Comparison of the Modified Centers for Disease Control and Prevention 2019-Novel Coronavirus Real-Time RT-PCR Method for Detection of Infectious and Heat-Inactivated Virus on Stainless Steel

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Abstract

Background: Infectious Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) was used in the validation of methods for detection of SARS-CoV-2 on stainless steel surfaces in the AOAC Research Institute Emergency Response Validation project. Handling infectious virus requires Biosafety Level (BSL)-3 facilities. Objective: To compare the recovery and detection of infectious and heat-inactivated (65°C for 30 min) SARS-CoV-2 from stainless steel by the modified US Centers for Disease Control and Prevention (CDC) 2019-Novel Coronavirus Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Diagnostic Panel. Methods: Viral stocks were diluted in viral transport medium and deposited onto stainless steel test areas at 2 x 10³ and 2 x 10⁴ genomic copies for low and high, respectively. Test areas were sampled, and aliquots of the resulting test solutions analyzed by RT-qPCR according to the CDC method. Results were analyzed by Probability of Detection (POD) statistics. Results: The low level, where fractional positive results (25–75 %) are expected, yielded PODᵢ = 0.80 (0.58, 0.92) for the infectious virus and PODᵢ = 0.15 (0.05, 0.36) for the heat-inactivated virus. The bias, dPODᵢ = -0.65 (-0.80, -0.35), demonstrated a statistical difference between infectious and heat-inactivated virus.
detection. No difference was observed at the high inoculation level. Conclusion: Despite the statistical difference observed, the use of the heat-inactivated virus is a viable alternative for matrix extension studies using a method comparison study design. Highlights: The use of heat-inactivated SARS-CoV-2 can mitigate the need for a BSL-3 facility for matrix extension validation of alternative methods in SARS-CoV-2 studies.

Introduction

The first case of Coronavirus disease 2019 (COVID-19) was reported from Wuhan, China in December 2019 (1). The causative agent, Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), is a ribonucleic acid (RNA) encapsulated virus. The first genomic sequence of SARS-CoV-2 was released by China on January 10, 2020 (2) and efforts were then underway to develop clinical methods to aid in the diagnosis of COVID-19. The U.S. Food and Drug Administration (FDA) issued an Emergency Use Authorization (EUA) for the real-time reverse transcriptase polymerase chain reaction (RT-qPCR) method developed by the US Centers for Disease Control and Prevention (CDC) on February 4, 2020 (3). The method originally included 3 target regions within the nucleocapsid gene, N1, N2 and N3, but the N3 target was later dropped, and the current method includes the N1 and N2 targets only (4). In addition, the CDC method includes a primer/probe set for detection of the human RNase P gene (RP), which serves as an internal control for human clinical specimens.

In May of 2020, the AOAC Research Institute initiated an Emergency Response Validation (ERV) process to validate multiple candidate methods for detection of SARS-CoV-2 on food-grade stainless steel surfaces through the Performance Tested MethodsSM (PTM) program. Several published studies have indicated the virus is able to survive on nonporous surfaces for up to 4 days depending on conditions (5, 6, 7). In order to use the CDC method as a reference method in the ERV-PTM project, the
method was modified for use with environmental swabs. A foam-tipped environmental swab was chosen based on internal CDC evaluations. In addition, the RP internal control was not performed since it requires a human clinical specimen.

At the time the ERV-PTM was initiated, there was little data available comparing the detection of infectious SAR CoV-2 to that of various attenuated forms of the virus. Thus, the ERV-PTM matrix study was performed in a BLS-3 facility using infectious virus. At the conclusion of the study, the AOAC Research Institute commissioned MRI Global (Kansas City, MO), the independent laboratory for this project, to perform a comparison of the infectious virus used for the ERV-PTM project to a heat-inactivated preparation of the virus. This would potentially allow method developers to perform matrix extension studies to add other environmental or food surface types to their claim without the need for BSL-3 facilities. We report here on the results of this comparison study.

Experimental

The comparison study design is consistent with the Performance Tested MethodSM validation program and the AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces (10). The comparison study was conducted in the laboratories of MRI Global in a BSL-3 facility.

