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Improved sensitivity using a dual target, E and RdRp assay for the diagnosis of SARS-CoV-2 infection: Experience at a large NHS Foundation Trust in the UK

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To The Editor,

We have prepared a letter for the *Journal of Infection* which we feel is of interest to many of your readers and is particularly timely.

It details our early experience of using a dual target (RdRp and E gene) real time PCR assay. As initial diagnostic algorithms for SARS-CoV-2 raised concerns of the specificity of E gene amplification in isolation due to batch contamination of reagents, we were interested in particular in analysing the samples where E gene amplified in the absence of RdRp amplification.

The principle findings are that E gene detection in the absence of RdRp detection was common (12.3%) amongst positive samples, the majority (96.9%) of which were considered true positives following further assessment. In addition, in a subset of samples where symptom onset was available (145 samples from 128 patients), it was clear that the cycle threshold values for both RdRp and E gene were lowest around 48 – 72 hours following symptom onset (Figure 2), and at each stage of infection the median CT values for RdRp were higher than those for the E gene.

We believe dual target testing, using the E gene as a second target, will help improve both laboratory diagnostic pick up and our clinical response to this pandemic. This may be particularly relevant due to the current difficulty in sourcing nucleic acid extraction kits globally, as alternative options such as PCR without a nucleic acid extraction step are being explored, which inherently reduces the sensitivity of RNA detection from clinical samples

Many thanks for your consideration.

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Improved sensitivity using a dual target, E and RdRp assay for the diagnosis of SARS-CoV-2 infection: Experience at a large NHS Foundation Trust in the UK

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Author Contributions

Hayley Colton & Michael Ankcorn – collated and analysed clinical and PCR data, prepared manuscript.

Mehmet Yavuz & Leeanne Tovey – collected data and validated E-RdRp assay.

Alison Cope – edited manuscript.

Mohammad Raza – edited manuscript.

Alexander J Keeley – collected data, edited manuscript.

Amy State – collected data, edited manuscript.

Bozena Poller– collected data, edited manuscript.

Matthew Parker – collated sequencing data, edited manuscript.

Thushan I de Silva & Cariad Evans – oversaw project, analysed data and edited manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Ethics

The project was undertaken by Laboratory Medicine as part of the evaluation, validation and verification of the diagnostic test as per the Quality Assurance requirements of the relevant ISO, BSI and UKAS accreditation system. The primary aim of this work is therefore not classed as research, therefore Research Ethics Committee opinion was not required.

Improved sensitivity using a dual target, E and RdRp assay for the diagnosis of SARS-CoV-2 infection: Experience at a large NHS Foundation Trust in the UK

Dear Editor,

We read with interest the letter from Hao *et al* highlighting the issues regarding the sensitivity of real time reverse-transcriptase polymerase chain reaction (RT-PCR) testing of upper respiratory tract samples for COVID19 disease [1]. Extensive RT-PCR testing by has been key to clinical decision-making, epidemiological analysis and policy development during the current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic. The majority of RT-PCR assays target the RNA-dependent RNA polymerase (RdRp), envelope protein (E) or nucleocapsid protein (N) genes [2]. However, initial testing algorithms and expert opinion from the European Centre for Disease Prevention and Control (ECDC) advised that E gene amplification in isolation should be treated cautiously, due to concerns of non-specificity and issues related to contamination of reagents [3]. Early experience at Sheffield Teaching Hospitals NHS Foundation Trust (UK) on serially sampled patients with confirmed SARS-CoV-2 infection suggested that E gene detection persists beyond RdRp detection, and may offer enhanced diagnostic sensitivity. Therefore we explored the significance of E gene detection in relation to RdRp, and in the absence of RdRp detection in a retrospective evaluation of SARS-CoV-2 RT-PCR testing.

