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Presence of pathogenic enteric viruses in illegally imported meat and meat products to EU by international air travelers

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ABSTRACT

One hundred twenty two meat samples confiscated from passengers on flights from non-European countries at the International Airport of Bilbao (Spain) were tested for the presence of the main foodborne viral pathogens (human noroviruses genogroups I and II, hepatitis A and E viruses) during 2012 and 2013. A sample process control virus, murine norovirus, was used to evaluate the correct performance of the method. Overall, 67 samples were positive for at least one enteric viruses, 65 being positive for hepatitis E virus (53.3%), 3 for human norovirus genogroup I (2.5%) and 1 for human norovirus genogroup II (0.8%), whereas hepatitis A virus was not detected in any sample. The type of positive meat samples was diverse, but mainly was pork meat products (64.2%). The geographical origin of the positive samples was wide and diverse; samples from 15 out 19 countries tested were positive for at least one virus. However, the estimated virus load was low, ranging from 55 to 9.0×10^4 PDU per gram of product. The results obtained showed the potential introduction of viral agents in travelers' luggage, which constitute a neglected route of introduction and transmission.

1. Introduction

Gastroenteritis caused by enteric viruses is an emerging and serious public health issue, as it is estimated that they are responsible of more than 5.5 million of foodborne illness episodes in USA annually (Scallan et al., 2011), and only one type, human noroviruses, accounts for 5,461,731 cases, representing an estimated annual cost of $2 billion for medical costs, productivity losses, and illness-related mortality (Hoffmann et al, 2012). Similarly, the number of outbreaks related to foodborne viruses has increased during the last years in Europe; enteric viruses were the third cause of foodborne outbreaks in 2012 (from 525 in 2011 to 756 in 2012), and the largest foodborne outbreak reported that year was due to frozen strawberries contaminated with norovirus in Germany (almost 11,000 people were affected) (European Food Safety Authority & European Centre for Disease Prevention and Control, 2014).

In this context, the potential source of foodborne pathogenic agents, and in particular of human enteric viruses, is widening, as neglected routes of transmission exist (e.g. cross-border routes). Microbial threats can be introduced by post or carried in the baggage of travelers arriving from countries outside the EU by personal consignments containing meat, milk or products thereof (Mangili and Gendreau, 2005). Nowadays, over 1 billion people travel in commercial flights every year (Mangili and Gendreau, 2005), and consequently the frequent global travel can facilitate the spread of infectious diseases by passengers and the introduction of infectious diseases from endemic countries to third ones. There have been cases described in the literature of viral diseases introduction within products carried on a plane (Pavlin et al., 2009; Smith et al., 2012). Furthermore, after the introduction of those products, they can be intended to be illegally sold in local markets with no sanitary control or verification, constituting a neglected route of introduction and transmission of foodborne pathogens. As a result, marketing of unauthorized food which has not passed the appropriate controls constitutes a neglected route of foodborne pathogen transmission. Regulatory measures as Commission Regulation (EC) No. 206/2009 try to prevent the entrance of animal-borne food products carried in the baggage of travelers arriving from countries outside the EU in the form of personal consignments. However, there is evidence of (neglected) illegal entrance to EU of different foodborne pathogens via foods introduced via international flights or cross-border routes (Beutlich et al., 2015; Oniciuc et al, 2015; Rodríguez-Lázaro et al., 2015a,b; Schoder et al., 2015), and recently a study reported the introduction of zoonotic viruses through illegally imported products in USA

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(Smith et al., 2012). In this study, we evaluated for the first time the presence of the main foodborne pathogenic viruses (i.e. human noroviruses, hepatitis A and E viruses) in food confiscated from non-EU flight passengers from April 2012 to June 2013 at an international airport (Bilbao, Spain). This information can provide an overview on the prevalence and the potential risk of foodborne viruses in illegally imported food.

2. Materials and methods

2.1. Food samples

The food samples consisted of illegally imported meats confiscated to passengers on flights from nineteen non-EU countries by the border authorities at the International Bilbao Airport (Spain) (www.aeropuertodebilbao.net) during April 2012 to June 2013, following the Commission Regulation (EC) No. 206/2009. In total, 122 meat samples of diverse animal origins (mainly dried or frozen raw meat samples including antelope, beef, chicken, duck, guinea pig, pork, rodents, and turkey) were tested. The origin of the meat samples was wide: Africa — Equatorial Guinea (7) and Morocco (2), Central and South America — Argentina (8), Bolivia (16), Brazil (11), Colombia (3), Dominican Republic (2), Ecuador (11), Paraguay (1), Peru (9), and Venezuela (1), Asia — People's Republic of China (31), Nepal (1), and Turkey (1), Eastern Europe — Bosnia and Herzegovina (3), Moldavia (7), Serbia (3), and Ukraine (3), Oceania-Australia (1), and one sample from an unknown origin (flight came from Paris-France).

2.2. Sample process control virus

A sample process control virus (SPCV) was added to each sample immediately before the start of the analysis. It was murine norovirus 1 (MNV-1) (Diez-Valcarce et al., 2011a), which had been propagated in RAW264.7 cells to a concentration of 10^7 $TCID_{50}$/ml, and an inoculum containing approximately 3×10^3 $TCID_{50}$ was added to each sample.

