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Supplementary appendix

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Tropism, replication competence and innate immune responses of the novel coronavirus SARS-CoV-2 in human respiratory tract and conjunctiva: an analysis in *ex vivo* and *in vitro* cultures

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Supplementary information

Materials and methods

Human alveolar epithelial cells isolation

Primary human alveolar epithelial cells were isolated using human non-malignant lung tissue obtained from patients undergoing lung resection in the Department of Surgery, Queen Mary Hospital, Hong Kong SAR, under a study approved by the Institutional Review Board of the University of Hong Kong and Hospital Authority Hong Kong West Cluster. After removing visible bronchi, the lung tissue was minced into pieces of > 0.5 mm thickness using a tissue chopper and washed with balanced salt solution (BSS) containing Hanks' balanced salt solution (Gibco, USA) with 0.7 mM sodium bicarbonate (Gibco, USA) at pH 7.4 three times to partially remove macrophages and blood cells. The tissue was digested using a combination of 0.5% trypsin (Gibco, USA) and 4 U/ml elastase (Worthington Biochemical Corporation, USA) for 40 min at 37°C in a shaking water-bath. The digestion was stopped by adding DMEM/F12 medium (Gibco, USA) with 40% FBS in and DNase I (350 U/ml) (Sigma, USA). Cell clumps were dispersed by repeatedly pipetting the cell suspension for 10 min. A disposable cell strainer (gauze size of 50 μm) (BD Bioscience, USA) was used to separate large undigested tissue fragments. The single cell suspension in the flow-through was centrifuged and resuspended in a 1:1 mixture of DMEM/F12 medium and small airway basal medium (SABM) (Lonza, USA) supplemented with 0.5 ng/ml epidermal growth factor (hEGF), 500 ng/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 0.5 μg/ml hydrocortisone, 30 μg/ml bovine pituitary extract and 0.5 mg/ml BSA together with 5% FBS and 350 U/ml DNase I. The cell suspension was plated on a tissue culture grade plastic flask (Corning, USA) and incubated in a 37°C water-jacketed incubator with 5% CO_2 supply for 90 min. The non-adherent cells were layered on a discontinuous cold Percoll

density gradient (densities 1.089 and 1.040 g/ml) and centrifuged at 25×g for 20 min without brake. The cell layer at the interface of the two gradients was collected and washed four times with BSS to remove the Percoll. The cell suspension was incubated with magnetic beads coated with anti-CD14 antibodies at room temperature (RT) for 20 min under constant mixing. After the removal of the beads using a magnet (MACS CD14 MicroBeads), cell viability was assessed by trypan-blue exclusion. The purified pneumocyte suspension was resuspended in small airway growth medium (Lonza, USA) supplemented with 1% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and plated at a cell density of 3×10^5 cells/cm^2. The cells were maintained in a humidified atmosphere (5% CO_2, 37°C) under liquid-covered conditions and growth medium was changed daily starting from 60 h after plating the cells. When the cell layer approached 75% confluence, the pneumocytes were trypsinised and detached into Hank's buffered saline solution

Isolation and culture of primary human peripheral blood monocyte derived macrophages

Peripheral blood leucocytes were separated from buffy coats of healthy blood donors from the Hong Kong Red Cross Blood Transfusion Service by centrifugation on a Ficoll-Paque density gradient and monocytes were purified by adherence on plastic petri dishes as described previously[1-3]. Monocytes were seeded onto tissue culture plates in RPMI 1640 medium supplemented with 5% heat-inactivated autologous plasma. The purity of the monocyte preparations was confirmed by immunostaining for the surface receptor CD14 (BD Biosciences, USA). Cells were allowed to differentiate for 14 days *in vitro* before use. Culture medium was changed to macrophage serum free medium SFM (Gibco, USA) a week before infection.

