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Short communication

Occurrence of hepatitis A and E and norovirus GI and GII in ready-to-eat vegetables in Italy

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ABSTRACT

Fresh vegetables and their ready-to-eat (RTE) salads have become increasingly recognized as potential vehicles for foodborne diseases. The EU Reg. 1441/2007 establishes microbiological criteria for bacterial pathogens for products placed on the market during their shelf-life (i.e. *Salmonella* spp., *Listeria monocytogenes*) for pre-cut fruits and vegetables (RTE) whilst it does not address the problem of contamination by enteric viruses.
In this study we investigated the contamination by hepatitis A virus (HAV), hepatitis E virus (HEV) and norovirus (NoV) in 911 ready-to-eat vegetable samples taken from products at retail in Apulia and in Lombardia.
The vegetable samples were tested using validated real-time PCR (RT-qPCR) assays, ISO standardized virological methods and ISO culturing methods for bacteriological analysis.
The total prevalence of HAV and HEV was 1.9% (18/911) and 0.6% (6/911), respectively. None of the samples analysed in this study was positive for NoV, *Salmonella* spp. or *Listeria monocytogenes*. The detection of HAV and HEV in RTE salads highlights a risk to consumers and the need to improve production hygiene.
Appropriate implementation of hygiene procedures is required at all the steps of the RTE vegetable production chain and this should include monitoring of emerging viral pathogens.

1. Introduction

Leafy green vegetables and their ready-to-eat (RTE) salads are important components of the current human diets but are accompanied by new food safety threats since they are eaten raw and usually without any further washing/decontamination procedures (Little and Gillespie, 2008). Apart from psychrotrophic pathogens and spoilage microorganisms, RTE vegetables can be contaminated with a number of human pathogens, including parasites, bacteria and viruses.

In Italy, the prevalence of bacterial pathogens in leafy green vegetables has been estimated between 3.7 for fresh and 1.8% for RTE products (Losio et al., 2015). Numerous foodborne virus outbreaks have been linked to the consumption of fresh produce, mostly attributable to enteric viruses such as norovirus (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), rotavirus (RV) and astrovirus (AstV) (European Food Safety Authority and European Centre for Disease Prevention and Control, 2013; Chiapponi et al., 2014; Collier et al., 2014; European Food Safety Authority, 2014; Kokkinos et al., 2012; Terio et al., 2015; Wheeler et al., 2005).

Enteric viruses may contaminate vegetables, during cultivation before harvest or post-harvest. During pre-harvest cultivation, there are various routes of contamination, which usually include application of organic wastes as fertilizer, contamination of water used for irrigation with faecal material, contact with inadequately-treated sewage or sewage-polluted water. In addition, direct contamination by livestock, wild animals and birds should be considered (Heaton and Jones, 2008).

Water is the main critical vehicle of contamination in the farm-to-fork continuum. Spraying, washing or immersion of fruits and vegetables in water are common practices during post-harvest processing (Gandhi et al., 2010). Moreover, minimal processing may induce cross-contamination of clean produce during cutting, washing and packaging (Lopez-Velasco et al., 2010). In addition, much emphasis has also been placed on the role of workers during collection, processing, storage, distribution or final preparation (European Food Safety Authority BIOHAZ Panel, 2014; Koopmans and Duizer, 2004; Rzezutka and Cook, 2004).

Currently, the European legislation on the safety of pre-cut (ready-to-eat) fruit and vegetables requires the assessment of *Salmonella* spp.

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and *Listeria monocytogenes* contamination at the end of the production chain (at retail). In addition, the *Escherichia coli* count is required during the manufacturing processes (Commission Regulation (EC) No. 1441/2007). The regulations do not take into consideration the risks deriving from food contamination by enteric or emerging viral pathogens, although there is a growing attention and interest for this important risk, as evidenced by recent NoV and HAV outbreaks in Europe (European Centre for Disease Prevention and Control and the European Food Safety Authority, 2014; Müller et al., 2016). Limited data exist in the literature on the prevalence of foodborne viruses in RTE vegetables. In Italy, a study on RTE at retail has been reported recently, revealing a very low rate (<0.1%) of NoV contamination (Losio et al., 2015). The purpose of this study was to investigate the presence of enteric viruses (HAV, HEV and NoV) in RTE vegetables available for sale in Italy.

2. Materials and methods

2.1. Sampling

A total of 911 samples of bagged ready-to-eat vegetables belonging to different brands and purchased from supermarkets in Apulia and Lombardy regions, Italy, were collected during 2014–2015. They included 619 mixed salads, 53 carrot (*Daucus carota* subsp. *sativus*), 40 valerian (*Valeriana officinalis*), 104 rocket (*Eruca vesicaria*), 10 spinach (*Spinacia oleracea*), 18 Iceberg (*Lactuca sativa*) and 67 Romaine lettuce (*Lactuca sativa* L var. longifolia). All samples were labelled as "Pre-washed and ready-to-eat". All samples were obtained in their original packaging and analysed before the expiration date (up to 8 days). Samples were transported to the laboratory in refrigerated boxes (<8 °C) and analysed on the day of purchase.

