Detection of norovirus capsid proteins in faecal and food samples by a real time immuno-PCR method

P. Tian and R. Mandrell

United States Department of Agriculture, Agricultural Research Service, Produce Safety and Microbiology Research Unit, Western Regional Research Center, Albany, CA, USA

Abstract

Aims: To develop a sensitive real time immuno-polymerase chain reaction (rtI-PCR) method for detecting norovirus (NV) capsid protein in food samples.

Methods and Results: The viral antigens were captured by two polyclonal antibodies against recombinant Norwalk viral-like particles (rNVLPs). Biotin-conjugated antibodies, avidin and biotin-conjugated DNA reporter were used to convert the protein signals into DNA signals. The reporter DNA was then amplified by addition of primers and PCR. A real time PCR method was used in order to perform a quantitative post-PCR analysis. One hundred rNVLPs (10 fg) and a NV sample containing 660 rNVLPs equivalent particle units (66 fg) could be detected by this method.

Conclusion: The PCR inhibitors present in the food samples had minimal effect on antigen capture and were removed by multiple wash steps during the rtI-PCR procedure. The sensitivity of rtI-PCR was >1000-fold higher than the standard enzyme-linked immunosorbent assay and approximately 10 times higher than reverse transcription PCR in detection of NV capsid protein in stool and food samples.

Significance and Impact of the Study: This is the first report of a rtI-PCR method to detect NV in contaminated food samples without concentration or purification of the virus.

Introduction

Noroviruses (NVs) cause millions of cases of sporadic and epidemic gastrointestinal disease in the US annually, accounting for 50–67% of foodborne illness (Daniels et al. 2000; Glass et al. 2001). NVs are the most frequent cause of outbreaks of acute gastroenteritis following ingestion of raw shellfish (Atmar et al. 1995; Schwab et al. 2000). In addition, various common-source food-borne vehicles that have been implicated in NV outbreaks include bakery frosting, salad, celery, melon, vermicelli consommé, fruit salad, coleslaw, frozen raspberries, sandwiches, lettuce, cold cooked ham, commercial ice and water (Seymour and Appleton 2001). In most outbreaks of food-borne viral gastroenteritis involving fresh produce, it has been challenging to detect the viruses directly from the food samples with current methods.

The virus cannot be grown in cell culture; therefore, reverse transcription-polymerase chain reaction (RT-PCR) is the primary tool for detection of these viruses (Jiang et al. 1992b). The concentration of virus in produce usually is not sufficient for detection by current methods. In addition, steps to remove inhibitors present in these types of samples are required for RT-PCR (Atmar and Estes 2001). Depending on the type of food sample, various complicated nucleic acid extraction methods are required to concentrate viral RNA and to remove RT-PCR inhibitors (Atmar et al. 1995; Schwab et al. 2000; Atmar and Estes 2001; Kingsley and Richards 2001; Sair et al. 2002). Most methods involve multiple extraction and elution procedures that create the potential for viral RNA loss preceding each step.

NV represents a diverse set of viruses. At present, most human NV have been classified genetically into two major
groups, genogroups I and II (GI and GII, respectively), with GI comprising more than seven subgroups and GII comprising more than 10 subgroups (Ando et al. 2000; Burton-MacLeod et al. 2004). The RT-PCR reaction requires a perfect match between the last nucleotide of the primers and the viral sequence in order to permit elongation. Therefore, it is a great challenge to design one or a few appropriate primer sets for detection of all NV strains by RT-PCR assays (Kageyama et al. 2003).

An enzyme-linked immunosorbent assay (ELISA) is an alternative method for detection of viral antigens present in stool samples. Recently, two commercial ELISA kits for detecting NV in stool samples have been evaluated (Burton-MacLeod et al. 2004). Although stool samples can be tested directly in these ELISA kits without complicated concentration and purification steps, the sensitivity of ELISA is much lower than RT-PCR (Burton-MacLeod et al. 2004). Therefore, ELISA is not suited for detecting NV contamination in food samples without a major improvement in sensitivity.

