Cell-based culture of SARS-CoV-2 informs infectivity and safe de-isolation assessments during COVID-19

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Summary: Isolation of SARS-CoV-2 in cell-based culture may be used as a surrogate marker for infectivity and inform de-isolation protocols in the management of COVID-19, particularly in persons where SARS-CoV-2 RNA is persistently detected by nucleic acid testing despite clinical recovery.

ABSTRACT

Background

The detection of SARS-CoV-2 RNA by real-time polymerase chain reaction (PCR) in respiratory samples collected from persons recovered from COVID-19 does not necessarily indicate shedding of infective virions. By contrast, the isolation of SARS-CoV-2 using cell-based culture likely indicates infectivity, but there are limited data on the correlation between SARS-CoV-2 culture and PCR.

Methods

One hundred and ninety-five patients with varying severity of COVID-19 were tested (outpatients [n=178]), inpatients [n=12] and critically unwell patients admitted to the intensive care unit [ICU; n=5]). SARS-CoV-2 PCR positive samples were cultured in Vero C1008 cells and inspected daily for cytopathic effect (CPE). SARS-CoV-2-induced CPE was confirmed by PCR of culture supernatant. Where no CPE was observed, PCR was performed on day four to confirm absence of virus replication. Cycle threshold (Ct) of the day four PCR ($Ct_{culture}$) and the PCR of the original clinical sample (Ct_{sample}) were compared, and positive cultures were defined where Ct_{sample} - $Ct_{culture}$ was $\geq$3.

Findings

Of 234 samples collected, 228 (97%) were from the upper respiratory tract. SARS-CoV-2 was only successfully isolated from samples with Ct_{sample} $\leq$32, including in 28/181 (15%), 19/42 (45%) and 9/11 samples (82%) collected from outpatients, inpatients, and ICU patients, respectively. The mean duration from symptom onset to culture positivity was 4.5 days (range 0-18). SARS-CoV-2 was significantly more likely to be isolated from samples collected from inpatients (p<0·001) and ICU patients (p<0·0001) compared with outpatients respectively, and in samples with lower Ct_{sample}.

Conclusion

SARS-CoV-2 culture may be used as a surrogate marker for infectivity and inform de-isolation protocols.

Keywords: COVID-19; SARS-CoV-2; viral culture; infection control; de-isolation

Introduction

In late December 2019, a cluster of pneumonia cases of unknown etiology were reported in Hubei Province, China [1]. The causative agent, a novel betacoronavirus, now known as SARS-CoV-2, was isolated on January 7, 2020 [2] and the genetic sequence January 10, 2020 enabling the rapid development of diagnostic assays for COVID-19 (the disease caused by SARS-CoV-2) to be available within a month of the outbreak's notification to the World Health Organization (WHO) [3]. Effective control of SARS-CoV-2 transmission requires widespread testing, isolation, contact tracing of infected persons and other non-pharmaceutical interventions [4], particularly in the absence of effective SARS-CoV-2-specific antivirals and vaccines. Nucleic acid testing such as real-time reverse transcriptase polymerase chain reaction (PCR) is the predominant diagnostic method to confirm infection, however, the detection of SARS-CoV-2 RNA may not always indicate the presence or shedding of viable virus with replicative capacity and, by implication, transmissibility [5]. The diagnostic value of culture in the routine management of COVID-19 is limited by requirements of isolation to be performed under physical containment laboratory level 3 (PC3) biosafety conditions [6], technical expertise, turnaround times (TATs), and costs. However, the ability to culture SARS-CoV-2 has important roles in providing adequate positive control material for the development, validation, evaluation and quality assurance of SARS-CoV-2 diagnostic assays; supporting the development of vaccines and therapeutic agents, and enabling research into viral virulence and transmission.

