



Analytical performance of norovirus real-time RT-PCR detection protocols in Canadian laboratories

Kirsten Mattison^{a,*}, Elsie Grudeski^b, Brian Auk^c, Julie Brassard^d, Hugues Charest^e, Kerry Dust^f, Jonathan Gubbay^g, Todd F. Hatchette^h, Alain Houde^d, Julie Jeanⁱ, Tineke Jones^j, Bonita E. Lee^k, Hiroshi Mamiya^{f,1}, Ryan McDonald^l, Oksana Mykytczuk^a, Xiaoli Pang^k, Astrid Petrich^{m,2}, Daniel Planteⁿ, Gordon Ritchie^o, Julie Wong^c, Tim F. Booth^b

^a Bureau of Microbial Hazards, Health Canada, Ottawa, ON, Canada

^b National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada

^c BCCDC Public Health Microbiology and Reference Laboratory, Vancouver, BC, Canada

^d Agriculture and Agri-Food Canada, St. Hyacinthe, QC, Canada

^e Laboratoire de santé publique du Québec, Ste Anne de Bellevue, QC, Canada

^f Cadham Provincial Laboratory, Winnipeg, MB, Canada

^g Ontario Agency for Health Protection and Promotion, Toronto, ON, Canada

^h Queen Elizabeth II Health Science Center, Halifax, NS, Canada

ⁱ Institute of Nutraceuticals and Functional Foods, Laval University, Québec, QC, Canada

^j Agriculture and Agri-Food Canada, Lacombe, AB, Canada

^k Provincial Public Health Laboratory, Edmonton, AB, Canada

^l Saskatchewan Disease Control Laboratory, Regina, SK, Canada

^m McMaster University, Hamilton, ON, Canada

ⁿ Health Canada, Longueuil, QC, Canada

^o Saint Paul's Hospital, Vancouver, BC, Canada

ARTICLE INFO

Article history:

Received 12 July 2010

Received in revised form 6 October 2010

Accepted 13 October 2010

Keywords:

Norovirus

Real-time reverse transcription PCR

Method comparison

ABSTRACT

Background: Noroviruses (NoVs) are the leading cause of infectious gastroenteritis worldwide. Real-time reverse transcription PCR (real-time RT-PCR) is the preferred method of NoV detection for the majority of testing laboratories. Although the accepted target region for molecular detection assays is the conserved ORF1/ORF2 junction, multiple variations have been published with differences in primers, probes, reagents, multiplexing, etc.

Objectives: We assessed the detection limit for GII.4 NoV real-time RT-PCR assays as well as the ability to detect the non-GII.4 NoV genotypes in each participating laboratory.

Study design: A panel of 25 RNA samples was circulated to 18 testing laboratories for comparison of their real-time RT-PCR procedures for NoV detection.

Results: Multiple protocols with slight differences in reagents or conditions successfully detected 10 genome equivalents or fewer of NoV per reaction. Multiplex procedures were significantly associated ($p=0.04$) with false negative results, particularly for a GI.2 strain. Sensitive detection was associated with false positive results ($p=0.03$).

Conclusions: Overall, the data indicate that comparable results are produced under slightly different assay conditions.

Crown Copyright © 2010 Published by Elsevier B.V. All rights reserved.

1. Background

Noroviruses (NoVs) are the most common cause of infectious gastroenteritis worldwide.¹ Symptoms last for 24–72 h and include vomiting and diarrhoea that resolve without long term sequelae.² Large outbreaks are a common feature of norovirus infections, with a variety of reported transmission routes including person-to-person contact, food, water and contaminated fomites.^{3–10} These outbreaks can be extremely difficult to control and have a major financial impact.¹¹

* Corresponding author at: Bureau of Microbial Hazards, Health Canada, 251 Sir FG Banting Drwy, PL2204E, Ottawa, ON K1A 0K9, Canada.

Tel.: +1 613 957 0887; fax: +1 613 941 0280.

¹ Current address: Department of Epidemiology, Biostatistics and Operational Health, McGill University, Montreal, QC, Canada.

² Current address: Department of Paediatric Laboratory Medicine, Hospital for Sick Children, Toronto, ON, Canada.