Immuno-PCR (I-PCR) methods that combine antibody capture of antigen and DNA amplification have been developed to detect small amounts of antigens such as cytokines, hormones and viral antigens. This method was first introduced by Sano et al. (1992) with reported detection limit of as few as 580 molecules of BSA. The combined ELISA and PCR methods use a sandwich ELISA to capture antigens with specific antibodies. Instead of using an enzyme [alkaline phosphatase (AP) or horseradish peroxidase] to catalyse substrates in ELISA, I-PCR uses a reporter DNA and PCR for amplification. Depending on the type of antigen captured, different formats of the assay have been developed. Antigen can be coated directly on the plate, or antigen is captured by a sandwich-style dual antibody method (Saito et al. 1999). Biotin can be directly conjugated with the second antigen detection antibody or conjugated to a third anti-(second antibody)-antibody (Sanna et al. 1995). Avidin is used as a bridge to connect the biotin-conjugated antibody and biotinylated reporter DNA. The increased sensitivity of I-PCR compared to traditional ELISA results from orders of magnitude increased amplification of the reporter DNA by PCR compared to traditional enzyme amplification (Mweene et al. 1996; Saito et al. 1999; Liang et al. 2003; Adler et al. 2003b; Barletta et al. 2004; Chao et al. 2004).

The I-PCR method has been used for detection of Clostridium botulinum neurotoxin type A (Chao et al. 2004), group A streptococcus (Liang et al. 2003), soluble proteins (Adler et al. 2003a,b), prions (Gofflot et al. 2004), viral proteins from herpes virus (Mweene et al. 1996), influenza virus (Ozaki et al. 2001), and HIV (Barletta et al. 2004). I-PCR methods are recognized as one of the most sensitive detection methods that exist (Barletta et al. 2004). Compared to ELISA, the sensitivity of I-PCR is increased three to five logs (Mweene et al. 1996; Saito et al. 1999; Liang et al. 2003; Adler et al. 2003b; Barletta et al. 2004; Chao et al. 2004). However, most I-PCR studies reported have tested clinical samples such as blood, body fluid, or purified proteins. In this study, we have reported an I-PCR method for detection of capsid protein of NV in food samples by using strain-specific polyclonal antibodies, and the detection limit of the method.

**Materials And Methods**

**NVs and recombinant Norwalk viral-like particles (rNVLPs)**

NV-positive clinical samples and prototype NV (8FIIa) were kindly provided by Dr David Schnurr (Department of Health Services, CA, USA), Dr G. Richards (ARS, USDA, DE, USA) and Dr Jason Jiang (Cincinnati Children’s Hospital, OH, USA). Genogroups of the virus were determined either with a Dako ELISA kit (Dako, Carpinteria, CA, USA) or by alignment of RT-PCR determined sequences (Burton-MacLeod et al. 2004). Recombinant baculovirus expressing capsid protein of rNVLPs was constructed from NV (8FIIa) by using the Bac-to-Bac recombinant baculovirus expression system (Invitrogen, Carlsbad, CA, USA). rNVLPs were purified from S9 insect cells (Invitrogen) infected with recombinant baculovirus expressing rNVLP as described previously (Jiang et al. 1992a). Briefly, 200 ml of cultured S9 cells in SF-900 II SFM media (Invitrogen) were grown in a 500 ml Corning flask at 28°C prior to infection at a multiplicity of infection (MOI) of 0.5 plaque-forming units (PFU) per cell and incubated at 28°C for 6 days. Culture supernatants were clarified by centrifugation at 1800 g for 30 min at 4°C. rNVLPs were concentrated by centrifugation through a sucrose cushion (30% w/v) at 141 000 g for 2 h in a SW28 rotor (Beckman Coulter, Fullerton, CA, USA). rNVLP were re-suspended in water and banded in cesium chloride at a density of 1.368 g cm⁻³ by centrifugation at 147 000 g in a SW50.1 rotor for 18 h at 4°C. The fraction containing rNVLPs was isolated, diluted in PBS and centrifuged at 147 000 g for 2 h to pellet the viral-like particles. Finally, purified rNVLPs were re-suspended in PBS and stored at 4°C. The concentration of rNVLP was determined in a micro BCA protein assay (Pierce, Rockford, IL, USA). The number of rNVLP particles was calculated based upon each virus particle being composed of 180 capsid protein molecules with a predicted molecular weight of 56 571 per capsid protein (Jiang et al. 1993; Prasad et al. 1994). Therefore, each particle is assumed to have a molecular weight of 10 182 780 Da,
which equates to 1 fg of purified rNVLP representing approximately 66 particles.

