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A Comparison of Two Methods for Detection of Norovirus RNA in Environmental Swab Samples

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Abstract: Standardised molecular methods are available for the detection of norovirus from water and specific food items. Detection of norovirus from stool samples also relies on molecular methods, but differences exist between nucleic acid extraction, reverse transcription, and amplification strategies recommended by the ISO 15216-1:2017, and those employed in clinical laboratories. Here, we conduct a direct comparison of two methods for the detection and quantitation of norovirus from a stool sample and from artificially contaminated swabs. We also compare use of linear dsDNA standards as recommended in ISO 15216:2017 against an *in vitro*-transcribed single-stranded RNA (ssRNA) for estimation of norovirus genome copy number. Our results show that the two methods have comparable sensitivity for the detection of norovirus RNA from a clinical sample or swab. The use of a ssRNA standard revealed that quantitation performed against a linear dsDNA standard consistently underestimated the genome copy numbers by 1.5 to 2 log due to the relative inefficiency of the reverse transcription step. This has important implications for the estimation of the sensitivity of norovirus detection methods, comparability of results across sites, and assessment of viral loads that may be clinically significant or estimated to constitute infectious doses.

Keywords: norovirus; molecular methods; method standardization; quantitative standard

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1. Introduction

Norovirus is the commonest cause of acute gastroenteritis worldwide. In the UK, there are over 3 million norovirus infections each year [1,2]. Onset of clinical illness usually begins 12–48 h post-infection, and presents as vomiting, non-bloody diarrhoea, abdominal cramps, and low-grade fever, with symptoms lasting 12–72 h, and usually self-resolving after this time.

Transmission of viruses via the food chain is an increasingly recognised public health problem (reviewed in [3]). Norovirus transmission occurs via a faecal–oral pathway, and there are multiple potential routes of transmission, including direct person-to-person and indirect via contaminated food, water, and the environment. The attribution of norovirus infections by these different routes of infection is poorly understood, however, it is likely that food-associated transmission of norovirus is important, and it has been estimated that between 14% and 23% of norovirus infections may be food-associated [4,5].

Several factors likely contribute to the transmission of norovirus through the food chain: (i) the low infectious dose [6,7], (ii) high viral shedding in stool [8], (iii) environmental stability of the virus [9], and (iv) a high rate of asymptomatic shedding [10,11]. Together, these factors mean that contamination of foods by food handlers or from surfaces

contaminated with the virus in kitchen environments is likely to be an important source of transmission and outbreaks.

To better understand the epidemiology and ultimately assess the contribution made by the food chain to the burden of norovirus infections, sensitivity in molecular detection of viruses in food and environmental samples is required, as well as development of tractable methods for deployment in public health laboratories. The validation of a methodology for detection of norovirus in shellfish and soft fruits as an international standard (ISO 15216-1:2017) [12,13] is significant progress in this field. However, even when laboratories apply methodologies that are compliant with ISO 15216-1:2017, there is room for differences in various steps of the method, and in some cases, the recommended methods may not be universally adoptable due to constraints on the technology available within differently resourced laboratories.

Here, we present a comparison of two nucleic acid extraction and two norovirus detection protocols: one representing methods widely used in diagnostic and public health laboratories for the detection of norovirus in clinical samples, and the second according to the standardised method described in ISO15216-1:2017 and as per recommendations in the related annexes. Further, we compare the use of in vitro transcribed, single-stranded RNA and linear dsDNA as external quantitation standards for estimating viral nucleic acid load by real-time PCR.

2. Methods

2.1. Preparation of Specimens and Swabs

For the purposes of method validation, two stool specimens, one each containing a GI and GII norovirus, which were prepared as 10% suspensions in PBS, and ten-fold serial dilutions of the suspension were prepared.

Aliquots of the ten-fold dilutions were either extracted directly or used to contaminate Viscose swabs (Technical Service Consultants Ltd., Heywood, UK). Swabs were contaminated in duplicates, and both aliquot and swab pairs were extracted using two different methods, as described below.

