European Community Reference Laboratory
for monitoring bacteriological and viral
contamination of bivalve molluscs

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Report on the Norovirus/Hepatitis A Ring Trial, 2006

CRL ring trial reference: RT15 (NoV/HAV 2006)

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Introduction

Regulation (EC) No 882/2004 designates the Centre for Environment, Fisheries and Aquaculture Science at Weymouth U.K. as the Community Reference Laboratory (CRL) for monitoring the viral and bacteriological contamination of bivalve molluscs. Under Article 4 (a and b) the laboratory is responsible for organising comparative testing by national reference laboratories.

At the 4th annual workshop of NRLs Nantes 2005 it was established that the Norovirus (NoV) and hepatitis A (HAV) ring trials were beneficial and should be offered in 2005/6. The workshop resolved that "the CRL should organise further ring trials for detection of NoV/HAV and that this should include shellfish analyses…." Resolution 4. 4th annual workshop of NRLs for the bacteriological and viral contamination of bivalve molluscs.

NRLs acknowledged that in the continuing absence of internationally or nationally accepted standards or procedures of equivalent standing methods used for analysis should be laboratory in-house procedures. Thus whilst not able to demonstrate specific method performance, the ring trial would generate information of comparative efficacy of laboratory developed procedures, and in the absence of other formal external quality assessment schemes provide laboratories with limited data in support of internal quality procedures.

Samples

Samples comprised laboratory constructed material (vials A-F), bio-accumulated and naturally contaminated Pacific oysters (*Crassostrea gigas*) (BM1 and 2). The intended contents of each vial (A-F) is included as Appendix I.

Samples positive for Norovirus (genogroups I and II)

Norovirus genogroup I (NoV GI) and genogroup II (NoVGII) positive samples were faecal material (stool samples) collected from patients presenting with acute gastro-enteritis. Faecal material was tested for NoV (NoV GI and NoVGII) using CRL standard methods. Ten percent faecal extracts were prepared in phosphate buffered saline (PBS). Extracts were then centrifuged at 3000 x *g* for 5 min. The supernatants were decanted; serially $\log_{10}$ diluted in PBS and analysed to determine NoV titre using the CRL standard method. Dilutions were aliquoted into 1ml volumes and stored at <-70ºC.

Samples positive for Hepatitis A

HAV, strain HM175 was cultured in FRKK-4 cells at 37±1ºC, 4-6%CO_2 for 3 days. Following visualisation of CPE (cytopathic effect) tissue culture flasks were frozen at <-15ºC overnight, thawed at room temperature and centrifuged at approximately 3000 x *g* for up to 1 min. Tissue supernatants were decanted and serially log_{10} diluted in PBS. HAV was assayed using CRL standard methods. Dilutions were aliquoted into 1ml volumes and stored at <-70ºC.

Samples positive for Norovirus and hepatitis A

Equal volumes of selected dilutions of NoV (GI and/or II) and HAV prepared as described above were mixed by inversion, aliquoted into 1ml volumes and stored at <-70ºC.

Negative samples

Virus negative samples were PBS. Prior to aliquoting into 1ml volumes, sub-samples were assayed using CRL standard methods. Aliquots were stored at <-70ºC.

Bivalve mollusc samples

A batch of approximately 600 Pacific oysters (*Crassostrea gigas*) was collected from a U.K. commercial producer classified under Regulation (EC) 854/2004 as B in December. Prior testing of the site had indicated that the likelihood of natural contamination with norovirus was high during the winter months. On arrival the oysters were inspected visually and split into two equal sub-samples. Sub-samples were designated BM1 and BM2. BM1 was further sub-divided randomly into 40 samples each comprising 5 oysters. Samples of BM1 were sealed in plastic bags and held at <-70ºC until quality control testing, dispatch or reference analysis.

Bioaccumulation with norovirus GII

BM2 were placed on trays and re-immersed in 550 litres of re-circulating natural seawater at 18±1˚C and 33ppt (Figure 1). The re-circulation rate was estimated at 20 litre/min. Following an acclimatisation period of 10h, 37.5ml of NoV GII (estimated titre 1 x 10^9 PCR units/ml) was added to the experimental unit. After 10h oysters were removed from the tank and rinsed in freshwater. Immediately 5 oysters per sample were selected

at random and sealed in clean plastic bags. Bags were held at <-70ºC until quality control testing, dispatch or reference analysis.

Figure 1. Bivalve shellfish bio-accumulation

Distribution

The NoV/HAV ring trial 2005-6 was designated as RT15. Laboratory constructed vials and bivalve mollusc samples were distributed together. Samples were packaged according to IATA regulations, UN3373 as diagnostic specimens, division 6.2 under the packing instruction code 650. All participating laboratories received a three sealed bio-pouches containing vials A-F, BM1 and BM2 within a single thermal control unit (CL-2/4) (Air Sea Containers Ltd., Birkenhead, U.K. CH42 1LE). Relevant transport documentation, import permits, instruction sheets and result forms were included with the package. Participants were requested to complete a protocol form describing the methods utilised for the ring trial. Accompanying paperwork is included as Appendix II of this report. Samples were distributed frozen on dry ice by City Sprint dedicated courier services on Tuesday 13th December 2005. On receipt, participants were requested to complete the analyses and return results by 3 February 2006.