Sandwich ELISA assay
Polyclonal guinea pig antiserum (GP140) and rabbit anti-serum (R183) against rNVLPs were produced by immunization with the prototype strain 8FIIa (GI) and kindly provided by Dr Patricia Reilly (Wyeth Research). Both antibodies were obtained from animals immunized with highly purified rNVLPs and have a high titre against purified rNVLP and NV determined by ELISA assay without significant background (Dr P. Reilly, personal communication). Nunc TopYield microtitre modules (VWR, Brisbane, CA, USA) were selected for this study since both ELISA and PCR reactions can be performed in the same wells. The modules were coated with 0.05 ml of GP140 antibody diluted 1 : 5000 in PBS and incubated at 4°C overnight. Unbound antibodies were removed by washing once with PBS (EMD Chemicals Inc., Gibbstown, NJ, USA), and the modules were blocked with 0.2 ml of OmniPur Bio-block (EMD Chemicals Inc.) for 2 h at 37°C. After three washes with PBS, 0.075 ml of testing samples or corresponding controls were added and the modules were incubated at 37°C for 1 h followed by eight washes with PBS containing 0.5% Tween 20 (PBS-T). Polyclonal rabbit anti-rNVLP antiserum (R183) diluted 1 : 10 000 in PBS was added and the modules were incubated at 37°C for 1 h, followed by eight washes with PBS-T. Goat anti-rabbit IgG conjugated to AP (Zymed Laboratories, South San Francisco, CA, USA) was diluted 1 : 10 000 in PBS and added, and the modules were incubated at 37°C for 1 h followed by eight washes with PBS-T. AP-goat anti-rabbit antibody bound to rNVLP in the modules was detected by adding 0.1 ml of p-nitrophenyl phosphate, disodium salt substrate at a concentration of 1 mg ml⁻¹ in diethanolamine substrate buffer (Pierce, Rockford, IL, USA). The absorbance was read at 405 nm on a Spectramax ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Reporter DNA and primers
The *Escherichia coli* beta-glucuronidase (GUS) gene was used as the reporter gene in our system (Liang et al. 2003). pCAMBIA-2201 plasmid that contains a GUS gene was kindly provided by Dr G. Chen (WRRC, Albany, CA, USA). The reporter DNA was generated by PCR using an Invitrogen PCR kit with a forward (5'-Biotin-AACTATGCCGGAATCCATCG-3') and a reverse primer (5'-ACATAATCCAGCCATGCACAC-3'). The PCR reaction was performed at the following conditions: 94°C for 3 min to completely denature the template; 35 cycles with 94°C for 45 s to denature, 55°C for 30 s to anneal and 72°C for 90 s to extend. An additional 10 min extension at 72°C was included at the end of the cycle. The 1 kb reporter DNA amplification product was purified with a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA) and stored at −20°C until used. Real time-PCR primers were designed with the Lux™ fluorogenic primer design software and obtained from Invitrogen. The forward primer is 5'-GTACCTTTGGAAACGGCAGAGG-FAM-AC-3' and the reverse primer is 5'-CGGCTGATG-CAGTTTCTCCT-3'.

Real time immuno-polymerase chain reaction (rtI-PCR)
A sandwich-type tri-antibody system (Sanna et al. 1995; Chao et al. 2004) was adapted in our rtI-PCR. Briefly, the rtI-PCR reaction was performed in a manner similar to the sandwich ELISA. However, instead of using AP-conjugated anti-rabbit antibody to detect the rabbit anti-NV antibody (R183) bound to NV antigens in the module, a biotin-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used at a final concentration of 1 : 5000 diluted in PBS (Chao et al. 2004). The biotin-conjugated affinity purified donkey anti-rabbit IgG (H + L) has minimal cross-reactivity with IgG from other species, including guinea pig IgG, according to the manufacturer. To further decrease the nonspecific binding between the biotin-conjugated anti-rabbit IgG and the guinea pig coating antibodies, the biotin-conjugated anti-rabbit antibodies were preincubated with 0.1% normal guinea serum (Jackson ImmunoResearch Laboratories). After eight washes of the modules with PBS-T, 0.075 ml of recombinant avidin (Pierce, Rockford, IL, USA) was added to each module at a final concentration of 100 ng ml⁻¹, and incubated at 37°C for 1 h. After eight washes with PBS-T, 0.075 ml of the reporter DNA was added to each module at a concentration of 5 ng ml⁻¹ (7.5 × 10⁵ copies) and incubated at 37°C for 1 h. After eight washes with PBS-T and eight washes with PBS, 0·025 ml of 1X Brilliant QPCR mix (Stratagene, La Jolla, CA, USA) was added to each module. The 1X QPCR mix was obtained by diluting the 2X Brilliant QPCR matrix with an equal volume of primers diluted in water for a final concentration of the primers of 100 nmol l⁻¹ each. The modules were then sealed with polyolefin sealing tape (VWR, West Chester, PA, USA) and run in a MX3000P real time-PCR system (Stratagene) using the following amplification protocol: 95°C for 2 min to completely denature the template, 50 cycles with 95°C for 10 s to denature, incubation at 50°C for 10 s to anneal, and 72°C for 30 s to extend.
RT-PCR for detection of viral RNA

