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Journal of Clinical Virology 152 (2022) 105166

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Usefulness of real-time RT-PCR to understand the kinetics of SARS-CoV-2 in blood: A prospective study

Nelly Daniela Zurita-Cruz [a,*], Alexandra Martín-Ramírez [a], Diego Aníbal Rodríguez-Serrano [b], Isidoro González-Álvaro [c], Emilia Roy-Vallejo [d], Rafael De la Cámara [e], Leticia Fontán García-Rodrigo [a], Laura Cardeñoso-Domingo [a]

[a] Department of Clinical Microbiology, Hospital Universitario de la Princesa, Madrid, Spain
[b] Intensive Care Department, Hospital Universitario de la Princesa, Madrid, Spain
[c] Rheumatology Department, Hospital Universitario de la Princesa, Madrid, Spain
[d] Internal Medicine Department, Hospital Universitario de la Princesa, Madrid, Spain
[e] Haematology Department, Hospital Universitario de la Princesa, Madrid, Spain

ARTICLE INFO

Keywords:
SARS-CoV-2
Plasma viral-load
Viremia-kinetics
RT-PCR
Intensive care unit
Mortality

ABSTRACT

Background: SARS-CoV-2 viral load and kinetics assessed in serial blood samples from hospitalised COVID-19 patients by RT-PCR are poorly understood.
Methods: We conducted an observational, prospective case series study in hospitalised COVID-19 patients. Clinical outcome data (Intensive Care Unit admission and mortality) were collected from all patients until discharge. Viremia was determined longitudinally during hospitalisation, in plasma and serum samples collected sequentially, using two commercial and standardised RT-PCR techniques approved for use in diagnosis of SARS-CoV-2. Viral load (copies/mL and log10) was determined with quantitative TaqPath™COVID-19 test. Persistent viremia (PV) was defined as two or more consecutive quantifiable viral loads detected in blood samples (plasma/serum) during hospitalisation.
Results: SARS-CoV-2 viremia was studied in 57 hospitalised COVID-19 patients. PV was detected in 16 (28%) patients. All of them, except for one who rapidly progressed to death, cleared viremia during hospitalisation. Poor clinical outcome occurred in 62.5% of patients with PV, while none of the negative patients or those with sporadic viremia presented this outcome ($p < 0.0001$). Viral load was significantly higher in patients with PV than in those with Sporadic Viremia ($p < 0.05$). Patients presented PV for a short period of time: median time from admission was 5 days (Range = 2–12) and 4.5 days (Range = 2–8) for plasma and serum samples, respectively. Similar results were obtained with all RT-PCR assays for both types of samples.
Conclusions: Detection of persistent SARS-CoV-2 viremia, by real time RT-PCR, expressed as viral load over time, could allow identifying hospitalised COVID-19 patients at risk of poor clinical outcome.

1. Introduction

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) was first described in December 2019 in Wuhan, China [1]. As of 19th of January 2022, 5542,359 deaths were reported to WHO [2].

While most COVID-19 patients present mild disease, others develop a severe disease [3]. Identifying patients at risk of developing severe COVID-19 is an unmet need to improve the management of these patients and prevent morbidity and mortality. Some risk factors of poor prognosis, such as elevated concentrations of interleukin (IL)−6, contribute to risk stratification of COVID-19 patients [4–6]

Detection of SARS-CoV-2 RNA by real-time reverse transcription polymerase chain reaction (RT-PCR) in nasopharyngeal swabs is the gold standard for COVID-19 diagnosis [7]. However, no consistent association has been reported between the viral load in these samples and disease severity [8].

Recently, detection of SARS-CoV-2 RNA in blood samples (viremia) has been considered as a potential predictor of poor prognosis, due to its association with rapid deterioration and death [9–13]. Nevertheless, there is a lack of reports analysing viral load quantification over time in

* Corresponding author.
E-mail address: nellydaniela.zurita@salud.madrid.org (N.D. Zurita-Cruz).

https://doi.org/10.1016/j.jcv.2022.105166
Received 7 February 2022; Received in revised form 2 April 2022;
Available online 26 April 2022

sequential samples, which could allow understanding viremia kinetics and its relationship with the patient clinical outcome.

The objectives of this prospective study were to analyse the kinetics of SARS-CoV-2 viremia, by qualitative and quantitative RT-PCR methods using standardised commercial reagents in sequential samples collected longitudinally from COVID-19 patients during hospital admission, and to determine its relationship with disease clinical course.

2. Material and methods

2.1. Study design and patients

This is a prospective longitudinal case series study, conducted at Hospital Universitario de La Princesa, Madrid (Spain), from November 2020 to January 2021. Fifty-seven consecutively admitted patients with COVID-19 diagnosed by RT-PCR from nasopharyngeal swabs were included. The inclusion criteria were (a) positive RT-PCR for SARS-CoV-2 in nasopharyngeal and throat swabs, at most 48 h prior to hospitalisation; (b) acceptance to participate in the study and oral or written informed consent; (c) age higher than 18 years; (d) need for hospitalisation. The exclusion criteria were (a) patients without a baseline viremia determination in the first 24–36 h after admission; (b) patients who could not be followed-up because they were candidates to be referred to other facilities. The research protocol was approved by the Institutional Ethics Review Board (register number 4267). We followed guidance from The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) standards for observational research, and those of the Updated List of Essential Items for Reporting Diagnostic Accuracy Studies (STARD) [14]. Sociodemographic and clinical characteristics such as symptom onset, hospital admission, hospital discharge, death and sample collection dates were recorded in a pseudonymized database. Qualitative variables were expressed as number and proportion, and quantitative variables were expressed as median and Range (R) or mean and standard deviation as appropriate.

