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Title: Evaluation of heating and chemical protocols for inactivating SARS-CoV-2

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Abstract

Abstract text

Clinical samples collected in COVID-19 patients are commonly manipulated in BSL-2 laboratories for diagnostic purpose. We used the French norm NF-EN-14476+A2 derived from the European standard EN-14885. To avoid the risk of exposure of laboratory workers, we showed that Sodium-dodecyl-sulfate and Triton-X100 must be added to guanidinium thiocyanate-lysis buffers to obtain a 6-log reduction of infectious virus. Heating protocol consisting of 92°C-15min was more effective rather than 56°C-30min and 60°C-60min to achieve 6-log reduction.

Keywords: SARS-CoV-2, coronavirus, heat inactivation, extraction buffer, COVID-19, biosafety

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Abstract

Clinical samples collected in COVID-19 patients are commonly manipulated in BSL-2 laboratories for diagnostic purpose. We used the French norm NF-EN-14476+A2 derived from the European standard EN-14885. To avoid the risk of exposure of laboratory workers, we showed that Sodium-dodecyl-sulfate and Triton-X100 must be added to guanidinium thiocyanate-lysis buffers to obtain a 6-log reduction of infectious virus. Heating protocol consisting of 92°C-15min was more effective rather than 56°C-30min and 60°C-60min to achieve 6-log reduction.

TEXT (1488 words)

Introduction

Coronavirus disease 19 (COVID-19), classified as pandemic by WHO, is a severe acute respiratory syndrome (SARS) caused by the virus designated SARS-CoV-2 (1). Since December 2019, measures to reduce person- to-person transmission of COVID-19 have been implemented to attempt control of the outbreak. Tremendous efforts are done by an increasing number of scientific personnel working daily with the live virus and / or infectious samples, and thus heavily exposed to the risk of infection (2–4). Accordingly, the WHO introduced laboratory guidelines to mitigate this risk for diagnosis and research activities (5). Nonetheless, laboratory workers processing clinical samples will continue to be exposed to infectious SARS-CoV-2 (6). SARS-CoV-2 direct diagnosis is based on RNA detection by RT-qPCR (7). The methods for nucleic acid (NA) extraction use buffers, which formulation intends to obtain high quality NAs. They are not primarily developed for inactivation. Automated NA extraction is generally performed outside of biosafety cabinets which demands that only non-infectious samples must be loaded. To achieve this objective, a prior inactivation step under appropriate biosafety conditions is an absolute requirement. Previous studies have addressed the ability of lysis buffers added to the samples in initial step of NA extraction to act as inactivation agents of several pathogenic viruses (including coronaviruses). However, discrepant results observed with dissimilar protocols led to controversial conclusions (8–10). On another hand, the Center for Disease Control and Prevention (CDC) recommends using Triton X-100 and to heat the sample at 60°C for 1 hour for samples suspect of containing Viral Hemorrhagic Fever (VHF) agent. This procedure has been adopted by many laboratories for handling samples that may contain Ebola virus. Others studies with SARS-CoV and MERS-CoV have established that heat treatment can inactivate beta-coronaviruses (11,12). Consequently, definitive validation to SARS-CoV-2 is still awaited. Soon or later during the COVID-19 pandemic, serological tests will be used for diagnostics and for seroprevalence studies aiming at measuring the penetration of SARS-CoV-2 infection at population level. Detection of past infection will be pivotal for allowing immune persons to take back their professional activity. Since SARS-CoV-2 was detected in blood during infection (13), samples will have to be inactivated prior to serological tests are performed (14). In this study, we have

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tested ten different protocols including three lysis buffers and six heat inactivation procedures on SARS-CoV-2 culture supernatant.

Materials and methods

Lysis buffers

Three lysis buffers produced by Qiagen (Hilden, Germany) were tested. Approximate composition of each buffer is provided by Qiagen. ATL (25-50% Guanidinium Thiocyanate [GITC] and 1-10% sodium dodecyl sulfate [SDS]), VXL (25-50% GITC, 2.5-10% Triton-X-100), and AVL (50-70% GITC).

Cell line

African green monkey kidney cells (Vero-E6; ATCC#CRL-1586) were grown at 37°C in 5% CO_2 with 1% Penicillin/Streptomycin (PS; 5000U.mL-1 and 5000µg.mL-1; Life Technologies) and supplemented with 1% non-essential amino acids (Life Technologies) in Minimal Essential Medium (Life Technologies) with 5% FBS.

Viruses

The Human 2019 SARS-CoV-2 strain (Ref-SKU: 026V-03883) was isolated at Charite University (Berlin, Germany) and obtained from the European Virus Archive catalog (EVA-GLOBAL H2020 project) (https://www.european-virus-archive.com). Experiments were performed in BSL3 facilities.

