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To the Editor,

In this study, we assessed viral factors of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) shedding trying to prove that a RT-PCR crossing threshold (Ct) value superior to 33 on nasopharyngeal swab (NPS) samples could be a major criterion to lighten infection prevention and control measures as duration of eviction, contact tracing, or discharge from hospital wards. The SARS-CoV-2 replication cycle includes after direct translation of genomic RNA into several viral proteins, synthesis of negative- and positive-stranded replicative intermediate RNAs (RIs), viral assembly, and release of mature virions [1].

To provide proof of active replication, we conducted real-time RT-PCR assays to detect specifically the presence of the viral E (envelope) subgenomic RNA and E negative-strand RNA in clinical samples. We also attempted virus isolation from samples to associate the presence of RIs and the detection of viable virus. Data were obtained from 61 immunocompetent healthcare workers (HCWs) diagnosed with SARS-CoV-2 infection by RT-PCR on NPS (12 asymptomatic ones and 49 with mild/moderate clinical forms). Detailed description of the characteristics of HCWs, clinical context, and sample processing methods are presented in the supplementary material.

Overall, the median age was 28 (interquartile range [IQR] =23–36; 38 females). SARS-CoV-2 viral loads ranged from 9.64 to 3.57 Log_{10} copies/mL (cp/mL) (Supplemental Table). Virus isolation was successful for 41.0% of clinical samples. Our data showed that the likelihood of recovering infectious virus correlates with high viral loads and the presence of RIs (Figure 1A). Strikingly, no isolate was recovered when viral load was below 5.83 Log_{10} cp/mL (i.e. Ct>28), which is similar to previously reported cut-offs [2-4]. Moreover, no RIs were detectable in samples when viral load was below 4.34 Log_{10} cp/mL (i.e. Ct>33). Interestingly, the ratios of mean normalized RIs per genome indicate a high level of viral replication during the first 5 days after the onset of symptoms followed by a significant decline thereafter, as

previously reported (Figure 1B) [4]. Among asymptomatic HCWs, high viral loads (>5 Log_{10} cp/mL) were associated with detection of significant signals of RIs and virus isolation.

While recent studies express reservations [5], our findings confirmed subgenomic viral RNAs as indicators of active replication in clinical samples [4]. Moreover, we also demonstrated that negative-strands detection -never used hitherto- perfectly correlates with subgenomic viral RNA detection. With accumulating evidence available thus far, our findings strengthen the possibility to use in association with a symptom-based strategy the RT-PCR Ct value cut-off of 33 as value above which individuals would no longer be contagious.

Conflict of interest disclosure

No potential conflict of interest relevant to this article was reported.

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Author Contributions

S.B. initiated the study and coordinated all work carried out. S.M. did the targeted and strand-specific molecular assays, and cell culture isolation trials. M.L. provided clinical information about HCWs. S.B., V.C., and AG.M. drafted the initial manuscript with inputs. All authors contributed to the final submitted version. All authors have read and agreed to the final version of the manuscript.

