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Original Article

Prolonged existence of SARS-CoV-2 RNAs in the extracellular vesicles of respiratory specimens from patients with negative reverse transcription-polymerase chain reaction☆

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ABSTRACT

Background and aim: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is primarily in the respiratory tract, particularly in patients with underlying comorbidities. This study aimed to investigate the presence of the virus inside the extracellular vesicles (EVs) in patients with and without chronic liver disease (CLD).

Methods: Eighty patients with positive SARS-CoV-2, including twenty-four patients with CLD and fifty-six patients without CLD, and five healthy controls with negative SARS-CoV-2 were enrolled. Nasal swab specimens were tested for the detection of SARS-CoV-2 using reverse transcription-polymerase chain reaction (RT-PCR). Patients with coronavirus disease 2019 (COVID-19) were followed up on days 7 and 14. Nasal swab, collected in viral transport media (VTM), and plasma samples were investigated at each time point. EVs were isolated from the nasal swabs (collected in VTM) and plasma using differential ultracentrifugation and estimated at each time point. The transmission or replication by the EVs was assessed in Vero E6 cells.

Results: In patients with baseline RT-PCR positive, SARS-CoV-2 RNAs inside the EVs were found in 68/80 (85%) patients with higher viral load in the nasal swabs than in the EVs (cycle threshold (Ct) value, 23.4 ± 5.7 *vs.* 30.3 ± 5.0, $P < 0.001$). On follow-up at day 7, of the 32 patients negative for COVID-19, 15 (46.9%) had virus persistence in the EVs (Ct value, 30.7 ± 2.7), and on day 14, of the 56 patients with negative SARS-CoV-2, 16 patients (28.6%) had positive SARS-CoV-2 RNAs in the EVs (Ct value, 31.4 ± 3.0). The mean viral load decreased on days 7 and 14 compared to baseline in the nasal swabs ($P < 0.001$) but not in the EVs. Additionally, SARS-CoV-2 RNAs were undetectable in the plasma, but 12.5% of patients were positive in the plasma EVs. Significantly prolonged and high viral load was found in the EVs on day 14 in COVID-19 patients combined with CLD compared with COVID-19 patients ($P = 0.0004$). We found significant higher levels of EV-associated with endothelial cells and hepatocytes in the COVID-19 + CLD group than COVID-19 group ($P = 0.032$ and $P = 0.002$, respectively), suggesting more endothelial cells and hepatocytes cellular injury in liver disease patients with COVID-19. Interestingly, we also found EVs could transmit SARS-CoV-2 RNAs into Vero E6 cells at 24 h post-infection.

Conclusions: The identification of SARS-CoV-2 RNAs in the EVs in patients with negative RT-PCR indicates the persistence of infection and likely recurrence of the infection. It is suggestive of another route of transmission as EVs harbor SARS-CoV-2 RNAs. EV-associated RNAs may determine the ongoing inflammation and clinical course of subjects with undetectable SARS-CoV-2 virus and this may have relevance to better management of patients with CLD.

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1. Introduction

A new coronavirus identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was the etiological agent responsible for the 2019–2021 viral pneumonia outbreak.[1] The coronavirus disease 2019 (COVID-19), the resulting disease, has a broad range of clinical manifestations, ranging from asymptomatic, or mild infection to a severe respiratory illness progressing to respiratory and multiorgan failure.[2] It should be noted that the comorbidities including liver disease can increase the risk of severe illness or complications. Individuals with underlying liver disease conditions, such as chronic liver disease (CLD), may be at increased risk of developing severe COVID-19 if they become infected, and may cause further damage to the liver.[2] Although the virus predominantly involves the respiratory tract epithelium, its existence beyond the respiratory tract is not yet explored. Reactivation/reinfection of the virus or prolonged detection of SARS-CoV-2 could be due to the coexistence of the virus in extra-respiratory sites.

Despite reports characterizing the clinical, epidemiological, laboratory, and radiological features and treatment and clinical outcomes of patients with COVID-19 pneumonia,[3,4] information on SARS-CoV-2 reactivation remains a mystery. The curative and eradicative therapy for COVID-19 is still in its infancy. Urgent questions that need to be addressed promptly include whether patients with COVID-19 pneumonia are at risk of reactivation or recurrence. Current detection tools have limitations, and risk factors that predict SARS-CoV-2 reactivation or prolonged detection in patients remain unknown. Therefore, there could be false negatives for oropharyngeal or nasopharyngeal (NP) swab tests, which could be affected by the sampling site, operator experience, and actual quantity of the virus.[5,6]

Recently, in the case of hepatitis B, we have shown that even in patients who were negative for the hepatitis B virus (HBV) DNA, the HBV DNA was present in low amounts inside their extracellular vesicles (EVs). We also found that EVs associated with HBV DNA could infect and transmit the virus in naïve hepatocytes.[7]

Presently, the reasons for COVID-19 recurrence are largely unknown, primarily because of its abrupt presentation not related to age, sex, or other physiological parameters.[8] Thus, to achieve a complete cure, the virus in any associated form must be identified because it could contribute to reactivation or recurrence. EVs, including microvesicles (MVs), or exosomes have served as vehicles for intercellular communication and transfer of genetic materials in several viral infections.[9] Therefore, we hypothesized that EVs may serve as reservoirs of COVID-19 and transmit SARS-CoV-2 RNAs to naïve cells and that the EV-associated SARS-CoV-2 RNAs might contribute to reactivation after viral clearance and more so in comorbid conditions such as CLD.

Therefore, this study aimed to identify EV-associated SARS-CoV-2 RNAs in both nasal swabs and plasma in COVID-19 patients with or without CLD. This study took advantage of the widely established centrifugation-based EV purification strategy and characterized the relationship between SARS-CoV-2 RNAs and EVs with follow-up of patients when RNAs are undetectable in the nasal swabs and plasma. The study also examined whether the EV-associated SARS-CoV-2 can transmit infection in naïve cells *in vitro*.

