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Development and validation of a duplex RT-qPCR assay for norovirus quantification in wastewater samples

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ABSTRACT

Norovirus (NoV) is a highly contagious enteric virus that causes widespread outbreaks and a substantial number of deaths across communities. As clinical surveillance is often insufficient, wastewater-based epidemiology (WBE) may provide novel pathways of tracking outbreaks. To utilise WBE, it is important to use accurate and sensitive methods for viral quantification. In this study, we developed a one-step duplex RT-qPCR assay to simultaneously test the two main human pathogenic NoV genogroups, GI and GII, in wastewater samples. The assay had low limits of detection (LOD), namely 0.52 genome copies (gc)/μl for NoVGI and 1.37 gc/μl for NoVGII. No significant concentration-dependent interactions were noted for both NoVGI and for NoVGII when the two targets were mixed at different concentrations in the samples. When tested on wastewater-derived RNA eluents, no significant difference between duplex and singleplex concentrations were found for either target. Low levels of inhibition (up to 32 %) were noted due to organic matter present in the wastewater extracts. From these results we argue that the duplex RT-qPCR assay developed enables the sensitive detection of both NoVGI and NoVGII in wastewater-derived RNA eluents, in a time and cost-effective way and may be used for surveillance to monitor public and environmental health.

1. Introduction

Norovirus (NoV) is one of the most common causes of gastroenteritis, resulting in nearly 700 million cases and approximately 200,000 deaths a year worldwide (Katayama and Vinje, 2017). It is a highly contagious non-enveloped ssRNA virus, with as few as 20 viral particles sufficient to cause an infection (Teunis et al., 2008). Symptoms of infection include diarrhoea, vomiting, stomach pains and headaches and, in some cases, the infection can be life-threatening (Katayama and Vinje, 2017). The virus usually spreads through a faecal-oral route via direct contact, contaminated food or water, contaminated surfaces and potentially via aerosols of vomit (de Graaf et al., 2016). Viral prevalence is seasonal with noticeable increases in case numbers during winter (Ahmed et al., 2013; Donaldson et al., 2022).

Infected individuals shed NoV in stool at high concentrations (up to 10^{10} virus particles/g) and shedding can occur even in asymptomatic cases (Atmar et al., 2008; Teunis et al., 2015). As the virus is very persistent outside the host (Desdouits et al., 2022; Kotwal and Cannon, 2014), it can be found in municipal wastewater at high concentrations (Eftim et al., 2017). Therefore, it is possible to monitor NoV abundance in wastewater for wastewater-based epidemiology (WBE) programmes. WBE is a well-established approach to monitor the prevalence of pathogens circulating in the community. For example, WBE has been used to monitor poliovirus abundance since the 1950s (Hovi et al., 2012). Since the start of the COVID-19 pandemic, WBE has been utilised in over 70 countries for spatio-temporal tracking and for early detection and prediction of SARS-CoV-2 infections (Ahmed et al., 2020; Medema et al., 2020; Peccia et al., 2020). Data from WBE can be used in conjunction with clinical case numbers to better identify outbreaks at early stages, evaluate geographical areas lacking clinical testing and vaccine uptake and monitor long-term trends. WBE can also be applied to other viral pathogens that shed in stool or urine, such as influenza viruses (Dumke et al., 2022) and NoV (Guo et al., 2022; Huang et al., 2022). The possibility to detect arboviruses through WBE techniques has also been

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discussed (Lee et al., 2022).

The viral WBE approach involves the concentration, extraction and quantification of genetic material in a known volume of wastewater. Real-time reverse transcription quantitative PCR (RT-qPCR) has become the gold standard method for viral RNA quantification. The advantages of RT-qPCR are numerous; it's rapidity, reproducibility as well as assays having the capability to be highly sensitive and specific are highly desired characteristics for both clinical and environmental monitoring programmes (Mackay et al., 2002; Rahman et al., 2013). The major disadvantage of qPCR is its sensitivity to inhibitors, which are often present in wastewater-derived extracts and can prevent amplification (Hedman and Rådström, 2013; Scott et al., 2023). Other disadvantages include cost of machinery and reagents (Rahman et al., 2013), as well as variation in specificity and sensitivity, with some assays having sensitivity as low as 38 % (Dramé et al., 2020; Liu et al., 2020). However, using a probe-based assay, multiple targets can be quantified within one run, reducing the time and costs of quantification compared with running consecutive singleplex assays. These assays should be carefully validated to reduce variability and maximise specificity and sensitivity (Bonvicini et al., 2013; Gibson et al., 2012; Hata et al., 2015; Ward et al., 2004).

The aim of this study was to validate a duplex one-step RT-qPCR assay to quantify NoV genogroups I and II (NoVGI and NoVGII) in wastewater samples, opening the possibility of using this method in future WBE programs. Cross-reactivity, limits of detection and quantification (LOD and LOQ), and concentration-dependent interactions between NoVGI and NoVGII using singleplex and multiplex assays were assessed using pre-quantified target RNA and DNA sequences. Further comparisons to investigate cross-reactivity and inhibition were made between the singleplex and duplex assays using wastewater samples.

