Food Microbiology 60 (2016) 124–130

Contents lists available at ScienceDirect

Food Microbiology

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

Comparison and optimization of detection methods for noroviruses in frozen strawberries containing different amounts of RT-PCR inhibitors

Christina Bartsch, Kathrin Szabo, Mai Dinh-Thanh, Christina Schrader, Eva Trojnar, Reimar Johne*

Federal Institute for Risk Assessment, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany

ARTICLE INFO

Article history:
Received 22 April 2016
Received in revised form
18 July 2016
Accepted 18 July 2016
Available online 19 July 2016

Keywords:
Norovirus
Strawberries
Detection method
PCR inhibitors

ABSTRACT

Frozen berries have been repeatedly identified as vehicles for norovirus (NoV) transmission causing large gastroenteritis outbreaks. However, virus detection in berries is often hampered by the presence of RT-PCR-inhibiting substances. Here, several virus extraction methods for subsequent real-time RT-PCR-based NoV-RNA detection in strawberries were compared and optimized. NoV recovery rates (RRs) between 0.21 ± 0.13% and 10.29 ± 6.03% were found when five different artificially contaminated strawberry batches were analyzed by the ISO/TS15216-2 method indicating the presence of different amounts of RT-PCR inhibitors. A comparison of five different virus extraction methods using artificially contaminated strawberries containing high amounts of RT-PCR inhibitors revealed the best NoV RRs for the ISO/TS15216 method. Further improvement of NoV RRs from 2.83 ± 2.92% to 15.28 ± 9.73% was achieved by the additional use of Sephacryl®-based columns for RNA purification. Testing of 22 frozen strawberry samples from a batch involved in a gastroenteritis outbreak resulted in 5 vs. 13 NoV GI-positive and in 9 vs. 20 NoV GII-positive samples using the original ISO/TS15216 method vs. the extended protocol, respectively. It can be concluded that the inclusion of an additional RNA purification step can increase NoV detection by the ISO/TS15216-2 method in frozen berries containing high amounts of RT-PCR inhibitors.

1. Introduction

Diseases induced by viral pathogens are increasingly recognized in food microbiology. Particularly, many outbreaks involving plant-derived food such as berries and lettuce contaminated with norovirus (NoV) or hepatitis A virus have been described recently (Mäde et al., 2013; Coudray-Meunier et al., 2015). For example a large NoV gastroenteritis outbreak involving more than 10,000 diseased people occurred in Germany in 2012, which could be traced to contaminated frozen strawberries (Mäde et al., 2013). Moreover, several outbreaks were recorded, in which contaminated raspberries were identified as the source of infection (Fell et al., 2007; Sarvikivi et al., 2012).

NoVs are members of the *Caliciviridae* family and can be subdivided into six genogroups, whereby only genogroups (G) I, II and IV are human pathogens (Karst et al., 2015). The virus is transmitted from human-to-human mainly by the faecal–oral route. The minimal dose of infection is as low as 10 to 100 virus particles (Caul, 1996). In addition, NoV is a highly stable virus, which can survive multiple days up to several months on various surfaces (Cheesbrough et al., 1997; Sattar et al., 2001). These properties of NoV enable indirect transmission of NoV through food, water or contact materials contaminated by infected persons or wastewater (Maunula et al., 2013; Brassard et al., 2012). For berries, irrigation with water contaminated with human sewage or direct contaminations by infected food handlers have been supposed (Mäde et al., 2013). To ensure customer protection, the diligent compliance of official hygienic standards is therefore essential during the food production or packaging process (Codex Alimentarius, 2012).

NoVs are non-enveloped single (+)-stranded RNA viruses with an icosaedric nucleocapsid, which consists of capsid proteins with shell (s) and protruding (p) domains (Koromyslova et al., 2015). It is suggested that the viral (p) domains specifically interact with human histo blood group antigens (HBGAs), which are oligosaccharide specific for the individual blood type (Tan and Jiang, 2008; Kato and Ishiwa, 2015). By this, the NoV particles may be able to enter

* Corresponding author.
E-mail address: reimar.johne@bfr.bund.de (R. Johne).

http://dx.doi.org/10.1016/j.fm.2016.07.005

the host cells and accomplish infection.

The detection of viruses in food can be difficult and is mainly dependent on the food matrix (Scherer et al., 2010). Particularly berries (i.a. strawberries and raspberries) have a fragile texture and contain various substances, which may inhibit RNA detection by reverse transcription (RT-) PCR (Schrader et al., 2012). Consequently, RT-PCR detection of viruses in berries is prone to false negative results (Mäde et al., 2013). Therefore, several methods for virus extraction, which should remove RT-PCR inhibitors and concentrate the virus before RNA extraction and real time RT-PCR analysis, have been developed. A protocol using polyethylene glycol (PEG) precipitation has been shown to be suitable for analysis of berries (Butot et al., 2007; Scherer et al., 2010; Mäde et al., 2013). This method has also been implemented in the ISO/TS 15216-2 standard for detection of NoV and hepatitis A virus in food (ISO, 2013). Another protocol uses porcine gastric mucin (PGM), which shows a similar structure as HBGAs, for binding of NoV particles to magnetic beads (Tian et al., 2005). In addition, an ultrafiltration method aiming at decreasing the buffer volume and thereby concentrating the viruses has been described (Mäde et al., 2013). Furthermore, Trizol®-based techniques, which release the viral RNA directly from the food matrix before RNA extraction (Baert et al., 2008; Szabo et al., 2015) or methods applying a direct RNA extraction from the food (Perrin et al., 2015) have been described recently. However, most of these techniques show variable virus recovery rates and many of the methods are poorly reproducible in other laboratories. This may be caused by the non-uniform application of complicated laboratory procedures; however, the use of different viruses and batches of food may also be considered.

