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VIROLOGY

Clinical associations of SARS-CoV-2 viral load using the first WHO International Standard for SARS-CoV-2 RNA

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Summary

SARS-CoV-2 viral load declines from the time of symptom onset; in some studies viral load is higher or persists longer in more severe COVID-19 infection, and viral load correlates with culture positivity. This was a retrospective cohort study of inpatients and outpatients during the first wave of COVID-19 infection in Western Australia, March to May 2020, of the relationship of SARS-CoV-2 viral load (using the First WHO International Standard for SARS-CoV-2 RNA) from symptom onset, by clinical subgroups determined from the public health database and hospital records, using regression analysis. We studied 320 samples from 201 COVID-19 cases: 181 mild, seven severe, 11 critical, and four cases who died (two were also critical cases). At symptom onset the mean viral load was 4.34 $\log_{10}$ IU/mL (3.92–4.77 $\log_{10}$ IU/mL 95% CI, cobas SARS-CoV-2 assay ORF1a Ct 28.9 cycles). The mean viral load change was -0.09 $\log_{10}$ IU/mL/day (-0.12 to -0.06 95% CI). R^2 was 0.08 and residual standard deviation 2.68 $\log_{10}$ IU/mL. Viral load at symptom onset was higher for those reporting fever compared to those not reporting fever. Viral load kinetics were not different for gender, age, shortness of breath, or those requiring oxygen. Mean viral load at usual release from isolation at 14 days was 2.5 $\log_{10}$ IU/mL or day 20 was 1.8 $\log_{10}$ IU/mL. Variability in respiratory sample SARS-CoV-2 viral load kinetics suggests viral loads will only have a role supporting clinical decision making, and an uncertain role for prognostication.

Key words: SARS-CoV-2; COVID-19; viral load; WHO international standard; Ct value.

INTRODUCTION

Globally SARS-CoV-2 continues to cause morbidity, death and overwhelm healthcare systems. There are many reports describing quantitative SARS-CoV-2 changes over time, associations with clinical outcomes, associations with various specimen types, correlations with infectivity, and correlations with culture positivity.[1–5] Most of these reports have studied cycle threshold (Ct) values which are semi-quantitative results and do not always accurately correlate with viral load,[6] and no studies to date have used an international standard to determine quantitative RNA in respiratory specimens. In 2021, the First WHO International Standard for SARS-CoV-2 RNA (First WHO IS SARS-COV-2 RNA) was released.[7] While time from admission to sample collection is sometimes reported, many studies have not described the time from symptom onset to specimen which is known to be strongly associated with viral load.[4] Some studies have pooled results from lower and upper respiratory tract specimens,[2,5] when viral load is higher in the lower respiratory tract.[4]

Western Australia had a single small wave of COVID-19 infection between March and May 2020 and has had sporadic cases thereafter. Taking into account the time from symptom onset, using viral loads determined retrospectively against the First WHO IS SARS-CoV-2 RNA, we examined for associations with demographic factors, symptoms and the severity of illness, and describe viral loads at release from isolation.

MATERIALS AND METHODS

Demographic and clinical data

The PathWest Laboratory Medicine Molecular Microbiology Department at Fiona Stanley Hospital is one of two public laboratories in Western Australia performing SARS-CoV-2 molecular testing. All routine testing for SARS-CoV-2 was performed in our laboratory with the cobas SARS-CoV-2 assay (cobas; Roche, Switzerland) on the cobas 6800 instrument according to the manufacturer's instructions. Positive COVID-19 cases were identified as those with positive (ORF1a positive with/without E-gene positive) or presumptive positive (E-gene only positive) cobas SARS-CoV-2 results in our laboratory. For hospitalised patients, the clinical records were examined for supplemental oxygen use, invasive ventilation and death. For all cases including non-hospitalised cases, the public health database was examined for the date of symptom onset and specific symptoms of the cases which had been captured by a standardised case report form (fields of arthralgia, chills/rigors, conjunctivitis, cough, diarrhoea, fatigue, fever, malaise, nausea, rhinorrhoea, shortness of breath, sore throat, vomiting). There were no asymptomatic cases. We chose to examine fever and shortness of breath as possible manifestations of more severe illness.

