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Exploring the relationship between anellovirus load and clinical variables in hospitalized COVID-19 patients: Implications for immune activation and inflammation

Marijn Thijssen[a], Timothy Devos[b], Geert Meyfroidt[c], Marc Van Ranst[a], Mahmoud Reza Pourkarim[a,d,e,*]

[a] KU Leuven, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Laboratory for Clinical and Epidemiological Virology, Leuven, Belgium
[b] University Hospitals Leuven, Department of Haematology, Department of Microbiology and Immunology, Laboratory of Molecular Immunology (Rega Institute), KU Leuven, Leuven, Belgium
[c] University Hospitals Leuven, Department of Intensive Care Medicine, Department of Cellular and Molecular Medicine, Laboratory of Intensive Care Medicine, KU Leuven, Leuven, Belgium
[d] Shiraz University of Medical Sciences, Health Policy Research Centre, Institute of Health, Shiraz, Iran
[e] Blood Transfusion Research Centre, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran

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ABSTRACT

Objectives: Anelloviruses have been linked with host-immunocompetence and inflammation. Here, we studied the anellovirus load in hospitalized COVID-19 patients.
Methods: We collected samples of patients recruited in the DAWN-Plasma trial that received convalescent plasma (CP) therapy (four plasma units) combined with standard of care (SOC) or SOC of alone. Plasma samples were collected on day 0 and 6 of hospitalization and we quantified anellovirus load. With multivariate models, clinical variables were associated with changes in anellovirus load.
Results: Samples were collected on day 0 and 6 of 150 patients (103 CP + SOC and 47 SOC). Anellovirus load was higher on day 0 compared to day 6 and we found a significant drop in SOC patients. Patients receiving immunosuppressive drug had a lower anellovirus load (coefficient: 1.021, 95% confidence interval [CI] 0.270-1.772, $P = 0.008$), while patients admitted to the emergency room displayed a higher abundance on day 0 (1.308, 95% CI 0.443-2.173, $P = 0.003$). Unspecific markers of inflammation and organ damage, D-dimer (0.001, 95% CI <0.001-0.001, $P = 0.001$) and lactate dehydrogenase (0.002, 95% CI 0.001-0.004, $P = 0.044$), were positively associated with anellovirus load. Finally, anellovirus load on day 0 (–39.9, 95% CI –75.72 to –4.27, $P = 0.029$) was negatively associated with SARS-CoV-2 antibody response on day.
Conclusion: The results showed associations between clinical variables and anellovirus load in COVID-19 patients. Many variables share properties related to host immunocompetence or inflammation. Therefore, we expect that anellovirus abundance displays the net state of immune activation.

Introduction

Previous studies have demonstrated the presence of viral communities in the blood of asymptomatically individuals, i.e., the blood virome [1]. Members of the *Anelloviridae* family are among the most frequently detected viruses in the blood [2]. This family of viruses has attracted attention due to their linkage with host immunocompetence across various clinical backgrounds [3,4]. Furthermore, studies have indicated that changes in host conditions associated with inflammatory responses and aging can influence the abundance of anelloviruses [5–7]. Consequently, there is growing interest to use these viruses as markers for assessing immunocompetence and inflammation.

An excessive disturbance of immune homeostasis has been observed in coronavirus disease 2019 (COVID-19) patients. Clinical outcomes of severe COVID-19 are related to a hyperinflammatory response driven by an overexuberant release of proinflammatory cytokines in infected patients. The association between anelloviruses and immunocompetence raises intriguing questions concerning the presence of these viruses and the susceptibility to develop severe COVID-19. A recent study has reported increased torque teno virus (member of the *Alphatorquevirus*

* Corresponding author.
E-mail address: mahmoudreza.pourkarim@kuleuven.be (M.R. Pourkarim).

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Table 1
Demographic characteristics of the included individuals.

Age (median)	65 ± 13.2 (66)				
	Female	**Male**			
Gender	49	101			
	Caucasian	**North-African**	**Middle Eastern**	**Sub-Saharan**	**Asia**
Race	128	14	3	1	2
	CP + SOC	**SOC**			
Treatment	103	47			
Comorbidities	**Present**	**Absent**			
Diabetes	47	103			
Hypertension	*75*	75			
Arrhythmia	31	119			
Pulmonary disease	11	139			
COPD	21	129			
Asthma	12	138			
Heart failure	10	140			
Ischemic Heart Disease	16	134			
Liver disease	3	147			
Kidney disease	19	131			
Cancer	8	142			
Haematological disorder	12	138			
Neurological disorder	9	141			
Other	39	111			

