Online Data Supplement:

Severe respiratory viral infection induces procalcitonin in the absence of bacterial pneumonia

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Supplemental methods:

Matching strategy

We used a matching strategy to test the hypothesis that elevated procalcitonin (PCT) in the co-infection group was attributable to severity rather than bacterial infection. In this approach, we assembled a subset of 179 patients from the group of 2,075 with pure respiratory viral infection that matched the 179 patients in the bacterial co-infection group with respect to four severity-related variables: gender, intensive care unit (ICU) admission status, mechanical ventilation, and in-hospital mortality. We used a matching algorithm to randomly select 75 discrete matched lists of 179 patients from the pure viral group. Receiver operator characteristic curves (ROCs) for PCT were plotted and areas under the curves (AUCs) were calculated for each matched list. The standard deviation of the AUCs for these 75 lists was low (0.019), and the 95% CI was 0.577 to 0.586. The list corresponding to the median AUC (0.584) was used as the matched viral group.

Gautam S, *et al. Thorax* 2020; 75:974–981. doi: 10.1136/thoraxjnl-2020-214896

The number of matching variables (four) was constrained by the size of the study population. We selected gender, ICU admission, mechanical ventilation, and mortality for the primary analysis as these variables were significantly different between the pure viral and bacterial co-infection groups in Table 1, and represent important markers of disease severity *a priori*. However, several other variables in Table 1 were also significantly different between these two groups. We therefore performed sequential matching controls for every combination of these variables. This produced median AUCs that were nearly identical to the group used in the primary analysis (AUC 0.584). For example, matching for i) gender, ICU admission, fever, and obesity; and ii) gender, ICU admission, white blood cell count, and creatinine produced median AUCs of 0.582 and 0.579, respectively. It is important to note that certain matching variables were recorded contemporaneously with PCT (see 'Timing of patient data collection' below), while others were recorded at the terminal point of the hospitalization (e.g. mortality). Nevertheless, the timing of variable had no appreciable effect on the AUC results. For instance, matching against a combination of contemporaneous and terminal variables (such as gender, ICU admission, mechanical ventilation, and mortality – as used throughout the primary analysis) produced a highly similar AUC to a purely contemporaneous set of variables (such as gender, ICU admission, white blood cell count, and creatinine): 0.584 vs. 0.579, respectively.

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We elected to utilize a matching strategy to control for the confounding effects of disease severity due to several factors that complicate more commonly-used adjustment analyses. For instance, the use of analysis of covariance (ANCOVA) proved difficult because of the high degree of collinearity between covariates, and the strong associations between group membership and nearly all of the covariates. Additional complexity arose from the presence of both dichotomous and continuous covariates, and from the large imbalance in sample size between groups. The matching strategy solved several of these problems, ensuring group size balance and simplifying subsequent analysis while maintaining statistical validity.

Timing of patient data collection

Patient laboratory tests and other clinical metrics (such as temperature and use of mechanical ventilation) were evaluated as close as possible to the time at which maximum PCT was measured. The median times between measurement of maximum PCT and evaluation of clinical metrics are recorded in the table below. All median times were below 12 hours, indicating that evaluation of clinical metrics was largely contemporaneous with the measurement of PCT.

Median time between procalcitonin measurement and evaluation of metric	
Metric	Median time (IQR)
Creatinine	3.4 hours (1.4, 5.0)
Lactate	6.0 hours (1.9, 18.9)
ALT	6.7 hours (2.6, 18.1)
INR	7.7 hours (2.9, 27.7)
WBC	3.8 hours (1.7, 5.5)
Maximum temperature	11.8 hours (4.1, 31.7)
Mechanical ventilation	1.9 hours (0.2, 28.5)

Missing data

In assembling Tables 1 and 3, patients lacking select data points were omitted from analysis related to that characteristic. Data points were missing due to an inability to extract the data from the electronic medical record or because the data were not obtained (e.g. an unmeasured laboratory lest). Multiple imputation was not deemed appropriate as Little's missing completely at random (MCAR) test rejected the MCAR assumption ($P < 0.001$). However, the low percentages of missing values, as seen in the table below, suggest a minimal effect on the data.