2. Material and methods

2.1. Primers, probes and quantification standards

We used established primers and probes for the detection and quantification of NoVGI and NoVGII, as described in Table 1, sourced from Eurogentec (Belgium). For quantification standards, plasmid DNA incorporating the target sequence (Farkas et al., 2017) and RNA Ultramers, sourced from Integrated DNA Technologies (IDT, USA) were used. The DNA standards were quantified using Qubit 4 (Invitrogen, USA) and droplet digital PCR (ddPCR) with the QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad, USA) with the primers detailed in Table 1 according to the manufacturer's instructions. All results confirmed the nominal concentration of the DNA standards. The RNA standards were quantified against the DNA standards in RT-qPCR. Dilution series (10^{10}-10^{0} copies/μl) for each standard were prepared in TE buffer with 0.1 mg/ml yeast tRNA (Invitrogen, USA), aliquoted and stored at − 80 °C (RNA) or − 20 °C (DNA) for up to six months.

2.2. Reaction conditions and duplexing

We used the TaqMan Viral 1-step RT-qPCR master mix (Applied Biosystems, USA) with 1 μg bovine serum albumin (BSA) in 20 μl reaction mixes. We trialled different forward (0.25, 0.5 mM) and reverse primer (0.5, 1 mM) and probe (0.125, 0.25 mM) concentrations and explored the effect of having at 0, 0.4, 0.8 and 1.6 mM $MgSO_4$ in the reaction mix. These experiments were set up in singleplex and duplex assays using RNA standards. Negative template controls were included in all experiments to monitor for external contamination.

All RT-qPCR reactions were performed using a QuantStudio Flex 6 real-time PCR machine (Applied Biosystems Inc., USA). The reaction conditions contained an RT step at 50 °C for 30 min, followed by RT inactivation at 95 °C for 20 s and 45 cycles of denaturation at 95 °C for 3 s and annealing extension at 60 °C for 30 s

The 6-point, ten-fold dilution series (10^{0}–10^{5} copies/μl) of standard RNA/DNA with duplicates of each dilution were used in all reactions for quantification. To compare the performance of the singleplex and duplex assays, an RNA standard dilution series with six replicates in each dilution was used.

LOD and LOQ were determined for both singleplex and duplex assays using an RNA standard dilution series using concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.8 gc/μl with ten replicates at each dilution. The LOD was the lowest concentration where all ten replicates were positive, whereas LOQ was the lowest concentration where the coefficient of variation calculated using $\log_{10}$-transformed concentrations was below 0.25.

2.3. Concentration-dependent interactions

To determine if any interactions between targets were present at different concentrations, RNA standards were mixed to create a concentration matrix between NoVGI and NoVGII and tested in three independent identical duplex RT-qPCR assay experiments (Fig. 1).

Table 1
DNA and RNA sequences used as primers, probes and standards for norovirus GI and GII (NoVGI and NoVGII) in RT-qPCR assays.

Target	Type	Sequence	Reference	Source
NoVGI	Forward primer	CGCTGGATGCGNTTCCAT	(da Silva et al., 2007)	Eurogentec
	Reverse primer	CCTTAGACGCCATCATCATTTAC	(Svraka et al., 2007)	Eurogentec
	Probe	**FAM**-TGGACAGGAGATCGC-**MGB**	(Hoehne and Schreier, 2006)	Eurogentec
	Target DNA	CGCTGGATGCGATTCCATGACTT AAGTTTGTGGACAGGAGATCGC GATCTCTTGCCCGATTATGTAAA TGATGATGGCGTCTAAGG	(Farkas et al., 2017)	Plasmid
	Target RNA	CGCUGGAUGCGAUUCCAUGACU UAAGUUUGUGGACAGGAGAUC GCGAUCUCUUGCCCGAUUAUGU AAAUGAUGAUGGCGUCUAAGG	N/A	IDT
NoVGII	Forward primer	ATGTTCAGRTGGATGAGRTTCTCWGA	(Loisy et al., 2005)	Eurogentec
	Reverse primer	TCGACGCCATCTTCATTCACA	(Kageyama et al., 2003)	Eurogentec
	Probe	**Dragonfly Orange**-AGCACGTGGGAGGGCGATCG-**DDQII**	(Loisy et al., 2005)	Eurogentec
	Target DNA	ATGTTCAGATGGATGAGATTCTCAGA TCTGAGCACGTGGGAGGGCGATCGC AATCTGGCTCCCAGTTTTGTGAATGAA GATGGCGTCGA	(Farkas et al., 2017)	Plasmid
	Target RNA	AUGUUCAGAUGGAUGAGAUUCUCA GAUCUGAGCACGUGGGAGGGCGAU CGCAAUCUGGCUCCCAGUUUUGUG AAUGAAGAUGGCGUCGA	N/A	IDT