Quality control

Prior to the distribution, 3 randomly selected samples were thawed at room temperature and analysed in triplicate using the CRL standard methods. The results are given in Table 1.

Table 1. Taqman™ quality control analyses of RT15 ring trial material

Samples	NoV (GI)	NoV (GII)	HAV
A	-	-	-
B	-	-	-
C	+ (33.14)	+ (28.05)	-
D	-	+ (38.16)	+ (37.69)
E	-	-	+ (38.46)
F	+ (27.59)	+ (31.75)	-
BM1	+ (42.20)	+ (39.62)	-
BM2	+ (40.30)	+ (29.30)	-

Average CT values in parentheses

Participation

Invitations inviting expressions of interest in the NoV/HAV ring trial were sent to all designated MS NRLs, candidate countries laboratories (Bulgaria, Turkey and Romania), Norway, U.S., New Zealand, Chile, Hong Kong, Canada and South Korea. Seventeen MS NRLs registered an interest in the ring trial, together with Norway, New Zealand, Canada (2 laboratories), Hong Kong, Chile, South Korea and U.S. Table 2 summarises the participation in the NoV/HAV ring trial distribution (RT15). Material was dispatched to twenty-seven laboratories. Addresses of participating laboratories are included as Appendix III. Twenty-three laboratories returned results for the trial (Table 2). Ireland, Latvia. South Korea and Italy carried analyses for norovirus only. NRL Finland and The Czech Republic were unable to participate due to lack of trained personnel and health and safety concerns over potential hepatitis A samples respectively. NRL Greece and Chile did not return results.

Confidentiality of results

To preserve anonymity of participants a confidential identification number was used to identify each laboratory. This number is circulated to each laboratory accompanied by this performance assessment report.

Reference results

Reference analyses on samples stored at >-20ºC were undertaken by the CRL following standard methods. On at least two separate occasions three extractions per vial/bivalve sub-sample were carried out on six randomly selected sample from each distribution set. The extracted viral RNA from each vial was pooled from each triplicate extraction/sample. Taqman™ analyses were then performed in triplicate on each of the pooled viral RNAs. The quantitative results of CRL reference testing are presented in Appendix IV. The intended results derived from these analyses are given as Appendix I.

Participants' results

Participant's results were assessed initially as discrete percentage correct returns per vial/bivalve mollusc sample for each viral determinant. In addition a simple scoring system was also applied to the returned results to facilitate assessments of combined performance across the whole ring trial exercise. Scores were assigned per vial according to the following algorithm:

Returned result	**Score**
-totally correct presence/absence result for GI, GII and HAV	3
-correct presence/absence result for GI and GII incorrect for HAV	2
-correct presence/absence result for GI and HAV incorrect for GII	2
-correct presence/absence result for GI, incorrect for GII and HAV	1
-correct presence/absence result for GII, incorrect for GI and HAV	1
-correct presence/absence result for HAV, incorrect for GII and GII	1
-no correct results reported	0

Scoring was adapted to incorporate laboratories that returned partial results sets.

Table 2. Participation in the NoV/HAV ring trial distribution (RT15)

Member State	Material dispatched	Results returned to the CRL	Comments
Austria	no	-	
Belgium & Luxembourg	yes	yes	
Czech Republic	yes	no	Not able to carry out HAV tests due to in-house health and safety issues
Denmark[3]	yes	yes	
Estonia	yes	yes	
Finland	no	-	Shortage of personnel prevented participation
France[3]	yes	yes	
Germany	yes	yes	2 laboratories participated both reported results
Greece	yes	yes	
Ireland[1,3]	yes	yes	
Italy[1]	yes	yes	
Latvia[1]	yes	yes	
Lithuania	no	-	
Netherlands	yes	yes	
Poland	yes	yes	
Portugal	yes	yes	
Slovakia[*]	yes	yes	
Slovenia	yes	yes	
Spain[2]	yes	yes	
Sweden	no	-	
United Kingdom[3]	yes	yes	
Candidate country			
Bulgaria	no	-	
Romania	yes	no	Holding samples
Turkey	no	-	Techniques not yet established in the laboratory

[1] norovirus only; [2]no norovirus differentiation; [3] reported quantitative data

Country	Material dispatched	Results returned to the CRL	Comments
EFTA			
Norway[3]	yes	yes	
Third Country			
New Zealand[3]	yes	yes	
United States	yes	yes	
Canada	yes	yes	2 laboratories participated both reported results
Hong Kong[3]	yes	yes	
South Korea	yes	yes	
Chile	yes	yes	

[1] norovirus only; [2]no norovirus differentiation; [3] reported quantitative data

Results

Twenty-three laboratories returned results, two of whom used methods that did not enable discrimination of NoV GI and NoV GII. Two laboratories did not return results for GI analysis whilst four did not analyse for HAV. One laboratory did not analyse bivalve shellfish samples. Tables 3 through 6 give the results for correct assignment of laboratory-constructed samples for NoV GI, NoV GII, HAV and PBS containing vials. Table 7 gives the results of virus results (all determinants assessed individually) for the *C. gigas* samples. No account is taken of false positive returns.