Despite clinical recovery, there are a proportion of patients where SARS-CoV-2 RNA remains detectable for many days to weeks [5]. The relationship between persistent PCR positivity and virus viability is important in understanding the natural history and pathogenesis of SARS-CoV-2 infection, and whether truly persistent infection occurs. This has significant implications for the release of infected persons from quarantine, particularly for those who reside in high-risk settings such as aged care, mental health and correctional facilities, or work with at-risk population groups such as

healthcare workers (HCWs). Persistent SARS-CoV-2 RNA detection also has implications for patient isolation and bed management, the safe use of non-invasive ventilation and ongoing requirements for appropriate use of personal protective equipment for HCWs when caring for such patients.

We investigated the correlation between SARS-CoV-2 culture and PCR results from respiratory tract samples collected from persons with COVID-19 to guide the safe de-isolation of those with persistently positive PCRs.

Methods

SARS-CoV-2 PCR and cell culture results of respiratory tract samples received between March 1 and April 22, 2020 were analysed. Samples were inoculated into cell culture as part of (i) routine laboratory protocol at the start of the epidemic; (ii) if collected from patients admitted to the ICU and (iii) if requested by attending physicians (usually due to persistent PCR positivity in an individual awaiting release from quarantine) or public health physicians as part of outbreak investigations.

Sample types

Samples were collected from the upper respiratory tract (URT; nasopharyngeal swabs and combined nose and throat swabs) and lower respiratory tract (LRT; sputum, endotracheal aspirate, non-bronchoscopic bronchoalveolar lavage). URT swabs were received in 1-3 mL of viral or universal transport media (UTM), Amies transport media, or dry; stored at +4°C and tested within 12 hours of specimen receipt.

SARS-CoV-2 nucleic acid detection

RNA was extracted from 200µL of respiratory tract samples using the MagNA Pure 96 instrument's Viral NA Small Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the "Pathogen Universal Protocol" (elution volume, 100µL) according to the manufacturers' instructions. SARS-CoV-2 nucleic acid amplification and detection were performed using the LightCycler® 480 II System

(Roche Diagnostics, Rotkreuz, Switzerland). The primers and probes, mastermix composition and cycling conditions used are outlined in (Table 2, Supplementary). Each PCR assay included a negative (RNAse-free water), positive (10^{-3} dilution of a SARS-CoV-2-positive cell culture supernatant) and internal control (human beta-globin gene). Cycle threshold (Ct) of positive samples were recorded.

SARS-CoV-2 gene targets included E, RdRp, N, M, and ORF1ab for samples from ICU patients; and between one and four of E, RdRp, N and ORF1ab for all other samples. Global shortages of reagents and consumables influenced the targets tested. The total number of samples tested per target were: one (n=24), two (n=115), three (n=22), four (n=62) and five targets (n=11).

SARS-CoV-2 culture of respiratory tract samples

Samples with detectable SARS-CoV-2 RNA were inoculated into cell culture under PC3 conditions [4] on the same day or stored at +4°C for inoculation the following day. Vero C1008 (Vero 76, clone E6, Vero E6 [ECACC 85020206]) cells were seeded at 1-3 x 10^4 cells/cm^2 with Dulbecco's minimal essential medium (DMEM; LONZA, Alpharetta, GA, USA) supplemented with 9% foetal bovine serum (FBS; HyClone™, Cytiva, Sydney, Australia) in 25cm^2 cell culture flasks or Costar® 24-well clear TC-treated multiple well plates (Corning®, Corning, NY, USA). The medium was changed within 12 hours for DMEM containing 1% FBS with the addition of penicillin, streptomycin and amphotericin B deoxycholate to prevent microbial overgrowth and then inoculated with 500µL (cell culture flasks) or 200µL (24-well plates) of clinical sample. Plates were sealed with AeraSeal™ Film (Excel Scientific, Inc., Victorville, CA, USA) to minimize evaporation, spillage, and well-to-well cross-contamination. Inoculated cultures were incubated at 37^0C in 5% CO_2 for 5 days (day 0 to 4) and observed daily for CPE, 200µL of viral culture supernatant was collected on days 1, 2, 3 and 4 for the ICU cohort and on day 4 for all other samples.

