical purposes as the plant-derived products have great potential as the source of

pharmaceuticals [13].

Microbial study on vegetables in Bangladesh generally revealed the association of a huge pathogenic load. Several reports showed the growth and proliferation of pathogenic bacteria causing enteric diseases in salad vegetables [14]. Previously we also noticed an array of bacterial pathogens among the commonly consumed salad vegetables including lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*) and carrot (*Daucus carota*) collected from Dhaka Metropolis [15]. Additional salad vegetables including green chili (*Capsicum annum*), onion (*Allium cepa*), capsicum (*Capsicum assum*) and coriander (*Coriandrum sativum*) were included in the study to portray a complete map of the overall microbiological traits of all salad vegetables found in Dhaka Metropolis. The drug resistance traits of the pathogenic isolates and the anti-microbial activity of all 8 types of salad samples were also examined to endow with an absolute insight on the food safety as well their possible role as natural antimicrobials.

2. Materials and Methods

2.1. Study Area, Sampling and Sample Processing

Samples of 8 categories of salad vegetables including lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), carrot (*Daucus carota*), chili (*Capsicum annum*), onion (*Allium cepa*), capsicum (*Capsicum assum*) and coriander (*Coriandrum sativum*) were randomly collected from different super shops, local markets and from street vans within a time frame of July 2012 to December 2012. Samples were collected early in the morning and quickly transported to the laboratory as soon as possible according to the method suggested by American Public Health Association [16]. For the identification and enumeration of pathogenic bacteria and fungi, 25 g of each sample was blended with 225 ml buffer peptone water (pH 7.2 ± 0.2) and serial dilutions were prepared up to 10^{-6} following the standard methods for plating purposes [17].

2.2. Microbiological Analysis

2.2.1. Enumeration of Total Viable Bacteria and Fungi

Total viable bacteria (TVB) were enumerated by spreading 0.1 ml of each sample suspension onto nutrient agar (Hi-Media Laboratories Pvt. Ltd., India). After incubation at 37 °C for 24 hours, plates were examined. For the estimation of fungal load, 0.1 ml of each sample was spread onto sabouraud dextrose agar (Hi-Media Laboratories Pvt. Ltd., India) followed by incubation at 25 °C for 48 hours.

2.2.2. Estimation of Total Fecal Coliform, Escherichia Coli and Klebsiella Pneumoniae

For enumerating total fecal coliform (TFC), 0.1 ml of each sample was spread onto membrane fecal coliform agar (Hi-Media Laboratories Pvt. Ltd., India). Plates were incubated at 44.5 °C for 24 hours. For the isolation of *Escherichia coli* and *Klebsiella pneumoniae*, 0.1 ml of suspension was spread over Mac Conkey agar (Hi-Media Laboratories Pvt. Ltd., India) for each sample and incubated at 37 °C for 18-24 hours. Presence of *E. coli* was further confirmed by the appearance of bluish-black colonies with green metallic sheen on the eosine methylene blue agar (Hi-Media Laboratories Pvt. Ltd., India). *Klebsiella* spp. was be identified by observing gummy pink color on Mac Conkey agar.

2.2.3. Isolation of Salmonella Spp., Shigella and Vibrio Spp

In case of *Salmonella*, *Shigella* and *Vibrio* spp, the absence of growth was assumptive of microbial cells of being in stressed condition or in viable but nonculturable (VBNC) state [18-19].

Therefore, enrichment technique was performed in selenite cystine broth (SCB) for *Salmonella* and *Shigella* spp. and in alkaline peptone water (APW) for *Vibrio* spp. For isolation of *Salmonella* and *Shigella* spp., one ml of homogenized sample suspension was transferred to SCB for enrichment followed by incubation at 37 °C for 6 hours and serial dilutions up to 10^{-6}, from which 0.1 ml was spread onto *Salmonella-Shigella* agar (Oxoid Ltd., Basingstoke, Hampshire, England) followed by incubation at 37 °C for 24 hours. In case of *Vibrio* spp., 1 ml of homogenized sample suspension was transferred to alkaline peptone water (APW) for enrichment and incubated at 37 °C for 6 hours. Serial dilutions up to 10^{-6} were made of the enriched broth, and 0.1 ml of suspension was spread onto TCBS agar, followed by incubation al 37 °C for 24 hours.