Severity was based on WHO categories.[8] Respiratory rate and oxygen saturations were not always reported, so supplemental oxygen was used to define severe COVID-19 disease, and invasive ventilation defined critical COVID-19 disease. All cases admitted to the intensive care unit had invasive ventilation. Non-invasive ventilation was not used for any patients. In all cases of death no other cause was evident apart from COVID-19, and all deaths were within 30 days of COVID-19 diagnosis. Mild cases were those not requiring oxygen, not requiring invasive ventilation, and not dying from COVID-19.

Conditions for release from isolation were taken from the most recent Australian COVID-19 series of national guidelines, where COVID-19 cases may be released from isolation 14 days after symptom onset in those with

resolution of fever and respiratory symptoms, and at 20 days after symptom onset in those without complete resolution of fever and acute respiratory symptoms.[9] As we could not determine when fever or respiratory symptoms resolved in our cases, we reported viral load data at day 14 and 20 of the whole cohort.

At the time of the cases in this study, PCR testing for release from isolation was performed at least 7 days after symptom onset in the group of patients who planned to visit high risk settings such as aged care facilities, healthcare facilities, childcare centres and correctional facilities.[10]

The study was approved by the Governance, Evidence, Knowledge, Outcomes system of the Western Australia Department of Health, GEKO activity 35397. The study was exempt from informed participant consent as it was a retrospective observational study with negligible risk to participants, de-identification of data, presentation as composite rather than individual data, and due to the impracticality of contacting participants.

Patient samples

We included all positive and presumptive positive samples run in our laboratory from March to May 2020 inclusive. Where positive/presumptive positive cases had a follow-up negative sample, the first negative sample ('not detected' by the cobas assay) was included with an assigned viral load of zero international units/mL (IU/mL). A combined deep nasal/throat swab from each patient was inoculated into 3 mL of either Copan UTM-RT media (Brescia, Italy), CITOSWAB (Citotest Scientific Jiangsu, People's Republic of China) or Virus Transport Media (VTM) prepared by PathWest Media.[11] All positive samples were stored as aliquots at $-80°C$. Samples were thermally treated for $75°C$ for 15 min in a Dri-bath, then tested with cobas SARS-CoV-2 assay within 2 hours.[12] The Ct values were recorded for ORF1a and E-gene.

Quantitative standards, external control and analysis

Quantitative standards were prepared from the First WHO International Standard for SARS-CoV-2 (NISBSC code 20/146), supplied as 7.7 $\log_{10}$ IU/mL (National Institute for Biological Standards and Control, UK). The standard was reconstituted with 0.5 mL of phosphate buffered saline following the manufacturer's instructions. Once reconstituted, the standard was 10-fold serially diluted in a naso-oropharyngeal matrix. This matrix consisted of pooled naso-oropharyngeal samples from samples previously tested as SARS-CoV-2 negative using cobas. Seven standards were prepared over the range of 0.7–6.7 $\log_{10}$ IU/mL. Each standard was tested in triplicate with cobas. The mean Ct value at each concentration was used to calculate ORF1a and E-gene standard curves and regression. The regression formulas were used to calculate the ORF1a and E-gene IU/mL for all positive samples retrospectively. Given the strong correlation of IU/mL for both targets (Fig. 1), for simplicity the results from a single target (ORF1a) were used as the reported viral load or Ct value unless otherwise stated. An external control (EQC) was also performed routinely to monitor reproducibility (Optitrol NAT SARS-CoV-2; DiaMex, Germany).[13]

Statistical analysis

Viral loads in $\log_{10}$ IU/mL were plotted against time from symptom onset. Comparison of regression lines (viral load at symptom onset and change of viral load over time) was used to compare subgroups of gender, age, shortness of breath, fever, supplemental oxygen requirement, invasive ventilation, or death. Regression analysis was also used to examine the relationship of Ct values for ORF1a or E-gene targets to viral load dilutions of the First WHO IS SARS-CoV-2 RNA. For the complete data set, in addition to simple linear regression a moving average trend line with window width of five samples was performed, with locally weighted scatterplot smoothing (LOESS) at 80% smoothing span. Comparison of medians was performed with the Mann–Whitney test for non-parametric data. A significance p value of <0.05 was used. Analysis was performed in MedCalc version 15.4 (MedCalc Software, Belgium).