The main purpose of our study was therefore the comparison and optimization of the mentioned virus extraction methods for use in berries. Frozen strawberries artificially contaminated with NoV GII were used for method development due to the known involvement of NoV-contaminated strawberries in (large) gastroenteritis outbreaks in the past. The above mentioned assumption that different batches of berries may contain different amounts of RT-PCR inhibitors should be tested by analyzing different strawberry samples derived from the market. The method giving the best results with strawberries containing high amounts of RT-PCR inhibitors should be further optimized and thereafter tested on field samples originated from an original gastroenteritis outbreak. The study should provide a suitable and sensitive method for NoV detection in berries, which can be used for outbreak investigations as well as for routine testing of berry batches from the market.

2. Materials & methods

2.1. Strawberry samples

Sealed 2–5 kg batches of fresh and frozen strawberries originating from Germany and Spain were purchased in food stores in Berlin, Germany. All of these batches were tested negative for NoV-RNA using the ISO/TS 15216-2 method as described below before starting of the experiments. A sealed 5 kg batch of frozen strawberries from a lot of imported strawberries involved in a large foodborne NoV outbreak in Germany (2012) (Mäde et al., 2013) was kindly provided by D. Mäde (State Office for Consumer Protection, Saxony-Anhalt, Germany).

2.2. Viruses

The bacteriophage MS2 was used as process control virus as described (Dreier et al., 2005). For all experiments, 10 μl of an MS2 solution containing 10^4 plaque forming units of the bacteriophage were utilized. A NoV II.3-containing stool sample from a child suffering from enteric symptoms was used for the inoculation experiments as described (Scherer et al., 2010). The stool sample was diluted 1:10 in phosphate-buffered saline (PBS), aliquoted and stored at −80 °C. The stool sample preparation contained 5.4×10^6 RNA copies/μl according to quantification by real time RT-PCR as described in the following sections.

2.3. Artificial contamination of strawberries

The strawberry batches were aliquoted into 25 g portions, cut into pieces and transferred into 50 ml tubes. The tubes were stored for up to 1 h at 4 °C until contamination with NoV. For the experiments comparing the different virus extraction protocols, the NoV-containing fecal sample was diluted with PBS to a concentration of 2.16×10^5 RNA copies/μl and 10 μl of this suspension (containing 2.16×10^6 NoV RNA copies) were spread on the fruit surfaces under a sterile bench. The tubes were left open for 45 min for efficient adsorption of NoV to the strawberry surfaces before closing the tubes and storing them at −20 °C. NoV process controls (10 μl each) were generated from the same contamination solution and stored similarly. For the determination of the detection limit of selected methods, tenfold serial dilutions of the NoV solution were prepared and the 25 g samples were contaminated with 10 μL aliquots containing 2.16×10^5 RNA copies, 2.16×10^4 RNA copies, 2.16×10^3 RNA copies, 2.16×10^2 RNA copies or 2.16×10^1 RNA copies. These samples were prepared in triplicates. Non-contaminated strawberry samples were analyzed in each experiment in parallel serving as negative controls.

2.4. Virus extraction according to ISO/TS 15216-2

The protocol included in the revised version of the ISO/TS 15216-2 (ISO, 2013) was used. Briefly, 25 g strawberries were placed in a 400 ml Stomacher bag with filter compartment. At this point, 10 μl of the MS2 phage suspension was added to the field samples and incubated at room temperature for 10 min. Thereafter, 40 ml of Tris Glycin Beef Extract (TGBE)-Buffer (pH 9.5) and 1140 units pectinase (*A. aculeatus*) (Sigma Aldrich, Darmstadt, Germany) were added. The sample was incubated on a horizontal shaker for 10 min at room temperature (RT) under soft tilting. This step was followed by checking the pH value and readjusting to 9.5 with NaOH. Thereafter, the sample was incubated on the horizontal shaker for additional 10 min and the pH was checked again. The procedure could be repeated one more time to stabilize the pH at 9.5. Then, the buffer solution was transferred to a 50 ml tube after passing through the bag filter compartment. The solution was centrifuged at 4 °C and 10,000 × *g* for 10 min to remove fruit debris. The cleared solution was transferred into a new tube, the pH was adjusted to 7.0–7.3 with HCl and 10 ml of 5 × PEG/NaCl solution (500 g/l PEG 8000 (Sigma Aldrich, 1.5 mol/l NaCl)) were added. The mixture was shaken vigorously for 30 s and incubated for 1 h in an over-head rotator at 4 °C and 60 rpm. This step was followed by centrifugation at 10,000 × *g* and 4 °C for 30 min. The PEG formed a pellet and the supernatant was discarded. Another centrifugation at 10,000 × *g* and 4 °C for 5 min was performed to compact the PEG pellet followed by removal of liquid residues by pipetting. The pellet was dissolved in 500 μl PBS by vortexing and repeated pipetting. The solution was transferred into a fresh Eppendorf tube and 500 μl of a chloroform/butanol mixture (1:1, v/v) were added. After vortexing and incubation for 5 min at RT, the sample was centrifuged at 10,000 × *g* and 4 °C for 15 min. Thereafter, the upper aqueous phase (400–500 μl) was transferred to a fresh tube by pipetting. The extract was either stored at −80 °C or directly used for nucleic acid extraction.