Viral mRNA was extracted by using QIAamp viral RNA mini kit (Qiagen). Briefly, a 0·14 ml of sample of a 10% human stool suspension was mixed with 0·56 ml of lysis buffer, vortexed for 15 s, and incubated at room temperature for 10 min. A 0·56 ml volume of 100% ethanol was added to each sample and the samples were passed through the QIAamp spin columns. After washes with the AW1 and AW2 wash buffers, viral mRNA was eluted with 0·06 ml of elution buffer and stored at −80°C. A modified p289/290 primer set was used for RT-PCR (Jiang et al. 1999). The primer pair was designed based on the RNA polymerase sequence of 25 prototype and currently circulating strains of human caliciviruses and was shown to be able to detect both NVs and Sapporo-like caliciviruses effectively (Jiang et al. 1999). The primer sequences are 5′-GATATTCCAAATGGGACTCCAC-3′ (P290), 5′-GATTATTCAAATGGGATTCCA-3′ (P290A), 5′-TGACAATGTAAATCATCCCCACA-3′ (P289) and 5′-TGACAATGTAAATCATCCCCGTA-3′ (P289A), respectively. A Prostar high fidelity single tube RT-PCR system (Stratagene) was used for detection of viral mRNA and the manufacturer’s protocol for RT-PCR was followed. Briefly, 0·005 ml of a purified RNA sample was used in a 0·05 ml reaction mixture with a final primer concentration of 100 nmol l−1 each. A 1·25 U of StrataScript reverse transcriptase and 2·5 U of TaqPlus precision DNA polymerase were used in each reaction. The RT-PCR was performed at 42°C for 15 min, 95°C for 1 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 68°C for 2 min. An additional 10 min incubation at 68°C was performed after 40 cycles ended. About 0·01 ml of RT-PCR product was electrophoresed on 1·5% agarose gel and visualized by ethidium bromide staining. The concentration of virus was determined by end-point titration RT-PCR as described previously (Schwab et al. 1997).

Processing of food samples spiked with rNVLP or NV

Food samples (oysters, various salads or fresh strawberries) were purchased from a nearby supermarket. Food samples were diluted 1 : 10 w/v in PBS and blended with a Waring commercial blender at low speed for 30 s. rNVLPs or NV were then added to samples at different concentrations or dilutions, respectively. Samples were clarified by centrifugation at 3000 rev min−1 for 15 min. Samples were collected before and after centrifugation. Except for 1% oyster samples, all NV or rNVLPs were diluted in the 10% clarified food sample supernatant from a nonspiked sample. This was used as diluents to control for nonspecific binding due to food in the sample. In some experiments, equal amounts of the clarified supernatant from different salads were mixed to make a ‘mixed salad sample’. In some experiments 1% oyster samples were prepared by further dilution of the 10% oyster suspension with PBS.

Statistical analysis

The results for rtI-PCR were analyzed for significance by two methods. The cut-off point for a significant value (above cycle threshold) was calculated as the mean of negative controls ± two times standard deviations (SD) or 0·99, whichever value was greater. The 0·99 or higher cut-off point was selected based on the average of 3·3-fold change in the cycle threshold (Ct) number for every 10-fold change in template DNA concentrations in standard real-time PCR assays. To achieve a signal to noise (S/N) ratio of two or greater, a difference between the positive and negative control must be greater than 0·66. We selected a S/N ratio of three to make the analysis more stringent. A Student’s t-test was used to analyse data from two groups and a one way ANOVA test was used for data comparisons for more than two groups. All samples analysed were at least triplicates and at least two to three individual experiments were performed to determine assay consistency.

When rNVLP were used as internal standards, the mean Ct was calculated from three wells and plotted against rNVLP concentrations ranging from 105 to 109 particles per well. Linear regression curves were generated by SIGMAPLOT programme (Systat, Point Richmond, CA, USA). NV concentration was converted to rNVLP concentration by fitting the mean Ct for NV into the standard curve of rNVLP generated in the same experiment.