A number of factors contribute to the spread of these viruses in the community. NoVs are non-enveloped 30 nm particles, which are resistant to drying, heating and disinfection.^{12–18} The infectious dose is less than 10 particles, but the virus is shed to high titres of 10^6 – 10^8 particles per gram in the feces of infected individuals.^{19,20} There are more than 26 genotypes of NoV that infect humans,²¹ with varying degrees of serological cross-reactivity.²² Previous infection with a strain of NoV does not confer long term protection from disease, even when the second infection is of the same strain type.²³ In addition, the most common genetic type, GII.4, is under constant selective pressure and new strain variants emerge periodically to cause epidemics.^{24,25}

NoV detection methods must meet several criteria to be useful for routine sample testing. They must be amenable to high throughput modifications. Some testing laboratories participating in this study receive as many as 200 samples per week during the winter months associated with higher NoV activity. Methods should also be as sensitive and specific as possible. It can be challenging to identify protocols that detect all of the diverse NoV genotypes. Capsid protein sequence variation separates NoV into 5 genogroups, with GI and GII causing the majority of human infections.²¹ The different NoV genogroups share less than 39% amino acid identity in the capsid region.²¹ Within each genogroup, capsid sequence identity ranges from 56% to 86% at the amino acid level.²¹ The current method of choice for NoV detection in most laboratories is probe based real-time reverse transcription PCR (real-time RT-PCR). Most real-time RT-PCR assays target a highly conserved region of the NoV genome at the ORF1/ORF2 junction, but there are slight differences that may affect method performance.^{26,27}

1.1. Objectives

In this study, we circulated a panel of NoV RNA samples for detection by real-time RT-PCR procedures in each participating laboratory. The objective of the study was to compare the detection methods from each participating laboratory. We assessed the detection limit for each real-time RT-PCR assay as well as the ability to detect the less common NoV genotypes.

2. Study design

2.1. Preparation of coded panels

Coded panels of 25 RNA samples previously tested for NoV by TaqMan real-time RT-PCR^{26,28} were assembled at Health Canada, Ottawa, ON. Fecal specimens were derived from outbreaks of acute gastroenteritis across Canada during 2007–2009 and have been stored as whole stools at either 4 °C, –20 °C or at –70 °C. A 10% stool suspension was clarified sequentially by centrifugation (6000 × g for 5 min), 0.45 μm filtration (Millipore, Etobicoke, ON) and 0.22 μm filtration (Millipore). RNA was extracted from mul-

tiplied aliquots of filtrate using the QIAamp Viral RNA extraction kit as per manufacturer's recommendation (QIAGEN, Mississauga, ON). RNA extracts from each aliquot were pooled and the entire batch was diluted 1:1 in RNA Storage Solution (Ambion, Austin, TX) prior to storage in 20 μL aliquots at –70 °C. The panel included four NoV-negative RNA samples: one rotavirus group A sample, one adenovirus 40/41 sample, one enterovirus sample and one sapovirus sample. Nine samples were a dilution series of RNA from a NoV GII.4 (2006b) strain variant: three samples at each concentration of 100, 10 and 1 genome equivalents (g.e.) per reaction (2 μL) as quantified by real-time RT-PCR with a standard curve generated from a RNA run-off transcript of the targeted region.²⁹ Based on probabilities of sampling, some samples should have less and some more than 1 g.e., this can be kept in mind when interpreting the results. The remaining twelve samples were RNA from NoV GI.2, GI.3, GI.4, GI.6, GI.8, GI.13, GII.2, two representatives of GII.3, GII.4 (2008), GII.5 and GII.7, based on sequence analysis of the region C and region D capsid fragments.^{30,31} Quantification of the RNA from other genotypes was based on transcripts of GII.4 (GII viruses) or GI.1 (GI viruses). The panel samples were frozen at –70 °C and shipped on dry ice to 18 participating laboratories.

Before beginning the complete RNA panel, a test panel of ten coded stool/filtrate samples and ten coded RNA samples derived from the same specimens was circulated to nine laboratories. RNA was extracted, stored and shipped in RNA Storage Solution (Ambion) according to the protocol proposed for the full panel study. Results confirmed that labs obtained the same test results for corresponding fecal and RNA samples, indicating that RNA was stable during shipment (data not shown). Distribution of the full panel of RNA samples proceeded as described above.