Infection of human alveolar epithelial cells, peripheral blood monocyte derived macrophages and Caco-2 cells

Cells were infected with viruses at a MOI of 0.1 or two for the analysis of replication kinetics and cytokine and chemokine expression, respectively. DMEM with 2% FBS medium (Gibco, USA) with 100 U/ml penicillin and 100 μg/ml streptomycin was used as inoculum in the mock infected cells. The cell cultures were incubated with the virus inoculum for one hour in a water-jacketed 37°C incubator with 5% CO_2. The cells were rinsed three times with warm PBS and replenished with the appropriate growth medium. The infected cells were harvested for mRNA collection at 24 and 48 hpi. SARS-CoV ORF1b,[4] MERS-CoV UpE,[5] influenza matrix gene were quantified using qPCR as described.[6,7]

Viral titration by $TCID_{50}$ assay

A confluent 96-well tissue culture plate of Vero-E6 or MDCK cells was prepared one day before the virus titration ($TCID_{50}$) assay. Cells were washed once with PBS and replenished with 2% DMEM or serum-free MEM medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and 2 μg/ml of TPCK (tosylsulfonyl phenylalanylchloromethyl ketone) treated trypsin for Vero-E6 and MDCK cells, respectively. Serial dilutions of virus supernatant, from 0.5 log to 7 log, were performed before adding the virus dilutions onto the plates in quadruplicate. The plates were observed for cytopathic effect daily. The end-point of viral dilution leading to CPE in 50% of inoculated wells was estimated using the Karber method.[8]