Results

Determination of the detection limit of rNVLP in a sandwich ELISA assay and by rtI-PCR assay

As previously demonstrated, rNVLP was similar to the native virus in antigenicity and immunogenicity (Jiang et al. 1992a, 2002). We applied rNVLP in our assay system to quantitatively analyse and compare the results from the rtI-PCR method. rNVLP 1 : 3·33 serially diluted was used for analysis. The mean OD reading for PBS controls, and for 3·3 × 104, 1 × 105, 3·3 × 105, 1 × 106, 3·3 × 106, 1 × 107 rNVLP particles were 0·301, 0·343, 0·378, 0·78, 1·174, 2·168, 3·825 with SD of 0·016, 0·111, 0·028, 0·176, 0·233, 0·233, 0·375, respectively. A cut-off value was calculated as the mean of negative controls + 2 SD, resulting in a detection limit for purified rNVLP of 3·3 × 107 particles (5 pg) per well (Fig. 1a).

rNVLPs serially diluted 10-fold in PBS ranging from 1 × 105 to 10 particles per well and PBS controls were measured in the rtI-PCR assay. Figure 1b shows
rtI-PCR method for norovirus detection

P. Tian and R. Mandrell

Figure 1 (a) Sensitivity of ELISA for rNVLP detection. The OD readings at 405 nm and rNVLP numbers are shown at Y- and X-axis, respectively. The OD readings represent the mean of three wells with standard deviations. The cut-off value was 0.477. (b) rtI-PCR amplification of rNVLP. Amplification plots of fluorescence intensities (ΔRn) vs PCR cycle numbers are displayed for serial 10-fold dilutions of samples containing 10–100,000 rNVLP particles or PBS controls. Each plot corresponds to a particular input target quantity marked by a corresponding symbol. Samples containing (●) 10⁵ particles; (■) 10⁴ particles; (▲) 10³ particles; (□) 10² particles; (X) 10⁴ particles and (○) negative control samples. The cut-off Ct value was calculated as 39.98.

representative results of the rtI-PCR. Each plot represents an average of three individual modules for each concentration of rNVLPs. Figure 1b shows plots from the individual modules for each concentration. The mean Ct number for the negative controls was 41.373 ± SD 0.696. The Ct number for 10, 10², 10³, 10⁴ and 10⁵ rNVLPs were 39.980 (±SD 0.569), 37.987 (0.443), 35.400 (1.004), 31.330 (0.471) and 28.417 (0.718), respectively. The cutoff Ct value was calculated as the mean of negative control values minus twice the SD representing a value of 39.98. Samples from groups with 10² to 10⁵ rNVLPs were considered positive as the Ct numbers for these concentrations were below the cut-off point of 39.98. The Ct number for the group containing 10 rNVLPs was the same as the cut-off value (39.98 vs 39.98). One way ANOVA analysis of the data indicated a significant difference between the PBS controls and all other rNVLP concentrations (P < 0.001) with the exception of the 10 particles per well (P = 0.1340). Although the mean Ct number in the 10 rNVLP per well group was the same as the cut-off point, the Ct numbers from two out of three sample were under the cut-off point.

To further evaluate the sensitivities of the rtI-PCR method, we tested additional wells containing 10 rNVLP. Eight wells containing 10 rNVLPs and eight PBS controls were coded for ‘blind’ testing. Four uncoded PBS control samples were used as negative controls to calculate the mean Ct number and SD. Four wells each containing 100 rNVLPs were used as positive controls. The mean Ct number for negative controls was 48.387 (±SD 0.777), with a cut-off point calculated as 46.833. The mean Ct number for positive controls was 45.76. The results indicated that all PBS negative controls were below the cut-off point with a mean of 47.85 and a SD of 0.393. Ct numbers from five out of eight samples from the group containing 10 rNVLPs were under the cut-off point and were designated positive. However, the mean Ct number from this group was 46.953 with a SD of 0.472, which was above the cut-off point of 46.833. Therefore, the 10 rNVLP per well sample group was considered overall negative. When the t-test was applied to the analysis, there was a significant difference between the 10 particles group and the PBS negative control group (P = 0.001) with the 95% confidence interval for difference of means ranging from 0.428 to 1.359. To ensure the accuracy of the sensitivities of the rtI-PCR assay, we designated a result as positive when both assays yield consistent results. Using this criteria, overall results from the 10 rNVLP per well sample group was designated as negative. Therefore, we have concluded that the detection limit for rNVLP by rtI-PCR was between 10 and 100 particles per well.