RESULTS

Demographic and clinical results

There were 201 COVID-19 cases between 15 March and 11 May 2020 diagnosed by our laboratory. Median age was 53 years [interquartile range (IQR) 33–68 years, range 8–85 years] and 50.2% were male. There were 320 samples (249 positive, five presumptive positive, and 66 negative samples), median one positive sample per patient (164 cases with one sample, 29 cases with two positive samples, three cases with three positive samples, one case with four positive samples, four cases with five positive samples), and 66 (32.5%) cases had a follow up negative test. There were 162 cases not admitted to hospital (235 samples), 39 cases admitted to hospital (85 samples); 181 were mild cases (272 samples), seven were severe cases (16 samples), 11 were critical cases (28 samples), and four patients died (two were also critical
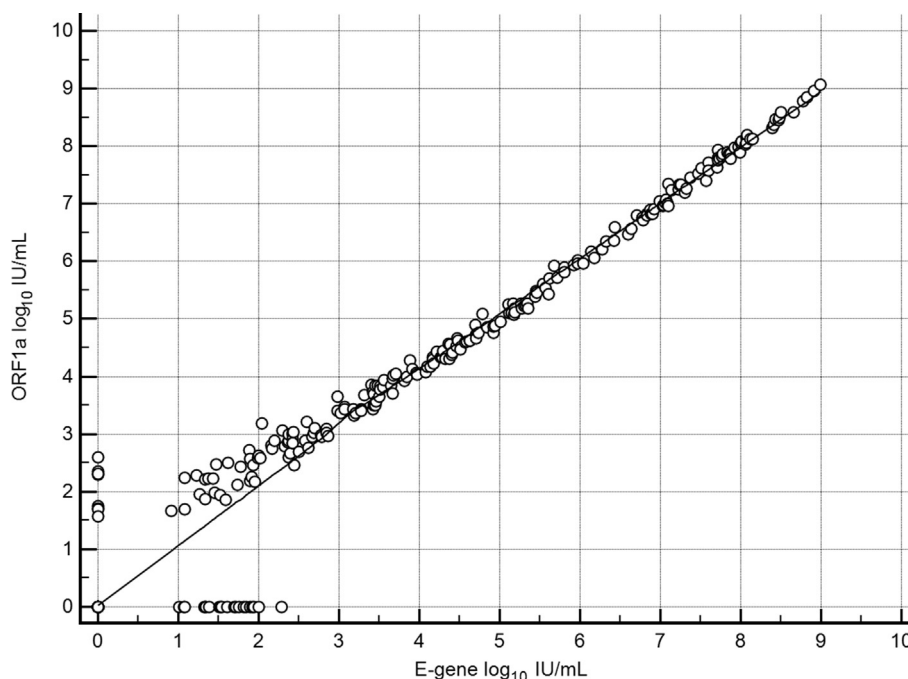

Fig. 1 Correlation of viral load in $\log_{10}$ IU/mL in each sample for the ORF1a and E-gene targets of the Cobas SARS-CoV-2 PCR assay.

cases, one was a severe case, one was a mild case; total six samples). The mild case who died was an elderly patient with comorbidities admitted with respiratory complaints. The patient tested SARS-CoV-2 PCR positive on combined nose/throat swab, also had myocardial infarction, did not receive oxygen and was palliated.

