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CEFAS

The Centre for Environment, Fisheries & Aquaculture Science

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

Report on the Norovirus Ring Trial, 2003/2004

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CRL ring trial reference: **RT7 (Norovirus, 2004)**

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General comments

The 'Centre for Environment, Fisheries and Aquaculture' (CEFAS) Weymouth, was designated as the co-ordinating European Community reference laboratory (CRL) for monitoring bacteriological and viral contamination of bivalve molluscs by the European Council on 29 April 1999. Each Member State was also required to designate a National Reference Laboratory (NRL) to co-ordinate activities in their country. One of the duties of the reference laboratory network is to organise comparative testing.

At the 2003 NRLs workshop it was agreed that the CRL would develop a ring trial for Norovirus, which would follow the same approach taken previously for HAV. The CRL therefore organised a three part Norovirus (NV) ring trial, all parts of which were distributed frozen to participants on the 3rd of October 2003. The package contained two distributions of clinical faecal material together with a distribution of artificially (laboratory) contaminated shellfish.

Ring Trial Material

Faecal extracts

10% faecal extracts were prepared from two stool samples one containing a NV genogroup I (GI) stain and the other a NV genogroup 2 (G2) strain. These faecal samples had previously been assayed by the CRL standard method (Boom nucleic acid extraction followed by nested RT-PCR; Green and Henshilwood et al., 1998) and shown to produce correct size NV PCR amplicons. Amplicons were cloned and sequenced to confirm presence of NV and to identify the NV strain present. Both the GI and G2 strains were confirmed as NV sequence with the GI sharing >95% homology with strain Pelelui (EMBL AY149297) and the G2 strain sharing > 95% homology with the common Grimsby (Lordsdale) strain. 10% faecal extracts were prepared by mixing a 1:10 dilution (wt/vol.) of faecal stool in PBSa followed by centrifugation at 3000g for 5 minutes. The

supernatants were serially diluted in PBSa and analysed for NV using the CRL method (as above) to determine the virus titre. Stock dilutions of a high and low concentration of each NV genogroup were made as detailed in Table 1. A number of 1ml aliquots of each stock dilution were prepared and stored frozen at –20^{o}C for distribution in the ring trial. A representative vial of each stock dilution was then defrosted and assayed to ensure NV was still detectable. Similar vials containing buffer only were also prepared and frozen (NV negative samples).

Samples distributed	Content (all dilutions in PBSa)
D	NV GI $^{-3}$ dilution (high titre)
A	NV GI $^{-5}$ dilution (low titre)
B	NV G2 $^{-3}$ dilution (high titre)
C	NV G2 $^{-5}$ dilution (low titre)
E, F, G, H	PBSa Only

Table 1. Contents of vials distributed in NV ring trials parts 1 and 2

Bio-accumulated Shellfish

Pacific oysters (*Crassostrea gigas*) were sourced from a grade 'A' harvesting area. A representative sample was analysed to ensure the absence of NV using the CRL shellfish method (as above). 100 animals were then shucked, packaged and stored at –20^{o}C prior to dispatch (negative shellfish). The remainder were placed in a recirculating seawater tank at 15^{o}C and 50ml of a 10% faecal suspension (1:10 dilution wt/vol of faecal stool in PBSa) of high titre G2 NV containing stool added to the recirculating system. The NV strain in this stool sample had been previously sequenced (as described above) and found to share >95% homology with the Grimsby (Lordsdale) G2 strain. Shellfish were removed from the system after 48h, washed externally, shucked, packaged and stored at

-20^{o}C prior to dispatch for the ring trial. A representative shellfish sample was defrosted and analysed by the CRL standard method for NV and was found strongly positive for G2 NV. The negative shellfish samples were similarly defrosted and tested and found negative for NV by the CRL method.

Package	Content
K	6 shucked NV G2 positive shellfish
J	6 shucked shellfish negative for NV by CRL method

Table 2. Package contents for norovirus ring trial part 3

Packaging and Transport

All participating NRLs received three sealed containers labelled 1-3. Sealed container 1 contained Packages 1 and 2. Package 1 contained vials E, B, F and A (clinical material ring trial part 1) and Package 2 contained vials C, G, D and H (clinical material ring trial part 2). Sealed container 2 contained Package J and sealed container 3 contained package K (shellfish material ring trial part 3). The three sealed containers were transported on dry ice to ensure samples remained frozen for the duration of transport. Transit times ranged between 1 and 3 days depending on destination.

