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**14. ABSTRACT**

The current COVID-19 disease pandemic is caused by the Coronavirus SARS-CoV-2. SARS-related viruses in humans are generally carried by animals, and human infections are only due to contact with infected animals. But in the case of SARS-CoV-2, human to human transmission is the most studied route of infection and primarily through fomites or respiratory particulates. The objective of this project was to determine feasibility of methods to test and detect infectious SARS-CoV-2 virus and viral RNA in stool. Once detected, the results were used to support the hypothesis that fecal/oral exposure to SARS-CoV-2 virus is a potential route of infection in animal models and humans and an area to address in the control and prevention of COVID-19 disease.

**15. SUBJECT TERMS**

RT-qPCR, SARS-CoV-2, Stool, Fecal material, Fecal/Oral transmission, Human Stool, Hamster Stool, COVID, RNA, TCID50 Assay

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# Establishing Human and Animal Stool Testing for SARS-CoV-2 to Determine Feasibility of Fecal/Oral Transmission

Project Report

**FOR**

**U.S. Food and Drug Administration**

**Robert Fisher, Ph.D.**
25 New Hampshire Avenue
Silver Spring, Maryland 20903

MRIGlobal Project No. 110890.01.208.06.01.02

April 16, 2021

# Preface

This Project Report was prepared at MRIGlobal for the work performed under MRIGlobal contract numbers:

- Homeland Defense Technical Area Task - HD TAT (HT 16-1348)
  - Contract No. FA8075-14-D-0006
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Funding for this review was provided through a contract with U.S. Food and Drug Administration.

The Study Director of the program was Dr. Shelton Bradrick and managed by William Sosna. The studies were performed in compliance with MRIGlobal quality procedures.

MRIGLOBAL

Shelton Bradrick, Ph.D.
Study Director

Approved by:

Kenton L Lohman, Ph.D.
Principal Science Advisor, Life Sciences

April 16, 2021

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## Figures



## Tables

# Section 1.
# Objective

The current COVID-19 disease pandemic is caused by the Coronavirus SARS-CoV-2. SARS-related viruses in humans are generally carried by animals, and human infections are only due to contact with infected animals. But in the case of SARS-CoV-2, human to human transmission is the most studied route of infection and primarily through fomites or respiratory particulates. The objective of this project was to determine feasibility of methods to test detect infectious SARS-CoV-2 virus and viral RNA in stool to support testing the hypothesis that fecal/oral exposure to SARS-CoV-2 virus is a potential route of infection in animal models and humans to address the control and prevention of COVID-19 disease.

# Section 2.
# Sponsor, Testing Laboratory, and Personnel Responsibilities

## 2.1 Sponsor

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## 2.3 Personnel Responsibilities

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# Section 3.
# Test Systems and Methods

## 3.1 Equipment

Test Equipment: RTPCR Instrumentation. $TCID_{50}$ Cell culture

## 3.2 Methods

Testing Description

### 3.2.1 Task 1: Establish Sensitivity of RT-qPCR and $TCID_{50}$ Assays in Stool

#### 3.2.1.1 Subtask 1: RT-qPCR Assay Optimization on Hamster Stool

MRIGlobal conducted testing in a high containment Biosafety Level 2 & 3 (BSL2-3) laboratories at MRIGlobal, Kansas City, MO.

Our current RT-qPCR singleplex assay for SARS-CoV-2 has been optimized for hamster stool. We will initially test the assay against contrived hamster stool to compare performance to human stool. To generate contrived samples, naïve matrix will be spiked with live titered SARS-CoV-2. Contrived samples will be frozen and then extracted using the QIAGEN PowerMicroBiome kit. MRIGlobal plans to follow our current procedures for preservation of RNA in matrix prior to extraction. Human stool samples will be purchased from established vendors. Hamster stool will be collected from naïve animals and preserved as described above.

- Construct contrived human and hamster stool samples.
- Construct 1mL stool stocks for each sample type.

#### 3.2.1.2 Subtask 2: $TCID_{50}$ Optimization on Human and Hamster Stool

Our current $TCID_{50}$ Assay will be optimized for human and hamster stool samples to reduce or eliminate bacterial or other contaminants that might interfere with viral growth on VeroE6 cells.

- Filtration of stool along with standard antibiotics used in cell culture will be tested in stool samples against purified live virus stocks.
- Assay will be tested on the highest possible stock dilution available using our in house SARS-CoV-2 stocks.