2.5. Virus extraction using porcine gastrin mucin (PGM)-coated magnetic beads

This method was based on a protocol described by Tian et al. (2005). Briefly, MagnaBind Carboxyl Derivatized Beads (Thermo Fisher Scientific, Schwerte, Germany) were agitated vigorously before 1 ml was transferred into a fresh Eppendorf tube and washed 3 times. Each washing step consisted of adding 1 ml PBS, detaching the beads from the tube side by careful pipetting and shaking, magnetic separation on a magnetic rack and subsequent removal of the liquid by pipetting. The PGM (Typ III PGM, Sigma-Aldrich) was dissolved in MES buffer (BupH MES Buffered Saline packs, Thermo Fisher Scientific) to a concentration of 10 mg/ml. Afterwards, 1 ml of this solution was added to the washed beads followed by soft agitation. Thereafter, 10 mg EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid, Sigma Aldrich), were dissolved in 1 ml MES buffer and 100 µl of the resulting solution were mixed with the PGM/bead suspension by shaking. After incubation for 30 min at RT, the unbound PGM was removed by magnetic separation including 3 washing steps. Finally, the PGM-conjugated beads were resuspended in 1 ml PBS. The strawberry samples (25 g each) were contaminated with NoV, the berries were washed with TGBE buffer including pectinase and the pH was adjusted as described above in the ISO protocol. Thereafter, 200 µl of the PGM-conjugated beads were added to the solution and the sample was incubated on a horizontal shaker for 1 h with soft tilting at RT followed by magnetic separation for 30 min at RT. The supernatant was aspirated, the bead pellet was dissolved in 1 ml PBS and transferred to a fresh Eppendorf tube. After 3 washings, the beads were resuspended in 250 µl PBS and either stored at −80 °C or used directly for RNA extraction. The nucleic acid extraction protocol was slightly modified in order to remove the PGM-coated magnetic beads, which may interfere with the magnetic silica beads used for nucleic acid extraction. Therefore, 1 ml of the lysis buffer (NucliSENS® easyMAG system, BioMérieux, Marcy I'Etoile, France) was directly added to the PGM-bead-containing samples, mixed by repeated pipetting and vortexing followed by an incubation for 5 min at RT. After magnetic separation for 5 min at RT, the supernatant was obtained, magnetic silica were added and the nucleic acid extraction protocol was followed as described below.

2.6. Virus extraction by direct lysis

This method was based on a protocol published by Perrin et al. (2015). Briefly, the 25 g strawberry samples were contaminated with NoV as described above. To avoid increased fruit juice discharge, the frozen contaminated strawberries were not completely defrosted, but used directly for the experiment. A total of 2 ml lysis buffer (NucliSENS® easyMAG system, BioMérieux, Marcy I'Etoile, France) was added to the contaminated strawberries and the tube was incubated in an over-head rotator at RT and 60 rpm for 10 min. Afterwards, large strawberry debris was removed with sterile tweezers and smaller debris was pelleted by centrifugation for 10 min at 10,000 × *g* and 4 °C. Finally, 3 ml of the cleared supernatant were used for nucleic acid extraction as described below.

2.7. Virus extraction using TRI®Reagent

This method refers to a recent publication by Szabo et al. (2015), where it was originally used for analysis of sausages. Therefore, modifications were introduced according to the requirements for soft fruits. Briefly, 25 g of contaminated strawberry samples generated as described above, were defrosted for 10 min. Thereafter, 5 ml TRI®Reagent Solution (Life Technologies GmBH, Darmstadt, Germany) were added directly to the strawberries followed by an incubation in an over-head rotator at RT and 60 rpm for 10 min. The larger debris was removed with sterile tweezers and the smaller debris was pelleted by centrifugation for 20 min at 10,000 × *g* and 4 °C. The supernatant was transferred to a fresh tube and 200 µl chloroform was added per 1 ml. After vortexing and incubation for 5 min at RT, a centrifugation for 15 min at 10,000 × *g* and 4 °C was performed. Subsequently, 1 ml of the upper aqueous phase was used for nucleic acid extraction as described below.

2.8. Virus extraction using ultrafiltration

This method is based on protocols of several publications using ultrafiltration for virus concentration (Cheong et al., 2009; Mäde et al., 2013; Esseili et al., 2015). Briefly, 5 ml PBS was added to the 25 g portions of frozen contaminated strawberries. The tubes were incubated in an over-head rotator at RT and 60 rpm for 10 min. Thereafter, large strawberry debris was removed with sterile tweezers and smaller debris was pelleted by centrifugation for 10 min at 10,000 × *g* and 4 °C. The cleared solution was filtrated through a polyethersulfone (PES) filter system containing one filter with pore size 0.45 µm and one with pore size 0.22 µm (SARSTED, Nümbrecht, Germany). The filtered solution was transferred into a Vivaspin 20 ultrafiltration device (50.000 MWCO, Sartorius, Göttingen, Germany) and was centrifuged in steps of 15 min at 4600 × *g* and 4 °C until 500 µl of the solution remained. The solution was transferred into a fresh Eppendorf tube and the ultrafiltration membrane was rinsed with 500 µl PBS, which were added to the remaining solution as well. Finally, 1 ml of the solution was used for nucleic acid extraction as described below.