Modified CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Method (mCDC)

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel was performed as described (4) with modification. Foam swabs (Harmony Lab and Safety Supplies 25-1607SC; Garden Grove, CA) were selected for environmental sampling based on a recommendation from CDC scientists. The sampling of surfaces was based on recommendations from the World Health Organization (WHO;
Briefly, swabs were moistened by dipping into a 15 mL conical tube containing 2.0 mL viral transport medium (VTM; 9). A 2” × 2” test area was sampled by swiping in two directions while applying pressure to the surface and rotating the swab. The swab was snapped at the break point and placed back in the tube containing VTM. Swab tubes were vortex mixed briefly and placed in a refrigerator at 2–8°C within 15 min of surface sampling until nucleic acid extraction was performed.

The swab tubes were vortex mixed briefly after removal from refrigeration and RNA was extracted from a 140-µL aliquot from the swab tube using the QIAamp Viral RNA Mini Kit (QIAGEN 52906; Germantown, MD) according to the manufacturer’s instructions. Extracted RNA was analyzed according to the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (Integrated DNA Technologies 10006713; Coralville, IA) with TaqPath™ 1-Step RT-qPCR Master Mix CG (Thermo Fisher Scientific A15299; Waltham, MA) on an Applied Biosystems 7500 Fast Dx Real-Time PCR instrument (Thermo Fisher Scientific 4406985; Waltham, MA). Prepared master mix consisted of 8.5 µL nuclease-free water, 1.5 µL primer/probe mix, and 5.0 µL TaqPath 1-step RT-qPCR Master Mix CG (15 µL total volume) per reaction. For the RT-qPCR reaction, 15 µL of prepared master mix was added to each well followed by 5 µL of control, standard, or sample extract. Thermal cycling parameters were as described in the method (4).

SARS-CoV-2

Infectious SARS-CoV-2 (isolate USA_WA1/2020) was sourced from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA, University of Texas Medical Branch, Galveston, TX). The infectious viral stock was received as a lyophilizate of a 1-mL aliquot of virus containing 3.6 × 10⁶ plaque-forming units according to product documentation. Upon receipt, the lyophilizate was resuspended in 2.0 mL Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12 medium,
Gibco 11330032; Thermo Fisher Scientific, Waltham, MA), divided into 50-µL aliquots, and frozen at -70°C.

Heat-inactivated (HI) SARS-CoV2 (isolate 2019-nCoV/USA-WA1/2020) was sourced from the American Type Culture Collection (ATCC VR-1986HK; Manassas, VA) and was received as a frozen stock. The HI virus was prepared at ATCC from cultured virus (1.6 × 10^5 TCID₅₀/mL) treated at 65°C for 30 min according to product documentation. The certificate of analysis reported no visible cytopathic effect when ≥10% of the heat-treated seed was incubated with Vero E6 cells at 37°C with 5% CO₂ for 7 days. Upon receipt, the HI viral suspension was thawed once to remove an aliquot for determination of genomic copy (GC) concentration, then refrozen and stored at -70°C until use.

**Determination of Genomic Copy Concentration**

RT-qPCR using the CDC N1 primer/probe set and a standard curve of synthetic SARS-CoV-2 RNA (ATCC VR-3276SD; Manassas, VA) was performed as described above to determine the GC concentration of the infectious and HI viral suspensions. The synthetic RNA standard curve consisted of 1 × 10^1, 1 × 10^2, 1 × 10^3, 1 × 10^4, and 1 × 10^5 GC/µL. Viral stocks were serially decimally diluted in nuclease-free water to a 10^-5 dilution. RNA standards and viral stock dilutions were analyzed in triplicate by RT-qPCR using the N1 primer/probe set as described above. A standard curve was generated by plotting Cq values against the log₁₀ RNA concentration. Linear regression analysis was performed and used to determine the RNA concentrations of the viral stocks based on the mean Cq values of triplicate analyses of all dilutions that were within the standard curve range.

**Verification of Viral Infectivity**

The presence of infectious virus in the WRCEVA stock was verified using cell culture. Approximately
3 × 10^6 Vero E6 cells (ATCC, Manassas, VA) were plated into a T75 flask with 15 mL DMEM/F12 and incubated overnight at 37 ± 1°C in a humidified incubator with 5% CO₂. The following day, the DMEM/F12 was replaced with fresh medium and inoculated with an aliquot of virus stock. The inoculated cells were incubated for 5 days at 37 ± 1°C in a humidified incubator with 5% CO₂. Determination of cytopathic effect (cpe) was made by microscopic examination.

