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Inactivation of Human Coronavirus OC43 (hCoV-OC43) by Dusmit Air-Purifying System

1. Aim:

To test the Dusmit air-purifying system for its ability to inactivate human coronavirus OC43 (HCoV-OC43), by environmental sampling and monitoring of cytopathogenic effect (CPE), in parallel to cell viability assay and verification by quantitative PCR (qPCR)

2. The Dusmit air-purifying system:

The system included the following components (see Appendix A):

- Nebulizer, allowing the spread of the required viral inoculum into the input chamber in aerosol droplets < 10μm (Appendix A; A)
- Input chamber, collecting the nebulizer aerosols (Appendix A; B)
- Air compressor, pushing the virus-inoculated air into the tank input pipeline (Appendix A; C)
- Compressor-to-tank input pipeline, delivering the inoculated air into the inactivation tank (Appendix A; D)
- Inactivation tank, serving as physical barrier to air-borne particles, and capable of heating (Appendix A; E)
- [redacted]
- Output chamber, collecting the air flowing through the system (Appendix A; G)

All parts and components of the Dusmit system were provided, and installed in a biological laminar hood at hylabs virology lab, by the customer, 24 hours prior to performing this study.

3. Related Documents:

3.1. Hy Laboratories Ltd. SOP No. 11-038- "General instructions for Real Time PCR procedure"

3.2. Hy Laboratories Ltd. SOP No. 11-033- "Operating instruction for real time PCR - Bio-Rad instrument"

3.3. Hy Laboratories Ltd. SOP No. 11-035- "General Maintenance in the Tissue Culture Laboratory"

4. Materials:

4.1. hCoV-OC43 ($TCID_{50}$/mL 10^7)

4.2. MEM NEAA (Biological Industries, Cat. 01-040-1A)

4.3. L-Alanyl-L-Glutamine Solution (200 mM; Biological Industries, Cat. 03-022-1B)

4.4. Penicillin-Streptomycin Solution (Biological Industries, Cat. 03-031-1B)

4.5. Fetal Bovine Serum (FBS; Biological Industries, Cat. 04-127-1A)

4.6. Human MRC5 cells (lung fibroblasts; ATCC, Cat. CCL171)

4.7. Human coronavirus OC43 (hCoV-OC43; stock titer: $1x10^7$ $TCID_{50}$/ml)

4.8. Hydrogen peroxide solution H_2O_2 (Sigma, Cat. 216763)

4.9. Lysis buffer (LB; hylabs, Cat. BP007/200)

4.10. MagCore Viral Nucleic Acid Extraction Kit (RBC Bioscience, Cat. MVN400-04; MagCore cartridge code: 203) including Proteinase K and Carrier RNA.

4.11. Hy RT PCR Kit (hylabs, Cat. ER1012).

4.12. Primers (hylabs): HCoV_OC43_F3: 5'-ATTGTCGATCGGGACCCAAG-3', HCoV_OC43_R3: 5'-TGTGCGCGAAGTAGATCTGG-3'.

4.13. Nuclease free water (hylabs, Cat. BP 556/100S).

4.14. Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Cat .11733-046).

4.15. ACD-200 “Bobcat” dry filter Air Sampler (InnovaPrep)

4.16. Rapid Filter Elution Kit with PBS "Wet foam elution 0.075% Tween 20 / PBS" (InnovaPrep, Cat. AC00201-P)

4.17. Consumables: Filter tips (Axygen, Cat. TF-200-R-SThermo fisher, Cat. 94052410), sterile 1.5 ml Microcentrifuge tubes (Sarstedt Cat. 72.690.001), 48-well plate (Greiner Bio One, Cat. 677180), RT-PCR reaction plate (USA Scientific Cat. 1402-9990), RT-PCR reaction plate Sealing Film (USA Scientific, Cat. 2921-0000).