2.9. Nucleic acid extraction

For nucleic acid extraction, the NucliSENS® easyMAG system (BioMérieux, Marcy I'Etoile, France) was used according to the manufacturer's instructions. The sample volumes amounted between 250 µl and 1000 µl, depending on the virus extraction protocol. Elution was done with 100 µl elution buffer and the extracts were stored at −80 °C until real-time RT-PCR analysis.

2.10. Purification using Mobispin S-400 columns

The MobiSpin S-400 columns (MoBiTec, Göttingen, Germany) were used according to the instructions of the supplier (MoBiTec GmbH, 2012). Briefly, the column was shortly vortexed, the bottom plug removed and the cap loosened. The column was then placed onto a 1.5 ml Eppendorf tube and centrifuged for 1 min at 800 × *g* at RT in order to compact the matrix. The column was placed onto a new 1.5 ml Eppendorf tube and 50 µl of the RNA extract was added to the center of the resin. After centrifugation for 2 min at 800 × *g* at RT, the purified RNA was present at the bottom of the tube. The purified RNA was directly used or stored at −80 °C until real-time RT-PCR analysis.

2.11. Real-time RT-PCR

All real-time RT-PCR analyses were performed using 5 µl of extracted nucleic acids in 25 µl reactions in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). In the experiments comparing the virus extraction methods, NoV GII detection was performed by real time RT-PCR according to Hoehne and Schreier (2006) in combination with the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany). In the experiments for determination of the detection limits and for analysis of the field samples, NoV GI and NoV GII detection strictly followed the method

described in the ISO/TS 15216-2 protocol including the use of the RNA Ultrasense One Step qRT-PCR System (Invitrogen, Carlsbad, USA). Standards for NoV GII quantification were prepared and used for generation of standard curves as described recently (Kreuzer et al., 2012). Briefly, *in vitro* transcribed RNA derived from a plasmid containing the RT-PCR product of a NoVII.3 genome was quantified using a NanoDrop device (Thermo Fisher). A tenfold dilution series of this preparation was thereafter used in the real-time RT-PCR for generation of the standard curve. The bacteriophage MS2 was detected by real-time RT-PCR as described by Dreier et al. (2005).

2.12. Calculation of recovery rate, detection limit and statistical analyses

The recovery rate (RR) was calculated by comparison of the ct-value of the process control (nucleic acid directly extracted from the virus solution used in the artificial contamination experiments) with the ct-value of samples (nucleic acid extracted from artificially contaminated strawberries after application of the respective virus extraction method). The recovery rate was calculated by the following formula (Scherer et al., 2009):

$$\mathrm{RR}\ (\%) = 2(-\hat{\Delta}\mathrm{ct}) \times 100, \text{ where } \Delta\mathrm{ct} = \mathrm{ct}(\text{sample}) - \mathrm{ct}(\text{process control}).$$

The detection limit of a method was defined as the lowest virus amount (expressed in RNA copy numbers) used for contamination of berries detectable by the method in at least one of three triplicates. The statistical analyses used for comparison of the different methods and food matrices included analysis of variance homogeneity, normal distribution and the Mann-Whitney-U-test and were performed with the SPSS Statistics 21 program (IBM, New-York, USA).

3. Results & discussion

3.1. Testing of different strawberry batches

Five different batches of strawberries were purchased from different stores, artificially contaminated with NoV GII.3, frozen and subsequently tested using the ISO/TS 15216-2 method. The batches differed in ripeness and matrix consistency and consisted either of fresh or frozen strawberries (Table 1). As evident from Table 1, the calculated NoV RRs differed remarkably between the batches despite the use of the identical contamination and detection protocol. Mean NoV RRs between 10.29± 6.03% and 0.21± 0.13% were evident, which showed a clear correlation with the ripeness and fresh/frozen condition at purchase. A statistical analysis showed that only the differences in the NoV RRs between batch 2 and 3 as well as between batch 4 and 5 were not significant ($p = 0.955$ and $p = 0.065$), whereas the RRs between all other batches were highly significant (p-values between <0.001 and 0.026). The highest NoV RRs were determined for fresh berries showing a light red color, whereas the lowest NoV RRs were found in frozen, dark red colored strawberries.

Berries are known to contain large amounts of real time RT-PCR inhibiting substances (Schrader et al., 2012; Mäde et al., 2013). It may be assumed that the amount of those substances increases during ripening and that repeated freezing and thawing increasingly releases these substances into the analyzed liquid. So far, the distinct substances involved in this process are not known. However, anthocyanins and other aromatic molecules present in berries can partially resemble nucleic acids structures by their aromatic ring structure and may therefore interfere with RT-PCR enzymes (Peist et al., 2001; Seeram et al., 2006; Wei et al., 2008). The differences of the inhibiting activities in different lots should generally be considered during the development of RT-PCR-based detection methods. Consequently, RRs and detection limits reported in different publications and assessed with different lots of berries cannot be directly compared to each other. In order to enable comparison of detection methods, two strawberry lots were therefore selected here for the following experiments. As a good detection method should work with all field samples, some of which may contain high amounts of RT-PCR inhibitors, batches 3 and 4 were selected.