**Preparation of Inoculated Stainless Steel Test Areas**

Dilutions of infectious viral stock were prepared in VTM from two pooled frozen aliquots. Dilutions of HI viral stock were made in VTM from an aliquot of a single frozen vial from ATCC. Infectious and HI virus stocks were diluted to 1.3 × 10^4 GC/mL for the low level and 1.3 × 10^5 GC/mL for the high level. These concentrations were based on prior range-finding experiments with infectious virus demonstrating fractional positive results for the low level after deposition on stainless steel plates and performance of the modified CDC method.

Stainless steel plates (grade 304, 12” × 12”) were selected to mimic food preparation surfaces. The plates were cleaned prior to use by first treating with Goo-Gone to remove tape residue (if needed) followed by washing with a dish soap solution and rinsing with water. The plates were then disinfected by wiping with a 10% bleach wipe, thoroughly rinsed with water, then wiped with a 70% isopropanol solution, and autoclaved (121°C for 30 min) prior to use. Test areas (2” × 2”) were delineated with laboratory tape. Each test area was inoculated with 150 µL of a low-level virus suspension, a high-level virus suspension, or VTM alone. The test areas, therefore, received 2.0 × 10^3 or 2.0 × 10^4 GC for the low and high levels, respectively. A sterile 10 µL inoculating loop was used to spread the inoculum evenly over the entire test area and the inoculated plates were left to air dry in a biosafety cabinet for up to 1 h until visibly dry. The plates were then sealed in plastic containers and stored overnight (20 to
21 h) at room temperature. During the inoculation and drying process, the room temperature was in the range 21.5 to 24.6°C and relative humidity varied from 36 to 56%.

**Study Design**

To compare the infectious and heat-inactivated SARS-CoV-2 virus, twenty test areas were inoculated at the low level and five test areas were inoculated at the high level for each virus preparation. In addition, 5 test areas received VTM alone as a negative control. The fifty-five test areas were sampled with swabs as described above, the resulting swab tubes were labeled with random ID numbers, and the swab tubes were held at 2–8°C for 24 h to simulate overnight shipment of swabs to a testing laboratory. A second analyst not familiar with the ID key code performed the nucleic acid extraction and RT-qPCR according to the modified CDC method as described above. Statistical analysis of results was performed according to the probability of detection (POD) model (11).

**Results and Discussion**

Infectious SARS-CoV-2 was confirmed to be infective by observance of cpe after microscopic examination of inoculated Vero E6 cells incubated for 5 days as described. Genomic content was calculated from RT-qPCR with the CDC method N1 primer/probe set. Results of triplicate analyses of viral stocks at 1:100, 1:1000, 1:10,000, and 1:100,000 dilutions were used in the calculations. Triplicate determinations were subjected to statistical analysis by the Grubbs’ test and one outlier in the 1:100,000 dilution of the heat-inactivated virus was removed. Viral stocks were thus determined to contain $1.6 \times 10^9$ GC/mL (SD, = 2.2 $\times 10^8$ GC/mL, RSD, = 13.8%) and $9.8 \times 10^7$ GC/mL (SD, = 2.4 $\times 10^7$ GC/mL, RSD, = 24.5%) for the infectious and heat-inactivated viral stocks, respectively. The latter value is slightly lower (within 2-fold) than the reported value of $1.9 \times 10^8$ GC/mL provided by ATCC as determined by Droplet Digital PCR for the heat-inactivated viral stock.
For the comparison study, viral stocks were diluted to $1.3 \times 10^4$ GC/mL for the low level and $1.3 \times 10^5$ GC/mL for the high level and 150 µL aliquots were applied to the test areas, resulting in $2.0 \times 10^3$ GC/test area for the low level and $2.0 \times 10^4$ GC/test area for the high level. Table 1 presents the results of the comparison study. Since the internal control, RP, was not applicable for surface testing (human DNA is not expected to be present), there was no determination of invalid results due to PCR inhibition. The external positive and negative controls produced expected results and all negative control test areas yielded negative results for both N1 and N2.

Using the same isolate (USA-WA1/2020) of SARS-CoV-2 inoculated and dried onto stainless steel at the same genomic copy concentrations, the modified CDC 2019-nCoV RT-qPCR method yielded positive results for both N1 and N2 for all high-level inoculated test areas for infectious virus and HI virus. At the low level, a concentration previously determined to provide fractional positive final results (25 to 75% positive) when testing infectious virus by the modified CDC method, more positive swabs were detected using infectious virus than heat-inactivated virus for both the N1 and the N2 targets. The CDC method requires both N1 and N2 to be positive in order to call the final method result positive for clinical diagnosis. Here we report the results for N1, N2, N1+N2 (both targets positive), and N1/N2 (either target positive). Table 1 shows the heat-inactivated virus at the low level yielded 5 positive test areas for the N1 target, 11 positive test areas for the N2 target, only 3 test areas positive for N1+N2, and 13 test areas positive for N1 or N2 (N1/N2). The infectious virus, however, yielded 16 positive test areas for the N1 target, 19 positive test areas for the N2 target, 16 test areas positive for N1+N2, and 19 test areas positive for N1/N2.