#### 3.2.1.3 Subtask 3: Sensitivity Testing of $TCID_{50}$ for Infectious Virus vs Viral RNA in Stool

Finally, we will use the optimized RT-qPCR and $TCID_{50}$ assays to establish sensitivity of the system to quantify both viral RNA and infectious virus in human and hamster stool.

- Construct contrived human and hamster stool samples.
- Construct 1mL stool stocks for each sample type.
- Assay will be tested by serial 10X dilutions beginning with the highest possible concentration of our in house SARS-CoV-2 stocks.

# Section 4.
# Results

## 4.1 SubTask 1: RT-qPCR of SARS-CoV-2 in Stool

### 4.1.1 RT-qPCR Optimization:

RT-qPCR was optimized and the Standard curve is shown in Figure 1.

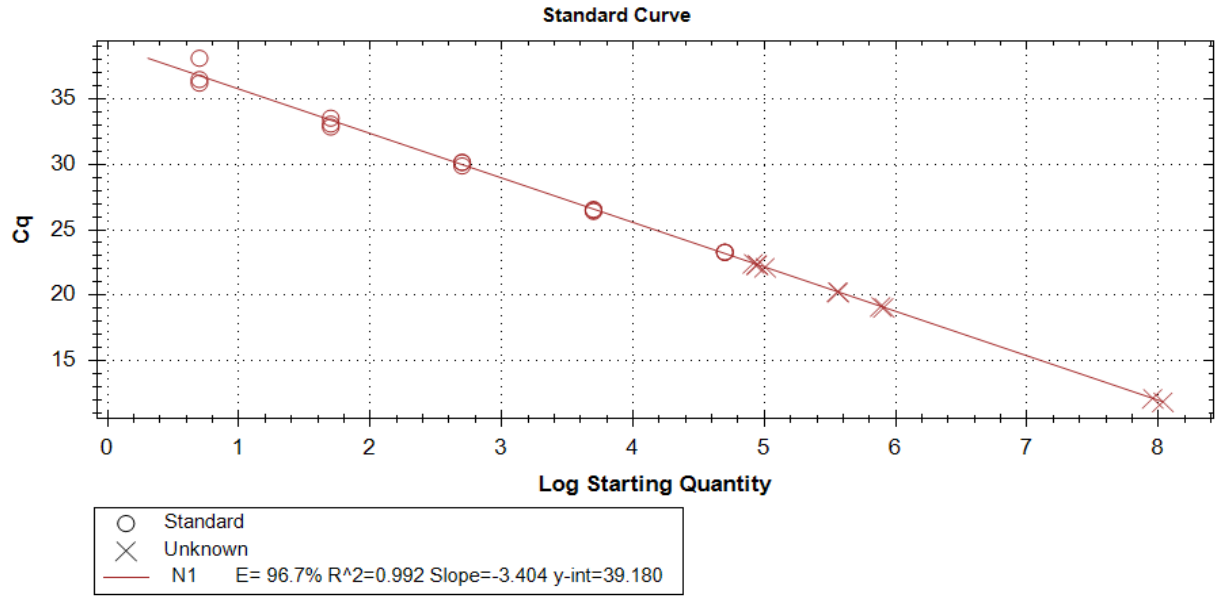


**Figure 1. Standard Curve for RT-qPCR Optimization Results SARS-CoV-2**

Serial ten fold dilutions of a synthetic RNA standard were tested to determine standard curve for RT-qPCR for SARS-CoV-2 RNA targeting the N1 gene. The standard curve allows quantification of viral copies per mL in experimental samples.

### 4.1.2 RT-qPCR Detection of Viral RNA from Hamster and Human Stool:

For the RT-qPCR and $TCID_{50}$ experiments, the same amount of virus (UK variant, B.1.1.7, Isolate hCoV-19/USA/CA_CDC_5574/2020) was used. This equates to 9×109 copies/ml [**NOTE** that the CDC N1 assay reacts with both genomic (gRNA) and subgenomic (sgRNA) present in crude virus preps]. The samples for RT-qPCR analysis from stool were spiked with virus and were also spiked with a synthetic MS2 RNA as an extraction control. To determine the effect of stool exposure to viral RNA (vRNA) recovery, a "no stool" control sample was processed side by side with spiked stool samples.

Each sample was extracted and analyzed by RT-qPCR in duplicate. Data in Table 1 show the mean Ct values and vRNA copies per mL for each condition. The results show that, compared to the no stool control, hamster and human stool reduced vRNA recovery to 0.58% and 0.23% of the control, respectively. Both human and hamster stool also reduced recovery of the MS2 RNA though the effects were not identical to that seen with vRNA: hamster was 23.3% recovery and human was 0.40% recovery compared to the no stool control.

