

## Persistent viral infectivity after 27 days from COVID-19 symptoms onset

The persistence of SARS-CoV-2 in pharyngeal swabs belonging to patients affected by COVID-19 is not a rare occurrence. Indeed, the viral load determined through real-time reverse-transcriptase PCR (rRT-PCR) test peaks at the onset of symptoms and decreases to undetectable level within 1–3 weeks.<sup>1</sup>

Nevertheless, few studies investigated the potential infectivity of the clinical specimens, and to date viral culture isolation failed after some weeks from the first symptoms,<sup>1</sup> being the 18<sup>th</sup> day the last timing in which virus replication *in vitro* was achieved in immunocompetent host.<sup>2</sup>

Here we report the case of SARS-CoV-2 infection positivity at the molecular test after 42 days, describing successful viral replication *in vitro* obtained after 27 days from the first positive swab.

A 58-year-old man resulted positive to SARS-CoV-2 during routine screening for health workers; he was initially asymptomatic. He underwent isolation and was treated with lactoferrin, vitamin C and D. The subsequent day after testing positive, swab specimens from his wife and daughter yielded positive for SARS-CoV-2 infection; however, they returned negative after 15 and 10 days, respectively. After 4 days he developed fever (max 38.7°C) and cough and was treated with paracetamol and levocloperastine. At the seventh day after the swab positivity he aggravated and presented dyspnoea exertional, although with 94%–97% saturation and no pulmonary abnormalities evidenced by direct lung auscultation; treatment with azithromycin (500 mg/day) for 6 days was started. After 3 days of treatment, he did not show fever. After 23 days he continued to have a cough that was successfully treated with

**Table 1** Molecular results from the swabs obtained during the COVID-19

Days	E gene	RdPR gene	N gene
0	37.06	37.65	33.43
20	37.68	ND	34.61
27	ND	ND	36.24
30	39.08	ND	36.92
36	36.42	35.06	39.08
42	40.8	ND	40.8
48	ND	ND	ND

The swabs were submitted to thermolysis (15 µL of samples mixed with 45 µL of distilled water protocol: 98°C for 3' and 4°C for 5'). Then, 5 µL were tested with the Allplex SARS-CoV-2 assay (Seegene, Seoul, South Korea) on the CFX Connect Real-Time PCR Detection System (Bio-rad, Hercules, California, USA) following manufacturer's instructions. The cycle threshold for E, RdPR and N genes are displayed. ND, not detected.

prednisone (50 mg/day for 4 days, 25 mg/day for 2 days, 12.5 mg/day for 2 days). At the haematologic analysis the unique alteration regarded an increment of D-dimer (D-dimer=996 ng/mL), a characteristic associated with a hypercoagulable status often found in severe cases of COVID-19.<sup>3</sup> Treatment with fodaparinux (2.5 mg/day for 10 days) was initiated. The leucocytes number were in the normal level range with a slight decrement of lymphocytes ( $8.82 \times 10^9/L$  leucocytes,  $6.56 \times 10^9/L$  neutrophils,  $1.39 \times 10^9/L$  lymphocytes). After 42 days he showed diffuse respiratory wheezing in forced expiration with pulmonary obstructive features deduced through direct lung auscultation, and therapy with symbicort 160/45 (two inhalations every 12 hours) was started. After 48 days, the molecular test was negative. After 49 days D-dimer returned to normal level (420 ng/mL), leucocytes decremented ( $6.14 \times 10^9/L$  leucocytes,  $2.48 \times 10^9/L$  neutrophils,  $2.83 \times 10^9/L$  lymphocytes) and antibody anti-SARS-CoV-2 S1/S2 (193 AU/mL) were detected (figure 1).

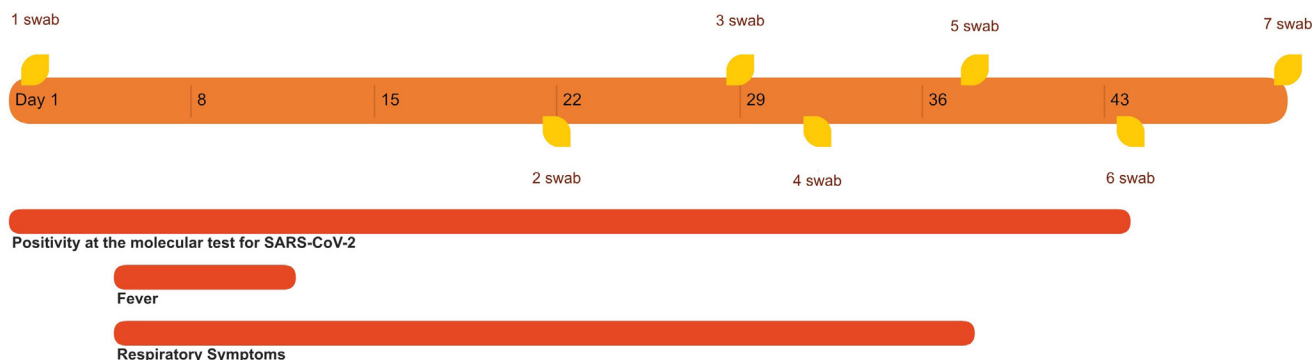
A total of seven swabs were collected during the course of the COVID-19 and tested for the viral E, RdR and N genes with the Allplex SARS-CoV-2 assay (Seegene, Seoul, South Korea) (table 1). The analysis showed a positivity with a cycle threshold above 35 for all the timings.