	1	2	3	4	5	6	7	8	9	10	11	12
A	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5	GI:10^5
	GII:10^5	GII:10^5	GII:10^4	GII:10^4	GII:10^3	GII:10^3	GII:10^2	GII:10^2	GII:10^1	GII:10^1	GII:10^0	GII:10^0
B	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4	GI:10^4
	GII:10^5	GII:10^5	GII:10^4	GII:10^4	GII:10^3	GII:10^3	GII:10^2	GII:10^2	GII:10^1	GII:10^1	GII:10^0	GII:10^0
C	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3	GI:10^3
	GII:10^5	GII:10^5	GII:10^4	GII:10^4	GII:10^3	GII:10^3	GII:10^2	GII:10^2	GII:10^1	GII:10^1	GII:10^0	GII:10^0
D	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2	GI:10^2
	GII:10^5	GII:10^5	GII:10^4	GII:10^4	GII:10^3	GII:10^3	GII:10^2	GII:10^2	GII:10^1	GII:10^1	GII:10^0	GII:10^0
E	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1	GI:10^1
	GII:10^5	GII:10^5	GII:10^4	GII:10^4	GII:10^3	GII:10^3	GII:10^2	GII:10^2	GII:10^1	GII:10^1	GII:10^0	GII:10^0
F	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0	GI:10^0
	GII:10^5	GII:10^5	GII:10^4	GII:10^4	GII:10^3	GII:10^3	GII:10^2	GII:10^2	GII:10^1	GII:10^1	GII:10^0	GII:10^0

Fig. 1. Plate layout for testing concentration-dependent interactions for NoV GI (GI – brown) and NoV GII (GII – green with each sample run in duplicates.

2.4. Comparison of singleplex and duplex RT-qPCR assays

The efficiency of singleplex and duplex reactions was further tested on wastewater-derived total nucleic acid extracts. For both assays, 143 extracts derived from influent wastewater taken at centralised urban treatment plants during April-July 2022 as part of the English COVID-19 wastewater surveillance programme (Wade et al. 2022) were tested. The samples were concentrated using ammonium sulphate precipitation, and then the RNA was extracted using the NucliSens extraction system (BioMerieux, France) as described previously (Farkas et al., 2022; Kevill et al., 2022).

2.5. RT-qPCR inhibition testing

Potential inhibition due to the presence of residual organic matter was assessed using two approaches. For both assays, 127 wastewater-derived RNA eluents, prepared as described in Section 2.4, were used. First, the samples were tested in duplex RT-qPCR assays with either 4 µl or 2 µl of the samples added to the reaction mix, resulting in a two-fold dilution of each sample. Then, duplex RT-qPCR assay was used (with 4 µl samples in the reaction mix) with the addition of approximately 10^5 gc/µl NoVGI and NoVGII RNA standards as an external control. The RNA concentrations observed in water controls were compared with the concentrations in the samples.

2.6. Data analysis

RT-qPCR data was analysed using the QuantStudio Real-Time PCR software v1.7 (Applied Biosystems Inc., USA). The standard curve efficiency, slope and R^2 were calculated in the software. Sample concentration was expressed as gc/µl RNA extract or standard solution.

One-way ANOVA was used to assess the effect of different primer, probe concentration on the RT-qPCR assay performance, whereas a t-test was used to explore the effect of the additional $MgSO_4$ in the reaction mix, using SPSS Statistics V27 (IBM, USA). A t-test was used to compare standard dilution concentrations in singleplex and duplex assays using SPSS Statistics V27 (IBM, USA).

To determine if similar concentrations were measured regardless of the concentration of each target in the reaction mix, concentration-dependent interactions were explored. Results were Log10 transformed before Shapiro-Wilk tests for Normality, Kruskal-Wallis rank sum tests and pairwise comparisons using Wilcoxon rank sum test were run. These analyses were undertaken in R version 4.1.1 and R Studio 2022.07.2 + 576 (R Core Team, 2021).

RT-qPCR inhibition in spiked samples (Section 2.4) was calculated using the equation:

$$\%inhibition = \frac{(MCC - CS)}{MCC} \cdot 100$$

Where MCC was the mean concentration of the target virus measured for the spiked control (representing 0 % inhibition) and the CS was the concentration of each target virus measured for the spiked sample.

Samples described in Section 2.4 were analysed to determine, for both singleplex and duplex assays, whether the same samples were detected positive above the LOD and if sample concentrations above the LOQ were consistent. Results were $\log_{10}$ transformed and Shapiro-Wilk tests for Normality, followed by pairwise comparisons using Wilcoxon rank sum test with continuity correction were undertaken on the positive sample viral quantities above the LOD and the LOQ. Association between assays was tested using Spearman's rank correlation tests. These analyses were undertaken in R version 4.1.1 and R Studio 2022.07.2 + 576 (R Core Team, 2021).