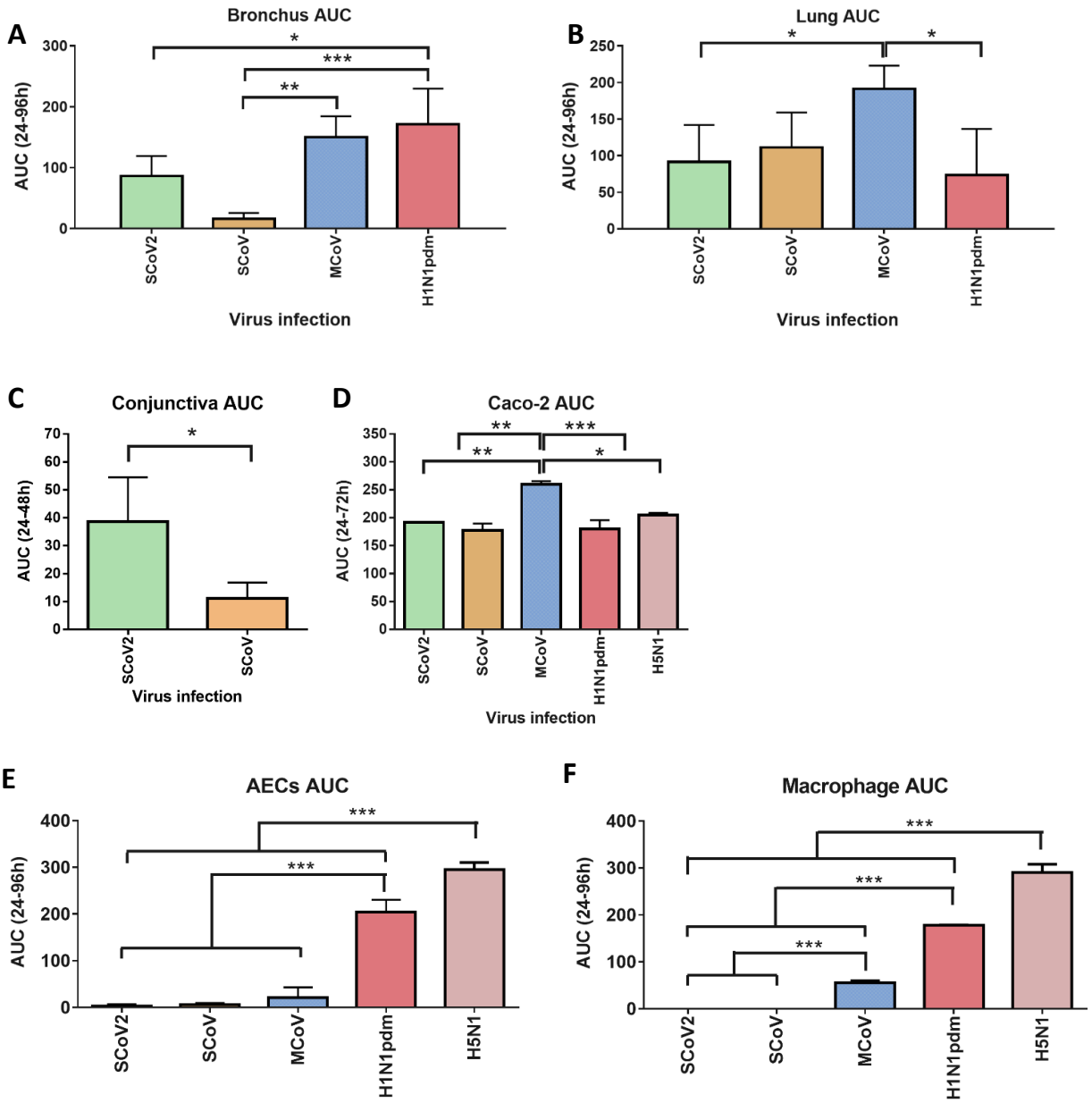
Immunohistochemical staining

Immunohistochemical staining of the respiratory tract tissue was carried out for the SARS-CoV-2 nucleoprotein as follows. The tissue sections were microwaved in 10 mM citrate buffer pH 6.0 for 15 min to expose antigens, blocked with 10% normal horse serum for 10 min at room temperature (RT). The sections were then incubated with 4D11 antibody for 60 min at RT followed by alkaline phosphatase (AP) conjugated anti-mouse antibody (Vector Laboratory, USA) for 60 min at RT, the sections were developed with VectorRed (VR) (Vector Laboratory, USA) for 3 min. After staining for the first antigen, the sections were blocked with 3% H_2O_2 in TBS for 10 min followed by 10% normal horse serum blocking for 10 min at RT. The sections were incubated with anti-CD68 (Dako), acetylated α Tubulin (Santa Cruz) or MUC5AC (ThermoFisher) for 60 min at RT followed by horseradish peroxidase (HRP) conjugated anti-mouse antibody (Vector Laboratory, USA) for 60 min at RT, and developed with DAB (Vector Laboratory, USA) for 10 min. The nuclei were counterstained with Mayer's Hematoxylin for 10 seconds. After bluing with Scott's tap water, the sections were dehydrated and mounted with Permount. The double staining of SCGB1A1/CC10 (Protein-tech), p63-alpha (Cell Signaling Technology) with 4D11 was performed similarly as mentioned above, except after VR visualization the sections were blocked with 10% normal goat serum, followed by incubating with HRP conjugated anti-rabbit antibody (Vector Laboratory, USA) for 60 min at RT and color developed with DAB.