After 3 weeks of swab positivity, the Division of Laboratory Medicine (ASUGI, Trieste, Italy) decided to attempt the viral culture in order to unravel if the patient still presented infectious secretions.

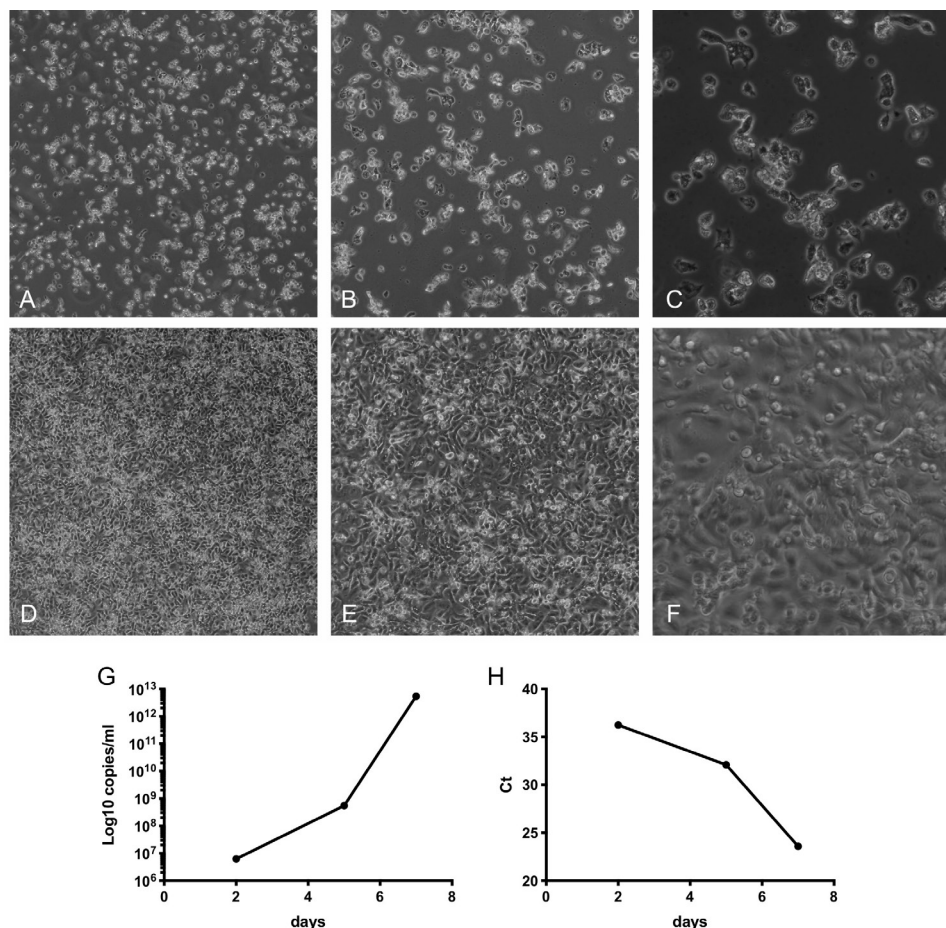
Briefly, the third swab (27 days after the first positivity) was filtered and transferred to a monolayer of Vero E6 cells and then monitored for 7 days. The molecular detection of SARS-CoV-2 by RT-PCR showed a decrement of cycle threshold and a relative increment of the viral load at days 2, 5 and 7 after the virus inoculation (from E+4 copies/mL to E+10 copies/mL, figure 2), as well as an evident cytopathic effect at the seventh day (figure 2).

Since the patient remained positive at the subsequent swab, the viral isolation was further tried but unsuccessfully. Concomitantly, the serologic test, performed with a chemiluminescent microparticle immunoassay (CMIA, SARS-CoV-2 IgG assay, Abbott, Chicago, Illinois, USA) displayed a signal to cut-off ratio of 7.74 for anti-nucleocapsid IgG. Therefore, the serum was tested against SARS-CoV-2 *in vitro*, showing the viral neutralisation even at a dilution of 1:100 (figure 3). At this time the patient still presented respiratory symptoms, but probably the immune system was now able to partially counteract the infection, since the viral isolation was not achieved. Nevertheless, the sixth swab (42 days) resulted in a new positivity, with a high Ct, however the virus replication *in vitro* failed again. Finally, the seventh swab (48 days) resulted as negative at the molecular test and the patient was released from isolation.