All specimens were spiked with Mengo virus (strain vMC_0) cell culture supernatant as a process control.

2.2. Extraction Method A

Extraction of total nucleic acid was performed using a manual guanidinium thiocynate (GTC)-silica method based on that described previously [14,15]. Briefly, either 200 µL of stool suspension or a contaminated swab tip were immersed into 1 mL of L6 (Severn Biotech, Kidderminster, UK) and incubated at room temperature, after which the swab was discarded where relevant, and to both stool and swab lysates, 20 µL of silica extraction matrix (Severn Biotech) was added, followed by incubation at room temperature with agitation for 15 min. Silica was pelleted by centrifugation and the pellet was washed twice in 1 mL of L2 (Severn Biotech), twice in 70% ethanol, and once in 100% acetone. Silica pellets were air-dried and suspended in 50 µL of molecular-grade water. Total nucleic acid was eluted from the silica by incubation at 56 °C for 15 min, after which silica was removed by centrifugation.

2.3. Extraction Method B

Extraction of total nucleic acid was performed using a semi-automated guanidinium isothiocynate (GTC)-silica method based on that described previously [13], and adopted as part of the published ISO standard 15216-1:2017 [12] and related annexes. Briefly, either 200 µL of stool suspension or a contaminated swab tip were immersed into 2 mL of NucliSENS Lysis Buffer (bioMérieux, Baisingstoke, UK) and incubated at room temperature, after which the swab was discarded where relevant. Total nucleic acid was extracted from both stool and swab lysates using the NucliSENS extraction system (bioMérieux) operated

either on the miniMAG or easyMAG system (bioMérieux) according to the manufacturer's instructions. Total nucleic acid was eluted into 100 µL of elution buffer.

2.4. Detection Method A

The RNA in the total nucleic acid was converted to randomly primed cDNA by reverse transcription (RT), as previously described [16], and norovirus was detected using genogroup-specific real-time PCR assays (qPCR), as previously described [17] (Figure 1). Detection of Mengo virus was performed as previously described [18].

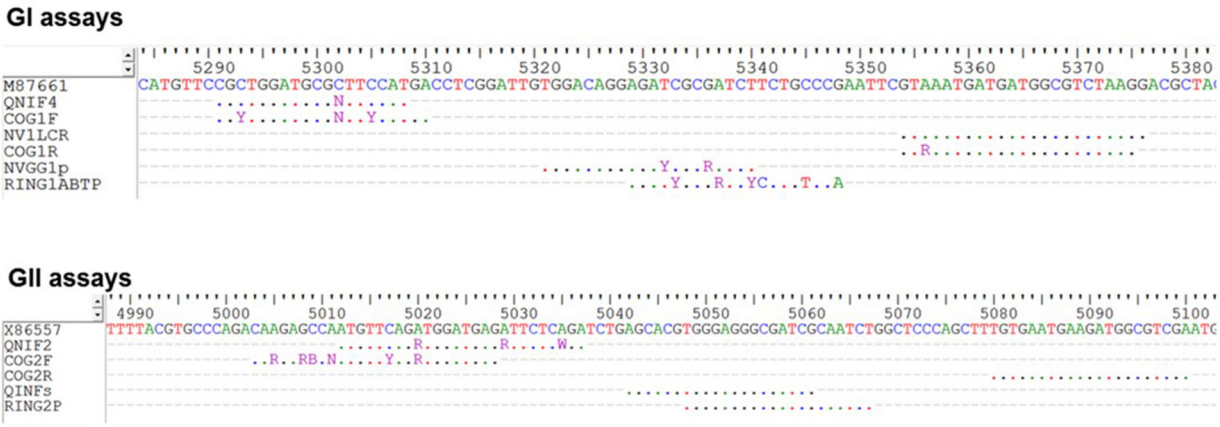

Figure 1. Alignment of primer and probe sequences used in detection method A (COG1F, COG1R, RING1ABTP, COG2F, COG2R, RING2P) and detection method B (QNIF4, NV1LCR, NVGG1p, QNIF2, COG2R, QINFs) real-time PCR methods against appropriate reference strains: GI using Norwalk virus (accession M87661) and GII using Lordsdale virus (accession X86557).