Participation in the 2003/4 norovirus ring trial

All NRLs were formally invited to take part in the norovirus ring trial. Austria, Ireland and Sweden declined to participate in the ring-trial. 12 NRLs agreed to participate and were sent a consignment of ring trial material. Table 3 shows the NRL participants and the time taken for delivery of the ring trial consignment.

Country	Date of dispatch	Date of arrival at lab *	Arrival temp °C**	Remarks
Belgium	03.10.03	07.10.03	<0	
Denmark	03.10.03	08.10.03	<0	
Finland	03.10.03	11.10.03	0±1	Airline misplaced shipment
France	03.10.03	08.10.03	<0	
Germany	03.10.03	06.10.03	<0	
Greece	03.10.03	09.10.03	<0	
Italy	03.10.03	10.10.03	<0	Customs and airline delays
Netherlands	03.10.03	07.10.03	<0	
Norway	03.10.03	08.10.03	<0	
Portugal	03.10.03	08.10.03	<0	
Spain	03.10.03	09.10.03	<0	Delivery refused by consignee
UK	03.10.03	Not applicable	<0	

Table 3: The receipt dates with consignment arrival temperatures for each participating NRL.

* Arrival dates reported by courier
** Arrival temperature reported by NRL recipient

On receipt containers 2 and 3 and Package 2 vials D, G, H, C were stored at -20°C. Package 1, containing vials E, B, F, A were stored at 4°C to be analysed within 48 hours. Participants were instructed to analyse 50 µl aliquots following their own 'in house' laboratory protocol for the detection of norovirus by RT-PCR. The order of analysis was specified (see appendix). Although most transit times exceeded those stipulated by the courier company, ten of eleven shipments arrived at their destination frozen. The consignment for Finland was delayed and may have been partially defrosted.

Analytical methods used

A summary of analytical methods reported by participants for ring trial parts 1 and 2 are shown in Table 4a. Table 4b display methods employed by participants for part 3 of the ring trial. Participants are coded to permit comparison with results of analysis.

NRL ID.	Nucleic Acid Ext.	RT – Protocol	PCR Protocol	PCR Cycling Parameters	Primers Used	Published Method	Additional Info
NRL 01	QIAamp Viral RNA Minikit (Qiagen)	OneStep RT-PCR Kit (Qiagen)	Titanium™ TaqDNA Polymerase (Clontech) Nested PCR	50°C 30mins, 95°C-15 mins and 35 cycles(94°C-30sec, 42°C-30sec, 72°C-45sec) then 72°C-10mins. Nested- 95°C-1mins and 35 cycles(95°C- 30 secs, 42°C-60sec, 68°C-60 sec) then 68°C-3mins	Schreir et al., 2000 ORF1: RNA polymerase gene (GI and G2) ORF 3: small basic protein region (G2) Vinje et al., 2003 Primer mixture: ORF1 and ORF1 with modifications	YES	
NRL 03	NucleoSpin RNA II Kit (Macherey-Nagel, Durren, Germany)	Booster Protocol	Booster - PCR	94°C-2mins and 40 cycles(94°C-1min, 37°C-90sec, 68°C-2min) then 68°C-7mins	Vinje Primers:JV-12 and JV-13	No	
NRL 04	QIAamp Viral RNA Minikit (Qiagen)	Two Phase, Random Hexamers (PdN6)	Nested	94°C-3 mins and 40 cycles(94°C-60sec, 50°C-30sec, 72°C-2mins) then 72°C-15mins. Nested- 94°C-3mins and 40 cycles(94°C- 30 secs, 50°C-30sec, 72°C-60 sec) then 72°C-7mins	PCR: Nishida et al 2003 GG1: COG1F +GISKR GG2:COG2F +G2SKR Nishida et al., 2003 + Kojima et al.,2002 GG1: G1SKF +G1SKR GG2: G2SKF + G2SKR	Yes/no	
NRL 05	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green & Henshilwood *et al*., (1998)	96°C-10mins and 35 cycles(94°C- 60 secs, 37°C-60sec, 72°C-60 sec) then 72°C-10mins. Nested Same	SM31, Ando, NI ,E3	Yes/No	
NRL 07	Vertrel, viral precipitation with PEG Viral RNA extraction with Trizol. RNA adsorption in an affinity RNA matrix	OneStep RT-PCR Kit	Single Round	-	-	-	-
NRL 08	Boom et al, (1990)	JV131 specific primers	Single Round	94°C-2mins and 40 cycles(94°C- 60 secs, 37°C-90sec, 74°C-60 sec) then 74°C-7mins	Vinje Primers: JV-12Y and JV-13I	Yes	GI and G2 determination of sample using hybridisation.