2.2. Sample collection

First, a blood sample (serum and plasma) was collected within the first 24–36 h upon admission; subsequently, additional blood samples were collected every 48–72 h during the first week. Later, samples were collected twice a week until discharge. Samples were frozen at −80 °C until RT-PCR performance.

2.3. Qualitative RT-PCR methods

All blood samples were assessed by two qualitative RT-PCR methods for SARS-CoV-2 detection. The Cobas®SARS-CoV-2 Test, (Roche Diagnostics, Basel, Switzerland) (Cobas®-test), detected two SARS-CoV-2 regions: the *orf1a/b* non-structural region, which is specific of SARS-CoV-2, and a region of the *e*-gene for pan-Sarbecovirus detection. This determination was performed on the Cobas® 6800 Systems (Roche Diagnostics, Mannheim, Germany), a fully automated nucleic acid extraction and purification system, from an initial volume of 800 μL of plasma and serum samples.

The TaqPath™COVID-19 CE IVD RT-PCR Kit, Thermo Fisher Scientific, Waltham, MA, USA (TaqPath™-test), was used to detect three specific SARS-CoV-2 genomic regions: *orf-1ab, s*, and *n* genes. A previous nucleic acid extraction was performed by the automatic eMAG® Nucleic Acid Extraction System (Biomerieux, Marcy-l'Etoile, France) from an initial volume of 400 μL, inactivated with 400μL of NUCLISENS® easyMag® Lysis Buffer (Biomerieux, Marcy-l'Etoile, France). Purified nucleic acids were obtained in 60 μL of elution buffer. This eluate was used for SARS-CoV-2 detection by TaqPath™ test according to manufacturers' indications.

For qualitative detection, samples were assessed in duplicate by TaqPath™-test and only once by Cobas®-test due to scarce volume. Detection of at least one target was considered as a positive result. Ct values of all detected targets for each technique were recorded and their mean value was calculated.

2.4. Quantification of SARS-CoV-2 viral load

Quantitative RT-PCR to determine viral load (qTaqPath-test) was performed using TaqPath™-test reagents in a QuantStudio™ 5 Real Time PCR System (Applied Biosystems, Whaltman MA, USA). Plasma and serum samples were analysed in duplicate. Viral load quantification of serum and plasma samples was obtained by plotting Ct values through the standard curve obtained using the QuantStudio™ Design and Analysis software version 2.4.3 (Applied Biosystems, Whaltman, MA, USA) and according to Thermocycler manufacturer specifications [15]. Detailed description of standard curve determination is shown in supplementary material. Samples were considered quantifiable when mean Ct in the duplicate test for each gene was $\leq$37 and standard deviation (SD) was $<$0.5. All results not fulfilling these criteria and/or those with detection in only one duplicate, were considered positive, but not quantifiable. To estimate a value for quantification limit, median of all non-quantifiable values was calculated, obtaining 17.75 copies/mL for serum samples and 14.89 copies/mL for plasma samples. For the genes that met quantification criteria, mean quantity was calculated from the duplicates, expressed as copies/mL and logarithm with base 10($\log_{10}$). Two positive controls (corresponding to 20,000 and 200 copies) and two negative controls were added in each run in duplicate.

Moreover, we studied the number of genes (*orf-1ab, s and n*) that were amplified in each plasma sample tested by q-RT-PCR.

2.5. Kinetics of SARS-CoV-2 in blood

The presence of SARS-CoV-2 RNA was detected by qualitative RT-PCR methods and viral load was determined by qTaqPath-test in plasma and serum of each patient collected throughout the follow-up. To analyse viremia kinetics, time course curves were obtained plotting viral load change over time. Specific patterns of viral load change were identified and their relationship with clinical evolution was analysed.

2.6. Variables

Three main outcomes in the considered study were: in-hospital all-cause mortality, Intensive Care Unit (ICU) admission and the combination of both (Poor outcome). Persistent Viremia (PV) was defined as SARS-CoV-2 RNA detected in two or more consecutive determinations.

2.7. Statistical analysis

Statistical analysis of qualitative variables was performed with a $\chi 2$ test or Fisher's exact test as appropriate. Comparison of quantitative variables was performed with Student's *t*-test, or by Mann-Whitney test as appropriate. Concordance analysis of both TaqPath™ and Cobas® tests, was performed calculating categorical agreement and kappa coefficient (κ). The relationship between Cts or viral loads was analysed using the Pearson correlation coefficient (r).