2.5. Detection Method B

Norovirus RNA was detected using genogroup-specific one-step combined RT-qPCR assays, as described previously [19] (Figure 1). Detection of Mengo virus was performed as previously described [18].

2.6. Production of IVT ssRNA

Partial ORF1 and complete ORF2 and ORF3 were amplified as a single amplicon from a GII.3 norovirus-positive faecal specimen and cloned into pCR2.1-TOPO vector (Thermo Fisher Scientific, Leicestershire, UK) according to the manufacturer's instructions. Plasmid DNA was prepared from overnight bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen, West Sussex, UK). The concentration of the eluted plasmid was measured using the Qubit® DNA BR Assay Kit (Thermo Fisher Scientific), and a dilution series of the plasmid was prepared in the range of 6×10^7 to 6×10^{-2} copies/µL.

Primers RNA-SC-F (5′-TAATACGACTCACTATAGAGGGTGAATGGATTTTT-3′) and RNA-SC-R (5′-AGGCCGGCGGCACCATCATTAGATGG-3′) were designed to produce an amplicon consisting of a T7 RNA polymerase promoter (underlined) upstream of the norovirus ORF1/ORF2 junction from this plasmid. Amplification was performed using the Expand High-Fidelity System (Roche, West Sussex, UK), with 0.4 µM of each primer. Thermal cycling conditions were: 95 °C for 5 min, followed by 3 cycles of 95 °C for 1 min, 60 °C for 30 s (decreasing by 2 °C every 3 cycles, with an additional 23 cycles at 50 °C), 72 °C for 3 min, and a final extension step of 72 °C for 5 min.

The resulting amplicon was purified using the QIAQuick Gel-Extraction Kit (Qiagen), and this was used as a template for in vitro transcription with the MEGAscript T7 High-Yield Transcription Kit (Fisher Scientific). The in vitro transcribed, single-stranded RNA (IVT ssRNA) was treated with Turbo DNase (Fisher Scientific) and precipitated using lithium chloride. Purified IVT ssRNA was suspended in 30 µL of DEPC-treated water (Fisher Scientific). The concentration of IVT ssRNA and residual DNA template was

measured using Qubit® RNA BR and DNA HS Assay Kits (Thermo Fisher Scientific), and subsequently the IVT ssRNA was diluted to a range of 6×10^5 to 6×10^{-2} copies/µL.

2.7. Validation of the IVT ssRNA and Linear dsDNA External Standards

Validation of the standards was performed using primers and probes as described by Kageyama et al. [17] (as detection method A, above) and used in a one-step and two-step assay format, described briefly below.

The final one-step assay included 1X Precision One-Step™ qRT-PCR Mastermix (Primerdesign, Hampshire, UK), 0.5 µM of each primer, 0.125 µM of probe, and 5 µL of IVT ssRNA. Alternatively, norovirus-specific primers and probe were replaced with 1 µL of the internal control (IC) primer–probe mix (as provided with kit). Thermal cycling conditions were as follows: 55 °C for 10 min, 95 °C for 8 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

The final two-step assay included 1X PrecisionPLUS Mastermix (Primerdesign), 0.4 µM of each primer, 0.1 µM of probe, and 5 µL of plasmid DNA. Thermal cycling conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 60 s.

The linear dsDNA standard was validated as a single standard curve in six replicates using the one-step assay, and three independent standard curves over twenty replicates using the two-step assay.

The IVT ssRNA standard was validated as three independent standard curves used across fourteen replicates in the one-step assay. A one-step (no RT control) and two-step assay was performed on each dilution to detect the presence of residual DNA.

For each respective standard, the mean C_q value and calculated genome copy number were averaged for each 10-fold dilution, the slope and y-intercept could then be calculated by linear regression analysis.