2.2.4. Estimation of Staphylococcus Aureus and Listeria Monocytogenes

For the estimation of *Staphylococcus aureus*, 0.1 ml of suspension was spread onto mannitol salt agar (Hi-Media Laboratories Pvt. Ltd., India) and the plates were incubated at 37 °C for 24 hours. The load of *Listeria monocytogenes* was enumerated by spreading 0.1 ml of suspension onto *Listeria* identification media (BD Diagnostic Systems, Europe) and plates were incubated at 37 °C for 24 hours. *Listeria monocytogenes* was identified as characteristic black gray color colonies surrounded by black halos on *Listeria* identification agar media [20].

2.2.5. Estimation of Clostridium Perfringens

One ml of each fresh salad vegetable sample blends were mixed in sterile normal saline in a ratio of 1:8 followed by heating at 80 °C for 15 minutes in order to kill vegetative cells of the microorganisms. From here, one ml of each samples were incubated for 4 hours at 37 °C with 9 ml of fluid thioglycolate broth. Then 0.1 ml of each enriched broth was subjected to 10-fold serial dilution for pouring on Clostridium isolation agar (Sigma-Aldrich, Inc., USA) plates, and was incubated at 37 °C within the anaerobic jar (2.5 L Anaero Jar, Oxoid Ltd., UK) for 48 hours.

2.3. Biochemical Tests for the Confirmative Identification

Finally, the standard biochemical tests were performed to confirm the identification of all the pathogenic isolates found in all 8 types of salad vegetable samples by the previously described methods [17,21].

2.4. Antibiotic Susceptibility Test

The pathogenic isolates were examined for antibiotic susceptibility traits (either drug resistant or sensitive) by disc diffusion assay on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA) against commonly used antibiotics following the standard protocol [22-28]. Antibiotics used in the study included trimethoprime/sulfamethoxazole 25 µg, erythromycin 15 µg, amoxicillin 30 µg, ceftriaxon 30 µg, ciprofloxacin 5 µg, streptomycin 10 µg, ampicillin 10 µg, tetracycline 30 µg, chloramphenicol 30 µg, cefixime 5 µg, polymyxin B (300 units), kanamycin 30 µg, vancomycin 30 µg, gentamycin 10 µg, nalidixic acid 30 µg, azythromycin 15 µg and penicillin G 10 µg (Oxoid Ltd., Basingstoke, Hampshire, England).

2.5. Determination of Antibacterial Activity of the Salad Vegetable Samples

The investigation of the antibacterial activity of the vegetable samples was performed by using agar well diffusion method [29-30]. Briefly, vegetable blends were used directly on the Mueller-Hinton agar media. At first, the pathogenic bacterial suspensions (*Pseudomonas* spp, *Listeria* spp, *Aeromonas* spp, *Vibrio* spp, *Salmonella* spp, *Klebsiella* spp, *Staphylococcus aureus*, *E. coli*) with the equivalent turbidity standard of McFarland (0.5) were introduced evenly over the Mueller-Hinton agar media separately using cotton swab and wells were made in the Mueller-Hinton agar media by cork borer. From each of the crude vegetables blends, 100 µl samples were then introduced separately in the specified well with a positive control (antibiotic disc) and a negative control (normal saline). Presence of clear zone around the sample solution (if any) indicated the antibacterial potential of vegetable samples.

2.6. Statistical Analysis

All the experiments were performed in triplicate. Statistical analyses were performed by determining the P-value through *t-test*. Errors were also calculated.

3. Result and Discussion

Outbreaks of human disease associated with the consumption of raw fruits and vegetables often occur in developing countries and have become more frequent in developed countries over the past decade. Vegetable products are the most common food items in Bangladesh. However, vegetable borne diseases may put the overall public health at a serious risk. Multiplication of bacterial pathogens as well as their drug-resistance properties mainly accounts for the health related problems [31]. Along these lines, the present study portrays the (i) pathogenic load of all salad vegetables consumed in Bangladesh, (ii) the drug-resistance traits of the pathogens, and finally (iii) the anti-microbial activities of these salad samples.