2.3. Virus concentration and nucleic acid extraction

The meat samples were collected (1 cm^3 from three different locations) and stored in a sterile plastic bag. The extraction procedure was based on a mechanical disruption of the tissues followed by a silica-membrane-based RNA extraction (Bouwknegt et al., 2007; Di Bartolo et al., 2012). Briefly, each sample (approximately 1 g) was finely chopped using a sterile razor blade, and then placed in a sterile RNase-free mortar together with 4 ml of buffer RLT (RNeasy Midi Kit, QIAGEN, Hilden Germany) containing 1:100 β-mercaptoethanol for homogenization. Two-hundred fifty milligrams of homogenate was transferred into a 1.5 ml microcentrifuge tube containing 1 ml buffer RLT and 2.5 g of sterile 1 mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA). Twenty microliters of MNV-1 (~3×10^3 $TCID_{50}$) was added to each tube. The tube was then placed into a mechanical disruptor (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA) and subjected to 2 cycles at a speed of 4 $m \times s^{-1}$ for 40 s. After centrifugation at 10,000 ×*g* for 20 min, approximately 800 μl of the aqueous phase was transferred to a new tube and centrifuged again. The resulting supernatant was used for immediate nucleic acid extraction using RNeasy Midi Kit (QIAGEN) following manufacturer instructions, and the final 300 μl RNA extract was assayed immediately or stored at −70 °C.

2.4. Virus detection by RT-qPCR

The presence of the target enteric viruses – hepatitis A virus, HAV; hepatitis E virus, HEV; human norovirus genogroups I and II, NoV GI and NoV GII – and the SPCV (MNV-1) was evaluated using reverse transcription real-time PCR (RT-qPCR). One-step duplex RT-qPCRs were performed using the oligonucleotides, controls and conditions previously described (Martínez-Martínez et al., 2011; Diez-Valcarce et al., 2011b, 2012). Nucleic acid obtained was assayed both undiluted and diluted ten-fold, and the RT-qPCRs were performed in duplicate. All RT-qPCRs were conducted in a duplex format, targeting the specific viruses (HEV, HAV, NoV GI, NoV GII, or MNV-1) with an FAM-labeled probe and the chimerical internal amplification control (IAC), using a VIC-labeled probe. All tests also included negative controls for viruses and for IACs.

2.5. Reporting and interpretation of data

For a proper interpretation of the results four different signals were considered: i. the target virus; ii. the SPCV virus; iii. the target IAC; and iv. the SPCV IAC (D'Agostino et al., 2011). When a PCR assay showed a Cq value ≤ 45, independently of the corresponding IAC Cq value, the result was interpreted as positive. When an assay showed a Cq value ≥ 45 with the corresponding IAC Cq value ≤ 45, the result was interpreted as negative. When both the target and its corresponding IAC showed Cq values ≥ 45, the reaction was considered to have failed. When at least one of the replicate target assays (for HAV, HEV, NoV GI, NoV GII) was positive, the sample was considered to be positive. In the absence of signals for SPCV and its IAC, the pre-amplification process (virus concentration and extraction steps) was concluded to have failed (D'Agostino et al., 2011). In case signals for SPCV and its IAC, and target IAC were present, the absence of target virus signal was conclusively considered as a test negative result.

2.6. Extraction efficiency

The extraction efficiency was calculated by comparing the Cq value (quantification cycle, previously known as the threshold cycle) of a meat sample containing the control (SPCV) with the Cq value of the SPCV alone, just spiked in the reagents used for concentration and extraction of the sample but without any food matrix (chopped meat sample) using the following formula: $2^{(Cq\ TNPC - Cq\ sample)} \times 100$ (Diez-Valcarce et al., 2012). Efficiency results were classified as insufficient (extraction efficiency <5%), acceptable (5–25%), good (25–50%) and very good (>50%). Extraction efficiencies lower than 5% were not acceptable and the pre-amplification process (virus concentration and extraction) of the given sample was repeated.

2.7. Estimation of virus concentration

The number of viruses per gram of meat sample was estimated using the most probable number-like approach (Teunis et al., 2005). Presence/absence profiles for target viruses were generated per sample by examining neat and serial 10-fold dilutions of nucleic extracts of samples until the end-point dilution, in duplicate. The unit of quantification was a PCR detectable unit (PDU), which represents an unknown number of target genomes (under ideal amplification conditions and a perfect assay, a single PDU would represent a single virus genome). It was assumed that when viruses were present, they were distributed homogeneously within the sample. The presence/absence pattern for the *n* examined dilutions per sample was combined according to the following formula:

$$l = \prod_{i=1}^{n} \left(1 - e^{-C \times V_i}\right)^{p_i} \left(e^{-C \times V_i}\right)^{1-p_i}$$

where *l* is the likelihood function, *n* is the number of examined dilutions, *C* is the estimated virus concentration, V_i is the actual volume of sample examined per reaction and p_i denotes presence ($p_i = 1$) or absence ($p_i = 0$) of target amplification in dilution *i*. For this estimation a confidence interval of 95% was applied, so limits, upper and lower, of the likely concentration of virus were obtained.