Where CPE was observed (generally within 2-3 days post-inoculation), SARS-CoV-2 replication was confirmed by PCR. Terminal sampling and PCR testing of culture supernatant was performed on day 4 as CPE may not be observed despite active viral replication, as seen with SARS-CoV [7]. Day 4 was chosen for terminal sampling as this was the optimal time for reading the endpoint as determined by the 50% tissue culture infective dose ($TCID_{50}$) assay below. Additionally, with no medium change and using a concentration of amphotericin B (2.5µg/mL), cell monolayer breakdown was observed from day 5 onwards in some cultures.

SARS-CoV-2 nucleic acid detection in cell culture supernatant

SARS-CoV-2 RNA was extracted from 200µL of culture supernatant using the EZ1 Advanced XL platform and EZ1 Virus Mini Kit v2.0 (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. SARS-CoV-2 PCR was then performed as above.

Determination of $TCID_{50}$

A primary isolate, SARS-CoV-2/01/Human/NSW Australia|EPI_ISL_407893|2020-01-24 (GISAID), was titrated in serial one log dilutions (from 10^{-1} to 10^{-8}) to obtain a $TCID_{50}$ using 24-well culture plates of Vero C1008 cells. The plates were observed daily for CPE using an inverted optical microscope for five days, and wells were considered positive if any characteristic altered cell foci were seen (Figure 4, Supplementary). The end-point titres were calculated according to the Reed and Muench method [8] based on four replicates for titration, and the infectious virus titre of our SARS-CoV-2 stock was 1 x $10^{6.5}$ infectious particles per 200µL (Figure 1).

Determination of virus replication and Ct cut-off for virus viability in cell culture supernatant

To clarify the significance of the Ct of culture supernatant in the absence of CPE, a dilutional series of virus stock was run (as per $TCID_{50}$) and SARS-CoV-2 RNA detection by PCR targeting six genes (E, RdRp, M, N, ORF1ab and ORF1b) at each dilution was performed (Figure 1). This allowed the limit of detection (LoD) of viral culture $TCID_{50}$ to be compared to the LoD of the various PCR gene targets

and demonstrated that the highest Ct reflective of non-viable virus was 37·29 with the ORF1b gene target. Using the same targets, the dilution series indicated a change in 1 log dilution of virus concentration correlated with a change in Ct of approximately ±3. This was also confirmed when testing synthetic plasmids using real-time quantitative PCR assays demonstrated that a 1 log difference in viral load corresponded to a change in Ct of 3·44 ± 0.16. Furthermore, a Ct of 34 and 37 correlated with viral loads of approximately 100 copies/mL and 10 copies/mL, respectively, a difference of 1 log (data not shown).

Positive viral cultures were defined as (i) CPE visualized and SARS-CoV-2 RNA detected from culture supernatant, or (ii) no CPE visualized but a decrease between the Ct of the original sample (Ct_{sample}) and day 4's terminal culture supernatant ($Ct_{culture}$) of ≥3 (equivalent to a 1 log increase in virus quantity), i.e. Ct_{sample} - $Ct_{culture}$ ≥3 = culture positive. Conversely, negative viral cultures were defined as (i) CPE not visualised, and no SARS-CoV-2 RNA detected from the culture supernatant, or (ii) no CPE visualized in culture and Ct_{sample} - $Ct_{culture}$ <3. Where SARS-CoV-2 RNA was detected in terminal sampling, we surmise that Ct_{sample} - $Ct_{culture}$ <3 was due to residual inoculated clinical sample and not replicating virus. This is supported by the initial rise in $Ct_{culture}$ on day 1 compared to Ct_{sample} on day 0 (Figure 2). In addition, no viral replication was identified with blind passaging of the first 20 cultures without CPE and Ct_{sample} - $Ct_{culture}$ <3, indicating the absence of viable virus. By contrast, SARS-CoV-2 replication was demonstrated by CPE and detectable RNA in subsequent passaging of samples without initial CPE but where Ct_{sample} - $Ct_{culture}$ was ≥3.