5. Methods and experimental procedures:

5.1. Dusmit system antiviral activity experiment:

5.1.1. Sample preparation: MRC5 cells (4.6) were grown in MEM medium (4.2) supplemented with 2mM L-Alanyl-L-Glutamine (4.3), 1% Penicillin-Streptomycin (4.4) and 10% FBS (4.5), in an incubator at 37^OC and 5% CO_2. 24 hours prior to the experiment cells were plated in a 48-well plate, and grown as described above. On the day of the experiment the Dusmit system was activated to generate the samples as follows: negative control (NC; 1

sample), positive control (PC; 2 samples), cleaning verification (CV; 1 sample), test samples (T-0.5, T-1, T-5; 3 samples).

5.1.2. Experimental procedure: the input chamber was inoculated using the nebulizer, for 3-4 minutes per sample, by: 1 ml of sterile growth medium (4.2), H_2O_2 (4.8) or hCoV-OC43 (4.1), followed by the Dusmit system activation to generate samples as elaborated below:

- **Negative control (NC):** nebulizer was loaded with 1ml MEM (4.2) supplemented with 2mM L-Alanyl-L-Glutamine (4.3), 1% Penicillin-Streptomycin (4.4), 2% FBS (4.5), and activated for 3-4 min into input chamber. Next, the Dusmit compressor was turned on, while inactivation tank heating was off, and all inner system air passages remained open. Air sampling was then performed by Bobcat air sampler (4.15) directly from the output chamber.
- **Positive control (PC):** nebulizer was loaded with 1ml hCoV OC43 (4.1) in MEM (MEM was supplemented as in the NC sample). Nebulizer was activated for 3-4 min into input chamber. Next, the Dusmit compressor was turned on, while inactivation tank heating was off, and all inner system air passages remained open. Air sampling was then performed by Bobcat air sampler (4.15) directly from the output chamber. The PC procedure was performed twice (biological duplicate)
- **Cleaning verification (CV):** nebulizer was loaded with 1 ml H_2O_2 (4.8), and activated for 3-4 min into input chamber. Next, the Dusmit compressor was turned on, while inactivation tank heating was off, and all inner system air passages remained open. No air sampling was performed from the output chamber, and the procedure was repeated twice more, allowing the air flowing within the system to be released into the laminar hood, and the evaporation of all residual H_2O_2. Next, the nebulizer was loaded with 1 ml MEM and the NC procedure was performed as elaborated above under "**Negative control (NC)**". Air sampling was then performed by Bobcat air sampler (4.15) directly from the output chamber.
- **Test samples (T-0.5 / T-1 / T-5):** the inactivation tank was pre-heated to [redacted] (~10min). Nebulizer was loaded with 1ml hCoV OC43 (4.1) in MEM (MEM was supplemented as in the NC sample). Nebulizer was activated for 3-4 min into input chamber. Next, the Dusmit compressor was turned on, while inactivation tank heating was on ([redacted]), and inner system air passages (valves) were shut closed to allow aerosolized virus remain within the inactivation tank. For T-0.5 sample, the incubation

time inside the tank was 30 seconds, i.e 0.5min tank size. Following incubation, the system valves were opened to allow air flow into the [redacted] and output chamber. Air sampling was then performed by Bobcat air sampler (4.15) directly from the output chamber.

For T-1 and T-5 samples the procedure above was repeated at 1min tank size (1 min incubation), and 5min tank size (5 min incubation), respectively, while the inactivation tank remained heated at >[redacted].

The input and output chambers were replaced 3 times during the entire procedure: between the PC samples and the CV sample, between the T-0.5 and T-1 sample, and between the T-1 and the T-5 sample. Following each of the above sample procedures air sampling was performed from output box by Bobcat air sampler (4.15) for 3min. Next, the system and Bobcat were turned off, and the Bobcat filter was removed and extracted with 6ml PBS using the Rapid Filter Elution Kit (4.16). 100µl of each filter elution were removed into 900µl MEM, followed by 5-fold dilution in MEM. The medium from the 48-well plate was then removed and 500µl of the 5-fold dilution were added instead (each in a duplicate).