Detection of rNVLP in food samples

An ELISA assay was used to determine the effect of food content on viral antigen binding to the antibody-coated plates. After food samples were spiked with rNVLPs, samples were collected and measured before and after centrifugation for clarification to determine the effect of clarification by comparing OD readings (Fig. 2a). In all samples tested, OD readings from samples after clarification were higher than the OD readings from the samples before clarification, indicating clarification was necessary to obtain the highest sensitivities. Therefore, only clarified samples were used for later experiments by rtI-PCR. The effect of different food content on ELISA was measured.
Detection of NV in faeces and food samples by rti-PCR method and RT-PCR

Clinical stool samples previously identified to contain prototype NV were used to evaluate the methods. ELISA results indicated samples were positive only in the undiluted 10% stool suspension (data not shown). Serially diluted stool suspensions were analysed also by rti-PCR. NV could be detected for samples diluted 1000-fold (Fig. 3a) determined by both cut-off point and one way ANOVA analysis. The Ct number with standard deviation (in parentheses) for groups with 0 (control), 10, 10^2 and 10^3 rNVLPs per well was 39.745 (0.621), 39.502 (2.244), 36.16 (0.324) and 34.425 (0.991), respectively. The cut-off point was calculated as 38.50. When the cut-off point was applied, samples containing 10^2 and 10^3 particles were considered positive and samples containing 10 rNVLPs were considered negative. One way ANOVA analysis of the data resulted in the same conclusions. There were significant differences between the controls and groups with 10^2 (P < 0.05), and 10^3 rNVLP particles (P < 0.05). However, there was no significant difference between the controls and the group with 10 rNVLP particles (P = 0.738).

Similar results were observed in detection of rNVLP in mixed salad samples (Fig. 2c). The Ct number with standard deviation (in parentheses) for groups with 0 (control), 10, 10^2 and 10^3 rNVLPs was 44.07 (1.614), 41.527 (0.117), 39.507 (0.250) and 36.093 (0.520), respectively. The detection limit in mixed salad was 100 particles per well as determined by the cut-off point (40.845) and by one way ANOVA analysis. Compared with the results with rNVLPs in PBS (Fig. 1b), variations in the Ct number are much greater for rNVLPs in oyster and mixed salad food matrices.

Detection of NV in food samples by ELISA (a) and rti-PCR (b and c). (a) The OD reading at 405 nm shown on the Y-axis represents the relative OD reading for total food samples containing rNVLPs before or after clarification. X-axis indicates the food sample type with each column representing the average data from three wells and the SD. OD readings from corresponding samples before clarification were taken as 100%. X-axis indicates samples after (in gray) or before clarification (in black). The food samples were 10% suspensions of (co) coleslaw; (ch) chicken; (po) potato; (st) strawberry; (se) seafood and (oy) oyster. (b and c) rti-PCR amplification of rNVLP in food samples. Amplification plots of fluorescence intensities (ΔRn) obtained at each PCR cycle are displayed for serial 10-fold dilutions of 1% oyster suspension (b) or 10% mixed salad suspension (c) containing 10–1000 rNVLP particles, or corresponding negative controls. Each plot represents the average results for three wells. Samples containing (●) 10^2 particles; (■) 10^3 particles; (□) 10 particles and (○) negative control samples. The cut-off points were 38.50 (b) and 40.845 (c) respectively.

by comparing the OD readings to PBS controls in samples with or without clarification. The effect of food on OD readings was minimal in most samples. The most significant effect observed was if rNVLP was spiked in samples containing 10% oyster. In this sample, the OD reading was about 17% lower than the control group. In all other food samples, the food contents either had no effect or less than 10% decrease in OD readings (data not shown).

The samples were diluted with either 1% clarified oyster supernatant (Fig. 2b) or 10% clarified mixed salad supernatant (Fig. 2c) to a final concentration range of 1 × 10^3–10 rNVLP particles per well. Clarified food supernatants containing no rNVLPs were used as negative controls. The Ct numbers with standard deviation (in parentheses) for groups with 0 (control), 10, 10^2 and 10^3 rNVLPs per well were 39.745 (0.621), 39.502 (2.244), 36.16 (0.324) and 34.425 (0.991), respectively. The cut-off point was calculated as 38.50. When the cut-off point was applied, samples containing 10^2 and 10^3 particles were considered positive and samples containing 10 rNVLPs were considered negative. One way ANOVA analysis of the data resulted in the same conclusions. There were significant differences between the controls and groups with 10^2 (P < 0.05), and 10^3 rNVLP particles (P < 0.05). However, there was no significant difference between the controls and the group with 10 rNVLP particles (P = 0.738).