Table 4 a: Summary of methods reported by NRLs for parts 1 and 2 of NV ring trial *(note : methods for part 3 to be confirmed).*

NRL ID.	Nucleic Acid Ext.	RT – Protocol	PCR Protocol	PCR Cycling Parameters	Primers Used	Published Method	Additional Info
NRL 09	QIAamp Viral RNA Minikit (Qiagen)	MuLV RT Protocol (Applied Biosystems)	Single roundTaq Polymerase Protocol (Applied Biosystems)	40 cycles (94°C-30sec, 50°C-30sec, 72°C-30sec) then 72°C – 7mins		YES	Hybridisation using Chemilumine sence
NRL 13	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green & Henshilwood *et al*., (1998)	96°C-10mins and 35 cycles(94°C- 60 secs, 37°C-60sec, 72°C-60 sec) then 72°C-10mins. Nested Same	SM31, Ando, NI ,E3	Yes/No	
NRL 14	QIAamp Viral RNA Minikit (Qiagen)	OneStep RT-PCR Kit (Promega)		48°C-45mins, then 94°C-2mins, then 40 cycles (94°C-30sec, 50°C-1min, 68°C-min) and 68°C-7mins.	JV12 and JV13 – Vinje, et all (1996)	YES	
NRL 15	Boom, (1990)	OneStep RT-PCR Kit (Qiagen)	QuantiTect SYBRGreen PCR Kit (Qiagen)	95°C–15mins then 40 cycles (94°C–20sec, 49°C-90sec) and 72°C-30sec.	RT/1st Rnd - *MJV12, RegA* 2nd Rnd PCR – *p290, Mp290*	NO	Smartcycler real-time PCR machine. (Cepheid)

Table 4a: Summary of methods reported by NRLs for parts 1 and 2 of NV ring trial

NRL ID.	Virus elution from tissue	Nucleic Acid Ext. and Purification	RT – Protocol	PCR Protocol	PCR Cycling Parameters	Primers Used
NRL001	Whole animal homogenised in glycine buffer. Precipitation using PEG. Resuspension of pellet in Tri-reagent (Sigma).	Addition of chloroform followed by incubation and centrifugation. Aqueous layer precipitated in isopropanol. Pellets washed in ice cold ethanol and resuspended in RNase free water. Addition of RNA binding buffer + Dynabeads-oligo($dT)_{25}$, followed by washing protocols. Final resuspension in Rnase-free water and heating to liberate RNA from Dynabeads.	OneStep RT-PCR Kit (Qiagen)	Nested PCR	50°C 30mins, 95°C-15 mins and 35 cycles(94°C-30sec, 42°C-30sec, 72°C-45sec) then 72°C-10mins. Nested- 95°C-1mins and 35 cycles(95°C-30 secs, 42°C-60sec, 68°C-60 sec) then 68°C-3mins	SM31, GI, GII Ando, NI ,E3
NRL003	Whole animal Homogenised in glycine buffer. PEG precipitation (twice). Pellets resuspended in PBSA. RNA extraction using chloroform. Aqueous phase for analysis.	NucleoSpin RNA II Kit (Macherey-Nagel, Durren, Germany)	Two Phase JV13 primer, AMV reverse transcriptase	Booster – PCR + Southern Blotting (Bergman et al., 1995)	94°C-2mins and 40 cycles(94°C-1min, 37°C-90sec, 68°C-2min) then 68°C-7mins	Vinje Primers: JV-12 and JV-13 Polymerase region
NRL004	Stomach and Digestive diverticula removed via dissection. Homogenised in PBSa + antifoam B + chloroform-butanol. Homogenate added to Cat-Floc T. Following centrifugation aqueous phase removed for PEG precipitation.	Digestion with proteinase k. Extraction using Phenol-Chloroform-Water. Aqueous phase precipitated in ethanol. Pellets resuspended in water + CTAB. Resuspension of pellets in water and cetyltrimethyl-ammoniam bromide + NACl. Following incubation and centrifugation pellets resuspended in saline and precipitated in a solution containing ethanol and sodium acetate.	Two Phase, Random Hexamers (PdN6)	Nested	94°C-3 mins and 40 cycles(94°C-60sec, 50°C-30sec, 72°C-2mins) then 72°C-15mins. Nested- 94°C-3mins and 40 cycles(94°C-30 secs, 50°C-30sec, 72°C-60 sec) then 72°C-7mins	PCR: Nishida et al 2003 GG1: COG1F +GISKR GG2:COG2F +G2SKR Nishida et al., 2003 + Kojima et al.,2002 GG1: G1SKF +G1SKR GG2: G2SKF + G2SKR
NRL005	Dissection of Glands (Stomach and Digestive diverticula). Homogenisation and addition of equal w/v Proteinase K. Incubation followed by inactivation of enzyme. Centrifugation and removal of supernatant for storage and analysis.	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green & Henshilwood *et al*., (1998)	96°C-10mins and 35 cycles(94°C- 60 secs, 37°C-60sec, 72°C-60 sec) then 72°C-10mins. Nested Same	SM31, GI, GII Ando, NI ,E3