A total of 12,015 clinical samples (combined nose/throat swabs or lower respiratory tract samples) were tested for SARS-CoV-2 as part of routine clinical diagnostics between 2nd March 2020 and 5th April 2020 at Sheffield Teaching Hospitals NHS Foundation Trust. Samples were extracted on the MagnaPure96 platform (Roche Diagnostics Ltd, Burgess Hill, UK). SARS-CoV-2 RNA was detected on 6µl of extract using a dual target (E gene and the RdRp gene) in-house PCR on ABI Thermal Cycler (Applied Biosystems, Foster City, United States) (supplementary material) [4]. The assay was modified to a multiplex single-well assay with the addition of PCR primers to detect a housekeeping gene, Ribonuclease P (RNAse P), which acts as an internal control and to assess sample quality.

Of the samples tested, 2,593 samples (21.6%) were positive with amplification curves for one or both target genes. Amongst positive results, we found E gene amplification alone to be common (n= 319, 12.3%), although the majority were positive for both RdRp and E gene targets (n = 2273, 87.7 %) and only 1 sample (<0.1 %) had RdRp gene amplification alone.

From the E-only positive group (n=319), 69 (21.6%) samples had low level amplification in the E gene (cycle threshold (CT) ≥35) and were investigated further. Within this subset, the majority (n=59, 85.5%) were considered to be true positives because they were either a) confirmed by an alternative assay (n=48) or b) a preceding or subsequent sample was positive for both E and RdRp (n=11) (Table 1). The alternative assay employed was a modified version of the Centers for Disease Control and Prevention (CDC) assay targeting the N gene (Micropathology Ltd, Coventry, UK) in most cases (n=47) or an alternative RdRp assay (n=1) [7]. Six samples (8.7 %) could not be confirmed in an alternative assay which had either high CT values for the E gene (n=4, CT values ≥39.0) or had good amplification curves not reaching the threshold (n=2). To further confirm the presence of SARS-CoV-2 RNA in samples with E gene only amplification, 11 samples were selected and successfully underwent whole genome sequencing (supplementary material). Analysis of the RdRp primer or probe binding sites in these samples did not reveal any mismatches to explain the lack of RdRp RT-PCR positivity (supplementary material).

We further explored the relationship between E gene detection and RdRp gene detection. Amongst samples with both RdRp gene and E gene amplification (n= 2273) , we found that CT values for the E gene target were significantly lower than the CT values for RdRP, with a mean difference of 5.8 (Paired t test, p-value < 2.2e-16, 95% CI 5.79-5.92) (supplementary material). In a subset of samples where symptom onset was available (145 samples from 128 patients), it was clear that the CT values for both RdRp and E gene were lowest around 48 – 72 hours following symptom onset (Figure 1). At each stage of infection, the median CT values for RdRp were higher than those for the E gene.

By using the E gene target in addition to the RdRp gene target we observed a significantly increased diagnostic pick up (11.9%). In one patient, E gene amplification was detected for three days beyond RdRp amplification, indicating a possible widening of the diagnostic window. Our findings confirm that clinical samples with E only amplification should not be dismissed as non-specific results. Not only were we able to obtain whole genome sequences for SARS-CoV-2 from a subset of this group, we also found that 85% of E only samples with high CT values were confirmed by a second assay targeting the N gene or an alternative RdRp only assay.

The enhanced sensitivity seen for the E gene in our dual target E-RdRP assay is yet to be explained. We observed a mean difference of over five CT values when comparing E gene to RdRp values, which may suggest the possibility of higher copy numbers of E gene being present in the primary or extracted sample. Due to the unique transcription strategy of coronaviruses, genes towards the 3' end of the genome would be present in higher copy numbers during active viral replication, which could explain these findings [5]. It is also possible that PCR optimised conditions in a multiplex system favours E gene amplification, however we found no significant loss of RdRp detection when comparing single and multiplex systems during validation, with observed CT rises averaging 1-2 cycles (data not shown). In addition, we found no evidence of primer or probe mismatches in the RdRp region.

We believe dual target testing, using the E gene as a second target, will help improve both diagnostic sensitivity and the appropriate clinical response to this pandemic. We urge testing laboratories to carefully consider the use of the E gene as a target in order to optimise SARS-CoV-2 diagnostics, including strategies to confirm samples with E gene only amplification as we have described.