2.2. Detection by real-time RT-PCR

Each laboratory submitted data as well as answers to a short questionnaire describing their protocols and operating procedures. Real-time RT-PCR assays were performed according to standard procedures in each laboratory. These protocols are summarized in Table 1 and Supplementary Tables 1–3. All primer and probe sets target the conserved ORF1/2 junction of the NoV genome. Briefly, most laboratories (16/18) used the COG1F/COG1R primer pair for GI detection, as described by Kageyama et al.²⁶ These laboratories (14/18) also used the RING1(a) and RING1(b) probe set for GI detection,²⁶ except for labs F and P, where RING1(c) (5'-AGA TYG CGI TCI CCT GTC CA) and RING1(a*) (5'-AGA TYG CGR TCY CCT GTC CA) were used, respectively. Labs R and U, used JJV1F/JJV1R primers and the JJV1P probe.²⁷ For GII detection, again most laboratories used primer and probe sets as developed by Kageyama et al., COG2F/COG2R/RING2 (16/18 labs).²⁶ Laboratory R used JJV2F/COG2R/RING2,²⁷ while laboratory U used JJV2F/QNIF2d/COG2R/RING2.^{27,32} Only four labs used internal amplification controls, as listed in Supplementary Table 2. There

Table 1
Detection limits calculated for each participating laboratory based on results from a dilution series of norovirus GII.4 2006b RNA. g.e.=genome equivalents based on comparisons to an RNA standard curve quantified by spectrometric analysis. n/c=not calculated because the data could not be fit to the four parameter logistic equation described in Section 2.

# Detected at 100 g.e. per reaction (n = 3)	# Detected at 10 g.e. per reaction (n = 3)	# Detected at 1 g.e. per reaction (n = 3)	50% detection limit (g.e. per reaction)	95% confidence interval (g.e. per reaction)	Laboratories
3	3	3	<1	n/c	B, D, V
3	3	2	0.9	0.3–2.8	M, N, O
3	3	0	3.2	n/c	Q, R
3	2	0	9.0	2.9–28	F
3	1	0	11.1	3.6–34	S
3	0	0	31	n/c	A, G, I, K, P, U
0	0	0	>300	n/c	J
2	2	2	n/c	n/c	H

Table 2

Detection of RNA representing different NoV genotypes from a coded panel of samples tested in eighteen laboratories. g.e. = genome equivalents per reaction based on run-off transcripts from a GI.1 strain (GI) or a GII.4 strain (GII).

Genotype	Quantity (g.e.)	# Detected (n = 16)	Laboratories with negative results
GI.2	2.3×10^2	12	J, N, O, P
GI.3	6.0×10^4	15	R
GI.4	2.0×10^4	15	H
GI.6	4.8×10^4	16	
GI.8	7.0×10^6	16	
GI.13	8.5×10^7	15	O
GII.2	1.0×10^5	16	
GII.3	1.2×10^6	16	
GII.3	5.7×10^5	16	
GII.4 (2008)	2.1×10^7	16	
GII.5	8.7×10^3	15	J
GII.7	5.7×10^5	16	

were differences in the fluorescent molecules and quenchers coupled to each probe, as well as in concentrations of primers and probes used for the assays (Supplementary Table 1). Differences in consumables, platforms and amplification conditions used for the reactions are noted in Supplementary Table 2. Cycling conditions were slightly different between laboratories and are shown in Supplementary Table 3.

2.3. Data analysis

Data from panel testing was collated and analyzed at Health Canada, Ottawa. Fractional positive data at each concentration were fitted to the equation for sigmoidal dose–response with a variable slope using Prism4 for Windows (GraphPad Software Inc., La Jolla, CA): $Y = 1 / (1 + 10^{(\log EC_{50} - X) \times HillSlope})$, where X is the logarithm of RNA concentration and Y is the likelihood of detection. Y is a fractional positive value that starts at 0 and goes to 1 with a sigmoid shape. This is identical to the “four parameter logistic equation”. Limits were set to indicate that the Hill Slope was equal for all labs, with variable EC_{50} (the 50% detection limit) between labs. Statistical evaluation of the association between test conditions and results was performed with a two-sided Fisher’s exact test. Associations were considered significant at a p value of less than 0.05.

3. Results

3.1. Detection limits for GII.4

When data from the 9 diluted GII.4 (2006b) samples were analyzed, a range of detection limits were observed, from <1 g.e. per reaction to >300 g.e. per reaction (Table 1). Of the 18 participants, one data set could not be assigned a 50% detection limit. Among the 17 remaining labs, 8 detected all samples at 100 and 10 g.e., resulting in calculated 50% detection levels of less than 5 g.e. per reaction. Only one participating lab failed to detect the most concentrated samples of 100 g.e. It was interesting to note that labs J and K used identical reaction reagents, parameters and conditions (Supplementary Tables 1–3).

