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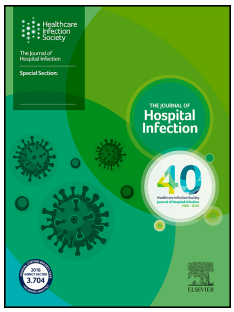

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Factors affecting stability and infectivity of SARS-CoV-2

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Keywords: SARS-CoV-2, stability, infectivity, high infection rate, route of transmission

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Summary

Background

In late 2019, a novel human coronavirus, SARS-CoV-2, emerged in Wuhan, China. This virus has caused a global pandemic involving more than 200 countries. SARS-CoV-2 is highly adapted to humans and readily transmits from person-to-person.

Aim

The aim of this study was to investigate the infectivity of SARS-CoV-2 under various environmental factors, disinfectants and different pH conditions. The efficacy of a variety of laboratory virus inactivation methods and home disinfectants against SARS-CoV-2 were investigated.

Methods

The residual virus in dried form or in solution was titrated on Vero E6 cell line at day 0, 1, 3, 5, and 7 after incubation at different temperatures. The viability of virus was determined after treatment with different disinfectants and pH solutions at room temperature (20~25^{o}C).

Findings

SARS-CoV-2 was able to retain viability for 3-5 days in dried form or 7 days in solution at room temperature. SARS-CoV-2 could be detected under a wide range of pH conditions from pH4 to pH11 for several days and 1 to 2 days in stool at room temperature but lost 5 logs of

infectivity. A variety of commonly used disinfectants and laboratory inactivation procedures were found to reduce viral viability effectively.

Conclusion

This study demonstrates the stability of SARS-CoV-2 on environmental surfaces and raises the possibility of faecal-oral transmission. Commonly used fixatives, nucleic acid extraction methods and heat inactivation were found to significantly reduce viral infectivity that could ensure hospital and laboratory safety during the COVID-19 pandemic.

Introduction

The first human coronavirus of confirmed zoonotic origin, SARS-CoV-1, rose in 2003. It spread in over 30 countries and caused severe acute respiratory syndrome (SARS) [1]. Sixteen years later, coronavirus disease 2019 (COVID-19) emerged in Wuhan, China. COVID-19 is caused by another zoonotic coronavirus: SARS-CoV-2 [2, 3]. SARS-CoV-2 belongs to the beta-coronavirus lineage B and shares ~ 80% identity to SARS-CoV-1. SARS-CoV-2 is currently causing a global pandemic which, as of mid-April 2020, has affected more than 2 million people and killed more than 150,000 people [4]. The actual number of infected cases is believed to be higher due to limitation of testing to persons requiring hospitalization in several countries during the early stages of the pandemic. It is estimated that 18% of infections are asymptomatic [5]. According to current estimates, the case fatality rate of COVID-19 infection is lower than that of SARS. However, due to its propensity to cause milder infections, SARS-CoV-2 spreads more efficiently in communities in the absence of rigorous social distancing measures. Previous findings showed that the viability of SARS-CoV-1 degraded and was rapidly lost at higher temperatures and higher relative humidity [6]. This may have impaired its transmission in tropical areas such as Malaysia, Indonesia or Thailand. Judging by the rapidity of its spread, SARS-CoV-2 infection appears less affected by hot weather and high humidity prevailing in Asian countries including Malaysia, Thailand and Singapore [4]. However, it is notable that their SARS-CoV-2 incidence rate appears lower than countries in Europe or USA [4].

Understanding the viability of SARS-CoV-2 in various environmental conditions and the effectiveness of disinfectants against it is crucial. This is particularly relevant to hospital settings, where highly effective viral inactivation methods are required in wards nursing

COVID-19 patients and laboratories processing samples from COVID-19 patients. In this study, stability of SARS-CoV-2 under various environmental factors and pH conditions were tested. We also investigated the effect of various disinfectant solutions and laboratory inactivation methods on SARS-CoV-2 viability. These factors could play a major role in transmission of disease and might suggest methods to stop the spread of the virus.