Word count 996

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106 **Tables & Figures**

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	n	%
Sent for confirmation at reference laboratory[ϕ]	**54**	**78.26**
- Confirmed by alternative assay	48	(88.89)
- Not confirmed	6	(11.11)
Repeat clinical sample positive	**5**	**7.25**
Previous clinical samples positive[Ψ]	**6**	**8.70**
Resulted without further testing[ǂ]	**4**	**5.80**
Total	69	

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112 **Table 1.** Summary of samples with low level E gene amplification alone (CT ≥ 35). CT, cycle threshold;
113 E, envelope gene.

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115 [ϕ] Most samples (n=53) were tested at Micropathology Ltd (Coventry) using a SARS-CoV-2 N gene
116 assay using a modified CDC assay[6]. The other sample confirmed positive at PHE Colindale using an
117 alternative SARS-CoV-2 RdRp assay.

118 Ψ As part of the High Consequences Infectious Diseases network, Sheffield received some of the first
119 positive patients in the United Kingdom, who had daily swabs taken. E gene amplification appeared
120 to persist in this cohort after the RdRp became negative.

121 ǂ Four results were authorised without further testing due to high pre-test probability e.g.
122 compatible symptoms with a confirmed household exposure to SARS-CoV-2.

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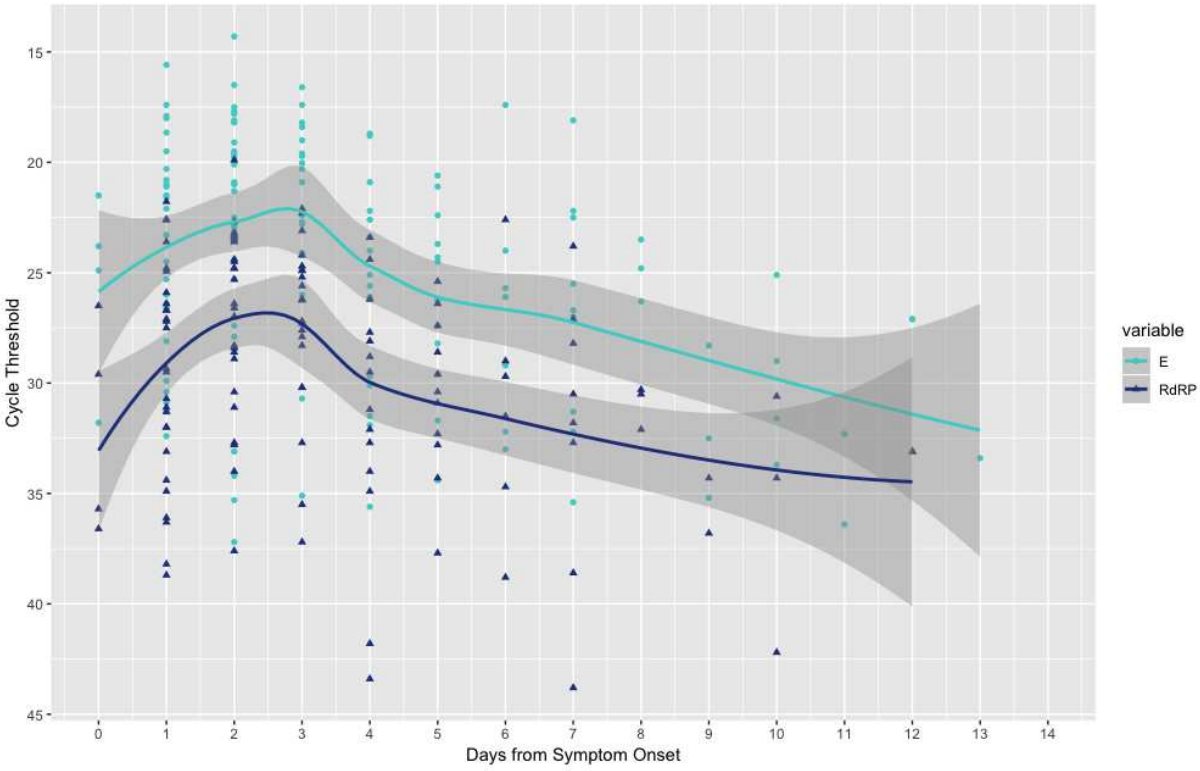

Figure 1. E and RdRp gene cycle threshold results in relation to symptom onset. E and RdRp amplification results plotted against days of symptom onset in 145 samples from 128 patients. Lowest CT values were seen around day 3 of symptoms, with mean RdRp CT higher at a given day compared to E gene CT value. The lines represent the smoothed conditional mean with 95% confidence intervals in the grey bars. E, envelope gene; RdRp, RNA-dependent RNA-polymerase.