Table 4b: Summary of methods reported by NRLs for part 3 NV ring trial

NRL ID.	Virus elution from tissue	Nucleic Acid Ext.	RT – Protocol	PCR Protocol	PCR Cycling Parameters	Primers Used
NRL008	150 mg of digestive diverticula and 850 µl lysis buffer RLT (Qiagen; Plant & fungi kit, Maryland, USA) were added to a 2 ml eppendorf tube filled for 50% with Zirconia beads (1.0mm Zirconia beads, BioSpec Products, Inc., Bartlesville, Canada) and homogenised for 40 seconds at 4.0 m/sec using the Hybaid ribolyser™ Cell Disrupter (Hybaid, Milford, USA).	RNeasy® Mini Kit (Qiagen, Plant & Fungi protocol).	Two phase JV13I primer AMV rev. transcriptase	Single round	94C-2min and 40 cycles(94C- 60 secs37C-90 sec, 74C-60 sec) then 74C-7min	Vennema et al., (2002) J Clin Virol 25:233-235 (modified Vinje primers)-
NRL009	Stomach and Digestive diverticula removed via dissection. Homogenised in PBSa + antifoam B + chloroform-butanol. Homogenate added to Cat-Floc T. Following centrifugation aqueous phase removed for PEG precipitation.	Following digestion with proteinase K, Samples extracted with phenol-chloroform-water and Aqueous phase precipitated in ethanol. Pellets resuspended in water + CTAB. Resuspension of pellets in water and cetyltrimethyl-ammoniam bromide + NACl. Following incubation and centrifugation. pellets resuspended in saline and precipitated in a solution containing ethanol and sodium acetate.	MuLV RT (Perkin Elmar) Specific Primers	Single Round + Hybridisation	40 cycles (94°C-30sec, 50°C-30sec, 72°C-30sec) then 72°C – 7mins	Polymerase: P110/NI, P110/36, P110/4562. Capsid: G1SKF/SKR and G2SKF/SKR
NRL0013	Dissection of Glands (Stomach and Digestive diverticula). Homogenisation and addition of equal w/v Proteinase K. Incubation followed by inactivation of enzyme. Centrifugation and removal of supernatant for storage and analysis.	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green & Henshilwood *et al*., (1998)	96°C-10mins and 35 cycles(94°C- 60 secs, 37°C-60sec, 72°C-60 sec) then 72°C-10mins. Nested Same	SM31, GI, GII Ando, NI ,E3

Table 4b: Summary of methods reported by NRLs for part 3 NV ring trial

Reporting of results

Denmark, Finland, France, Germany, Netherlands, and the UK reported results for all 3 parts of the ring trial. Belgium, Italy, Norway, Spain reported results for some but not all parts of the ring trial. Greece and Portugal did not report results for any parts of the ring trial. The reporting of results by NRLs is detailed in table 5.