2. Patients and methods

2.1. Ethical approval

Ethical approval for this study was provided by the Institutional Ethics Committee of Institute of Liver and Biliary Sciences, New Delhi, India (Ref. No. F.37/(1)/9/ILBS/DOA/2020/20217/513). The study protocol was conducted in accordance with the Declaration of Helsinki. All participants were enrolled after providing written informed consent. Biobank samples were collected as per the Institute of Liver and Biliary Sciences biobank guidelines, and samples from in-house hospitalized patients and hospital staff were obtained after obtaining written informed consent from the participants following the national bioethical guidelines.

2.2. Patients

Patients infected with SARS-CoV-2 between December 2020 and May 2021 were enrolled. Patients with COVID-19 (aged >18 years) were selected based on a confirmed diagnosis via real time-polymerase chain reaction (RT-PCR) assays (2 viral genes, envelope protein or E gene, and RNA-dependent RNA polymerase (RdRp) or *RdRp* gene) of nasal swab samples. Patients with CLD hospitalized in the intensive care unit (ICU) of the Institute of Liver and Biliary Sciences were also included in the study. Eighty patients with positive SARS-CoV-2, including twenty-four patients with CLD and fifty-six patients without CLD, and five healthy controls (HCs) with negative RT-PCR were enrolled. Patients with COVID-19 were also followed up on days 7 and 14. Nasal swab (collected in viral transport media (VTM)) and plasma samples were investigated at each time point. All participants were negative for other viral infections, including HBV, hepatitis C virus (HCV), hepatitis D virus (HDV), and human immunodeficiency virus (HIV); had no autoimmune liver diseases, sepsis, or septic shock; and were not on ventilator support.

2.3. EV isolation using differential ultracentrifugation

Circulating EVs were isolated from the plasma and nasal swabs (collected in VTM) using differential ultracentrifugation. Then, the collected pellet was characterized for the EVs based on cell size, morphology, and origin. A detailed protocol of the sample collection, processing, and isolation was implemented following the special guidelines by ISTH for EVs to avoid any artifacts as described previously by our group.[10]

2.4. Characterization of COVID-19-associated EVs using transmission electron microscopy

Transmission electron microscopy was performed to confirm the size and shape of the EVs. Moreover, 2–5 μL of the EV sample was placed on formvar carbon-coated, copper grids, and incubated for 10 min. After 10 min, the excess sample was washed twice with 100 μL of decarbonated water and dried at room temperature for 2 min. Then, the grids were examined in Talos F200C transmission electron microscopy (Thermo Fisher Scientific, Waltham, MA, USA) and captured using software, camera, or device.

2.5. Quantification of EVs using the nanoparticle tracking assay (NTA)

EVs were quantified, and sizes were determined using NanoSight 3000 (Malvern Instruments Ltd, Malvern, UK). EVs from the plasma and nasal swabs were diluted in a 1:500 ratio in endotoxin-free phosphate-buffered saline (PBS). Moreover, a 60-s video was recorded and analyzed using NTA 3.1 version 3.1.46, camera level 15, and detection threshold of 3. The temperature was monitored during the recording. The mean size (nm) concentration (particles/mL) was calculated and plotted as particle size versus the number of particles per mL.

2.6. Cell of origin of the circulating EVs using multicolor flow cytometer

EVs were characterized by flow cytometry. Briefly, the isolated EVs were resuspended in Annexin V binding buffer (10 × concentrate composed of a 0.2-μm sterile filtered 0.1 mol HEPES (pH 7.4), 1.4 mol NaCl, and 25 mmol $CaCl_2$ solution). All buffers were filtered with a 0.2-μm syringe filter. Annexin V -APC (diluted 1:10) was added. EVs were resuspended in Annexin V mix and then added with 10 μL of each fluorescent-labeled antibody (BioLegend, San Diego, CA, USA). Surface markers of epithelial cells (EpCAM-PE-Cy7$^+$, CK19-FITC$^+$, and CDh1-PE$^+$), endothelial cells (CD31-PerCP-Cy5.5$^+$ and CD45-BV510$^-$), and hepatocytes (ASGPRII-APC-Cy7$^+$) were used as markers of EVs to confirm the cell origin, which was described in detail previously.[10] Liquid-counting beads (Becton Dickinson, Franklin Lakes, NJ, USA) with a known number of latex beads were used for quantification. The samples were incubated for 30 min in the dark at room temperature, washed twice with PBS, and immediately analyzed on a BD FACS verse (BD Biosciences, San Jose, CA, USA). For EV detection by flow cytometry, an initial EV-size gate was set using calibrating Spherotech latex beads (Chicago, IL, USA), a mixture of microbeads of four sizes (0.22, 0.44, 0.88, and 1.00 μm), which was developed to confirm the size of the EVs. The absolute count of the EVs was measured by setting the stop condition for the liquid counting beads at 50,000 events, and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). The absolute count beads were added to determine the absolute count of EVs/μL using the following calculation.

$$\text{Absolute cell count} / \mu L = \frac{\text{Cell count obtained}}{\text{Bead count obtained}} \text{ X } \frac{\text{Total bead count}}{\text{Total volume of sample}}$$

2.7. SARS-CoV-2 RNA quantification

Total viral RNAs were extracted using 300 μL of EVs (normalized to 1 × 10^6 EVs for each sample) using Chemagic Viral DNA/RNA kit (PerkinElmer, Waltham, MA, USA) in a Chemagic 360 instrument (PerkinElmer) following manufacturer's instructions. Then, 10 μL of the extracted viral RNA elute was further subjected to RT-PCR to detect SARS-CoV-2 using TRUPCR® SARS-CoV-2 real-time kit (3B Black Bio Biotech India Ltd., Bhopal, India), which is an *in vitro* nucleic acid amplification assay used for the qualitative detection of SARS-CoV-2 through RT-PCR. The assay targets the E gene (for Sarbecovirus) and the RdRp gene (for SARS-CoV-2). The cutoff cycle threshold (Ct) of the assay is 35. The test specimen with Ct value of <35 was considered positive and >35 as negative for SARS-CoV-2. To remove any viral particles in the suspension that might be adsorbed on the cell surface, isolated EVs were treated with RNAse H (Sigma Aldrich, St Louis, MO, USA) before viral RNA isolation.