Materials and methods

1. Virus strains and cell line.

Vero E6 cell line was cultured in minimal essential medium (MEM, Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA), penicillin and streptomycin (Gibco, USA). Virus strains used in the study were SARS-CoV-2 HKU-SZ-005b and SARS-CoV-1 HKU39849 [6,7]. Virus propagated in Vero E6 was maintained in MEM with 1% FBS, and was stored at -80°C until use.

2. The Median Tissue Culture Infectious Dose ($TCID_{50}$) assay.

Confluent Vero E6 cells on 96-well plates were incubated with 100 µl of serial 10-fold dilutions of virus in MEM containing 1% FBS for 1 hour at 37°C. Then, the virus was removed from 96-well plates and 100ul of fresh MEM with 1% FBS was added to the cells. After the change of medium, cells infected with SARS-CoV-2 underwent an incubation of 5 days, while SARS-CoV-1 infected cells underwent a 3-day incubation, and cytopathic effect (CPE) was recorded. $TCID_{50}$ was determined by the Reed and Muench method [8].

3. Effect of drying and heat.

Ten µl of virus (SARS-CoV-2, $10^{6.5}$TCID50/ml; SARS-CoV-1, $10^{7}TCID_{50}$/ml) was placed on a glass slide within a shell vial, kept at room temperature (20~25^{o}C and relative humidity of 63%) and allowed to dry according to our previous study with slight modifications (6). One hundred microliters of MEM were used to re-suspend the virus for 0, 1, 3, 5, and 7 days after incubation at different temperatures: refrigerator (4^{o}C), room temperature (25^{o}C) and two incubators with different temperatures (33^{o}C and 37°C). All the time points were set up in

triplicate and was undertaken in the dark. The residual virus infectivity was titrated (8). Controls were viruses in solution, and stored in closed screw cap tubes with similar treatment.

4. Effect of pH on viability

Viral transport medium with different pH from 2 to 13 using 5M and 1M HCl or 5N and 1N NaOH were prepared as described (9). One hundred microliters of SARS-CoV-2 with $10^{6.5}TCID_{50}$/ml was added into each bottles of 0.9 ml VTM and incubated at room temperature (20-25^{o}C). All the tests were done in triplicates. The viability of virus was tested on day 1, day 3 and day 6. On each testing day, the pH of the VTM bottles were neutralized to pH 7 and viral titre was measured using the TCID50 assay (8). An untreated virus stock solution as the viral load for the positive control was included.

5. Stability in stool

One hundred microliters of virus with $10^{6.5}TCID_{50}$/ml was added to 0.9 ml watery stool derived from a human patient (10). Antibiotics (Vancomycin 100 µg/ml, Amikacin 90 µg/ml and nystatin 40 units/ml) were added to suppress any potential bacterial or fungal growth. The experiment was set up in duplicates. The viability of the virus was titrated as described above [8]. An untreated virus stock solution as the viral load for the positive control was included.

6. Stability in disinfectants

Thirty microlitres of SARS-CoV-2 ($10^{6.5}TCID_{50}$/ml) and 270 µl of various disinfectants were mixed and incubated at room temperature (Table 1). After incubation for 1 minute and 5

minutes at room temperature (20~25°C), 900 µl of MEM with 1% FBS was added in 100 µl of virus-disinfectant mixture to dilute the disinfectants effect immediately before determination of residual virus infectivity by the $TCID_{50}$ assay as described [8]. All disinfectants without virus was titrated in parallel to determine the cytotoxicity effect. An untreated virus stock solution as the viral load for the positive control was also included.

7. Heat inactivation of SARS-CoV-2

Thirty microlitres of SARS-CoV-2 ($10^{5.5}TCID_{50}$/ml) and 270 µl of FBS were mixed and incubated at 56°C for 30min. then, the residual infectivity of the virus was determined by TCID50 assay as described above. The test was set up in triplicates.