2.8. Absolute Quantitation of Lenticule® Disc Reference Standards

Lenticule® Discs (batch ID: PHE-RMNOROG2/231415) (Public Health England, Porton Down, UK) containing GII norovirus were prepared according to the manufacturer's instructions. For validation, up to three Lenticule® Discs were rehydrated per experiment, and this was performed three times to afford seven replicates overall. Lenticule® Disc suspensions were serially diluted from 1.1×10^4 to 1.1×10^{-2} copies and nucleic acid extracted, as described in extraction method A (above).

2.9. Statistical Analysis

Statistical analysis was performed in R (version 4.0.5) and significance ($p < 0.05$) was determined using the paired Student's *t*-test.

3. Results

3.1. Comparison of the Sensitivity of the Two Methods

Ten-fold serial dilutions of a suspension of a stool specimen containing a GII norovirus were used to contaminate swabs. Duplicate swabs contaminated with the GII norovirus were extracted using extraction methods A and B, and nucleic acid extracts from both extraction methods were tested in both detection methods A and B (Figure 1) using a Rotor-gene Q (Qiagen) (Table 1).

The results show that extraction protocol A is marginally more sensitive than extraction protocol B, regardless of the RT-PCR method used; however, the difference in C_q was <3.32, which would correspond to a ten-fold difference, and is therefore not significant. The process control (Mengo virus) was detected in all samples with either extraction/detection protocol combination (data not shown).

Table 1. Comparison of extraction protocol A vs extraction protocol B, and detection protocol A and detection protocol B.

RT-PCR Method	Detection A		Detection B	
Nucleic Acid Extraction Method	**Extraction A**	**Extraction B**	**Extraction A**	**Extraction B**
Swab Contaminated with 200 µL of:	**C_q** [1]	**C_q**	**C_q**	**C_q**
10% suspension	23.11	25.61	21.03	23.14
10^{-1} dilution	26.82	29.12	25.16	26.42
10^{-2} dilution	32.11	34.26	30.04	30.78
10^{-3} dilution	34.08	38.02	32.44	33.38
10^{-4} dilution	37.14	ND [2]	35.23	37.35
10^{-5} dilution	ND	ND	ND	ND
10^{-6} dilution	ND	ND	ND	ND
10^{-7} dilution	ND	ND	ND	ND
10^{-8} dilution	ND	ND	ND	ND
10^{-9} dilution	ND	ND	ND	ND
10^{-10} dilution	ND	ND	ND	ND
Negative control (PBS)	ND	ND	ND	ND

[1] C_q—Quantification cycle (cycle threshold); [2] ND—no virus RNA detected.

To further compare the sensitivity of the two detection protocols, a stool specimen containing a GI norovirus was prepared as a series of ten-fold dilutions and total nucleic acid was extracted and norovirus RNA was detected using both protocols. The results were calibrated against a dsDNA standard curve (kindly supplied by Dr James Lowther, Centre for the environment, fisheries, and aqua science (Cefas), Weymouth, UK) (Tables 2–4).

Table 2. Sensitivity comparison of the norovirus GI one-step vs. the two-step RT-PCR using 200 µL of a 10% suspension of a clinical sample.

Stool Sample	Detection Protocol A		Detection Protocol B	
	C_q [1]	**Genome Copies/ Reaction**	**C_q**	**Genome Copies/ Reaction**
10% suspension	18.1	1.8×10^5	17.3	4.8×10^4
10^{-1} dilution	22.4	1.0×10^4	20.0	9.7×10^3
10^{-2} dilution	25.5	1.4×10^3	24.3	7.8×10^2
10^{-3} dilution	29.7	8.8×10^1	27.0	1.5×10^2
10^{-4} dilution	34.2	4.8×10^0	31.2	1.3×10^1
10^{-5} dilution	38.4	3.0×10^{-1}	33.7	3.1×10^0
10^{-6} dilution	ND [2]		ND	
10^{-7} dilution	ND		ND	
10^{-8} dilution	ND		ND	
10^{-9} dilution	ND		ND	
10^{-10} dilution	ND		ND	
Negative control (PBS)	ND		ND	

[1] C_q—Quantification cycle (cycle threshold); [2] ND—no virus RNA detected.

