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Metagenomic analysis of viruses associated with field-grown and retail lettuce identifies human and animal viruses

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ABSTRACT

The emergence of culture- and sequence-independent metagenomic methods has not only provided great insight into the microbial community structure in a wide range of clinical and environmental samples but has also proven to be powerful tools for pathogen detection. Recent studies of the food microbiome have revealed the vast genetic diversity of bacteria associated with fresh produce. However, no work has been done to apply metagenomic methods to tackle viruses associated with fresh produce for addressing food safety. Thus, there is a little knowledge about the presence and diversity of viruses associated with fresh produce from farm-to-fork. To address this knowledge gap, we assessed viruses on commercial romaine and iceberg lettuces in fields and a produce distribution center using a shotgun metagenomic sequencing targeting both RNA and DNA viruses. Commercial lettuce harbors an immense assemblage of viruses that infect a wide range of hosts. As expected, plant pathogenic viruses dominated these communities. Sequences of rotaviruses and picobirnaviruses were also identified in both field-harvest and retail lettuce samples, suggesting an emerging foodborne transmission threat that has yet to be fully recognized. The identification of human and animal viruses in lettuce samples in the field emphasizes the importance of preventing viral contamination on leafy greens starting at the field. Although there are still some inherent experimental and bioinformatics challenges in applying viral metagenomic approaches for food safety testing, this work will facilitate further application of this unprecedented deep sequencing method to food samples.

1. Introduction

Fresh produce, particularly leafy greens, is an important component of a healthy and balanced diet, yet one that is increasingly being recognized as an important vehicle for the transmission of human pathogens including bacteria, viruses and parasites. Most recently, fresh produce has been identified as a major contributor to foodborne outbreaks worldwide (Berger et al., 2010; Lynch et al., 2009; Newell et al., 2010).

The number of foodborne outbreaks in the United States (U.S.) has continued to serve as an indicator of the vulnerability of our food systems to contamination and public health risk. The Centers for Disease Control and Prevention (CDC) has estimated, based on the data from 2000 to 2008, that each year 31 major pathogens caused 9.4 million episodes of foodborne illness in the U.S., with most illnesses caused by viruses (59%), particularly noroviruses (Scallan et al., 2011b). Although norovirus is currently the leading foodborne virus of concern, all pathogenic viruses of fecal origin can potentially cause foodborne illnesses ranging from acute gastroenteritis to chronic disease complications such as myocarditis, meningitis, liver disease and neurological symptoms (Newell et al., 2010). In addition, CDC estimates that approximately 80% of foodborne disease cases in the U.S. are caused by unspecified agents (Scallan et al., 2011a) which suggests that a better foodborne disease surveillance system is needed to fill a current knowledge gap concerning unknown and unidentified foodborne agents.

While numerous studies of bacterial contamination of fresh produce have been reported (Allen et al., 2013; Tango et al., 2014; Wijnands et al., 2014; Wood et al., 2015), our understanding of viral contamination of commercial fresh produce remains very limited (Kokkinos et al., 2012; Mattison et al., 2010), partly due to the difficulty in detecting viruses in food matrices. Detection of foodborne viruses has undergone a significant transition from traditional cell culture to molecular techniques, particularly PCR-based assays (Ceuppens et al., 2014; De Medici et al., 2015). However, PCR-based detection is largely restricted to known and well-characterized viruses, which have been sequenced, and there is currently no universal PCR assay that can target all viruses in a single sample. The rapid development of culture- and sequence-independent metagenomics coupled with next generation sequencing (NGS) offers novel and exciting opportunities to enhance our understanding of complex, diverse and dynamic microbial communities including viruses associated with food environments, and thus improve foodborne pathogen identification and potential contamination source tracking (Bergholz et al., 2014). Previous studies investigating microbial

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communities associated with fresh produce using the NGS, mostly 16S rRNA pyrosequencing, have primarily focused on bacteria (Jackson et al., 2013; Leff and Fierer, 2013; Telias et al., 2011) and no study has been conducted to assess the composition of virus communities of raw fresh produce. Until recently, viral metagenomic approaches have only been used to address bacteriophages in fermented food (Park et al., 2011) and plant pathogenic viruses in diseased plants (Coetzee et al., 2010).

A firm understanding of the virus ecology is needed for better defining of the key risk associated with fresh produce vegetables, which are usually consumed raw or minimally processed. In this study, we describe the diversity and composition of virus communities (or viromes), with a particular focus on potential foodborne viruses, associated with lettuce collected from commercial production fields and a produce distribution center using shotgun metagenomic sequencing.

2. Material and methods

2.1. Lettuce sample collection for viral metagenomics

Samples of field-grown iceberg and romaine lettuces were collected directly from the fields in the U.S. during December of 2013. A total of 42 (21 iceberg and 21 romaine) lettuce heads were collected at different stages of farm-level production. A detailed sample description is provided in Table 1. A total of 13 iceberg and 5 romaine lettuce samples were hand cut by field workers and placed on a packaging machine for bagging prior to sampling. A total of 8 romaine lettuce samples were collected after washing and cutting by workers on a processing machine in the field for bagged salad production. In addition, a total of 8 iceberg and 8 romaine lettuce samples were hand cut at ground level by the research team using gloves and a sterile harvesting knife as control samples. The outer leaflets were removed before placing the heads in large sterile Whirl-pak bags (Nasco). The purpose of this was to determine potential sources of viral contamination in lettuce field pre- and post-harvest.