CP, convalescent plasma; SOC, standard of care.

genus of the *Anelloviridae* family TTV) titers during early detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and a decrease when symptoms resolved [8]. Interestingly, TTV load remained unchanged in patients with respiratory symptoms unrelated to COVID-19. Another study reported that TTV could serve as a potential tool to predict severe nosocomial infections in critically ill COVID-19 patients [9]. These findings highlight the potential prognostic value of anelloviruses in assessing disease severity and the risk of complications in COVID-19 patients. However, it is still unclear whether anelloviruses only reflect the host immunocompetence or actively modulate the immune response to infection. To gain a deeper understanding of the interaction between anelloviruses and the host, we studied the anellovirus community in patients hospitalized for COVID-19. We used samples collected from patients who were recruited for the multicentre donated antibodies working against nCoV (DAWn-Plasma) trial (NCT04429854) launched across Belgium [10].

Methods

Study population

The patient population was selected from individuals that participated in the DAWn-Plasma trial [10]. This was a prospective, randomized open-label multicentre clinical trial initiated to evaluate the efficacy and safety of convalescent plasma therapy in addition to standard of care in hospitalized COVID-19 patients across Belgium. Briefly, recruited patients were adults (≥18 years) with confirmed COVID-19. Patients that received mechanical ventilation during screening were excluded from participation. Individuals were randomized according to a 2:1 allocation scheme to receive convalescent plasma and standard of care (CP and SOC) or SOC alone. The CP group received two units of convalescent plasma within 12 hours of randomization followed by a second treatment of two units 24-36 hours after the first administration. Plasma samples were collected on day 0 (baseline) and day 6 to determine SARS-CoV-2 neutralizing antibody titers. Residual samples were collected and used in the present study. Furthermore, demographic, clinical and laboratory data were retrieved from the DAWn-plasma trial records. This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics committee research UZ/KU Leuven, Belgium (S66515). All participants gave informed consent and were able to withdraw from the study at any moment.

Nucleic acid extraction and anellovirus quantification

Nucleic acid extraction was done by a semi-automated system. Briefly, 200 µl of plasma directly was added to 275 µl lysis buffer in a 96 well lysis plate for extraction with the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit (Applied Biosystems, ThermoFisher Scientific), according to the protocol for KingFisher Flex. To detect and quantify anellovirus in the extracted nucleic acid samples, we used an optimized quantitative polymerase chain reaction (qPCR) assay directed against the conserved untranslated region (UTR) [11]. The following primers were used: AMTS fwd (5′-GTGCCGNAGGTGAGTTTA-’3), AMTAS rev (5′-AGCCCGGCCAGTCC-’3), AMTASgr4 rev (5′-AGCCCGGCCAGACC-’3) and the AMTPTU probe (5′-FAM-TCAAGGGGCAATTCGGGCT-BHQ1-’3). The samples were subjected to a qPCR program that started with a preheating step up to 95˚C for 10 minutes, followed by 95˚C for 10 seconds and 55.0˚C for 30 seconds for 40 cycles.

Statistical analysis

To evaluate the differences in anellovirus load between day 0 and day 6 and the impact of treatment (SOC vs CP and SOC) a paired Wilcoxon rank sum test was used. Multiple linear regression (MLR) models were used to assess the influence of demographic, clinical and laboratory variables on anellovirus load on day 0 and day 6. Backward elimination was applied to build models with relevant variables and effect sizes, and 95% confidence intervals (CIs) were computed with the *stats* package (v 4.1.1) in R (v 4.1.1) [12].

Results

Anelloviridae quantification and treatment group

We were able to retrieve complete (day 0 and day 6) sampling from 150 patients recruited in the DAWn-Plasma trial. Baseline and demographic data are summarized in Tables 1 and 2. Anellovirus qPCR was