3. Results

3.1. Method optimisation

In order to maximize sensitivity and precision, different concentrations of primer, probe and $MgSO_4$ concentrations in the reaction mix were trialled. The NoVGI assays performance was significantly better when high concentration primer and probe were added to the reaction mix. However, the difference was significant only at 100–1000 gc/µl sample concentrations (one-way ANOVA $p = 0.017$; 0.002). Therefore, 0.5 mM, 1 µM and 0.25 mM NoVGI forward, reverse primer and probe concentrations were used in subsequent applications. In contrast, the NoVGII assay was not affected by reduced oligo concentrations, so the same oligo concentrations as applied for NoVGI were used in all assays for consistency and easy preparation. The addition of $MgSO_4$ to the mastermix had no significant effect on assay performance (t-test $p > 0.05$), therefore, no $MgSO_4$ was added to the reaction mix in subsequent assays.

For all RT-qPCR assays, the standard curves parameters were within the expected range of slope: − 3.1- − 3.6, R^2: > 0.98 and efficiency percentile: 90–100 %. To assess the performance of the singleplex and duplex assays, standards dilutions were run with six replicates in each dilution. The duplex assays had slightly lower slope values (Table 2). No statistical differences in the results for the standards in singleplex vs. duplex assays were found for the NoVGI standards (t-test, $p > 0.05$). Significant differences were shown in a similar comparison for the NoVGII target, however, the differences were inconsistent, only affecting the 10^4, 10^2, 10^0 copies/µl concentrations.

The LOD and LOQ values calculated for the singleplex and duplex assays are shown in Table 3. Both assays were extremely sensitive

Table 2
Standard curve quality for norovirus GI (NoVGI) and GII (NoVGII) singleplex and duplex RT-qPCR assays.

Target	Assay type	Slope	R^2	Efficiency %
NoVGI	Singleplex	-3.396	0.998	97.02
NoVGI	Duplex	-3.440	0.997	95.30
NoVGII	Singleplex	-3.308	0.997	100.59
NoVGII	Duplex	-3.417	0.998	96.18

Table 3
Limit of detection (LOD) and limit of quantification (LOQ) values for NoVGI and NoVGII singleplex and duplex RT-qPCR assays.

Target	Assay type	LOD (gc/µl)	LOQ (gc/µl)
NoVGI	Singleplex	0.841	4.259
NoVGI	Duplex	0.519	3.837
NoVGII	Singleplex	1.640	11.680
NoVGII	Duplex	1.369	11.680

enabling the detection of 2.0–6.6 RNA copies in each reaction. The LOQ for the NoVGI assay was low, however, the NoVGII assays had a higher LOQ, indicating quantification of low concentration samples may have more variation, though it is usually not significant when tested in wastewater samples which has high viral titres.

3.2. Method validation

When RNA standards were mixed at different concentrations in duplex RT-qPCR assays, no cross-dependent interactions were present (Fig. 2). Despite $\log_{10}$ transformation, results were not normally distributed for NoVGI ($W = 0.943$, $p < 0.001$) nor for NoVGII ($W = 0.929$, $p < 0.001$). No significant difference was found between quantities regardless of the concentration of each target for both NoVGI (Kruskal-Wallis chi-squared = 201, df = 199, $p = 0.448$) and for NoVGII (Kruskal-Wallis chi-squared = 215, df = 215, $p = 0.487$). All pairwise comparisons confirmed there were no significant differences in NoVGI quantities measured regardless of the varying NoVGII standard concentration (all p values > 0.05, Fig. 2A). This finding was the same NoVGII quantities measured with respect to varying NoVGI standard concentration (all p values > 0.05, Fig. 2B).

3.2.1. *Performance of singleplex and duplex RT-qPCR assays for wastewater eluents*

The performance of the singleplex and duplex RT-qPCR assays was further assessed in RNA eluents derived from wastewater concentrates. Log_{10} transformed results were not normally distributed (LOD dataset: $W = 0.982$, $p < 0.001$; LOQ dataset: $W = 0.965$, $p = 0.0018$) hence non-parametric testing was undertaken. For NoVGI, the duplex assay yielded

Table 4
The number of samples that tested positive for each assay type above the LOD and the LOQ. n = 143.

Assay	Number of positive samples above the LOD	Number of positive samples above the LOQ
NoVGI singleplex	39	18
NoVGI duplex	32	16
NoVGI both	30	15
NoVGII singleplex	44	16
NoVGII duplex	62	31
NoVGII both	43	16

