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Real-life lack of evidence of viable SARS-CoV-2 transmission via inanimate surfaces: The SURFACE study

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ABSTRACT

Introduction: Although the potential role of inanimate surfaces in SARS-CoV-2 transmission has yet to be adequately assessed, it is still routine practice to apply deep and expensive environmental disinfection protocols. The aim of this study was to verify the presence of viable virus on different surfaces exposed to droplets released by coughing in SARS-CoV-2 RNA positive patients.

Methods: Patients admitted to hospital with a positive SARS-CoV-2 real-time (RT)-PCR swab were asked to cough on steel, cardboard, plastic and their hands. Surfaces were tested at baseline (T_0) and at different timepoints thereafter using swabs dipped in medium, and quickly seeded on VERO E6 cells that were checked every other day for cytopathic effect (CPE). Laboratory-propagated SARS-CoV-2 strains were examined at the same time points and on identical materials.

Results: Ten RNA-positive patients were enrolled into the study. The median cycle threshold value was 20.7 (range 13–28.3). Nasopharyngeal swabs from 3 of the patients yielded viable virus 2–10 days post-inoculation. However, in none of the patients was it possible to isolate viable SARS-CoV-2 from sputum under identical experimental conditions. A CPE was instead already visible using laboratory-propagated SARS-CoV-2 strains at 20′, 60′, 180′ while an effect at 24 h required a 6-day incubation.

Conclusion: The evidence emerging from this real-life study suggests that droplets delivered by SARS-CoV-2 infected patients on common inanimate surfaces did not contain viable virus. In contrast, and in line with several laboratory-based experiments, *in vitro* adapted viruses could survive and grow on the same fomites.

Introduction

Severe acute respiratory Coronavirus 2 (SARS-CoV-2) is a human coronavirus first isolated in China in December 2019 that rapidly spread worldwide to become a pandemic in March 2020 as declared by the World Health Organization [1]. Since then, several public health interventions were implemented to contrast the pandemic without tangible success [2]. The development and distribution of vaccines, and the implementation of barrier measures were instrumental in reducing SARS-CoV-2 spreading [3,4]; however, the virus keeps circulating in the community and in hospital settings [5]. A better understanding of the modes of transmission is of paramount importance to help reducing the spread of the infection [6]. One hypothetical transmission mode is *via* fomites since SARS-CoV-2 has been shown to survive for several hours on different surfaces under strict laboratory conditions. Indeed, most studies used aerosols of SARS-CoV-2 strains that were propagated and stocked in the laboratory and used at high concentrations [8–10]. Such potentially contaminated inanimate surfaces may be able to transmit the infection upon touching [7]. Sterilising with UV irradiation or high

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temperatures is generally not possible in a communal setting, thus making these countermeasures unfeasible to contrast the spread of SARS-CoV-2. Last but not least, as the development of new variants hinders the efficacy of the vaccines changing the virus behaviour [11], it is even more important to assess the ability of SARS-CoV-2 to survive in the environment.

Materials and methods

Virus handling and SARS-CoV-2 isolation

All procedures of SARS-CoV-2 isolation, propagation and titration were performed by experienced personnel in a Biosafety Level 3 (BSL3) laboratory. The SARS-CoV-2 European Wild Type strain (D614G, B.1) and omicron variant (BA.1) used in this study for the *in vitro* evaluation of virus survival on fomites, were isolated from nasopharyngeal swabs of symptomatic patients, while, for the *ex-vivo* evaluation of SARS-CoV-2 survival, COVID-19 patients' sputum was used. The patients were asked to cough on the designated surfaces, including their own hands, to produce enough sputum to cover about one cm^2, which was then sampled at each defined time point. Swabs were preserved in transport medium for a maximum of 24 h at 4 °C, then 200 μL from each sample were decontaminated with 50 μL of antibiotics and antifungals (penicillin [50 μg/mL], streptomycin [50 μg/mL], gentamicin [50 ug/mL], neomycin [50 ug/mL], amphotericin B [2.5 μg/mL], glutamine 0.1 %, foetal calf serum 0.05 %, $NaHCO_3$ 0.4 %) for 30 min at room temperature to remove contaminants. The decontaminated samples were then seeded on confluent VERO E6 cells (VERO C1008 (Vero 76, clone E6, Vero E6); ATCC® CRL-1586™) in a 24-well flat-bottomed tissue-culture microtitre plate (COSTAR, Corning Incorporated, Corning, NY, USA) and incubated at 33 °C in an atmosphere of 5 % CO_2 in air for 1 h. After incubation and inoculum removal, fresh Eagle's MEM (EMEM, Lonza Group Ltd, Basel, Switzerland) supplemented with 1 % v/v penicillin, streptomycin and glutamine (Euroclone SpA) and 0.1 % v/v trypsin, was added before incubation, under the same conditions. The plates were then incubated at 33 °C in 5 % CO_2 and checked every other day for the appearance of a cytopathic effect (CPE).

