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Comparison of three extraction methods to detect noroviruses in dairy products

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ABSTRACT

Noroviruses (NoV) are currently the most common cause of viral foodborne diseases and RT-qPCR is widely used for their detection in food because of its sensitivity, specificity and rapidity. The ISO/TS (15216-1, 15216-2) procedures for detecting NoV and HAV in high-risk food categories such as shellfish, bottled water and vegetables were published in 2013. Milk products are less implicated in foodborne viral outbreaks but they can be contaminated with fruit added to these products or by the food handler. Thus, the development of sensitive and reliable techniques for the detection of NoV in dairy products is needed to ensure the safety of these products. The aim of this study was to develop a RT-qPCR based method for the detection of NoV in milk products. Three methods were tested to recover NoV from artificially contaminated milk and cottage cheese. The selected method was based on the use of proteinase K and the recovery efficiencies ranged from 54.87% to 98.87% for NoV GI, 61.16%–96.50% for NoV GII. Murine norovirus and mengovirus were used as process controls and their recovery efficiencies were respectively 60.59% and 79.23%. The described method could be applied for detecting NoV in milk products for routine diagnosis needs.

1. Introduction

Gastroenteritis is a major public health problem worldwide and human enteric noroviruses (NoV), which belong to the *Caliciviridae* family, are the most common agents of foodborne gastroenteritis. Human NoV belong to genogroups GI, GII and GIV; GII is the most prevalent among cases of NoV infection. NoV are predominant among young children and the elderly. NoV are responsible for 58% of domestically-acquired foodborne illnesses in the United States (CDC http://www.cdc.gov/norovirus/php/illness-outbreaks.html) and their importance has been recognized in European countries.

NoV are highly contagious with a very low infectious dose of less than 10 particles (Teunis et al., 2008). Transmission of NoV occurs primarily *via* the fecal-oral route, including direct person-to-person contact, consumption of contaminated food or water, or contact with contaminated environmental surfaces. NoV transmission is facilitated by the shedding of infectious viral particles (from asymptomatic individuals as well as before, during, and after the manifestation of symptomatic infections) (Barrabeig et al., 2010) and the high stability of NoV in the environment. NoV are most often associated with contamination in food-service settings. Food contamination mainly occurs in restaurants during preparation by infected food workers.

In France, in addition to high-risk food categories such as bivalve mollusks, fruits, vegetables, meat or water, dairy products are responsible for 7% of foodborne illnesses and about 2% of foodborne disease outbreaks due to viral agents (InVS, 2012).

ISO procedures (ISO/TS 15216-1, 15216-2) only propose standard methods for detecting NoV in high-risk food categories. The development of sensitive and reliable techniques for the detection of NoV in dairy products, such as cream, yogurt, smoothies, ice cream and in dairy-based desserts containing one or more types of fruit (in pureed and/or juice form), is needed to ensure the safety of these products. The general strategy for the detection of enteric viruses in food samples consists of three steps: virus extraction, purification of viral RNA and quantitative molecular detection of the purified RNA. The detection of enteric viruses in food is difficult due to the low level of viral contamination in the food and the presence of substances that can inhibit PCR amplification (Lee et al., 2012; Maunula et al., 2013; Suffredini et al., 2014). During virus extraction and RNA purification, molecules such as

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polysaccharides, proteins and fatty acids are removed to prevent inhibition of RNA extraction and molecular detection. Due to these inhibitory substances, it is important to implement process controls and external amplification controls (EAC) in sample analysis. The process control is added to samples before any sample processing occurs. It is co-extracted and co-concentrated with viruses of interest and detected from the same extract to monitor the efficiency of the entire sample process throughout elution, virus concentration, RNA purification and molecular detection (Lees, 2010). The virus selected as the process control should not be associated with food, should provide a good morphological and physicochemical model and exhibit similar robustness to be extracted in a similar way, and at comparable efficiency, as the viral targets (Lees, 2010). Mengovirus (MC0 strain), a member of the *Picornaviridae* family, sharing structural characteristics with the hepatitis A virus (HAV), has been used as a process control virus for the detection of NoV in shellfish (Costafreda et al., 2006; Le Guyader et al., 2009; Uhrbrand et al., 2010) as well as during the development of the Technical Specification ISO/TS 15216. The murine norovirus (MNV-1), a member of the *Caliciviridae* family, is morphologically and genetically similar to human noroviruses, and shows considerable promise as a human norovirus surrogate (Wobus et al., 2006). It has also recently been successfully tested as a process control virus for detecting enteric virus in food samples (Hennechart-Collette et al., 2014; Stals et al., 2011a, 2011b; Martin-Latil et al., 2012a, 2012b, 2014).

The aim of this study was to compare RT-qPCR based methods for the detection of NoV in milk products. Two process controls (mengovirus, MNV-1) were tested and the limit of detection (LOD) of NoV was determined in spiked milk and cottage cheese by using the selected method.