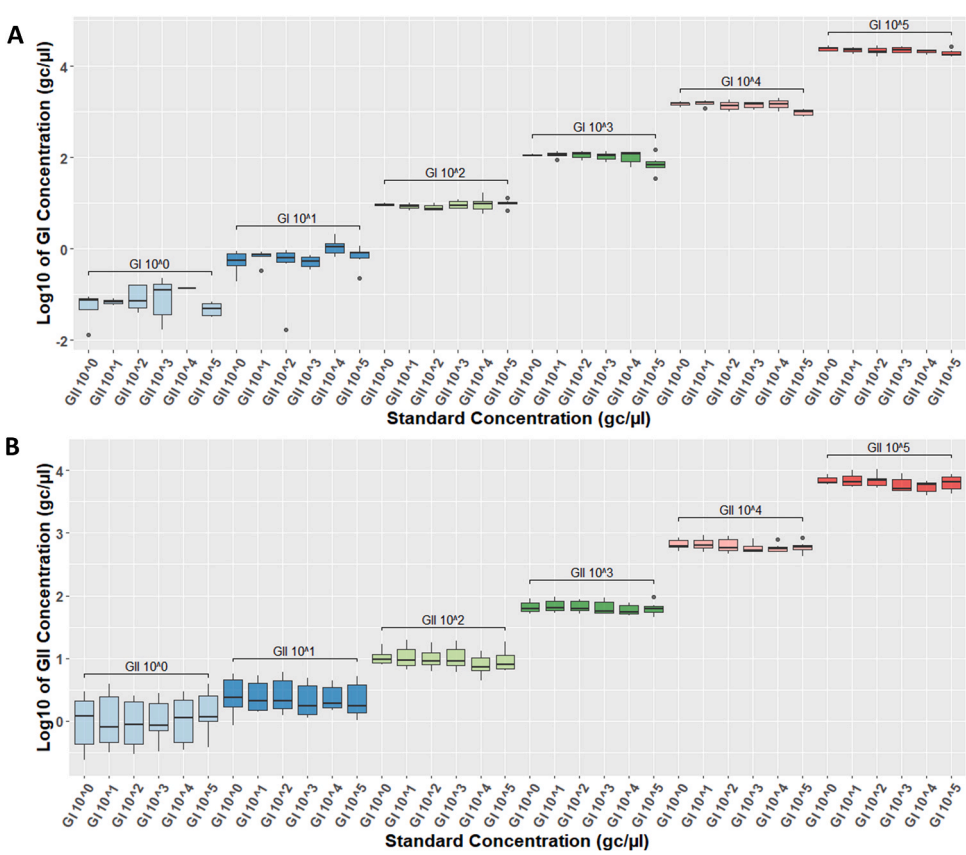

Fig. 2. Boxplots demonstrating the mean concentrations measured for (A) NoVGI (GI) and (B) NoVGII when standards were mixed at various concentrations in duplex RT-qPCR assays. Brackets group NoVGI standard concentrations (A) and NoVGII standard concentrations (B).

similar counts of positive samples compared to the singleplex assay (Table 4). For NoVGII, the number of positive samples detected was higher in the duplex assay compared to the singleplex assay, which may indicate an increased sensitivity in the duplex assay (Table 4), though not significantly so (LOD p = 0.70, LOQ p = 0.074).

For both targets, little difference in the concentrations measured between duplex and singleplex assays was found (Fig. 3). Pairwise comparisons demonstrated there was no significant difference between duplex and singleplex concentrations above the LOD for NoVGI (p = 0.87) and NoVGII (p = 0.70) (Fig. 3A). Furthermore, similar results were seen between duplex and singleplex concentrations above the LOQ for NoVGI (p = 0.614) and NoVGII (p = 0.074) (Fig. 3B). There was a strong positive correlation between the concentrations measured above LOQ in both singleplex and duplex assays for NoVGI (r(28) = 0.953, p < 0.001). Similar associations were found in concentrations above LOQ measured in NoVGII singleplex and duplex assays (r(30) = 0.979, p = <0.001). This suggests, for both targets, positive samples were likely to test positive with similar concentrations in both singleplex and duplex assays.

Inhibition was first assessed by comparing the assay performance on wastewater-derived RNA eluents and their dilutions. Overall, more samples tested positive for NoVGI before than after dilution, however, opposite trends were observed for NoVGII (Table 5). The relationship between the NoV concentrations assessed when both the undiluted and diluted samples gave a positive result for both targets (Fig. 4). The regression line for both NoVGI and NoVGII show that the quantifications are largely similar between diluted and undiluted samples, but at the lower RNA concentrations, the relationship was weaker as the intrinsic variability was too high to give an accurate measure.

Inhibition was further assessed by spiking the wastewater RNA eluents with known concentrations of NoVGI and NoVGII RNA standards and calculating the recovery percentiles (Fig. 5). The maximum inhibition levels for NoVGI and NoVGII were 31.4 % and 32.0 %, respectively. The mode, median and mean average > 0 % inhibition for NoVGI was 13 % in all cases. The mode, median and mean average > 0 % inhibition for NoVGII was 10 %, 11 % and 13 % respectively. The inhibition level calculated for the outlying sample in the dilution test were − 14 % for NoVGI and 9 % for NoVGII.