WHO criteria for patient discharge recommended the ending of isolation 10



**Figure 1** Schematic representation of the disease course of the patient.



**Figure 2** Cytopathic effect of the swab at the seventh day postinoculation on the Vero E6 cells. Vero E6 cells infected with the swab are displayed: (A) 10× magnification, (B) 20× magnification, (C) 40× magnification. Vero E6 cells not treated are shown for comparison: (D) 10× magnification, (E) 20× magnification, (F) 40× magnification. The viral load assessed through reverse-transcriptase-PCR at days 2, 5 and 7 post virus inoculation on Vero E6 cell culture. The viral load was displayed as viral copies/mL (G) and cycle threshold (H). About 15 µL of supernatant were mixed with 45 µL of distilled water and thermolysed (98°C for 3' and 4°C for 5'), then the viral load was determined using primers and probe for N gene (nucleocapsid, 500 nM forward primer GGG AGC CTT GAA Tac ACC AAA a, 500 nM reverse primer TGT AGC ACG att GCA GCA ttg, 125 nM probe FAM-AYC ACA ttg GCA CCC GCA ATC CTG-BHQ1), with the Luna Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, Massachusetts, USA) on the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following manufacturer's instruction. The nCoV-CDC-Control Plasmid (Eurofins, Luxembourg) was employed to generate the standard curve.<sup>6,7</sup>

days after symptom onset, plus at least three additional days without symptoms (including fever and respiratory features),<sup>4</sup> nevertheless prolonged viral RNA presence can occur.

In the present case report, viral infectivity was observed after 27 days from the first swab obtained in an asymptomatic phase. Noteworthy, the subsequent attempts to isolate the virus failed, probably due to a positive tail of viral RNA not linked with the presence of infectious virions, and therefore not able to establish infection *in vitro* and probably not contagious. Moreover, it can be speculated that initially the patient was not able to produce neutralising antibodies but after some weeks the immune response became efficient in counteracting the virus. An immunophenotypic characterisation (not available for this study), like the analysis

of the CD8+ T cells<sup>5</sup> could add more information about the cellular immune response activated by the patient. Possible issue related to technical aspects should be also considered, as the swab collection, storage and processing that may have not maintained the virus viable.

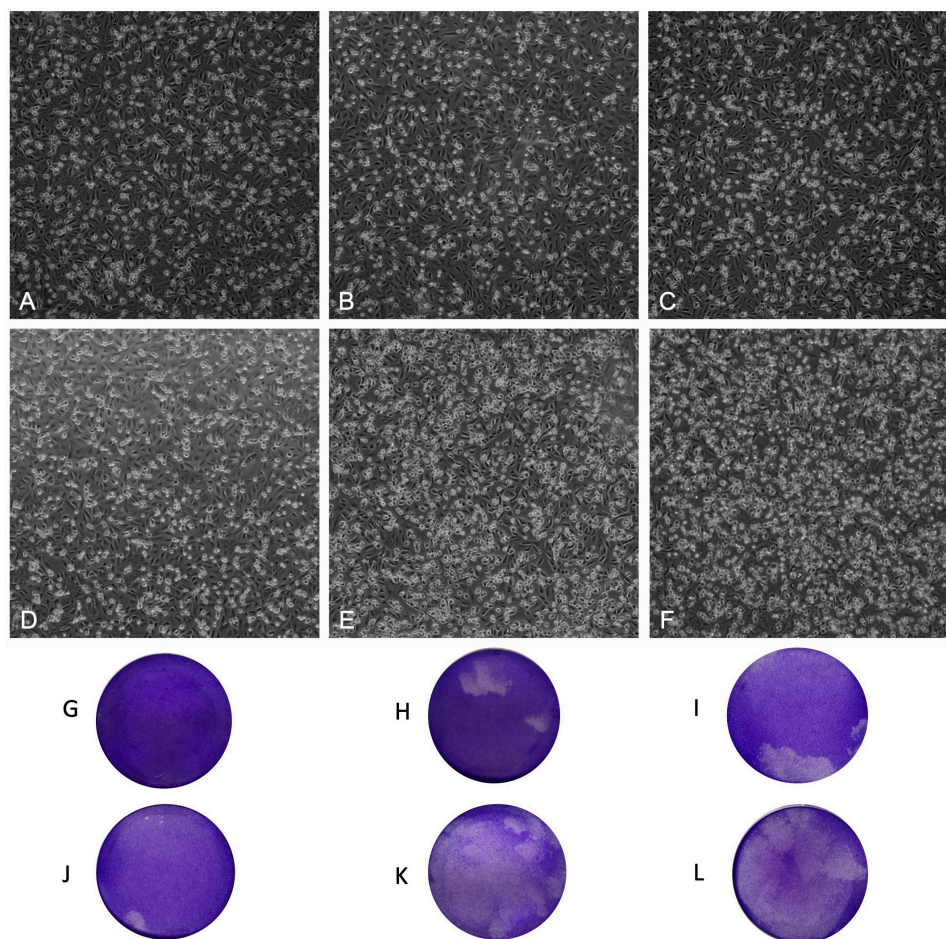
The duration of infectivity after the symptoms upset is still a matter of debate in the scientific community, indeed, the positivity at the molecular test does not imply that the virus is intact (the RT-PCR investigated a small viral genome fraction) and still infectious. A deep understanding of the COVID-19 infection course will help to interpret the virus shedding kinetics and the potential contagiousness of the affected individuals, indeed, in the case here described, the transmission of SARS-CoV-2 to patient's cohabitants occurred, although the Ct at