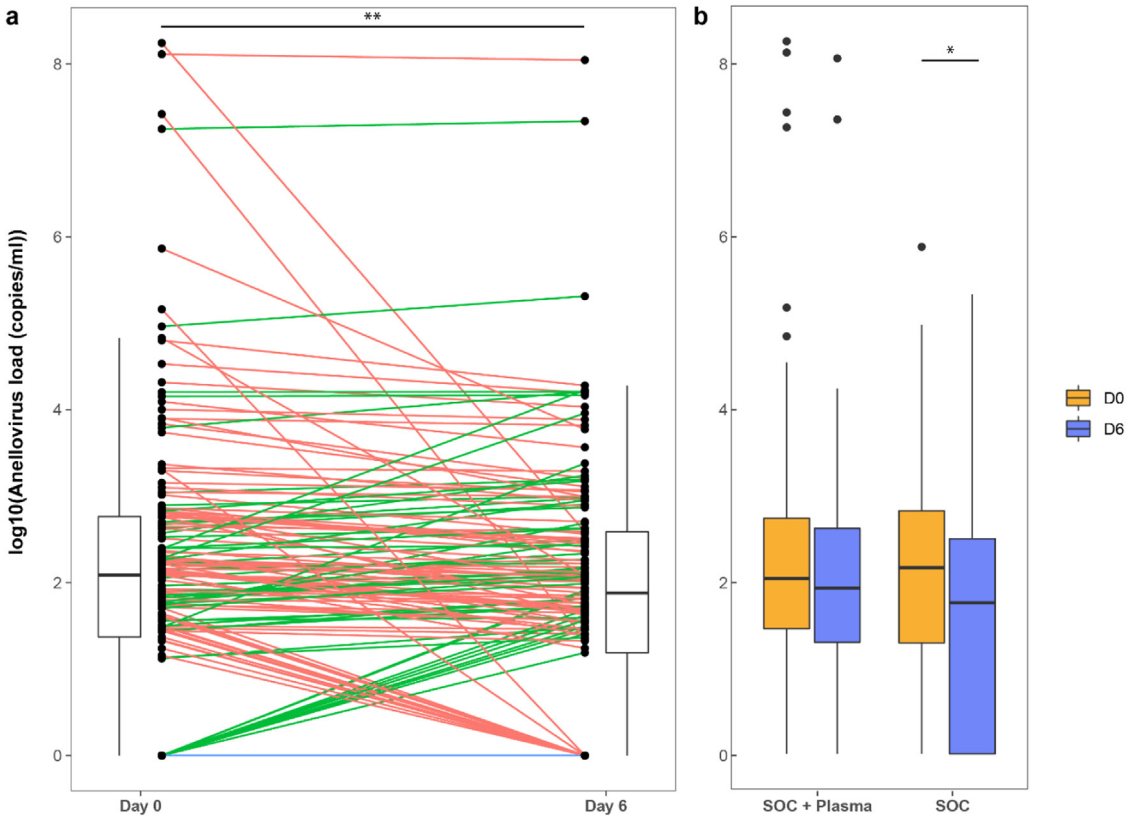

Figure 1. Anellovirus dynamics in hospitalized COVID-19 patients. (a) Change in anellovirus load (copies/ml) from inclusion (day 0) to day 6 of hospitalization. (b) Change in anellovirus load (copies/ml) between day 0 and day 6 grouped according to treatment group. SOC: standard of care; Plasma: convalescent plasma therapy. * P <0.05, ** P <0.01, paired Wilcoxon rank sum test. Green lines: increase, red lines: decrease and blue lines: equal. Boxplots show the median and interquartile ranges.

Table 2
Baseline characteristics of the study population.

	Present	Absent
O2 therapy	127	23
Respiratory distress	97	53
	Ward	**Intensive care unit**
Hospital admission status	134	16
COVID-medication	31	119
Hydroxychloroquine	3	147
Remdesivir	15	135
Lopinavir	1	149
Other	13	137
Anti-inflammatory		
Corticosteroids	80	70
Anti-microbial		
Antibiotics	46	104
Antifungals	1	149

positive for 85% of the included individuals that had one of both samples positive (130/153 patients). The overall anellovirus load was higher on day 0 compared to day 6 of hospitalization (2.09 ± 1.60 and 1.85 ± 1.43 respectively, paired Wilcoxon rank test, P < 0.01) (Figure 1a). A sub analysis based on treatment group, SOC and SOC plus convalescent plasma therapy, reveals a significant drop in anellovirus load in the SOC but not in the treatment group (Wilcoxon rank test, P <0.05 and P = 0.05 respectively) (Figure 1b). In total, in 82 individuals a decrease in anellovirus load was observed compared to 46 that showed an increase and 22 remained undetected.

Screening variables and day 0 anellovirus load

A MLR analysis was applied to associate clinical data collected during screening of patients with anellovirus load (logarithmic) on day 0. Backward elimination on completed cases (n = 150) for anellovirus load and screening variables resulted in a model with race (grouped Caucasian vs non-Caucasian), use of antihypertensive drugs, and the use of immunosuppressive therapy as significant explanatory variables (Table 3). A univariate model demonstrated an increase of anellovirus load on day 0 in patients with a Northern-African background (Kruskal-Wallis test, P < 0.05, Post-hoc Dunn's test with Bonferroni correction). Patients with a Belgian and Asian background seemed to have a lower anellovirus load on day 0. The use of antihypertensive and immunosuppressive drugs were positively associated with anellovirus load, while heart failure was negatively associated.