SARS-CoV-2 titration

SARS-CoV-2 was first propagated and titrated on Vero-E6 cells. Virus stock was diluted to infect Vero-E6 cells at a MOI of 0.001; then cells were incubated at 37°C for 24-48 hours after which medium was changed and incubation was continued for 24 hr; then supernatant was collected, clarified by spinning at 1500 × g for 10 min, supplemented with 25mM HEPES (Sigma), and aliquoted. Aliquots were stored at -80°C before titration. Virus infectivity was measured using 50% tissue culture infectivity dose ($TCID_{50}$); briefly, when cells were at 80% confluence, six replicates were infected with 150µL of tenfold serial dilutions of the virus sample, and incubated for 3-5 days at 37°C under 5% CO_2. CPE was read using an inverted microscope, and infectivity was expressed as $TCID_{50}$/ml based on the Karber formula (15).

Inactivation assays with lysis buffer (Table 1)

The French norm NF EN 14476+A2 derived from the European standard EN 14885 was used (16). For simulating "dirty" conditions, 3 g/L BSA was added before inactivation (Table 1). Each sample was incubated in duplicate with the lysis buffer at room temperature for 10 min; then lysis buffer was discarded via ultrafiltration with Vivaspin 500 columns (Sartorius, Göttingen, Germany) as described (17);

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column was washed with 500 µL PBS three times, and eluted in 20 µL of PBS; 0.1mL was inoculated onto Vero-E6 monolayer (70% confluence). Controls consisted of uninoculated Vero-E6 cells, Vero-E6 cells inoculated with the tested lysis buffer (cytotoxicity), and Vero-E6 cells inoculated with SARS-CoV-2 only. Cells were incubated at 37 °C under 5% CO_2 for 5 days. The read-out was the presence of CPE together with SARS-CoV-2 RNA detection through RT-qPCR at day 5; in the absence of CPE at day 5, 100 µL of supernatant was passaged with the same read-out 5 days later (day 10).

Heat inactivation assays (Table 2)

A 400-µL volume of SARS-CoV-2 supernatant ($3.3x10^6$ $TCID_{50}$/mL) was incubated in a pre-warmed dry heat block and immediately tested for measuring $TCID_{50}$ and RNA copies. Virus titration was performed in duplicate before and after heating to measure viral load reduction factor.

Integrity of SARS-CoV-2 RNA after heat inactivation

Heat inactivated samples were extracted using the Qiacube HT and the Cador pathogen extraction kit (both from Qiagen). Viral RNA was quantified by RT-qPCR (qRT-PCR EXPRESS One-Step Superscript™, ThermoFisher Scientific) (10min-50°C, 2 min-95°C, and 40 times 95°C-3 sec / 60°C-30 sec] using serial dilutions of a T7-generated synthetic RNA standard. Primers and probe target the N gene (Fw: GGCCGCAAATTGCACAAT; Rev : CCAATGCGCGACATTCC; Probe: FAM-CCCCCAGCGCTTCAGCGTTCT-BHQ1. The calculated limit of detection is 10 RNA copies per reaction.

Results

Inactivation assays with lysis buffer (Table 3)

VXL and ATL buffers were able to inactivate SARS-CoV-2 with viral loads as high as 10^6 $TCID_{50}$/ml. In contrast, AVL buffer (GITC 50-70%) either alone or in the presence of absolute ethanol or 1% Triton X-100 resulted in a partial inactivation (50-75%). In addition, our results show that GITC alone (AVL buffer) or GITC mixed with absolute ethanol also cannot guarantee SARS-CoV-2 inactivation as previously described (10). Finally, there was no difference observed between clean and dirty (3 g/L BSA) conditions.

Heat inactivation assays (Table 4)

Only the 92°C-15min protocol was able to inactivate totally the virus (>6 Log_{10} decrease), whereas the two other protocols resulted in a clear drop of infectivity (5 Log_{10} reduction) but with remaining infectivity equal or lower than 10 $TCID_{50}$/ml (Table 4). These results were consistent with previous studies on SARS-CoV and MERS-CoV (11,12). There was no difference between clean or dirty conditions.

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Integrity of SARS-CoV-2 RNA after heat inactivation

The analysis of the Ct values (instead of the $TCID_{50}$) showed that 56°C-30min and 60°C-60min did not affect significantly the number of detectable RNA copies (Δ Ct <1) (Table 4). In contrast, 92°C-15min resulted in a significant drop of the number of RNA copies (Δ Ct >5) (Table 4).

Discussion

Despite the previous emergence of SARS and MERS CoV, there are few studies on the inactivation protocols aiming at mitigating the risk of exposure for medical and laboratory personnel (18).

