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Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

ONLINE SUPPLEMENTARY MATERIAL

Tropism and replication of Middle East respiratory syndrome coronavirus from dromedary camels in the human respiratory tract: and in-vitro and ex vivo study

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METHODS:

Viral titrations

Vero-E6 (ATCC CRL-1586, Manassas, VA) were cultured using DMEM with 10% FBS and with 100 units/ml penicillin and 100 μg/ml streptomycin. MDCK were cultured using MEM with 10% FBS and with 100 units/ml penicillin and 100 μg/ml streptomycin. Viruses were titrated in serial half-$\log_{10}$ dilutions on 96-well tissue culture plates of Vero-E6 cells to obtain 50% tissue culture infectious dose ($TCID_{50}$) for each virus. The highest viral dilution leading to cytopathic effect (CPE) in ~50% of inoculated wells was estimated using the Karber method.

Ex vivo organ cultures and infection

Fresh human bronchus and lung parenchyma tissues were obtained from patients undergoing surgical resection of lung tissue at Queen Mary Hospital as part of clinical care that were surplus for routine diagnostic requirements. This study was approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW-13-104). Ex vivo cultures of human bronchial and lung tissues were performed as previously described.[1] The bronchial mucosae were placed on a surgical sponge with their apical epithelial surface facing upwards, while the lung parenchymal tissues were placed into a 24-well plate with 1 ml of F12K culture medium and with 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technology, Carlsbad, CA) at 37°C. The bronchial and lung tissues were infected with each MERS-CoV at a titre of 10^6 $TCID_{50}$/ml. After incubation for 1 h at 37°C, the cultures were washed three times with 5 ml of warm PBS to remove unbound virus. Mock-inoculated tissue was used as controls. Culture supernatants from the infected cultures were collected at 1, 24, 48, and 72 hpi and titrated in parallel for infectious virus using the $TCID_{50}$ assay.

Quantification of viral and host cytokine and chemokine mRNAs by quantitative RT-PCR