Statistical significance was defined at $p \leq 0.05$. Analyses were carried out with SPSS 25.0 (IBM Corp., USA)

3. Results

3.1. Characteristics of the study population

Fifty-seven patients consecutively admitted to our hospital were included in this study (Fig. 1). A median of 4 samples per patient (R = 2–18) was collected. Median age was 64 years (R = 31–94 years) and 61.4% were male. Median time from symptom onset to hospital admission was 6 days (R = 0–22 days). During hospital admission, 8

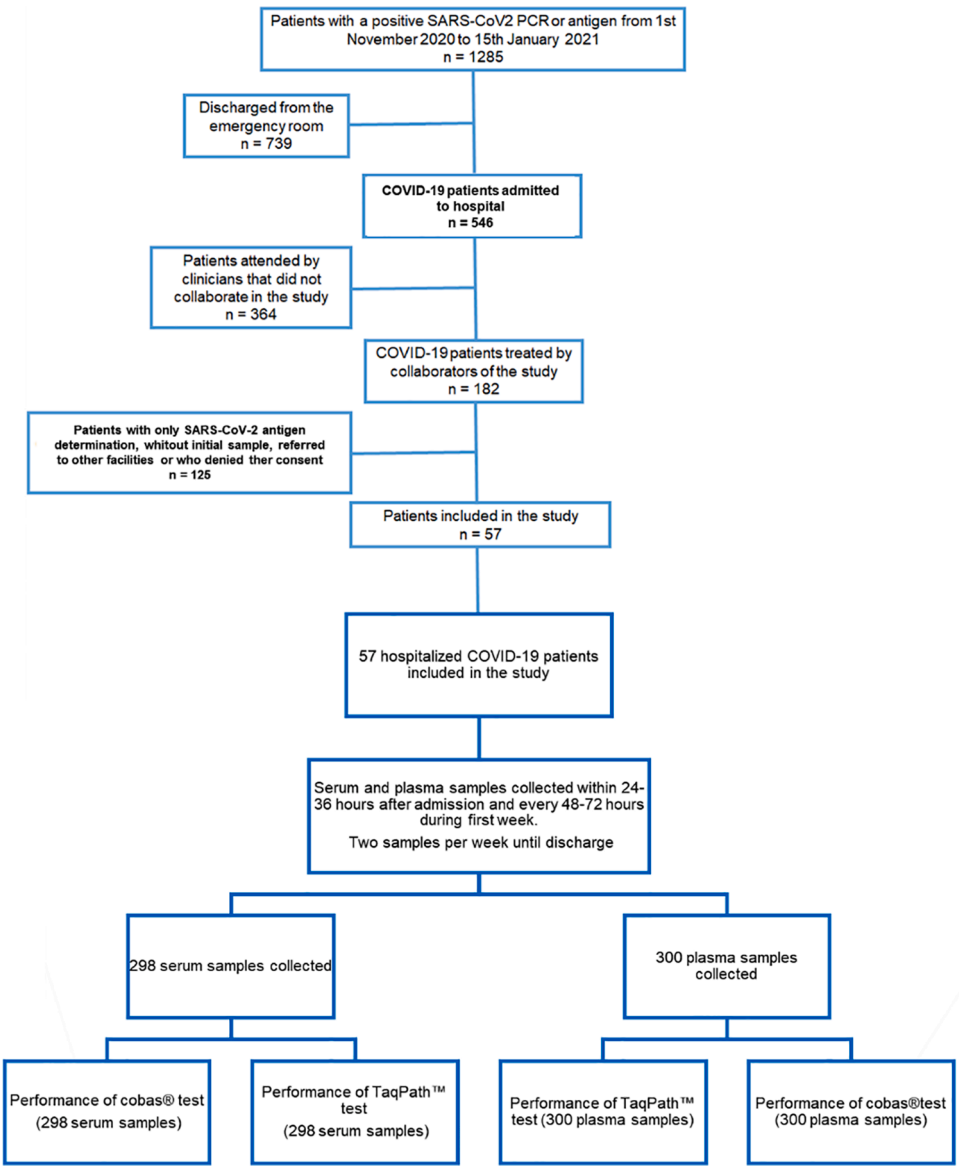

Fig. 1. STROBE flow chart of the study including participation, sample collection and tests.

(14.0%) patients required ICU admission (median age 59.5 years; R = 52–76 years; 87.5% male); and 5 (9%) died during hospitalisation (median age 76; R = 59–86 years; 80% male). Some patients presented more than one clinical condition. Patients included in the Poor Outcome group (n = 10; 17.5%) had a median age of 60.5 years; R = 52–86 years, 85.7% were male.

3.2. Analysis of samples and RT-PCR techniques

A total of 598 samples (298 serum and 300 plasma samples) were assessed (Fig. 1).

Concordance of qualitative detection for both types of samples, was similar for the Cobas® and the TaqPath™ tests ($\kappa = 0.613$ and $\kappa = 0.604$, respectively). However, both tests showed statistically significant higher positive detection in plasma samples (Table 1).

Mean Ct values obtained from plasma and serum by TaqPath™-test (33.99 ± 2.05 and 34.21 ± 1.94, respectively) and those obtained by Cobas®-test (35.34 ± 2.12 and 35.16 ± 2.33, respectively) did not show significant differences ($p > 0.5$). Pearson correlation analysis of mean Ct showed correlation coefficients (r) of 0.86 and 0.76 for plasma and

Table 1
Agreement of viremia qualitative detection in serum and plasma samples according to RT-PCR technique.

	Cobas®-test (n = 298)			TaqPath™-test (n = 300)		
	Plasma positive n (%)	Plasma negative n (%)	Total (%)	Plasma positive n (%)	Plasma negative n (%)	Total (%)
Serum Positive n (%)	75 (25.2)	17 (6)	**92 (30.9)**	76 (25.3)	13 (4.6)	**89 (29.7)**
Serum Negative n (%)	35 (11.7)	171 (57.8)	**206 (69.1)**	41 (13.3)	170 (56.7)	**211 (70.3)**
Total (%)	**110 (36.9)**	**188 (63.1)**	**298 (100)**	**117 (39)**	**183 (61)**	**300 (100)**
Agreement	82.55%			82%		
Kappa coefficient	0.613			0.604		
p value	<0.0001			<0.0001		

serum samples, respectively (Fig. 2). Results of the correlation of Ct obtained by both techniques in serum samples are consistent with findings described in our previous series [16].

