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Demonstration of the source of microbial contamination of freshly cultivated cabbage, cauliflower, potato and squash collected from rural farms of Bangladesh

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Abstract

Several researches have been conducted so far to detect the level of microbial contamination within the commonly consumed vegetables; however, the source of contamination has not been investigated except a few studies. A recent study revealed huge microbial contamination within bottle gourd, pumpkin, radish and eggplant together with the microbial load of the plantation soils, fertilizers and irrigation waters. Present study further endeavored to identify such contamination level among potato, cauliflower, cabbage and squash samples and depicted on the contamination sources. Microbial load within the vegetable samples (around 10^6 cfu/g) was estimated near or lower than those within surroundings (around 10^8 cfu/g). Thus the data reveals that the microbiological attributes within the vegetable samples largely depended on the microorganisms inhabiting soils, fertilizers and on the type and quantity of the aquatic microorganisms within the water bodies surrounding the sampling sites.

Introduction

Recent interests in maintaining a healthy lifestyle around the globe has contributed to the increased consumption of fruits and vegetables in fresh, raw or undercooked forms (Abadias *et al*., 2007; Feroz *et al.,* 2013). The increased demands for healthy foods have substantially risen in current days due to the "Five a day" and "Nine a Day" campaigns advocated by the UK and the USA Governments (Heaton and Jones, 2007). However, a converse impact on public health upon eating, especially the fresh produces, underlies the risk of microbiological contamination of these foods which in turn may cause the onset of food borne illnesses (Clark *et al*., 2010; Nipa *et al.*, 2011; Scannell, 2011; Nawas *et al*., 2012; Rahman and Noor, 2012; Roy *et al*., 2013; Khan *et al.*, 2014; Todd, 2014). This is particularly a problem due to the ease of availability of the prepared/processed fresh produces such as bagged salads, for example, which indeed favors the necessary growth conditions for the survival and continued proliferation of human pathogens (Heaton and Jones, 2007). Although several studies placed such fresh foods in a low risk category in context to the risk of food- borne illnesses, the abundant increase of pathogenic bacteria in these fresh produces may render these foods to be ranked in high-risk category (FSANZ, 2001a and b; Adak *et al.*, 2005). Contamination and growth of spoilage microorganisms usually limit the shelf life of vegetables with subsequent loss in their nutritional values (King and Bolin, 1989; Robbs *et al*., 1996; Feroz *et al*., 2013). Loss in quality of vegetables through microbial contamination may lead to microbial surface, visual microbial growth/colonies, discoloration, wetness and soft rot (O'Connor-Shaw *et al*., 1996).

Food and water have long been the principal vehicles of various enteric diseases in Bangladesh, and lots of previous studies have demonstrated the existence of harmful microorganisms from vegetables (Beuchat, 1996; Fain, 1996; Abadias *et al*., 2008; Cordano and Jacquet, 2009; Rahman and Noor, 2012; Nawas *et al*., 2012; Ahmed *et al*., 2014). However, the sustainability of the contamination, and more specifically, the exact sources of microbiological contamination has not been intensely investigated. Very recently our study on microbial contamination of bottle gourd *(Lagenaria siceraria)*, pumpkin *(Cucurbita pepo)*, radish *(Raphanus sativus)* and eggplant *(Solanum melongena)* revealed that the agricultural sites (soils), fertilizers, and the water used for irrigation may serve as the source of an array of microorganisms (Alam *et al*., 2015). One vital reason of such microbial contamination may be the unhygienic maintenance of the agricultural

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commodities (Telias *et al*., 2011). Contamination can also be due to various sources including unhygienic environments during preparation and storage (Nawas *et al*., 2012). Other sources may include the plantation soils and fertilizers, which has long been associated with the contamination of vegetables with *E. coli* and *V. cholerae* (Yao *et al.*, 2007; Uddin *et al*., 2012; Alam *et al.*, 2015). Even the eco-friendly bio-fertilizers, generally the resultant compost of animal- and human excreta together with kitchen wastes, may harbor an array of harmful microorganisms if not treated properly before the application in fields (Uddin *et al.*, 2012)