Mock and MERS-CoVs infected Calu-3 cells were harvested at 6, 24, and 30 hpi to quantitate MERS-CoV RNA and host cytokine and chemokine gene mRNA expression. Methods have been described previously.[1] The host gene expression profiles for proinflammatory cytokines (tumor necrosis factor alpha (TNF-α), interferon-β (IFN-β), interleukin (IL)-6, chemokines IFN-γ-induced protein 10 (CXCL10), and the housekeeping (β-actin) gene were determined and absolute copy numbers quantified using a standard curve generated from a plasmid with a known copy number run in parallel. The quantitative PCR (qPCR) SYBR Premix Ex Taq (Takara Shiga, Japan) was used and the real-time qPCR assays were run on a ViiA™ 7 Real Time PCR System (Life technology, Carlsbad, CA). Expression of these genes was normalized by using the product of the β-actin housekeeping gene mRNA.
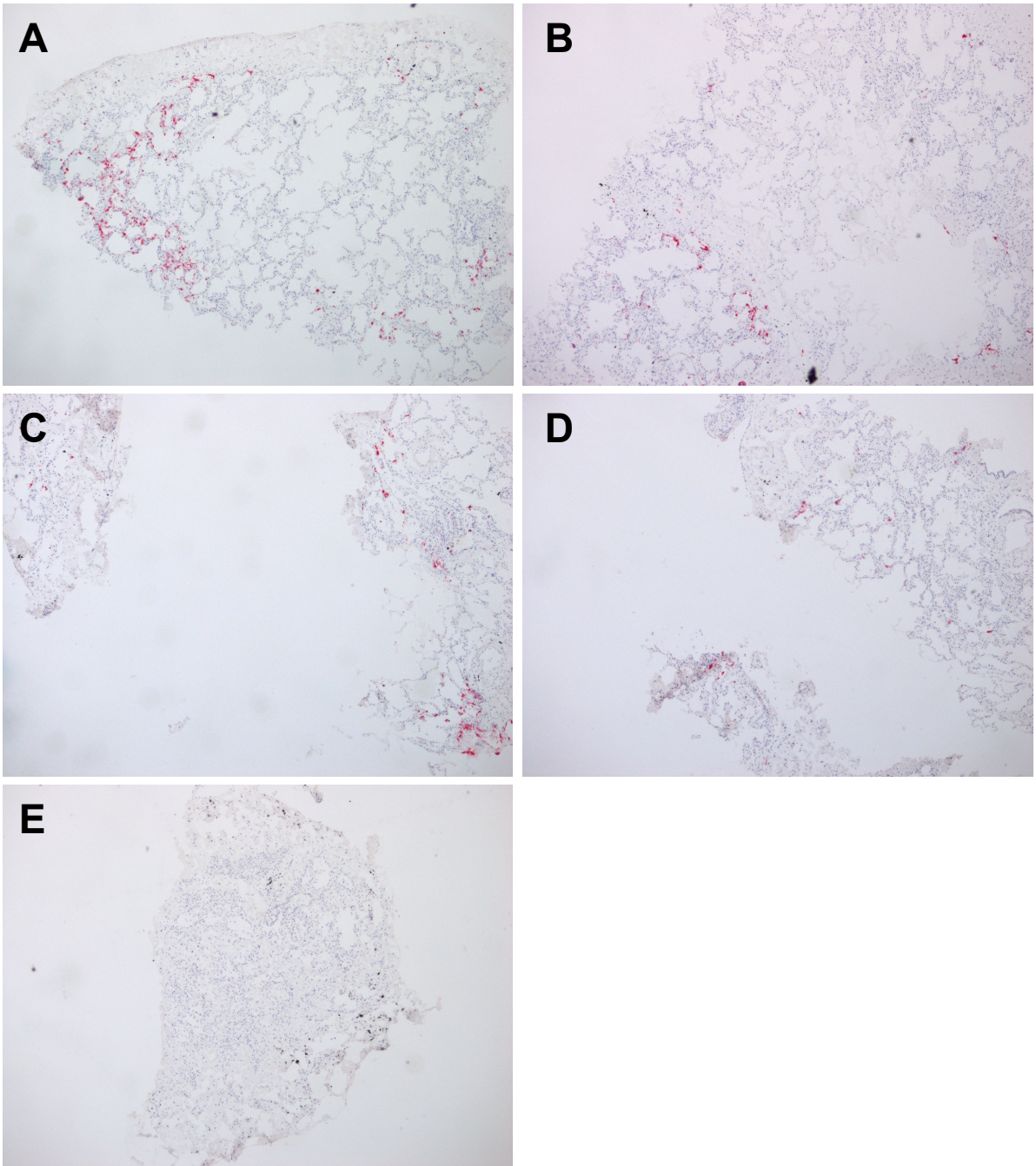
Immunohistochemistry

The 10% formalin-fixed tissues were embedded in paraffin and stained using a polyclonal mouse antibody raised against coronavirus HKU4·2 N glycoprotein (1:1000), which shares 71% identity with the N protein of hMERS-CoV EMC and cross-reacts with MERS-CoV by Western blotting and immunofluorescence assays as

previously described.[2] The reaction of the primary antibody was revealed by the use of biotinylated goat anti-mouse antibody (1:500; 115-065-146; Jackson ImmunoResearch, West Grove, PA) and developed using a VECTOR Red Alkaline Phosphatase substrate kit (SK-5100; Vector Labs, Peterborough, UK). Antibodies to CD68 (M0814; Dako, Glostrup, Denmark) for macrophages, AE1/AE3 (M3515; Dako, Glostrup, Denmark) for epithelial cells, β-tubulin (F-2043; Sigma, Vector Labs, Peterborough, UK) for ciliated bronchial epithelial cells, MUC5AC (18-2261; Life Technology, Carlsbad, CA) for goblet cells, and pro-surfactant C (ab3786, Millipore, Billerica, MA) for type II pneumocytes were used for double labeling using immunofluorescence with secondary antibody conjugated with fluorescein isothiocyanate (FITC). MERS-CoV was detected using mouse antiserum to HKU4·2 N protein kindly provided by Dr Ralph Baric, The University of North Carolina, Chapel Hill, North Carolina. Vector Red substrate was used in these double-labeling experiments as this substrate is also fluorescent when using a tetramethyl rhodamine isocyanate filter. The stained preparations were examined using a Nikon N*i* immunofluorescence microscope, and images were captured using a SPOT Slider 2-megapixel camera.