Qiagen is a prominent actor in the field of nucleic acid purification. Most of other manufacturers of NA purification kits use similar lysis buffer as ATL, AVL and VXL. The ability of AVL to inactivate pathogenic viruses was debated (8–10) but there is no data for ATL and VXL. A total of ten different protocols using AVL, ATL and VXL alone or in association with ethanol or Triton-X100 were studied on SARS-CoV-2 according to the French version of the European recommended procedure (NF EN 14476+A2) (16), as previously shown for other viruses such as Ebola virus or Foot and Mouth Disease virus (8,10,19,20). Our results are in line with data reported for Zaire Ebolavirus (10). They strongly suggest that ATL or VXL should be preferred to AVL. Our findings corroborate and expand recent results (21).

Considering that low SARS-CoV-2 viremia is observed in COVID-19 patients even at the acute stage of the disease (18), the 56°C-30min and 60°C-60min protocols commonly used before serology appears as sufficient for inactivating SARS-CoV-2 as recommended before serological assay for other enveloped RNA viruses (22). Samples treated accordingly will also be amenable for viral RNA detection. In contrast, when processing respiratory samples commonly exhibiting much higher viral loads (23), only the 92°C-15min protocol showed total inactivation; however, whether this protocol is more efficient for inactivation than the two other, the drastic reduction of RNA copies that are detectable thereafter precludes its utilization for subsequent RT-qPCR detection of SARS-CoV-2. For the latter, inactivation using VXL, ATL or similar lysis buffer should be preferred.
Since clinical samples collected in COVID-19 suspect patients are commonly manipulated in BSL-2 laboratories, the results presented in this study should help to choose the best suited protocol for inactivation in order to prevent exposure of laboratory personnel in charge of direct and indirect detection of SARS-CoV-2 for diagnostic purpose.

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References

1. Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Di Napoli R. Features, Evaluation and Treatment Coronavirus (COVID-19). In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 [cited 2020 Mar 10]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK554776/

2. Otter JA, Donskey C, Yezli S, Douthwaite S, Goldenberg SD, Weber DJ. Transmission of SARS and MERS coronaviruses and influenza virus in healthcare settings: the possible role of dry surface contamination. J Hosp Infect. 2016 Mar;92(3):235–50.

3. Xiao S, Li Y, Wong T, Hui DSC. Role of fomites in SARS transmission during the largest hospital outbreak in Hong Kong. Shaman J, editor. PLOS ONE. 2017 Jul 20;12(7):e0181558.

4. Ong SWX, Tan YK, Chia PY, Lee TH, Ng OT, Wong MSY, et al. Air, Surface Environmental, and Personal Protective Equipment Contamination by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) From a Symptomatic Patient. JAMA [Internet]. 2020 Mar 4 [cited 2020 Mar 10]; Available from: https://jamanetwork.com/journals/jama/fullarticle/2762692

5. Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV) - Interim Guidance. World Health Organization (WHO); 2020.

6. Sagripanti J-L, Hülseweh B, Grote G, Voß L, Böhling K, Marschall H-J. Microbial Inactivation for Safe and Rapid Diagnostics of Infectious Samples. Appl Environ Microbiol. 2011 Oct 15;77(20):7289–95.

7. Han Y, Yang H. The transmission and diagnosis of 2019 novel coronavirus infection disease (COVID-19): A Chinese perspective. J Med Virol. 2020 Mar 6;jmv.25749.

8. Blow JA, Dohm DJ, Negley DL, Mores CN. Virus inactivation by nucleic acid extraction reagents. J Virol Methods. 2004 Aug;119(2):195–8.

9. Ngo KA, Jones SA, Church TM, Fuschino ME, George KSt, Lamson DM, et al. Unreliable Inactivation of Viruses by Commonly Used Lysis Buffers. Appl Biosaf. 2017 Jun;22(2):56–9.

10. Smither SJ, Weller SA, Phelps A, Eastaugh L, Ngugi S, O'Brien LM, et al. Buffer AVL Alone Does Not Inactivate Ebola Virus in a Representative Clinical Sample Type. Caliendo AM, editor. J Clin Microbiol. 2015 Oct;53(10):3148–54.

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11. Leclercq I, Batéjat C, Burguière AM, Manuguerra J-C. Heat inactivation of the Middle East respiratory syndrome coronavirus. Influenza Other Respir Viruses. 2014 Sep;8(5):585–6.

12. Darnell MER, Subbarao K, Feinstone SM, Taylor DR. Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV. J Virol Methods. 2004 Oct;121(1):85–91.

13. Pan Y, Zhang D, Yang P, Poon LLM, Wang Q. Viral load of SARS-CoV-2 in clinical samples. Lancet Infect Dis. 2020 Feb;S1473309920301134.

14. Meyer B, Drosten C, Müller MA. Serological assays for emerging coronaviruses: Challenges and pitfalls. Virus Res. 2014 Dec;194:175–83.

15. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Epidemiol. 1938 May;27(3):493–7.