Viral load associations with demographic factors

At symptom onset the mean viral load was 4.34 $\log_{10}$ IU/mL [3.92 to 4.77 $\log_{10}$ IU/mL 95% confidence interval (CI), Ct 29.0]. The mean viral load change was -0.09 $\log_{10}$ IU/mL/day (-0.12 to -0.06 95% CI). The highest viral load was 9.1 $\log_{10}$ IU/mL (Ct 15.7) at day 1.5 and lowest viral load was 1.57 $\log_{10}$ IU/mL (Ct 36.8) at day 36. The earliest and latest positive results were at day -4 (1.33 $\log_{10}$ IU/mL for E-gene, negative for ORF1a so not seen in Fig. 2) and day 49 (2.82 $\log_{10}$ IU/mL), respectively. R^2 was 0.08 and residual standard deviation 2.68 $\log_{10}$ IU/mL. See Fig. 2 for simple linear regression with 95% CIs and Fig. 3 for moving average trend line with LOESS.

Subgroup regression analysis suggested those patients reporting fever had a higher viral load at symptom onset (Fig. 4). The finding of higher viral load at symptom onset in those who died and lower viral load of those who required invasive ventilation should be interpreted cautiously due to the small numbers in these subgroups. Additionally, samples were taken later from symptom onset in those who required invasive ventilation compared to those who did not require invasive ventilation (median 14.5 days, IQR 8.4–20 days, compared to median 6.4 days, IQR 3.3–10.6 days, $p<0.001$), and for those who required oxygen compared to those who did not require oxygen, nor ventilation, and did not die (median 12.2 days, IQR 8.5–33 days, compared to median 6 days, IQR 2.6–9.8 days, $p<0.001$). There was no significant difference when samples were taken from symptom onset for those who died compared to those who did not die (median 9.5 days, IQR 0.4–14.4 days, compared to 6.5 days, IQR 3.4–11.6 days, $p=0.97$). Subgroup regression analysis did not demonstrate significant differences in viral load at symptom onset or change in viral load over time for gender, age >60 vs ≤60 years, age >70 vs ≤70 years (data not shown), age >80 vs ≤80 years (data not shown), shortness of breath, or oxygen requirement (Table 1).

Serial viral load results for individual cases

Figure 5 demonstrates significant heterogeneity in viral load changes with time for those eight cases with more than two positive PCR results during the course of their infection.

Viral load at release from isolation

At 14 days from symptom onset, viral load by moving average trend with LOESS was 2.5 $\log_{10}$ IU/mL, and +/− 0.3 $\log_{10}$ IU/mL 95% CI of the regression line. At 20 days from symptom onset, viral load by moving average trend with LOESS was 1.8 $\log_{10}$ IU/mL, and +/− 0.5 $\log_{10}$ IU/mL 95% CI of the regression line.