2.8. Co-infection of Vero E6 cells by infected EVs from the cell culture of patients with COVID-19

Vero E6 cells are immortalized kidney epithelial cells, in which SARS-CoV-2 can be replicated easily. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO/BRL, Grand Island, NY, USA), supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum. The EVs isolated from the nasal swab (VTM) of patients were co-infected with Vero E6 cell line (1:10 ratio). SARS-CoV-2 RNAs were quantified by the E gene and RdRP gene 12, 24, and 72 h after infection. The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay was performed to investigate cytotoxicity.

2.9. Statistical analysis

All data were analyzed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS version 20 (SPSS Inc., Chicago, IL, USA). When appropriate, univariate statistics applied included Student's *t*-test/Mann–Whitney-*U* test and one-way analysis of variance to determine statistically significant differences among the control and experimental groups. Fisher's test was used to determine the odds ratio. Significance was defined as $P < 0.05$.

3. Results

3.1. Baseline clinical characteristics of patients with COVID-19

A total of 80 patients with RT-PCR-confirmed COVID-19 were examined; among them, 56 (70%) had no CLD and 24 (30%) had CLD. All patients were followed up from baseline to days 7 and 14. No significant difference was found among the groups with respect to age and sex. Among laboratory variables, the neutrophil-to-lymphocyte ratio, aspartate aminotransferase, alanine aminotransferase, and creatinine levels were not significantly different between the COVID-19 and COVID-19 + CLD group. The platelet counts were lower, whereas the bilirubin levels were higher in the COVID-19 + CLD group than in the COVID-19 group. Platelet counts may be low in the COVID-19 + CLD group because of the effects of both conditions. As regards comorbidities, diabetes was the most common in both groups (17.8% and 41.7% in the COVID-19 and COVID-19 + CLD groups, respectively). The clinical characteristics of the COVID-19 patients with and without CLD are summarized in Table 1.

3.2. EV size and morphological characterization from patients with COVID-19

After isolation of EVs from the VTM of nasal swabs and also from the plasma, characterized using NTA, the majority of the EVs were in the range of 80–400 nm in size, and concentration was between 1 × 10^6 and 9 × 10^6/mL with no differences among the two groups of patients with COVID-19 (Fig. 1A). The morphology was determined using transmission electron microscopy, which clearly shows the membranous structures, with size ranging from 75 to 380 nm/field as visible at 73,000 × and 120,000 × with the zeta potential of 27.1 mV (Fig. 1B and C).

3.3. EV-derived SARS-CoV-2 RNAs in patients with COVID-19

The schematic representation of the workflow is represented in Fig. 2A. At the baseline (at the time of presentation with symptoms), nasal swabs in the VTM and EVs isolated from VTM were analyzed (Fig. 2A). In patients with positive baseline RT-PCR, EV-derived SARS-CoV-2 RNAs were present in 68/80 (85%) patients with lower viral load in the EVs than in the nasal swabs (Ct value, 30.3 ± 5.0 *vs.* 23.4 ± 5.7, $P < 0.001$) (Fig. 2B). The mean viral load decreased on days 7 and 14 in the nasal swab from baseline ($P < 0.001$) (Fig. 2C), whereas no viral load differences were seen in the EVs (Fig. 2D). On follow-up on day 7, of the 32 patients negative for COVID-19, 15 (15/32, 46.9%) had virus persistence in the EVs (Ct value, 30.7 ± 2.7) (Fig. 2E), and on day 14, of the 56 patients with negative RT-PCR, 16 (28.6%) had EV-associated SARS-CoV-2 RNAs (Ct value, 31.4 ± 3.0) (Fig. 2F). SARS-CoV-2 RNAs were undetectable

Table 1
Baseline characteristics of COVID-19 patients with and without chronic liver disease (CLD).

Variable	COVID-19 ($n = 56$)	COVID-19 + CLD ($n = 24$)	*P*-value
Age (years)	54.0 (34.0–65.0)	58.0 (34.0–76.0)	ns
Male, *n* (%)	46 (82.1)	19 (79.2)	ns
Neutrophil-to-lymphocyte ratio	7.25 (2.40–38.10)	6.80 (4.20–17.00)	ns
Platelet count ($\times 10^9$/L)	215 (59–448)	73 (21–146)	<0.0001
Bilirubin (mg/dL)	0.82 (0.45–4.14)	3.01 (0.82–28.7)	0.0090
AST (U/L)	62 (35–216)	68 (39–281)	ns
ALT (U/L)	69 (17–173)	64 (22–103)	ns
Creatinine (mg/dL)	0.80 (0.42–1.55)	1.21 (0.50–3.60)	ns
ICU, *n* (%)	12 (21.4)	12 (50.0)	ns
ARDS, *n* (%)	13 (23.2)	10 (41.7)	ns
MELD score	–	23 (16–28)	–
Comorbidities, *n* (%)			
Hypertension	10 (17.8)	5 (20.8)	ns
Diabetes mellitus	10 (17.8)	10 (41.7)	ns
Coronary artery disease	7 (12.5)	5 (20.8)	ns
Obesity	3 (5.4)	2 (8.3)	ns

Data were expressed as median (interquartile range) or *n* (%).
Abbreviations: ALT, alanine aminotransferase; ARDS, acute respiratory distress syndrome; AST, aspartate aminotransferase; COVID-19, coronavirus disease 2019; ICU, intensive care unit; MELD, model of end-stage liver disease; ns, not significant.