Reference

1.	Chan RW, Chan MC, Agnihothram S, et al. Tropism of and innate immune responses to the novel human betacoronavirus lineage C virus in human ex vivo respiratory organ cultures. J Virol. 2013; **87**(12): 6604-14.

2.	van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. MBio. 2012; **3**(6).

Supplementary Figure 1. Tissue tropism of human and camel MERS-CoVs in ex vivo formalin-fixed paraffin-embedded sections of lung in low power magnification. Immunohistochemical staining of MERS-CoV N protein (stained by Vector Red in pink) in the human lung cultures were determined after infection by (**A**) hMERS-CoV EMC from human, (**B**) dcMERS-CoV AH13, (**C**) dcMERS-CoV AH19D and (**D**) dcMERS-CoV NRCE-HKU270, and (**E**) Mock from camel at 72 hpi.

hMERS-CoV EMC   **dcMERS-CoV AH13**

dcMERS-CoV AH19D   **dcMERS-CoV NRCE-HKU270**
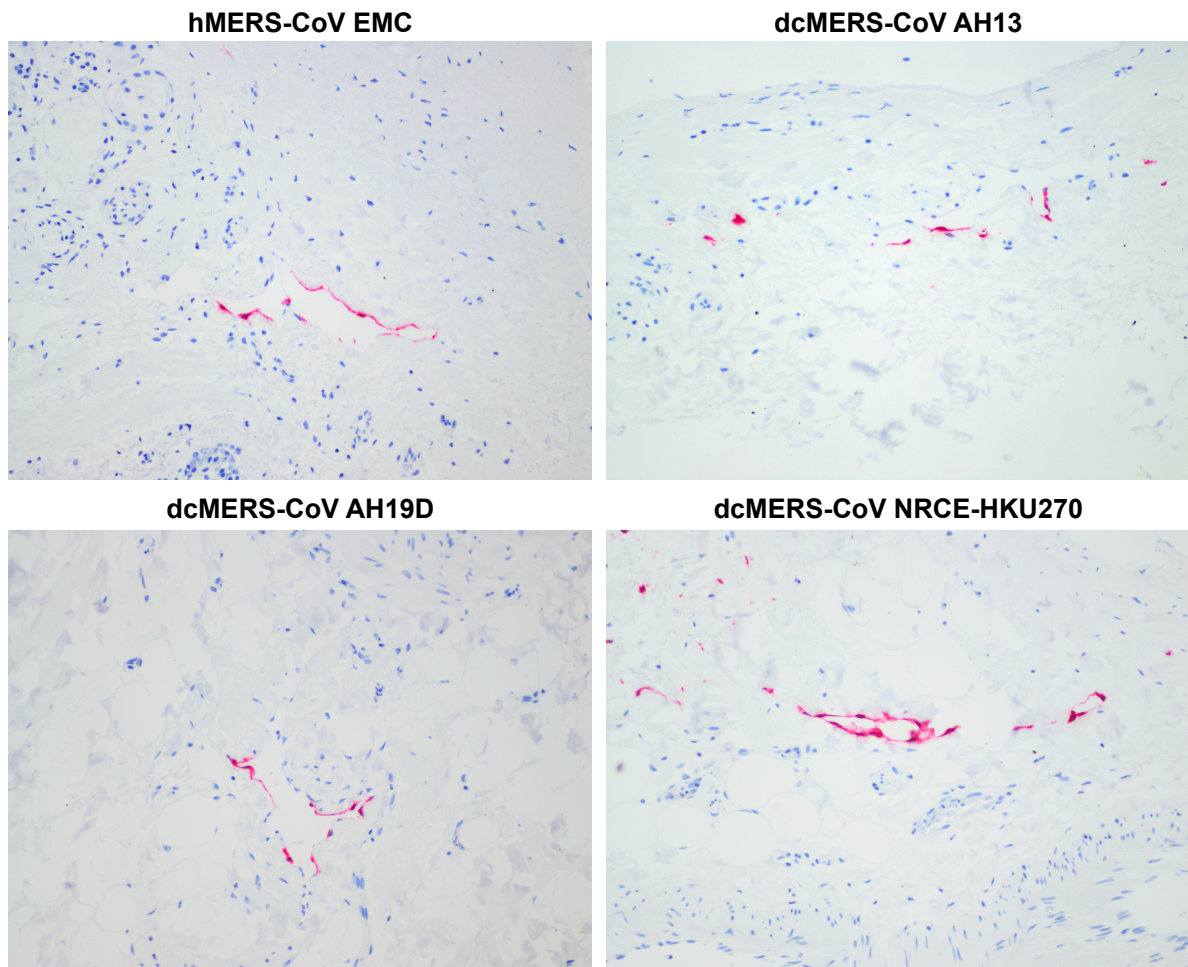
Supplementary Figure 2. Endothelial cell tropism of MERS-CoVs in human bronchial tissue at 72hpi. Immunohistochemical staining of MERS-CoV N protein (stained by Vector Red in pink) in the endothelial cell of human bronchial tissue were determined after infection by (**A**) hMERS-CoV EMC from human, (**B**) dcMERS-CoV AH13, (**C**) dcMERS-CoV AH19D, and (**D**) dcMERS-CoV NRCE-HKU270 from camel at 72 hpi.
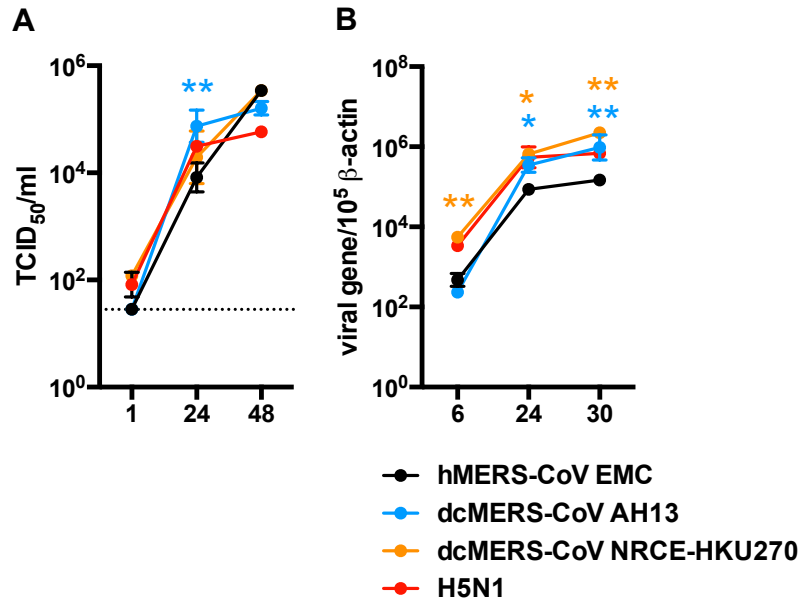