A total of 54 (27 iceberg and 27 romaine) lettuce samples were also collected from a produce distribution center monthly from January to March 2014. Fresh produce vegetables are usually stored in the distribution center no longer than three days before they are distributed to retail stores. During each sampling event, three cartons of each iceberg and romaine lettuce were opened and three samples were taken from each carton. The iceberg lettuce collected in this study was wrapped and sealed in a plastic bag at harvest. All lettuce samples were collected using sterile, disposable gloves and placed into large Whirl-pak bags. All Samples were immediately transported to the laboratory for processing. A total of 96 lettuce samples were processed for viral metagenomics.

2.2. Recovery of viruses from lettuce

The recovery of viruses from lettuce was performed using a concentration method as previously described with modifications (Dubois et al., 2006). Briefly, the outer leaflets were cut with a scalpel 2.5 to 5 cm from the core under sterile conditions. Fifty grams of each sample was washed in a Whirl-pak bag with 250 ml sterile 100 mM Tris — 50 mM glycine buffer at a pH of 9.5 and gently mixed for 20 min at room temperature. The wash solution was recovered and immediately adjusted to neutral pH (7.2 ± 0.2). Viral particles contained in the wash solution were further concentrated and purified by polyethylene glycol (PEG) precipitation. The samples were mixed with 10% (wt/vol) molecular biology grade PEG 8000 (Promega Corporation, Madison WI) and 0.3 M NaCl (*w*/*v*). The samples were incubated at 4 °C for approximately 18 h before centrifuging the sample at 10,800 × *g* (8000 rpm) for 30 min at 4 °C. The supernatant was then carefully poured off and the remaining pellet was dissolved in 20 ml of sterile phosphate buffered saline (PBS) by soaking at room temperature for 1 h. An equal volume of chloroform was added to each PEG precipitate to remove the PEG and purify the sample. The solution was then vortexed gently for 30 s and centrifuged at 3000 × *g* (4300 rpm) for 15 min at 4 °C to collect the supernatant containing virus particles. The remaining supernatant was then passed through 0.45- and 0.22-μm filters and further concentrated to approximately 1 ml by Amicon centrifugal ultrafiltration (30 kDa, Millipore, Billerica, MA).

2.3. Nucleic acid extraction and sequencing

The final concentrates (1 ml) were treated with 100 units of DNase-I (Roche) for 1 h at 37 °C before nucleic acid extraction to remove free nucleic acids from the concentrated virus samples. Viral DNA and RNA were simultaneously extracted using a PureLink viral RNA/DNA mini kit (Life Technologies) following the manufacturer's instructions. For each viral concentrate, three individual nucleic acid extracts were prepared to minimize nucleic acid extraction bias. Following extraction, the samples were screened by 16S ribosomal DNA (rDNA) PCR with 27F/1492R universal primers to ensure the absence of any contaminating microbial DNA. To obtain a sufficient DNA and cDNA (for RNA viruses) for metagenomics sequencing, the viral nucleic acids were reverse transcribed and amplified as previously described (Wang et al., 2002; Wang et al., 2003). Briefly, RNA was reverse transcribed with Primer A (5′-GTTTCCCAGTCACGATCNNNNNNNNN-3′) using Superscript III reverse transcriptase (Life Technologies). Sequenase 2.0 (USB/Affymetrix, Cleveland, OH, USA) was used for second-strand cDNA synthesis and for random-primed amplification of viral DNA. Each sample was then subjected to 40 cycles of PCR amplification with Primer B (5′-GTTTCCCAGT CACGATC-3′) using AmpliTaq Gold (Life Technologies). Three PCR reactions were performed from the same nucleic acid extract to minimize amplification bias and the PCR products were pooled. PCR products were purified using Promega Wizard SV Gel and a PCR Clean-Up System (Promega Corporation).

Libraries from each sample were prepared using a Rubicon ThruPLEX DNA-seq kit (Rubicon Genomics) with a unique dual index adapter pair for each sample. Samples were sequenced in a 2 × 100-base pair (bp) paired end format using two lanes of an Illumina HiSeq 2500 Rapid Run flow cell at the Research Technology Support Facility, Michigan State University.

Table 1
Description of the lettuce samples collected for viral metagenomics, the percentage of viral contigs and number of contig identified as viral pathogens of human and animal for each sample category.

Lettuce type	Sample description	No. of sample	No. (%) of contigs assigned to virus[a]	No. of contig identified as viral pathogens of human and animal
Romaine	Lettuce field (control), lettuce harvested by research team	8	2366 (17.9)	1
	Lettuce field — lettuce harvested by field worker	5	1175 (13.6)	4
	Lettuce field — wash and cut for bagged salad (value-added produce)	8	1658 (10.5)	1
	Produce distribution center	27	3110 (12.5)	56
Iceberg	Lettuce field (control), lettuce harvested by research team	8	2122 (10.9)	2
	Lettuce field — lettuce harvested by field worker	13	2598 (10.0)	1
	Produce distribution center	27	3726 (19.7)	80

[a] Based on the BLASTX analysis against the NCBI Viral Reference Sequence database.