First WHO IS SARS-CoV-2 RNA standard curves

Detection of ORF1a was log-linear over the range of 2.70–6.70 $\log_{10}$ IU/mL with an R^2 value of 0.99 (regression equation $y = -0.3557x + 14.649$, where y=viral load in $\log_{10}$ IU/mL and x=Ct value). Detection of E-gene was log-linear over the range of 1.70–6.70 $\log_{10}$ IU/mL with an R^2 value of 0.99 (regression equation $y = -0.3451x + 14.502$) (Supplementary Data, Appendix A). The EQC inter-assay reproducibility analysed retrospectively over 20 consecutive runs for ORF1a was 4.09 $\log_{10} \pm 0.21$ IU/mL and E-gene was 3.93 $\log_{10} \pm 0.21$ IU/mL. There was a strong correlation of IU/mL for both targets, $R^2=0.98$ (Fig. 1).
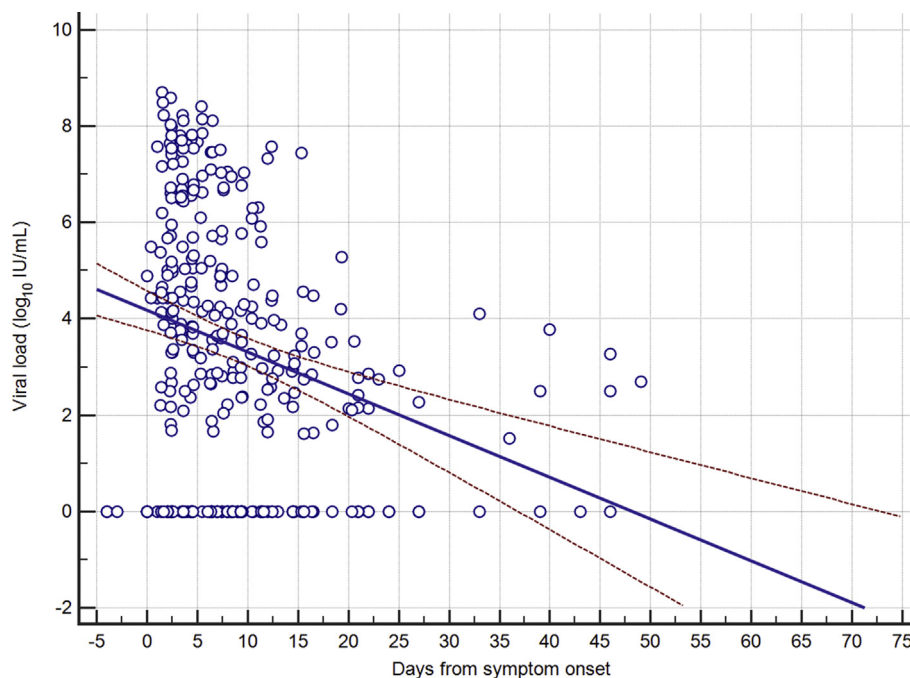

Fig. 2 Viral load in $\log_{10}$ IU/mL against time from symptom onset, with 95% CI of the regression line intervals shown by dashed lines.
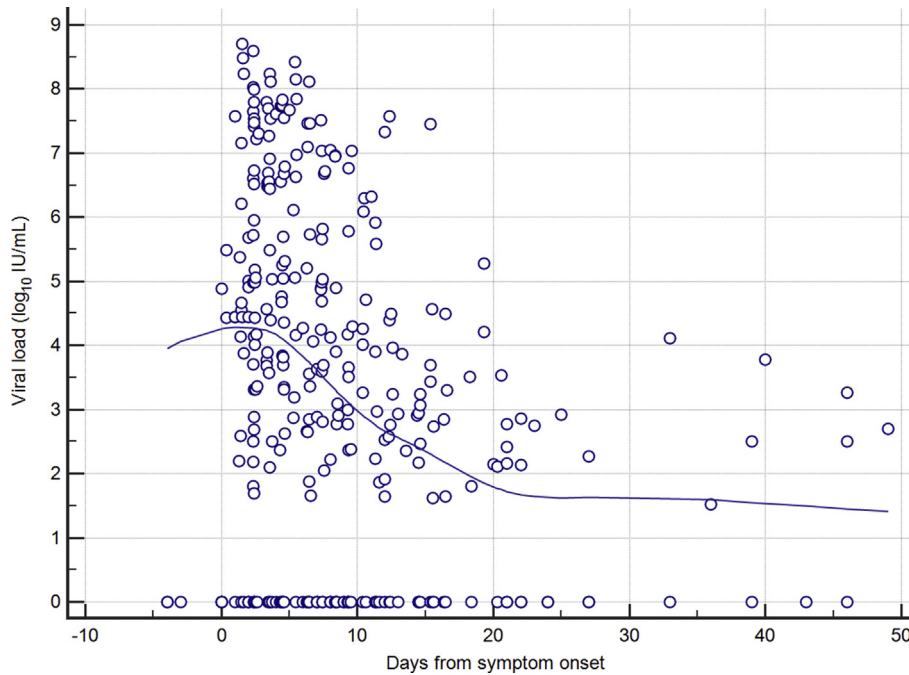
Fig. 3 Viral load in $\log_{10}$ IU/mL against time from symptom onset with a moving average trend line and locally weighted scatterplot smoothing (LOESS).
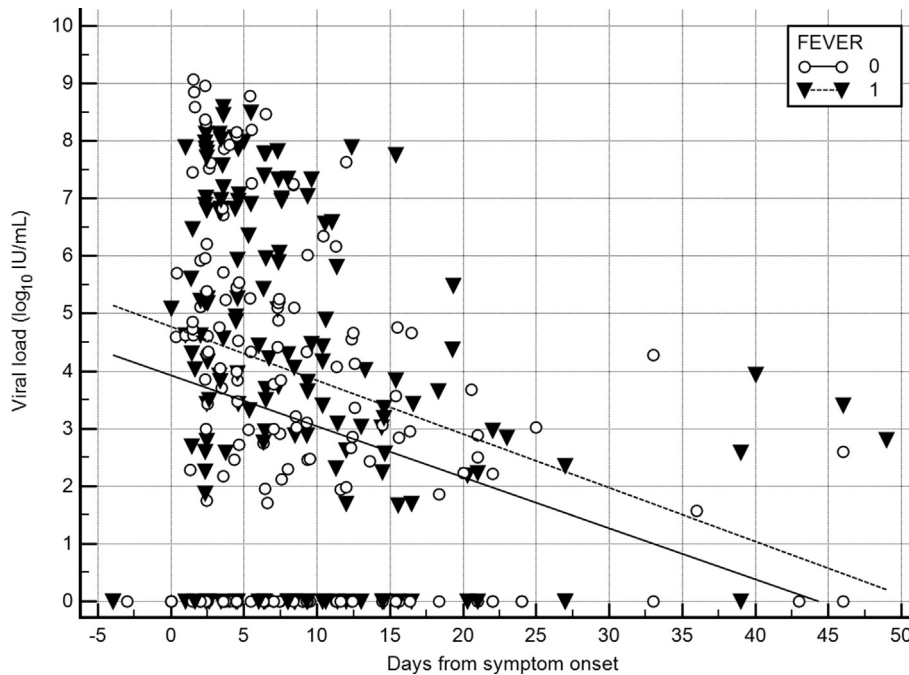