Based on the recent findings of bacterial contamination sources for bottle gourd, pumpkin, radish and eggplant, present study further incremented the knowledge on bacterial proliferation among potato, cauliflower, cabbage and squash samples. The objectives of the current study were (1) to obtain the base-line information on the occurrence of the heterotrophic bacteria and the specific bacterial pathogens within the vegetables commonly cultivated, and (2) to identify the most probable causes and sources of microbial contamination from the rural farms of Bangladesh, prior to marketing and sales towards the consumers.

Materials and Methods

Study area and sampling

The study was confined to local agricultural lands where different crops and vegetable are produced each year. Four categories of vegetable samples (Potato, Cauliflower, Cabbage and Squash), the associated soil and fertilizer samples, and the surrounding water samples were collected from diverse agricultural fields of nine different districts of Bangladesh in accordance with the area from which the vegetables were collected. Around 100 g of surface soil samples and fertilizer samples were scraped within 2 inches depth with a sterile spatula within each site. One liter each of source and end of line irrigation water samples were also collected from each site. The samples were placed in sterile plastic bags and stored at 4^{o}C. Samples were collected early in the morning and transported quickly to the laboratory and immediately processed for further tests (APHA, 1998; Ahmed *et al.*, 2014).

Isolation and quantification of bacteria and fungus from vegetables, soils, fertilizers and water samples

Identification and enumeration of microorganisms were carried out according to standard procedures (Cappuccino and Sherman, 1996; Rahman and Noor, 2012; Feroz *et al.*, 2013; Ahmed *et al.*, 2014). Ten (10) grams of vegetable samples were homogenized with 90 ml of buffer peptone water and diluted up to 10-4, followed by plating in different selective and differential culture media using the standard methods. For the quantification of total viable bacteria (TVB) and fungi, 0.1 ml of each sample was introduced onto the Nutrient agar and Sabouraud's dextrose agar (Oxoid Ltd., Basingstoke, Hampshire, England) plates, respectively, by means of spread plate technique. Plates were incubated at 37^{o}C for 24 hours and at 25^{o}C for 48 hours for total viable bacteria and fungi, respectively (Rahman and Noor, 2012).

From the dilutions 10^{-4}, 0.1 ml of each sample was spread onto the Membrane fecal coliform (MFC) agar and MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England) for the enumeration of total fecal coliform (TFC), and coliforms (*Escherichia coli* and *Klebsiella* spp.), followed by incubation at 44.5^{o}C and 37^{o}C for fecal coliform and coliforms, respectively for 24 hours. Likewise, *Staphylococcus* spp. was isolated by adding 0.1 ml of diluted sample onto the Mannitol salt agar (MSA) (Oxoid Ltd., Basingstoke, Hampshire, England) following incubation at 37^{o}C for 24 hours. For the enumeration of *Listeria* spp., 0.1 ml of suspension was spread onto *Listeria* identification media and plates were incubated at 37^{o}C for 24 hours. After homogenization of 10 g of vegetable samples with 90 ml of buffer peptone water, 10 ml of samples were transferred into 90 ml of selenite cysteine broth and alkaline peptone water (Oxoid Ltd., Basingstoke, Hampshire, England) for the enrichment of *Salmonella, Shigella,* and *Vibrio* spp., respectively and incubated at 37^{o}C for 4 to 6 hours. Samples were then diluted up to 10^{-4} and 0.1ml of samples from each of the 10^{-4} dilutions were spread onto *Salmonella-Shigella* agar and thiosulfate citrate bile salt sucrose agar (Oxoid Ltd., Basingstoke, Hampshire, England) for the isolation of *Salmonella* spp. and *Shigella* spp., and *Vibrio* spp., respectively. Plates were incubated at 37^{o}C and the appearance of typical colonies (if any) was noticed within for 24 to 48 hours. Finally, the identity of all the isolates was confirmed by conducting a series of biochemical tests as described previously.