Statistical analyses

Welch's t-test, two-tailed hypothesis with 95% confidence intervals (95% CI) was used to compare the mean Ct of URT and LRT samples (Figure 6, Supplementary) of culture positive (with or without CPE) and culture negative samples (Figure 5, Supplementary), and to determine the mean Ct of the N gene in each week of culture (Figure 3). Fisher's exact test was used to determine significance in

culture positivity rates between different cohorts with 95% CI, (Table 1) and the difference between the culture positive samples per week (Figure 3). Mann-Whitney U test, two-tailed hypothesis (95% CI) was used to compare the date of symptom onset in culture positive and negative patients (Figure 7, Supplementary) and to compare the median Ct of the N gene in each week (Figure 3). *p*-values <0·05 were considered statistically significant.

Results

Of 234 samples (228 [97%] from the URT) collected from 195 persons with COVID-19, 11 (5%), 42 (18%) and 181 (77%) were from 178 outpatients, 12 inpatients not requiring ICU and five ICU patients, respectively (Table 1). SARS-CoV-2 was isolated from 56 (24%) samples, with CPE observed and SARS-CoV-2 RNA detected in 46 (20%) samples. No CPE was observed in 10 (4%) samples but fulfilled the Ct_{sample} - $Ct_{culture}$ ≥3 criteria to define positivity. Whole genome sequencing failed to identify specific SARS-CoV-2 lineages A (A, A.1-A.5) or B (B, B.1-B.9 and B.11) where CPE was absent [9]; furthermore the samples were not collected from any epidemiologically linked clusters (data not shown). Mean Ct of the N gene were significantly lower in CPE positive cultures when compared to both CPE negative but PCR positive cultures (25·01 vs 27·75, *p* <0·001) and negative cultures (25·01 vs 36·87, *p* <0·001) (Figure 5, Supplementary).

The highest Ct for the N gene target in any clinical sample where SARS-COV-2 was successfully cultured was 32. Based on this result and the Ct cut-off value of 37 determined by PCR of $TCID_{50}$ dilutions, and incorporating a 1 log margin of error, we were confident that any clinical sample with a Ct of ≥37 was not indicative of viable virus (Figure 1). SARS-CoV-2 was significantly more likely to be isolated in inpatients compared to outpatients (45% vs 15%, *p*<0·001), and ICU compared with other inpatients (82% vs 45%, *p*=0·04) or outpatients (82% vs 15%, *p*<0·001) (Table 1). Serial sampling of ICU patients and testing multiple gene targets demonstrated the variability in Ct when SARS-CoV-2 RNA was detected, with the E gene generally giving the lowest Ct and N gene the

highest (Figure 2 and Figure 6, Supplementary) in this cohort. There was consistent correlation over time between targets and concordance with the selected definition for culture positivity (Figure 2).

Of note, SARS-CoV-2 was isolated 17 and 18 days post-symptom onset in a 36 year old female patient with hypothyroidism; no other samples were culture-positive beyond 10 days after symptom onset (Figure 3). The mean Ct across all cultures increased as time from symptom onset increased (Figure 3). Using the N gene as a representative target, cultures were significantly more likely to be positive from samples collected within the first week after symptom onset compared to the second week (80% vs 45%, p=0·002), and between the second and third week (45% vs 4%, p<0·001) (Figure 3).

Ct values of the most frequently used target (N gene) ranged from 17·56 to 40, mean=34·16, IQR (29·21 –39·37). Mean Ct of the N gene was significantly lower in LRT compared with URT samples (26·76 vs 34·41, p<0·001) (Figure 6, Supplementary). No significant difference was observed for the other targets, potentially due to a type II error.