For the detection of norovirus RNA directly from a stool suspension or from contaminated swabs, the results showed that both methods had an identical end point, and that the C_q value differences for each dilution were <3.32 cycles (or within a log if expressed as DNA quantity), and therefore, not significantly different.

In contrast, for detection of dsDNA, protocol A can detect a single norovirus genome copy, and this suggests that this PCR protocol is marginally more sensitive than detection protocol B. However, comparisons between ten-fold dilutions of dsDNA by protocols A and B were not statistically significant.

Table 3. Sensitivity comparison of the norovirus GI one-step vs. the two-step RT-PCR using 200 µL of a 10% suspension of the same clinical sample to contaminate swabs.

Swab Contaminated with:	Detection Protocol A		Detection Protocol B	
	C_q	Genome Copies/ Reaction	C_q	Genome Copies/ Reaction
10% suspension	21.7	1.66×10^4	19.3	1.48×10^4
10^{-1} dilution	25.8	1.15×10^3	23.2	1.44×10^3
10^{-2} dilution	29.7	8.59×10^1	25.4	4.03×10^2
10^{-3} dilution	34.4	4.21×10^0	30.5	2.01×10^1
10^{-4} dilution	37.4	5.66×10^{-1}	34.1	2.44×10^0
10^{-5} dilution	39.7	1.28×10^{-1}	38.3	2.04×10^{-1}
10^{-6} dilution	ND		ND	
10^{-7} dilution	ND		ND	
10^{-8} dilution	ND		ND	
10^{-9} dilution	ND		ND	
10^{-10} dilution	ND		ND	
Negative control (PBS)	ND		ND	

Table 4. Sensitivity comparison of the norovirus GI one-step vs. the two-step RT-PCR using the Cefas dsDNA standard curve.

dsDNA Standard Curve Dilution:	Detection Protocol A		Detection Protocol B	
	Mean $C_q \pm$ SD	Genome Copies/ Reaction	Mean $C_q \pm$ SD	Genome Copies/ Reaction
10^{-1}	22.0 ± 0.28	1.00×10^4	19.75 ± 0.63	1.00×10^4
10^{-2}	26.3 ± 0.28	1.00×10^3	24.15 ± 0.78	1.00×10^3
10^{-3}	29.8 ± 0.28	1.00×10^2	27.8 [2]	1.00×10^2
10^{-4}	33.5 ± 1.34	1.00×10^1	31.55 ± 0.07	1.00×10^1
10^{-5}	36.1 ± 0.35	1.00×10^0	ND	-
10^{-6}	ND [1]	-	ND	-

[1] ND—no virus RNA detected; [2] Not mean, one replicate only as one replicate had no virus detected.

3.2. Absolute Quantitation with a Plasmid Standard Can Underestimate Viral Load

Additionally, we examined whether differences in sensitivity were observed between the use of IVT ssRNA or linear dsDNA as an external standard for estimation of viral load by qPCR.

Both IVT ssRNA and linear dsDNA were titrated at ten-fold dilutions between 3×10^6 and 3×10^3 copies per reaction, and tested using one-step and two-step assays, respectively (Figure 2). Comparison of the two external standards indicated that the IVT ssRNA generated higher C_q values at an identical predicted genome copy number to the linear dsDNA (Figure 2)

Lenticule® Discs are standardised reference materials which contain a geometric mean of 4.04 $\log_{10}$ GII HuNoV genome copies/disc. Lenticule® Discs were diluted and quantitated with the IVT ssRNA and linear dsDNA standards. Ten-fold dilutions of the Lenticule® Disc preparation were estimated to be at 3.39 ± 0.17, 2.78 ± 0.14, and 1.57 ± 0.089 $\log_{10}$ genome copy number/reaction against the dsDNA standard, whereas the IVT ssRNA standard estimated higher titres of 5.31 ± 0.17, 4.72 ± 0.13, and 3.57 ± 0.082 $\log_{10}$ genome copy number/reaction (Figure 3), a difference of between 1.5 and 2 $\log_{10}$, and consistent with the 6–7 C_q value differences observed when both standards (dsDNA and ssRNA) were compared against each other. The genome copy number/reaction measured between the dsDNA and IVT ssRNA standard was significantly different ($p < 0.05$) if each respective ten-fold dilution was compared.