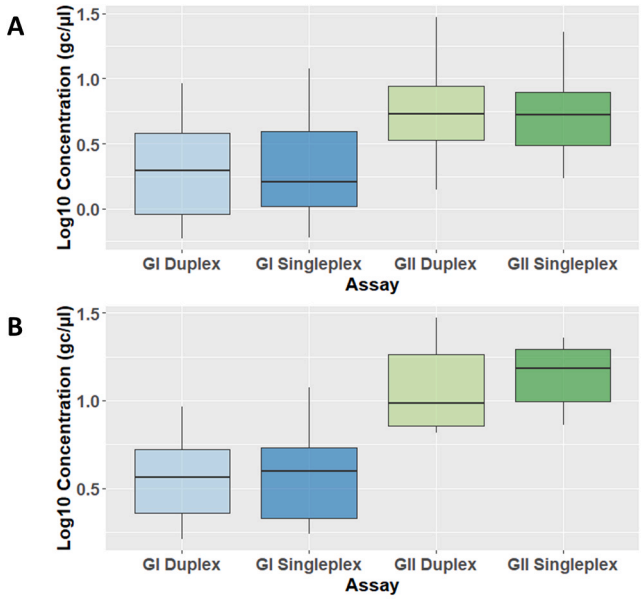

Fig. 3. Box plot demonstrating the mean concentration of positive samples above the LOD (A) and above the LOQ (B) for each assay NoVGI (GI) and NoVGII (GII).

Table 5
The number of wastewater-derived RNA eluents (original and diluted) which tested positive for NoVGI and NoVGII (n = 127).

Target	Dilution	Number of positive samples
NoVGI	4x	72
NoVGI	Undiluted	78
NoVGI	4x and undiluted	61
NoVGII	4x	118
NoVGII	Undiluted	112
NoVGII	4x and undiluted	103

4. Discussion

In this study, we developed and validated a duplex RT-qPCR assay targeting NoVGI and NoVGII, that is suitable for the detection and quantification of NoV in wastewater-derived samples. We used primers and probes that has been widely used for the targets in environmental studies (Bounagua and Bouderra, 2021; Ferland et al. 2023; Korajkic et al., 2022; Prado et al., 2019). The RT-qPCR mix has also been proven to be suitable for wastewater-derived samples with the reaction conditions we used in this study (Ahmed et al., 2022; Barrios et al., 2021; Hasing et al., 2021; Lee et al., 2021; Maksimovic Carvalho Ferreira et al., 2022; Zhou et al., 2021), hence no other master mixes or conditions were trialled. The primer, probe and magnesium concentrations were carefully adjusted in both singleplex and duplex assays to ensure the standard curves are within the expected limits (Table 2). The developed assays were extremely sensitive, with a low detection limit of 2.0–6.6 RNA copies in each reaction. In comparison, previous studies have found the potential detection limit of NoVGII and NoVGI TaqMan RT-PCR assays to be less than 10 and 100 copies per reaction, respectively (Trujillo et al., 2006) and 5–50 copies of viral RNA per reaction from stool samples (Chhabra et al., 2021). This demonstrates our duplex assay is highly sensitive and therefore an excellent option for maximising targets while minimising costs.

While there were significant differences in LOD and LOQ between NoVGI and NoVGII, each genogroup performed similarly when run as singleplex vs. duplex with no interaction between the two primer/probes or interference of the individual fluorescence signals. Similar performance between singleplex and multiplex assays for NoVGI and NoVGII have been found in faecal samples (Feeney et al., 2011), stool suspension supernatant (Farkas et al., 2015), shellfish and sediment (Farkas et al., 2017) despite differences in RNA extraction and assay run conditions to the present study. Furthermore, similar to other multiplex research, we found little competition or cross-amplification between the targets regardless of their concentration (De Keuckelaere et al., 2013; Molenkamp et al., 2007). However, previous studies have detailed a mutual competitive affect between NoVGI and NoVGII in a multiplex PCR assay, where a 2-log concentration difference resulted in significant shifts of the Ct values for the target with the lowest concentration (Stals et al., 2009). Competition between individual PCR reactions within multiplex assays is common (Candotti et al., 2004; Cook et al., 2002; Stals et al., 2009) and needs to be considered during assay selection for samples that may have extreme target concentrations.

The singleplex and duplex assays were further tested on wastewater-derived RNA extracts to assess performance and the effect of inhibitions derived from residual organic matter in the RNA eluent. We found that if a sample was positive in a singleplex assay it was highly likely to be positive in the duplex assay and vice versa for both targets. Our finding of the NoVGII assays having a higher LOQ may be a limitation for research interested in the reduction of NoV by wastewater treatment processes. Further research on challenging the primers and probes against strains within the NoV genogroups could refine the duplex assay we propose here. Future work could include additional platforms of detection, such as ddPCR, to support the accuracy and sensitivity of the duplex RT-qPCR assay we developed (Meregildo-Rodriguez et al., 2023; Nuraeni et al., 2023) but also explore virus viability in wastewater or