In clinical samples where SARS-CoV-2 was isolated, the mean duration between symptom onset and sample collection was 4·5 days (median 3·5, range 0-18). By contrast, the mean duration between symptom onset and sample collection in culture negative samples was 20 days (median 18·4, range 3-29; p<0·001) (Figure 7, Supplementary).

Discussion

Detection of SARS-CoV-2 RNA by PCR has been the primary diagnostic method to confirm COVID-19. As an indicator of infectivity, the absence of detectable SARS-CoV-2 RNA in respiratory tract samples is utilized in many jurisdictions as a requirement for release of persons with COVID-19 from quarantine [10]. In Australia, infected persons from high-risk settings (including but not limited to HCWs) are required to have two consecutive negative SARS-CoV-2 tests at least 24 hours apart

before being allowed to return to such settings [11]. Whilst there are reports of persistent or prolonged PCR positive results in recovered persons [12,13], few have provided robust data to support that they are no longer infectious. Furthermore, there is substantial variability in the duration of PCR positivity (up to 28 days after symptom onset) [12], resulting in individuals (including HCWs) being isolated for prolonged periods given the potential risks of virus transmission. As the detection of SARS-CoV-2 RNA in respiratory samples does not necessarily indicate viable infectious virions, we examined the correlation between the Ct of PCR positive samples and viral culture, a surrogate marker for infectivity. The Australian National Guidelines for Public Health Units also recommends that viral culture results be considered when deciding on the release of persons from quarantine following COVID-19 [11].

We successfully isolated SARS-CoV-2 from upper and lower respiratory tract samples collected from persons with acute COVID-19 and correlated the yield of cell culture with PCR Ct values. Similar to other reports [14,15] we demonstrate that the threshold Ct where culture will be successful is <32 for the N gene target. However, this Ct is specific for our laboratory and we would recommend that diagnostic laboratories correlate their Ct values with culture results due to the variable analytical performance between different SARS-CoV-2 PCR assays [16,17].

The cell culture method described herein relies not only on detection of CPE but incorporates PCR testing into the workflow. We demonstrate that virus with replicative capacity can be isolated and detected despite the absence of CPE by correlating the Ct of cell culture supernatant with the Ct of the original clinical samples where SARS-CoV-2 was detected. This effect was not due to any SARS-CoV-2 genotypes, however further genetic analysis may be warranted. This provides a more objective and sensitive measure of virus isolation as the detection of CPE can be subtle or absent due to low levels of virus that result in a delayed rise in virus load beyond day four in the cell lines used. This may assist in improving workflows in laboratories processing large numbers of cell

cultures or those with reduced capacity to detect CPE. A Ct_{sample} cut-off >32 in our laboratory correlates with non-viable virus; and in the absence of CPE, a Ct_{sample} - $Ct_{culture}$ value <3 also indicates an absence of viral replication, suggesting these individuals could be safely released from isolation.

We have demonstrated that culture yield is improved when samples are collected as close to the date of symptom onset as possible, and that virus isolation is more likely in patients with more severe illness requiring admission to hospital or the ICU compared to outpatients. A previous study determined that virus may be isolated up to six days prior to symptom onset [18], however our study did not include such a cohort. Given the TATs , culture results of samples collected from persons during the pre-symptomatic stage would not be available in a meaningful timeframe to guide clinical management but would provide laboratory evidence of pre-symptomatic or asymptomatic SARS-CoV-2 transmission. We showed culture positivity out to 18 days post-symptom onset in one patient, which is ten days longer than previous findings from Singapore [15]. As paired URT and LRT samples were not collected concurrently in this study, we were not able to ascertain the relationship between the two in the context of de-isolation assessments. In the majority of persons that have clinically recovered from COVID-19, LRT samples may not be available, particularly in the absence of sputum production.