A similar approach was applied for the baseline variables dataset on completed cases (n = 150). A backward elimination of variables with anellovirus load on day 0 as the dependent variable resulted in a model with only hospital admission status as a significant independent variable (Table 3). Patient admitted to the intensive care unit had on average a higher anellovirus load on day 0 compared to patients admitted to the ward.

Paraclinical data

The anellovirus load was modeled with laboratory data collected on day 0. Backward elimination on the data including imputed observations showed a significant positive association between anellovirus load and eosinophils, alanine transaminase, lactate dehydrogenase (LDH), and D-dimer concentrations (Table 3). In contrast, negative associations were

Table 3
Multiple linear regression models with significant independent variables associated with anellovirus load on day 0 (dependent variable).

	Coefficient (95% confidence interval)	Adjusted R^2	*P*-value
Screening variables		0.125	<0.001
Race	0.996 (0.296-1.696)		0.006
Anti-hypertensive drugs	1.120 (0.459-1.782)		0.001
Immune suppressive drugs	1.021 (0.270-1.772)		0.008
Baseline variables		0.051	0.003
Hospital admission status	1.308 (0.443-2.173)		0.003
Laboratory parameters			
Imputed data		0.286	
Eosinophils (10^9/l)	5.275 (2.514-8.036)		<0.001
Platelets (10^9/l)	–0.004 (–0.006 to –0.002)		<0.001
Aspartate aminotransferase (U/l)	–0.030 (–0.048 to –0.012)		0.001
Aalanine transaminase (U/l)	0.024 (0.010-0.038)		<0.001
Lactate dehydrogenase (U/l)	0.002 (0.001-0.004)		0.044
D-dimer (g/l)	0.001 (<0.001-0.001)		0.001
Completed cases (n = 68)		0.286	<0.001
Platelets (10^9/l)	–0.003 (–0.006 to –0.001)		0.035
Aspartate aminotransferase (U/l)	–0.029 (–0.053 to –0.006)		0.014
Alanine transaminase (U/l)	0.022 (0.005-0.039)		0.013
Lactate dehydrogenase (U/l)	0.003 (0.001-0.006)		0.039
D-dimer (g/l)	0.001 (<0.001-0.001)		0.001

Table 4
Multiple linear regression models with significant independent variables associated with SARS-CoV-2 NT50 on day 6 (dependent variable).

	Coefficient (95% confidence interval)	Adjusted R^2	*P*-value
SARS-CoV-2 NT50 day 6		0.179	<0.001
Anellovirus load (day 0)	–39.9 (–75.72 to –4.27)		0.029
Age	–6.63 (–11.20 to –2.06)		0.005
Arrhythmia	–162.95 (–28.23 to –297.17)		0.018
Statins	–195.80 (–319.90 to –71.69)		0.002
Immunosuppressive drug	–358.00 (–171.62 to –544.38)	0.051	<0.001

NT50, neutralizing antibody titer.

observed for platelet and aspartate aminotransferase concentration. After removal of the imputed datapoints and inclusion of only completed cases (n = 68), eosinophil count was dropped from the resulting model (Table 3).

With all significant covariates, we performed an additional MLR analysis with backward elimination (Supplementary Table 1) and anellovirus load on day 0 as the dependent variable. The final model did not include race and the use of hypertensive drugs, which were significant in the separate models.

SARS-CoV-2 antibody response

SARS-CoV-2 antibody titers were determined on day 0 and day 6 of patient inclusion. A correlation analysis showed a negative correlation between anellovirus load on day 0 and neutralizing antibody titer (NT50) on day 6 (Spearman's rank correlation, rho = -0.23, $P = 0.005$), i.e. a high anellovirus load on day 0 corresponded to a lower NT50 on day 6. This correlation was maintained in patients that received convalescent plasma therapy (Spearman's rank correlation, rho = -0.22, $P = 0.02$), while the correlation in the SOC treatment group was non-significant (Spearman's rank correlation, rho = -0.26, $P = 0.08$).

A MLR with backward elimination and NT50 on day 6 as dependent variable, revealed associations between baseline variables on day 0 and anellovirus load on day 0 (Table 4). In addition to anellovirus load on day 0, a higher age was associated with a lower NT50 on day 6. Furthermore, arrhythmia, statin and immunosuppressive drug use were associated with a lower NT50 on day 6. Patient randomization in either the SOC + plasma or SOC group was not associated with day 6 NT50.