Country	Part 1	Part 2	Part 3
Belgium	✓	✓	x
Denmark	✓	✓	✓
Finland	✓	✓	✓
France	✓	✓	✓
Germany	✓	✓	✓
Greece	x	x	x
Italy	✓	x	✓
Netherlands	✓	✓	✓
Norway	✓	x	x
Portugal	x	x	x
Spain	✓	x	x
UK	✓	✓	✓

Table 5: Detailing the reporting of results by NRLs for parts 1,2 and 3 of the NV ring trial

Ring trial results

Results for ring trial part 1 – vials E, B, F and A (faecal sample)

Results for ring trial part 1 are shown in Table 6. All NRLs reporting results detected the presence of NV in at least one of the samples to which NV had been added. All NRLs except NRL 07 reported the absence of NV in vials to which NV had not been added. NRL 07 reported the presence of NV in vials to which NV had not been added. All NRLs except NRL 15 reported the presence of NV in vial B, which contained a high titre of NV G2. NRLs 05, 07, 08, 09, 13, and 15 reported the presence of NV in vial A, which contained a low titre of NV GI. NRL

08 and 15 reported this in only 1 of 2 replicates. NRLs 01, 03, 04, 14 did not report the presence of NV in vial A (low titre GI strain). NRLs 04, 05, 08, and 09, reported a genogroup I and correctly distinguished between NV GI and G2 strains. NRL 14 reported genogroup but did not correctly distinguished between NV GI and G2 strains. NRLs 03, 07, and 15 did not report NV genogroup. Results for part 1 of the NV trial were sent to participants prior to commencement of part 2.

Results for ring trial part 2 – vials C, G, D and H (faecal samples)

Results for ring trial part 2 are shown in Table 7. NRLs 03, 07, 15 reported results for part 1 but not part 2 of the ring trial. All other NRLs participating (except NRL 14) correctly reported the presence of NV in vials to which NV had been added. NRL 14 did not report the presence of NV in any of the vials. All NRLs reported the absence of NV in vials to which NV had not been added. All NRLs (except NRL 14) reported the presence of NV in both the high titre GI sample (vial D) and the low titre G2 sample (vial C). All NRLs correctly distinguished between NV genogroups.

Results for ring trial part 3 – packages J and K (shellfish samples)

Results for ring trial part 3 are tabulated in table 8. NRLs 07, 14 and 15 reported results for part 1 and/or part 2 but not for part 3 of the ring trial. Only 7 NRLs of the 12 agreeing to participate reported results for part 3 of the ring trial. Of the NRLs reporting results all correctly reported the presence of NV in package K (shellfish contaminated in the laboratory with a NV G2 strain). Of those NRLs reporting results all corrected reported the genogroup as NV G2. Of those NRLs reporting results all reported the anticipated result for package J (grade A shellfish found not to contain NV by the CRL method) which was absence of NV.

	E			B			F			A			E			B			F			A		
	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-
NRL001	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
NRL002*																								
NRL003			-			+			-			-			-			+			-			-
NRL004	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
NRL005	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
NRL007						+			+			+			+			+			+			+
NRL008	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-
NRL009	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
NRL012*																								
NRL013	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
NRL014	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
NRL015			-			-			-			-			-			-			-			+

* These laboratories did not return results for the ring trial.

Table 6. Tabulated results for NV ring trial part 1 (faecal samples).

Note: Results reported by NV genogroup are indicated in columns headed GI and G2. +/- indicates result as presence or absence of NV. Results reported as just presence or absence of NV are reported only in columns headed +/-

	C			G			D			H			C			G			D			H		
	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-
NRL001	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL002*																								
NRL003*																								
NRL004	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL005	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL007																								
NRL008	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL009	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL012*																								
NRL013	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL014	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NRL015*																								

* These laboratories did not return results for the ring trial.

Table 7. Tabulated results for NV ring trial part 2 (faecal samples).

Note: Results reported by NV genogroup are indicated in columns headed GI and G2. +/- indicates result as presence or absence of NV.