8. Viability after fixation treatment

Vero E6 cells were infected with SARS-CoV-2 at one multiplicity of infection (MOI) in a 6 well-plate for two days. The infected cells were scraped, spotted on slides and dried. The fixed smears were fixed with chilled acetone (VWR Chemicals BDH, USA) for 10 minutes at -20°C or 4% paraformaldehyde for 30 minutes at room temperature. The dried acetone and paraformaldehyde fixed smears were washed twice in PBS to remove residual fixatives. The inactivation effects of these fixative were monitored by scraping cells from fixed smears onto culture tube with VeroE6 cells. Cytopathic effect was examined up to 7 days and then antigen expression of NP of COVID-19 was tested [11].

Results

Dried SARS-CoV-2 retained viability for 3~ 5 days at room temperature (20 ~25^{o}C) with prolonged survival for more than 14 days at 4^{o}C (Fig 1). The virus lost its infectivity within 1 day at warmer temperatures (~37°C). SARS-CoV-2 in solution retained viability for 7 days at room temperature (20~25°C) and remained viable up to 14 days at 4^{o}C. The virus suspended in solution retained viability for 1~2 days at hot temperature 33~37^{o}C. In comparison, SARS-CoV-1 had similar viability as SARS-CoV-2 at the same environmental conditions except that dried SARS-CoV-1 had better survival rates for 7 to 14 days at room temperature (20~25°C).

When SARS-CoV-2 was added in VTM with pH ranging from 2 to 13, the virus remained viable up to 6 days but lost between 2.9 and 5.33 logs of infectivity from pH5 to pH9 and up to 1~2 days in pH4 and pH11 (Table 2). The virus lost infectivity within 1 day at pH extremes (pH2~3 and pH11~12). The virus lost 5.25 logs of infectivity in stool over a 3-day period.

Laboratory or domestic disinfectants, including two commonly used as a lysis buffer for nucleic acid extraction, were tested for their effects on SARS-COV-2 on Vero E6 (Table 3). Due to the cytotoxicity of certain disinfectants, detection limit of inactivation had been found to range from 0.83 to 3.25 log10 reduction for 1 minute and 0.92 to 3.75 log10 reduction for 5 minutes. This showed that SARS-CoV-2, like SARS-CoV-1, can be inactivated by common laboratory or domestic disinfectants [10, 12, 13].

When the virus was added to 90% FBS or MEM and was heated at 56°C for 30 minutes, virus viability in both FBS and MEM was reduced by at least 3 logs (3.58±0.29). This mimics the

conditions of heat inactivation, which should effectively inactivate SARS-CoV-2 in human serum for use in immunoassays.

After treatment with chilled acetone or 4% paraformaldehyde, the viability of fixed culture cells was tested. No CPE was observed or virus was detected by NP antigen expression. As for SARS-CoV-1, chilled acetone is required to complete inactivation of SARS-CoV-2 infected cell smears [10]. In this study, both chilled acetone and 4% paraformaldehyde completely inactivated SARS-CoV-2, rendering fixed slides safe for further processing in a Biosafety Level 2 laboratory.

Discussion

The main transmission routes of SARS-CoV-2 are believed to be via (1) inhaling aerosols generated by infected persons, (2) direct contact with infected persons and, (3) contact with environmental fomites [13, 14]. Our study investigates infectiousness of the virus under a variety of environmental conditions. In this study, the dynamic rate of decay of SARS-CoV-2 was similar to SARS-CoV-1 (Fig 1). Dried SARS-CoV-2 virus on glass can retain viability for over 3~4 days at room temperature (22–25°C) and 14 days at cold temperature (4°C), but loses viability rapidly within one day at warm temperatures (37°C). However, SARS-CoV-2 in solution remained viable for longer under the same different temperature conditions compared with dried SARS-CoV-2. Our data demonstrated that SARS-CoV-2 could survive on environmental surfaces and that such contaminated surfaces may act as a reservoir for transmission of this virus if not adequately cleaned and disinfected.

These could explain large SARS-CoV-2 outbreaks such as the one on the Diamond Princess cruise ship. This outbreak caused 712 out of 3711 passengers to become infected with 12 deaths. This ship had been placed under quarantine orders from 5 February 2020 [5]. All passengers were confined in the ship with close contact during the quarantine period and shared common food and facilities such as buffet, water supply, shared sanitation and air-conditioning systems for many days. SARS-CoV-2 has been shown to have a longer half-life on stainless steel and plastic surfaces [14]. SARS-CoV-2 outbreaks also occurred in military warships in USA and France. Our study clearly illustrates how SARS-CoV-2 can cause long lasting environmental contamination in such settings.