Clinical outcome

Patients with a shorter hospital stay (0-6 days) had a significantly steeper decline in anellovirus load compared to patients that were hospitalized for more than 6 days (Supplementary Table 2). Furthermore, patients that stayed shorter (0-6 days) tended to be younger compared to other age groups, while no direct association was found between anellovirus load and age. Differences in treatment group did not affect these associations. Finally, no associations were observed with respiratory distress and need for oxygen support on day 15.

Discussion

We reported the wide prevalence of anellovirus in hospitalized COVID-19 patients. The inclusion of comprehensive clinical background data allowed us to thoroughly assess potential variables associated with anellovirus. We found a wide variety of variables that were associated with anellovirus load. Overall, these variables seem to be associated with functioning of the immune system, a common predictor of anellovirus load.

Anellovirus viremia has been linked to host-immunocompetence [3,13]. Our multivariate analysis found a positive association between the use of immunosuppressive drugs and anellovirus load on day 0. Furthermore, we observed higher anellovirus loads in patients admitted to the emergency room on day 0 (within 24 hours of hospitalization). Current data on anellovirus abundance and inflammation seems inconclusive [9,13]. Some studies found a high load in inflammatory conditions while other report decreased loads. A report in severe COVID-19 patients demonstrated an association between severe disease and low anellovirus load [13]. This contradicts our findings, albeit fundamental differences

in patient recruitment and timing of sample collection might underlie these differences.

Anellovirus load on day 0 was higher in patients that admitted to the intensive care unit. Even though, severity of COVID-19 has been linked to age and underlying comorbidities including type II diabetes and hypertension, predicting disease course has been extremely difficult [14]. In addition to the patients' clinical condition, laboratory parameters like D-dimer and LDH have also been associated with disease severity [15,16]. These parameters can be useful, even though these are often unspecific clinical markers. However, the fact that anellovirus might reflect immune activation and seems elevated in patients with more severe disease, strengthens the potential case for the use of these viruses in triaging patients for hospital admission.

We observed that anellovirus load on day 0 was associated with lower SARS-CoV-2 NT50 on day 6. Multiple studies have investigated the predictive role of TTV in vaccine responses. A study in lung transplant recipients found a strong association between a high pre-vaccination TTV load and lack of antibody response [17]. Another study reported a higher response to vaccination in individuals with lower TTV loads at baseline [18]. Interestingly, in addition to anellovirus load, our multivariate model included age, the use of statins and immunosuppressive drugs. These variables were all negatively associated with SARS-CoV-2 NT50 on day 6. All variables have previously demonstrated potential immune modulatory effects, which could attenuate the ability to raise antibodies against infections [19,20]. Based on previous data, we do not expect anelloviruses to actively modulate immune response, but merely reflect the net state of the immune system. Further research should focus on the potential predictive role of anellovirus load in infectious disease outcome.

We found that the anellovirus load remained more stable in patients receiving convalescent plasma therapy compared to the SOC group. Previous studies have demonstrated the presence of anellovirus in blood transfusion products [21]. Furthermore, donor derived anellovirus lineages have been found over the course of months in blood recipients, which suggests blood transfusion as a potential source of anellovirus transmission [22]. How the body deals with newly introduced anelloviruses is still unknown. However, some studies suggest a peak in viral load after infection, which might explain why we observed a lower drop in anellovirus load in CP treated patients. More long-term studies in both blood products and blood recipients are needed to confirm this hypothesis.

This study provided with its prospective aim and controlled design a unique setting to study anellovirus in the context of convalescent plasma therapy in hospitalized COVID-19 patients. Combined with a detailed clinical dataset we were able to link certain variables to anellovirus load. However, this study also has some limitations. For instance, the high genetic variability between anellovirus species might negatively affect PCR sensitivity. Even though this might result in an underestimation of anellovirus load, the method applied in this study is widely used in the field and has proven its ability to detect changing trends in anellovirus quantities. Finally, plasma samples provide a snapshot of the overall anellovirus community in the body, which might ignore viruses present in remote body sites.

In conclusion, multiple variables associated with anellovirus load might have an indirect effect on the viral abundance through modulation of the immune system. In light of previous findings anellovirus load might be an indirect marker of host-immunocompetence. To obtain a comprehensive understanding of the role of anelloviruses, future studies should consider analyzing samples from multiple body sites.

Declarations of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Author contributions

MT, TD, MVR, and MRP conceptualized and designed the study. TD and GM were investigators involved in the data collection and recruitment of patients. Formal analysis was done by MT, TD and MRP. MT and MRP wrote the first draft of the manuscript with the support off all co-authors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijregi.2023.09.005.

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