3.3. Analysis of SARS-CoV-2 viral loads

Viral load could be quantified with qTaqPath-test in 50 (16.7%) plasma and 41 (13.7%) serum samples. Median viral load was 462.88 copies/mL (R = 37.26–21,277) for plasma, and 370 copies/mL (R = 29.42–15,257) for serum samples. Viral load expressed in $\log_{10}$ showed a normal distribution. Mean value of viral load in plasma was 2.65±0.63 $\log_{10}$ and 2.6 ± 0.58 $\log_{10}$ for serum samples. No statistically significant differences were found between both types of samples (p = 0.65). Furthermore, correlation analysis of viral load obtained from plasma and serum showed a Pearson correlation coefficient r = 0.89 (Fig. 3).

3.4. Detection of SARS-CoV-2 genes in plasma samples

Detection of SARS-CoV-2 genes in plasma of positive viremia patients, by TaqPath™ Kit showed that at least two assay targets could be detected in 79% of the patients: all 3 targets were detected in 64.3%, 2 targets in 14,3% and only the N gene in 21.4% of the cases. (Fig. 4).

3.5. Kinetics of SARS-CoV-2 viremia in hospitalised patients

Viremia analysis over time was performed in consecutive plasma and serum samples of individual patients (n = 57) through the qualitative methods, Cobas® and TaqPath™ tests, and viral load was assessed using qTaqPath-test. Three different viremia patterns were identified: i) Persistent Viremia (PV), viremia was detected in two or more consecutive determinations, n = 16 (28%); ii) Sporadic Viremia (SV), viremia was only detected in isolated samples, n = 34 (60%); and, iii) Negative Viremia (NV), viremia was not detected in any of the samples during hospitalisation, n = 7 (12%).

Patients presented PV for a short period of time: median time was 5 days (Range = 2–12) for plasma samples and 4.5 days (Range = 2–8) for serum. Viremia clearance occurred spontaneously in PV patients during hospitalisation, except for one patient who progressed rapidly to death.

Analysis of viral load in consecutive samples of all patients with PV (n = 16) showed different trends. Nine (56.2%) patients showed a parabolic curve, with an initial increase of viral load followed by a decrease. Six (37.5%) patients, 5 of them admitted to ICU, showed a decreasing viral load curve. Conversely, one patient (6.3%) showed increasing viral load until decease. (Fig. 5)

To further characterise the kinetics of SARS-CoV-2 in the blood of patients with PV, both viremias assessed by the 2 qualitative techniques (expressed a Ct) and viral load assessed by the q-TaqPath test (expressed as copies/mL) were determined in plasma and serum samples. Kinetics curves represent the change of these parameters in individual PV patients during hospitalisation for the qualitative (Fig. 6A) and quantitative (Fig. 6B) determinations. Curves obtained for each individual patient showed similar kinetic behaviour regardless of the technique or the type of sample used for the analysis. These results suggest that PV could be monitored using plasma or serum samples and both, qualitative and quantitative RT-PCR methods.

3.6. Relationship of persistent viremia with clinical outcome and viral load in blood

Analysis of viral load in consecutive samples over time and its relationship with clinical outcome showed that 100% of those patients with Poor Outcome (ICU admission and/or death) during hospitalisation (N = 10) presented PV. Of the 8 patients who required admission to the ICU, 6 (75%) met criteria for Persistent Viremia a median of 3.5 days (R = 1 – 8 days) before being admitted. The remaining 2 (25%) patients required direct admission to the ICU at the time of hospitalisation. None of those patients with SV and NV presented a Poor Outcome and all of them were discharged. Although 6 PV patients did not present any characteristic of poor clinical outcome, those patients represented only the 13% of all patients without Poor Outcome (N = 47), that is, poor clinical outcome was significantly associated with PV compared to no PV (p < 0.0001; Sensitivity 100%; Specificity 87.2%), as shown in Fig. 7.

No difference was detected in age (p = 0.2), sex (p = 0.4) or time from symptom onset to hospital admission (p = 0.19) between patients with different viremia patterns.

The viral load had a normal distribution when it was expressed as $\log_{10}$. Regarding viral load in patients with different viremia patterns, SV patients showed lower viral loads than PV. Median viral loads for SV were 77.3 copies/mL (IQR = 37.3–77.3; mean = 1.82 ± 0.22 $\log_{10}$) and 59.4 copies/mL (IQR = 29.4–59.4; mean = 1.71 ± 0.34 $\log_{10}$) for plasma and serum, respectively, whereas for PV median viral load was 558 copies/mL (IQR = 170.3–1145.4; mean = 2.71 ± 0.61 $\log_{10}$) for plasma and 370.4 copies/mL (IQR = 180.92–1233.6; mean = 2.57 ± 0.55 $\log_{10}$) for serum. Viral load was significantly higher for PV compared to SV for both types of samples (Fig. 8).