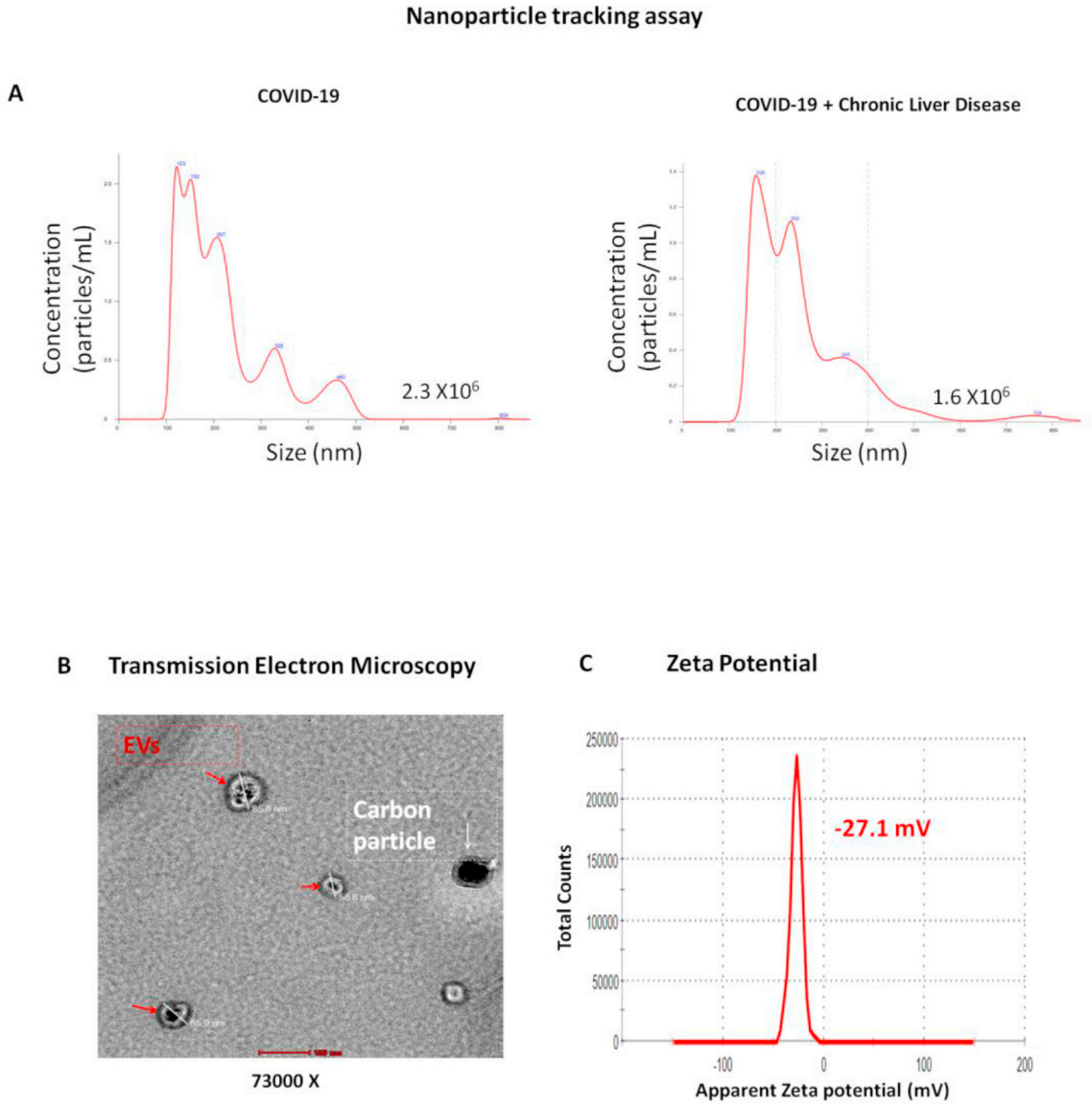

Fig. 1. Characterization of extracellular vesicles (EVs) from patients with COVID-19. (A) Nanoparticle transmission assay depicting the size and concentration of the EVs. **(B)** Size and morphological features of the EVs by transmission electron microscopy. **(C)** Membrane potential of EVs (mV) by Zeta analyzer. Abbreviation: COVID-19, coronavirus disease 2019.