Identification and enumeration of microorganisms from soils and fertilizer samples were carried out according to standard procedures as described earlier (Cappuccino and Sherman, 1996; Uddin *et al*., 2012; Alam *et al.*, 2015). Ten (10) grams of soil samples were homogenized with 90 ml of BPW (Buffer Peptone Water) and diluted up to 10^{-7}, followed by plating in different selective and differential culture

Table 1. Load of bacteria, fungi and specific pathogens within potato samples (cfu/g)

Location	Total Viable Bacteria	Total Fungal Count	*E. coli*	*Klebsiella* spp.	Fecal coliform	*Staphylococcus* spp.	*Psuedomonas* spp.	*Salmonella* spp.	*Shigella* spp.	*Vibrio* spp.	*Listeria* spp.	*Bacillus* spp.
Gazipur	24	4.5×10^2	1.2×10^2	0	0	76	0	0	0	1.0×10^2	4.0×10^2	0
Boro Monohorpur	1.3×10^7	3.5×10^3	1.5×10^2	0	0	0	0	0	0	0	0	0
Comilla	3.3×10^7	3.2×10^3	0	6.7×10^2	0	7.6×10^2	9.0×10^2	0	0	0	5.0×10^2	7.0×10^2
Manikgonj	2.4×10^7	4.5×10^3	1.2×10^2	0	0	0	0	0	0	1.0×10^2	4.0×10^2	0
Pabna	26	1.1×10^2	0	67	0	5	0	0	0	0	0	0
Joypurhat	3.6×10^8	2.8×10^2	3.0×10^1	1.8×10^2	0	0	0	2.5×10^2	0	0	0	1.8×10^2
Bogra	3.1×10^7	1.8×10^4	0	4.0×10^2	0	0	0	0	0	3.0×10^2	1.1×10^2	0
Khulna	6.7×10^8	3.4×10^3	0	1.1×10^2	0	0	2.8×10^2	0	0	0	0	1.2×10^2
Jessore	2.6×10^6	1.1×10^2	0	6.7×10^2	0	0	0	0	0	0	0	0

media using the standard methods as stated above. Pathogenic determination was also conducted as described above. Microbiological analysis of water samples were done as described by Acharjee *et al.*, 2013. Finally, the identity of all the isolates was confirmed by conducting a series of biochemical tests as described previously.

Results

Microbial load in the vegetables samples studied

In potato samples, the heterotrophic bacterial load was observed to be around 10^6 to 10^8 cfu/g in 8 out of 10 cases while samples from 4 sites were found to harbor fungi within the range of 103 to 10^4 cfu/g (Table 1). While the fecal coliforms and *Shigella* spp. were completely absent, proliferation of *E. coli, Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Vibrio* spp., *Listeria* spp. and *Bacillus* spp. was observed in few cases (Table 1). The presence of *Salmonella* spp. was observed in one sample as well. In squash samples, the heterotrophic bacterial load and the fungal load were found within the range of 10^5 to 10^6 cfu/g and 10^2 to 10^3 cfu/g, respectively except in samples from one site (Table 2). Presence of *Staphylococcus* spp., *Vibrio* spp., *Listeria* spp. and *Bacillus* spp. was observed in few cases while the proliferation of *Klebsiella* spp. was noticed to be significant (Table 2). In cabbage samples, the heterotrophic bacterial load was up to 10^6 cfu/g in all cases while samples from 9 sites were found to harbor fungi up to the load of 102 cfu/g (Table 3). While the fecal coliforms, *E. coli, Salmonella* spp., *Shigella* spp. and *Shigella* spp. were completely absent, proliferation of *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Vibrio* spp., *Listeria* spp. and *Bacillus* spp. was observed within the samples from a few sites (Table 3). In cauliflower samples, the heterotrophic bacterial and fungal load was observed to be 10^7 cfu/g and 10^3 cfu/g, respectively in the samples from almost all sites (Table 4). Proliferation of *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Vibrio* spp., *Listeria* spp. and *Bacillus* spp. was observed within the samples from a few sites (Table 4).