Similar results were observed in detection of rNVLP in mixed salad samples (Fig. 2c). The Ct number with standard deviation (in parentheses) for groups with 0 (control), 10, 10^2 and 10^3 rNVLPs was 44.07 (1.614), 41.527 (0.117), 39.507 (0.250) and 36.093 (0.520), respectively. The detection limit in mixed salad was 100 particles per well as determined by the cut-off point (40.845) and by one way ANOVA analysis. Compared with the results with rNVLPs in PBS (Fig. 1b), variations in the Ct number are much greater for rNVLPs in oyster and mixed salad food matrices.

To compare the sensitivities of rT-PCR and RT-PCR, viral RNA was extracted from 10-fold serially diluted stool samples and analysed by RT-PCR with the modified p289/290 primer set. The viral RNA could be detected in samples diluted 100-fold (data not shown), which represented a concentration of approximately $6 \times 10^5$ rNVLP EPU.

**Discussion**

The I-PCR developed for clinical samples has been shown to be very sensitive. An I-PCR method developed for HIV detection reported that as few as two viral particles could be detected with anti-gp120 antibody compared to the 50 copies of HIV–RNA necessary for detection by RT-PCR (Barletta et al. 2004). However, it was not clear if an I-PCR would be useful for other samples, in particular for food samples that contain complex organic compounds and strong inhibitors of PCR reactions. The amount of the virus present in NV-contaminated ready-to-eat food is lower usually than the detection level of current RT-PCR methods. Additionally, the inhibitors present in many food samples must be removed prior to
RT-PCR, thus necessitating complicated concentration and purification procedures (Atmar et al. 1995; Schwab et al. 1997; Abu Al-Soud and Radstrom 2000; Schwab et al. 2000; Atmar and Estes 2001; Seymour and Appleton 2001). We have demonstrated that PCR inhibitors in food samples interfered with the assay by running real-time PCR reactions in the presence of serially diluted clarified food or stool samples. No amplification was observed unless the samples were diluted at least 1:1000 (data not shown). Our results were consistent with others (Abu Al-Soud and Radstrom 2000) who reported positive PCR could be obtained when less than 0.4% faecal samples or 0.2% meat samples were used. In this paper, we tested if this method could be used for detection of NV in food samples and assessed the sensitivity of the assay.

In most I-PCR papers published, the results were assessed by visualization of the corresponding PCR product on electrophoresed agarose gels stained by ethidium bromide. Unlike the standard PCR reaction, the negative controls for I-PCR assay were always ‘contaminated’ with the reporter DNA. Although extensive washes were applied between each step, it was impossible to remove all the contaminating DNA reporter molecules from the negative controls. Therefore, false positive results can occur with negative controls due to DNA reporter that could not be removed completely even by multiple washes. In addition, although it is possible also that multiple antibodies used in this system might bind nonspecifically to E. coli present in contaminated food and faecal samples thus amplifying signal by PCR, these antibodies were negative for E. coli by ELISA (data not shown). We have used two sets of controls in all tests presented in the paper as negative controls. The first set lacks viral antigen but with all other elements including coating Ab, blocking, second capture Ab, biotinylated anti-second Ab, avidin and biotinylated DNA reporter. The second set negative control has all elements but no coating Ab. By using these two sets of negative controls, the false positive results should be minimized. In addition, the results must be judged by a significant signal to noise ratio (S/N ratio). However, with the current methods by visualization of PCR products on gels, it has been difficult to perform a quantitative post-PCR analysis. Therefore, we have combined a real time PCR method with an immunochromical method for our I-PCR assay.

A method similar to the principle used in ELISA assays was applied to our rtI-PCR. In a standard ELISA assay, samples are considered positive when the S/N is equal or greater than 2:0. In our assays, the end point was set as the mean ± 2 SD with a 95% confidence interval. When the value of 2 SD was smaller than 0·99, the cut-off point was calculated as the mean ± 0·99. Therefore, in our assays, the S/N ratio was set as 3:0 or greater, which was a more stringent standard than the ratio of 2:0 used for ELISA, or the criteria for visualization of a positive PCR product on the gel. To make the criteria even more stringent, significance of the results was analysed by a t-test (for two groups) or by one way ANOVA test (for multiple groups). The results were considered positive when both assays were consistent.