	J			K			J			K		
	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-
NRL001	-	-	-	-	+	+	-	-	-	-	+	+
NRL002*												
NRL003	-	-	-	-	+	+	-	-	-	-	+	+
NRL004	-	-	-	-	+	+	-	-	-	-	+	+
NRL005	-	-	-	-	+	+	-	-	-	-	+	+
NRL007*												
NRL008	-	-	-	-	+	+	-	-	-	-	+	+
NRL009	-	-	-	-	+	+	-	-	-	-	+	+
NRL012*												
NRL013	-	-	-	-	+	+	-	-	-	-	+	+
NRL014*												
NRL015*												

* These laboratories did not return results for the ring trial.

Table 8. Tabulated results for NV ring trial part 3 (shellfish).

Note: Results reported by NV genogroup are indicated in columns headed GI and G2. +/- indicates result as presence or absence of NV.

Comments

Transport of samples

The courier used for this ring trial was DGI, Global Logistics Management Ltd. The CRL specified temperature and time transport criteria to DGI for these temperature critical samples. DGI were contracted to maintain all samples in a frozen state using dry ice and deliver them within a maximum three day delivery period. Although many of the packages exceeded the specified delivery times, all samples with the exception of one arrived at their destination frozen. Prolonged delivery times incurred were as a result of delays by Customs at the port of arrival and/or consignment misplacement by the airline. However as all samples, with the possible exception of that for Finland, arrived frozen, the delays in transport are not considered to have affected the results of the ring trial.

Laboratory participation in ring trial

Although 12 NRLs agreed to participate only 7 completed the final part 3 of the ring trial (shellfish samples). Only 6 NRLs (50%) completed all 3 parts of the ring trial. This is a high drop-out rate for a ring trial. As the reasons for not reporting results were not given to the CRL this also potentially effects interpretation of overall laboratory performance in the ring trial. It is noticeable that laboratories performing less well in part 1 did not report results for parts 2 or 3 of the ring trial. Thus the good performance of remaining NRLs in part 3 of the ring trial cannot be regarded as representative of all NRLs. Non reporting of results requires further discussion as it is both wasteful of resources and potentially distorts the interpretation of ring trial findings. Non reporting of results undermines the primary purpose of ring trials, which is to assess comparability of analytical performance and to identify and improve poor performance.

Laboratory performance

Most NRLs consistently reporting results performed very well in the ring trial. These NRLs were clearly able to detect NV in faecal sample dilutions, at both high and low titre, and were able to discriminate correctly between positive and negative samples. These NRLs were also able to detect NV in shellfish samples contaminated in the

laboratory and to distinguish between samples which had been contaminated and shellfish samples which had not been contaminated. These NRLs were also able to correctly distinguish between genogroups of NV.

The most difficult sample to analyse was the low titre NV GI faecal sample (vial A) with only 6 of 10 participants successfully reporting the presence of NV. This sample had the lowest virus titre of all samples used in this ring trial. Only weak bands were visualised by gel electrophoresis by the CRL prior to dispatch, and, this was duly noted by other NRL participants. Thus assay sensitivity may not be equivalent in all laboratories.

Several participants in ring trial part 1 experienced problems with the analysis. One NRL reported presence of NV in vials to which no NV had been added, indicating a problem with cross contamination. One NRL failed to detect NV in the high titre G2 sample and only in 1 of 2 replicates of the GI sample. This probably indicates a problem with assay sensitivity or sample processing. Four NRLs were only able to detect the presence of NV in the high titre G2 sample and not the low titre GI sample. This could indicate a problem with either assay sensitivity or broadness of reactivity for NV strains. One NRL incorrectly reported the G2 strain as a GI strain. These 6 NRLs experiencing problems are advised to review their procedures. Of these 6 laboratories experiencing problems 4 did not report results for ring trial part 2.

Ring trial part 2 was more successful with 6 of 7 participants reporting the anticipated results. One NRL failed to detect NV in either of the samples to which NV had been added and is advised to review their procedures. This NRL did not report results for part 3 of the ring trial.

All remaining 7 NRLs reporting results for part 3 of the ring trial correctly reported the anticipated result of presence of NV G2 in one shellfish sample and absence of NV in the other shellfish sample. This is a good result for a first ring trial distribution of shellfish material. However, it should be noted that the CRL has analysed the NV containing shellfish sample by the recently developed NV real-time assay - which can give quantitative information – and the NV titre was found to be very high. The level of NV

template found in the laboratory bio-accumulated shellfish was in excess of 10^6 times the limit of sensitivity of the real-time assay (CT value 26.2). This contrasts with environmentally contaminated shellfish samples, which typically contain no more than 10-100 times the limit of assay sensitivity (CT value 37.5 – 41). Thus an unrepresentatively high titre of NV was present in this laboratory contaminated sample. Clearly it would be advantageous to repeat this exercise with shellfish containing lower titres of NV template or with naturally contaminated samples.