2.2. Isolation of Salmonella spp. and Listeria monocytogenes

Vegetable samples were subjected to isolation of the pathogenic foodborne microorganisms *Salmonella* spp. and *Listeria monocytogenes*, using EN/ISO 6579 and EN/ISO 11290-1, respectively.

2.3. Virus concentration and nucleic acid extraction

Viral RNA was extracted following the ISO/TS 15216-2:2013 method for NoV and HAV detection in foodstuffs. In brief, 25 g of each sample were cut into small pieces and homogenized with TGBE buffer pH 9.5 (100 mM Tris–HCl, 50 mM glycine, 1% beef extract) and 10 μl of process control virus material (Mengovirus). The eluate was concentrated with 5× PEG/NaCl solution (50% (w/v) PEG 8000, 1.5 M NaCl) and the viral nucleic acids were extracted and purified using commercial kits (NucliSENS miniMAG kit, bioMérieux) according to the manufacturer's instructions.

2.4. Reverse transcriptase-polymerase chain reaction

Reverse transcription of viral RNA was performed using High-Capacity cDNA Reverse transcription Kit (Applied Biosystems, Italy), containing 10× RT Buffer II, 5 mM $MgCl_2$, 1 mM dNTPs, 2.5 μM random hexamers, 20 U RNAse inhibitor and 50 U Reverse transcriptase according to the manufacturer's instructions. The reaction conditions were 42 °C for 30 min and 99 °C for 5 min. The obtained cDNA was used for specific real-time PCR (RT-qPCR) for each target virus: HAV, HEV, NoV GI and NoV GII.

The RT-qPCR reactions were performed in duplicate. All tests included negative controls for virus and for an internal amplification control (IAC).

2.5. Hepatitis A virus qPCR

This assay was performed using the primers and conditions described in the ISO/TS 15216-2:2013 method with the inclusion of an internal amplification control (IAC). The reaction contained 1× iTaq™ Universal Probes Supermix (Bio-Rad), 0.5 μM primer HAV68, 0.9 μM primer HAV240; 0.25 μM probe HAV150 (−) (FAM labelled) (Costafreda et al., 2006), 50 nM IAC probe (VIC labelled) and 300 copies of IAC (Martinez-Martinez et al., 2011). 20 μl of cDNA was added to make a final reaction volume of 50 μl. The thermocycling conditions were 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C and 1 min at 65 °C.

2.6. Hepatitis E virus qPCR

This assay used the primers and conditions described by Jothikumar et al. (2006) with the inclusion of an internal amplification control (IAC). The reaction contained 1× iTaq™ Universal Probes Supermix (Bio-Rad), 0.25 μM for each primer (JVHEVF and JVHEVR), 0.1 μM probe JVHEV-P (labelled with FAM), 50 nM IAC probe (labelled with VIC) and 300 copies of IAC (Martinez-Martinez et al., 2011). 20 μl of cDNA was added to make a final reaction volume of 50 μl. The thermocycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

2.7. Norovirus GI and GII qPCR

This assay was performed using the primers and conditions described in the ISO/TS 15216-2:2013 method with the inclusion of an internal amplification control (IAC). The reaction was carried out using 1× iTaq™ Universal Probes Supermix (Bio-Rad), 250 nM probe (labelled with FAM), 500 nM forward primer and 900 nM reverse primer, 50 nM IAC probe (labelled with VIC) and 300 copies of IAC (Martinez-Martinez et al., 2011).

Primers targeted the ORF2 region; for NoV GI: forward primer QNIF4, reverse primer NV1LCR and probe NV1LCpr were employed; for NoV GII, forward primer QNIF2d, reverse primer COG2R and probe QNIFS were used (da Silva et al., 2007; Kageyama et al., 2003; Loisy et al., 2005; Svraka et al., 2007).

20 μl of cDNA was added to make a final reaction volume of 50 μl. The thermocycling conditions were 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C and 1 min at 65 °C.

2.8. Extraction efficiency

For each sample, two aliquots of 5 μl nucleic acid (NA) extract were added to adjacent wells of a 96-well optical reaction plate and made up to 25 μl with Mengovirus-specific TaqMan reaction mix. A dilution series prepared from the Mengovirus process control material was also tested. The percentage extraction efficiency for each sample was determined by comparing the Ct values for the sample NA extract with those for the Mengovirus dilution series. Any sample with an extraction efficiency of <1% was subjected to retesting, in a first instance by re-extracting the viral RNA from stored homogenate, then by testing the stored RTE salads. Results for any sample providing three extraction efficiency results of <1% were considered invalid.

3. Results

3.1. Isolation of Salmonella spp. and Listeria monocytogenes

The pathogenic foodborne microorganisms *Salmonella* spp. and *L. monocytogenes*, were not detected in any RTE salads.