Microbial proliferation in the surrounding environment

In a recent experiment while analyzing the microbial load within bottle gourd, pumpkin, radish and eggplant vegetable samples (collected from the same sites as in the present study) the plantations soils, fertilizers applied within the agricultural fields, and the irrigation waters were found to harbor lots of bacterial species (Alam *et al*., 2015). In the water samples, the heterotrophic bacterial load was observed to be 10^5 to 10^8 cfu/ml in case of 9 out of 10 sites cases while the fungal burden also peaked up to 10^2 cfu/ml (Alam *et al*., 2015). Existence of the fecal coliforms, *E. coli, Salmonella* spp. and *Listeria* spp. was noticed at least in 2 sites, while the presence of *Klebsiella* spp. was observed in 8 cases (Alam *et al*., 2015). The proliferation of *Vibrio* spp. was observed in one water sample as well. As in cases of the water samples, in the soil samples too, massive bacterial and fungal growth was observed. Proliferation of *Klebsiella* spp. was observed in 8 cases followed by the abundance of *Pseudomonas* spp. and *Bacillus* spp. in 8 and 5 soil samples, respectively (Alam *et al.*, 2015). Existence of *Listeria* spp. and *Vibrio* spp. was also observed in few cases, while the fecal coliforms, *E. coli, Salmonella* spp. and *Shigella* spp. were found to be completely absent (Alam *et al*., 2015).

Finally in the fertilizers, huge proliferation of the total viable and culturable bacteria and fungi was observed. Existence of *Bacillus* spp. and

Table 2. Load of bacteria, fungi and specific pathogens within squash samples (cfu/g)

Location	Total Viable Bacteria	Total Fungal Count	*E. coli*	*Klebsiella* spp.	Fecal coliform	*Staphylococcus* spp.	*Psuedomonas* spp.	*Salmonella* spp.	*Shigella* spp.	*Vibrio* spp.	*Listeria* spp.	*Bacillus* spp.
Gazipur	3.3×10^6	3.2×10^5	0	6.7×10^2	0	9.0×10^2	ND	0	0	7.0×10^2	5.0×10^2	0
Boro Monohorpur	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Comilla	4.5×10^6	1.2×10^3	0	2.8×10^3	0	8.0×10^2	0	0	0	7.0×10^2	0	3.2×10^2
Manikgonj	2.6×10^5	1.1×10^2	0	1.1×10^1	0	0	0	0	0	1.0×10^2	0	0
Pabna	3.1×10^5	2.6×10^2	0	1.1×10^1	0	1.2×10^2	0	0	0	Nil	0	0
Joypurhat	6.9×10^6	2.8×10^2	0	2.1×10^2	0	0	0	0	0	1.1×10^1	0	0
Bogra	5.1×10^5	2.2×10^2	0	3.1×10^2	0	0	0	0	0	0	0	1.1×10^1
Khulna	5.8×10^5	3.1×10^2	0	1.3×10^2	0	0	0	0	0	0	0	1.3×10^1
Jessore	7.3×10^6	4.1×10^2	0	3.3×10^2	0	2.8×10^2	0	0	0	0	3.6×10^2	0

ND: Not done (In Boro Monohorpur site, samples were not available)

Table 3. Microbial proliferation within cabbage samples (cfu/g)