In parallel, in the same 48-well plate, six (6) additional wells (in triplicates) were used to serve as reference curve for assaying cell viability of all filter samples following the above described experimental procedure. In each triplicate of wells the media was replaced (in the same manner as above) by 500µl per well of serial 10-fold dilutions of hCoV-OC43 (starting at 10^6 $TCID_{50}$, and ending at 10^1 $TCID_{50}$). Three (3) additional wells were used as calibration curve negative control (cNC) for the viability assay, in which the media was replaced by 500µl sterile MEM supplemented with 2mM L-Alanyl-L-Glutamine, 1% Penicillin-Streptomycin, 2% FBS, containing no hCoV-OC43.

Following media replacement cells were incubated for 6 days at 35^{O}C and 5% CO_2, and monitored every 24 hours under the microscope. Cell viability was determined by MTT assay on day six (6) of incubation.

5.1.3. MTT viability assay: on day 6, the growth medium was removed from each well. Next, 5 mg/ml MTT compound (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, in PBS) was diluted 1:7.5 in MEM, and 200µl of the diluted MTT were added per well as replacement medium. The plate was then incubated for 2 hours at 35^{O}C and 5% CO_2.

Following incubation, medium + MTT was removed, and 300µl DMSO were added per well. The plate was incubated for 15 minutes at room temperature following DMSO addition, and was read by SPECTRAFluor Plus plate reader (Tecan) at 560nm. MTT assay results are presented in Table 1.

5.2. qPCR molecular verification test:

5.2.1. Sample preparation: of the filter extractions (of 6ml PBS obtained as elaborated in section 5.1.2), 250µl samples were removed for qPCR analysis as follows: 3x 250µl of the NC filter extract, 6x 250µl of the second PC filter extract, and 6x 250µl of the T-5 filter extract. In parallel, 1x 250µl sample of each viral reference curve serial 10-fold dilution, were also taken for qPCR analysis. Each 250µl were pipetted into a MagCore sample tube, supplemented in advance with 130µl lysis buffer (LB; 4.9), 10µl carrier RNA and 20µl of proteinase K (provided with the MagCore Viral Nucleic Acid Extraction Kit; 4.10). MagCore sample tubes were then capped and left in the laminar hood at room temperature for 10 minutes, to allow for complete viral inactivation. Next, tubes were spun down for 30 sec, sprayed thoroughly with 70% ethanol on their outer side, and taken for viral nucleic acid extraction.

5.2.2. Viral nucleic acid extraction: hCoV-OC43 viral RNA was extracted from each sample, using the MagCore extraction device and the MagCore Viral Nucleic Acid Extraction Kit (4.10). RNA was eluted in 60µl elution buffer. Each 3 extractions of the same sample were then mixed together, except for the reference curve samples which were not mixed.

5.2.3. cDNA preparation: 12.4µl of each mixture of extracted RNA samples were used for first-strand synthesis using the Hy RT PCR Kit (4.11). Control reactions, to which no reverse transcriptase was added (-RT) were performed as well. cDNA was then used as template for qPCR (5.2.5)

5.2.4. Primers used: HCoV_OC43_F3 and HCoV_OC43_R3 (4.12).

5.2.5. qPCR: each generated cDNA sample was diluted 1:2, and 5µl of each diluted cDNA (+/-RT) served as a template for qPCR, performed per each cDNA sample in duplicates (for the reference curve samples – in triplicates), using the Platinum SYBR Green qPCR SuperMix (4.14) in a total volume of 20µl per reaction, utilizing the CFX96 real-time instrument (Bio-Rad). qPCR results are presented in Table 2.