Supplementary Material

Table S1. Primer and Probe sequences used for RdRp and E gene detection.

Name	Sequence
RdRp gene F	GTG TGA RAT GGT CAT GTG TGG CGG
RdRp gene R	CAR ATG TTA AAS ACA CTA TTA GCA TA
RdRp gene P	6-FAM- CAG GTG GAA CCT CAT CAG GAG ATG C- BHQ1
E gene F	ACA GGT ACG TTA ATA GTT AAT AGC GT
E gene R	ATA TTG CAG CAG TAC GCA CAC A
E gene P	HEX-ACA CTA GCC ATC CTT ACT GCG CTT CG-BHQ1

Abbreviations: BHQ-1, black hole quencher; F, forward primer; HEX, HEX flourophore; P, probe; R, reverse primer; 6-FAM, 6-Carboxyfluorescein flourophore.

Initial Result	**N**	**%**
Both RdRp+ and E+	2273	18.92
RdRp- and E+	319	2.66
RdRp+ and E-	1	<0.01
Invalid (negative internal control)	8	0.07
RdRp- and E-	9414	78.35
Total	12015	

Table S2. Summary results for all diagnostic samples tested for SARS-CoV-2. A total of 12,015 clinical samples (combined nose and throat swabs or lower respiratory tract specimens) were tested for SARS-CoV-2 by PCR as part of routine clinical diagnosis over a period of approximately four weeks. The results are summarised above. RdRp, RNA-dependent RNA-polymerase gene; E, envelope gene.

192 **Table S3. Whole Genome Sequencing of Samples with Unamplified RdRp**

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GISAID Identifier	E gene CT	RdRp gene CT	Coverage across genome	ARTIC Primer set
hCoV-19/England/SHEF-C0488/2020	31.2	NA	0.988	V3
hCoV-19/England/SHEF-BFD09/2020	32.2	NA	0.965	V2
hCoV-19/England/SHEF-BFF30/2020	32.3	NA	0.959	V1
Not Submitted Due To Low Coverage	34.7	NA	0.791	V3
hCoV-19/England/SHEF-C0567/2020	34	NA	0.946	V3
hCoV-19/England/SHEF-C05D0/2020	33.9	NA	0.995	V3
hCoV-19/England/SHEF-BFECA/2020	33.5	NA	0.885	V1
hCoV-19/England/SHEF-BFF5E/2020	31.7	NA	0.962	V1
hCoV-19/England/SHEF-C01DC/2020	34.6	NA	0.944	V1
hCoV-19/England/SHEF-BFE51/2020	32.4	NA	0.988	V1
hCoV-19/England/SHEF-BFCA2/2020	32.9	NA	0.988	V1