Fig. 4 Viral load in $\log_{10}$ IU/mL against time from symptom onset for patient reporting fever compared to those not reporting fever. Fever=1, no fever=0.

DISCUSSION

We examined SARS-CoV-2 viral load changes according to the time from symptom onset for subgroups of demographic factors, symptoms, and severity of illness. We showed a higher viral load at symptom onset in those who reported fever, a finding of uncertain significance which should be confirmed with further data. Our findings of higher viral load at symptom onset in those who died and a lower viral load at symptom onset in those who had invasive ventilation are to be taken with caution due to small numbers, and the potential bias introduced by the later sampling in those requiring invasive ventilation.

We have controlled a number of factors which are lacking in other studies such as including only one sample type (combined deep nasal/throat swab), controlling for the time of sample collection from symptom onset, using a single PCR assay, using viral load as a more accurate quantitative assessment than Ct values, for the first time using the First WHO IS SARS-CoV-2 RNA, and providing statistical assessment by regression analysis. It is difficult to directly compare with other studies due to the variable methods they have employed, including pooling of lower and upper respiratory tract samples,[2,5] and using Ct values rather than a formal

Table 1   Extrapolated viral load ($\log_{10}$ IU/mL) at the time of symptom onset (VL_0), and slope of viral load change (change in $\log_{10}$ IU/mL/day) according to subgroups