Location	Total Viable Bacteria	Total Fungal Count	*E. coli*	*Klebsiella* spp.	Fecal coliform	*Staphylococcus* spp.	*Psuedomonas* spp.	*Salmonella* spp.	*Shigella* spp.	*Vibrio* spp.	*Listeria* spp.	*Bacillus* spp.
Gazipur	3.1×10^6	0	0	2.2×10^2	0	2.9×10^2	0	0	0	0	7.0×10^2	3.2×10^2
Boro Monohorpur	3.3×10^5	1.7×10^2	0	2.0×10^2	0	0	3.1×10^2	0	0	0	0	0
Comilla	4.5×10^6	2.6×10^2	0	2.0×10^2	0	1.1×10^2	0	0	0	1.3×10^2	0	0
Manikgonj	4.3×10^5	3.1×10^2	0	0	0	1.9×10^2	0	0	0	0	2.0×10^2	0
Pabna	4.1×10^5	2.0×10^2	0	2.2×10^2	0	1.2×10^2	0	0	0	0	0	0
Joypurhat	3.1×10^5	1.3×10^1	0	2.3×10^2	0	2.1×10^2	0	0	0	0	0	0
Bogra	3.5×10^6	1.2×10^1	0	3.0×10^2	0	2.0×10^2	0	0	0	0	3.7×10^2	0
Khulna	4.1×10^6	2.2×10^2	0	2.4×10^2	0	1.2×10^2	0	0	0	0	0	0
Jessore	1.3×10^6	7.7×10^2	0	3.8×10^2	0	0	0	0	0	0	0	0

Pseudomonas spp. was noticed in almost all cases while the proliferation of *E. coli* was observed in the 50% of the fertilizer samples. However, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Listeria* spp., and the fecal coliforms were found to be completely absent (Alam *et al*., 2015). Proliferation of *Klebsiella* spp. was observed in two samples.

Discussion

An array of pathogenic bacteria, fungi, parasites, viruses, toxins, trace elements have been recurrently reported both locally and globally to contaminate the fresh foods triggering the food borne illnesses or infections (Bennett, 2003; Lynch *et al*., 2006; Frazier and Westhoff, 2007; CDC, 2010; Clark *et al*., 2010; Nipa *et al*., 2011; Scannell, 2011; Rahman and Noor, 2012; Roy *et al.*, 2013; Ahmed *et al*., 2014; Khan *et al*., 2014; Todd, 2014). However, the sources originating the propagation of the microbial contaminant have not been chalked out yet. Current study has further established the presence of pathogens among freshly cultivated vegetables in Bangladesh, along with its presence in soil, water and fertilizers.

In order to identify the source of contamination, present study attempted to pinpoint water as the most likely source of contamination, but it still could not sufficiently rule out the role of soil and fertilizer (Alam *et al*., 2015). Interestingly, the fecal coliforms were absent from all samples of vegetables, soil and fertilizers, but were found to be present in 2 samples of water, which is assumptive of possible dissemination of the fecal coliforms within the vegetables. The soil and the fertilizer samples were found to be dominated by *Bacillus* spp. (while being absent in the vegetables samples), *Staphylococcus* spp. and

Klebsiella spp., which might pose massive public health risk if propagated into the vegetables. Indeed the bacterial load of soil was found to be significantly higher than that of vegetables and water. Moreover, as water samples and the vegetables samples showed, for majority of the samples, results matching the load of vegetables for both total viable bacteria and fungi. It is also worth mentioning that in most samples the enumeration of specific bacteria between water and vegetables were within the nearly similar range. Additionally, this is to ponder that although collected from separate areas, vegetables of the same species showed similar pathogenic growth patterns.