**Table 1. RT-qPCR data for SARS-CoV-2 RNA in Hamster or Human Stool**

| Sample ID | Description | N1 | | MS2 | | Estimated copies/mL of vRNA |
|---|---|---|---|---|---|---|
| | | Avg Ct* | Std Dev | Avg Ct* | Std Dev | |
| QCS1 | human stool blank (MS2 spike only) | N/D | N/A | 27.1 | 0.06 | N/A |
| QCS2 | hamster stool blank (MS2 spike only) | N/D | N/A | 21.2 | 0.04 | N/A |
| 1 | human stool spike (virus + MS2) | 22.2 | 0.11 | 26.8 | 0.05 | 1.9E+07 |
| 2 | human stool spike (virus + MS2) | 22.4 | 0.05 | 27.1 | 0.11 | 1.7E+07 |
| 3 | hamster stool spike (virus + MS2) | 20.3 | 0.01 | 20.9 | 0.09 | 7.3E+07 |
| 4 | hamster stool spike (virus + MS2) | 19.1 | 0.04 | 21.3 | 0.00 | 1.6E+08 |
| 5 | no stool (virus + MS2) | 12.0 | 0.14 | 19.0 | 0.02 | 2.0E+10 |

* n=2.

Data from Table 1 (graphed in Figure 2 below) shows a two log reduction of RT-qPCR SARS-CoV-2 signal in hamster stool and approximately 2.5 log signal reduction in human stool. Note that the human sample was not pooled but was from a single individual donor. We speculate that the loss of RNA in stool samples is likely due, at least in part, to action of endogenous RNases on free sgRNA and MS2 control.