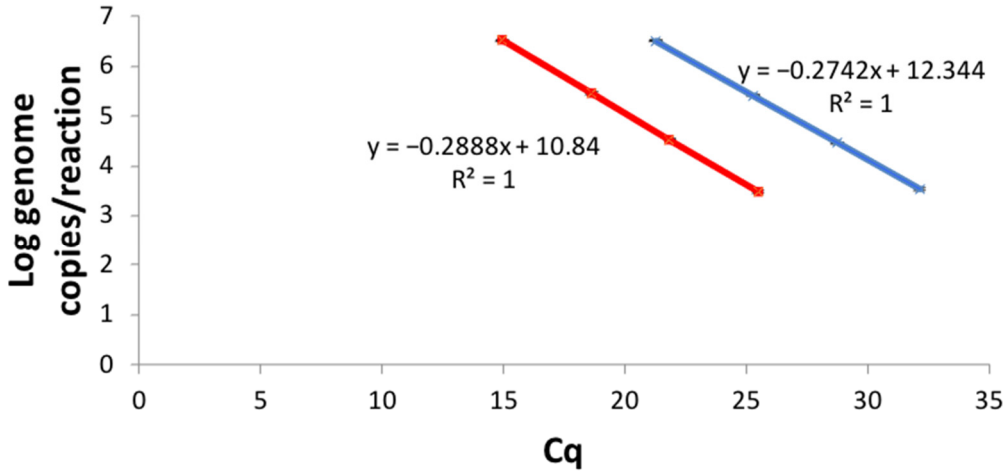

Figure 2. Standard curve validation of the RNA and plasmid template in one- and two-step qPCR assays (Red—linear dsDNA, Blue—IVT ssRNA). Cq—Quantification cycle (cycle threshold).

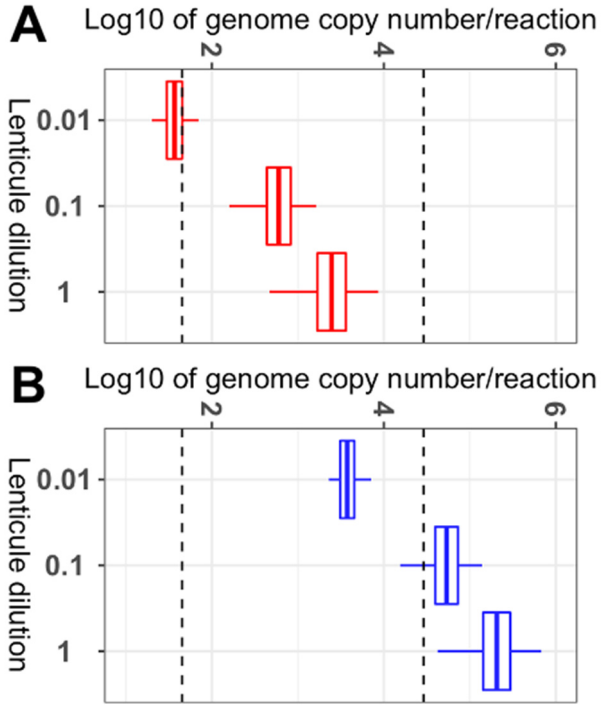

Figure 3. Quantitation of Lenticule® Disc reference materials with different standards: (**A**) linear dsDNA and (**B**) IVT ssRNA (boxes and whiskers represent the standard error and range around the mean, respectively. The dashed lines correspond to the upper and lower expected range of the Lenticule® Disc batch).

4. Discussion

We presented data that demonstrate two different nucleic acid extraction protocols—one manual and one semi-automated—and two different norovirus qPCR detection methods—a one-step and a two-step protocol—which have comparable sensitivity for the detection of GI and GII norovirus in environmental swab samples and clinical specimens.