Supplementary Figure 3. MERS-CoVs replication kinetics in Calu-3 cells. (**A**) Culture were infected at a MOI of 0·01 and supernatants were harvested at the 1, 24, and 48 hpi, and virus titres were determined by $TCID_{50}$ assay. Results are presented as mean titre with error bars show standard error of mean (SEM) from three independent experiments. The horizontal dotted line denotes the limit of detection in the $TCID_{50}$ assay. (**B**) Expression of the coronavirus E protein gene or influenza Matrix protein gene at 6, 24, and 30 hpi in Calu-3 were shown. Means and SEMs of the mRNA copies expressed per 10^5 β er 10e were shown. Statistical significance of viral titre and gene expression amongst hMERS-CoV and dcMERS-CoVs infected Calu-3 at each time point were compared using pairwise one way ANOVA followed by *Bonferroni* multiple-comparison test. Asterisk denote significant difference between hMERS-CoV and dcMERS-CoV with its corresponding color key * $p < 0·05$, and ** $p < 0·01$. Key: Black – human MERS-CoV EMC infected; Blue – camel MERS-CoV AH13 infected; Orange – camel MERS-CoV NRCE-HKU270; and Red- influenza H5N1 virus infected.
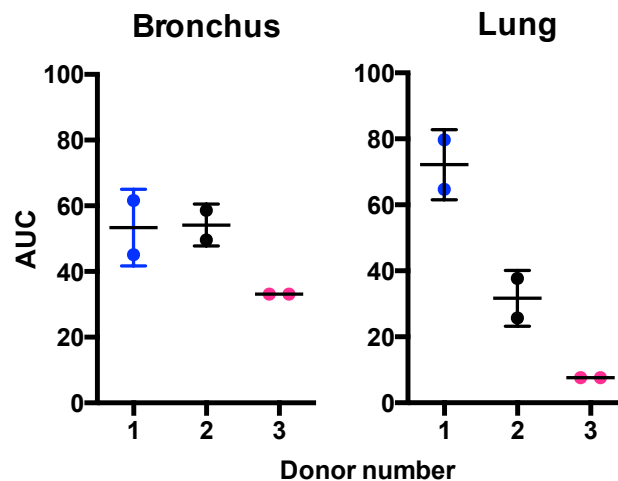
Supplementary Figure 4. Technical variation in ex-vivo bronchial and lung cultures. The data points on the graph showed AUC values of human MERS-CoV EMC calculated from two replicates of (A) bronchial and (B) lung ex vivo cultures per donor from three independent donors. The line showed the mean and the error bar showed the SD of the data set.