Performance assessment

Application of the simple scoring system enabled a crude assessment of the performance for laboratory constructed samples vials A-F and bivalve shellfish with respect to accurate assignment of total sample results. In this assessment presence/absence data was used and no consideration of quantitative measurements (C_T values) was made. It should be noted that assessments are provisional and should not be interpreted as true measures of laboratory performance. For example no account is made of the probability of recording at either a positive or negative result by chance, which for participants' returning all or mainly positive or negative data may be significant. Neither has any consideration been given to within and between sample constituent variability. No statistical analysis of the dataset was undertaken as the numbers of participating laboratories and samples were deemed too small to enable any power to determine false positive and negative rates.

Performance assessment- laboratory constructed samples (Table 8)

Sixteen laboratories analysed vials A-F for all determinants. Of these six (37.5%) returned results corresponding exactly to the intended contents. Seven (81%) laboratories scored >70%. Three laboratories achieved scores of less than 70%. However, of these one recorded 2 NoV GI and 1 HAV positive results in intended negative vials and failed to detect 1 NoV GII and 1 HAV positive vials. One laboratory failed to detect any HAV. One laboratory did not return any positive norovirus results for any vial, interestingly this laboratory detected both genogroups in bivalve samples. HAV contamination appeared to present as a problem for this laboratory with detection recorded in all intended negative vials (and bivalve samples), HAV was not reported in the intended positive vials. Seven laboratories did not carry out all analyses, all achieved

scores of >70% for the analyses undertaken. Four did not assay for HAV, all four returned accurate results with respect to norovirus and negative controls.

Performance assessment- bivalve shellfish samples (Table 8)

Twenty-two laboratories analysed bivalve samples -sixteen laboratories analysed the bivalve samples for all determinants. Only two laboratories returned negative results for the high titre NoV GII sample (BM2). One laboratory returned positive results corresponding to the intended viral content of the samples. However, this laboratory also recorded two of norovirus positive results in laboratory-constructed samples intended as negative controls thus the data may be indicative of laboratory contamination. Four laboratories, that analysed for all determinants, achieved scores of 67%, two detected NoV GII in both low and high titre bivalve samples but did not detect low titre NoV GI in either. One laboratory detected both genogroups of norovirus in BM1 and BM2, but also reported HAV presence in both samples. One laboratory reported BM2 in accordance with the intended results but did not detect low titre NoV GI or NoV GII in BM2. Of the remaining eleven laboratories, ten recorded scores of 50% amongst these a majority did not detect the low titre NoV GI in either sample. Two laboratories analysed for NoV GII and HAV only, one returned results corresponding exactly to the intended sample content. The other did not report the low titre NoV GII but recorded HAV in one sample. Four laboratories did not analyse for HAV. One failed to detect any norovirus (high or low titre) in the samples.

Performance assessment-combined

Taken together the overall performance of laboratories for both laboratory constructed samples and bivalve shellfish appeared good with just four laboratories achieving scores of less than 70% (considered as a level denoting satisfactory performance in other proficiency testing schemes (e.g. CRL/HPA EQA). However, it is clear from Table 8 and that the overall performance of laboratories is heavily skewed by the generally lower performance in bivalve shellfish analyses. This is masked in the overall scores by the high level of accurate returns from laboratory-constructed samples.

Table 3. Participants' performance norovirus GI (vials C, F)

Laboratory ID	RT15 % score
019	100
041	100
025	100
037	100
033	100
055	100
010	100
032	100
024	100
027[1]	100
039	100
007	100
015	100
044	100
011	100
003	100
017[1]	(100)*
049	50
002	50
022[1]	0
029	100
009	0
021	0
048[2]	-
014[2]	-

[1] laboratory tested for norovirus GI and GII together
[2] GI tests not reported
* vial C empty on arrival

Table 4. Participants' performance norovirus GII (vials C, D, F)

Laboratory ID	RT15 % score
019	100
041	100
025	100
037	100
055	100
033	100
010	100
032	100
024	100
027[1]	100
039	100
044	100
011	100
003	100
048	100
014	100
021	100
017[1]	(100)*
007	67
022[1]	0
029	100
002	33
015	33
049	33
009	0

[1] laboratory tested for norovirus GI and GII together
* vial C empty on arrival

Table 5. Participants' performance HAV (vials D, E)

Laboratory ID	RT15 % score
014	100
019	100
041	100
025	100
037	100
033	100
049	100
010	100
032	100
024	100
055	100
027	100
039	50
007	50
021	50
015	0
048	0
009	0
002	0
022	0
029	50
011	-
003	-
044	-
017	-

Table 6. Participants' performance negative samples (vials A, B)

Laboratory ID	RT15 % score
041	100
025	100
037	100
049	100
055	100
024	100
039	100
021	100
015	100
002	100
007	(100)*
044	(100)*
019	(100)*
011[3]	100
017[1]	100
003[1]	100
014[2]	100
010	83.3
033	83.3
022	100
029	100
027[1]	83.3
048[2]	83.3
032	66.8
009	66.8

[1] laboratory analysed for norovirus only
[2] did not return results for GI
[3] laboratory tested for norovirus GI and GII together
* vial A empty on arrival

Table 7. Participants' performance bivalve shellfish samples (BM1 and BM2)