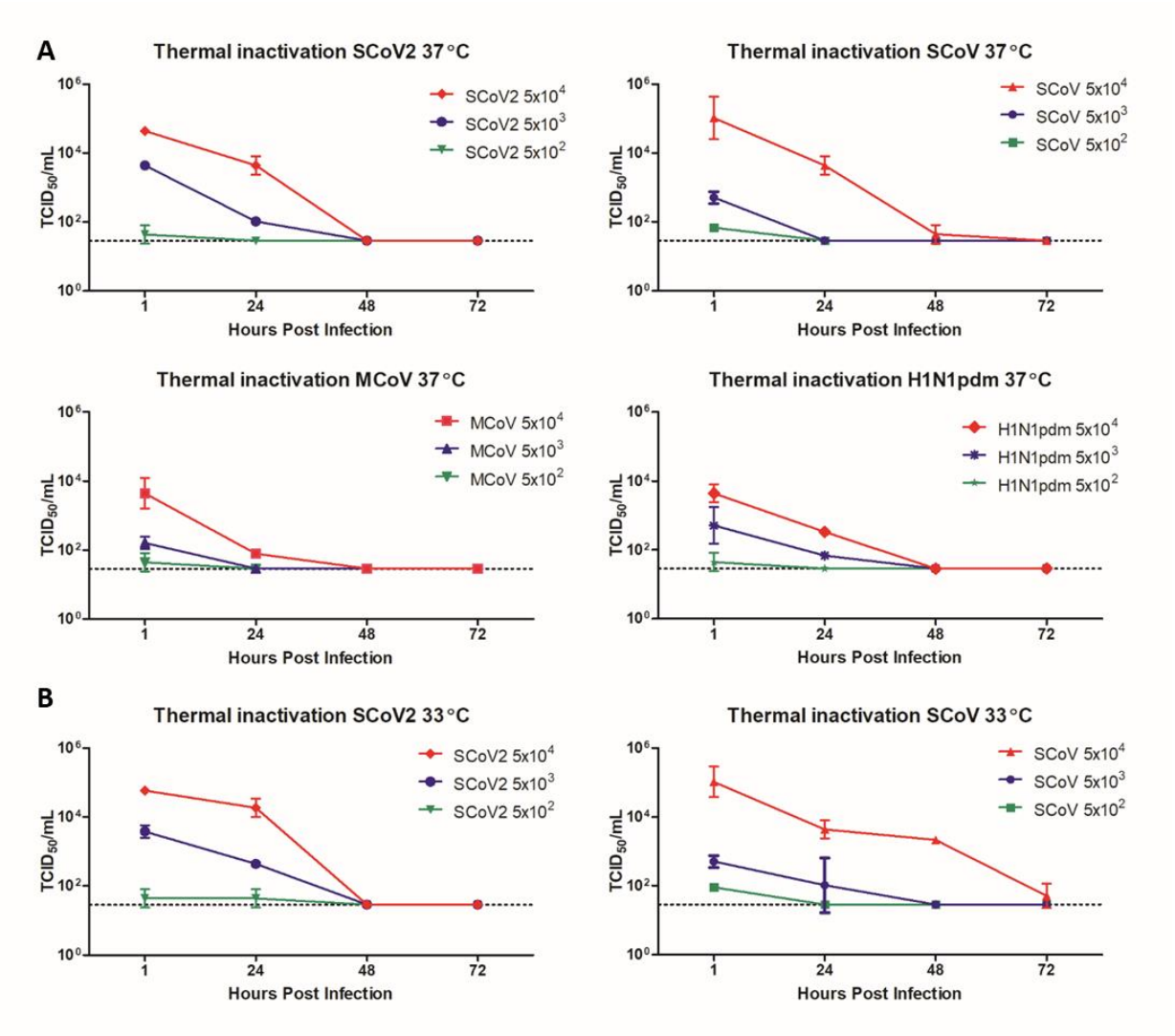
Real-time PCR assay

The RNA of infected cells were extracted at 24 h post infection using a MiniBEST universal RNA extraction kit (Takara Biotechnology). RNA was reverse-transcribed by using oligo-dT primers with

RT-PCR kit (Takara). mRNA expression of target genes was performed using an ABI ViiA™ 7 real-time PCR system (Applied Biosystems). The gene expression profiles of cytokines and chemokines were quantified and normalized with β-actin as previously described[1,3,9].



Supplementary Figure 1. Viral replication kinetics of SARS-CoV-2 and SARS-CoV viruses in *ex vivo* cultures of human respiratory tract, conjunctiva, Caco-2 cells, AECs and macrophages. Data from the e*x vivo* cultures of human (A) bronchus (n=5), (B) lung (n=4), (C) conjunctiva (n=3), *in vitro* cultures of (D) Caco-2 cells (n=3), (E) AECs (n=3) and (F) macrophages (n=3) in Figure 3 A-F respectively are depicted as area under the curve (AUC). Bar-charts show the data at indicated time points denoted as mean ± standard deviation (SD). *p<0·05. **p<0·01. ***p<0·001.



Supplementary Figure 2. Thermal inactivation curves at 37°C and 33°C.

Virus cultures in tissue culture wells without tissues or cells were incubated at (A) 37°C, at which human bronchus and lung tissues were incubated, and (B) 33°C, at which human conjunctiva tissues were incubated. Virus supernatants were harvested at indicated time-points for titration by $TCID_{50}$ assay to define the thermal inactivation of the virus in the absence of replication. Graphs show the mean virus titer from three independent experiments ± standard deviation (SD). The horizontal dotted line denotes the limit of detection in the $TCID_{50}$ assay.