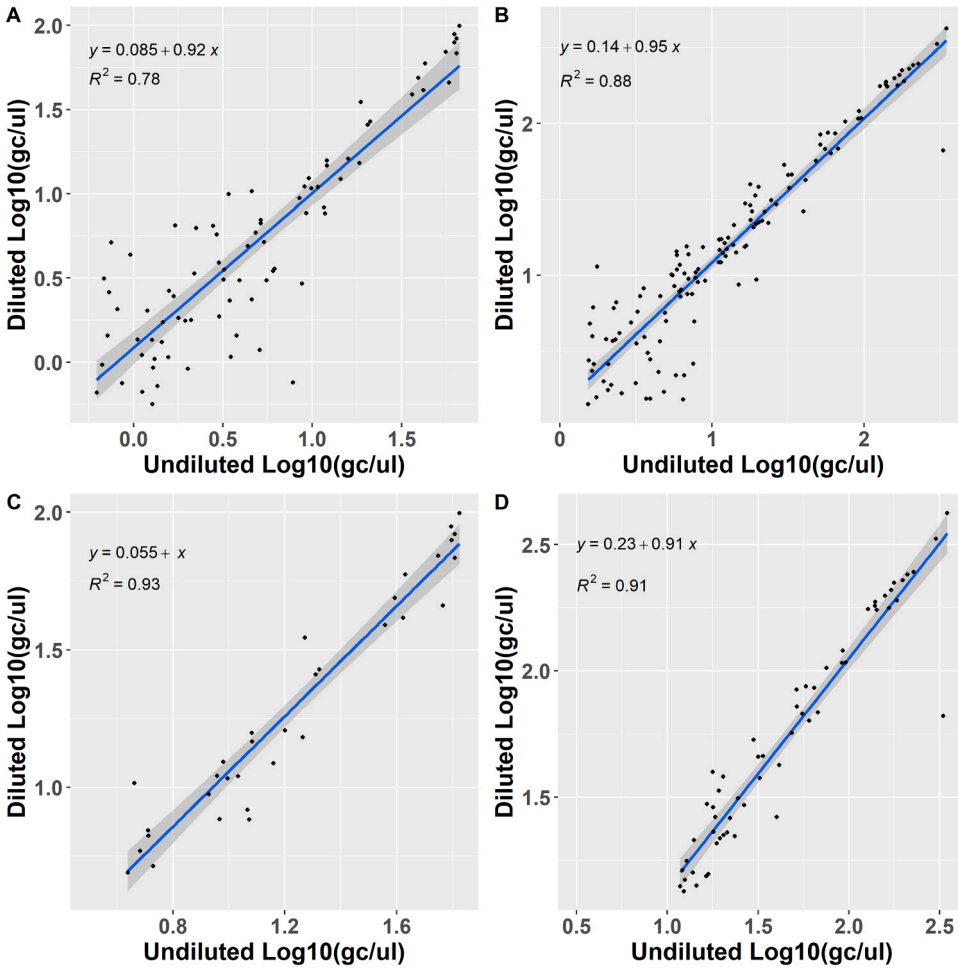

Fig. 4. Regression analysis to assess the effect of dilution on the duplex RT-qPCR assay targeting (A) NoVGI above LOD, (B) NoVGII above LOD, (C) NoVGI above LOQ and (D) NoVGII above LOQ levels in wastewater-derived RNA eluents. Diluted concentrations have been back-adjusted to neat (undiluted) concentrations to allow direct comparison.

capsid integrity (Raymond et al., 2023).

The presence of inhibitory compounds in RT-qPCR reactions affecting the amplification of the target signal has been well documented and reviewed (Hedman and Rådström, 2013). Inhibition can occur with non-target compounds interfering with the RT and DNA polymerase enzymes, preventing of nucleic acid binding and interfering with the fluorescence signal. Many compounds that can cause PCR inhibition are likely to be found in wastewater. Compounds from anthropogenic sources include heme (from hemoglobin), lactoferrin (Al-Soud and Rådström, 2001) and melanin, found in skin and hair (Eckhart et al., 2000). From environmental sources polyphenolic compounds such as tannic, humic and fulvic acids which originate from plants (Kreader, 1996) can easily be washed into sewage networks. Faeces also contain phenolic compounds as well as large amounts of complex polysaccharides which have been shown to inhibit PCR reactions (Monteiro et al., 1997; Wilson, 1997). The presence of wastewater-derived urine can also change the ion content of the qPCR reaction mixture (Mahony et al., 1998), with additional ions, such as Ca^{2+} (Bickley et al., 1996), K^{+} and Na^{+} (Abu Al-Soud and Rådström, 1998) competing with Mg^{2+} for binding sites therefore inhibiting DNA amplification by supressing the activity of the DNA polymerase. The addition of a protein, such as BSA, in excess can reduce the effects of inhibition from naturally occurring compounds (Al-Soud and Rådström, 2001; Eckhart et al., 2000; Kreader, 1996) that may occur in our wastewater samples. When BSA was added to the reaction mix, we noted up to 30 % inhibition in wastewater-derived samples which was not specific to either virus. The inhibition was lower than what was noted using different RT-qPCR mixes in wastewater samples processed the same way as described here (Scott et al., 2023). This may indicate that monitoring RT-qPCR inhibition on a sample-by-sample basis is unnecessary when using this assay. Where monitoring inhibition is necessary, diluting samples before performing RT-qPCR reduced inhibition. However, assay sensitivity was also reduced and hence that approach may not be suitable for samples with low target concentrations. Alternative methods to assess inhibition include use of an internal amplification control (IAC), though these require extensive optimisation, or serial dilution of samples which is consumable and labour intensive and prone to cross-contamination (Laverick et al., 2004; Scott et al., 2023).

