International Journal of Food Microbiology 261 (2017) 73–81

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Application of next generation sequencing toward sensitive detection of enteric viruses isolated from celery samples as an example of produce

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ARTICLE INFO

Keywords:
Foodborne virus detection
RNA isolation
Next generation sequences
Metagenomics

ABSTRACT

Next generation sequencing (NGS) holds promise as a single application for both detection and sequence identification of foodborne viruses; however, technical challenges remain due to anticipated low quantities of virus in contaminated food. In this study, with a focus on data analysis using several bioinformatics tools, we applied NGS toward amplification-independent detection and identification of norovirus at low copy ($< 10^3$ copies) or within multiple strains from produce. Celery samples were inoculated with human norovirus (stool suspension) either as a single norovirus strain, a mixture of strains (GII.4 and GII.6), or a mixture of different species (hepatitis A virus and norovirus). Viral RNA isolation and recovery was confirmed by RT-qPCR, and optimized for library generation and sequencing without amplification using the Illumina MiSeq platform. Extracts containing either a single virus or a two-virus mixture were analyzed using two different analytic approaches to achieve virus detection and identification. First an overall assessment of viral genome coverage for samples varying in copy numbers (1.1×10^3 to 1.7×10^7) and genomic content (single or multiple strains in various ratios) was completed by reference-guided mapping. Not unexpectedly, this targeted approach to identification was successful in correctly mapping reads, thus identifying each virus contained in the inoculums even at low copy (estimated at 12 copies). For the second (metagenomic) approach, samples were treated as “unknowns” for data analyses using (i) a sequence-based alignment with a local database, (ii) an “in-house” k-mer tool, (iii) a commercially available metagenomics bioinformatic analysis platform cosmosID, and (iv) an open-source program Kraken. Of the four metagenomics tools applied in this study, only the local database alignment and in-house k-mer tool were successful in detecting norovirus (as well as HAV) at low copy (down to $< 10^3$ copies) and within a mixture of virus strains or species. The results of this investigation provide support for continued investigation into the development and integration of these analytical tools for identification and detection of foodborne viruses.

1. Introduction

Food has been proposed as an excellent vehicle for virus transmission (Newell et al., 2010), and foodborne transmission has been either documented, implicated or suspected for viruses belonging to at least 10 out of 24 virus families currently known to infect humans (Supplemental Table 1) (Iturriza-Gomara and O'Brien, 2016; Mead et al., 1999; Medicine, 2012; Newell et al., 2010; Scallan et al., 2011). Among these viruses, human norovirus (NoV) and hepatitis A virus (HAV) are the most frequently documented while others such as rotavirus (RV), sapovirus, and astrovirus have been documented with a rarer frequency (Felix-Valenzuela et al., 2012; Iturriza-Gomara and O'Brien, 2016; Medicine, 2012; Scallan et al., 2011). NoV and HAV are the species most frequently associated with, and/or identified in foodborne outbreaks. According to data from 2000 to 2008 reported by the Centers for Disease Control and Prevention (CDC), 59% of foodborne illness in the US is caused by viruses (Scallan et al., 2011). Worldwide, NoV is responsible for at least 95% of nonbacterial gastroenteritis outbreaks, and GII.4 strains have been reported to cause 80% of the norovirus infections (Karst, 2010; Lindesmith et al., 2011; Martella et al., 2013), thus creating a burden and challenge to establishing a safe food supply in the United States as well as the worldwide market.

In the area of food safety, quick and accurate methods to isolate, identify, and sub-type foodborne viruses are important to effectively

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.07.021
Received 19 April 2017; Received in revised form 15 July 2017; Accepted 30 July 2017
Available online 03 August 2017
0168-1605/ Published by Elsevier B.V.

attribute the causes of foodborne illness and their sources, thereby preventing and potentially mitigating outbreaks. These efforts along with methods to purify typically cell culture recalcitrant foodborne viruses are also important for enhanced food surveillance. Thus, development of specific virus extraction methods from the variety of food matrixes (either a particular contaminated food item or complex matrices), along with low viral particle and/or multiple viral species in the same contaminated food presents additional stratification and inherently more complex challenges. A study on shellfish contaminated with NoV and HAV showed that overall quantification levels ranged from 10^2 to 10^6 genomic copies per gram of digestive tissues (Polo et al., 2015). While studies presenting quantitative data with regard to foodborne virus contamination of produce are scarce, there are several investigations providing some information, albeit limited in the number of viral species examined, in this area. Felix-Valenzuela et al. (2012) detected several foodborne viruses by RT-qPCR in fresh produce (46 samples including cilantro, parsley, lettuce, green onions and coriander) from packinghouse facilities. They reported that HAV, RV and NoV were present in 28.2%, 13.0% and 32.6% of the samples, respectively, and concentrations ranged from as low as 10^2 copies/g for HAV up to 10^5 copies/g for RV and in between for NoV. Eleven out of 46 (22%) samples were positive for two viruses, suggesting the occurrence of multiple virus contamination in produce. El-Senousy et al. (2013) examined fresh produce items (included green onion, watercress, radish, leek, and lettuce, 144 samples of each type) and reported that NoV was presented in 20.8–34.0% of each type of produce with genome copy numbers around 10^2 copies per gram. Maunula et al. (2009) examined frozen raspberries associated with an outbreak of NoV illness and demonstrated the presence of this virus, although at low levels. The viral load ranged from 10^2 to 10^6 genomic copies per gram of food specimens from the studies above could potentially pose risks/threats for foodborne transmission, since minimum infective dose (minimum dose of virus particles that can initiate infection) could be as low as 10 and 100 viral particles for HAV and RV and even less for NoV (as reviewed Yezli and Otter, 2011).