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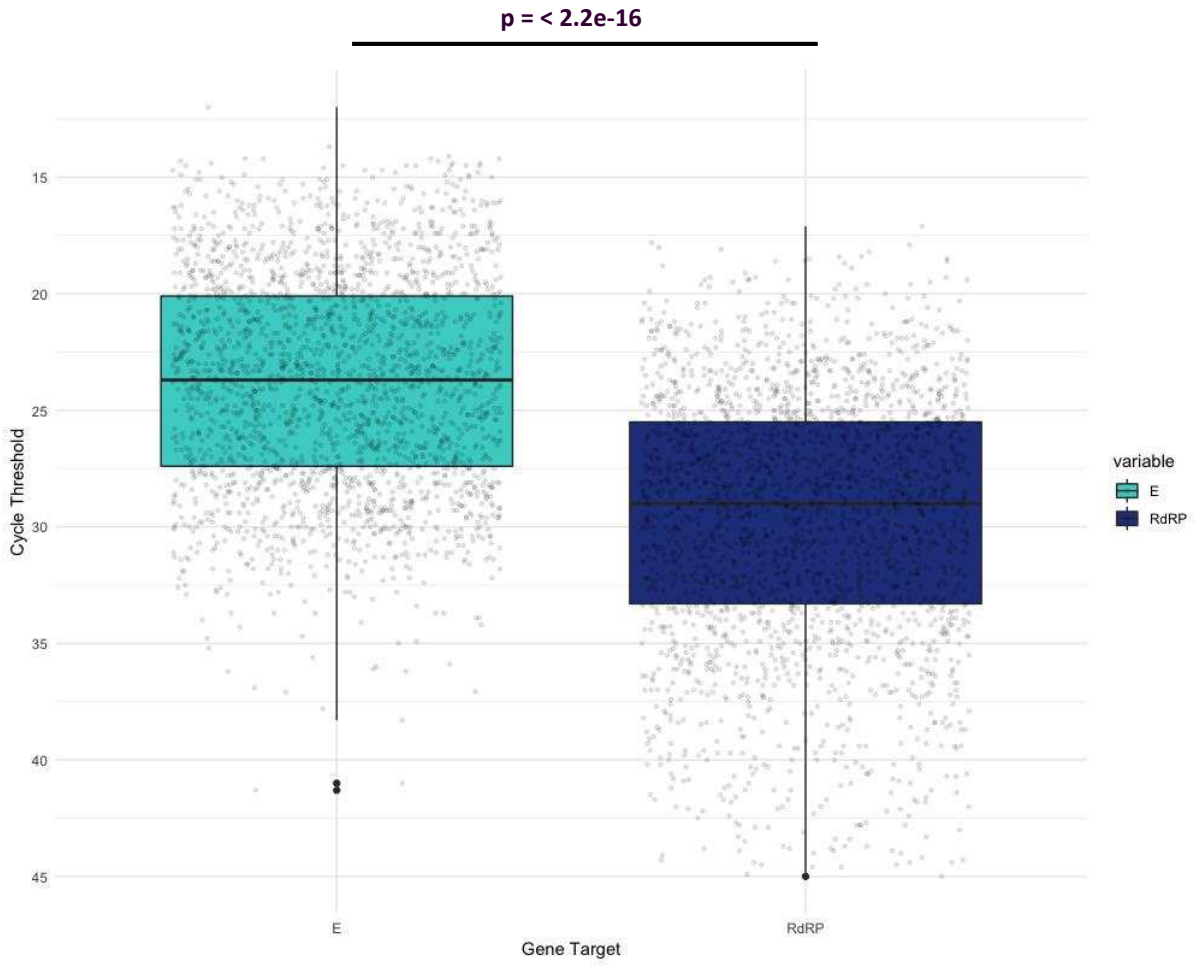

Figure S1. Boxplot of E gene CT values and RdRp CT values in samples with PCR amplification curves for both targets. The box is defined by the interquartile range and the median value is represented by the central horizontal line. CT values for the E gene target were significantly lower than the CT values for RdRP, with a mean difference of 5.8 (Paired t test, p-value < 2.2e-16, 95% CI 5.79-5.92). CT, cycle threshold; RdRp, RNA-dependent RNA-polymerase gene; E, envelope gene.

