International Journal of Food Microbiology 257 (2017) 80–90

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

A metagenomic assessment of viral contamination on fresh parsley plants irrigated with fecally tainted river water

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ARTICLE INFO

Keywords:
Food metagenomics
Viral pathogens
Food safety
Caliciviridae
Picornaviridae
Next generation sequencing

ABSTRACT

Microbial food-borne diseases are still frequently reported despite the implementation of microbial quality legislation to improve food safety. Among all the microbial agents, viruses are the most important causative agents of food-borne outbreaks. The development and application of a new generation of sequencing techniques to test for viral contaminants in fresh produce is an unexplored field that allows for the study of the viral populations that might be transmitted by the fecal-oral route through the consumption of contaminated food. To advance this promising field, parsley was planted and grown under controlled conditions and irrigated using contaminated river water. Viruses polluting the irrigation water and the parsley leaves were studied by using metagenomics. To address possible contamination due to sample manipulation, library preparation, and other sources, parsley plants irrigated with nutritive solution were used as a negative control. In parallel, viruses present in the river water used for plant irrigation were analyzed using the same methodology. It was possible to assign viral taxons from 2.4 to 74.88% of the total reads sequenced depending on the sample. Most of the viral reads detected in the river water were related to the plant viral families *Tymoviridae* (66.13%) and *Virgaviridae* (14.45%) and the phage viral families *Myoviridae* (5.70%), *Siphoviridae* (5.06%), and *Microviridae* (2.89%). Less than 1% of the viral reads were related to viral families that infect humans, including members of the *Adenoviridae*, *Reoviridae*, *Picornaviridae* and *Astroviridae* families. On the surface of the parsley plants, most of the viral reads that were detected were assigned to the *Dicistroviridae* family (41.52%). Sequences related to important viral pathogens, such as the hepatitis E virus, several picornaviruses from species A and B as well as human sapoviruses and GIV noroviruses were detected. The high diversity of viral sequences found in the parsley plants suggests that irrigation on fecally-tainted food may have a role in the transmission of a wide diversity of viral families. This finding reinforces the idea that the best way to avoid food-borne viral diseases is to introduce good field irrigation and production practices. New strains have been identified that are related to the *Picornaviridae* and distantly related to the *Hepeviridae* family. However, the detection of a viral genome alone does not necessarily indicate there is a risk of infection or disease development. Thus, further investigation is crucial for correlating the detection of viral metagenomes in samples with the risk of infection. There is also an urgent need to develop new methods to improve the sensitivity of current Next Generation Sequencing (NGS) techniques in the food safety area.

1. Introduction

Food-borne diseases remain a significant cause of illness worldwide, and consumers are exposed to microbiological and chemical contaminants. From a microbiological point of view, food can be a vehicle for protozoan, bacterial, viral, and prion infections. Although most fecally excreted microorganisms cause gastroenteritis or acute hepatitis, other pathologies such as meningitis, myocarditis, and neurological disorders are also possible.

Food contamination can occur at several stages of food chain production, from the irrigation and collection stages on farms to contamination during food processing in industrial settings, food

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.06.001
Received 24 December 2016; Received in revised form 30 May 2017; Accepted 3 June 2017
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preparation at a restaurant, or at home. In high income countries, measures have been implemented to reduce the risk of fecal contamination of water and food such as proper sewer pipeline systems and hygienic measures during food handling, manufacturing, and preparation. Countries use legislation to measure the microbiological quality of water and food, yet food-borne outbreaks are still reported (Bernard et al., 2014; Ethelberg et al., 2010).

Among all the food-borne etiological agents, viruses are the most important causative agents of food-borne outbreaks with noroviruses accounting for 125 million cases a year (Kirk et al., 2015; Painter et al., 2013). The increase in fresh food consumption, probably as a way for consumers to develop a healthier diet, has been linked to an increase in viral food-borne outbreaks (Callejón et al., 2015; Kozak et al., 2013). Coleman et al. (2013) noted that of the 127 outbreaks associated with leafy greens in the United States from 2004 to 2008, 64% of cases were attributed to a viral infectious agent such as noroviruses, sapoviruses, and hepatitis A virus. As mentioned, viruses can be accidentally introduced at different steps in food chain production, and crop irrigation with fecally contaminated water is one of the most critical points. Recently, Maunula et al. (2013) reported that 9.5% of irrigation water samples used to water berries were positive for human adenoviruses (HAdV), underlining the presence of fecal contamination. Although there are laws to control the microbiological water quality, none of them currently include specific viral parameters, and, therefore, water safety monitoring relies only on the use of fecal indicator bacteria (FIB). The usefulness of these laws for minimizing the viral presence in food matrices is unclear because the FIB do not always correlate with the presence of viral pathogens, and viruses are more resistant to water treatments than bacteria (Gerba et al., 1979; Pusch et al., 2005; Savichtcheva and Okabe, 2006).