Water is generally known to harbor diverse range of microorganisms leading to the public health fatality (Munshi *et al*., 2012). The site map of Bangladesh brought about the propinquity of her greatest extent of agricultural land with facade waters, rendering the probable propagation of water borne microorganisms (including the pathogenic strains of *E. coli, Salmonella* spp., *V. cholerae*, *Shigella* spp., *Cryptosporidium parvum, Giardia lamblia, Cyclospora cayetanensis, Toxiplasma gondii,* Norwalk viruses, Hepatitis A viruses and etc.) into the associated commodities with plantation soils. The degree of microbial attack is further aided due to the dense population with extreme unhygienic sanitary conditions (Faruque *et al.*, 2003; FAO, 2012; Ali *et al*., 2013; Khairuzzaman *et al*., 2014; Khan *et al.*, 2014). Surface water has long been used for agricultural sources, but it contains the risk of contamination due to animal defecation, malfunctioning sewage and septic systems, storm water drainage and urban runoff (Chigor *et al*., 2013). By considering the metabolic process and life sustainability of human and animal, pure and safe water is an important issue especially in developing countries where many enteric diseases are related to the contamination of water (Solomon *et al.*, 2002; Wagg *et al*. 2013).

Besides water, soils host a variety of organisms, many of which play a vital role in maintaining its fertility and productivity (Bao *et al.*, 2012). Soil borne diseases can be a major limitation to crop production, particularly for vegetables (Adak *et al*., 2005). Soil born plant pathogens including *Bacillus* spp., *E. coli, Staphylococcus aureus, Pseudomonas* spp. and *Listeria* spp. can significantly reduce yield and quality in vegetable crops. These pathogens are particularly challenging because they often survive in soil for many years and each vegetable crops may be susceptible to several species. Simultaneous infections from multiple soil borne pathogens sometimes result in a disease complex that can further damage the crop. Many diseases caused by soil borne pathogens are difficult to predict, detect and diagnose. In the current study, soil and fertilizer samples were found to exhibit significantly higher growth rates compared to those in water samples as observed earlier (Alam *et al*., 2015). Additionally the total viable bacteria and fungi within the vegetables samples were found nearly similar to that in the water samples tested which is also in consistent to the data found earlier on different samples (Alam *et al.*, 2015). Hence, the present data were suggestive of identifying water as the principal source of contamination of the vegetable samples (Alam *et al*., 2015). The microbial load of water might have been transferred to the vegetables during washing. Certain pathogens such as *Bacillus* spp. and *Psuedomonas* spp. were could have been propagated into the vegetable samples from fertilizer or soil.

Indeed, microbiologically contaminated water used for the processing of the fresh produces (i.e., fruits and vegetables) generated from the defective water distribution system is often responsible for triggering the enteric diseases (Faruque *et al*., 2003; Alam *et al*., 2015). Besides, the over usage of agricultural lands, underprivileged management of animal feeing operations, overgrazing, ineffective application of pesticides, lack of worker hygiene, employment of manures and irrigation water harboring pathogenic microorganisms are the agricultural activities triggering the microbial contamination of vegetables (CAC, 2007; van Schothorst *et al.*, 2009). Another aspect underlies the impact of polluted runoff (carrying natural and manmade impurities) usually created by rainfall as in Bangladesh, which in turn deposit the pollutants into ponds, rivers, lakes and other water reservoirs (EPA, 2005). Such pollutants are generally termed as the nonpoint source (NPS) pollutants, which differ from the point sources like the industrial and sewage effluents (EPA, 2005; Vasanthavigar *et al*., 2011; Paul *et al*., 2013). Indeed the prompt increase in populations with an associated boosting in urbanization and fresh food demand in the developing countries like in Bangladesh is mounting. The situation is unfortunately deteriorating with the employment of polluted waters (although unintended) for the processing of fresh vegetables, and the dearth in acquaintance in the maintenance of general cleanliness and professional hygiene.

Conclusion

Together with the recent study conducted by Alam *et al*., 2015, current study further pointed the discrete reasons of microbial contamination of vegetables by determining the contamination sources. The

investigation achieved the basic information on the propagation means of harmful microorganisms into the vegetables commonly cultivated and consumed afterwards. The microbial analysis of the vegetable samples obtained the critical information not only on the microbial load but also on their dissemination originators (i.e., soil, fertilizer and water). Such knowledge is expected to contribute to preventing microbial contamination and reducing the extent of food borne diseases especially in the agriculture based developing countries round the globe.

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