the rRT-PCR were high and the patient still asymptomatic at the beginning of the COVID-19.

Our results also showed that in this case there is no correlation between the Ct and the residual infectivity, since all the swabs yielded a weak positivity during the course of COVID-19. Moreover, very interestingly, although the specimen collected at the third analysis revealed a high Ct value at the rRT-PCR (above 35), it was still able to lead to viral isolation *in vitro* even after 27 days from the first positive molecular test.

Being aware that we just described a single case, our findings suggested that it is necessary to not underestimate individuals with weak positivity and to consider all the patients affected with COVID-19 as potential spreaders.





**Figure 3** Neutralisation assays. Pictures at 20× magnification and crystal violet staining are shown. SARS-CoV-2 was neutralised with serum diluted at 1:10 (A, G), 1:50 (B, H), 1:100 (C, I). Panels D, J showed the not treated well, meanwhile panel E, K and F, L the wells infected with SARS-CoV-2 (100 PFU). A signal to cut-off ratio of 1.5 of antinucleocapsid IgG was detected by using the SARS-CoV-2 IgG chemiluminescent microparticle immunoassay (CMIA, Abbott, Chicago, Illinois, USA) on the architect i2000SR instrument (Abbott, Chicago, Illinois, USA). Therefore, neutralisation assay was performed. Briefly, serum was inactivated for 30' at 56°C and then diluted at 1:10, 1:50 and 1:100 with MEM (Minimum Essential Medium with Earle's Salts, supplemented with 2% fetal bovine serum, 8 mM glutamine, 100 U/mL penicillin, 100 mg/L streptomycin and 2.5 µg/mL amphotericin B, Euroclone, Pero, Italy). About 200 µL of diluted serum was incubated with 200 µL of SARS-CoV-2 (200 PFU) for 1 hour at 37°C, then 200 µL were transferred to Vero E cells (200 000 Vero cells in 12 multiwell plates) for 1 hour at 37°C. At the end of the experimental procedures, the supernatants were removed and replaced with a new one composed by MEM (Minimum Essential Medium with Earle's Salts, 2% fetal bovine serum, 8 mM glutamine, 100 U/mL penicillin, 100 mg/L streptomycin and 2.5 µg/mL amphotericin B, Euroclone, Pero, Italy): carboxy methylcellulose (C5678, Sigma Aldrich, Saint Louis, Missouri, USA) (1:1) for 3 days. At the end of the 3 days, the supernatants were removed and the cells were fixed in paraformaldehyde 4% in phosphate buffer saline for 20' and stained with crystal violet for 30'.

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**Handling editor** Tahir S Pillay.

**Acknowledgements** We are grateful to TCR Tecora—Pollution Check (Cogliane, MB) for providing the BLS3 facility with a microscope.

**Contributors** LZ and LC were involved in performing the experiments and writing of original draft. FF and MR were involved in supervision of the experiments conducted in the BLS3 facility, writing the review and editing. PB-B was involved in patient management. SC was involved in conceptualisation of the study, writing the review and editing.

**Funding** This work was supported by IRCCS Burlo Garofolo/Italian Ministry of Health (RC 15/2017, 03/2020, 47/2020).

**Competing interests** None declared.

**Patient consent for publication** Obtained.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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**To cite** Zupin L, Fontana F, Clemente L, et al. *J Clin Pathol* Epub ahead of print: [please include Day Month Year]. doi:10.1136/jclinpath-2021-207394

Received 5 January 2021

Accepted 14 June 2021

*J Clin Pathol* 2021;0:1–4.  
doi:10.1136/jclinpath-2021-207394

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