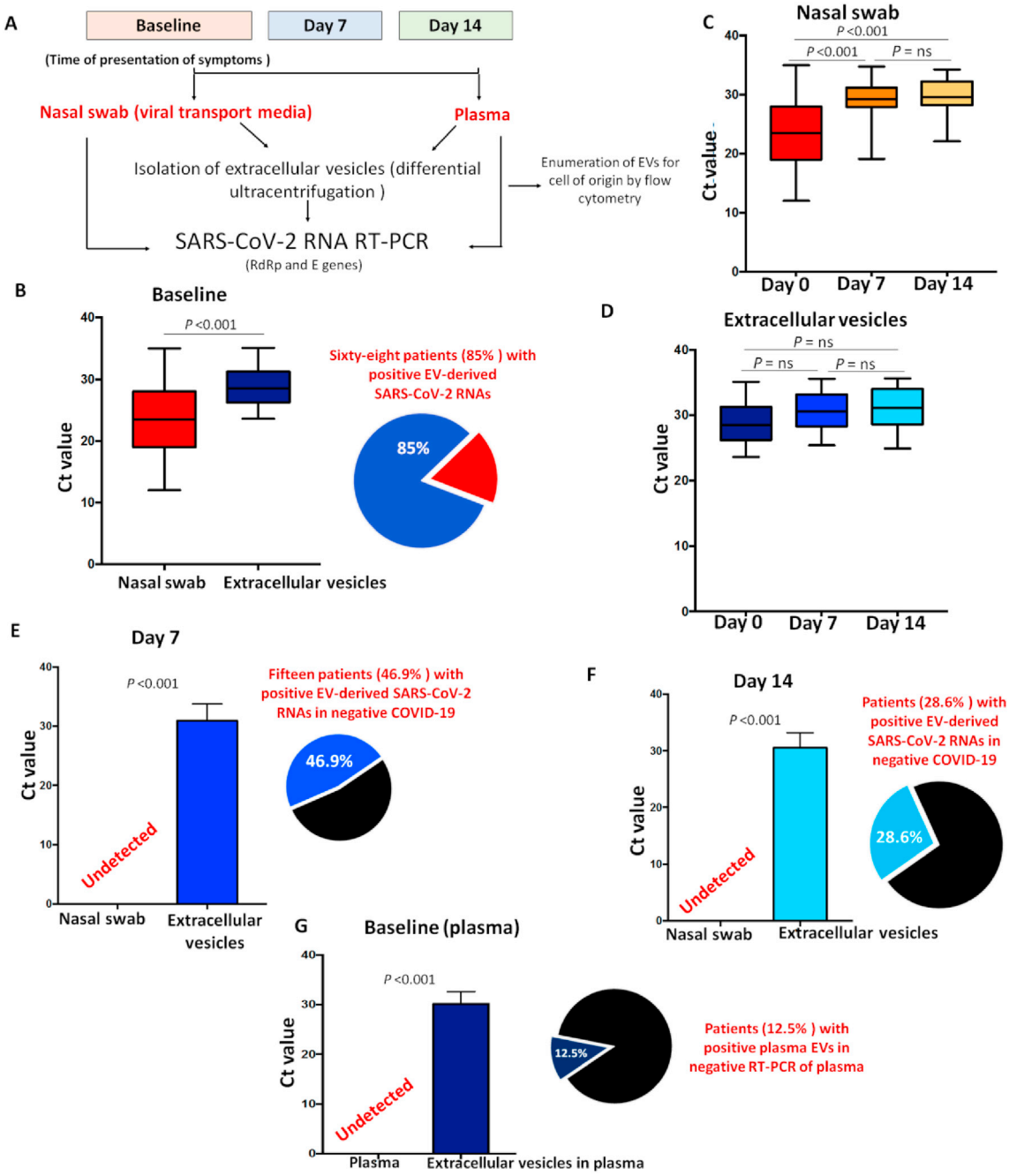

Fig. 2. Extracellular vesicles (EVs) from patients with COVID-19 contain SARS-CoV-2 RNAs. (A) Schematic workflow representing analysis of nasal swabs and EVs at different time points. **(B)** Box plots show the viral loads in nasal swabs and EVs at baseline. **(C, D)** Follow-up data of patients with a significant decrease in the viral load in the nasal swabs (C) and no change in the EVs (D) on days 7 and 14. **(E, F)** EV-containing SARS-CoV-2 RNAs were positive, even in patients who recovered from COVID-19 as detected using the RT-PCR in EVs conducted on days 7 (E) and 14 (F). **(G)** SARS-CoV-2 RNA was undetected in plasma and found to be present in plasma-EVs of 12.5% patients. $P < 0.05$ defined as statistically significant; ns, not significant. Abbreviations: COVID-19, coronavirus disease 2019; Ct, cycle threshold; RT-PCR, reverse transcription-polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

in the plasma; however, 12.5% of patients were found to be positive in the plasma EVs (Fig. 2G).

3.4. EV-associated SARS-CoV-2 RNAs in patients with COVID-19 and CLD

Interestingly, upon comparison between the COVID-19 groups with or without CLD, no significant differences in Ct values of EVs were found at baseline, whereas a significantly prolonged and a high viral load in the EVs was noted on day 14 in the COVID-19 + CLD group than COVID-19 group ($P = 0.0004$) (Fig. 3).

3.5. Cellular origin of EVs in patients with COVID-19

To determine the cellular origin of EVs and cell injury in patients with COVID-19, we analyzed the EVs and confirmed epithelial cells ($EpCAM^+$, $CK19^+$, and $CDh1^+$), endothelial cells ($CD31^+$ and $CD45^-$), and hepatocytes ($ASGPRII^+$) using flow cytometry in all

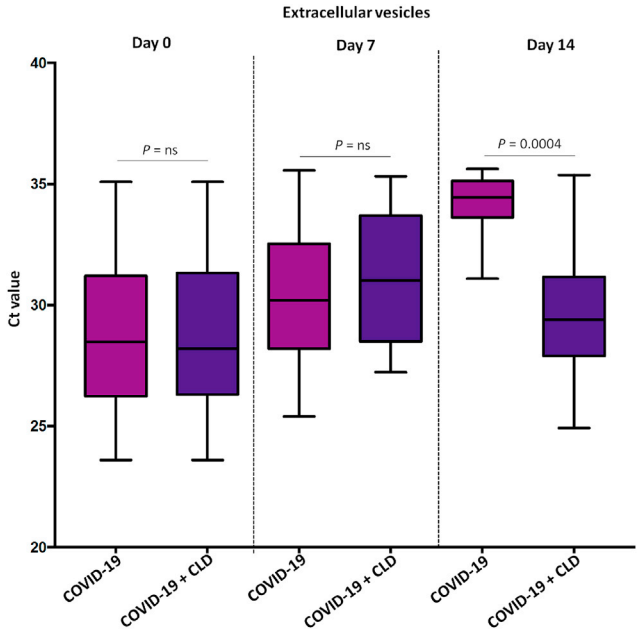

Fig. 3. SARS-CoV-2 RNAs in patients with and without chronic liver disease (CLD). Box plots depict similar viral load in the COVID-19 group *vs.* COVID-19 + CLD group at baseline and day 7 and increased viral load in the EVs of the COVID-19 + CLD group on day 14. $P < 0.05$ defined as statistically significant; ns, not significant. Abbreviations: COVID-19, coronavirus disease 2019; Ct, cycle threshold; EVs, extracellular vesicles; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