3.2. Detection of GI and GII strains

GI NoVs were not detected as consistently as GII viruses, based on the data from 12 samples with different NoV genotypes (Table 2). In particular, the GI.2 strain was not detected in four laboratories. Other strains that were occasionally not detected are GI.3, GI.4, GI.13 and GII.5. All samples that were occasionally not detected had less than 10^5 g.e. of RNA per reaction, with the exception of the GI.13 strain. Sequence analysis reveals two mismatches between the JJV1F primer and the GI.13 RNA as well as a single mismatch with the GI probes (Fig. 1). Five of the six laboratories that had a

false negative result use a multiplex GI and GII detection protocol. The use of a multiplex assay was significantly associated with false negative results ($p = 0.04$).

3.3. Non-NoV samples

No participating laboratories obtained NoV positive results from the adenovirus or enterovirus RNA. Two labs detected the rotavirus sample as positive for GII NoV and five labs detected the sapovirus sample as a GII norovirus. All of these laboratories had good detection limits, with a 50% probability of detecting less than 10 g.e. per reaction. Sensitive reactions may have a higher risk for false positives, as only 4 labs were able to achieve detection limits less than 10 g.e. without detecting one of the negative controls, while none of the 8 labs with detection limits above 10 g.e. obtained a false positive result. There was a significant association between a limit of detection of less than 10 g.e. per reaction and false positive results ($p = 0.03$). The false positive reactions do not correlate with primer and probe sets or with cycling parameters or platforms.

One of the amplicons obtained from the sapovirus-containing sample was sequenced to determine if this false detection was associated with NoV cross-contamination. The sequences amplified were not viral; they were derived from bacterial or plant components of the stool matrix. Regions of complementarity were identified with the COG2 primers and the RING2 probe (each between 6 and 12 bases). Ct values obtained for the false amplicons were the same or less than those obtained for 1 g.e. of NoV RNA.

4. Discussion

This comparative study is the largest that has been reported for NoV RT-PCR detection. A coded panel of RNA samples was circulated in order to isolate the real-time RT-PCR detection protocol from upstream sample processing effects that confounded the interpretation of previous results.³² The data were analyzed to determine the impact of procedural differences on the analytical performance of NoV RNA detection. Among the primer/probe sets and amplification conditions tested here, all were capable of providing sensitive detection of less than 10 g.e. of NoV RNA per reaction. Detection of less than 10 g.e. per reaction, rather than a specific reaction condition, was associated with false positive detection ($p = 0.03$). The use of a multiplex assay was significantly associated with false negative results when a range of NoV types were tested ($p = 0.04$).