3.2. Comparison of methods for NoV detection in strawberries

Five virus extraction methods were selected from the published literature, which were based on ultrafiltration, direct lysis in RNA extraction buffer, lysis with TRI®Reagent or the use of PGM-coated magnetic beads, and compared to the ISO/TS 15216-2 method. Strawberries of batches 3 and 4 (at least 3 samples of each) were artificially contaminated with NoV GII.3, frozen and thawed, and the respective virus extraction protocol was applied. Thereafter, an identical protocol for RNA extraction and real-time RT-PCR was applied in all cases for NoV detection. As evident from Table 2, the NoV RRs differed remarkably between the methods showing mean values from 0.01 ± 0.03% to 1.71± 2.31%. The highest NoV RR was achieved by using the ISO/TS 15216-2 method.

The ultrafiltration method aims at the virus concentration and removal of inhibiting substances by filtration devices. The results

Table 2
Method comparison for NoV detection on strawberries.

Method	RR mean ± SD (%)	Reference
ISO/TS 15216-2	1.71 ± 2.31	ISO (2014)
Ultrafiltration	0.98 ± 0.95	Esseili et al. (2015)
Direct lysis	0.52 ± 0.54	Perrin et al. (2015)
PGM magnetic Beads	0.04 ± 0.1	Tian et al. (2005)
TriReagent	0.01 ± 0.03	Szabo et al. (2015)

Strawberries of batches 3 and 4 were artificially contaminated with NoV GII.3, frozen and subsequently analyzed by the mentioned methods. At least 3 samples from each batch were analyzed with each method and the mean NoV recovery rates (RRs) along with the standard deviation (SD) are indicated.

Table 1
Testing of five strawberry batches artificially contaminated with NoV GII using the ISO/TS 15216-2 method.

	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Sample number	6	6	9	6	6
Condition at purchase	fresh	fresh	fresh	frozen	frozen
Harvest time	November	September	May	unknown	unknown
Fruit color	light red	red	red	dark red	dark red
RR mean value ± SD (%)	10.29 ± 6.03	2.95 ± 2.44	2.77 ± 2.39	0.59 ± 0.49	0.21 ± 0.13

The strawberries were originally purchased in different stores, contaminated with NoV GII.3 and frozen before analysis. The characteristics of the berries from the different batches and the resulting NoV recovery rate (RR) along with the standard deviation (SD) are shown.

show that the RRs are lower than using the ISO/TS 15216-2 method, which is in concordance with another study comparing these methods with strawberries involved in a NoV outbreak (Mäde et al., 2013). Either the virus concentration is ineffective by ultrafiltration, or inhibitory substances bind to the filter matrix and are therefore not efficiently removed. The direct lysis method showed results comparable with the ultrafiltration method. This method is very rapid and easy to perform and has previously been shown to produce good results with frozen raspberries (Perrin et al., 2015). The low RRs determined in our study may be explained by the use of strawberries containing high amounts of RT-PCR-inhibiting substances, which were presumably not removed by this quick method. The method using PGM-coated magnetic-beads showed only very low recovery rates. PGM was used as binding agent because of its resemblance to HBGAs, which are known interacting partners of NoVs. Indeed, different NoV types have been shown to exhibit different binding activities to HBGAs (Koromyslova et al., 2015). As this method depends on specific binding of NoV particles to sugar residues of the PGM (Tian et al., 2005), an inefficient binding may be supposed. The binding activity of the used NoV GII.3 strain to PGM is not known. Also, the TRI®Reagent protocol showed very low RRs. It is based on rapid denaturation of the viruses in the food sample by a phenol-like substance, which should lead to efficient release of viral RNA. Although this method has previously been shown to be very effective for meat products (Szabo et al., 2015), it seems to be less appropriate for the analysis of berries. Maybe, the absence of thorough purification steps in this protocol leads to inefficient removal of RT-PCR inhibitors present in the berry extracts.

The ISO/TS 15216-2 method is based on a PEG precipitation protocol (Dubois et al., 2002; Butot et al., 2007), which has been optimized during development of the standard method. By application of PEG at a specific pH, the virus particles are efficiently precipitated and concentrated. At a later step, the virus pellet is treated with chloroform/butanol in order to remove RT-PCR-inhibiting substances. Although this protocol is laborious and time-consuming, it seems to be well optimized and efficient for the use with strawberries. Based on the results, we further focused on the optimization of the ISO/TS 15216-2 protocol, because it appeared to be the best established method for virus detection in strawberries.

