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Batch testing for noroviruses in frozen raspberries

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ABSTRACT

Berries, in particular raspberries, have been associated with multiple recalls due to norovirus contamination and were linked to a number of norovirus (NoV) outbreaks. In the present study a total of 130 samples of frozen raspberries were collected from 26 batches in four different raspberry processing companies. In two companies the samples consisted of bulk frozen raspberries serving as raw material for the production of raspberry puree (an intermediate food product in a business to business setting). In two other companies, the samples consisted of bulk individually quick frozen (IQF) raspberries serving as raw material for the production of frozen fruit mixes (as a final food product for consumer). Enumeration of *Escherichia coli* and coliforms was performed as well as real-time reverse transcription PCR (RT-qPCR) detection of GI and GII NoV (in 2 × 10 g). In addition, in cases where positive NoV GI or GII RT-qPCR signals were obtained, an attempt to sequence the amplicons was undertaken.

Six out of 70 samples taken from the 14 batches of frozen raspberries serving raspberry puree production provided a NoV RT-qPCR signal confirmed by sequencing. Four of these six positive samples clustered in one batch whereas the other two positive samples clustered in another batch from the same company. All six positive samples showed NoV RT-qPCR signals above the limit of quantification of the RT-qPCR assay. These two positive batches of frozen raspberries can be classified as being of insufficient sanitary quality. The mean NoV level in 20 g of these raspberry samples was 4.3 log genomic copies NoV GI/20 g. The concern for public health is uncertain as NoV RT-qPCR detection is unable to discriminate between infectious and non-infectious virus particles. For the IQF raspberries, one batch out of 12 tested NoV positive, but only 1 out of the 5 samples analyzed in this batch showed a positive RT-qPCR GI NoV signal confirmed by sequencing. The RT-qPCR signal was below the limit of quantification of the assay used (<3.7 log genomic copies/20 g). It was shown that the applied protocol for sequencing of the amplicon to confirm the specificity of the RT-qPCR signal was successful for GI NoV amplicons but often failed and provided an inconclusive result for GII NoV amplicons.

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1. Introduction

Soft red fruits such as raspberries and strawberries have been repeatedly linked with food borne outbreaks due to human norovirus (NoV) (Ponka et al., 1999; Mäde et al., 2013; Sarvikivi et al., 2012) and hepatitis A virus (HAV) (Reid and Robinson, 1987; Nordic outbreak investigation team, 2013). In the period 2009–2012 the EU Rapid Alert System for Food and Feed (RASFF) reported 10, 1 and 2 recalls/withdrawals due to the presence of NoV in frozen raspberries, frozen strawberries and other frozen (mixes of) berries, respectively.

Within that same period (2009–2011) at least 28 outbreaks due to NoV contaminated raspberries and strawberries were identified in Europe (EFSA, 2013). Furthermore in 2012 a huge outbreak affecting approximately 11,000 people occurred in Germany due to NoV contaminated strawberries (Mäde et al., 2013).

The increasing number of alerts and reported outbreaks has prompted national competent authorities and the fruit-based processing industry to establish a testing regime for NoV. The assessment of environmental conditions, good agricultural and hygienic practices in primary production and berry collection centers is important (FAO/WHO, 2012) especially because subsequent steps in the production of frozen berries (or derived purees) may not be adequate to remove contamination. Nonetheless, it is well established that end product testing for food safety assurance has limitations, both due to the confidence one can have that the samples are representative of the batch, and also because methods of detection of pathogens, including the RT-qPCR methods for NoV are imperfect (ICMSF, 2011). Nevertheless, this type of batch testing regime is often applied in import controls or inspections by competent authorities (Ferrier and Buzby, 2014). For example, as a consequence of the large 2012 NoV outbreak in Germany associated with frozen strawberries (Mäde et al., 2013), European Regulation (618/2013) was updated and mandated testing of imported frozen strawberries from China for NoV (n = 5, c = 0, absence of NoV in 25 g). It is also common practice in supplier-buyer transactions, to

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monitor, by analysis, incoming raw material, or to provide a certificate of analysis per batch to its buyers. The intensity of such testing tends to increase when outbreaks or alerts occur related to specific commodities. This is presently the case for NoV in (frozen) raspberries. Although sampling plans are intrinsically limited in assessing the quality and safety of sampled foods, sampling might be useful to reveal major non-compliances and be a basis for analyzing performance trends so that corrective actions can be taken before loss of control (Lahou et al., 2014; ICMSF, 2011). Detection of NoV genomic copies in frozen raspberries could be considered a useful parameter to be used for verification of good hygienic practice applied to berries at primary production and processing premises.

There are few reports of NoV screening in non-outbreak related samples. Maunula et al. (2013) sampled frozen raspberries, fresh raspberries and fresh strawberries at point of sale in three European countries (i.e. Czech Republic, Poland and Serbia). They reported that none of the 39 frozen raspberry samples (95% CI 0–9%), none of the 60 fresh raspberry samples (95% CI 0–6%) and none of the 21 fresh strawberry samples (95% CI 0–15%) were positive for NoV using RT-qPCR. Baert et al. (2011) reported 6.7% (10/150) of soft red fruits in a France study (unreported whether frozen or not) positive for NoV. Stals et al. (2011a) reported 4 of 10 raspberry samples (originating from Poland/Serbia) testing positive for NoV. These surveys are reporting single-sample subunit analysis, and there are currently no data available on NoV screening based upon a multiple-sample subunit approach (n = 5) as is commonly used in setting microbiological standards.