Whole genome sequencing

All variants for *in vitro* study were identified by complete genome sequencing [12] in order to confirm the presence of variant-defining mutations. Sequences were submitted to GISAID.

Virus titration for in vitro evaluation of SARS-CoV-2 survival

After propagation in a 25 cm^2 cell flask (Corning, NY14831, USA), the titre of each variant was defined as the 50 % tissue culture infectious dose (TCID50) in six replicate wells of a 96-well flat-bottomed tissue-culture microtitre plate. Logarithmic dilutions of stock virus were incubated for 72 h in the presence of 3×10^4 VERO E6 at 33 °C in 5 % CO_2. Cells were observed by light microscopy for CPE development and stained with Gram's crystal violet solution (Merck KGaA, Darmstadt, Germany) plus 5 % v/v formaldehyde 40 % m/v (Carlo Erba SpA, Arese, Italy). The value of TCID50 mL^{-1} was calculated with the Reed–Muench method [13].

Quantitative real-time PCR (RT-PCR)

To identify SARS-CoV-2 positive patients a RT-PCR was performed using in house developed primer sets as previously reported [14]. Briefly, total RNA was extracted using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions. The extracted RNA was subjected to a one-step RT-PCR using the SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Cycle threshold (Ct) values were used to screen eligible patients. All patients with a Ct below 30 were included in the study after providing their written informed consent.

Sampling

Patients were asked to provide a sputum sample, immediately after diagnosis of SARS-CoV-2 positivity. The sputum was subsequently distributed on different surfaces, including the palm of the patient's hand, a metal hospital food trail, a piece of cardboard and a piece of plastic. The contaminated surfaces, including the patient's hand, were then secured in plastic bags to preserve the sputum. At each defined time point (20′, 60′, 180′ and 24 h) the residual sputum was sampled using a swab, dipped in 3 mL of medium and immediately transported to the BSL3 laboratory for further processing.

Viral isolation from sputum and fomites

At each time point, 200 μL from each sampled swab were used to verify SARS-CoV-2 viability as reported in the section 'SARS-CoV-2 isolation'. Every day, the plates were observed under an inverted microscope, 10 × magnification, to assess the appearance of CPE. Plates were kept for a maximum of 10 days, and fresh medium was added every 3–4 days to the wells in which no CPE was visible. For the *in vitro* analysis using in-lab propagated SARS-COV-2 variants, at the same time points, 40 μL of 100, 1000 and 10,000 TCID50 were placed on 2×4 cm^2 stainless steel, plastic and cardboard surfaces. These were kept inside sterile sealable containers and secured in the Glow-Box hood for the duration of the experiment without ventilation. At each time point the inoculum was resuspended in 400 μL Eagle's MEM, supplemented with 1 % v/v penicillin, streptomycin and glutamine (Euroclone SpA) and 0.1 % v/v trypsin, optimal for respiratory virus growth, of which 200 μL were used for SARS-COV-2 isolation.

Results

A total of 10 patients (6 males and 4 females, median age 61.5 years, range 23.2–79.4 years) were enrolled in the study. Ct value range was 13–28.3, median 20.7. Viable SARS-CoV-2 was isolated from the nasopharyngeal swabs of three patients at T_0, but not from the remainder. The three nasopharyngeal swabs had Ct values equal to 13, 28.3 and 18.7 and were positive at 10-, 2- and 6-days post-inoculation, respectively. A SARS-COV-2-like CPE was observed in only one patient at T_0 on cardboard, but molecular analysis on the sample failed to amplify viral RNA. In none of the patients was it possible to isolate viable SAR-COV-2 from sputum at any of the predefined time points (Table 1). In contrast, laboratory-adapted SAR-COV-2 strains, the European Wild Type strain D614G, B.1

Table 1
Overall results of SARS-COV-2 Isolation from sputum on fomites.

Ex vivo Isolation					
Time	20 min	60 min	180 min	24 h	Days post-inoculum
Plastic	0/10	0/10	0/10	0/10	1–5
	0/10	0/10	0/10	0/10	6–10
	0/10	0/10	0/10	0/10	> 10
Paper	0/10	0/10	0/10	0/10	1–5
	0/10	0/10	0/10	0/10	6–10
	0/10	0/10	0/10	0/10	> 10
Metal	0/10	0/10	0/10	0/10	1–5
	0/10	0/10	0/10	0/10	6–10
	0/10	0/10	0/10	0/10	> 10
Skin	0/10	0/10	0/10	0/10	1–5
	0/10	0/10	0/10	0/10	6–10
	0/10	0/10	0/10	0/10	> 10

Table 2
In vitro isolation of lab-adapted SARS-COV-2 Wild type B.1 strain from fomites.