In this study, we have demonstrated that SARS-CoV-2, like SARS-CoV-1, can survive in stool for up to 1 to 2 days but with a 5-log loss of viability [10]. This suggests that the viability is quickly lost in faecal material. SARS-CoV-2 is frequently shed in the stool of infected patients [15]. Due to the virus remaining viable under a wide range of pH and environmental conditions, we anticipate that it would be able to retain its infectivity in environmental surfaces and potentially even in infected food handlers shedding SARS-CoV-2 in faeces. Transmission via the faecal-oral route is theoretically possible, especially in individuals with reduced gastric acidity due to medications like proton pump inhibitors. In fact, the SARS-CoV-2 host receptor was found in the cytoplasm of gastrointestinal epithelia cells of infected patients [15] and 17.6% of patients with COVID-19 had gastrointestinal symptoms and virus RNA was detected in stool samples from 48.1% patients [16].

In this study, the results showed that a variety of commonly used disinfectants and laboratory inactivation procedures can reduce viral viability. This is particularly significant for healthcare settings including laboratories that require highly reliable inactivation methods to safeguard staff working with COVID-19 patients and samples. PCR assays, immunofluorescence staining and serology are all core components of the BSL-2 virology laboratory. Our study has confirmed that commonly used fixatives, nucleic acid extraction methods and heat inactivation can significantly abrogate viral infectivity. This study, therefore, has a direct impact on hospital and laboratory safety during the COVID-19 pandemic.

A limitation of our study is that residual cytotoxicity from disinfectants might have been present as we performed dilution rather than neutralization of active compounds before virus titration.

Conclusion

Our data presented here contribute to a better understanding of the stability of SARS-CoV-2 in different environmental situations. The stability of SARS-CoV-2 is similar to SARS-CoV-1. This study showed that SARS-CoV-2 can survive for days on contaminated environmental surfaces and for prolonged periods of time when in fluid suspensions. This has implication for infection transmission in healthcare, but also in terms of transmission related to food handlers and workers in meat and poultry processing facilities [17]. Finally, we show that commonly used viral inactivation methods in the clinical virology laboratory and disinfectant solutions used in healthcare settings are sufficient to drastically reduce viability of SARS-CoV-2, as a contribution to improve hospital safety

Conflict of Interests

None declared

Funding Source

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315

316 Legends

317 Figure 1 Stability of SARS-CoV-2 and SARS-CoV-1

318 a) Stability of SARS-CoV-2 in dried form b) Stability of SARS-CoV-2 in solution

319 c) Stability of SARS-CoV-1 in dried form d) Stability of SARS-CoV-1 in solution

320

321 Table 1 Disinfectants used in the study

322 USA: United States of America

323 UK: United Kingdom

324 HKSAR: Hong Kong Special Administrative Region

325 HKU: University of Hong Kong

326 DMDM Hydantoin: 1,3-Bis(hydroxymethyl)-5,5-dimethylimidazolidine-2,4-dione

327

328 Table 2 Effects of disinfectants on viability of SARS-CoV-2

329 * Include untreated virus stock solution as the viral load for the positive control

330 (TCID50/ml =6.50±0.61).

331 All tests were neutralized before testing and was set up in triplicates.

332 Positive = Culture positive

333 Negative = Culture negative

334 ND = Not done

335

336

337 Table 3 Effects of different pH condition on infectivity of SARS-CoV-2

338 *Include untreated stock solution as the viral load for the positive control ($TCID_{50}$/ml