RT-qPCR is currently the accepted standardized method for the detection of NoV in food including frozen berries, but is technically complex. The use of small analysis volumes of the nucleic acid extract produced after sample treatment, combined with the variable extraction efficiency, can result in the method being unable to detect virus below e.g. 10^2–10^3 genomic copies per sample. Also the production of the nucleic acid controls is also challenging and overall experience with implementation of RT-qPCR methods in surveys indicates that these methods might be further refined with regard to sampling, sample preparation, limit of detection and interpretation of results (EFSA, 2014; Stals et al., 2013).

Overall, NoVs can be detected in frozen raspberries, but prevalence studies in particular using a multi-sample approach are limited, and quantitative data on viral load are scarce. Moreover, in many studies the focus has been on the technical elaboration of the NoV RT-qPCR detection method rather than the approach to interpretation of RT-qPCR results. The present study presents data on occurrence and levels of NoV in batches of frozen berries including the elaboration of a decision-tool for interpretation of NoV RT-qPCR and indicating batches of insufficient sanitary quality. In this study, sampling was performed on 26 batches (5 samples/batch) of frozen raspberries from four different frozen raspberry processing companies in Belgium. All batches originated from Poland, which is one of the biggest berry producing country in the EU. The outcome of the survey, limitations of this batch testing approach in assuring food safety and concerns of technical nature associated with RT-qPCR implementation and interpretation of results are discussed.

2. Materials and methods

2.1. Sampling plan

Sampling was selected to investigate the suitability of a multi-sample approach (n = 5) and a decision-tool for interpretation of NoV RT-qPCR results on frozen berries, based on the resources available. A total of 130 convenience samples were collected from 26 batches in four different raspberry processing companies. In two companies (A and B) the incoming cooled and mixed/minced raspberries served as raw material for the production of raspberry puree (an intermediate food product). Samples were taken after initial storage of the incoming raspberries at −20 °C at the processing site. In two other companies (C and D), the samples consisted of bulk individually quick frozen (IQF) raspberries serving as raw material for the production of frozen fruit mixes (as a final food product for consumers) or were intended for further distribution. IQF raspberries were sampled upon arrival in these companies. All 26 sampled batches, which originated from Poland, were picked in the summer of 2011 and 2012. From each batch, 5 samples of ca. 100 g raspberries were randomly taken by the companies themselves from different vessels/boxes of a batch. All samples were stored at −20 °C in the lab and analyzed within two months. The total of 130 samples tested consisted of 14 batches of frozen bulk raspberries (n = 70; 5 samples/batch) intended for the production of puree and 12 batches (n = 60; 5 samples/batch) of IQF raspberries.

From each sample, 20 g (2 × 10 g) was used for analysis of the presence of NoV using RT-qPCR detection as described below. Another 10 g was used for enumeration of *Escherichia coli* and coliforms by plating (pour plate method) a 10-fold diluted suspension on RAPID′ *E. coli 2* chromogenic agar medium (BioRad, France) and counting of typical colonies after 24 h incubation at 37 °C. The frozen raspberries were thawed overnight at 4 °C before the NoV extraction procedure.

2.2. NoV RT-qPCR detection

2.2.1. Detection strategy

The detection strategy was adopted, with minor adjustments, from the one previously described by Stals et al. (2011a) and is schematically represented in Fig. 1. In detail, the detection of NoV (in 20 g, 2 × 10 g subsamples) was performed according to the elution-concentration method described earlier (Stals et al., 2011b; Baert et al., 2008). The first subsample (10 g) was spiked with 100 μl of an MNV-1 solution containing ca. 10^7 genomic copies of MNV-1 (first batch and second batch of aliquots contained initially 2.2×10^7 and 1.4×10^7 genomic copies of MNV-1, respectively) as a process control (PC). After spiking, the subsample with MNV-1 PC was incubated for 30 min at room temperature before starting the virus extraction. The PC was used to calculate the recovery efficiency of the extraction process.

Virus extraction of the second subsample (10 g) was performed in parallel with the first subsample, but during the reverse transcription (RT) step one micro liter of MNV-1 genomic RNA (containing 10^3 to 10^4 copies) was added to the reaction mix as RT-control (MNV-1 RTC). No internal amplification control (MNV-1 IAC) was used during qPCR as previous results indicated that the RT-step is the most prone to PCR inhibition and previous recovery efficiencies of the MNV-1 IAC were ca. 100% for raspberries (Stals et al., 2011a). The purpose of this MNV-1 RTC was to control the amplification efficiency and hence inhibition.

For each subsample, qPCR was performed for the detection of MNV-1, either as PC or RTC. For each subsample, detection of GI and GII NoV was performed according to a duplex RT-qPCR as described earlier (Stals et al., 2009a).

2.2.2. Virus elution and concentration method

Virus extraction and elution using 30 ml tris/glycine/beef extract (TGBE) buffer (0.1 M Tris-HCl, 3% beef extract, 0.05 M glycine, pH 9.5 adjusted with 10 M NaOH) was performed as previously described by Baert et al. (2008) and Stals et al. (2011b). To prevent gel formation, 150 μl of Pectinex (Sigma-Aldrich, Steinheim, Germany) was added to the elution buffer. Afterwards, viruses were concentrated using the PEG 6000/NaCl precipitation technique. The final pellet was dissolved in 1.5 ml of phosphate-buffered saline (Lonza, Verviers, Belgium) of which 1 ml was subjected to further purification by the use of a chloroform–butanol purification step. The supernatant was stored at −80 °C until further RNA purification.

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