6. Results and conclusion:

6.1. Antiviral activity experiment results (MTT assay results; presented in Table 1):

6.1.1. Recovery of hCoV-OC43 from the Dusmit air-purifying system: based on the PC sample group the system displayed a recovered viral $TCID_{50}$ lower than 10^1, which was the lowest $TCID_{50}$ used for the viral reference curve in this assay. Therefore, it is concluded that, using hCoV-OC43 titer of $TCID_{50} = 10^7$, the recovery of viral particles from the Dusmit system, when heat inactivation was off, is near 0, under the conditions described in this report.

6.1.2. The results presented in this study report indicate that the Dusmit air-purifying system completely abolishes hCoV-OC43 infectivity, both under heating and under no heating conditions, and regardless of the heating duration. This is concluded both by the MTT assay results, and by our daily observations where no CPE of the cells was seen during the 6 days of the experiment. These results imply that the Dusmit system serves as a physical barrier, preventing viral particles passing through it from exiting.

6.2. qPCR results (presented in Table 2):

6.2.1. All qPCR negative controls and no RT controls exhibited negative results for the presence of hCoV-OC43 nucleic acid.

6.2.2. The results displayed by the qPCR show that while a very low viral RNA signal was detected in the PC sample (i.e. no tank heating; Cq = 37), no viral RNA was detected following 5 minutes heating of the inactivation tank (5min tank size). These data indicate that the tank heating damages to some extent the hCoV-OC43 RNA.

6.3. In conclusion, in this study, the Dusmit air-purifying system was tested for its ability to hamper the infectivity of hCoV-OC43. To that end two methods were employed: a direct method assaying cell viability following their infection by system-treated virus, and a molecular, non-direct method, providing information as to the viral nucleic acid that can be detected exiting the system. Results of both methods indicate that the tested system indeed reduces the viral load entering it from its input end.

It is noteworthy, that the molecular method only detects viral nucleic acid, and a very small portion of it. Thus, any qPCR observed effect of the system should not be regarded as indicative of viral activity, and not as direct indication of an actual infection capability.

Table 1: Dusmit system antiviral activity experiment - CPE & MTT assay results

Sample group	Group % viability	Calculated viral $TCID_{50}$	Viral log reduction	CPE
NC (no tank heating; 1 sample)	92	< 1.00E+01	> 6	---
PC (no tank heating; 2 samples)	88.5	< 1.00E+01	> 6	---
CV (no tank heating; 1 sample)	90	< 1.00E+01	> 6	---
T-[redacted] tank size, 1 sample)	90	< 1.00E+01	> 6	---
T-[redacted] tank size, 1 sample)	87	< 1.00E+01	> 6	---
T-[redacted] tank size, 1 sample)	74	< 1.00E+01	> 6	---

Group % viability: indicating viable cells per sample group. Group % viability was calculated as percentage of the average MTT result of the cNC group wells, which was regarded as representing 100% cell viability.

Calculated viral $TCID_{50}$: was assessed for each sample group compared to the reference curve performed in triplicates of 6 serial 10-fold dilutions (5.1.2).

Viral log reduction: was calculated per sample group by dividing the input chamber inoculated viral $TCID_{50}$ (i.e. 1.00E+07) by the 'Calculated viral $TCID_{50}$' of each sample group.

CPE: the viral cytopathogenic effects as observed under the microscope during the 6 days of experiment. '+++' = massive CPE, '++-' = mild CPE, '+--' = minimal CPE, '---' = no CPE.