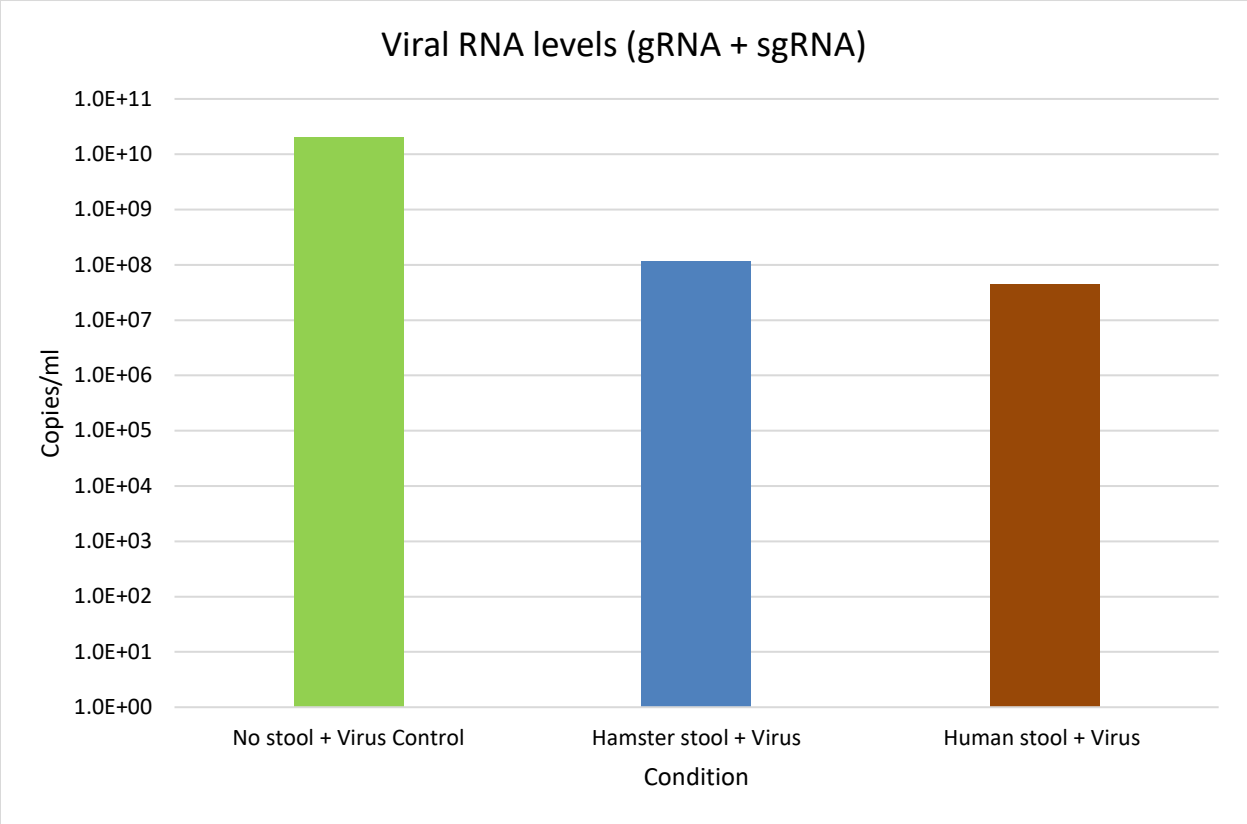


**Figure 2. Graphical Representation of data from Table 1**

## 4.2 Results for SubTasks 2 & 3

### 4.2.1 SubTask 2: Optimization Plan for TCID50 Assay in hamster and human stool

1. The following steps were followed for each sample type:

   Stool Samples in triplicate plus one no virus control:

   a. Weigh out 0.25 g of stool in to a 2.0 mL tube. Tabular format is below.

| Replicate No. | Human Stool Weight (g) | Hamster Stool Weight (g) |
|---|---|---|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 (no virus control) | | |

   b. Spike feces with 100 µL of SARS-CoV-2 stock virus, UK variant, B.1.1.7, Isolate hCoV-19/USA/CA_CDC_5574/202 ($1.1 \times 10^7$ $TCID_{50}$/ml)
   c. Incubate for 10 minutes at room temperature.
   d. Add 900 µL of PBS and vortex.
   e. Proceed with Step 2.

   A no stool control was also performed in triplicate:

   a. Pipette 100 µL of SARS-CoV-2 (UK) in to a 2.0 mL tube.
   b. Incubate for 10 minutes at room temperature.
   c. Add 900 µL of PBS and vortex thoroughly.
   d. Proceed with Step 2.

2. Centrifuge the sample at 1000 × g for 10 minutes.
3. Transfer the supernatant to an Ultrafree-MC filter and centrifuge at 12,000 × g for 1-4 minutes.
4. Use the flow-through for $TCID_{50}$ Testing.

### 4.2.2 SubTask 3: $TCID_{50}$ Testing of SARS-CoV-2 in Hamster and Human Stool

1. The day prior to testing, seed five 96-well plates with Vero E6 cells at 10,000 cells per well.
2. Perform serial dilutions of each sample in DMEM/F12 + antibiotics (Pen/Strep) using the scheme below:

   a. 100 µL Sample in to 900 µL of media = $10^{-1}$ dilution.
   b. 100 µL $10^{-1}$ diluted sample in to 900 µL of media = $10^{-2}$ dilution.
   c. Perform dilutions until $10^{-8}$ is reached.

3. Decant media on 96-well plate in to a bleach bath.
4. Plate 100 µL of each sample dilution in 5 wells.
5. Incubate plate at 37°C with 5% $CO_2$ for 4-6 days.
6. Read plate for cytopathic effect (CPE) after incubation is complete.
7. Calculate titer using the Reed and Muench method.

#### 4.2.2.1 $TCID_{50}$ Testing of Spiked Stool

Results of SubTask 3 are shown below in Table 2 and Figure 3.

**Table 2. $TCID_{50}$/mL on Hamster and Human stool Spiked with SARS-CoV-2**

| Rep | No Stool + Virus Control | Hamster Stool + Virus | Human Stool + Virus |
|---|---|---|---|
| 1 | 2.37E+05 | 2.37E+03 | 3.16E+01 |
| 2 | 4.22E+04 | 1.47E+04 | 6.81E+02 |
| 3 | 1.47E+05 | 3.16E+04 | 4.22E+02 |
| Mean | 1.42E+05 | 1.62E+04 | 3.78E+02 |
| SD | 9.75E+04 | 1.47E+04 | 3.27E+02 |
| P (t-test) | | 0.099521 | 0.064432 |

The results show that hamster stool reduced virus titer by an average of 8.77-fold (15% remaining infectious virus) and human stool reduced titer by 376-fold (0.27% remaining infectious virus) compared to the no stool control. **NOTE** that the human stool is from a single donor (not pooled). Also note that the $TCID_{50}$ result for replicate #1 of the Human Stool + Virus samples was significantly lower than the other two.