4. Discussion

The hypothesis of this study was that detectable SARS-CoV-2 viremia in successive samples over time (persistent viremia) could identify hospitalised COVID-19 patients with high risk of poor clinical outcome. To verify this, we studied methods for qualitative detection of SARS-CoV-2 RNA and for quantification of viral load in plasma and serum, using commercially available RT-PCR kits, marked with CE and FDA

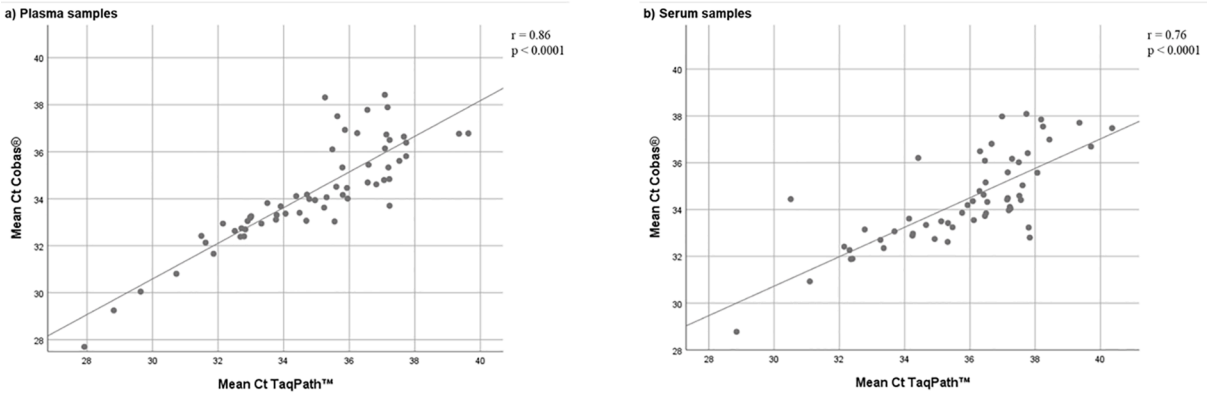

Fig. 2. Scatter plot of mean Ct obtained by Taqpath™ and Cobas® RT-PCR tests in plasma (a) and serum (b). Correlation between results was analysed by Pearson correlation (r = 0.86 and 0.76, respectively. P < 0.0001 for both techniques).

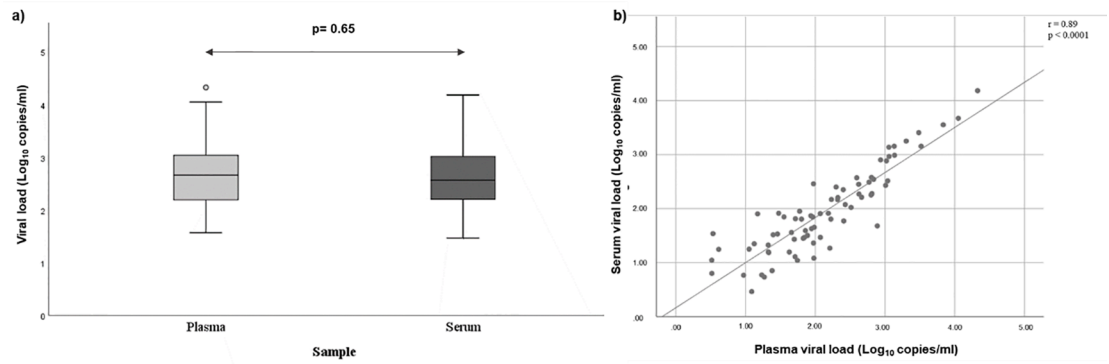

Fig. 3. Comparison of viral load in plasma and serum samples. Viral loads were determined by qTaqPath-test. Differences between plasma and serum were analysed by student's *t*-test ($p = 0.65$) (a). Correlation between data was evaluated by Pearson correlation coefficient ($r = 0.89$; $p < 0.0001$) (b).

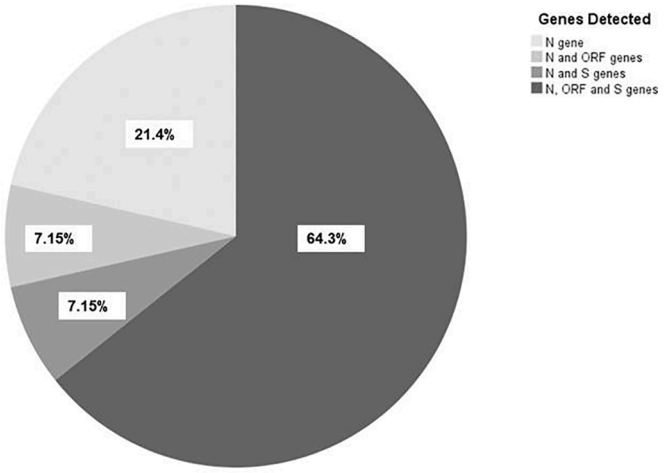

Fig. 4. Distribution of genes detection by TaqPath™ kit in patients with positive viremia.

authorisation. In addition, the association of viremia evolution with patient outcome was also evaluated.

Regarding the relationship between disease severity and the pattern of viremia kinetics, our results showed that all patients with ICU admission and/or death during hospitalisation had PV, while none of the patients with SV or NV had poor clinical outcome in this cohort. This supports our hypothesis that patients with PV are more likely to have a poor clinical outcome, in agreement with other authors. [17–23].

