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Quantitative microbial risk assessment to estimate the risk of diarrheal diseases from fresh produce consumption in India

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ABSTRACT

This study estimates illness (diarrhea) risks from fecal pathogens that can be transmitted via fecal-contaminated fresh produce. To do this, a quantitative microbial risk assessment (QMRA) framework was developed in National Capital Region, India based on bacterial indicator and pathogen data from fresh produce wash samples collected at local markets. Produce wash samples were analyzed for fecal indicator bacteria (*Escherichia coli*, total *Bacteroidales*) and pathogens (*Salmonella*, Shiga-toxin producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC)). Based on the *E. coli* data and on literature values for *Cryptosporidium* and norovirus, the annual mean diarrhea risk posed by ingestion of fresh produce ranged from 18% in cucumbers to 59% in cilantro for *E. coli* O157:H7, and was <0.0001% for *Cryptosporidium*; for norovirus the risk was 11% for cucumbers and up to 46% for cilantro. The risks were drastically reduced, from 59% to 4% for *E. coli O157:H7*, and from 46% to 2% for norovirus for cilantro in post-harvest washing and disinfection scenario. The present QMRA study revealed the potential hazards of eating raw produce and how post-harvest practices can reduce the risk of illness. The results may lead to better food safety surveillance systems and use of hygienic practices pre- and post-harvest.

1. Introduction

Foodborne disease outbreaks are well recognized around the globe and involve high costs as well as loss of productivity and even lives in some cases. Challenges in identifying specific sources of foodborne outbreaks include: i) the vehicle of infection is not readily identified, as many foodborne pathogens have a relatively long incubation period (several days to months); ii) possible sources of food-contamination are discarded or consumed before the diagnosis is made; iii) patients have a hard time remembering what they consumed during the past days to weeks; and iv) inadequate diagnostic tools or inexperienced staff may be involved in food sample pathogen detection (Robertson et al., 2015). In India, foodborne diseases are rarely investigated though gastroenteritis symptoms are common (Bhat and Rao, 1987). Moreover, India is a country where 28% of males and 29% of females over the age of 15 eschew fish, meat, and eggs (General, 2014), and people use many types of fresh produce in their daily lives (Chourasiya et al., 2015; Khillare et al., 2012). India is the second largest producer of fruits and vegetables in the world and world's third largest economy after the US and China (Food and Agriculture Organization of the United Nations (FAO, 2017).

Fresh produce quality in markets can be compromised at numerous points along a food system from farm to consumption. Reasons for contamination can be related to i) insufficient sanitation facilities on the farm and in the wholesale produce markets; ii) presence of vectors such as flies in the food vicinity; iii) poor water quality used for washing fresh produce; iv) improper holding temperatures of fresh produce; and v) poor hygiene conditions where food handlers/vendors frequently handle the produce resulting in contamination and microbial proliferation during produce display or manipulation (Viswanathan and Kaur, 2001). A better understanding of improved food safety can be obtained using a risk assessment and risk management approach that includes hazard identification and produce consumption scenarios using a stochastic modeling approach called quantitative microbial risk

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assessment (QMRA). The QMRA framework is used to estimate the foodborne disease risk given variables such as pathogen concentration, consumption rate of produce, and dose-response data for an exposed population. The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations are playing an important role in the deliberations of international standard-setting bodies (such as Codex-Alimentarius) in the application of QMRA in addressing complex food safety issues (Dennis, 2002). It has also been widely applied in different water sectors such as wastewater, surface water, and drinking water in developing countries such as Nepal, Vietnam, Ghana etc. (Enger et al., 2012; Howard et al., 2006; Kouame et al., 2017; Nguyen-Viet et al., 2012; Petterson, 2016). The present study presents an application of QMRA for food safety purposes in resource-limited setting that can help in managing public health risks from foodborne pathogens.

Testing fresh produce quality to consider health risks can be approached by identifying the presence of a particular disease-causing pathogen, and/or by identifying more general fecal contamination markers. The identification of pathogens in complex environmental matrices is difficult because: i) pathogen prevalence and concentration are low in the environment, therefore large sample sizes may be required to identify low levels of pathogens; ii) some pathogens are still not cultivatable in laboratories (e.g. norovirus), iii) some pathogens have very low infectious dose to cause disease, and iv) pathogens are not uniformly distributed in the environment. Therefore, an alternative approach is based on the fecal indicator bacteria (FIB) *Escherichia coli* (*E. coli*), as it is present in all fecal material and can be readily detected by use of simple cultivation methods. However, there can be a lack of correlation between the presence of *E. coli* and pathogens (Benjamin et al., 2013; Haack et al., 2009; McEgan et al., 2013; Won et al., 2013). Another important limitation is that *E. coli* cannot easily identify the source of fecal contamination. Therefore, we also considered an alternative, highly host-specific fecal indicator: the gram-negative obligate anaerobe *Bacteroidales* (Layton et al., 2006). The polymerase chain reaction (PCR) can be used to selectively identify 16S rRNA genome of *Bacteroidales,* whereas *E. coli* can be enumerated using a culture-based most probable number (MPN) method. *Bacteroidales* and pathogens can be detected simultaneously using molecular methods that are becoming quick and more cost-effective, and regardless of detection method, data on presence and concentration of indicator bacteria and pathogens can be incorporated into risk assessment frameworks that aim to detect, control, and prevent foodborne disease outbreaks.