While bacterial contamination in food has been widely reported, notifications of viral outbreaks have been hampered in many cases by a lack of specific, sensible, and standardized concentration/detection methods. These important factors are mandatory for the inclusion of viral parameters in food legislation. Recently, a standard ISO 15216-1:2017 was published for the concentration and quantification of the two food-borne viruses hepatitis A virus (HAV) and human noroviruses (HNoV) (https://www.iso.org/standard/65681.html), and qualitative detection is under revision (ISO/TS 15216-2:2013). The concentration methods are limited by their low recoveries while detection and quantification methods, which are usually based on RT-PCR or RT-qPCR, are restricted to specific targeted viruses. Many different pathogens may contaminate water and food simultaneously, especially if fecally contaminated irrigation water is used on fresh vegetables. The introduction of next generation sequencing (NGS) techniques in the food safety field allows for the simultaneous analysis of diverse viral pathogens in a single assay. To date, NGS has been applied to study the viral species that are present in all types of environmental and clinical samples as follows: oceans (Hurwitz et al., 2013), lakes (Djikeng et al., 2009), raw sewage (Cantalupo et al., 2011), reclaimed water (Rosario et al., 2009), and infectious clinical samples with unknown etiological agents (Greninger et al., 2015). However, the use of NGS in the food safety field has not been exhaustively explored (Aw et al., 2016; Park et al., 2011; Zhang et al., 2014).

The focus of the present study is to provide more information on the applicability of NGS techniques in the food safety field by studying viral contamination in fresh vegetables, with parsley plants (*Petroselinum crispum* L.) that were irrigated with river water containing fecal contamination as a model.

2. Materials and methods

2.1. River water samples

The Besòs River (Sant Adrià de Besòs, Barcelona, Catalonia, Spain) is a 17.7 km long, and it displays irregular discharge due to the Mediterranean climate. Along its path, the river collects the secondary effluent of 27 wastewater treatment plants (WWTP) and ends in the Mediterranean Sea next to Barcelona. Since the mid-1990, it has been subjected to a recovery process to improve its water quality. As a consequence of these efforts, the Besòs River is being used to irrigate crops by some local farmers. Twenty liter river water samples from the Besòs River were collected in May 2014. Ten liters of each river sample was used to irrigate the parsley plants, and the remaining 10 L was used to concentrate the viral particles to characterize the viruses that were present through qPCR and NGS. After two weeks, the procedure was repeated. The river water samples collected for parsley irrigation were kept in the dark at 4 °C for 15 days and used to irrigate the plants.

2.2. River water viral particle concentration

The viruses present in the 10 L river water samples were concentrated using skimmed milk organic flocculation (SMF). This method has a recovery efficiency of 50% (20–95%) and it was applied as previously described by Calgua et al. (2013). Viral concentrates were suspended in 8 mL of phosphate buffer (pH 7.5) and stored at −80 °C until further use. The two river water viral concentrates were labeled as the Besòs River water (BRW1 and BRW2) samples.

2.3. Parsley plant growth and irrigation

Parsley (*Petroselinum crispum* L.) seeds were planted and cultivated in a climate room at 22 °C, 60% relative humidity, and light conditions equivalent to 110 μmol of photosynthetically active radiation (PAR) at the “Serveis de Camps Experimentals” at the University of Barcelona. The seeds were irrigated using a nutritive solution containing iron, nitrogen and phosphorus (Hoagland solution 50%). After 6 weeks, a total of 12 different parsley pots were moved to a greenhouse from the same facility.

All 12 parsley pots were irrigated twice a week during the whole growth process (from May to June) by using 4 L of the same nutritive Hoagland solution. This irrigation procedure was performed by inundating the tray containing the parsley pots, and thus the parsley leaves were not washed. Half of the 12 pots were irrigated daily, in addition to receiving nutritive solution, by spraying the leaves with 15 mL of Besòs River water. These samples were labeled as Besòs River Parsley (BRP).

In the same way, the remaining pots were used as a Negative Control Parsley group (NCP), and they were irrigated daily by spraying 15 mL of the same nutritive solution that was used for irrigation, but by inundation, into both the control and test pots. The negative control was used as a blank sample to identify viral sequences that could be naturally present in the parsley plants or due to other external factors (greenhouse, irrigation nutritive solution, facility users, manipulation, reagent contaminants or equipment).

Two weeks later, a fresh BRW sample was collected to continue the irrigation process for another 15 days. At the end of the study, both plant groups were irrigated with 450 mL of river water or control nutritive solution water. After one month of daily irrigation, 25 g of parsley leaves from NCP and BRP were hand-cut by investigators who were wearing sterile gloves, and the leaves were placed in sterile bags (BagPage® filter-bag, Interscience, France). The samples were kept at 4 °C for < 48 h until the concentration method was applied.

2.4. Viral concentration from plants

Twenty-five grams of NCP and BRP were washed in a sterile filter-bag with 50 mL of glycine buffer (pH of 9.5, 0.25 N) for 40 min using a stomacher. Afterwards, the sample pH was adjusted to 7.0 (± 0.2) by using HCl 0.1 N. To remove bacteria and other suspended organic material, the samples were centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was carefully collected without disturbing the pellet

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