Overall, the real-time RT-PCR methods used by these 18 testing laboratories give sensitive and accurate results for the detection of NoV RNA. This indicates that results from different testing laboratories are of similar dependability, even if there are slight variations in reaction conditions. Only one laboratory (J) failed to detect the NoV

```

COG2F      CARGARBCNATGTTYAGRTGGATGAG-----
JJV2F      CAAGAGTCAATGTTTAGGTGGATGAG-----
QNI F2d    -----ATGTTTCAGRTGGATGAGRTTCTCWGA-----
COG2R1     -----TGGGAGGGCGATCGCAATCT-----TGTGAATGAAGATGGCGTCGA
RING2      -----TGGGAGGGCGATCGCAATCT-----

GII_4A_Richmond CAAGAGGCCATGTTTAGGTGGATGAGATTCTCGACCTCAGCACATGGGAGGGCGATCGCAATCTTGCTCCCAGGGTGTGAATGAAGATGGCGTCGA
GII.2       CAAGAACCATATGTTTAGGTGGATGAGATTCTCAGATTTGAGCACGTGGGAGGGCGATCGCAATCTTGCTCCCAGTCTTGTGAATGAAGATGGCGTCGA
GII.3       CAAGAACCAATGTTTAGGTGGATGAGATTCTCAGATTTGAGCACGTGGGAGGGCGATCGCAATCTTGCTCCCAGTCTTGTGAATGAAGATGGCGTCGA
GII.4       CAAGAACCAATGTTTAGGTGGATGAGATTCTCAGATTTGAGCACGTGGGAGGGCGATCGCAATCTTGCTCCCAGTCTTGTGAATGAAGATGGCGTCGA
GII.4 (2006b) CAAGAACCAATGTTTAGGTGGATGAGATTCTCAGATTTGAGCACGTGGGAGGGCGATCGCAATCTTGCTCCCAGTCTTGTGAATGAAGATGGCGTCGA
GII.4 (2008) CAAGAGCCAATGTTTAGGTGGATGAGATTCTCAGATTTGAGCACGTGGGAGGGCGATCGCAATCTTGCTCCCAGTCTTGTGAATGAAGATGGCGTCGA
GII.5       CAAGAGCCAATGTTTAGGTGGATGAGATTCTCAGATTTGAGCACGTGGGAGGGCGATCGCAATCTTGCTCCCAGTCTTGTGAATGAAGATGGCGTCGA
GII.7       CAAGAGGCCATGTTTAGGTGGATGAGATTCTCGACCTCAGCACATGGGAGGGCGATCGCAATCTTGCTCCCAGGTTGTGAATGAAGATGGCGTCGA

COG1F      -----CGYGGATGCGNNTTYCATGA-----
JJV1F      GCCATGTTGCGITGGATG-----
COG1R1     -----GTRAATGATGATGGCGTCTAAG--
JJV1R1     -----ATGATGATGGCGTCTAAGGA
RING1 (a)  -----TGGACAGGRGATCGCRATCT-----
RING1 (b)  -----TGGACAGGAGACCGCGATCT-----
RING1 (c)  -----TGGACAGGRGATCGCRATCT-----
RING1 (a*) -----TGGACAGGRGAYCGCRATCT-----
JJV1P      -----TGTGGACAGGAGATCGCAATCTC-----

GI_1_Norwalk  GCCATGTTCCGCTGGATGCGGTTCCATGACCTCGGATTGTGGACAGGAGATCGCGATCTTCTGCCCGAATTCGTAATGATGATGGCGTCTAAGGA
GI.2       GCCATGTTCCGCTGGATGCGGTTCCATGACCTCGGATTGTGGACAGGAGATCGCGAATCTCCTGCCCGAATTTGTAATGATGATGGCGTCTAAGGA
GI.3       GCCATGTTCCGCTGGATGCGGATTCCATGATCTAAGTTTGTGGACAGGGGACCGCGATCTCCTGCCCGATTATGTAATGATGATGGCGTCTAAGGA
GI.4       GCCATGTTCCGCTGGATGCGGTTCCATGACCTCGGATTGTGGACAGGAGATCGCAATCTCCTGCCCGAATTCGTAATGATGATGGCGTCTAAGGA
GI.6       GCCATGTTCCGCTGGATGCGGTTCCATGATCTTGCTTGTGGACAGGAGATCGCAATCTCCTGCCCGAATTCGTAATGATGATGGCGTCTAAGGA
GI.8       GCCATGTTCCGCTGGATGCGGATTCCATGACTTAAAGTTTGTGGACAGGAGATCGCGATCTTCTGCCCGATTATGTAATGATGATGGCGTCTAAGGA
GI.13      CCCATGTTTCGCTGGATGCGGTTCCATGATCTGAGCTTGTGGGCAGGAGATCGCAATCTCCTGCCCGATTATGTAATGATGATGGCGTCTAAGGA

```

Fig. 1. Sequences of the primers and probes used for real-time RT-PCR detection of NoV aligned with reference strains and with the RNA samples included in this panel. Reference strains are GI.1 Norwalk and GII.4 Richmond (GenBank accession numbers M87661 and AF414419, respectively). R=A or G, Y=T or C, W=A or T, B=C or G or T, N=A or C or G or T. Mismatches between panel RNA samples and primers/probes are indicated by bold face and underlined font. (1) These sequences are written as the reverse complement of those used in the real-time RT-PCR assays to allow for direct sequence comparisons.

GII.4 (2006b) RNA diluted to 100 g.e. The less sensitive detection of laboratory J as compared to K could be attributed to technician training time and familiarity with protocols, where the more experienced technician achieved more sensitive detection. The “J” dataset also showed false negative results for GI.2 and GII.5 samples at less than 10^4 g.e. per reaction. Six of the remaining laboratories did not detect the GII.4 (2006b) sample diluted to 10 g.e. Only one of these six labs had a false negative result for the different NoV strains. This suggests that detection of a standard GII.4 RNA diluted to 100 g.e. could be used as an indicator of good method performance.