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Figure

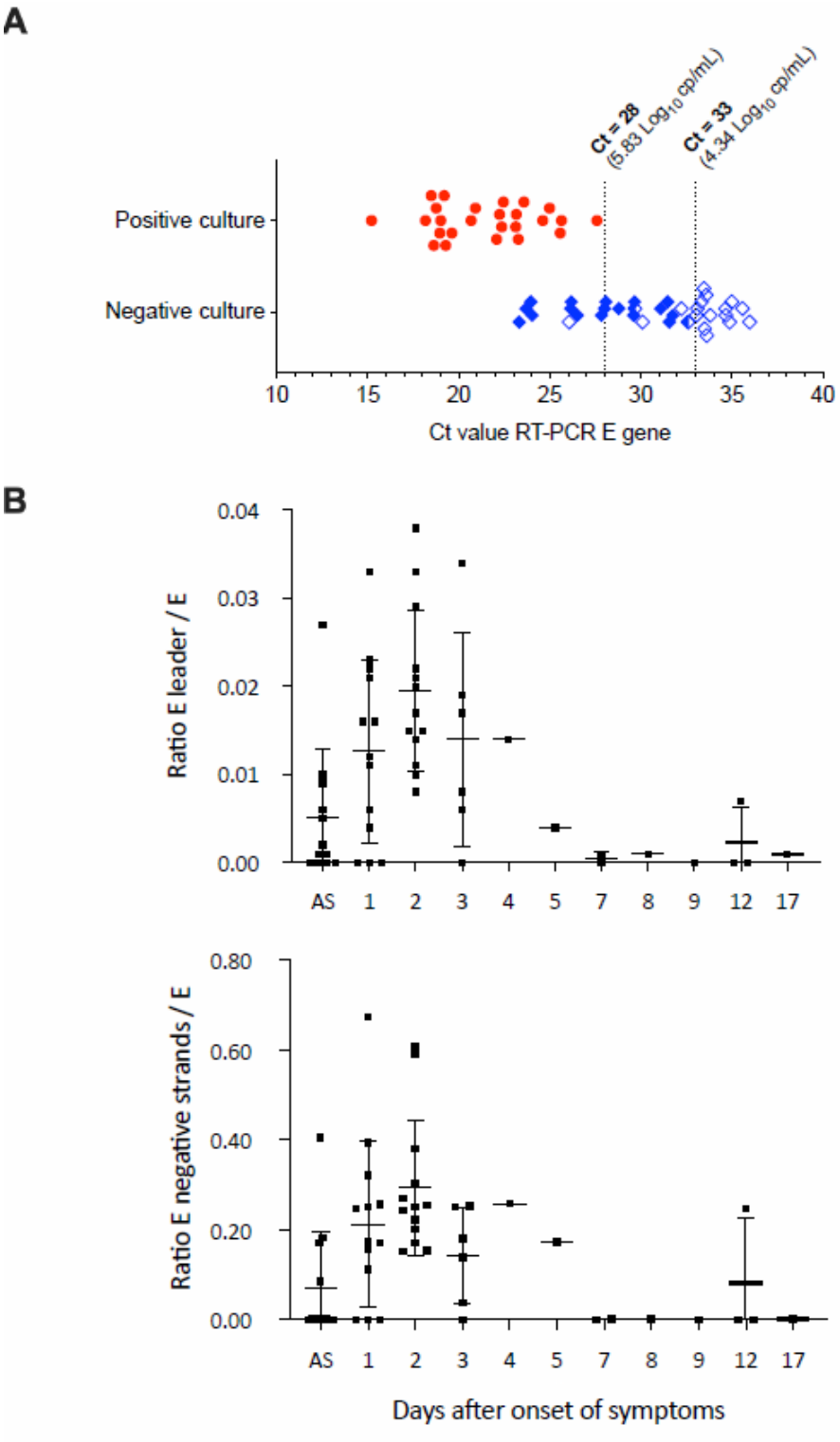
A
Ct = 28
(5.83 Log10 cp/mL)
Ct = 33
(4.34 Log10 cp/mL)
Positive culture
Negative culture
10
15
20
25
30
35
40
Ct value RT-PCR E gene
B
Ratio E leader / E
0.04
0.03
0.02
0.01
0.00
AS
1
2
3
4
5
7
8
9
12
17
Ratio E negative strands / E
0.80
0.60
0.40
0.20
0.00
Days after onset of symptoms

Figure legends

Figure 1A. Virus isolation success in relation to Ct value of the RT-PCR targeting E viral gene.

Red circle and blue diamond symbols represent positive and negative cell culture assays, respectively. Empty and half-filled symbols showed clinical samples with no E subgenomic or E negative-strand RNA signal detected, respectively. Crossing threshold (Ct) values of 28 and 33 correspond to 5.83 and 4.34 Log_{10} copies/mL (cp/mL), respectively.

Figure 1B. Subgenomic viral and negative-strand RNAs in relation to viral E genomic RNA.