We did not assess whether SARS-CoV-2 replication in the absence of CPE occurs in cell lines other than Vero C1008 cells, However, this has been described in MEK and COS cell lines during the isolation of SARS-CoV [7]. The culture-positive samples demonstrating no CPE but detectable virus replication by PCR were predominantly seen in the ICU cohort with approximately half collected from the LRT. Given the small sample size we were unable to determine whether other factors (e.g. concurrent infections, antimicrobial use) may have affected detection of CPE in these samples. Due to the limited volume of clinical sample received, culture was not set up in duplicate however quality

control to ensure the sensitivity of the cells was undertaken with a stock of titred virus to ensure a consistent LoD was maintained.

The performance of our inhouse SARS-CoV-2 assay was comparable to the Roche cobas® SARS-CoV-2 (Roche Diagnostics, Rotkreuz, Switzerland), (data not shown). Due to reagent shortages, not all samples or culture supernatants were able to be tested with all five SARS-CoV-2 gene targets (E, RdRp, M, N and ORF1ab). Nevertheless, we hypothesize that the findings using the N target would be similar for the other targets as a similar pattern was observed where testing of multiple targets were performed (Figure 2). Nucleic acid extraction from the primary sample and culture supernatant was performed on the MagNA Pure 96 and EZ1 Advanced XL instruments, respectively. Previous evaluations of automated nucleic acid extraction instruments for non-SARS-CoV-2 respiratory viruses showed superiority of the EZ1 Advanced XL compared to the MagNA Pure 96 [19], but there are limited data on the performance of extraction instruments for SARS-CoV-2. We were unable to ascertain if different swab types or UTM affected either PCR or culture results. This study also did not investigate why certain individuals shed SARS-CoV-2 RNA for longer periods compared to others, nor were we able to determine whether there were any factors such as immunosuppression that may have explained prolonged viral shedding, and further research is recommended to address these questions.

In summary, viral culture may assist clinicians to release from isolation individuals who have recovered clinically but remain persistently or intermittently positive SARS-CoV-2 by PCR. Whilst there is limited availability of cell culture in most clinical diagnostic laboratories, this capacity adds important insights not provided by molecular methods and should be maintained in reference laboratories to guide clinical management and de-isolation.

NOTES

Author Contributions

Literature search: Basile K, Maddocks S and Kok J

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Acknowledgments

We thank Jimmy Ng[1] and Basel Suliman[1] for their assistance with inactivation of cell culture supernatant and nucleic acid extraction. Clinical specimens were received and processed at the NSW Health Pathology-Institute of Clinical Pathology and Medical Research (NSWHP-ICPMR) laboratory for standard of care diagnostic purposes and deemed not research. A non-research determination for this project was granted by the Health Protection NSW as it was a designated communicable disease control activity.

Funding statement: This study was supported by the Prevention Research Support Program funded by the NSW Ministry of Health and the National Health and Medical Research Council (APPRISE 1116530). The funders had no role in the study design, data collection, data analysis and interpretation, or writing of the report. The corresponding author had full access to study data and final responsibility for the decision to submit for publication.

Conflict of interest

None declared

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Table 1: Patient demographics and sample characteristics.

	ICU	Inpatients (non-ICU)	Outpatients (Non-admitted)	Total
Number of patients	5	12	178	195
Average age in years (range)	58 (36-76)	43 (22-78)	40 (18-71)	40 (8-78)
Male gender	3 (60%)	8 (70%)	136 (77%)	147 (75.4%)
Number of samples	11	42	181	234
URT	6	40	181	227 (97%)
LRT	5	2	0	7 (3%)
Average days from symptom onset to sample collection (range)	7 (0-20)	7 (1-16)	12 (0-29) **	11 (0-29)
Culture Positive with CPE	6 (55%)	17 (40%)	23 (12%)	46 (20%)
Culture Positive without CPE	3 (27%)	2 (5%)	5 (3%)	10 (4%)
TOTAL Culture Positive	9 (82%)	19 (45%)	28 (15%)	56 (24%)
TOTAL Culture Negative	2 (18%)	23 (55%)	153 (85%)	178 (76%)

*ICU: intensive care unit; URT: upper respiratory tract; LRT: lower respiratory tract; CPE: cytopathic effects

** Time of symptom onset was unavailable for 56 samples collected from outpatients as part of contact investigations who were either asymptomatic or had no symptom onset date recorded.