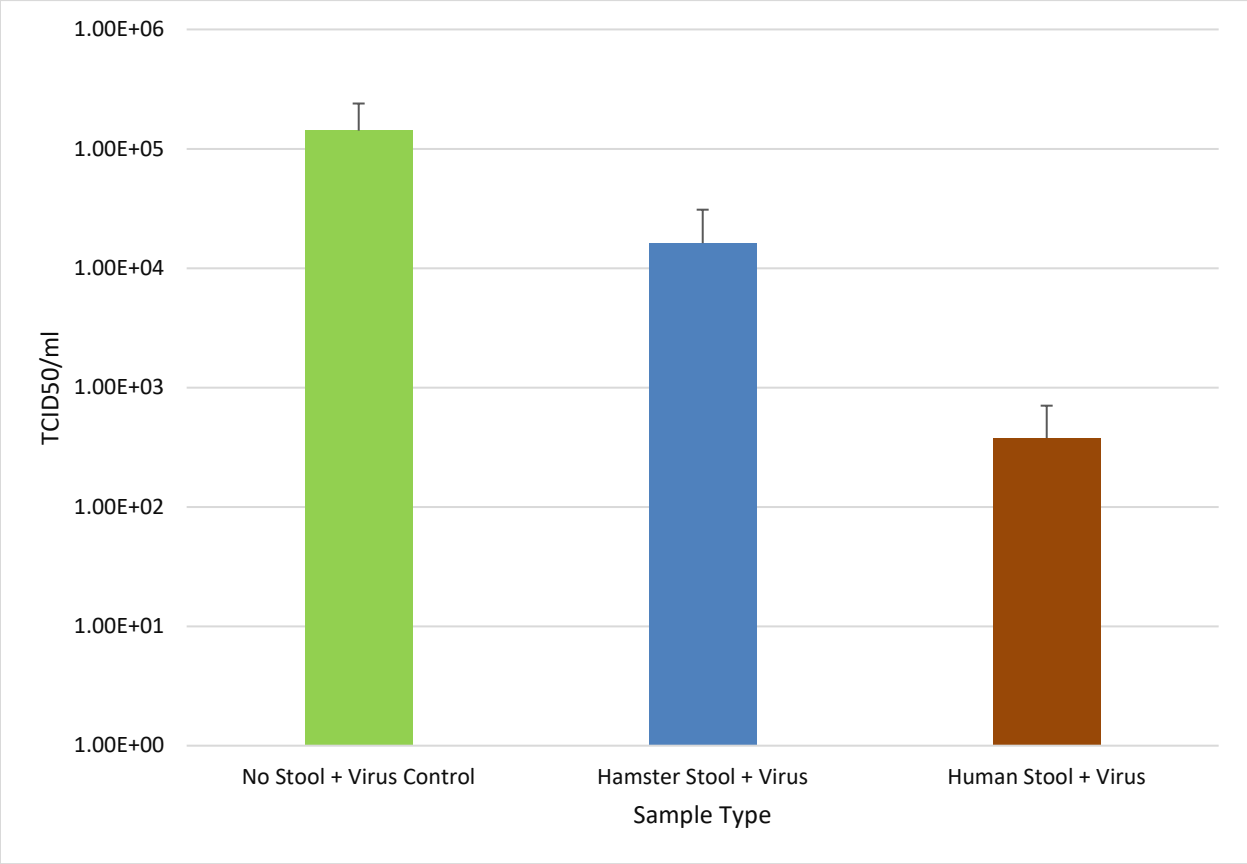


**Figure 3. $TCID_{50}$ on Contrived Hamster and Human Stool Samples**

Graphical representation of average $TCID_{50}$/mL results for Hamster and Human stool spiked with $1.1\times10^{6}$ $TCID_{50}$ infectious units of SARS-CoV-2 is shown in Figure 3 above.

# Section 5.
# Conclusions

## 5.1 Outcomes:

### 5.1.1 RT-qPCR:

- The RT-PCR portion of Subtask 1 has been successfully completed. Our assay has been shown to work on RNA extracted from stool of both hamster and human stool. There is clearly loss of RNA due to RNase activity, or inhibition of RT-qPCR by extracted material, in both hamster and human stool with human stool showing half a log larger effect. This may limit the sensitivity of vRNA detection when tested on clinical samples from infected hamsters or humans. We could address the possibility of assay inhibition this by adding extracted blank material to RT-PCR reactions.

### 5.1.2 $TCID_{50}$:

- Our ability to detect live, infectious SARS-CoV-2 spiked into hamster or human stool was successful. However, in this preliminary study, there was a marked reduction in the virus infectivity in human stool and, to a lesser extent, hamster stool. Without more extensive study, we cannot determine the source of this inhibition of infectivity. The stool sample was only from a single human and not pooled samples, so it is possible that this result is not representative.
- In live animal model challenge studies, there may be a need to collect stool samples temporally (every 12-24 hours perhaps) to determine the timing of maximum shedding of virus in stool post infection.