Laboratory ID	RT 15: BM1			RT 15: BM2		
	NoV GI	NoV GII	HAV	NoV GI	NoV GII	HAV
041	✓	x	✓	✓	✓	✓
025	x	✓	✓	x	✓	✓
037	x	x	✓	x	✓	✓
049	✓	✓	✓	✓	x	x
055	x	x	✓	x	✓	x
024	x	x	✓	x	✓	✓
039	x	✓	x	x	✓	✓
021	x	x	✓	x	✓	✓
015	x	x	✓	✓	✓	✓
002	x	✓	✓	x	✓	✓
007	x	x	✓	x	✓	✓
044	nt	nt	nt	nt	nt	nt
019	x	x	✓	x	✓	✓
011[3]	x	x	-	x	x	-
017[1,3]		x	-		✓	-
003[1]	x	x	-	✓	✓	-
014[2]	-	x	x	-	✓	✓
010	✓	✓	✓	✓	✓	✓
033	x	x	✓	x	✓	✓
027[1]		x	x		x	x
048[2]	-	✓	✓	-	✓	✓
032	x	x	✓	x	✓	✓
009	x	✓	x	✓	✓	x
022		x	x		x	x
029	✓	✓	✓	✓	✓	✓

nt- not tested
[1] laboratory analysed for norovirus only
[2] did not return results for GI
[3] laboratory tested for norovirus GI and GII

Table 9. Performance assessment of laboratories

Laboratory ID	RT 15 % score[a]		
	Laboratory constructed vials[a]	Bivalve shellfish[a]	Combined performance[a]
019	100	50	88
041	100	83	96
025	100	67	92
037	100	50	88
055	94	33	79
033	94	50	83
049	78	67	75
010	83	100	88
032	89	50	79
024	100	50	88
027[1]	75	25	63
039	94	50	83
044[2,4]	100	nt	100
011[2]	100	0	75
003[2]	100	50	88
048[3]	75	100	81
014[3]	100	50	88
021	83	50	75
017[1,2]	100	50	88
007	67	50	63
002	77	83	78
015	67	67	71
009	39	50	42
022[1]	33	0	25
029	89	100	92

nt not tested
[a] scored according to algorithm for total vial content
[1] laboratory analysed for norovirus only (no differentiation)
[2] no analysis for HAV undertaken
[3] no analysis for GI
[4] bivalve mollusc samples not tested

Participants' methods

Laboratories reported a wide range of protocols. Frequently 'QIAmp Viral RNA Minikit and 'Boom' based extraction methods were described. At least ten laboratories reported use of real time PCR. Both one and two-step formats were utilised. A number of laboratories used multiple protocols. A summary of analytical methods reported by participants for the ring trial part is as Appendix V.

Comments

The NoV/HAV ring trial was successful with respect to participation. Material was dispatched to twenty-seven laboratories- twenty-five laboratories (92%) returned results. Lack of trained personnel, health and safety concerns and changes in laboratory prioritisation (due to avian flu) were cited as reasons for not completing the proficiency testing exercise within the specified time. Notwithstanding this the 2005-6 NoV/HAV ring trial was the most comprehensive CRL ring

trial to date with a 92% increase in uptake from 2004-5. This demonstrates the increasing competence and interest in the area.

Of the twenty-five laboratories returning results twenty-four analysed bivalve samples indicative of the increase in the number of laboratories with the capacity to undertake this type of sample. The number and type of methods employed in this study was diverse with respect to extraction procedures, PCR formats and primer selection, with some laboratories employing more than one procedure. However, at least ten laboratories returned quantitative real time PCR data compared with six in 2004-5 demonstrating the rise in numbers of institutes with the ability to generate real time quantitative data. The generation of quantitative results greatly enhances the ability to assess both method and laboratory performance compared with presence/absence testing.

The courier used for this ring trial was City Sprint Ltd. The CRL specified temperature and time transport criteria to for these temperature critical samples. The courier was contracted to maintain all samples in a frozen state and deliver them within a maximum three days. Most packages arrived within the specified delivery times and all arrived at their destination frozen. However, costs of transportation of labile samples requiring specialised carriage conditions remain very high and it is important to continue development of stable reference materials that can be easily and effectively distributed (preferably at ambient temperatures) for future external quality assurance purposes.

Extensive parallel reference testing by the CRL generated a large dataset describing the nature of the samples sent to participants. Quantitative analysis using Taqman^{TM} PCR for detection of NoV and HAV using the Applied Biosystems SDS 7000 was undertaken. The results indicated that even at low titres vial contents were relatively homogeneous. Thus differences in laboratory performance, particularly with respect to detection of low titre virus, did not appear to be attributable to between vial variability. However, the potential for stochastic variance in viral content of individual samples cannot be ruled out. Between sub-sample repeatability in GII bioaccumulated bivalve samples was high. However, naturally contaminated *C. gigas* were considered as contaminated at levels around the limit of detection (according to the CRL standard method) thus the probability that individual sub-samples were virus negative cannot be discounted entirely.

Summary

The NoV/HAV ring trial was a successful exercise. The data generated has identified a number of key issues and recommendations with respect to the application of Norovirus and hepatitis A:

- The methods of analysis utilised by laboratories were numerous and diverse.