FIGURE LEGENDS

Figure 1: PCR Ct cut-off value for non-viability of clinical samples and for determination of culture positive versus negative in the absence of CPE. A. Culture positivity rate by clinical sample PCR Ct value: No clinical samples with PCR Ct values >32 resulted in positive viral cultures. Safety margin of 1 log viral load less than this and concordant with B. was 37. **B. Culture supernatant PCR Ct values by gene target for $TCID_{50}$ dilutions:** indicates differential sensitivity of PCR depending on target gene. Red line at $10^{-6.5}$ indicates limit of detection by $TCID_{50}$. Ct for ORF1b target of 37.29 (bold) chosen as highest Ct representative of non-viable virus.

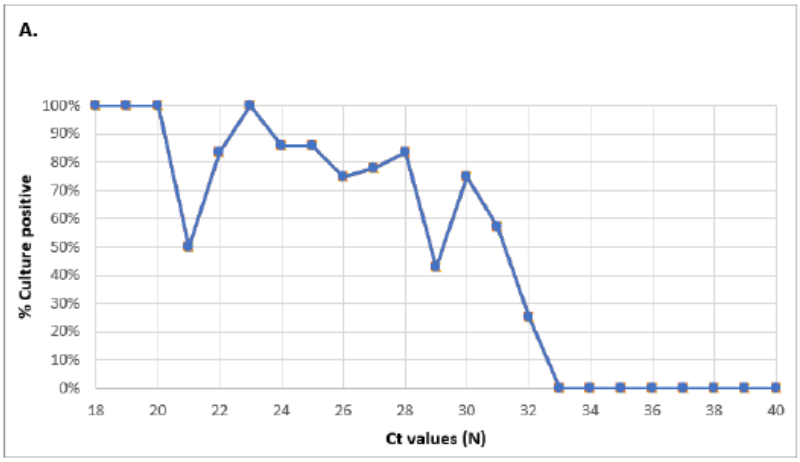
Figure 2: Representative samples from ICU cohort demonstrating target-dependent change in Ct values over four days of culture in **A**. A positive culture *with* CPE, **B.** A positive culture *without* CPE but Ct_{sample} - $Ct_{culture}$ ≥3, and **C.** a negative culture (no CPE, PCR positive but Ct_{sample} - $Ct_{culture}$ <3). Target genes: E, RdRp, M, N and ORF1ab.

Figure 3: Comparison of Culture Positive and Negative samples and Days since symptom onset with the clinical sample N gene Ct value.

** Note: This figure excludes 56 samples where date of symptom onset was unknown (patient asymptomatic, or the date of symptom onset not recorded).

Figure 1A - B

A.

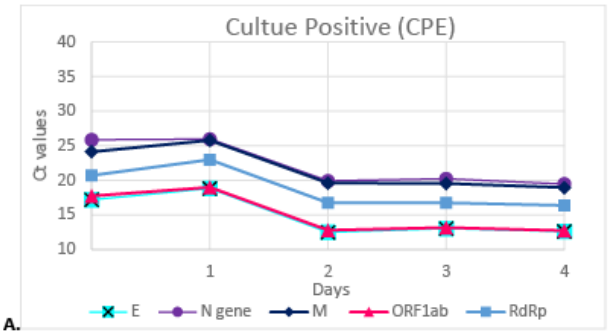


B.