The graphical representation of viremia kinetics patterns in patients with PV, assessed by two qualitative and one quantitative RT-PCR methods showed similar kinetic patterns regardless of the use of different reagents and RT-PCR platforms. This analysis suggests that the dynamics of viremia in longitudinal samples correlates with the presence of SARS-CoV-2 RNA in blood and is reproducible. However, some discrepancy values were detected in isolated cases where the Ct value or viral load was lower than those in the paired sample from the same blood extraction (plasma vs. serum). We hypothesised that those discrepancies could be due to a loss of sample integrity [24]. However, the fact that different techniques can be used to analyse the kinetics of the SARS-CoV-2 viremia increases the versatility of this determination when it comes to its implementation in clinical microbiology laboratories.

Our previous results suggested that the Ct value is a good approximation for the stratification of severely ill patients [12]. However, the variation due to the sample type and handling, the technique used, and the intrinsic variability of RT-PCR makes it necessary to develop a standardised method to obtain more reproducible results [25]. Even in this series, both assays detected a higher number of positives in plasma than in serum. In this sense, it is well known that the quantification of viral load in plasma has been standardised in other pathologies associated to viral infections such as HIV, HCV, HBV, CMV [26–29], and provides a more precise and reliable monitoring of viremia than Ct values. For this reason, we propose the use of SARS-CoV-2 viral load from plasma samples for the analysis of viremia kinetics.

Different authors have quantified viremia using new technologies such as Droplet Digital PCR [18,30] using internally developed methods with primer design [17], or ultrasensitive quantitative RT-PCR(31). In this context, the objective of this work was to assess the usefulness of a quantification method using RT-PCR to monitor SARS-CoV-2 viral load in hospitalised COVID-19 patients. We used commercial and standardised reagents with CE and FDA marking for authorisation, making a standard curve and taking advantage of the options offered by the QuantStudio 5 thermal cycler interpretation software. Taken all together, these characteristics provide a robust and reproducible

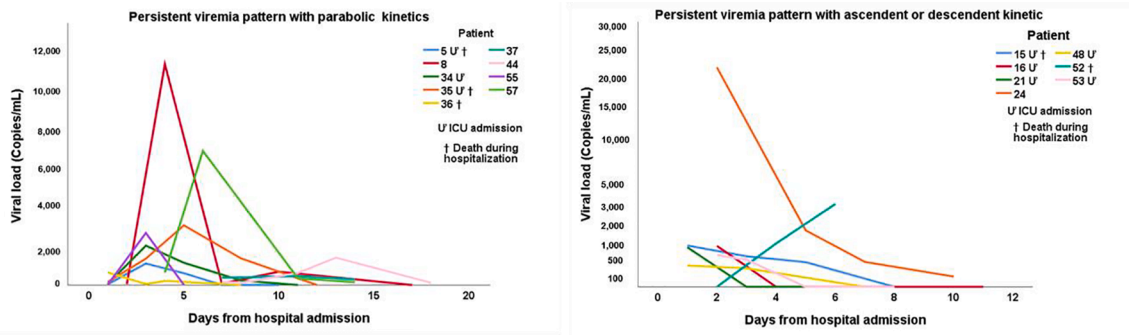

Fig. 5. Viral load kinetics curves throughout hospitalisation in plasma from patients with persistent viremia. Clinical characteristics defining poor outcome are included within the graphics for each patient. ICU: intensive care unit.