- The increase in numbers of laboratories generating real-time quantitative data was encouraging and greatly enhances the ability to assess both method and laboratory performance.
- Laboratory constructed samples of both moderate and low viral titre can be effectively and correctly analysed using a number of approaches.
- Most methods can be employed to successfully detect high titre virus (GII norovirus) in bivalve shellfish matrix.
- Low titre virus (GI and GII) in bivalve shellfish (representative of natural levels of contamination, typically found in harvesting areas) presents a significant challenge to laboratories.
- Standard reference material is required to enable accurate method assessment.
- A standardised reference method is required to enable accurate assessment of between laboratory performance.

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Appendix I

FDR 3416
by e-mail

23rd February 2006

Dear Participant

NOROVIRUS/HAV RING TRIAL (RT15)

The distribution due for examination by 3rd February 2006 consisted of six vials (A-F) containing 1 ml of test material and two samples of Pacific oysters (*Crassostrea gigas*)

The intended results were:

Sample	Norovirus		HAV
	GI	GII	
A	-	-	-
B	-	-	-
C	+ (39.02, low)	+ (35.71, moderate)	-
D	-	+ (37.35, moderate)	+ (41.52, low)
E	-	-	+ (39.34, low)
F	+ (39.09, low)	+ (35.07, moderate)	-
BMS1	+ (41.06, low)	+ (39.03, low)	-
BMS2	+ (42.87, low)	+ (29.30, high)	-

Average viral titres as C_T values derived from CRL testing with interpretation in parentheses

A full report including an analysis of all participants' results will be sent shortly.

Yours sincerely

Rachel Rangdale

CEFAS
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Appendix II

RT15 norovirus/hepatitis A ring trial instructions:

Within your package you will find one labelled sealed container marked 1 and two sealed bags.

Sealed container 1

Contains vials A - F. Each vial contains a 1ml aliquot of test material

Sealed bag 1

Contains 5 *Crassostrea gigas* labelled BM1

Sealed bag 2

Contains 5 *Crassostrea gigas* labelled BM2

On receipt of ring trial material

1. All samples have been sent frozen on dry ice. Please take the temperature inside the box and record it on the RT15 report form. Inspect the contents to insure you have all of the material listed above.

2. Store all samples at 2-6^{o}C, and analyse within 48h. If samples cannot be processed within 48h and have arrived frozen they can be held at <-15°C until analysis. If samples cannot be processed within 48h and are not completely frozen on arrival please inform the organisers immediately.

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RT15: Norovirus / HAV ring trial report form 2005/6

Date of Analysis:

1. Name and Country of Laboratory / Institute:

2. Contact name:

3. E–mail address:

4. Contact telephone:

*Were the samples frozen on arrival? yes/no

*Was the labelling clearly discernible? yes/no

5. For each vial and bivalve mollusc sample please indicate a positive or negative result for norovirus (please include genogroup if possible) and/or HAV. If using a real-time assay format please give C_T values. If you wish to make any additional comments about the sample please use the box provided.

Sample	Norovirus		HAV	COMMENTS
	GI	GII		
A				
B				
C				
D				
E				
F				
BM1				
BM2				

Please return this form by post to Rachel Rangdale, CEFAS, Weymouth laboratory, The Nothe, Barrack Road, Weymouth, DT4 8UB or electronically to r.e.rangdale@cefas.co.uk by 3rd February 2006.

If this form is not appropriate to your method please attach your standard worksheet clearly labelled with the name of your laboratory and the sample.

Please see overleaf

RT-PCR procedure information 2005/6

Please include details of the procedures used to analyse the ring trial material. If different procedures are used for the bivalve mollusc samples and the faecal/tissue culture material please indicate this clearly.

Virus extraction procedure **(include the initial volume/weight of material processed)**

Nucleic acid extraction procedure (include the volume material processed)

RT PCR-procedure (e.g. real-time one step RT-PCR, conventional semi-nested etc)

Confirmation (include details of amplimer confirmation if appropriate)

Primers

Published Method Yes/No

If yes reference of publication

Additional Information

Appendix III

Participating laboratories

1. Departement des Sciences des denrees alimentaires
Service de microbiologie
Bd de Colonster no. 20
Bat B43Bis
4000 Liege
BELGIUM

2. Veterinary Faculty
National Veterinary Institute
Gerbiceva 60
SI-1115 Ljubljana
SLOVENIA

3. Canadian Food Inspection Agency
Burnaby Laboratory
2250 Boundary Road
Burnaby,
British Columbia
V5M 4L9
CANADA

4. National Institute for Public Health and the Environment (RIVM)
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3721 MA bilthoven
THE NETHERLANDS

5. Food Virology Research Unit
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Canadian Food Inspection Agency
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CANADA

6. State Veterinary and Food Institute
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6. Japan Food Safety Centre
Olivos 943
Independencia
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CHILE