3.1. Prevalence of Pathogenic Microorganism

Our previous study showed that *E. coli*, *Salmonella* spp., *Shigella* spp., *S. aureus*, *Vibrio* spp. and *Listeria* spp. were common in carrot (*Daucus carota*), tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*) and cucumber (*Cucumis sativus*) samples [15]. The load of *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. in those vegetables were nil before enrichment; however, after enrichment *Vibrio* spp. was estimated within a range of 2.0×10^4 to 8.3×10^7 cfu/g in carrot, lettuce and tomato samples, while *Salmonella* and *Shigella* spp. were found within a range of 1.0×10^3 to 3.1×10^7 cfu/g and 3.0×10^4 to 4.8×10^8 cfu/g, respectively in carrot, cucumber, lettuce, tomato samples. The similar results were observed in current study in case of the above mentioned vegetables. Moreover in the present study, we included chili (*Capsicum annum*), onion (*Allium cepa*), capsicum (*Capsicum assum*) and coriander (*Coriandrum sativum*) salad vegetables where VBNC state bacteria [15], i. e., *Vibrio*, *Salmonella* and *Shigella* spp. were found to be absent (Table 1). *E. coli* and *Pseudomonas* spp. were also absent in all 4 new samples. *Aeromonas* spp. was found in capsicum, chili and coriander within the range of 1.2×10^6 to 2.7×10^6 cfu/g. Staphylococci (1.2×10^8 cfu/g) in onion indicates health risk upon consumption of this raw vegetable. Moreover, the presence of pathogenic organisms such as *Aeromonas* spp. and *Klebsiella Pneumoniae*. Revealed the possibility of spreading enteric diseases to the consumers. *Clostridium* spp. was absent in all samples, while fungal growth was observed in all the vegetable samples tested.

Table 1. *Microbial load (cfu/g) of fresh salad vegetables.*

Samples	TVBC	Fungi	*Klebsiella* spp.	*Aeromonas* spp.	*Staphylococcus aureus*
Capsicum	6.3×10^6	6.6×10^6	0	1.2×10^6	0
Onion	2.6×10^7	1.3×10^8	1.0×10^4	0	1.2×10^8
Chili	1.9×10^7	1.4×10^8	0	2.2×10^6	0
Coriander	1.3×10^7	1.5×10^7	0	2.7×10^6	0

TVBC: Total Viable Bacterial Count.
All data were statistically analyzed and were found significant ($P < 0.1$).

Overall, our study revealed the presence of a huge range of microorganisms in the commonly consumed salad vegetables. The pathogens might be introduced from the crop land, organic fertilizers, irrigating water, packaging materials, transport vehicles etc. Besides, unhygienic personnel handling and processing of the vegetables and their storage in such a condition which favors microbial growth might also account for such spoilage of vegetables. The contaminating pathogens are responsible for various

types of enteric diseases as well as serious intoxications in human health and hence the further detection of the virulent genes would be interesting.

3.2. Antibiotic Susceptibility Patterns of Pathogens Found in Salad Vegetables

Drug resistance is a serious problem in these days that is becoming more and more risky for the global public health. Our study of antibiogram revealed that most of the isolates were susceptible towards some antibiotics, whereas resistance towards several antibiotics indicated the risk of the emerging resistant isolates causing health hazards difficult to eradicate by those antibiotic therapy (Table 2).

Table 2. *Antibacterial susceptibility of the pathogenic isolates.*

Antibiotic	Disc Content	*E. coli*	*Klebsiella* spp.	*Salmonella* spp.	*Listeria* spp.	*Staphylococcus aureus*	*Shigella* spp.	*Aeromonas* spp.	*Vibrio* spp.
Trimethoprime/ Sulfamethoxazole	25 µg	S	S	S	S	R	S	R	ND
Erythromycine	15 µg	R	S	ND	ND	R	ND	R	ND
Amoxicillin	30 µg	S	R	R	ND	S	ND	S	R
Ceftriaxone	30 µg	S	R	ND	ND	S	S	I	I
Ciprofloxacin	5 µg	S	S	S	S	S	S	S	ND
Streptomycin	10 µg	I	R	ND	ND	S	ND	S	I
Ampicillin	10 µg	R	R	R	R	S	ND	S	R
Tetracycline	30 µg	S	S	ND	S	S	ND	S	R
Chloramphenicol	30 µg	S	S	S	ND	S	S	R	R
Cefixime	5 µg	S	R	ND	ND	S	ND	R	ND
Polymyxin B	300 units	S	S	ND	ND	R	ND	S	ND
Kanamycin	30 µg	ND	ND	S	S	S	ND	ND	I
Streptomycin	10 µg	ND	ND	ND	S	ND	ND	ND	ND
Vancomycin	30 µg	ND	ND	ND	S	ND	R	ND	R
Gentamycin	10 µg	ND	ND	S	S	ND	S	ND	ND
Nalidixic acid	30 µg	ND	ND	S	S	ND	S	ND	R
Azythromycin	15 µg	ND	ND	S	S	ND	ND	ND	R
Penicillin G	10 µg	ND	ND	ND	R	ND	ND	ND	R

ND= Not done, R= Resistant, S= Sensitive, I= Intermediate.