The ratios are depicted according to the number of days after the onset of symptoms. Dots represent mean values of RT–PCR data obtained from at least two independent experiments on samples from individual healthcare workers (HCWs). Plots show median values with interquartile ranges. Data concerning date of symptom onset were confidently available for 54 (88.5%), HCWs with no precise data were excluded from the analysis. No E subgenomic ('E leader') and E negative-strand RNAs were never detected after 7 days after the onset of symptoms, except at 12 days after the onset of symptoms for HCW N°35 who experienced persistent clinical signs at the time of sampling. Moreover, no isolate was recovered from the clinical sample of this patient (viral load = 5.83 Log_{10} copies/mL). AS: asymptomatic HCWs.

Supplemental Material

Interpretation of SARS-CoV-2 replication according to RT-PCR crossing threshold value

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This file contains Supplementary Material, which include a description of RNA extraction and RT-PCR methods, a description of cell culture, and Supplemental Figure.

Methods

Molecular analysis of clinical samples

Nasopharyngeal swabs (NPS) were collected from the upper respiratory tract from healthcare workers (HCWs) of the Pitié-Salpêtrière University Hospital (Paris, France) for diagnostic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection between August, 1 and September, 18, 2020. Flocked swabs packaged with 1 mL of viral transport medium (VTM) were used for specimen collection. This standard VTM is suitable for conventional cell culture methods and molecular biology techniques. Initial real-time RT-PCR detection of SARS-CoV-2 was performed using the SARS-CoV-2 test on a Cobas®6800 system (Roche Diagnostics). Information about the HCW (demographic characteristics, days of sample collection after symptoms onset, clinical symptoms) included in this study are summarized in the Supplemental Table (see below). All patient samples and data used in this study were collected in the context of routine clinical patient care. Additional analyses were performed only on surplus of clinical material collected in the context of routine clinical patient care. Our institutional review board approved the use of these data and samples. To provide proof of active replication, we conducted real-time RT-PCR assays to detect specifically the presence of the viral E (envelope) genomic and subgenomic RNA, and E negative-strand RNA in NPS samples. Retrospectively, RNA was extracted from clinical samples by using the automated nucleic acid extraction eMAG platform following the manufacturer´s instructions (bioMérieux, France) to perform a real-time RT-PCR for SARS-CoV-2 detection targeting the viral E gene (i.e. genomic RNA designed below as ‘E amplicon’; amplicon size: 113 base pairs [bp]) and a real-time RT-PCR for E subgenomic RNA (sgRNA) detection (designed below as ‘E leader amplicon’; amplicon size: 171 bp), as partially previously described (Wölfel et al., 2020). Forward (Fw) and reverse (Rv) primer and probe sequences are detailed in the tables hereinafter. In addition to a leader-specific primer (sgLeadSARSCoV2-Fw), we used a specific-probe targeting sequences upstream of the start codon of the E gene to ensure full specificity (see Supplemental Figure below for primers and probes positions).

RT-PCR targeting E gene (E amplicon):

Name	5'– 3'
E-primer-Fw	ACAGGTACGTTAATAGTTAATAGCGT
E-primer-Rv	ATATTGCAGCAGTACGCACACA
E-probe	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ-1

E subgenomic RNA specific RT-PCR (E leader amplicon):

Name	5'– 3'
sgLeadSARSCoV2-Fw*	CGATCTCTTGTAGATCTGTTCTC
E-primer-Rv	ATATTGCAGCAGTACGCACACA
E-probe-sgLead	HEX-CTTATGTACTCATTCGTTTCGGA-BHQ-1

*As previously published.

Both RT-PCR assays used the Superscript®III one-step RT-PCR system with Platinum®Taq Polymerase (Invitrogen) with 500 nM concentrations of each of the primers, as well as 250 nM of probe. Thermal cycling involved 20 min at 50°C for reverse transcription, followed by 3 min at 95°C and 45 cycles of 10 s at 95°C, 15 s at 56 °C, and 5 s at 72°C. For negative-strand specific detection, we used the same nucleic acid extracts as for RT-PCR assays.