A

Clade	nsp1-3, 8217 nt	NRCE-HKU270	NRCE-HKU205	Jordan-N3	EMC_2012	FRA/UAE	**KFU-HKU13**	Al-Hasa_4	Riyadh_9	Riyadh_1	Jeddah_1	England_1	Riyadh_14
Egypt	**MERS-CoV_NRCE-HKU270_2013/12/30**												
Egypt	MERS-CoV_NRCE-HKU205_2013/11/06	0.9954											
A	Human_betacoronavirus_2c_Jordan-N3_2012/04	0.9942	0.9944										
A	Human_betacoronavirus_2c_EMC_2012/06/13	0.9944	0.9946	0.9983									
B	MERS-CoV_isolate_FRA/UAE_2013/05/07	0.9921	0.9923	0.9950	0.9953								
B	**MERS-CoV_Al-Hasa_KFU-HKU13_2013/12/30**	0.9923	0.9926	0.9953	0.9955	0.9988							
B	MERS-CoV_isolate_Al-Hasa_4_2013/05/01	0.9927	0.9929	0.9956	0.9959	0.9987	0.9989						
B	MERS-CoV_isolate_Riyadh_9_2013/07/17	0.9923	0.9926	0.9953	0.9955	0.9983	0.9985	0.9989					
B	MERS-CoV_isolate_Riyadh_1_2012/10/23	0.9929	0.9932	0.9959	0.9961	0.9987	0.9989	0.9993	0.9989				
B	MERS-CoV_isolate_Jeddah_1_2013/11/06	0.9925	0.9927	0.9956	0.9959	0.9977	0.9979	0.9983	0.9979	0.9985			
B	Betacoronavirus_England_1_2012/09/11	0.9932	0.9934	0.9961	0.9963	0.9989	0.9991	0.9995	0.9991	0.9998	0.9988		
B	MERS-CoV_isolate_Riyadh_14_2013/08/15	0.9927	0.9929	0.9959	0.9959	0.9977	0.9979	0.9983	0.9979	0.9983	0.9976	0.9985	