Variable	VL_0	95% CI	*p* value	Slope	95% CI	*p* value
Sex						
Male (*n*=152)	4.35	3.74 to 4.95		−0.08	−0.13 to −0.03	
Female (*n*=168)	4.36	3.75 to 4.97	0.47	−0.10	−0.16 to −0.05	0.46
Age, years						
>60 (*n*=126)	4.66	3.99 to 5.32		−0.09	−0.13 to −0.05	
≤60 (*n*=194)	4.30	3.69 to 4.91	0.07	−0.11	−0.18 to −0.05	0.55
SOB						
Yes (*n*=50)	4.62	3.16 to 6.08		−0.12	−0.29 to 0.05	
No (*n*=270)	4.32	3.86 to 4.77	0.85	−0.09	−0.12 to −0.05	0.71
Fever						
Yes (*n*=160)	4.76	4.16 to 5.37		−0.09	−0.14 to −0.04	
No (*n*=160)	3.93	3.33 to 4.53	0.008	−0.09	−0.14 to −0.03	0.91
Death						
Yes (*n*=6)	5.73	2.08 to 9.38		0.02	−0.33 to 0.38	
No (*n*=314)	4.31	3.88 to 4.74	0.03	−0.09	−0.13 to −0.06	0.52
Invasive ventilation						
Yes (*n*=28)	3.38	1.76 to 4.99		−0.09	−0.19 to 0.01	
No (*n*=292)	4.38	3.93 to 4.82	0.047	−0.08	−0.12 to −0.05	0.95
Oxygen						
Yes (*n*=16)	2.37	0.48 to 4.27	0.15	−0.03	−0.11 to 0.05	0.23
No (and not ventilation and not death) (*n*=272)	4.42	3.95 to 4.89		−0.09	−0.13 to −0.04	
Total (*n*=320)	4.34	3.92 to 4.77		−0.09	−0.12 to −0.06	

n, number of samples; SOB, shortness of breath.

viral load.[1] Many studies did not take into account the sample collection time with respect to the onset of symptoms, instead reporting an average Ct or viral load for a subgroup of patients.[1] Many studies have recorded the time from admission to sample collection but this may be biased by a variable time from onset of symptoms to presentation to hospital for specific subgroups.[1,2,4] Indeed we found cases requiring oxygen or invasive ventilation had sampling later from symptom onset compared to those cases which did not require these interventions. Some studies of larger numbers of severe and critical cases have shown lower Ct values (higher viral loads) in these subgroups while others have not.[1,4] One study demonstrated a higher peak viral load and longer duration of viral shedding in 71 ventilated compared to 90 non-ventilated hospitalised patients, though initial viral loads were similar.[14] Like our findings, a large study of 3712 positive samples showed no association of viral load with age, and there has not been clear association of viral load with gender.[15] While not the focus of our study, quantitative SARS-CoV-2 results have been examined for associations with infectivity, most recently for the Delta SARS-CoV-2 variant for which Ct values on the day of first detection were lower compared to clade 19A/19B
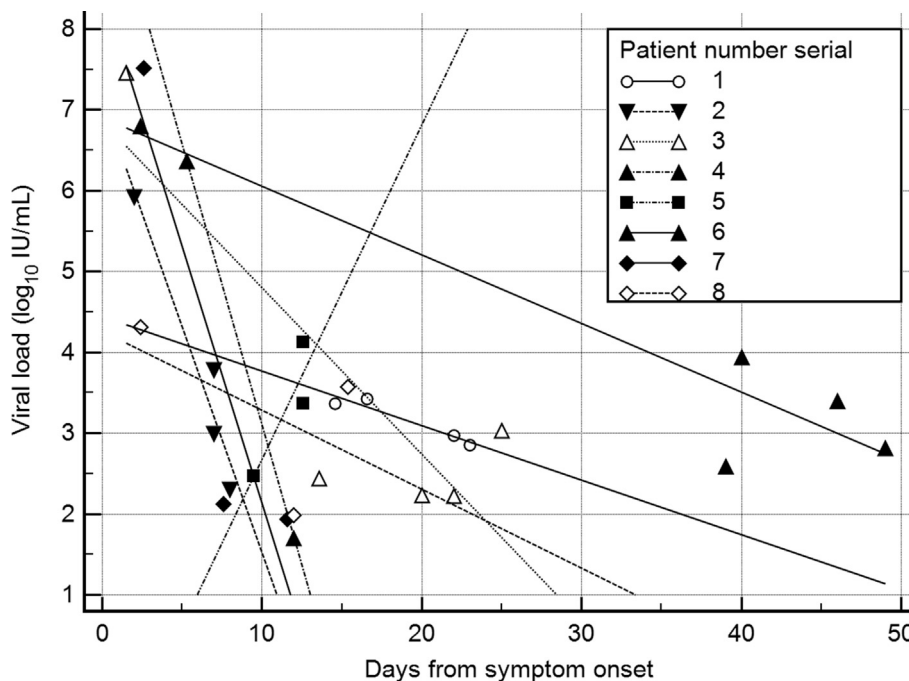