The majority of methods, published or validated, for foodborne virus analysis, incorporate viral (particle or genome) extraction typically applied in conjunction with concentration/isolation procedures for subsequent detection and identification by methods such as RT-qPCR or derivatives of this technique. As the current techniques often involve an amplification step with virus specific primers, as well as inclusion of a nucleotide probe for quantitative reactions, there is a priori knowledge required of the viral agent in question; thus potentially limiting the number of targetable viral species and/or strains that can be detected and identified by this approach. The use of degenerate primer/probe sequences and random primers, individually or in combination, has broadened the "capture range" of these techniques. However, this approach may further lead to amplification bias which favors the dominant target, resulting in underrepresentation of a minor target(s). Subsequently, nucleotide sequencing is often applied to the standard and quantitative RT-PCR amplicons for identification or genotyping of a foodborne viral contaminant. There are a number of technologies envisioned as adjuncts to RT-qPCR in our and other laboratories (Coudray-Meunier et al., 2016). Whole viral genome sequencing is one of these techniques and there is great interest in its potential application to foodborne virus detection.

Next generation sequencing (NGS) technologies are capable of comprehensively interrogating genomes without prior knowledge of their sequence of interest (e.g., foodborne virus) or annotation, thus offering great potential for foodborne virus applications after their extraction from diverse origins, including both clinical (Capobianchi et al., 2013; Rutvisuttinunt et al., 2013) and environmental samples (Lopez-Bueno et al., 2009; Pantaleo et al., 2010; Rodriguez-Brito et al., 2010; Wong et al., 2013). However, these studies have incorporated amplicon-sequencing of the partial capsid N/S region by RT-PCR. For example, NGS has been used to profile norovirus in pre- and post-depurated oysters (Imamura et al., 2016a), as well as to investigate the diversity of norovirus genotypes/genogroups present in shellfish (Imamura et al., 2016b). In another example, NGS was also applied to detect and identify HAV isolated from frozen berries implicated in an outbreak of infectious hepatitis in Northern Italy (Chiapponi et al., 2014). In that study, HAV extracted from berry samples was first inoculated onto Frp/3 (Fetal rhesus monkey kidney, FRhK-4 derivative) cells, and then isolated from culture supernatant after passaging. NGS was performed on amplicons from four PCR reactions targeting conserved regions of HAV full-length genomes, and a full-length HAV genomic sequence was obtained in an assembled contig. Hence, amplification was incorporated into the analytic procedure using a combination of (limited) replication in culture and molecular based (PCR) amplification to achieve sufficient material for sequencing. Aw et al. (2016) applied a metagenomic approach for the analysis of viruses associated with field-grown and retail lettuce. The viral nucleic acids recovered from lettuce samples were reverse transcribed, amplified and then re-amplified using a combination of random and defined priming strategies before NGS was performed. Following de novo assembly, contigs larger than 200 bp were BLASTed against viral RefSeq database in NCBI in order to achieve identification of rotavirus, as well as several other animal viruses, in the samples.

As an alternative approach to generating pooled amplicons, our laboratory has focused on direct extraction of virus from foods without prior amplification of the viral target using shotgun NGS (Yang et al., 2016a). However, little has been published in the area of culture- and amplification-independent sequencing of extracted material with the purpose of identifying specific viral target(s). The objective of this study was to optimize sequencing protocols for data generation, and its bioinformatic analysis in order to obtain and develop sensitive yet amplification-independent virus detection by sequencing. In this investigation we used inoculums of virus mixtures containing varying ratios of virus over a range of virus concentrations comprised of either two NoV genotypes, a GII.4 and a GII.6, or two different virus species, NoV GII.4 and the HAV strain HM175/18f.1. We provide results demonstrating the successful application of our amplification-independent sequencing method and in-house bioinformatics tools toward detection and identification of norovirus at high and low copy as a single or as a multi-strain mixture extracted from food.

2. Materials and methods

2.1. Viruses

The human norovirus strains used in this study were GII.P4-GII.4 Minerva 2006 (identified as GII.4 in this study for brevity) obtained from Dr. J. Vinje (Yang et al., 2016b) and GII.P7-GII.6 (identified as GII.6) obtained from a sporadic case of gastrointestinal illness (Yang et al., 2016c). The HAV strain HM175/18f.1 variant is a rapidly replicating cytopathic variant derived from a culture-adapted strain of HAV/HM175 (Cromeans et al., 1987; Lemon et al., 1991) purchased from the American Type Culture Collection (ATCC) (Manassas, VA), subjected to several sequential passages and generation of a purified, high stock for experimental usage (GenBank accession number KX035096).

2.2. Viral inoculation and extraction from foods, and RNA isolation

Celery was purchased from a grocery store and stored at 4 °C until use. The stalk of the celery, trimmed of leafy section, was cut into pieces each approximately 3 in. in length where 3–4 pieces comprised a 50 g sample. Norovirus positive stool samples were suspended in 10% phosphate-buffered saline and centrifuged at 9000 × *g* for 3 min to obtain clarified supernatant containing norovirus. Fifty gram celery samples were inoculated with a 100 µl of solution (either stool suspension or culture stock diluted in water) distributed containing either

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