339 = 6.50±0.61). The experiment was set up in triplicate

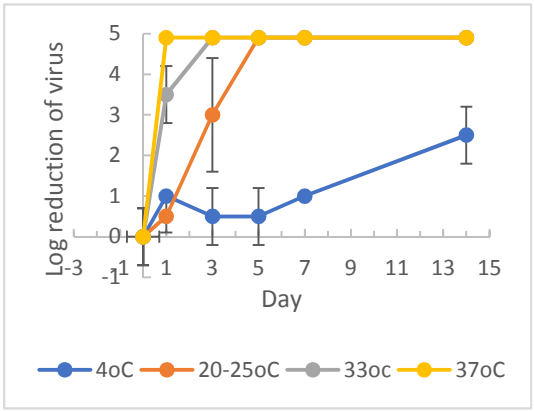
340

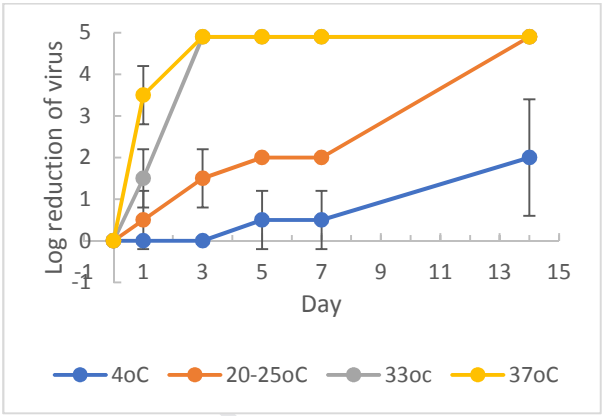
341

342 Figure 1 Stability of SARS-CoV-2 and SARS-CoV-1

343

344 a) Stability of SARS-CoV-2 in dried form b) Stability of SARS-CoV-2 in solution





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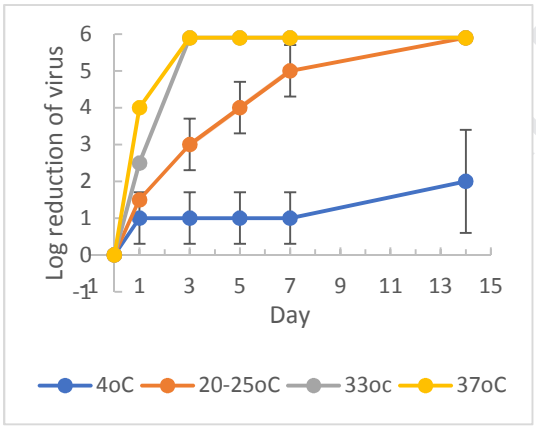
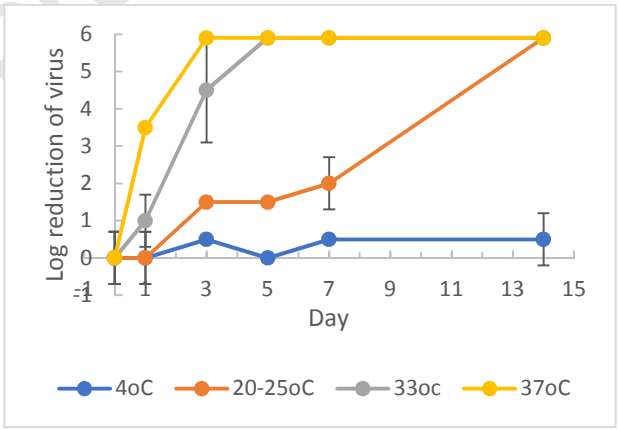
347 c) Stability of SARS-CoV-1 in dried form d) Stability of SARS-CoV-1 in solution