16. French standard NF EN 14476+A2 October 2019 - Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity in the medical area - Test method and requirements. AFNOR; 2019.

17. Burton JE, Easterbrook L, Pitman J, Anderson D, Roddy S, Bailey D, et al. The effect of a non-denaturing detergent and a guanidinium-based inactivation agent on the viability of Ebola virus in mock clinical serum samples. J Virol Methods. 2017 Dec;250:34–40.

18. Chang L, Yan Y, Wang L. Coronavirus Disease 2019: Coronaviruses and Blood Safety. Transfus Med Rev. 2020 Feb;S0887796320300146.

19. Wood BA, Mioulet V, Henry E, Gray A, Azhar M, Thapa B, et al. Inactivation of foot-and-mouth disease virus A/IRN/8/2015 with commercially available lysis buffers. J Virol Methods. 2020 Apr;278:113835.

20. Haddock E, Feldmann F, Feldmann H. Effective Chemical Inactivation of Ebola Virus. Emerg Infect Dis. 2016 Jul;22(7):1292–4.

21. van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, et al. Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. N Engl J Med. 2020 Mar 17;NEJMc2004973.

22. Roehrig JT, Hombach J, Barrett ADT. Guidelines for Plaque-Reduction Neutralization Testing of Human Antibodies to Dengue Viruses. Viral Immunol. 2008 Jun;21(2):123–32.

23. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, et al. SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. N Engl J Med. 2020 Mar 19;382(12):1177–9.

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Table 1. Protocols tested for assessing inactivation using lysis buffers

Lysis buffer	Composition[a]	Nucleic acid extraction kit (catalog #)	Interfering substance / added	Lysis buffer / sample	Temperature (°C)	Contact time (min)
Buffer ATL	25-50% GITC[b] 1-10% SDS[c]	QIAsymphony DSP Virus/Pathogen Kits (#937036) or QIAsymphony DSP DNA Mini Kit (#937236)	± BSA[e] (3g/L)	1:1	20	10
Buffer VXL	25-50% GITC 2.5-10% Triton X-100[d]	QIAmp cador Pathogen Mini kit (#54104) or QIAmp 96 DNA QIAcube HT kit (#51331)	± BSA (3g/L)	1:1	20	10
Buffer AVL	50-70% GITC	QIAamp Viral RNA Minikit (#52904)	± BSA (3g/L)	4:1	20	10
			± BSA (3g/L) + 1 volume ethanol 100%	4:1	20	10
			± BSA (3g/L) + 1% Triton X-100[f]	4:1	20	10

[a] as provided by Qiagen; [b] guanidium thiocyanate, [c] Sodium dodecyl sulfate, [d] vol/vol, [e] Bovine serum albumin, [f] final concentration (vol/vol).

Table 2. Protocols tested for assessing heat inactivation.

Sample tested	Interfering substance	Volume sample (µL)	Temperature (°C)	Time (min)
SARS-CoV-2 cell supernatant ($3.3 \pm 2.3 \times 10^6$ $TCID_{50/ml}$)[a]	± BSA (3g/L)[b]	400	56	30
			60	60
			92	15

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Table 3. SARS CoV-2 inactivation using lysis buffers with additional reagents and with / without

Lysis buffer protocol	Virus detection (CPE + RT-qPCR) after inactivation			
	Without interfering agent		With interfering agent (BSA 3g/L)	
	Replicate#1	Replicate#2	Replicate#1	Replicate#2
ATL buffer	No VR[a]	No VR	No VR	No VR
VXL buffer	No VR	No VR	No VR	No VR
AVL buffer	VR	VR	VR	VR
AVL buffer + 100% ethanol	VR	No VR	VR	No VR
AVL buffer + Triton X-100	VR	VR	VR	No VR

[a] VR, viral replication; no VR defined as absence of CPE at passage#1 and passage#2 confirmed by RT-qPCR showing a Ct value >40; VR defined by CPE at passage#1 or passage#2 confirmed by RT-qPCR showing a Ct value <40.

Table 4. SARS-CoV-2 heat inactivation

Heating protocol	Viral titer ($TCID_{50}$/ml)[a]			Log_{10} reduction factor	Number of RNA copies before *vs* after ($x10^6$)
	before heat inactivation	After heat inactivation			
		no BSA	3g/L BSA		
56°C, 30 min	$3.3 \pm 2.3 \times 10^6$	8.5 ± 7	No VR	> 5	8.01 / 5.16
60°C, 60 min	$3.3 \pm 2.3 \times 10^6$	No VR	5 ± 2.8	> 5	8.01 / 4.54
92°C, 15 min	$3.3 \pm 2.3 \times 10^6$	No VR	No VR	> 6	8.01 / 0.16

[a] Mean value ± SD; no VR defined as absence of CPE at passage#1 and passage#2 confirmed by RT-qPCR showing a Ct value >40.