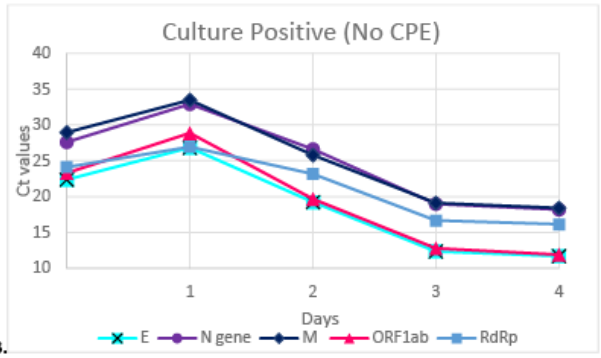
TCID50	WHO E GENE	WMD M GENE	WMD N GENE	RDRP	CHINA CDC ORF1AB	WMD ORF1B
NEAT	12.45	18.6	18.14	16.22	12.09	14.54
1.00E-01	15.81	21.87	22.35	19.97	15.3	18.22
1.00E-02	18.31	25.12	25.52	23.12	18.63	21.56
1.00E-03	20.13	28.24	28.86	25.8	22.2	24.81
1.00E-04	21.58	31.57	32.21	27.62	25.49	28.19
1.00E-05	NEG	34.65	34.7	28.04	28.64	31.54
1.00E-06	NEG	35.59	36.71	NEG	32.19	34.63
1.00E-07	NEG	36.46	36.74	NEG	34.88	**37.29**
1.00E-08	NEG	NEG	NEG	NEG	NEG	NEG

Figure 2A – B – C

A.



B.



C.

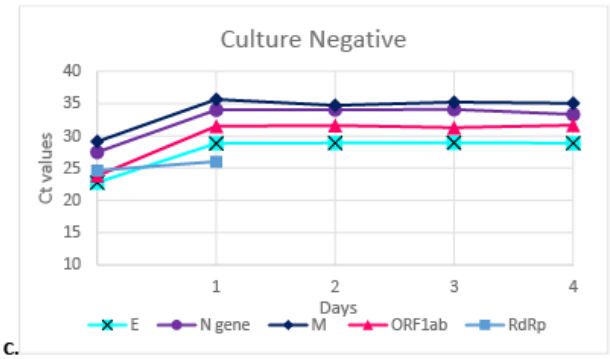
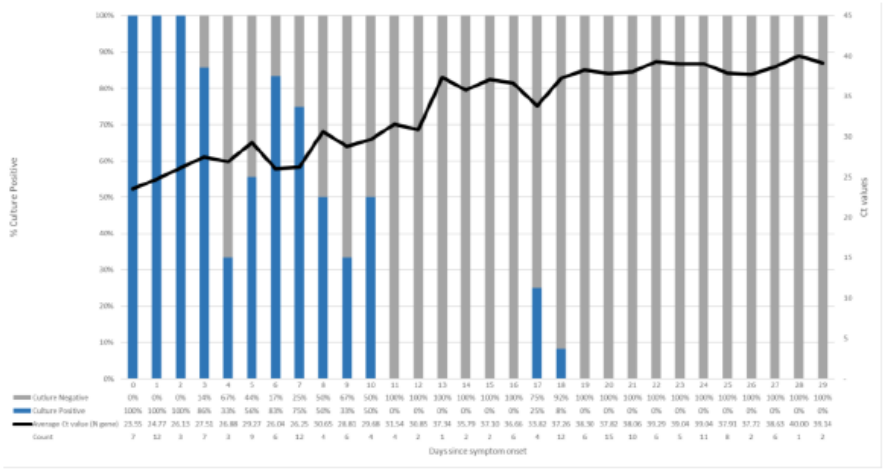


Figure 3


% Culture Positive
Ct values
Days since symptom onset
Culture Negative
Culture Positive
Average Ct value (N gene)
Count