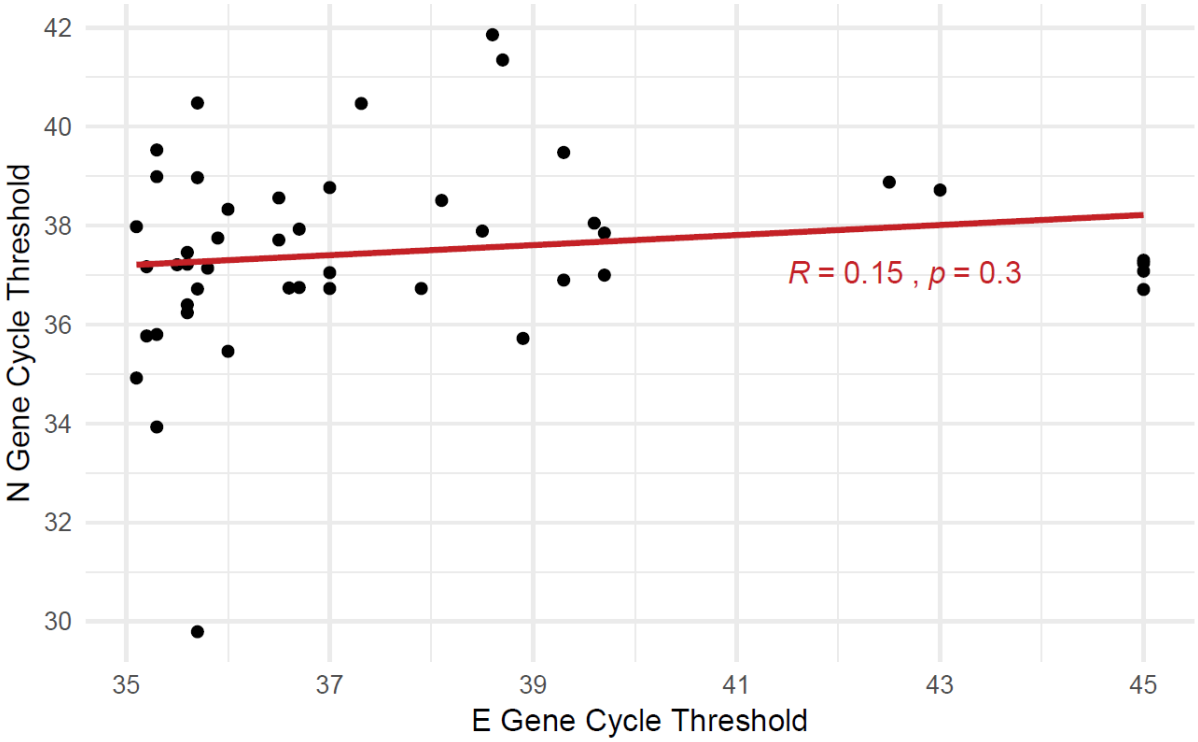

Figure S2. Correlation of E and N gene cycle thresholds. A total of 53 extracts were sent to another laboratory (Micropathology Ltd, Coventry) for confirmatory testing in an N gene assay. Forty seven samples were confirmed as harbouring SARS-CoV-2 RNA with N gene amplification, 46 of which are plotted above. No significant correlation was seen between the CT values for the E gene and the confirmatory N gene assay (Pearson correlation 0.15, p = 0.3).

Abbreviations: CT, cycle threshold; RdRp, RNA-dependent RNA-polymerase gene; E, envelope gene.

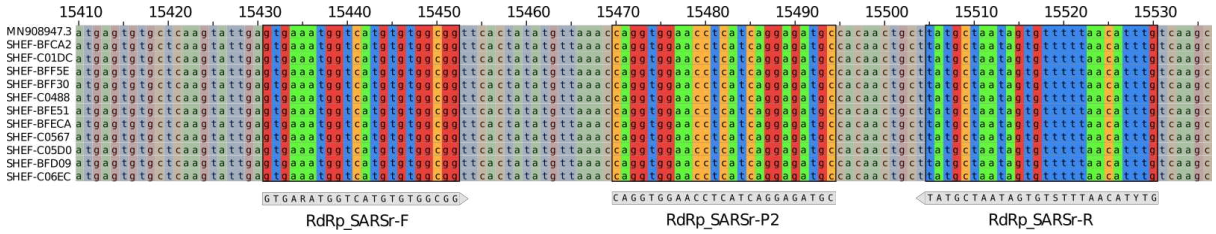

Figure S3. Multiple Sequence Alignment of SARS-CoV-2 Consensus Sequences. 11 samples reactive for E and not the RdRp gene were subjected to Oxford Nanopore sequencing using the artic network protocol[1] to determine variation that may be present in the RdRp primer or probe sites that could lead to a failure of the assay. A multiple sequence alignment was carried out for the resulting consensus sequence for these samples using MAFFT (v7.450) (1) including the Wuhan-Hu-1 (MN908947.3) reference sequence. Numbers represent position in genome. There was no sequence variation present in either primer site or the probe. Figure was produced using Jalview (2).

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