8. IPMAR
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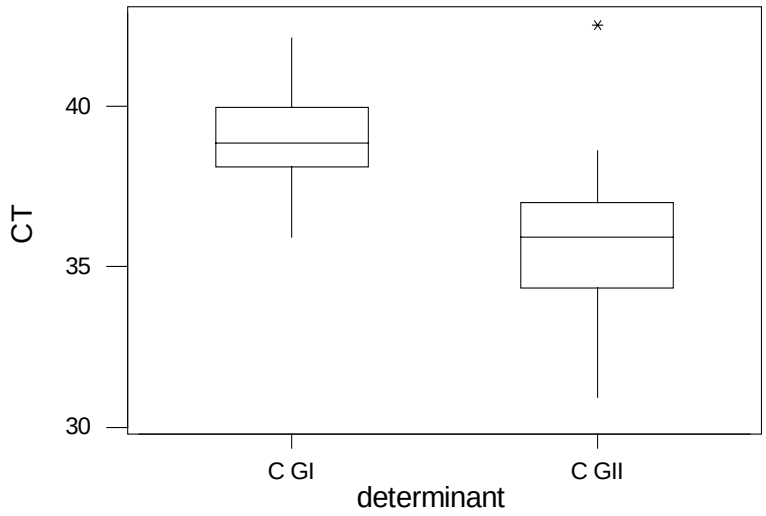
Appendix IV

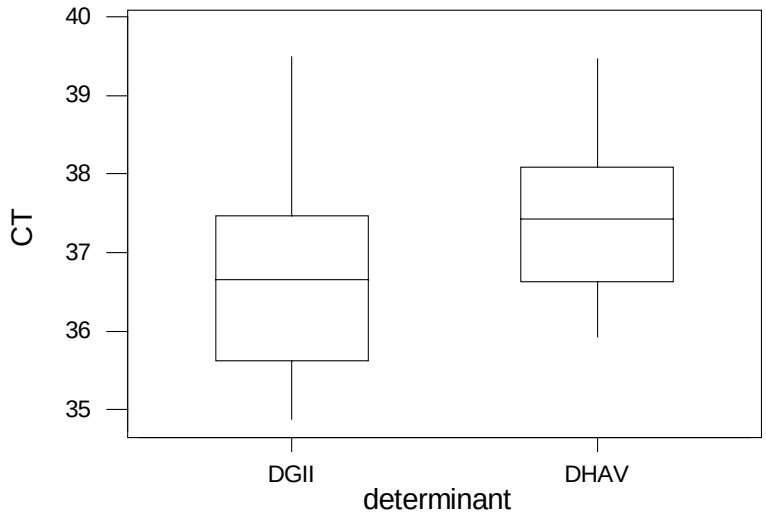
Reference results

CRL Reference results- Displayed as boxplots
The central line denotes the median Ct value. The bottom of the box is at the first quartile (Q1), and the top is at the third quartile (Q3) value. The whiskers are the lines that extend from the top and bottom of the box to the adjacent values. The adjacent values are the lowest and highest observations that are still inside the region defined by the following limits:
Lower Limit: Q1 - 1.5 (Q3 - Q1)
Upper Limit: Q3 + 1.5 (Q3 - Q1)
Outliers are points outside of the lower and upper limits and are indicated with asterisks (*).