Table 2 presents the statistical analysis comparing detection of heat-inactivated virus to that of infectious virus using the POD model. The POD is the fraction positive ($x/n$) and $dPOD_{HI}$ is the bias for detection of heat-inactivated virus compared to infectious virus ($dPOD_{HI} = POD_{HI} - POD_{I}$). Bias is
observed only at the low level (2.0 × 10^3 GC/test area) where fractional positive results are obtained. The smallest bias is observed for the N1/N2 results with dPOD_{HI} = -0.3 (-0.52, -0.05) and the largest bias is observed for the N1+N2 results with dPOD_{HI} = -0.65 (-0.80, -0.35). When the 95% confidence interval of the dPOD does not include zero, the bias is considered significant at the 5% level. Therefore, for the low level of inoculum, the bias observed between the infectious and heat-inactivated virus as measured by the modified CDC method is statistically significant for all targets and combinations of targets. Since the quantification of the viral stocks was based on the N1 primer/probe set and the detection of virus from the inoculated stainless-steel surfaces was based on the N1 and N2 primer/probe sets, the expectation is that the N1 POD results would therefore be equivalent, but this is not the case. Even for the N1 target alone, the dPOD_{HI} is statistically significant at the low inoculation level. It is important to note that no difference in detection rate was observed at the high inoculation level for N1, N2, N1+N2, or N1/N2.

There are several possible factors contributing to the observed significant difference between detection of heat-inactivated and infectious virus at the low level. Unlike a method comparison study, this study compares the detection of two distinct preparations of virus by a single method. When working at fractionally positive concentrations, small changes or errors in concentration could have large effects on the probability of detection, depending on the slope of the POD curve. Since we don’t know the shape of the POD curve for either of the virus preparations, we cannot estimate the effect of the error in the estimation of genomic copy concentration by RT-qPCR (RSD, 13.8% and 24.5% for the infectious and heat-inactivated preparations, respectively) on the POD results. Other potential contributing factors come from the difference between the determination of GC concentration of viral stocks and detection of virus on dried surface areas. These factors include differential recovery of the viral preparations from the stainless-steel surface, differential release from the foam-tipped swab,
differential stability of the viral preparations when dried onto stainless steel, and differential stability during the 24-h hold of the swabs in VTM. Additional research would be needed to investigate the contribution of each of these factors to the observed statistical difference in this case.

While the heat-inactivated virus yields a lower detection rate than the infectious virus in the mCDC method when dried on stainless steel, we cannot extrapolate whether this holds true for other surface types or other isolates of the virus. Kim et al. (12) treated SARS-CoV-2 isolate CBNU-nCoV01 at 65°C and demonstrated a reduction of viral titer to below the limit of detection of the 50% tissue culture infectious dose (TCID$_{50}$) assay within 15 min, while RNA copy number determined by RT-qPCR was unchanged for up to 15 min for ORF1a and E gene targets and up to 30 min for S and N gene targets. Using SARS-CoV-2 isolate England/2/2020, Burton et al. observed variable reduction in virus titer without complete inactivation at 60°C for up to 60 min, but little effect on Ct values ($\Delta$Ct ≤0.5) by RT-PCR targeting the E gene (13).

For the purposes of environmental method validation where the study design is a method comparison, we propose that the heat-inactivated virus preparation is a viable alternative to the infectious virus because method comparison studies provide a measure of relative reactivity of the candidate method to the reference method, not an absolute reactivity of the candidate method to the analyte concentration. Thus, for each virus preparation (isolate, treatment, and diluent) and each surface type, a range-finding experiment must be performed to determine the concentration of virus preparation that yields fractional positive results for at least one of the methods in the validation study. This may not be the same concentration for each study. Case in point, when validating a method for detection of Salmonella spp., one does not expect all Salmonella species to be equally reactive, only that the candidate method detects each serovar/matrix combination as well or better than the reference method. In addition, when inoculating environmental surfaces, factors such as temperature and
humidity can affect the stability of the virus and its RNA on surfaces (5, 6, 7). It is important, therefore, when interpreting data from validation studies to keep in mind that the AOAC study design for microbiological methods is intended for method comparison, not for determination of the limit of detection of a method. Any estimation of limit of detection would only be valid under the exact conditions (surface type, surface preparation, virus isolate and preparation, diluent, environmental conditions, etc.) of the experiment performed and cannot be generalized. By assessing relative method performance, the diminished POD of the heat-inactivated SARS-CoV-2 virus at the low level on stainless steel surfaces is, therefore, of little consequence and we can recommend its use for extending the matrix scope of validated methods.