In conclusion, the one-step RT-qPCR duplex assay developed here enables the sensitive detection of NoVGI and NoVGII in wastewater-derived RNA eluents. Although inaccuracy in quantification may exist due to inhibition and low viral load, using a multiplex assay allows the test for both targets in a time and cost-effective way. Wastewater-based epidemiology has been extremely useful for monitoring SARS-CoV-2 outbreaks globally, where RT-qPCR assays have been one of the many tools used to rapidly assess community outbreaks (Farkas et al., 2020; Vitale et al., 2021; Wade et al., 2022). We demonstrate that our assay can be used on a wide range of urban wastewater samples and can therefore contribute to on-going public health screening for disease outbreaks as well as quantifying wastewater-derived releases of NoV to the environment.

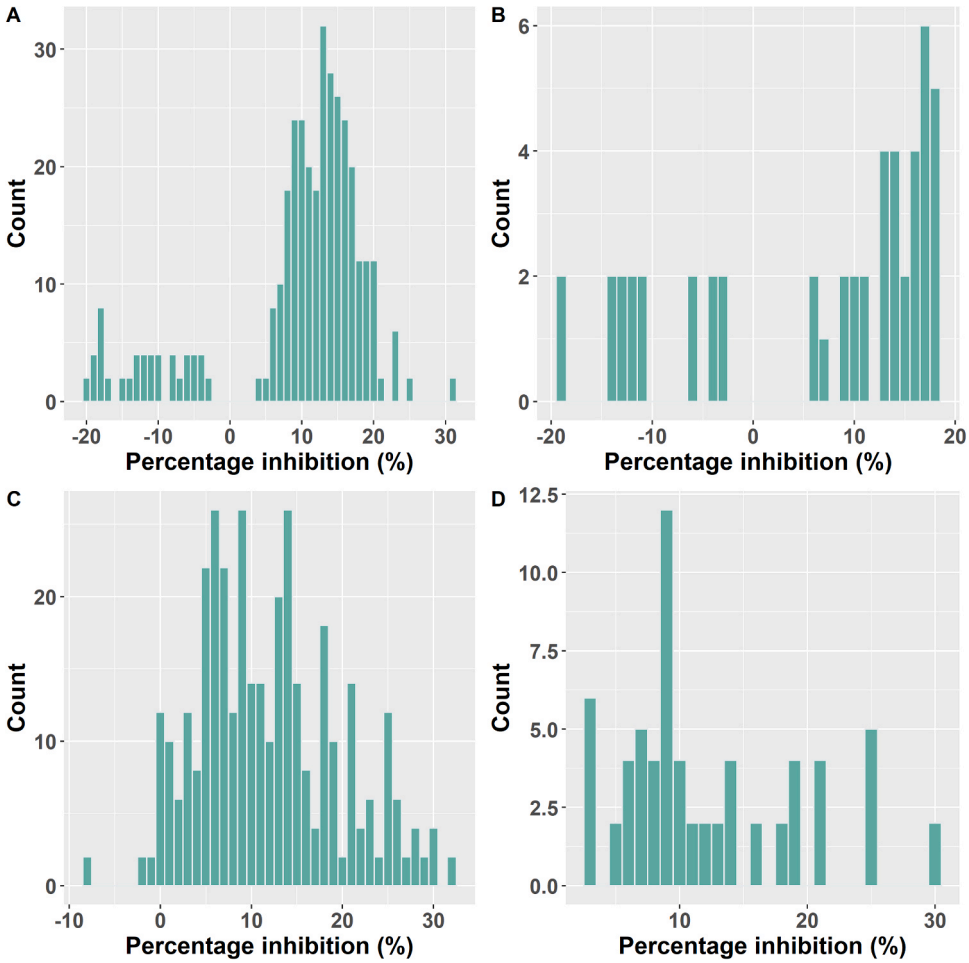

Fig. 5. Histograms demonstrating the frequency of samples with a particular range of inhibition for (A) NoVGI and (B) NoVGI showing samples with concentrations above LOQ, (C) NoVGII and (D) NoVGII samples with concentrations above LOQ.

CRediT authorship contribution statement

Natasha Alex-Sanders: Methodology, Investigation, Formal analysis, Writing – review & editing. **Nick Woodhall:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Kata Farkas:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Funding acquisition, Supervision. **George Scott:** Conceptualization, Writing – review & editing. **Davey L. Jones:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision. **David I. Walker:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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