348

349 Table 1. Disinfectants used in the study

Disinfectant	Active ingredient	Supplier	Country or region
Ethanol (75%)	Ethanol 75%	VWR Chemicals BDH®	USA
Bleach (10%)	Sodium hypochlorite 10%,	Kao	Japan
Virkon (2%)	Potassium Peroxymonosulfate 21.41%, Sodium Chloride 1.5%	Lanxess	UK
Formalin (10%)	Formaldehyde 4%	Thermo fisher	USA
Lysis buffer (EasyMAG)	Guanidine thiocyanate 50%, Triton X-100 <2%, EDTA <1%	Biomerieux	France
AVL (viral lysis buffer)	Guanidine thiocyanate 50~70%	Qiagen	USA
Liquid hand soap	Biodegradable amphoteric surfactants and DMDM Hydantoin	Funchem	HKSAR
Hand wash	Sodium Laureth Sulfate, Cocamidopropyl betaine	Manning	China
Hand rub (WHO formula 1)	Ethanol 80% v/v, Glycerol 1.45% v/v, Hydrogen peroxide (H_2O_2) 0.125% v/v	HKU in-house	HKSAR
Advanced hand sanitizer	Ethyl Alcohol 70%	Purell	USA
Disinfection solution	Sodium hypochlorite 0.002% and hypochlorous acid 0.013%	Dermo Dacyn	USA
Hand wash	Chloroxylenol (PCMX)	Walch	Germany

350 USA: United States of America

351 UK: United Kingdom

352 HKSAR: Hong Kong Special Administrative Region

353 HKU: University of Hong Kong

354 DMDM Hydantoin: 1,3-Bis(hydroxymethyl)-5,5-dimethylimidazolidine-2,4-dione

355

Table 2 *Effects of different pH condition on infectivity of SARS-CoV-2

pH	Day 1 (Log_{10} Reduction ±SD)	Day 3 (Log_{10} Reduction ±SD	Day 6 (Log_{10} Reduction ±SD
2	Negative (6.50±0.00)	Negative (6.50±0.00)	ND
3	Negative (6.50±0.00)	Negative (6.50±0.00)	ND
4	Positive (2.67±0.29)	Negative (6.50±0.00)	Negative (6.50±0.00)
5	Positive (1.08±0.52)	Positive (2.33±0.29)	Positive (3.50±0.50)
6	Positive (1.00±0.50)	Positive (1.67±0.58)	Positive (4.10±0.85)
7	Positive (0.67±0.29)	Positive (1.50±0.50)	Positive (2.90±0.96)
8	Positive (1.23±0.25)	Positive (2.73±0.64)	Positive (3.92±0.63)
9	Positive (1.50±0.87)	Positive (3.23±0.68)	Positive (5.33±0.58)
10	Positive (2.40±0.36)	Positive (5.13±0.40)	Negative (6.50±0.00)
11	Positive (3.00±0.70)	Negative (6.50±0.00)	Negative (6.50±0.00)
12	Negative (6.50±0.00)	Negative (6.50±0.00)	ND
13	Negative (6.50±0.00)	Negative (6.50±0.00)	ND

* Untreated virus stock solution as the viral load for the positive control $TCID_{50}^{/}$/ml =6.50±0.61. All tests were neutralized before testing and conducted in triplicates.

Positive = Culture positive

Negative = Culture negative

ND = Not done

365 Table 3 Effects of disinfectants on viability of SARS-CoV-2

366

Disinfectants*	Log_{10} reduction	
	1 min	5 min
Ethanol (75%)	≥ 1.83 ±0.29	≥ 2.00 ±0.00
Bleach (10%)	≥3.25 ±0.00	≥3.25 ±0.00
Virkon (2%)	≥3.00 ±0.00	≥3.00 ±0.00
Formalin (10%)	≥1.25 ±0.00	≥1.25 ±0.00
Lysis buffer (EasyMAG)	≥2.00 ±0.43	≥2.25 ±0.00
AVL (Viral lysis buffer, Qiagen)	≥3.00 ±0.43	≥3.25 ±0.00
Liquid hand soap (Funchem)	≥2.00 ±1.56	≥2.25 ±0.00
Hand wash (Mannings)	≥ 0.83±0.29	≥ 0.92 ±0.38
Hand rub (WHO Formulation 1)	≥2.17 ±0.14	≥2.25 ±0.00
Advanced hand sanitizer (Purell)	≥2.50 ±0.0	≥2.50 ±0.0
Disinfecting solution (Dermo docyn)	2.30 ±0.50	3.75 ±0.43
Hand wash (Walch)	≥ 0.83 ±0.29	≥ 0.92 ±0.14

367

368 *Untreated virus stock solution as the viral load for the positive control ($TCID_{50}$/ml =

369 6.50±0.61). The experiment was set up in triplicates.

370