Fig. 5   Viral load in $\log_{10}$ IU/mL against time from symptom onset, showing serial viral loads in eight patients with more than two positive results.

viruses (average Ct 24 versus 34 for an in-house ORF1ab gene assay).[16]

At the usual times of release from isolation, the viral load was 2.5 $\log_{10}$ IU/mL at 14 days and 1.8 $\log_{10}$ IU/mL at 20 days from symptom onset, with +/− 0.3−0.5 $\log_{10}$ IU/mL 95% confidence intervals of the linear regression line. Overall, there was wide variation in viral loads compared to time from symptom onset with a residual standard deviation of 2.68 $\log_{10}$ IU/mL, and heterogenous individual kinetics for the small number of cases with serial PCR positive results. These findings are consistent with a report from New Zealand where Ct values ranged from <20 to >35 more than 10 days from symptom onset in non-hospitalised patients.[17] Apart from differences in the virus behaviour between individuals, the heterogeneity of respiratory samples and variability in collection method likely contribute to these poorly reproducible quantitative results which is not a problem encountered with quantitative testing of blood, serum or plasma. Nucleic acid extraction, amplification and detection are not the cause of quantitative variability, as we found excellent performance of serial dilution of the First WHO IS SARS-CoV-2 RNA, and very strong correlation of the two gene targets of the cobas SARS-CoV-2 PCR assay. Generally, previous studies have shown high Ct values or low viral loads to be associated with culture negativity,[3] but culture may not be sensitive enough to correlate with infectivity, requires increased laboratory precautions, is not routinely available, and is laborious.[18]

A strength of our study is reporting viral loads at times of interest in a moderate sized data set with the First WHO IS SARS-CoV-2 RNA, which allows comparison to other data examined with this standard. However, the designation of 'zero' IU/mL when nucleic acid targets are not detected is dependent on the limit of detection of the assay, so there may be a different threshold for 'zero' IU/mL with other assays. Our data only include upper respiratory tract samples so we cannot comment on viral load kinetics in lower respiratory tract samples and includes a limited number of cases with severe and critical COVID-19 illness, and no cases of asymptomatic infection (during the period of the study, Western Australian state policy restricted testing to symptomatic people). Our study of the first wave of COVID-19 infection may not represent viral load kinetics of variants of concern which have since evolved. Follow-up testing for positive cases was not routine or protocolised which leads to an incomplete picture of viral load dynamics in all patients and the potential for bias. We have determined viral loads at usual times of release from isolation, though it is acknowledged there are additional conditions to be met for release from isolation in public health guidelines and the time frames represent the earliest possible time of release.

We analysed SARS-CoV-2 viral load from symptom onset in mild, severe and critical COVID-19 cases. We found higher viral load at symptom onset for those reporting fever, a finding which needs further validation. Findings of higher viral load at symptom onset in those who died and lower viral load at symptom onset in those who required invasive ventilation are interpreted cautiously due to small numbers. SARS-CoV-2 quantitative measurement should evolve with routine viral load assessment rather than Ct values, and to reporting in IU/mL as assays are calibrated to the international quantitative standard. However, the variability of viral load kinetics between individuals and the poor quantitative reproducibility inherent in respiratory samples suggests that viral load assessment will only ever be able to support clinical decision making rather than be determinative in prognostication or infection control.

APPENDIX A. SUPPLEMENTARY DATA
Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2021.11.006.

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