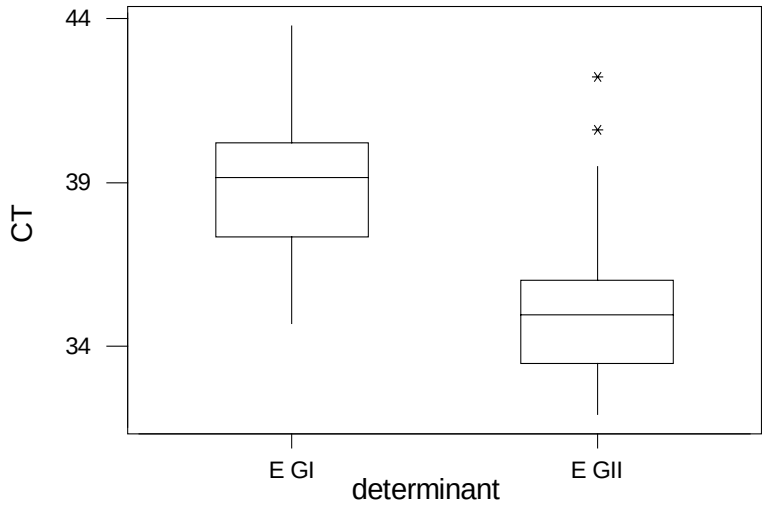
1. Vial C



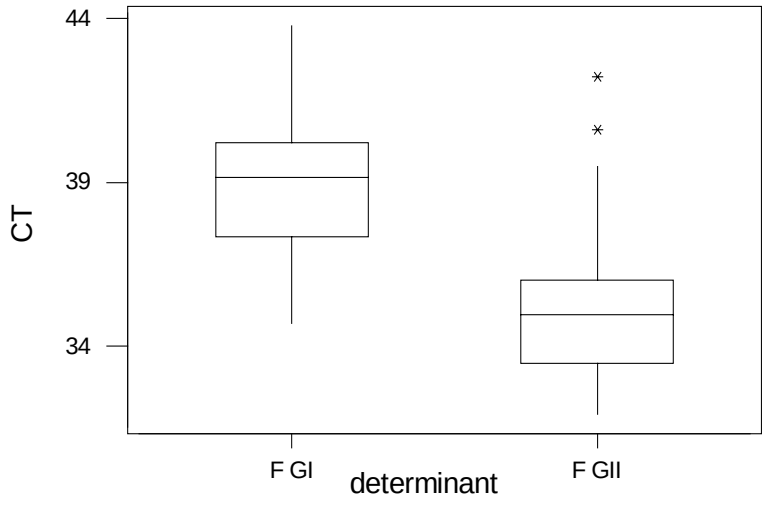
2. Vial D



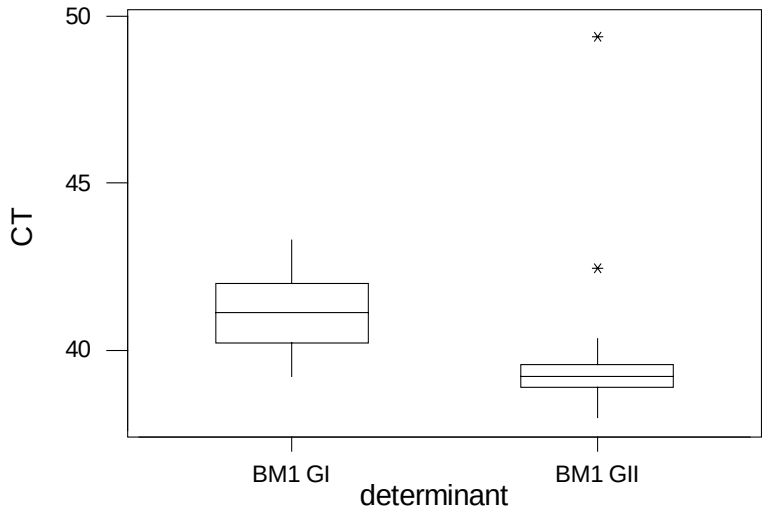
3. Vial E



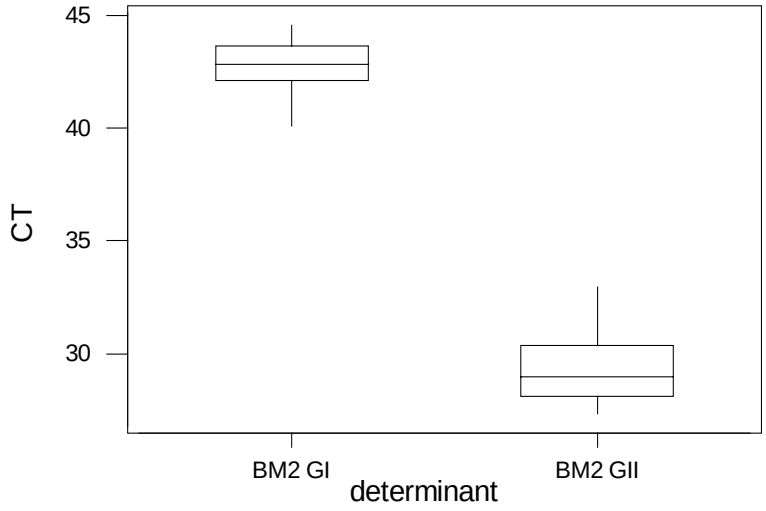
4. Vial F



5. BM1


50
45
40
CT
BM1 GI
BM1 GII
determinant

6. BM2


45
40
35
30
CT
BM2 GI
BM2 GII
determinant

Appendix V

Participant's methods

Laboratory ID	Virus Extraction Method	Viral RNA Extraction Method	RT-PCR /PCR Protocol	Primers	Confirmation
015	Digestive gland/Proteinase K	NoV Modified Boom HAV Nucleospin RNA viruses	**NoV** 2 Phase random hexamers nested PCR **HAV** Access Quick RT-PCR (Promega)	**NoV** Green *et al* 1998 **HAV Gentsch *et al* 1992** **Mannua *et a*l 1994**	-
010	Digestive gland/Proteinase K	Modified Boom extraction	**Real Time** TaqMan	NoV JTMG1F/ JTMG1R COG2F/COG2R Jothikumar et al 2005 HAV Costafreda et al	Taqman probe JTMG1/RING
002	OPFLP-01 digestive glands only	OPFLP-01	**www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume5/index_e.html** **HAV Conventional RT-PCR Qiagen One step RT-PCR kit**	NoV Kageyama et al 2003 Anderson et al 2001 HAV HAV1/2 neHAV1/2 Kingsley and Richards 2001	Not stated
041	0.9g pancreatic tissue PEG ppt	Boom Biomerieux Boom Biomerieux on PEG pellet (BM)	Real time one step RT-PCR (Platinum Invitrogen kit)	**NoV and HAV (as CEN TAG4)**	Classic RT-PCR
032	Modified Kingsley and Richards 2001	QIAampViral RNA minikit TriReagent	**NoV** One-step RT-PCR kit (Qiagen) Real time RT-PCR system: Quantitect Probe RT-PCR Kit/Quiagen **HAV** nested RT-PCR Real time PCR: One-step RT-PCR	**NoV** Single round –S. Monroe Nested RT-PCR Green *et al* 1998 **Real time GII** one step Hohne, Schreier **HAV**Goswami et al 2002	**Taqman probe**