3.3. Optimization of the ISO/TS 15216-2 method using MobiSpin S-400 columns

In order to further purify the RNA extracted from the artificially contaminated strawberries, the use of MobiSpin S-400 columns in addition to the ISO/TS 15216-2 protocol was tested. Three different batches of strawberries were analyzed, which exhibited different RT-PCR-inhibiting activities as assessed in the first set of experiments. As shown in Table 3, the NoV RRs could be largely improved by the use of the columns in case of the strawberry batches 3 and 4. For these batches, the improvement also turned out to be highly significant (Table 3). In contrast, no improvement in the NoV RRs could be obtained in case of batch 1.

The MobiSpin S-400 columns contain a Sephacryl®-based matrix (MoBiTec GmbH, 2012). It can be used in a quick and easy centrifugation procedure. During the purification process, the sample interacts with the Sephacryl®-based matrix leading to a binding of small molecules, whereas larger molecules are able to pass the column. Most of the RT-PCR-inhibiting substances are small molecules (Schrader et al., 2012), which should therefore be removed by this purification step, whereas the larger RNA molecules should not be affected by the procedure. Indeed, the results of our experiments show that the NoV RRs could be improved for the batches 3 and 4, which were considered to contain high amounts of RT-PCR inhibitors. This indicates that some RT-PCR-inhibiting substances were still in the RNA preparation after application of the ISO/TS 15216-2 method and RNA extraction, which thereafter could be efficiently removed by the use of the columns. In contrast, batch 1 contained only low amounts of RT-PCR inhibitors as already suggested by the results of the first experiments. Therefore, removal of inhibitors was not necessary and the use of the columns could not further improve the NoV RR in this case.

3.4. Determination of detection limits

Tenfold dilutions of the NoV GII.3 suspension were used for artificial contamination of strawberries of batch 2, which were subsequently tested by the ISO/TS 15216-2 method with or without the use of MobiSpin S-400 columns. As shown in Table 4, the detection limit was about 10-fold lower by the use of the columns as compared to the original ISO/TS 15216-2 method. The results confirm the better performance of the optimized method and indicate a higher sensitivity of this method for strawberries containing high amounts of RT-PCR inhibitors.

3.5. Testing of field samples

In order to test the performance of the method with naturally contaminated samples, a batch of frozen strawberries, which were involved in a large NoV gastroenteritis outbreak in Germany in 2012, was analyzed. A total of 22 subsamples were derived from the batch and tested with the ISO/TS 15216-2 method with or without the use of MobiSpin S-400 columns. The addition of the MobiSpin S-400 column-based RNA purification increased the detection rate of NoV GI from 22.3% to 59.1% and for NoV GII from 40.1% to 90.9% (Table 5). Furthermore, the RRs for the used process control bacteriophage MS2 were significantly improved by use of the columns. However, the RRs for MS2 were generally very low thus questioning the use of this bacteriophage as appropriate process control for analysis of strawberries.

The results show that the ISO/TS 15216-2 method is suitable for analysis of field-origin frozen strawberry samples and that an additional purification of the extracted RNA can increase the detection NoV rate. The analyzed batch has been suspected to be contaminated with human sewage explaining the presence of multiple viruses in it (Mäde et al., 2013). In the original analysis of this strawberry batch using a method similar to ISO/TS 15216-2, detection rates of 10.7% and 53.6% were reported for NoV GI and NoV GII, respectively (Mäde et al., 2013). The increased NoV detection rates and the increase in the RRs of the process control virus after additional RNA purification argue for the presence of high amounts of RT-PCR inhibitors in this field sample, which could be efficiently removed by the extended procedure. Therefore, methods enabling the removal of inhibiting substances should be applied in outbreak investigations and routine monitoring under field conditions in order to avoid false negative results.

4. Conclusions

This study shows large differences between the NoV RRs of artificially contaminated strawberries with different degrees of ripeness and matrix conditions. Consequently, the distinct sample type has a strong impact on the results of analytical tests. This should be taken into consideration when method performances and detection limits of methods are compared to each other.

The recently standardized method for NoV detection in soft fruit as laid down in ISO/TS 15216-2 could be shown to be better suited for the analysis of frozen strawberries than any of the other virus

Table 3
Optimization of the ISO/TS 15216-2 method using MobiSpin S-400 columns.

Batch	ISO/TS 15216-2 method	ISO/TS 15216-2 method + MobiSpin columns	p-value
	RR mean ± SD (%)	RR mean ± SD (%)	
1	9.85 ± 6.88	9.42 ± 5.22	1.00
3	2.83 ± 2.92	15.28 ± 9.73	0.09
4	0.59 ± 0.49	5.60 ± 1.58	0.02

Strawberries of batches 1, 3 and 4 were artificially contaminated with NoV GII.3, frozen and subsequently analyzed by the ISO/TS 15216-2 method with or without a further purification step of the extracted RNA using MobiSpin S-400 columns. A total of 6 samples were analyzed for each batch and the mean NoV recovery rates (RRs) along with the standard deviation (SD) are indicated. The p-values obtained by Mann-Whitney-U-test indicating the significance of differences between the results of both methods are indicated right.

Table 4
Comparison of detection limits for NoV GII.3 on artificially contaminated strawberries using the ISO/TS 15216-2 method with and without using MobiSpin S-400 columns.