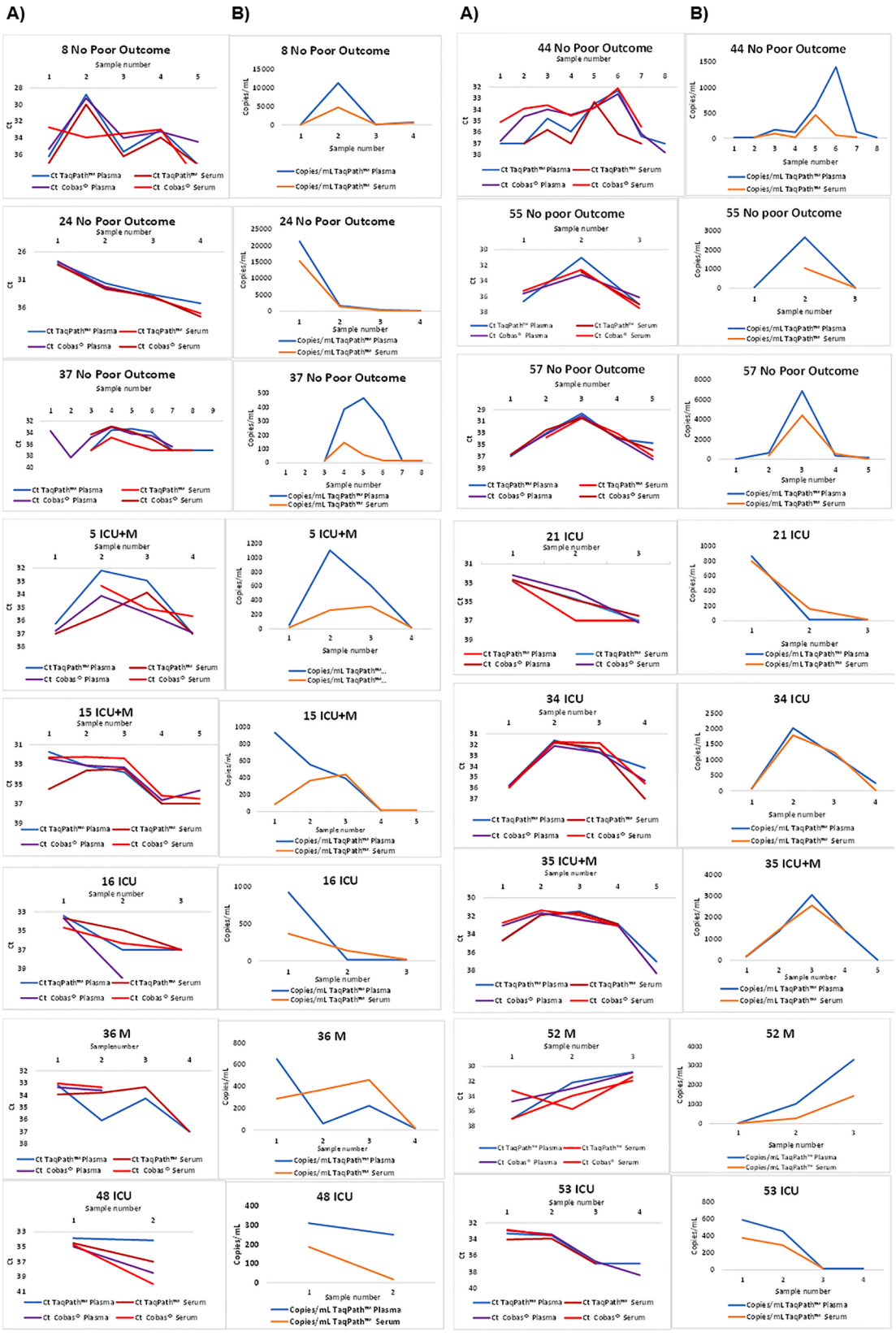

Fig. 6. Analysis of SARS-CoV-2 viremia kinetics assessed by different RT-PCR methods in plasma and serum samples. The headings of the graphs represent the code of the anonymised patients, followed by their clinical outcome (ICU: Intensive Care Unit admission; M: Death during hospital admission). The curves show the kinetics of viremia determined by the TaqPath™-test and Cobas®-test, expressed as Ct values (A), and the q-TaqPath test, expressed in copies/mL (B).

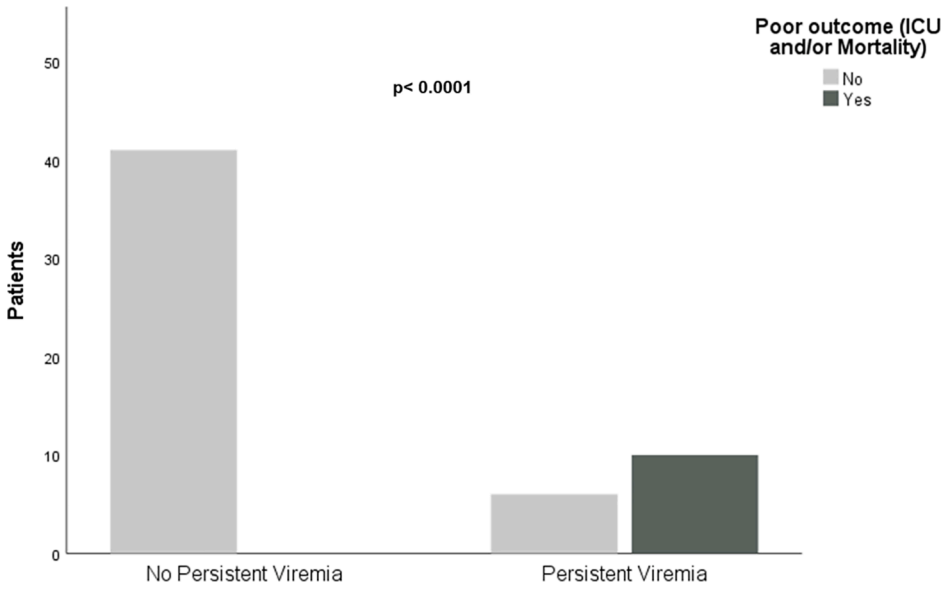

Fig. 7. Relationship of persistent viremia with clinical outcome. Distribution of patients according to the presence or absence of persistent viremia is shown. The comparison between both groups was analysed with the $\chi 2$ test.

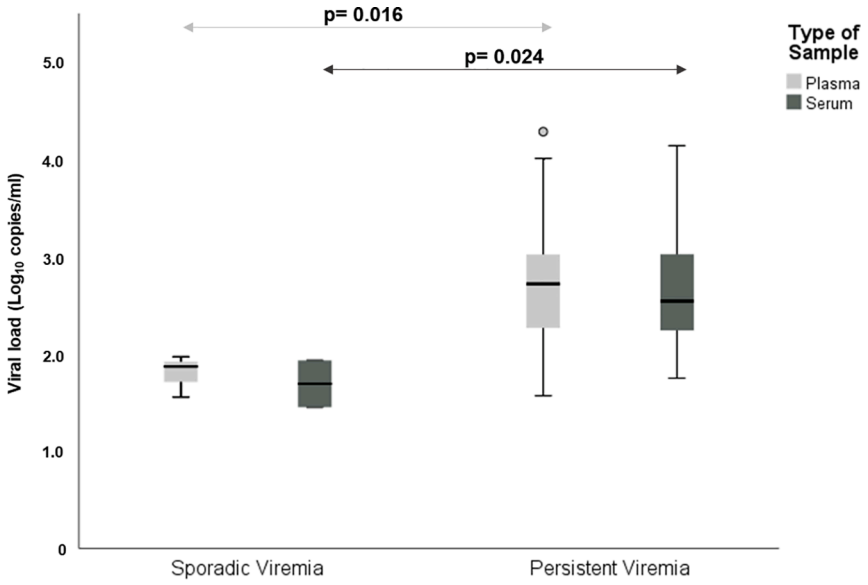

Fig. 8. Viral load is significantly higher in patients with persistent viremia than those with sporadic viremia in serum and plasma samples. The viral load expressed as log10 showed a normal distribution, so the difference of viral loads between both groups was analysed by student's *t*-test.

quantification method that allows monitoring viremia in hospitalised COVID-19 patients. Also, we agree with other authors on the need to develop standardised techniques for quantifying viral load in blood, with FDA or CE approval, that could contribute to improve the management of patients with COVID-19 [17,21].