024	Digestive gland/Proteinase K	Roche High Pure viral Nucleic acid kit	NoV Real time two step RT-PCR assay HAV conventional nested RT-PCR	**NoV** Kageyama *et al* 2003 **HAV 2949,3192 (region VP1/2A junction) nested dKA24, dKA25 Robertson et 1992** **Kingsley and Richards 2001**	
019	Hybaid ribolyser	Boom extraction	Single round	**NoV** Venema et al 2002 **HAV** Bosch et al 2001	Southern Blotting
025	Atmar et al 1995 LeGuyader et al 2000	QIAampViral RNA minikit Proteinase K phenol-clhorfrom ethanol ppt.	**RT Invitrogen SuperscriptIII PCR Taqman probe based**	**NoV JJV1NF modified Jothikumar et al 2005 JJV1R Jothikumar et al 2005 RING1b Kageyama et al 2003, Loisy et al 2005** HAV Costafreda et al	
014	Digestive gland/Proteinase K	QIAampViral RNA mini kit	**Real time one step RT-PCR**	**NoV JJVIFJJV1R, COG2F, COG2R** HAV HEP A1F, HEP A2F HEP AR	
009	Digestive gland/Proteinase K	Boom then total quick RNA cells and tissue kit (Talent) or Rneasy Plus mini kit (QIAGEN)	Two step conventional RT-PCR One step SuperscriptIII RT-PCR Platinum (Invitrogen)		
021	Digestive gland equal vol glycine	QIAampViral RNA minikit* Rneasy Plant Minin kit	QuantiTect probe RT-PCR kit (Quiagen) Real time RT-PCR run on Smartcycler	NoV Jothikumar et al 2005 HAV Costa-Mattoili et al 2002	Real time probe
055		RNeasy Qiagen kit GCSL method published by Mullendore et al.	Real-time one Step RT-PCR-TaqMan Style Prob	NoV Primer COG1F Primer COG1R, Probe GI-P-1 Probe GII-P HAV Gardner, S. N., T. A. Kuczmarski, et al. (2003)	
007		Chloroform-Butanol extraction (le Guyader 2003)	**NoV** semi nested RT- PCR **HAV** Single round Rt-PCR	**NoV** Kojima *et al* 2002 Le Guyader *et al* 2003 **HAV** Bosch 2001	Southern Hybridisation **NoV** Le Guyader *et al* 2003 **HAV** Bosch *et al* 2001
039		Trizol method (Gibco INoVitrogen)	Access RT- PCR system **NoV** GGI one step RT-PCR Vinje *et al* 2004 NoV GGII one step RT-PCR **HAV** One step RT-PCR and Semi-nested	**NoV** GGI Vinje *et al* 2004 GGII Kojima *et al* 2002 **HAV** Le Guyader 1994	

017	Glycine – PEG DeMedici *et al* 2001	NOV Nucleospin RNA II kit (MACHEREY-NAGEL)	NoV-Booster PCR -double PCR same primer set	NoV Vinje & Koopmans 1996	Southern hybridisation Vinje & Koopmans 1996
027	VERTREL and PEG	Guanidinium tiocianate/phenol (Trizol) followed by silica matrix adsorption System	One step RT-PCR	**NoV** JV12Y, JV13I **HAV** HAV240, HAV68	
039		Trizol (Invitrogen) method from 250 microlitres faecal material Nucleospin RNA Plant (MACHEREY-NAGEL) 100mg digestive gland	Access one step RT- PCR system (PROMEGA)	**NoV** GGI Kojima *et al* 2002 GGII Kojima *et al* **HAV** Le Guyader 1994	
011	1:10 tryptose phosphate –0.05M glycine, sonication, PEG 6000, centrifugation, sonication, ultracentrifugation	AGPC with modifications with TRIZOL	Nested RT-PCR	**NV35, NV36, NV81, NV82, SM82, Yuri52F, Yuri52R, MR3, MR4 COG1F, G1SKR, G1SKF, COG2R, G2SKF, G2SKR, G1, G2, SM31, Ando, NI, E3**	Sequencing
007	Chloroform-Butanol extraction (le Guyader 2003)	Chloroform-Butanol extraction (le Guyader 2003)	**NoV** Single round RT- PCR **HAV** Single round RT-PCR	**NoV** Kojima et al 2002 Le Guyader et al 2003 **HAV** Bosch 2001	Southern Hybridisation **NoV** GI Le Guyader *et al* 200 NoV GII P157 unpublished **HAV** Bosch *et al* 2001
048	PEG	TRI-reagent	One step RT-PCR	**Schreier et al 2000 Beurat et al 2002**	
003	Digestive gland/Proteinase K	Modified Boom extraction	**Real Time two –step** TaqMan	NoV NoV JTMG1F/ JTMG1R COG2F/COG2R Jothikumar *et al* 2005	Taqman probe JTMG1/RING
044	Amplisens N0V	-	One step RT-PCR	**Not known**	Gel electrophoresis
037	Chloroform PEG6000, elution in Na_2HPO_4	QIAGEN UltraSens RNA kit, RNA mini kit	Two step conventional nested HAV, real time NoV.	**HAV VP1 capsid region NoV Gp(I) Gp(II) capsid region**	Sequencing
033	-	QIAGEN QIAamp Viral RNA mini kit RNeasy protect mini kit	One step RT-PCR kit (QIAGEN) conventional semi nested	**HAV HAV1, HAV2, neHAV1, neHAV2 NoV SM31, GI, GII, Ando, MON433, MON434, MON435, MON436 NLV-GG2-F NLV-GG2-R Made et al 2005**	-

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