2. Materials and methods

2.1. Viruses and cells

Stool samples of NoV GI (E8050) and NoV GII (E6929) from infected humans were provided by the French National Reference Centre for Enteric Viruses in Dijon, France. The fecal samples were suspended in 10 mM phosphate buffered saline (PBS), pH 7.4 to obtain a final 10% suspension (w/v), and then vortexed and centrifuged at 4000×*g* for 20 min at 4 °C. Aliquots of 100 µL were kept frozen at −80 °C for later use. The genomic titers of the clarified fecal suspensions were determined by RT-qPCR by using an RT-qPCR standard curve obtained with the 10-fold diluted *in vitro* RNA transcripts as previously described (Hennechart-Collette et al., 2014). Based on this approach, the clarified suspension stocks of NoV GI and NoV GII had titers of approximately 7.4×10^8 genome copies/mL and 7×10^6 genome copies/mL, respectively.

MNV-1 (CW1 strain) was provided by Dr. H. Virgin from Washington University (Saint Louis, MO, USA) to the ANSES Fougères Laboratory (Fougères, France) and was propagated in a mouse leukemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell line (Cannon et al., 2006). RAW 264.7 was grown at 37 °C in an atmosphere containing 5% CO_2 in DMEM supplemented with GlutaMAX™, 1% non-essential amino acids and 10% fetal bovine serum (Life Technologies, Saint Aubin, France). The extracted RNA was confirmed with RT-qPCR and quantified by measuring absorbance at 260/280 nm with a NanoDrop ND-1000 spectrophotometer. Based on this approach, the production stock of MNV-1 had titers of approximately 10^{12} genome copies/mL.

A non-virulent mutant strain of mengovirus (vMC0 strain) (kindly provided by Dr. Albert Bosch, Department of Microbiology, Enteric Virus Group, University of Barcelona, Spain) was grown on HeLa cells (ATCC, CCL-2™) as described (Costafreda et al., 2006). HeLa cells were grown at 37 °C in an atmosphere containing 5% CO_2 in Minimum Essential Media GlutaMAX™ (MEM), 1% non-essential amino acids and 10% fetal bovine serum (Life Technologies). The extracted RNA was confirmed with RT-qPCR and quantified by measuring absorbance at 260/280 nm with a NanoDrop ND-1000 spectrophotometer. Based on this approach, the production stock of mengovirus had titer of approximately 10^{12} genome copies/mL.

2.2. Artificial contamination of milk products

For spiking experiments, milk (semi-skimmed milk) and cottage cheese (3.2% fat content with respect to total weight) were purchased from a local market.

All the food samples were weighed in quantities of 2.5 g (or 2.5 mL) and placed in a 50 mL centrifuge tube (Falcon). Virus contamination was carried out by spiking 100 µL of 10-fold dilutions in DEPC-treated water (Fisher Bioblock Scientific, Illkirch, France) of NoV clarified suspension stock and left overnight at 4 °C as previously described by Fraisse et al. (2011) and Stals et al. (2011a).

To compare three different elution-concentration methods, the inoculation of milk and cottage cheese was performed on inoculation levels of 7.4×10^5 genome copies of NoV GI and 7×10^4 genome copies of NoV GII.

To assess the LOD of the selected method, milk and cottage cheese were spiked with inoculation levels ranging from 0 to 7.4×10^6 genome copies of NoV GI and levels ranging from 0 to 7×10^4 genome copies of NoV GII. Each food sample was co-inoculated with 10^6 genome copies of MNV-1 or 10^7 genome copies of mengovirus (process control virus) just before adding elution buffer. One unspiked sample was used as a negative control. Three repetitions of each experiment set were performed.

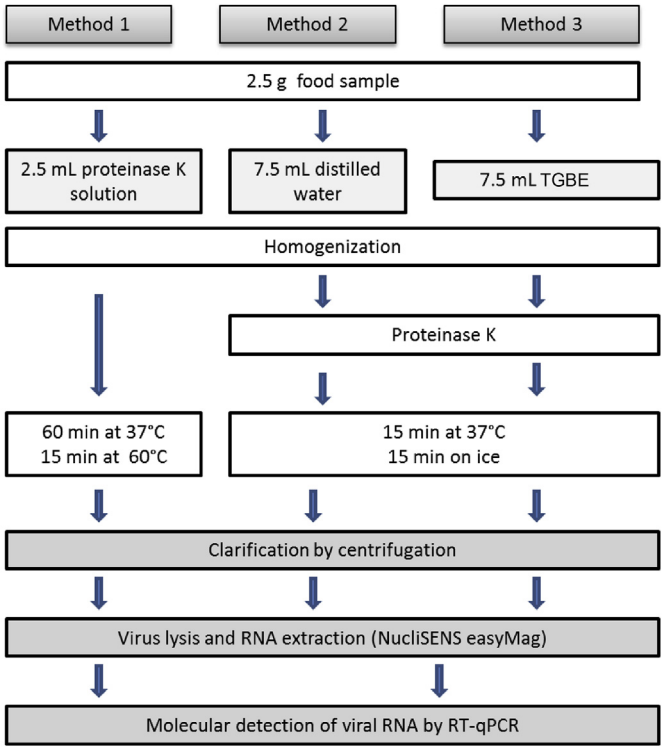

Figure 1. Flowchart of Methods 1, 2 and 3 evaluated for recovery and detection of noroviruses in dairy products.