Only two of the eighteen participating laboratories detected every NoV sample and none of the non-NoV samples, for an estimated 50% detection limit of <1 g.e. per reaction (labs D and V). Other laboratories with very sensitive detection identified a positive amplification of one or more of the non-NoV samples. Sequencing of one non-NoV amplicon revealed that bacterial or plant components in stool might cross-react with the real-time RT-PCR primers and probes to yield false positive results. Another possibility is that very sensitive reactions may detect low levels of cross-contamination during the procedure.

Previous collaborations for the evaluation of NoV detection methods have been complicated by the use of different extraction procedures in participating laboratories.^{32,33} This study circulated RNA panels to standardize the method input and provide a multi-centre comparison of real-time RT-PCR procedures in isolation of other factors. We have confirmed that the ORF1/ORF2 region identified in 2003²⁶ still provides good detection of 11 distinct NoV genotypes. This is in agreement with the published results of the real-time RT-PCR assay being assessed by the European CEN/TC275/WG6/TAG4 group as a standard method.³⁴ For the GII.4 (2006b) strain included as a dilution series, we did not observe any difference in detection limit between laboratories using the COG²⁶ or JJV²⁷ primer and probe sets. The previously reported detection limit of <10 g.e. for NoV GII^{27,28,35} was achieved in 9/18 labs and

the method was robust to minor variations in probe labelling or reagent concentration. Since these results were obtained, some laboratories have implemented changes to their primers or protocols to improve detection sensitivity. The use of a multiplex assay was associated ($p=0.04$) with false negative reactions, in contrast to a number of publications that report sensitive detection for multiplex protocols.^{27,36–39} Labs that include a control reaction in multiplex or that wish to multiplex multiple reactions should be aware of the possible decrease in sensitivity. This may be strain dependent, as noted in a previous study for multiplex assay development.³⁹

In the analysis presented here, we could only determine the efficacy of methods currently in use in the participating laboratories. Consequently, the data does not provide a validation of all published real-time RT-PCR protocols for NoV detection and the statistical power to compare the Kageyama²⁶ and Jothikumar²⁷ protocols was limited. The analysis confirmed that participating laboratories obtain sensitive and specific results using methods with slight variations in conditions. We conclude that there is no need to implement identical protocols in order to achieve comparable results by real-time RT-PCR between testing labs. In addition, the laboratories in this study could not commit the resources to test serial dilutions for each targeted strain. As a result, the differences in detection of the various genotypes are confounded by differences in sample concentration. However, our data are derived from undiluted stool filtrates and the concentration range is relevant to clinical testing labs. Finally, this targeted RNA panel project did not address methods of RNA extraction or control of inhibition, both of which are critical for effective NoV detection. These questions are the proposed targets of future studies for our group.

For this study, the participants designed the panel and submitted samples to be included for testing. As a result, they were able to cooperatively access a wide range of genotypes. This provides an excellent model for cooperative method validation that is particularly useful with non-culturable organisms, where reference sets are not readily available. We will use these findings to

develop a federal standard that can be compared to participant data in future proficiency studies, which are planned on a yearly basis to ensure that these assays stay current. Most laboratories detected less than 100 g.e. per reaction and were able to detect less common NoV genotypes. The most sensitive reactions were associated with false positive detection of non-NoV samples ($p = 0.03$). Reducing the sensitivity of these assays slightly may increase their specificity. Multiplex reactions were associated with failure to detect some strains ($p = 0.04$). Overall, slight variations in reagents or amplification conditions were not associated with differences in sensitivity or specificity.

Funding

Most laboratories used internal operating funds for this study. JB: Agriculture and Agri-Food Canada (AAFC) Research Branch Peer Reviewed Research Projects F.1401.02.11, AH: AAFC Grant/Growing Forward RBPI-233, JJ: Natural Sciences and Engineering Research Council of Canada.

Ethical approval

Not required.

Conflicts of interest

None declared.