For E negative-strand specific detection, the oligonucleotide E-primer-Fw was used to prime complementary DNA (cDNA) synthesis from positive-sense viral RNA using Superscript®III system (Invitrogen) according to manufacturer's instructions. cDNA synthesis reaction also contained 500 nM of E-primer-Fw, 6.25 µL of reaction mix with MgSO4, 3.25 µL of RNA template and RNase-free water up to 10 µL. The reaction was carried out at 50°C for 20 minutes, stopped by heating at 70°C for 20 minutes. A 6.5 µL amount of cDNA product from the RT step was added to a reaction mixture as described above for 'E amplicon' detection. PCR thermal cycling involved initial heating to 95°C for 3 min followed by 45 cycles of 10 s at 95°C, 15 s at 56°C, and 5 s at 72°C. Molecular assays were proven to give reliable results by intra-assay and inter-assay variability assessment. Analytical validation of RT-PCR E assays was partially previously published (Wölfel et al., 2020; Corman et al., 2020). To confirm assay reliability in our experimental conditions, a SARS-CoV-2 positive NPS specimen was serially diluted and subjected to the RT-PCR assays in duplicate repeats. Both assays demonstrated good linearity over a wide range of 3–9 Log10 cp/mL ($R^2 > 0.98$). Specificity of negative-strand detection was also validated using a SARS-CoV-2 cell culture model (data not shown). Cycle threshold (Ct) values are inversely correlated to the genomic viral RNA load. Subsequently, Ct values were converted to Log10 RNA copies/mL (cp/mL) using calibration curves based on quantified E-gene in vitro RNA transcripts as previously described (Wölfel et al., 2020; Jones et al., 2020).

Cell culture method for virus isolation from clinical samples in biosafety level 3 laboratory

Samples were stored at -80°C until further use. Aliquots were thawed once before being processed in parallel for virus isolation and RNA extraction. Vero CCL-81 cells were maintained in Dulbecco's minimal essential medium (DMEM, ThermoFisher Scientific) supplemented with 5% heat inactivated fetal bovine serum (FBS, ThermoFisher Scientific) and 1% penicillin/streptomycin (ThermoFisher Scientific) before used. Vero cells were seeded into 24-well plates at 70-80% confluency. Nasopharyngeal samples were two-fold diluted in serum free DMEM containing 1% penicillin/streptomycin, 1% amphotericin B and 0.8% TPCK-tryspin (ThermoFisher Scientific) and filtered on a 0.45-µm pore sized centrifugal filter (Ultra-free®-MC, Merck Millipore). Then, 150 µL were inoculated onto Vero cells and incubated at 37°C for 1 hour. Then, final volume of medium was adjusted to 1 mL DMEM composite as described above. The inoculated cells were grown in a humidified 37°C incubator with 5% CO_2 and checked for cytopathic effect (CPE) every 2 days for 7 days. Two subcultures were performed if no CPE were observed until virus isolation was stopped.

Analyses and graphic drawing

Analyses and graphic drawing were performed using GraphPad Prism v.9.

Data availability

All other data are available from S.B. upon reasonable request.

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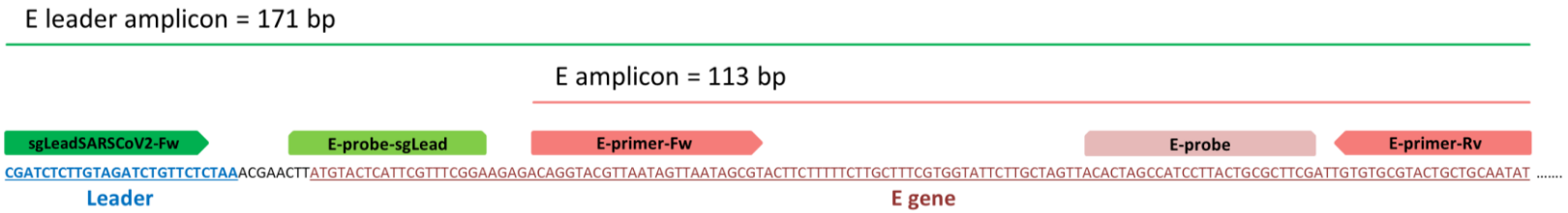
Supplemental Table