Inoculation level (NoVII RNA copies/25 g strawberries)	ISO/TS 15216-2 method positive/samples tested	ISO/TS 15216-2 method + MobiSpin columns positive/samples tested
2.16×10^5	3/3	3/3
2.16×10^4	3/3	3/3
2.16×10^3	1/3	3/3
2.16×10^2	0/3	1/3
2.16×10^1	0/3	0/3

1:10 serial dilutions of a quantified NoV GII.3 solution were used for artificial contamination of strawberries from batch 2. The detection limit was defined as the highest dilution showing a positive detection in at least one of 3 samples.

Table 5
Analysis of frozen strawberry field samples involved in a NoV outbreak in Germany (2012) using the ISO/TS 15216-2 method with and without using MobiSpin S-400 columns.

Sub-sample number	NoV GI ISO/TS 15216-2 method	NoV GI ISO/TS 15216-2 method + MobiSpin columns	NoV GII ISO/TS 15216-2	NoV GII ISO/TS 15216-2 method + MobiSpin columns	MS2 RR (%) ISO/TS 15216-2 method	MS2 RR (%) ISO/TS 15216-2 method + MobiSpin columns
1	−	−	−	+	0.0037	0.16
2	+	+	−	−	0.0022	0.15
3	−	−	−	−	0.0026	0.13
4	−	−	+	+	0.0025	0.10
5	−	+	+	+	0.0024	0.16
6	−	+	−	+	0.0023	0.15
7	−	+	+	+	0.0031	0.16
8	−	+	−	+	0.0012	0.08
9	+	−	+	+	0.0001	0.01
10	−	−	−	+	0.0013	0.07
11	+	+	+	+	0.0019	0.10
12	−	+	+	+	0.0034	0.25
13	−	−	−	+	0.0026	0.13
14	−	+	−	+	0.0044	0.36
15	+	+	−	+	0.0027	0.11
16	+	+	+	+	0.0052	0.19
17	−	−	−	+	0.0002	0.02
18	−	−	−	+	0.0002	0.04
19	−	+	+	+	0.0013	0.08
20	−	+	−	+	0.0020	0.22
21	−	+	+	+	0.0017	0.17
22	−	−	−	+	0.0004	0.05
Positive/samples tested (%)	5/22 (22.3%)	13/22 (59.1%)	9/22 (40.1%)	20/22 (90.9%)	22/22 (100%)	22/22 (100%)
RR mean ± SD (%)	NA	NA	NA	NA	0.0023 ± 0.0013	0.13 ± 0.08

22 sub-samples were analyzed and the detection rates for NoV GI and NoV GII as well as the calculated recovery rate (RR) for the process control (bacteriophage MS2) are shown. NA − not applicable.

extraction methods tested here. However, it could be further improved for frozen strawberries containing high amounts of RT-PCR inhibitors by inclusion of an additional RNA purification step. This optimized method should be tested in other laboratories and its suitability for other matrices, e.g. raspberries or food types with a known high amount of RT-PCR inhibitors, should be assessed in future. Considering that the presence or absence of inhibitory substances is usually not known when samples are analyzed under field conditions, a method efficiently removing such substances should generally be applied in order to avoid false negative results.

Acknowledgements

We thank D. Mäde and B. Jahn (State Office for Consumer Protection, Saxony-Anhalt, Germany) for providing the frozen strawberry field sample.

This study was supported by an intramural grant of BfR (No. 1322-647).

References

Baert, L., Uyttendaele, M., Debevere, J., 2008. Evaluation of viral extraction methods on a broad range of Ready-To-Eat foods with conventional and real-time RT-PCR for Norovirus GII detection. Int. J. Food Microbiol. 123, 101–108.

Brassard, J., Gagné, M.J., Généreux, M., Côté, C., 2012. Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries. Appl. Environ. Microbiol. 78, 3763–3766.

Butot, S., Putallaz, T., Sánchez, G., 2007. Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. Appl. Environ. Microbiol. 73, 186–192.

Caul, E.O., 1996. Viral gastroenteritis: small round structured viruses, caliciviruses and astroviruses. Part II. The epidemiological perspective. J. Clin. Pathol. 49, 959–964.

Cheesbrough, J.S., Barkess-Jones, L., Brown, D.W., 1997. Possible prolonged environmental survival of small round structured viruses. J. Hosp. Infect. 35, 325–326.

Cheong, S., Lee, C., Choi, W.C., Lee, C.H., Kim, S.J., 2009. Concentration method for the detection of enteric viruses from large volumes of foods. J. Food Prot. 72, 2001–2005.

Codex Alimentarius, 2012. Guidelines on the Application of General Principles of Food Hygiene to the Control of Viruses in Food. CAC/GL 79.

Coudray-Meunier, C., Fraisse, A., Martin-Latil, S., Guillier, L., Delannoy, S., Fach, P., Perelle, S., 2015. A comparative study of digital RT-PCR and RT-qPCR for quantification of Hepatitis A virus and Norovirus in lettuce and water samples. Int. J. Food Microbiol. 201, 17–26.

Dreier, J., Störmer, M., Kleesiek, K., 2005. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. J. Clin. Microbiol. 43, 4551–4557.

Dubois, E., Agier, C., Traoré, O., Hennechart, C., Merle, G., Crucière, C., Laveran, H., 2002. Modified concentration method for the detection of enteric viruses on fruits and vegetables by reverse transcriptase-polymerase chain reaction or cell culture. J. Food Prot. 65, 1962–1969.