Supplementary Table 1. Tissue donor information

Patient	Tissues used	Age	Gender	Smoking behaviour	Diagnoses
1	Bronchus and lung	75	F	Non-smoker	Lung cancer
2	Bronchus	44	M	Unknown	Lung cancer
3	Bronchus and lung	67	M	Ex-smoker	Lung cancer
4	Bronchus and lung	60	M	Non-smoker	Lung cancer
5	Bronchus and lung	64	F	Non-smoker	Lung cancer
6	Conjunctiva	70	F	Unknown	pterygium, cataract
7	Conjunctiva	57	F	Unknown	pterygium
8	Conjunctiva	85	F	Unknown	pterygium, cataract

Supplementary Table 2. Exact p values for Figure 3

Figure	3A (Bronchus)		3B (Lung)	
	Sum.	P Value	Sum.	P Value
1h				
MCoV vs. SCoV2	ns	>0.9999	ns	>0.9999
MCoV vs. SCoV	ns	0.5435	ns	>0.9999
MCoV vs. H1N1pdm	ns	>0.9999	ns	>0.9999
SCoV2 vs. SCoV	ns	>0.9999	ns	>0.9999
SCoV2 vs. H1N1pdm	ns	>0.9999	ns	>0.9999
SCoV vs. H1N1pdm	ns	>0.9999	ns	>0.9999
24h				
MCoV vs. SCoV2	ns	>0.9999	ns	0.0799
MCoV vs. SCoV	ns	>0.9999	ns	0.4713
MCoV vs. H1N1pdm	*	0.0174	ns	>0.9999
SCoV2 vs. SCoV	ns	>0.9999	ns	>0.9999
SCoV2 vs. H1N1pdm	**	0.0056	ns	>0.9999
SCoV vs. H1N1pdm	*	0.011	ns	>0.9999
48h				
MCoV vs. SCoV2	ns	>0.9999	****	<0.0001
MCoV vs. SCoV	ns	0.0593	**	0.0045
MCoV vs. H1N1pdm	ns	0.2411	***	0.0009
SCoV2 vs. SCoV	ns	>0.9999	ns	>0.9999
SCoV2 vs. H1N1pdm	*	0.0232	ns	>0.9999
SCoV vs. H1N1pdm	***	0.0002	ns	>0.9999
72h				
MCoV vs. SCoV2	ns	>0.9999	*	0.044
MCoV vs. SCoV	*	0.0427	ns	0.1665
MCoV vs. H1N1pdm	ns	>0.9999	**	0.0017
SCoV2 vs. SCoV	*	0.047	ns	>0.9999
SCoV2 vs. H1N1pdm	ns	>0.9999	ns	>0.9999
SCoV vs. H1N1pdm	ns	0.0921	ns	0.5811
96h				
MCoV vs. SCoV2	ns	>0.9999	ns	0.1148
MCoV vs. SCoV	****	<0.0001	ns	0.2505
MCoV vs. H1N1pdm	ns	>0.9999	***	0.0002
SCoV2 vs. SCoV	**	0.0017	ns	>0.9999
SCoV2 vs. H1N1pdm	ns	>0.9999	ns	0.1285
SCoV vs. H1N1pdm	*	0.0332	ns	0.0949

Figure	3C (Conjunctiva)	
	Sum.	P Value
SCoV2 - SCoV		
1h	ns	>0.9999
24h	ns	0.9644
48h	**	0.0017