Conclusion

Heat-inactivated SARS-CoV-2 is a viable alternative to using infectious virus for matrix extension studies where the study design is a method comparison of molecular-based detection for environmental sampling applications, mitigating the need for a BSL-3 facility. It is recommended that the initial validation study, however, be performed with infectious virus.

CRediT Authorship Statement

Sharon L. Brunelle: Conceptualization, Formal Analysis, Methodology, Visualization, Writing – original draft.

Patrick M. Bird: Conceptualization, Methodology, Writing – review and editing

Jeremy Boone: Data curation, Investigation, Resources, Writing – review and editing

Maria Nelson: Methodology, Visualization, Writing – review and editing

Zerlinde Johnson: Conceptualization, Methodology
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**Conflict of Interest**

All authors declare no conflict of interest.

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Tables for “Comparison of the Modified Centers for Disease Control and Prevention 2019-Novel Coronavirus Real-Time RT-PCR Method for Detection of Infectious and Heat-Inactivated Virus on Stainless Steel”

Table 1. Comparison study results by primer/probe set

| Matrix       | Inoculation strain | GC\(^a\) per test area | n\(^b\) | HI\(^c\) Virus Positives | Infectious Virus Positives\(^d\) |
|--------------|--------------------|--------------------------|---------|--------------------------|-------------------------------|
| Stainless    | SARS-CoV-2         | 0                        | 5       | 0                        | 0                             |
| Steel (2” × 2”) | USA-WA1/2020       | 2 x 10\(^3\)             | 20      | 5                        | 3                             |
|              |                    | 2 x 10\(^4\)             | 5       | 5                        | 5                             |

\(^a\)GC = Genomic copies

\(^b\)n = Number of test areas

\(^c\)HI = heat-inactivated

\(^d\)Only one set of negative control test areas was analyzed as they serve as controls for both infectious and HI virus.

\(^e\)N1+N2 indicates results positive for both the N1 and N2 targets.

\(^f\)N1/N2 indicates results positive for either the N1 or N2 target.
| Matrix            | Inoculation Strain | Target | GC\(^a\) per test area | HI\(^c\) Virus | Infectious Virus |
|-------------------|--------------------|--------|-------------------------|----------------|-----------------|
| Stainless Steel   | SARS-CoV-2 USA-WA1/2020 | N1     | 0                       | 0.00, 0.43     | 0.00, 0.43      |
|                   |                    |        | 2 x 10\(^3\)            | 0.25, 0.47     | 0.80, 0.92      |
|                   |                    |        | 2 x 10\(^4\)            | 1.00, 1.00     | -0.55, -0.73, -0.24 |
|                   |                    | N2     | 0                       | 0.00, 0.43     | 0.00, 0.43      |
|                   |                    |        | 2 x 10\(^3\)            | 0.34, 0.74     | 0.95, 1.00      |
|                   |                    |        | 2 x 10\(^4\)            | 0.57, 1.00     | -0.40, -0.61, -0.13 |
|                   |                    | N1+N2  | 2 x 10\(^3\)            | 0.15, 0.36     | 0.80, 0.92      |
|                   |                    |        | 2 x 10\(^4\)            | 0.57, 1.00     | -0.65, -0.80, -0.35 |
|                   |                    | N1/N2  | 2 x 10\(^3\)            | 0.65, 0.82     | 0.95, 1.00      |
|                   |                    |        | 2 x 10\(^4\)            | 0.57, 1.00     | -0.30, -0.52, -0.05 |

\(^a\)GC = Genomic copies  
\(^b\)n = Number of test areas  
\(^c\)HI = heat-inactivated  
\(^d\)Only one set of negative control test areas was analyzed as they serve as controls for both infectious and HI virus. Therefore, dPOD is not applicable.  
\(^e\)N1+N2 indicates results positive for both the N1 and N2 targets.  
\(^f\)N1/N2 indicates results positive for either the N1 or N2 target.