Unlike traditional methods based on detection of the viral genome, our method was designed to detect the viral capsid proteins by using a DNA fragment as a detection label for viral antigen. Since NV contains 180 capsid proteins per genome, the sensitivity of I-PCR should be higher than RT-PCR; RT-PCR measures only the viral genome. In our rtI-PCR system, we could measure the presence of approximately 100 purified rNVLP, indicating the sensitivity of the rtI-PCR was about 1000-fold higher than the ELISA method. When NV from faecal samples was used, the rtI-PCR method was about 10 times more sensitive than the RT-PCR method with stool and food samples. Although the sensitivity of our method is comparable to the RT-PCR method, the major advantage of our assay is that it does not require complicated concentration and purification procedures to remove the PCR inhibitors. To
the best of our knowledge, this is the first report to use the rtI-PCR method to detect contaminating viruses in food samples without initial purification or concentration of the virus. Although the method described here is similar to what might occur with naturally contaminated food samples, it is not identical. It is possible that viruses in food samples might be sequestered or bound tightly in food, and thus not efficiently released during pre-treatment of food samples by methods such as blending. Therefore, the sensitivity of the rtI-PCR method might be lower in naturally contaminated food samples. We have tested the impact of the blending process on viral protein binding to GP 140 coated plates by ELISA. The results suggested that there is no significant difference in OD readings when the rNVLP was added before or after blending (data not shown). However, it remains unclear if all viruses biologically accumulated in oyster tissue could be completely released by the simple blending process. In addition, this method was designed to detect the viral capsid proteins. Therefore, both noninfectious soluble proteins and proteins on the infectious viral particles will be detected and are not distinguished by this method.

The variation of results in rtI-PCR assays was greater than in RT-PCR assays, occurring both intra- and interexperimentally. Therefore, the Ct number was determined by averaging at least three wells at each concentration in order to reflect the intra experimental variations. Interexperimental variation also occurs. Inter-experimental Ct values for the internal standard might not be the same in each experiment. For example, the cut-off (Ct) values in Figs 2b,c and 3b,c were not the same due to variations that occurred between experiments. Therefore, the inter-experiment variation must be normalized by using an internal control as a standard in each experiment to allow comparison of results of subsequent assays. In our system, purified rNVLP was included as the internal standard to measure the NV concentration in samples as rNVLP EPU. The sensitivity of the rtI-PCR method for the virus was about approximately 660 rNVLP EPU.

At present, most human NV have been classified into either GI or GII. The virus could be further divided into different genotypes or subgenogroups (Burton-MacLeod et al. 2004). The enormous genetic diversity of the viruses makes it difficult to design NV primers to cover all strains for RT-PCR. Different approaches have been applied to design primers at different regions (regions A–D) of the viral genome, however, none of them could cover all strains, even strains within the same genogroup. Unlike the RT-PCR approach to detect the viral genome, the rtI-PCR method depends on the specific interaction between viral capsid protein and corresponding antibodies. Diversity within NVs, resulting from the accumulation of point mutations, may lead to lack of detection by a RT-PCR method, but that will be detectable by this antigen capture method. Therefore, rtI-PCR will provide an additional approach to detect such mutants.

Although we have noted that the strain-specific antibody-coated plate could capture other GI strains (data not shown), more NV strains could be captured if broadly reactive genogroup antibodies are used. Detection of NV from stool samples by ELISA by using GI and GII polyclonal or monoclonal antibodies has been reported (Burton-MacLeod et al. 2004). Monoclonal antibodies have been shown to be able to recognize epitopes common to either GI or GII, or both GI and GII (Hardy et al. 1996; Yoda et al. 2000; Kitamoto et al. 2002). Therefore, it is possible to develop a sensitive and broadly reactive rtI-PCR method to detect both genogroups of NV from food samples without complicated purification and concentration approaches by using broadly reactive reagents or two sets of genogroup-specific reagents. Currently, we are applying this method to detect both genogroups of NV by using two sets of genogroup-specific antibodies and with internal rNVLP standards to validate the Ct numbers for different concentrations of rNVLPs to make comparison and quantification of results possible.

Acknowledgements

We thank Dr Patricia Reilly (Wyeth Research) for providing two polyclonal antibodies; Dr D. Schnurr (Department of Health Service, CA, USA), Dr G. Richards (ARS, USDA) and Dr X. Jiang (Cincinnati Children’s Hospital) for providing clinical samples; Dr Maria Brandl and Anna Korn (WRRC, ARS, USDA) for helpful discussion and critical reading; and Guilin Wang, Anna Bates, and Aileen Haxo (WRRC, ARS, USDA) for some technical support.

References