Acknowledgements

We thank Kristin Agopsowicz, Mark Cardona, Jasmine Chamberland, Brian Detwiler, Christine Ellis, Marie-Josée Gagné, Maryline Girard, Michael Johns, Stacie Langner, Wenyang Li, Vanessa Morton, Elyse Poitras, Candy Rutherford, Chris Sherlock and Tom Stowe for expert technical assistance. We thank Catherine Carrillo and Nathalie Corneau for helpful comments on the manuscript and Stephen Hayward for expert statistical advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2010.10.008.

References

- Koopmans M. Progress in understanding norovirus epidemiology. *Curr Opin Infect Dis* 2008;**21**:544–52.
- Green KY. Caliciviridae: The noroviruses. In: Knipe DM, Howley PM, editors. *Fields Virology*. Philadelphia, PA: Lippincott Williams & Wilkins; 2007. pp. 949–979.
- Isakbaeva ET, Widdowson MA, Beard RS, Bulens SN, Mullins J, Monroe SS, et al. Norovirus transmission on cruise ship. *Emerg Infect Dis* 2005;**11**:154–8.
- Nygard K, Torven M, Ancker C, Knauth SB, Hedlund KO, Giesecke J, et al. Emerging genotype (GGIIB) of norovirus in drinking water, Sweden. *Emerg Infect Dis* 2003;**9**:1548–52.
- Lopman B, Vennema H, Kohli E, Pothier P, Sanchez A, Negrodo A, et al. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. *Lancet* 2004;**363**:682–8.
- Maunula L, Miettinen IT, von Bonsdorff CH. Norovirus outbreaks from drinking water. *Emerg Infect Dis* 2005;**11**:1716–21.
- Ng TL, Chan PP, Phua TH, Loh JP, Yip R, Wong C, et al. Oyster-associated outbreaks of norovirus gastroenteritis in Singapore. *J Infect* 2005;**51**:413–8.
- Friedman DS, Heisey-Grove D, Argyros F, Berl E, Nsubuga J, Stiles T, et al. An outbreak of norovirus gastroenteritis associated with wedding cakes. *Epidemiol Infect* 2005;**133**:1057–63.
- Simon A, Schildgen O, Maria Eis-Hubinger A, Hasan C, Bode U, Buderus S, et al. Norovirus outbreak in a pediatric oncology unit. *Scand J Gastroenterol* 2006;**41**:693–9.
- Estes MK, Prasad BV, Atmar RL. Noroviruses everywhere: has something changed? *Curr Opin Infect Dis* 2006;**19**:467–74.
- Johnston CP, Qiu H, Ticehurst JR, Dickson C, Rosenbaum P, Lawson P, et al. Outbreak management and implications of a nosocomial norovirus outbreak. *Clin Infect Dis* 2007;**45**:534–40.
- Bidawid S, Malik N, Adegbunrin O, Sattar SA, Farber JM. Norovirus cross-contamination during food handling and interruption of virus transfer by hand antiseptics: experiments with feline calicivirus as a surrogate. *J Food Prot* 2004;**67**:103–9.
- Clay S, Maherchandani S, Malik YS, Goyal SM. Survival on uncommon fomites of feline calicivirus, a surrogate of noroviruses. *Am J Infect Control* 2006;**34**:41–3.
- Malik YS, Maherchandani S, Goyal SM. Comparative efficacy of ethanol and isopropanol against feline calicivirus, a norovirus surrogate. *Am J Infect Control* 2006;**34**:31–5.
- Jimenez L, Chiang M. Virucidal activity of a quaternary ammonium compound disinfectant against feline calicivirus: a surrogate for norovirus. *Am J Infect Control* 2006;**34**:269–73.
- Cannon JL, Papafragkou E, Park GW, Osborne J, Jaykus LA, Vinje J. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. *J Food Prot* 2006;**69**:2761–5.
- Hewitt J, Greening GE. Effect of heat treatment on hepatitis A virus and norovirus in New Zealand greenshell mussels (*Perna canaliculus*) by quantitative real-time reverse transcription PCR and cell culture. *J Food Prot* 2006;**69**:2217–23.
- Mattison K, Karthikeyan K, Abebe M, Malik N, Sattar SA, Farber JM, et al. Survival of calicivirus in foods and on surfaces: experiments with feline calicivirus as a surrogate for norovirus. *J Food Prot* 2007;**70**:500–3.
- Chan MC, Sung JJ, Lam RK, Chan PK, Lee NL, Lai RW, et al. Fecal viral load and norovirus-associated gastroenteritis. *Emerg Infect Dis* 2006;**12**:1278–80.