groups. Using different latex size beads and Annexin V detected EVs. Fig. 4A is a representative dot blot of flow cytometry with gating at different sizes and counting beads. The number of EVs-associated with hepatocytes, epithelial cells, and endothelial cells was significant higher in patients with COVID-19 + CLD than in HCs ($P = 0.001$) (Fig. 4B–D). Moreover, a higher cellular injury is indicated in hepatocytes and endothelial cells in COVID-19 + CLD group than COVID-19 group as significantly high levels of EVs-associated with hepatocytes and endothelial cells were observed ($P = 0.002$ and $P = 0.032$, respectively) (Fig. 4B and D).

3.6. EV-associated SARS-CoV-2 RNAs are transmissible and infectious

To understand that EVs from patients with COVID-19 can infect naïve cells, EVs isolated from the patients with COVID-19 were co-infected with Vero E6 cells (Fig. 5A). We examined the SARS-CoV-2 RNAs at 0, 24, 48, and 72 h in Vero E6 cells and found that EVs could transmit SARS-CoV-2 RNAs into naïve cells at 24 h post-infection as suggested by RT-PCR for the SARS-CoV-2 E and RdRp genes (Fig. 5B). Further, SARS-CoV-2 RNA levels in Vero E6 cells were prolonged existence with time as determined by Ct values (E-gene, 20.1 ± 5.6 at 24 h and 23.6 ± 6.8 at 72 h) indicating SARS-CoV-2 RNA replication post-transmission from infected EVs (Fig. 5B). Infected EVs may induce apoptosis in immortalized cells, and the effect of the infected EVs on Vero E6 cells was assessed using an MTT assay. The treatment had no significant effect on the viability of Vero E6 cells even after 24, 48, and 72 h (Fig. 5C).

4. Discussion

The novelty of the study is related to it being the first to identify SARS-CoV-2 RNAs in the EVs of patients with negative SARS-CoV-2, which indicates the persistence and likely recurrence of the infection. Moreover, we found that infected EVs can transmit and infect naïve Vero E6 cells. It is suggested as another route of transmission as EVs harbor SARS-CoV-2 RNAs. EV-associated RNAs may determine the presence of an ongoing inflammation and the clinical course of patients with undetectable SARS-CoV-2; this may also have relevance in the management of patients with CLD.

SARS-CoV-2, previously known as 2019-nCoV, is the cause of the pandemic that started in 2019. Presently, the origins, and possible intermediate animal vectors of SARS-CoV-2, as well as the mechanism that this virus spread among humans, remain to be determined. Despite reports characterizing the clinical, epidemiological, laboratory, and radiological features and the treatment and clinical outcomes of patients with COVID-19 pneumonia,[11,12] information on SARS-CoV-2 reactivation remains a mystery. The curative and eradicative therapy for COVID-19 is not currently available. Urgent questions that must be addressed promptly include whether patients with COVID-19 pneumonia are at risk of reactivation or recurrence. Current detection tools have limitations, and the risk factors that predict SARS-CoV-2 reactivation are not known. In a recent report of a significant proportion of patients with COVID-19 (9%), SARS-CoV-2 reactivation developed after hospital discharge.[3] Moreover, the reported clinical characteristics of these patients with SARS-CoV-2 reactivation were similar to those without COVID-19 reactivation.[13] In our findings, the presence of SARS-CoV-2 in the EVs reveals for the first time the hidden form of RNAs. Notably, based on a few reports, current evidence suggests that a proportion of patients who recovered from COVID-19 may be test positive again.[13] In addition, even in patients with negative RT-PCR, SARS-COV-2 RNAs were detectable in the EVs after 14 days.

The results of the SARS-CoV-2 RNA tests in such cases are fluctuating, which may be because no study has yet accurately established the contagious period of COVID-19. Our study will be the first of its kind to identify a new route of transmission or infectivity. Besides patients and asymptomatic carriers, those in convalescence may be infectious. SARS-CoV-2 RNAs from respiratory tract specimens may be persistent or cause recurrent positivity during the disease course.[14,15]

Furthermore, angiotensin-converting enzyme-2 (ACE-2), identified as the cell entry receptor of SARS-CoV-2, is highly expressed in the lungs rather than in the upper respiratory tract.[16] The result of the SARS-CoV-2 RNA test likely depends on the viral load of the specimen. Therefore, false negatives could occur on occasion for oropharyngeal or NP swabs tests, which can be affected by the sampling site, operator experience, and actual quantity of the virus. The bronchoalveolar lavage fluid specimen test is considered more accurate but with a higher exposure risk. In addition to the above specimens, SARS-CoV-2 RNAs can be detected in the sputum, blood, or stool swabs by RT-PCR.[17] Running multiple tests and collecting different specimens are more effective approaches to maximize sensitivity. Therefore, we proposed investigating SARS-CoV-2 RNAs in the EVs, which will decipher the alternative form of infection and transmission of COVID-19 and may pave the way for newer and better prognostic tools to detect COVID-19. Moreover, EVs are miniatures of cells, and every cell type releases them during an illness. EVs are present in all body fluids and are abundant in circulation. Thus, even in the plasma, where SARS-CoV-2 RNAs are undetectable, SARS-CoV-2 RNAs were found in the plasma EVs.