Time	20 min			60 min			180 min			24 h			Days Post inoculum
TCID50	100	1000	10,000	100	1000	10,000	100	1000	10,000	100	1000	10,000	
Plastic	–	–	–	–	–	–	–	–	–	–	–	–	1
	–	–	–	–	–	–	–	–	–	–	–	–	2
	3/3	3/3	3/3	3/3	3/3	3/3	–	3/3	3/3	–	–	–	3
	–	–	–	–	–	–	–	–	–	–	–	3/3	4
	–	–	–	–	–	–	3/3	–	–	3/3	3/3	–	5
	–	–	–	–	–	–	–	–	–	–	–	–	6
	–	–	–	–	–	–	–	–	–	–	–	–	7
Cardboard	–	–	–	–	–	–	–	–	–	–	–	–	1
	–	–	–	–	–	–	–	–	–	–	–	–	2
	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	–	–	–	3
	–	–	–	–	–	–	–	–	–	–	–		4
	–	–	–	–	–	–	–	–	–	–	–	3–3	5
	–	–	–	–	–	–	–	–	–	2/3	3/3	–	6
	–	–	–	–	–	–	–	–	–	1/3	–	–	7
Metal	–	–	–	–	–	–	–	–	–	–	–	–	1
	–	–	–	–	–	–	–	–	–	–	–	–	2
	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	–	–	–	3
	–	–	–	–	–	–	–	–	–	–	–	–	4
	–	–	–	–	–	–	–	–	–	3/3	1/3	3/3	5
	–	–	–	–	–	–	–	–	–	–	2/3	–	6
	–	–	–	–	–	–	–	–	–	–	–	–	7

Table 3
In vitro isolation of lab-adapted SARS-CoV-2 BA.1 strain from fomites.

Time	20 min			60 min			180 min			24 h			Days Post inoculum
TCID50	100	1000	10,000	100	1000	10,000	100	1000	10,000	100	1000	10,000	
Plastic	–	–	–	–	–	–	–	–	–	–	–	–	1
	–	–	–	–	–	–	–	–	–	–	–	–	2
	3/3	3/3	3/3	3/3	3/3	3/3	–	3/3	3/3	–	–	–	3
	–	–	–	–	–	–	3/3	–	–	–	–	3/3	4
	–	–	–	–	–	–	–	–	–	3/3	3/3	–	5
	–	–	–	–	–	–	–	–	–	–	–	–	6
	–	–	–	–	–	–	–	–	–	–	–	–	7
Cardboard	–	–	–	–	–	–	–	–	–	–	–	–	1
	–	–	–	–	–	–	–	–	–	–	–	–	2
	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	–	–	–	3
	–	–	–	–	–	–	–	–	–	–	–	3/3	4
	–	–	–	–	–	–	–	–	–	3/3	3/3	–	5
	–	–	–	–	–	–	–	–	–	–	–	–	6
	–	–	–	–	–	–	–	–	–	–	–	–	7
Metal	–	–	–	–	–	–	–	–	–	–	–	–	1
	–	–	–	–	–	–	–	–	–	–	–	–	2
	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	–	–	–	3
	–	–	–	–	–	–	–	–	–	–	–	–	4
	–	–	–	–	–	–	–	–	–	–	–	2/3	5
	–	–	–	–	–	–	–	–	–	3/3	3/3	1/3	6
	–	–	–	–	–	–	–	–	–	–	–	–	7

(Table 2) and omicron subvariant BA.1 (Table 3) remained viable and produced CPE in each of the *in vitro* experimental conditions at different time points after inoculation.

Discussion

In this study, we provide definitive real-life evidence that contamination of inanimate surfaces with viable SARS-CoV-2 should be regarded as exceedingly rare and very short lasting. This is in contrast with data using laboratory-generated viruses, which showed robust and long-lasting virus viability.

Patient enrolment began in March 2022 when the prevalence of the omicron variant reached 98.3 %. The omicron variant (subvariants BA.1, BA.2, BA.2.12.1, BA. 4 and BA.5) involved new mutations in the spike protein, most of which are in the receptor binding domain (RBD), increasing its transmissibility which resulted in a decreased monoclonal antibody and vaccine response due to reduced fusogenesis [15]. Moreover, omicron shows a wider diffusion potential than previously isolated variants of concern (VOCs) and does not preferentially affect the lower respiratory tract, being characterised by a non-TMPRSS-dependent cellular entry mechanism [15]. Furthermore, it is usually associated with milder symptoms than other VOCs in immunocompetent individuals [16–18].