Some authors have referred to the detection of SARS-CoV-2 RNA in blood as RNAemia [13,24,30]. However, we use the expression "viremia" for this determination, as the presence of viral RNA in the blood, in line with other published studies [17,23,31–33], since in approximately 80% of the studied plasma samples 2 or 3 SARS-CoV-2 genes were detected.

Overall results of single viremia determinations within the first week do not allow analysing the association with clinical outcome whereas persistent viremia could be associated with Poor Outcome. This preliminary result suggests that this variable, which reflects early viral load kinetics, may be suitable for assessment of outcome in clinical practice. Nevertheless, this possibility should be further analysed in studies with higher sample size and using a more detailed experimental approach, which are beyond the scope of this study.

The present study has certain limitations. First, the number of patients included in the study was moderate (57 patients). Second, although two RT-PCR techniques were performed, only the TapPath™ test allowed us to quantify the viral load (in copies/ml and log10 viral load). Third, prolonged hospitalization may increase the number of viremia determinations and therefore the likelihood of detecting persistent viremia. However, persistent viremia was detected within the first week of hospitalisation in our cohort thereby strongly suggesting that prolonged hospitalisation could not cause this bias in our results.

In summary, we conclude that persistent SARS-CoV-2 viremia in blood samples may be potentially used as indicator of poor prognosis in hospitalised COVID-19 patients. Both qualitative and quantitative RT-PCR techniques are suitable for the characterisation of viremia

kinetics in plasma and serum samples. Knowledge of SARS-CoV-2 kinetics in blood allows stratification of hospitalised COVID-19 patients.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

The work of E. Roy-Vallejo has been funded by a Rio-Hortega grant CM19/00,149 from the Ministerio de Economía y Competitividad (Instituto de Salud Carlos III) and co-funded by The European Regional Development Fund (ERDF) "A way to make Europe"

Author contribution

All the authors have revised and approved the final version of the manuscript

CRediT authorship contribution statement

Nelly Daniela Zurita-Cruz: Methodology, Formal analysis, Investigation, Writing – original draft. **Alexandra Martín-Ramírez:** Methodology, Formal analysis, Investigation, Writing – original draft. **Diego Aníbal Rodríguez-Serrano:** Resources, Writing – original draft. **Isidoro González-Álvaro:** Resources, Writing – original draft. **Emilia Roy-Vallejo:** Resources, Writing – original draft. **Rafael De la Cámara:** Resources, Writing – original draft. **Leticia Fontán García-Rodrigo:** Methodology, Formal analysis, Writing – original draft. **Laura Cardeñoso-Domingo:** Conceptualization, Investigation, Methodology, Writing – original draft, Supervision.

'Declarations of 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.

Acknowledgments

Special thanks to our patients and relatives for agreeing with the use of pseudonymized clinical data and surpluses of clinical samples to perform this study, to Manuel Gomez Gutierrez, PhD for his excellent editing assistance, and Ancor Sanz for assistance with statistical analysis.

PREINMUN—COVID group includes:

Anaesthesiology: Rosa Méndez, Julia Hernando, David Arribas.

Haematology: Javier Ortiz, Isabel Iturrate, Rafael de la Cámara.

Immunology: Francisco Sánchez-Madrid, Ildefonso Sánchez-Cerrillo, Pedro Martínez-Fleta, Celia López-Sanz, Ligia Gabrie, Luciana del Campo Guerola, Elena Fernández, Reyes Tejedor, Cecilia Muñoz, Aranzazu Alfranca. Ana Triguero-Martínez.

Intensive Care Unit: Marta Chicot Llano, Pablo Patiño Haro, Marina Trigueros Genao, Begoña Quicios, Begoña González, Diego Aníbal Rodríguez Serrano.

Internal Medicine-Infectious Diseases: Emilia Roy-Vallejo, Eduardo Sánchez, Fernando Moldenhauer, Marianela Ciudad, Ana Barrios, Beatriz Sánchez, Almudena Villa, Jesús Álvarez, Cristina Arévalo Román, José María Galván-Román, Jesús Sanz, Carmen Suárez Fernández.

Microbiology: Nelly Daniela Zurita, Diego Domingo García, Teresa Alarcón Cavero, María Auxiliadora Semiglia Chong, Ainhoa Gutiérrez Cobos, María Diez Aguilar, Isabel García Arata, Laura Cardeñoso Domingo.

Pneumology: Elena Ávalos, Celeste Marcos, Elena García Castillo, Ana Sánchez Azofra, Tamara Alonso, Carolina Cisneros, Claudia Valenzuela, Francisco Javier García Pérez, Rosa María Girón, Javier Aspa, Enrique Zamora, Mar Barrio Mayo, Rosalina Henares Espi, Olga Rajas, Joan B. Soriano, Julio Ancochea.

Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2022.105166.

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