Recently, many assays are developed using quantitative RT-PCR approaches for virus detection. However, the typical turnaround time for screening and diagnosing patients with suspected SARS-

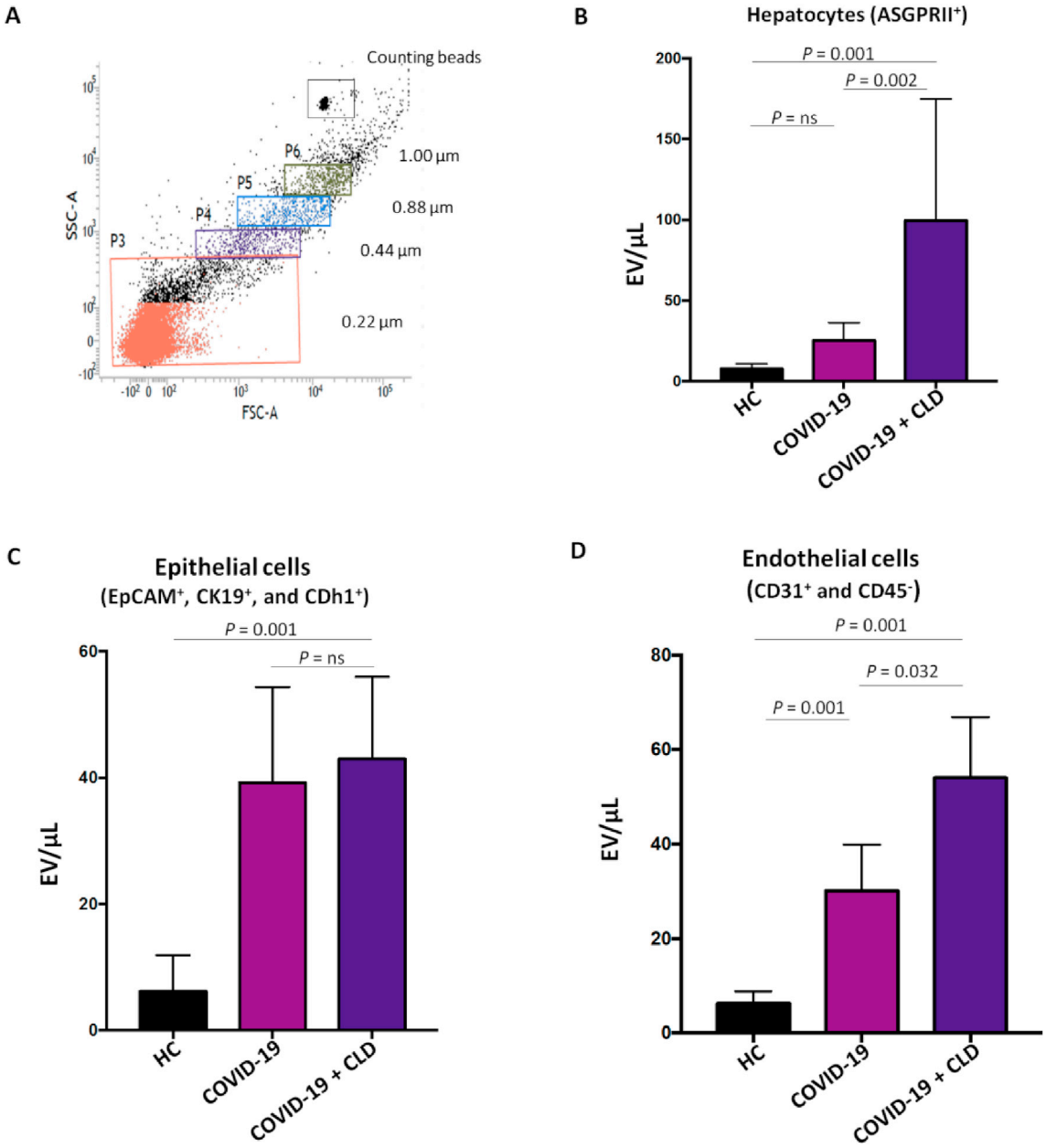

Fig. 4. Cellular origin of EVs from patients with COVID-19. **(A)** Dot blot of flow cytometry with counting bead gating at different sizes to analyze the cellular origin of EVs. **(B)** Bar graphs showed the levels of hepatocytes-derived EVs and found to be higher in all COVID-19 patients than healthy controls (HCs) and more so in patients with chronic liver disease (CLD). **(C)** Epithelial cell-derived EV levels were significant higher in patients with COVID-19 and CLD than in HCs. **(D)** Significant association of endothelial cell-derived EVs in CLD patients with increased cellular injury rates was found as depicted in bar graphs. $P < 0.05$ defined as statistically significant; ns, not significant. Abbreviations: CD31, platelet endothelial cell adhesion molecule; CK19, cytokeratin 19; COVID-19, coronavirus disease 2019; EpCAM, epithelial cell adhesion molecule; EVs, extracellular vesicles; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

CoV-2 has been >24 h,[17] given the need to ship samples overnight to reference laboratories. Although serology tests are rapid and require minimal equipment, their utility may be limited for the diagnosis of acute SARS-CoV-2 infection because a detectable antibody response can take several days to weeks following symptom onset. EV-associated SARS-CoV-2 RNA detection may solve the above problems.

In our study, we lack longitudinal follow-up of patients to find out how long SARS-CoV-2 RNA remains inside the EVs. Also, to conclude on recurrence of COVID-19 infection and role of EVs will need further investigations. Therefore, an urgent public health need is to develop rapid and more precise diagnostic tests for SARS-CoV-2.