Previous studies found that the recovery of infectious virus from cell culture inversely correlated with the presence of SARS-CoV-2 specific IgG in the respiratory tract, specific for both prior VOCs or monovalent vaccines [19]. Moreover, recovery of infectious virus was more frequent from samples with lower mean Ct values, regardless of the subvariant whereas, when Ct values < 20 were considered, BA.5 showed a statistically higher recovery rate of infectious virus compared with other subvariants [20]. In our study, we did not find viable virus on different surfaces, even if a low Ct was obtained from nasal swabs of the enrolled individuals. We can speculate that vaccine-induced or hybrid immunity may have played a role, even though previous findings failed to show viable virus on inanimate surfaces in hospital settings, before the advent of mRNA vaccines

[21]. Indeed, our patients were all vaccinated, enjoying robust protection by hybrid immunity.

Another interesting hypothesis regarding reduced virus viability in real-life conditions is provided by a recent study showing that mucin inhibits the infection of cells by human betacoronavirus OC43 in a concentration- and glycan-dependent manner [21]. This observation is consistent with low rates of fomite transmission of SARS-CoV-2 in the real-world [22], and suggests that mucins may be one culprit.

Despite dichotomous findings were clearly produced in real-life and laboratory conditions, there is still uncertainty on the presence of SARS-CoV-2 in the nosocomial and household settings, as some studies found extensive SARS-CoV-2 RNA contamination on inanimate surfaces in hospitals dedicated to patients with COVID-19 [24,25], while others did not [23]. Furthermore, a recent systematic review found that there is currently a lack of studies assessing SARS-CoV-2 viability on inanimate surfaces [26]. Therefore, until now, it was impossible to assess the real transmission potential of SARS-CoV-2 *via* surfaces.

Our study has been designed to precisely fill this gap of knowledge, comparing real-life with laboratory-based conditions. Indeed, some of the existing studies in the field are apparently at variance with our findings, providing direct evidence of SARS-CoV-2 viability on fomites for a length of time consistent with the possibility of onward transmission [27,28]. However, it must be emphasised that in those studies viable SARS-CoV-2 could be retrieved from fomites that were in continuous and long-lasting close contact with the patients, such endotracheal tubes [27]. Furthermore, hands, which in another study had shown a high infectious potential [29], were consistently negative for viable virus in our real-life study. omicron's markedly different behaviour from previously isolated VOCs, may partially explain discrepancies with previous VOCs that were apparently characterised by a more prolonged viral persistence on fomites, using both laboratory adapted and infected patients' sputum-derived SARS-CoV-2 [3].

Although prudence suggests not to let our guard down, our findings provide evidence in support of somehow debunking the role of inanimate surfaces in SARS-CoV-2 transmission.

Conclusions, strengths and limitations

To our knowledge, this is the first study reporting a head-to-head comparison of *in vitro* propagated and *ex vivo* isolated SARS-CoV-2 survival in the environment under similar experimental conditions. With respect to the *ex vivo* experiments we aimed at reproducing real-life conditions using different inanimate materials and the patients' own hands. Moreover, the initial studies on SARS-CoV-2 contamination of inanimate surfaces were performed early in the course of the pandemic, before the appearance of VOCs. Thus, this study, which was designed at a time in which omicron subvariants prevailed, represents a significant new addition to the field. Finally, the study also focused on the patients' skin and not only on fomites, therefore mimicking a previously unexplored real-life setting.

It may be argued that the role of superspreaders has not been investigated. There are several factors that determine how someone transmits a virus. There are some people who, because of their own personal biology, they produce a higher percentage of aerosols *versus* respiratory droplets. The hypothesis is that some of these people are super-emitters, and they are responsible for superspreading events. We believe that in this setting, in which patients were asked to cough on surfaces under controlled conditions, data variability according to different virus-emitting potential is somehow attenuated.

Our main limitation is the small sample size, which is mostly attributable to low patients' compliance, and stringent Ct values cut-off. Moreover, we were unable to collect enough material from the sputum samples to perform both isolation of viable virus and molecular quantitation of SARS-COV-2 copies. However, the high reproducibility of the data significantly attenuates this relative limitation. We also lacked an *in vitro* replacement for the skin contamination setting, for obvious reasons. However, in consideration of the results obtained by Hirose R. et al. [3] and our own previous data [23], we see this as a minor limitation of the study.

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Conflict of interest

We have no conflict of interest to declare.

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