Table 2: qPCR molecular verification test - results

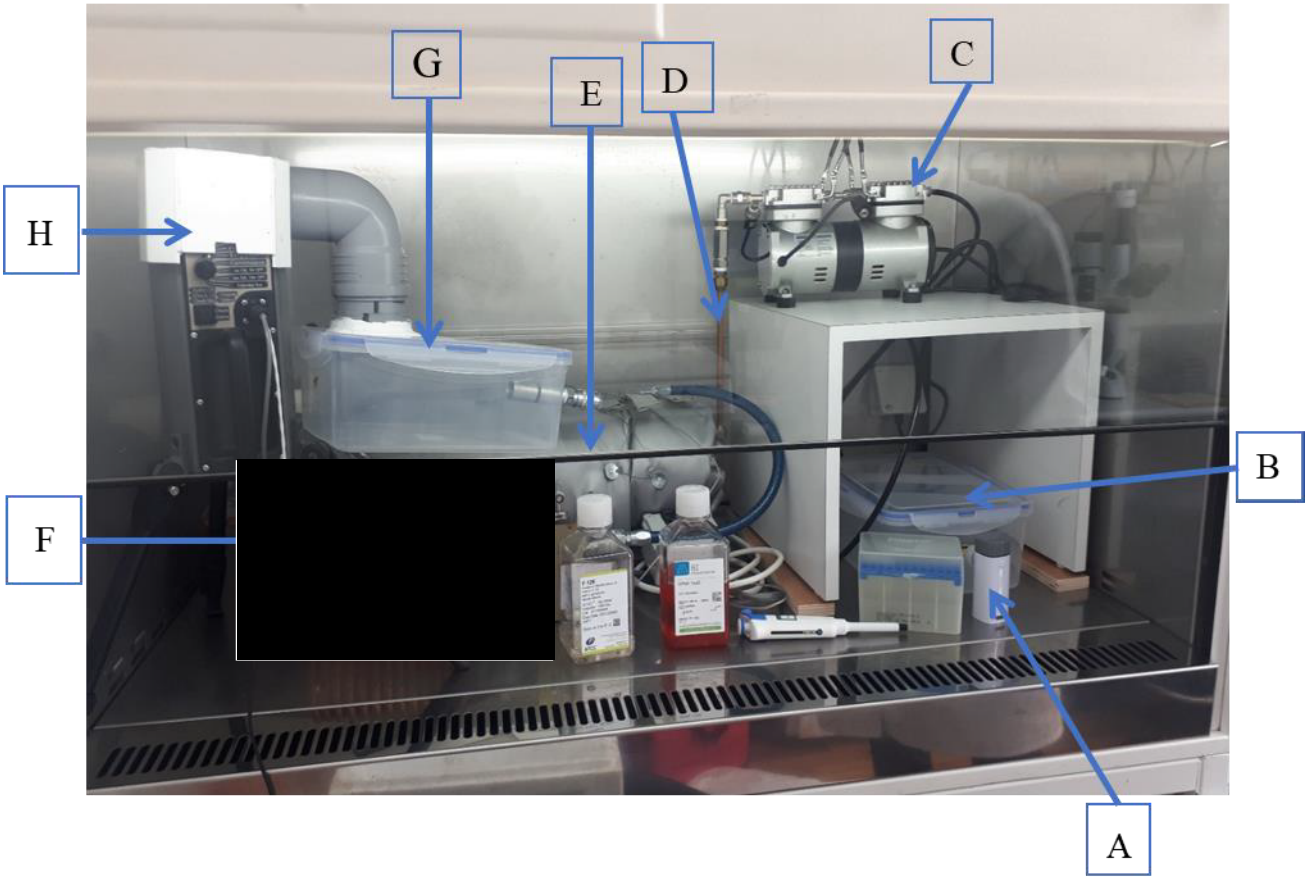
Sample Group	Group mean Cq
NC	NA
PC	37
T-5	NA

All control reactions with no reverse transcriptase added during cDNA synthesis gave: Cq = 0

Performed by: **M.Sc Salem Sirhan** **(Name & Sign)** DATE: **25.10.2020**

Reviewed by: **Dr. Maya Amichay** **(Name & Sign)** DATE: **25.10.2020**

Appendix A: Components of the Dusmit Air-Purifying System



(A) Nebulizer, **(B)** Input chamber, **(C)** Air compressor, **(D)** Compressor-to-tank input pipeline, (**E**) Inactivation (heating) tank, **(F)** [redacted], **(G)** Output chamber, (**H**) Bobcat air sampler