- Teunis PF, Moe CL, Liu P, Miller SE, Lindesmith L, Baric RS, et al. Norwalk virus: how infectious is it? *J Med Virol* 2008;**80**:1468–76.
- Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS. Norovirus classification and proposed strain nomenclature. *Virology* 2006;**346**:312–23.
- Hansman GS, Natori K, Shirato-Horikoshi H, Ogawa S, Oka T, Katayama K, et al. Genetic and antigenic diversity among noroviruses. *J Gen Virol* 2006;**87**:909–19.
- Graham DY, Jiang X, Tanaka T, Opekun AR, Madore HP, Estes MK. Norwalk virus infection of volunteers: new insights based on improved assays. *J Infect Dis* 1994;**170**:34–43.
- Siebenga JJ, Vennema H, Zheng DP, Vinje J, Lee BE, Pang XL, et al. Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001–2007. *J Infect Dis* 2009;**200**:802–12.
- Lindesmith LC, Donaldson EF, Lobue AD, Cannon JL, Zheng DP, Vinje J, et al. Mechanisms of GII.4 norovirus persistence in human populations. *PLoS Med* 2008;**5**:e31.
- Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, et al. Broadly reactive and highly sensitive assay for norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J Clin Microbiol* 2003;**41**:1548–57.
- Jothikumar N, Lowther JA, Henshilwood K, Lees DN, Hill VR, Vinje J. Rapid and sensitive detection of noroviruses by using taqman-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Appl Environ Microbiol* 2005;**71**:1870–5.
- Trujillo AA, McCaustland KA, Zheng DP, Hadley LA, Vaughn G, Adams SM, et al. Use of taqman real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. *J Clin Microbiol* 2006;**44**:1405–12.
- Logan C, O'Leary JJ, O'Sullivan N. Real-time reverse transcription PCR detection of norovirus, sapovirus and astrovirus as causative agents of acute viral gastroenteritis. *J Virol Methods* 2007;**146**:36–44.
- Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, et al. Genogroup-specific PCR primers for detection of norwalk-like viruses. *J Virol Methods* 2002;**100**:107–14.
- Vinje J, Hamidjaja RA, Sobsey MD. Development and application of a capsid vp1 (region d) based reverse transcription pcr assay for genotyping of genogroup I and II noroviruses. *J Virol Methods* 2004;**116**:109–17.
- Mattison K, Grudeski E, Auk B, Charest H, Drews SJ, Fritzinger A, et al. Multi-center comparison of two norovirus ORF2-based genotyping protocols. *J Clin Microbiol* 2009;**47**:3927–32.
- Vinje J, Vennema H, Maunula L, von Bonsdorff CH, Hoehne M, Schreier E, et al. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. *J Clin Microbiol* 2003;**41**:1423–33.
- Butot S, Le Guyader FS, Krol J, Putallaz T, Amoroso R, Sanchez G. Evaluation of various real-time RT-PCR assays for the detection and quantitation of human norovirus. *J Virol Methods* 2010;**167**:90–4.
- Houde A, Leblanc D, Poitras E, Ward P, Brassard J, Simard C, et al. Comparative evaluation of RT-PCR, nucleic acid sequence-based amplification (NASBA) and real-time RT-PCR for detection of noroviruses in faecal material. *J Virol Methods* 2006;**135**:163–72.
- Mohamed N, Belak S, Hedlund KO, Blomberg J. Experience from the development of a diagnostic single tube real-time pcr for human caliciviruses, norovirus genogroups I and II. *J Virol Methods* 2006;**132**:69–76.
- Pang XL, Preiksaitis JK, Lee B. Multiplex real time RT-PCR for the detection and quantitation of norovirus genogroups I and II in patients with acute gastroenteritis. *J Clin Virol* 2005;**33**:168–71.
- Antonishyn NA, Crozier NA, McDonald RR, Levett PN, Horsman GB. Rapid detection of norovirus based on an automated extraction protocol and a real-time multiplexed single-step RT-PCR. *J Clin Virol* 2006;**37**:156–61.
- Wolf S, Williamson WM, Hewitt J, Rivera-Aban M, Lin S, Ball A, et al. Sensitive multiplex real-time reverse transcription-PCR assay for the detection of human and animal noroviruses in clinical and environmental samples. *Appl Environ Microbiol* 2007;**73**:5464–70.