Esseili, M.A., Saif, L.J., Farkas, T., Wang, Q., 2015. Feline calicivirus, murine norovirus, porcine sapovirus, and tulane virus survival on postharvest lettuce. Appl. Environ. Microbiol. 81, 5085–5092.

Fell, G., Boyens, M., Baumgarte, S., 2007. Frozen berries as a risk factor for outbreaks of norovirus gastroenteritis. Results of an outbreak investigation in the summer of 2005 in Hamburg. Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz 50, 230–236.

Hoehne, M., Schreier, E., 2006. Detection of Norovirus genogroup I and II by multiplex real-time RT- PCR using a 3'-minor groove binder-DNA probe. BMC Infect. Dis. 10, 69.

ISO, 2013. Microbiology of the Food Chain — Horizontal Method for Determination of Hepatitis a Virus and Norovirus in Food Using Real-time RT-PCR. ISO/TS 15216-2:2013.

Karst, S.M., Zhu, S., Goodfellow, I.G., 2015. The molecular pathology of noroviruses. J. Pathol. 235, 206–216.

Kato, K., Ishiwa, A., 2015. The role of carbohydrates in infection strategies of enteric pathogens. Trop. Med. Health 43, 41–52.

Koromyslova, A.D., Leuthold, M.M., Bowler, M.W., Hansman, G.S., 2015. The sweet quartet: binding of fucose to the norovirus capsid. Virology 483, 203–208.

Kreuzer, S., Machnowska, P., Aßmus, J., Sieber, M., Pieper, R., Schmidt, M.F., Brockmann, G.A., Scharek-Tedin, L., Johne, R., 2012. Feeding of the probiotic bacterium Enterococcus faecium NCIMB 10415 differentially affects shedding of enteric viruses in pigs. Vet. Res. 27, 43–58.

Maunula, L., Kaupke, A., Vasickova, P., Söderberg, K., Kozyra, I., Lazic, S., van der Poel, W.H., Bouwknegt, M., Rutjes, S., Willems, K.A., Moloney, R., D'Agostino, M., de Roda Husman, A.M., von Bonsdorff, C.H., Rzeżutka, A., Pavlik, I., Petrovic, T., Cook, N., 2013. Tracing enteric viruses in the European berry fruit supply chain. Int. J. Food Microbiol. 167, 177–185.

Mäde, D., Trübner, K., Neubert, E., Höhne, M., Johne, R., 2013. Detection and typing of norovirus from frozen strawberries involved in a large-scale gastroenteritis outbreak in Germany. Food Environ. Virol. 5, 162–168.

MoBiTec GmbH, 2012. MobiSpin Columns, S-400, Standard Protocol, p. 7.

Peist, R., Honsel, D., Twieling, G., Löffert, D., 2001. PCR inhibitors in plant DNA preparations. QIAGEN News 3, 7–9.

Perrin, A., Loutreul, J., Boudaud, N., Bertrand, I., Gantzer, C., 2015. Rapid, simple and efficient method for detection of viral genomes on raspberries. J. Virol. Methods 224, 95–101.

Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., 2012. Multiple norovirus outbreaks linked to imported frozen raspberries. Epidemiol. Infect. 140, 260–267.

Sattar, S.A., Tetro, J., Springthorpe, V.S., Giulivi, A., 2001. Preventing the spread of hepatitis B and C viruses: where are germicides relevant? Am. J. Infect. Control 29, 187–197.

Scherer, K., Mäde, D., Ellerbroek, L., Schulenburg, J., Johne, R., Klein, G., 2009. Application of a swab sampling method for the detection of norovirus and rotavirus on artificially contaminated food and environmental surfaces. Food Environ. Virol. 1, 42–49.

Scherer, K., Johne, R., Schrader, C., Ellerbroek, L., Schulenburg, J., Klein, G., 2010. Comparison of two extraction methods for viruses in food and application in a norovirus gastroenteritis outbreak. J. Virol. Methods 169, 22–27.

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors - occurrence, properties and removal. J. Appl. Microbiol. 113, 1014–1026.

Seeram, N.P., Adams, L.S., Zhang, Y., Lee, R., Sand, D., Scheuller, H.S., Heber, D., 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. J. Agric. Food Chem. 54, 9329–9339.

Szabo, K., Trojnar, E., Anheyer-Behmenburg, H.3, Binder, A., Schotte, U., Ellerbroek, L., Klein, G., Johne, R., 2015. Detection of hepatitis E virus RNA in raw sausages and liver sausages from retail in Germany using an optimized method. Int. J. Food Microbiol. 215, 149–156.

Tan, M., Jiang, X., 2008. Association of histo-blood group antigens with susceptibility to norovirus infection may be strain-specific rather than genogroup dependent. J. Infect. Dis. 198, 940–941.

Tian, P., Brandl, M., Mandrell, R., 2005. Porcine gastric mucin binds to recombinant norovirus particles and competitively inhibits their binding to histo-blood group antigens and Caco-2 cells. Lett. Appl. Microbiol. 41, 315–320.

Wei, T., Lu, G., Clover, G., 2008. Novel approaches to mitigate primer interaction and eliminate inhibitors in multiplex PCR, demonstrated using an assay for detection of three strawberry viruses. JVirol Methods 151, 132–139.