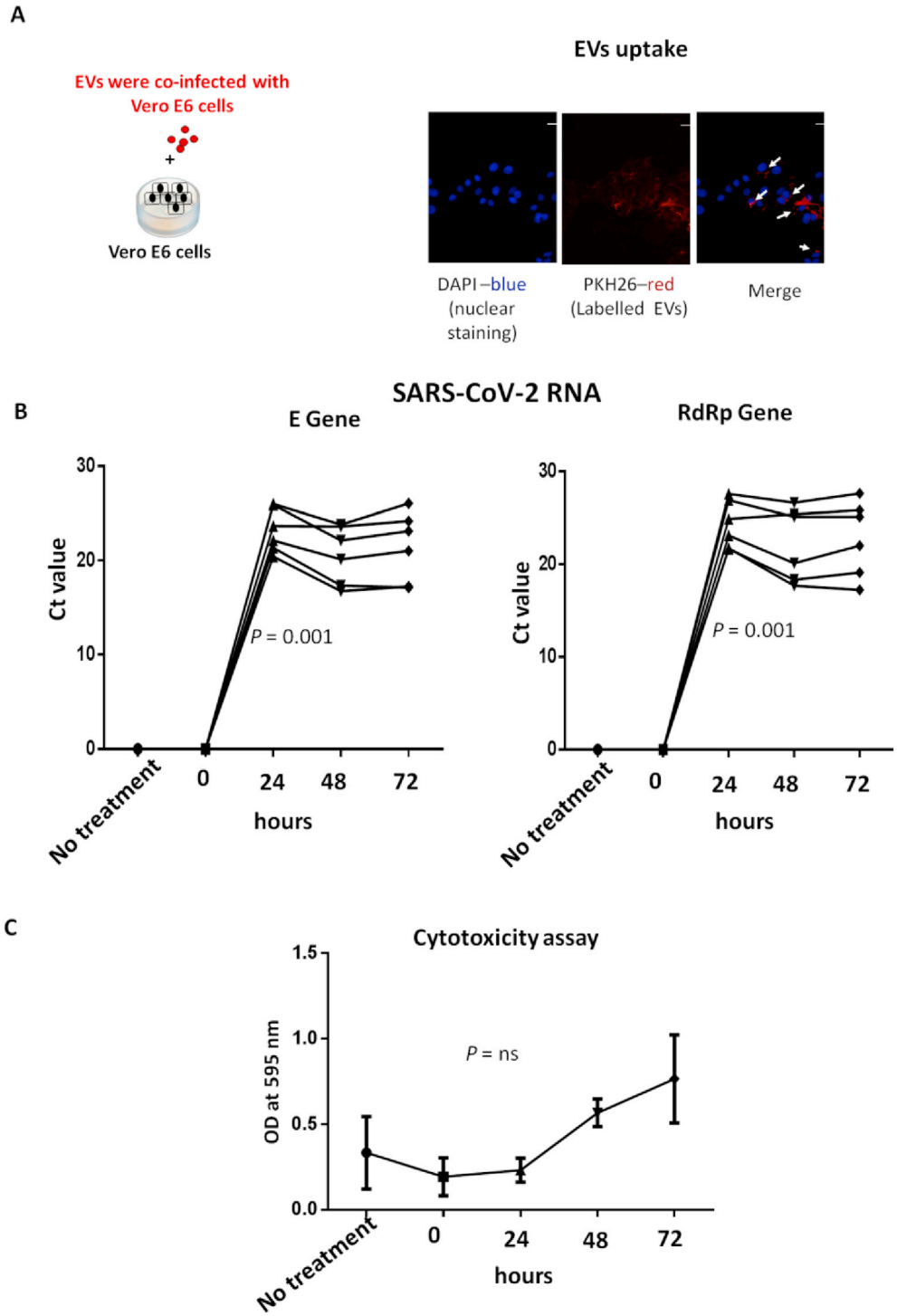

Fig. 5. SARS-CoV-2 RNAs are transmissible and infectious via EVs. (A) Co-culturing of EVs with naive Vero E6 cells and confocal microscopy showing cellular uptake using PKH26 labeled EVs and DAPI used as a nuclear stain for naive Vero E6 cells. Scale bar = 200 μm. **(B)** Detection of SARS-CoV-2 RNAs in naive Vero E6 cells at 24, 48, and 72 h of co-culturing EVs isolated from COVID-19 patients. **(C)** SARS-CoV-2-associated EVs had no significant effect on the viability of naive Vero E6 cells even after 72 h of treatment. $P < 0.05$ defined as statistically significant; ns, not significant. Abbreviations: COVID-19, coronavirus disease 2019; Ct, cycle threshold; DAPI, 4′, 6-diamidino-2-phenylindole; EVs, extracellular vesicles; RdRp, RNA-dependent RNA polymerase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

5. Conclusions

Although this study is not directly related to liver disease, the identification of SARS-CoV-2 in EVs is a highly important and relevant to COVID-19 infection in liver disease patients and for better management of these patients. The identification of SARS-CoV-2 RNAs in the EVs isolated from patients with negative RT-PCR indicates the persistence of infection and likely recurrence of the infection, which suggests another route of transmission as EVs harbor SARS-CoV-2 RNAs. Therefore, in the case of COVID-19 where we are unable to understand the mechanism of recurrence or false negativity, we may find the prolonged association of SARS-CoV-2

with EVs, which can easily be detected even in patients with negative RT-PCR. This could pave way for future directions to determine the cause of reactivation and also post-COVID-19 mortality.

Authors' contributions

P. Debishree Subudhi and Sheetalnath Rooge contributed equally to this work. Sukriti Baweja contributed to hypothesis, conceptualisation, experimental design, supervised the study, and manuscript writing. P. Debishree Subudhi, Sheetalnath Rooge, Sivang Goswami, and Swati Thangariyal performed the experiments and data collection. Swati Thangariyal, Reshu Agarwal, and Ekta Gupta did the patient selection, sample collection, and RT-PCR tests. Chhagan Bihari and Savneet Kaur gave the intellectual support. All authors read and approved the final version of this manuscript.

Declaration of competing interest

The authors declare that there is no conflicts of interest.

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