Majority of the isolates showed susceptibility against ciprofloxacin, tetracycline, kanamycin . However, most of the pathogenic isolates were resistance against ampicillin. Such resistance of the bacterial pathogens could be due to mechanistic-, epidemiologic- and genetic factors [32-34]. The resistance traits of the pathogens may result in the ineffective chemotherapy during food borne disease outbreaks.

Table 3. *Antimicrobial activity of the salad vegetables.*

Vegetable samples	Zone size of inhibition (mm)	
	Staphylococcus aureus	*Aeromonas* spp.
Carrot	0	0
Lettuce	11mm	0
Tomato	0	0
Cucumber	11mm	12mm
Chili	0	0
Onion	0	0
Capsicum	0	0
Coriander	0	0

All data were statistically analyzed and were found significant ($P < 0.1$).

3.3. Antibacterial Activity of Salad Vegetable Samples

Natural antimicrobials from the vegetables could be potential future candidates to be used as safe anti infective pharmaceutical products without causing any side effects like chemical medicines. Antimicrobial activity of different vegetables such as spinach, ghuniya, pumpkin, suran, cabbage, has been tested around the world in different times [13,30,35]. Along these lines, we determined the anti-microbial activity of the salad vegetables for the first time in Bangladesh (Table 3). Interestingly in present study, antibacterial activity was found in the lettuce and cucumber samples against *Staphylococcus aureus*. Apart from that cucumber samples also showed antibacterial activity against *Aeromonas* spp. (Table 3, Figure 1). However, the other vegetable samples (carrot, tomato, capsicum, onion, green chili, coriander) showed no antimicrobial activity against these bacterial isolates.

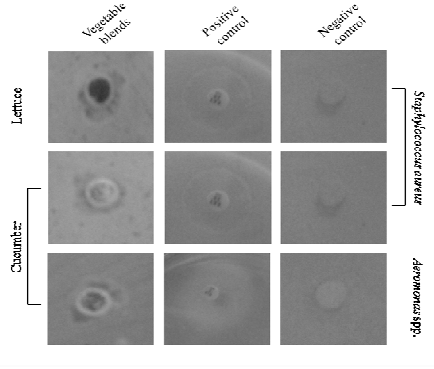

Figure 1. *Antibacterial activity of lettuce and cucumber. In the first column, lettuce and cucumber samples have been found to show antibacterial activity against Staphylococcus aureus and Aeromonas spp. In the second column, the first two images stand for positive controls using trimethoprime/sulfamethoxazole and the bottom image indicates the positive control using ciprofloxacin. In the third column, all the images are indicative of negative controls (normal saline).*

In cohort to our recent findings on food borne pathogens in within Bangladesh, the outcomes of the current study further added the insight of public health risk associated with fresh produce [36-39]. Moreover, the study might be further extended by identifying the genes responsible for the activity as well as to find out the way of using those gene products as anti-infective drugs to reduce the infection rates.

4. Conclusion

In the current study, we found that the common salad vegetable samples were largely populated with various microorganisms leading to serious public health hazards. The pathogenic bacteria present in the commonly consumed salad vegetables showed resistance against the regular antibiotics which is significant from the view point of public health. Our study thus imparted not only a complete picture on pathogenic profile of the salad vegetables but also presented a hopeful result on the antibacterial activity of lettuce and cucumber against microorganisms with pathogenic potential like *Staphylococcus aureus* and *Aeromonas* spp. Such antimicrobial trait of salad vegetables could further fortify the natural medication and an extended investigation could play role in preventing disease outbreaks caused by vegetable spoiling micro-flora. Nevertheless, our study contributed a detailed pathogenic profile of the salad vegetables which would be of significance for other countries/ regions with resource-poor settings.

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