Clade	nsp5-10, 3390 nt	NRCE-HKU270	NRCE-HKU205	Jordan-N3	EMC_2012	FRA/UAE	KFU-HKU13	Al-Hasa_4	Riyadh_9	Riyadh_1	Jeddah_1	England_1	Riyadh_14
Egypt	**MERS-CoV_NRCE-HKU270_2013/12/30**												
Egypt	MERS-CoV_NRCE-HKU205_2013/11/06	0.9979											
A	Human_betacoronavirus_2c_Jordan-N3_2012/04	0.9962	0.9965										
A	Human_betacoronavirus_2c_EMC_2012/06/13	0.9962	0.9965	0.9988									
B	MERS-CoV_isolate_FRA/UAE_2013/05/07	0.9956	0.9959	0.9970	0.9970								
B	**MERS-CoV_Al-Hasa_KFU-HKU13_2013/12/30**	0.9956	0.9959	0.9970	0.9970	1.0000							
B	MERS-CoV_isolate_Al-Hasa_4_2013/05/01	0.9950	0.9953	0.9965	0.9965	0.9994	0.9994						
B	MERS-CoV_isolate_Riyadh_9_2013/07/17	0.9950	0.9953	0.9965	0.9965	0.9994	0.9994	0.9994					
B	MERS-CoV_isolate_Riyadh_1_2012/10/23	0.9941	0.9944	0.9962	0.9962	0.9985	0.9985	0.9979	0.9979				
B	MERS-CoV_isolate_Jeddah_1_2013/11/06	0.9941	0.9944	0.9962	0.9962	0.9985	0.9985	0.9979	0.9979	0.9988			
B	Betacoronavirus_England_1_2012/09/11	0.9944	0.9947	0.9965	0.9965	0.9988	0.9988	0.9982	0.9982	0.9991	0.9991		
B	MERS-CoV_isolate_Riyadh_14_2013/08/15	0.9947	0.9950	0.9962	0.9962	0.9991	0.9991	0.9985	0.9985	0.9976	0.9976	0.9979	

Clade	S, 4095 nt	NRCE-HKU270	NRCE-HKU205	Jordan-N3	EMC_2012	FRA/UAE	KFU-HKU13	Al-Hasa_4	Riyadh_9	Riyadh_1	Jeddah_1	England_1	Riyadh_14
Egypt	**MERS-CoV_NRCE-HKU270_2013/12/30**												
Egypt	MERS-CoV_NRCE-HKU205_2013/11/06	0.9924											
A	Human_betacoronavirus_2c_Jordan-N3_2012/04	0.9958	0.9936										
A	Human_betacoronavirus_2c_EMC_2012/06/13	0.9953	0.9931	0.9975									
B	MERS-CoV_isolate_FRA/UAE_2013/05/07	0.9933	0.9911	0.9956	0.9970								
B	**MERS-CoV_Al-Hasa_KFU-HKU13_2013/12/30**	0.9926	0.9904	0.9948	0.9963	0.9978							
B	MERS-CoV_isolate_Al-Hasa_4_2013/05/01	0.9931	0.9909	0.9953	0.9968	0.9978	0.9970						
B	MERS-CoV_isolate_Riyadh_9_2013/07/17	0.9933	0.9909	0.9953	0.9968	0.9978	0.9970	0.9990					
B	MERS-CoV_isolate_Riyadh_1_2012/10/23	0.9941	0.9924	0.9963	0.9978	0.9983	0.9975	0.9980	0.9980				
B	MERS-CoV_isolate_Jeddah_1_2013/11/06	0.9946	0.9928	0.9968	0.9978	0.9958	0.9951	0.9956	0.9956	0.9970			
B	Betacoronavirus_England_1_2012/09/11	0.9943	0.9931	0.9965	0.9975	0.9956	0.9948	0.9953	0.9953	0.9968	0.9993		
B	MERS-CoV_isolate_Riyadh_14_2013/08/15	0.9928	0.9911	0.9951	0.9965	0.9970	0.9963	0.9968	0.9968	0.9983	0.9963	0.9961	