Methods

A variety of sample extraction protocols were reported by NRLs. Most common were the 'QIAmp Viral RNA Minikit' and 'Boom' based extraction methods. RT-PCR protocols also varied with both nested and single round protocols being described. Summaries of approaches are given in table 4a & 4b. It is however clear from ring trial reports that the various methods currently in use cannot be interpreted as giving equivalent performance. This clearly points to the need to address the development of standard methods for detection of NV in shellfish.

Further ring trials

Clearly the Norovirus ring trial was a useful exercise and demonstrated both good and less good laboratory performances. However, over interpretation of a single ring trial series should be avoided. A further programme of ring trials will be required to determine more reliably the effectiveness of laboratories and techniques for the detection of NV in shellfish. It is recommended that NRLs endorse a further ring trial series for NV. It will however be important to address the issue of non reporting of results by laboratories.

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Norovirus Ring Trial Instructions:

Within your package you will find three clearly labelled sealed containers marked 1, 2 and 3 respectively.

Sealed Container 1

This container contains 2 packages: -

Package 1 and Package 2.

Package 1 contains 4 vials of faecal material, marked E, B, F, and A

Package 2 contains 4 vials of faecal material marked D, G, H, C

Sealed Container 2

This container contains Package J

Package J has 2 x bags of 6-shucked shellfish.

Sealed Container 3

This container contains Package K

Package K has 2 x bags of 6-shucked shellfish.

On Receipt Of Your Container

1. All samples have been sent frozen on dry ice. On receipt of your package inspect the contents to insure you have all of the material listed above.

2. Freeze Sealed Containers 2 and 3 and Package 2 vials (D, G, H, C.) Immediately at -20◦C

3. The CRL will be in contact later about analysing these packages in relation to future ring trials

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Norovirus Ring Trial 1

Please store Package 1; containing vials **E, B, F, A** at 4°C and analysis within 48 hours of receipt.

Analyse using RT-PCR following the protocol in use in your laboratory.

The samples must be analysed in the following order using 50µl from each vial:

1. E
2. B
3. F
4. A
5. E
6. B
7. F
8. A

Please complete the attached results form and also the protocol form. Return to Sam Bark by post, fax (01305 206601) or e-mail. s.j.bark @cefas.co.uk If you have any queries with regards to these instructions, please do not hesitate to contact myself or Dr Kathy Henshilwood(k.henshilwood @cefas.co.uk)at the laboratory

Please return your results to the CRL by October 31st 2003

Thank-you.

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Norovirus Ring Trial 2

Package 2; containing vials **C, D, G, H**.

Analyse using RT-PCR following the protocol in use in your laboratory.

The samples must be analysed in the following order using 50µl from each vial:

1. C
2. G
3. D
4. H
5. C
6. G
7. D
8. H

Please complete the attached results form and also the protocol form. Return to Sam Bark by post, fax (01305 206601) or e-mail. s.j.bark @cefas.co.uk If you have any queries with regards to these instructions, please do not hesitate to contact myself or Dr Kathy Henshilwood (k.henshilwood@cefas.co.uk)at the laboratory

Please return your results to the CRL by 20th February 2004.

Thank-you.

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Norovirus Ring Trial 3

Sealed container 2 and 3; contains packages J and K respectively.

Sealed Container 2

This container contains Package J

Package J has 2 x bags of 6 shucked shellfish.

Sealed Container 3

This container contains Package K

Package K has 2 x bags of 6 shucked shellfish.

Extract and analyse tissues using the protocol in use in your laboratory for the analysis.

The samples must be analysed in the following order:

1. J
2. k
3. J
4. k

Please complete the attached results form and also the protocol form. Return to Sam Bark by post, fax (01305 206601) or e-mail. s.j.bark @cefas.co.uk If you have any queries with regards to these instructions, please do not hesitate to contact myself or Dr Kathy Henshilwood (k.henshilwood@cefas.co.uk)at the laboratory.

Please return your results to the CRL by 19th March 2004.