Although the principles of both extraction methods are the same, there are some minor differences in relation to the extraction platforms, reagents, as well as in the reverse transcription and polymerase chain reaction (RT-PCR) reagents and conditions. The differences between the two methods are as follows.

Extraction method A is a manual method, whereas extraction method B is semi-automated. Both involve lysis of the virus capsid using a high concentration of the chaotropic agent guanidinium isothiocyanate and detergents, followed by binding of the released virus nucleic acid to fractionated silica. Whilst extraction method B uses magnetic silica as a means for recovery of the silica-bound nucleic acid, extraction method A relies on centrifugation. It should be noted that any comparisons are limited by extraction method B requiring twice the volume of elution buffer in comparison to extraction method A. However, this cannot compensate for the 10-fold differences in the concentration (i.e., 3.32 cycle threshold values).

For amplification of norovirus RNA, detection method A uses a two-step approach, by which RNA is reverse transcribed using random hexamers, hence producing cDNA of all the RNA present in the sample, and then this is followed by separate norovirus genogroup-specific (GI and GII) PCRs and a third PCR for amplification and detection of Mengo virus. Detection method B employs a one-step approach, in which the same primers are used for generating cDNA in the reverse transcription step prior to the amplification in the same tube/well on three different reactions to detect norovirus GI or GII, or Mengo virus.

Although the primers used in all norovirus-specific assays amplify the same region of the virus genome (ORF1/ORF 2 junction), there were minor differences in the sequence of some of the norovirus-specific primers and probes used (Figure 1). Primers and probes used to amplify and detect Mengo virus were identical (data not shown).

Comparison of viral load estimation by qPCR against external standards indicated that norovirus load may be underestimated when using a linear dsDNA standard compared to an IVT ssRNA standard. The Lenticule® Discs batch tested had, according to the manufacturer's information, an expected range of 1.65 to 4.46 $\log_{10}$ genome copy number/reaction, which was in close agreement with the quantitation obtained against the linear dsDNA standard; hence, it is likely that the quantity of norovirus in the Lenticule® Discs was calculated using a DNA standard. Quantification of the Lenticule® reference material against the IVT ssRNA standard yielded an increase in the genome copy number of between 1.5 and 2 log. This can be explained by the relative poor efficiency of the RT step, as the sensitivity-limiting step in the reaction [20]. The implications of this are that the sensitivity of RT-PCR methods commonly reported against DNA standards may be overestimated. Additionally, as different reverse transcription enzymes can have different efficiencies, it is likely that the degree of overestimation will also differ depending on the enzyme/RT system used. For norovirus, the ISO standard (ISO 15216-1:2017) [12,13] adopted in this study provided a useful reference for evaluation of an alternative method, which was found not to differ significantly in sensitivity. Methods with standardised approaches and defined quantitative controls have recently been used in quantitating the recovery of norovirus from food, which is important in understanding the role of food and food handlers in norovirus transmission pathways [21–23].

5. Conclusions

Standardisation of quantitative molecular methods through the development of reference materials is an important step toward supporting comparable research outputs and surveillance data. However, the output of a molecular assay for absolute quantitation of a target can only be as accurate as the reference materials used, and therefore thorough technical validation of standards is essential. The choice of standard has important implications for the interpretation of data obtained using PCR-based methods, as estimation of viral loads may be considered in surveillance systems, measuring potential for infectiveness, or in clinical management to monitor infection progression or response to therapy. Further, the choice of standard is also an important factor to consider in quality assurance and of data, for example when intending to undertake a comparison of data from different laboratories, in the evaluation of methodologies, and/or in comparisons of data between studies.

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Conflicts of Interest: D.J.A. and M.I.-G. are affiliated with the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Gastrointestinal Infections at the University of Liverpool, in partnership with Public Health England (PHE), in collaboration with the University of East Anglia, the University of Oxford, and the Institute of Food Research. D.J.A. is based at The London School of Hygiene and Tropical Medicine, and PHE, and M.I.-G. is based at the University of Liverpool. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health, or Public Health England.

Appendix A

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