B

Clade	nsp1-3, 2739 aa	NRCE-HKU270	NRCE-HKU205	Jordan-N3	EMC_2012	FRA/UAE	KFU-HKU13	Al-Hasa_4	Riyadh_9	Riyadh_1	Jeddah_1	England_1	Riyadh_14
Egypt	**MERS-CoV_NRCE-HKU270_2013/12/30**												
Egypt	MERS-CoV_NRCE-HKU205_2013/11/06	0.9931											
A	Human_betacoronavirus_2c_Jordan-N3_2012/04	0.9894	0.9901										
A	Human_betacoronavirus_2c_EMC_2012/06/13	0.9898	0.9905	0.9967									
B	MERS-CoV_isolate_FRA/UAE_2013/05/07	0.9890	0.9898	0.9949	0.9953								
B	**MERS-CoV_Al-Hasa_KFU-HKU13_2013/12/30**	0.9890	0.9898	0.9949	0.9953	0.9993							
B	MERS-CoV_isolate_Al-Hasa_4_2013/05/01	0.9887	0.9894	0.9945	0.9949	0.9989	0.9989						
B	MERS-CoV_isolate_Riyadh_9_2013/07/17	0.9880	0.9887	0.9938	0.9942	0.9982	0.9982	0.9978					
B	MERS-CoV_isolate_Riyadh_1_2012/10/23	0.9890	0.9898	0.9949	0.9953	0.9989	0.9989	0.9985	0.9978				
B	MERS-CoV_isolate_Jeddah_1_2013/11/06	0.9880	0.9887	0.9938	0.9942	0.9974	0.9974	0.9971	0.9963	0.9978			
B	Betacoronavirus_England_1_2012/09/11	0.9894	0.9901	0.9953	0.9956	0.9993	0.9993	0.9989	0.9982	0.9996	0.9982		
B	MERS-CoV_isolate_Riyadh_14_2013/08/15	0.9883	0.9890	0.9949	0.9945	0.9985	0.9985	0.9982	0.9974	0.9982	0.9967	0.9985	

Clade	nsp5-10, 1130 aa	NRCE-HKU270	NRCE-HKU205	Jordan-N3	EMC_2012	FRA/UAE	KFU-HKU13	Al-Hasa_4	Riyadh_9	Riyadh_1	Jeddah_1	England_1	Riyadh_14
Egypt	**MERS-CoV_NRCE-HKU270_2013/12/30**												
Egypt	MERS-CoV_NRCE-HKU205_2013/11/06	0.9973											
A	Human_betacoronavirus_2c_Jordan-N3_2012/04	0.9947	0.9956										
A	Human_betacoronavirus_2c_EMC_2012/06/13	0.9965	0.9973	0.9982									
B	MERS-CoV_isolate_FRA/UAE_2013/05/07	0.9965	0.9973	0.9982	1.0000								
B	**MERS-CoV_Al-Hasa_KFU-HKU13_2013/12/30**	0.9965	0.9973	0.9982	1.0000	1.0000							
B	MERS-CoV_isolate_Al-Hasa_4_2013/05/01	0.9965	0.9973	0.9982	1.0000	1.0000	1.0000						
B	MERS-CoV_isolate_Riyadh_9_2013/07/17	0.9965	0.9973	0.9982	1.0000	1.0000	1.0000	1.0000					
B	MERS-CoV_isolate_Riyadh_1_2012/10/23	0.9956	0.9965	0.9973	0.9991	0.9991	0.9991	0.9991	0.9991				
B	MERS-CoV_isolate_Jeddah_1_2013/11/06	0.9965	0.9973	0.9982	1.0000	1.0000	1.0000	1.0000	1.0000	0.9991			
B	Betacoronavirus_England_1_2012/09/11	0.9965	0.9973	0.9982	1.0000	1.0000	1.0000	1.0000	1.0000	0.9991	1.0000		
B	MERS-CoV_isolate_Riyadh_14_2013/08/15	0.9956	0.9965	0.9973	0.9991	0.9991	0.9991	0.9991	0.9991	0.9982	0.9991	0.9991	