The present study was conducted to evaluate fecal contamination in common types of vegetables that are grown and consumed in the peri-urban area of National Capital Region (NCR), India. Studies have previously determined microbial quality of fresh produce (Mathur, 2014; Pingulkar et al., 2001; Viswanathan and Kaur, 2001), and the current study aims to take the next steps to integrate microbial data into a risk assessment framework in India as a threshold country. It also considers changes in risk associated with cost-effective interventions to help reduce the risk of foodborne illnesses and outbreaks.

2. Materials and methods

2.1. Description of the study area and microbiological testing

The study was carried out in three different wholesale markets (WM1, WM2, and WM3), 5 different foodmarts, and a road-side food seller in the National Capital Region (NCR), India. Fresh produce was collected upon delivery to fresh produce markets during the month of April 2014 in Delhi city. On each sampling day, two cantaloupes, two cucumbers, 100 g of green peppers, one bunch (approx.100 g) of mint and cilantro, and 500 g of English cucumber were collected to create produce wash rinsates. A total of 85 samples were analyzed: 17 samples of cantaloupe, 20 samples of green peppers, 15 samples of English cucumbers, 18 samples of cucumbers, and 15 samples of cilantro, were collected, respectively. The produce samples were collected into a sterilized sample bags (VWR, Radnor, PA) containing 350 mL of 0.15% sterile peptone water (PW) which were shaken for 30 s, massaged for 30 s, and again shaken for 30 s. The sample bags were stored on ice in a cooler and transported to the laboratory for analysis of *E. coli* within 6 h of collection. A total of 85 samples were collected during the summer season for microbiological analysis: bacterial fecal indicator *E. coli* (IDEXX Colilert® Quanti-Tray® 2000), total *Bacteroidales* 16S ribosomal RNA gene (an alternative fecal indicator (GeneSig® kit, Primerdesign Ltd., U.K), and pathogen testing for *Salmonella* (Atlas® *Salmonella* SEN Detection Assay), Shiga toxin producing *E. coli,* and enterohemorrhagic *E. coli* (EHEC) (Atlas® STEC EG2 Combo Detection Assay).

The bacterial indicator (*E. coli*) was estimated using the IDEXX Colilert® Quanti-Tray® 2000 defined substrate method (IDEXX, Westbrook, ME, USA) and the microbial levels were expressed as Most Probable Number (MPN)/100 mL. Briefly, upon receipt of samples in the laboratory, each sample was massaged for 60 s in the sample collection bag followed by pipetting 100 mL of each sample into a sterile bag (HACH, Loveland, CO); then reagents from the IDEXX kit were added to each 100 mL of sample. The samples were gently mixed to dissolve the media, and the contents were transferred into sterile Quanti-Tray 2000 trays. Then, each Quantitray was heat-sealed and incubated at $35 \pm 0.5\,^{\circ}C$ for 24 h. Negative control blanks were run daily in the laboratory using autoclaved bottled drinking water and always tested negative as expected. The samples below the assay lower bound of detection were reported as 1 MPN/100 mL, and samples above the upper limit of detection were reported as 2419.6 MPN/100 mL.

For *Bacteroidales* testing, produce-washes of 100 mL were filtered through a 47-mm, 0.45-μm-pore-size Millipore S filters. The filters were stored in 15-mL sterile tubes at $-20\,^{\circ}C$ until DNA extraction. DNA extractions were performed on bacterial concentrates using the PowerWater DNA Isolation kit (Catalog No. 14900, Qiagen, Carlsbad, CA) with a minor modification. Briefly, the filter was removed from the 15-ml tube, cut in half, rolled, and placed into the bead tube of the kit. DNA concentrates were stored at $-80\,^{\circ}C$ before qPCR analysis. The internal amplification control (IAC) was added to the extracted DNA sample once it was resuspended in lysis buffer. Quantitative qPCR was performed using a Bio-Rad CFX96 Touch™ real-time PCR detection system (Bio-Rad, Hercules, CA). The SsoAdvanced Universal Probes Super mix (Bio-Rad, Catalog No. 172−5281) was used as a master mix in running *Bacteroidales* assay. A *Bacteroidales*-specific primer and probe mix was provided in the kit, and amplicons were detected through the FAM channel (GeneSig® kit, Primerdesign Ltd., U.K). Standard curves were made using known concentrations (2×10^5 copy number (CN)/μl to 2 copy number (CN)/μl) in triplicates as provided in the kit. A non-template control (NTC) and a positive template control were included before running 96-well plates. Each reaction was run containing 5 μL of DNA template. The qPCR cycling conditions were $95\,^{\circ}C$ for 3 min, followed by 45 cycles of $95\,^{\circ}C$ for 15 s, and $60\,^{\circ}C$ for 60 s, performed on a CFX96 real-time system (Bio-Rad, Hercules, CA). The limit of detection of *Bacteroidales* assay was 5.36 genecopies/μl based on a Ct cut-off value at 40.

The presence and absence of *Salmonella*, enterohemorrhagic *E. coli* (EHEC), and STEC (Shiga toxin-producing *E. coli*) were determined in the fresh produce samples. As previously mentioned, produce-washes of 100 mL were filtered through a 47-mm, 0.45-

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