Figure	3D (Caco-2		3E (AECs)		3F (Macrophage)	
	Sum.	P Value	Sum.	P Value	Sum.	P Value
1h						
MCoV vs. SCoV2	ns	>0.9999	ns	>0.9999	ns	>0.9999
MCoV vs. SCoV	ns	>0.9999	ns	>0.9999	ns	>0.9999
MCoV vs. H1N1pdm	ns	0.5113	ns	>0.9999	****	<0.0001
MCoV vs. H5N1	ns	0.4958	ns	0.6731	****	<0.0001
SCoV2 vs. SCoV	ns	>0.9999	ns	>0.9999	ns	0.426
SCoV2 vs. H1N1pdm	ns	>0.9999	ns	>0.9999	****	<0.0001
SCoV2 vs. H5N1	ns	>0.9999	ns	>0.9999	**	0.0072
SCoV vs. H1N1pdm	ns	>0.9999	ns	>0.9999	****	<0.0001
SCoV vs. H5N1	ns	>0.9999	ns	>0.9999	****	<0.0001
H1N1pdm vs. H5N1	ns	>0.9999	ns	>0.9999	****	<0.0001
24h						
MCoV vs. SCoV2	ns	0.0879	ns	>0.9999	****	<0.0001
MCoV vs. SCoV	**	0.0042	ns	>0.9999	****	<0.0001
MCoV vs. H1N1pdm	****	<0.0001	***	0.0007	****	<0.0001
MCoV vs. H5N1	ns	0.9788	****	<0.0001	****	<0.0001
SCoV2 vs. SCoV	ns	>0.9999	ns	>0.9999	ns	>0.9999
SCoV2 vs. H1N1pdm	*	0.0499	***	0.0003	****	<0.0001
SCoV2 vs. H5N1	ns	>0.9999	****	<0.0001	****	<0.0001
SCoV vs. H1N1pdm	ns	0.555	***	0.0002	****	<0.0001
SCoV vs. H5N1	ns	>0.9999	****	<0.0001	****	<0.0001
H1N1pdm vs. H5N1	*	0.0223	****	<0.0001	****	<0.0001
48h						
MCoV vs. SCoV2	****	<0.0001	ns	>0.9999	****	<0.0001
MCoV vs. SCoV	****	<0.0001	ns	>0.9999	****	<0.0001
MCoV vs. H1N1pdm	****	<0.0001	****	<0.0001	****	<0.0001
MCoV vs. H5N1	**	0.0015	****	<0.0001	****	<0.0001
SCoV2 vs. SCoV	ns	>0.9999	ns	>0.9999	ns	>0.9999
SCoV2 vs. H1N1pdm	ns	>0.9999	****	<0.0001	****	<0.0001
SCoV2 vs. H5N1	ns	>0.9999	****	<0.0001	****	<0.0001
SCoV vs. H1N1pdm	ns	>0.9999	****	<0.0001	****	<0.0001
SCoV vs. H5N1	ns	>0.9999	****	<0.0001	****	<0.0001
H1N1pdm vs. H5N1	ns	>0.9999	****	<0.0001	****	<0.0001
72h						
MCoV vs. SCoV2	*	0.0174	ns	>0.9999	****	<0.0001
MCoV vs. SCoV	ns	>0.9999	ns	>0.9999	****	<0.0001
MCoV vs. H1N1pdm	*	0.0222	****	<0.0001	****	<0.0001
MCoV vs. H5N1	ns	0.2099	****	<0.0001	****	<0.0001
SCoV2 vs. SCoV	ns	0.9631	ns	>0.9999	ns	>0.9999
SCoV2 vs. H1N1pdm	ns	>0.9999	****	<0.0001	****	<0.0001
SCoV2 vs. H5N1	ns	>0.9999	****	<0.0001	****	<0.0001
SCoV vs. H1N1pdm	ns	0.9454	****	<0.0001	****	<0.0001
SCoV vs. H5N1	ns	>0.9999	****	<0.0001	****	<0.0001
H1N1pdm vs. H5N1	ns	>0.9999	ns	>0.9999	****	<0.0001
96h						
MCoV vs. SCoV2	--		ns	0.7809	****	<0.0001
MCoV vs. SCoV	--		ns	0.7809	****	<0.0001
MCoV vs. H1N1pdm	--		ns	0.2837	****	<0.0001
MCoV vs. H5N1	--		****	<0.0001	****	<0.0001
SCoV2 vs. SCoV	--		ns	>0.9999	ns	>0.9999
SCoV2 vs. H1N1pdm	--		**	0.003	****	<0.0001
SCoV2 vs. H5N1	--		****	<0.0001	****	<0.0001
SCoV vs. H1N1pdm	--		**	0.003	****	<0.0001
SCoV vs. H5N1	--		****	<0.0001	****	<0.0001
H1N1pdm vs. H5N1	--		****	<0.0001	****	<0.0001

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