Clade	S gene, 1353 aa	NRCE-HKU270	NRCE-HKU205	Jordan-N3	EMC_2012	FRA/UAE	KFU-HKU13	Al-Hasa_4	Riyadh_9	Riyadh_1	Jeddah_1	England_1	Riyadh_14
Egypt	**MERS-CoV_NRCE-HKU270_2013/12/30**												
Egypt	MERS-CoV_NRCE-HKU205_2013/11/06	0.9904											
A	Human_betacoronavirus_2c_Jordan-N3_2012/04	0.9948	0.9911										
A	Human_betacoronavirus_2c_EMC_2012/06/13	0.9948	0.9911	0.9970									
B	MERS-CoV_isolate_FRA/UAE_2013/05/07	0.9919	0.9881	0.9941	0.9970								
B	**MERS-CoV_Al-Hasa_KFU-HKU13_2013/12/30**	0.9911	0.9874	0.9933	0.9963	0.9963							
B	MERS-CoV_isolate_Al-Hasa_4_2013/05/01	0.9933	0.9896	0.9956	0.9985	0.9970	0.9963						
B	MERS-CoV_isolate_Riyadh_9_2013/07/17	0.9933	0.9889	0.9948	0.9978	0.9963	0.9956	0.9978					
B	MERS-CoV_isolate_Riyadh_1_2012/10/23	0.9933	0.9896	0.9956	0.9985	0.9970	0.9963	0.9985	0.9978				
B	MERS-CoV_isolate_Jeddah_1_2013/11/06	0.9941	0.9904	0.9963	0.9993	0.9970	0.9963	0.9985	0.9978	0.9985			
B	Betacoronavirus_England_1_2012/09/11	0.9933	0.9896	0.9956	0.9985	0.9963	0.9956	0.9978	0.9970	0.9978	0.9993		
B	MERS-CoV_isolate_Riyadh_14_2013/08/15	0.9941	0.9904	0.9963	0.9993	0.9978	0.9970	0.9993	0.9985	0.9993	0.9993	0.9985	

Supplementary Table 1. Pairwise sequence similarities between MERS-CoVs from Egypt camels, clade A human viruses and clade B human viruses. Three most variable regions (nsp1-3, nsp5-10 and S gene), which cover more than 50% of the genome, were selected from the genomes of these viruses for the analysis. The similarity values were estimated using nucleotide sequences (table A) and amino acid sequences (table B). Viruses used in this study are bolded.

A Bronchus

	hMERS-CoV EMC	dcMERS-CoV AH13	dcMERS-CoV AH19D	dcMERS-CoV NRCE-HKU270
Donor 1	112.8	63.3	55.8	42.3
Donor 2	184.8	147.3	96.3	139.8
Donor 3	21.3	40.8	31.8	30.3
Donor 4	178.8	73.8	42.3	103.8
Donor 5	112.8	61.8	46.8	87.3
Mean	122.1	77.4	54.6	80.7
Std. Deviation	66.1	40.9	24.9	45.0
Std. Error of Mean	29.6	18.3	11.1	20.1
Lower 95% CI of mean	40.0	26.6	23.7	24.9
Upper 95% CI of mean	204.2	128.1	85.5	136.5

B Lung

	hMERS-CoV EMC	dcMERS-CoV AH13	dcMERS-CoV AH19D	dcMERS-CoV NRCE-HKU270
Donor 1	178.8	49.8	34.8	0.3
Donor 2	115.8	90.3	70.8	57.3
Donor 3	4.8	9.3	21.3	39.3
Donor 4	118.8	42.3	19.8	43.8
Donor 5	49.8	4.8	0.3	0.3
Mean	93.6	39.3	29.4	28.2
Std. Deviation	67.4	34.7	26.2	26.3
Std. Error of Mean	30.2	15.5	11.7	11.8
Lower 95% CI of mean	9.8	-3.8	-3.2	-4.5
Upper 95% CI of mean	177.3	82.3	61.9	60.9

C Friedman test

	Bronchus	Lung
P value	0.151	0.270
Exact or approximate P value?	Exact	Exact
P value summary	ns	ns
Are means signif. different? (P < 0.05)	No	No
Number of groups	4	4
Friedman statistic	5.40	4.10
Data summary		
Number of treatments (columns)	4	4
Number of subjects (rows)	5	5
Number of families	1	1
Number of comparisons per family	6	6
Alpha	0.05	0.05

Supplementary Table 2. Table shows the area under curve (AUC) data values of each donor after MERS-CoVs infection calculated from 24 hpi to 72 hpi from figure 4B and C. Only that area above the detection limit of the assay (denoted by dotted line in figure 4) is considered. The statistical analysis summary shows the data in (**A**) bronchus and (**B**) lung ex vivo cultures from all 5 donors. (**C**) Summary table shows the Friedman test result of the AUC values obtained from bronchus and lung cultures.