Demographic characteristics of healthcare workers (HCWs), clinical symptoms, days of sample collection after symptoms onset, and viral load determined after 'E amplicon' detection

Patient number	Age (years)	Gender	Clinical symptoms	Days after symptom onset	Viral load (Log10 cp/mL)
1	30	M	Fever, cough	2	9.64
2	38	M	Fever, cough	1	8.75
3	28	F	Asymptomatic		8.66
4	34	M	Fatigue	2	8.62
5	27	F	Myalgia, cough toux	2	8.58
6	23	M	NA		8.52
7	26	M	Faintness, anorexia, diarrhea, fever, cough	1	8.50
8	25	F	Fever, cough	1	8.45
9	29	M	Fever, cough	1	8.42
10	23	F	Fatigue	1	8.32
11	22	F	Myalgia, odynophagia	3	8.01
12	32	M	Asymptomatic		7.94
13	19	F	Fever, fatigue	1	7.59
14	44	F	Nausea	1	7.54
15	47	F	Fever, fatigue	3	7.51
16	38	M	Fever	1	7.28
17	24	F	Fever, fatigue	4	7.27
18	36	F	Myalgia, rhinitis	2	7.22
19	25	F	Fever	2	7.24
20	44	F	Asymptomatic		7.14
21	29	F	Fever, cough	2	7.11
22	20	F	Myalgia, fever	1	7.11
23	23	F	Fever, rhinitis, fatigue	5	7.03
24	11	M	NA		7.01
25	24	M	NA		6.84
26	32	F	Fever, cough	3	6.72
27	25	M	Myalgia, headache, fever	2	6.55
28	55	M	Fever	2	6.53
29	33	F	Asymptomatic		6.41
30	87	F	Asthenia, confusion, dyspnea	2	6.38
31	69	F	Fever, dyspnea	3	6.37
32	71	M	Asthenia, fever, dyspnea, diarrhea, nausea	2	6.27
33	19	M	Odynophagia	3	5.95
34	31	F	Anosmia, ageusia, diarrhea	2	5.88
35	36	F	Asthenia, pneumonia, cough, arthralgia, myalgia	12	5.83
36	40	M	NA		5.81
37	25	M	Fever, cough	2	5.59
38	24	M	Asymptomatic		5.35
39	11	M	NA		5.34
40	33	M	Fever, asthenia	1	5.33
41	19	F	NA		5.21
42	29	M	Fever, fatigue	1	4.91
43	29	M	Cough, anosmia	2	4.80
44	19	F	NA		4.77
45	49	F	Headache	12	4.71
46	20	F	Asymptomatic		4.57
47	21	F	Asymptomatic		4.46
48	32	F	Fever, cough, fatigue	7	4.33
49	23	F	Anosmia, ageusia, headache	3	4.31
50	24	F	Asymptomatic		4.24
51	25	F	Asymptomatic		4.20
52	48	M	Fever, dyspnea	12	4.19
53	23	F	Headache, cough	1	4.17
54	24	M	Myalgia	1	4.16
55	59	F	Myalgia, asthenia, fever, dyspnea	9	4.10
56	28	F	Asymptomatic		3.85
57	33	F	Odynophagia, anosmia, ageusia	7	3.85
58	37	F	Rhinitis	8	3.79
59	27	F	Asymptomatic		3.75
60	23	F	Fever, myalgia, fatigue	17	3.57
61	51	F	Asymptomatic		3.46

cp: copies/mL; F: female; M: male; NA: not available.

Supplemental Figure. Primers and probes used for molecular assays



The leader sequence (blue nucleotides) and nucleotides coding for the 5′-proximal part of the viral *E* gene (red nucleotides) on the subgenomic RNA are shown. Primers and probes are depicted using colored arrows or boxes alongside nucleotide sequence. bp: base pairs.