Table	Group	Characteristic	# of patients missing data for characteristic (% of total)
1	Matched viral group	BMI	3 (0.8%)
1	Matched viral group	WBC	1 (0.3%)
1	Matched viral group	Creatinine	1 (0.3%)
1	Matched viral group	**Comorbidities**	**10 (2.8%)**
1	Unmatched viral group	**BMI**	**70 (3.1%)**
1	Unmatched viral group	Temperature	1 (< 0.1%)
1	Unmatched viral group	WBC	6 (0.3%)
1	Unmatched viral group	Creatinine	5 (0.2%)
1	Unmatched viral group	**Comorbidities**	**39 (1.7%)**
3	Unmatched viral & bacterial co-infection groups	Temperature	1 (< 0.1%)
3	Unmatched viral & bacterial co-infection groups	Creatinine	3 (0.8%)

Gautam S, *et al. Thorax* 2020; 75:974–981. doi: 10.1136/thoraxjnl-2020-214896

Definition of immunocompromising comorbidities:

ICD10 code	Disease
B20-24	Human immunodeficiency virus [HIV] disease
C81	Hodgkin lymphoma
C82	Follicular lymphoma
C83	Non-follicular lymphoma
C84	Mature T/NK-cell lymphomas
C85	Other specified and unspecified types of non-Hodgkin lymphoma
C86	Other specified types of T/NK-cell lymphoma
C88	Malignant immunoproliferative diseases and certain other B-cell lymphomas
C90	Multiple myeloma and malignant plasma cell neoplasms
C91	Lymphoid leukemia
C92	Myeloid leukemia
C93	Monocytic leukemia
C94	Other leukemias of specified cell type
C95	Leukemia of unspecified cell type
C96	Other and unspecified malignant neoplasms of lymphoid, hematopoietic and related tissue
D46	Myelodysplastic syndromes
D47	Other neoplasms of uncertain behavior of lymphoid, hematopoietic and related tissue
D48	Neoplasm of uncertain behavior of other and unspecified sites
D61	Other aplastic anemias and other bone marrow failure syndromes
D70	Neutropenia
D71	Functional disorders of polymorphonuclear neutrophils
D72	Other disorders of white blood cells
D75.81	Myelofibrosis
D80	Immunodeficiency with predominantly antibody defects
D81	Combined immunodeficiencies
D82	Immunodeficiency associated with other major defects
D83	Common variable immunodeficiency
D84	Other immunodeficiencies
T86	Complications of transplanted organs and tissue
Z92.25	Personal history of immunosuppression therapy

Sacrifice and bronchoalveolar lavage

Mice were euthanized via injection with 100 mg/kg and 10 mg/kg of ketamine and xylazine, respectively. The abdominal aorta was cannulated for arterial blood testing via the i-STAT 1 Handheld Analyzer (Abaxis, Union City, USA) and CG4+ cartridges as per the manufacturer's instructions. Due to occasional cartridge failure, certain samples were not accurately analyzed and were therefore excluded. The trachea was then exposed by making a small midline incision on the neck and cannulated with a 22g

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catheter. Two sequential aliquots of 750 μL cold PBS were instilled, retrieved, and pooled. Lungs were harvested, snap frozen in liquid nitrogen, and analyzed by qPCR as described below. White blood cell count in bronchoalveolar lavage (BAL) fluid was measured using a coulter counter (Beckman Coulter, Brea, USA). BAL was streaked out on sheep's blood agar plates (Thermo Fisher Scientific, Waltham, USA) to assess for bacterial growth after 48 hours. Cells in BAL were pelleted by centrifugation at 2,000 x g for 2 minutes and the protein content of the supernatant was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) following manufacturer's instructions. IFN-γ enzyme-linked immunosorbent assay (ELISA) was performed with the DuoSet Mouse IFN-gamma ELISA DY485 (R&D systems, Minneapolis, USA).

Cultivation of A549 Cells

A549 cells CCL-185 (ATCC, Old Town Manassas, USA) were grown in DMEM supplemented with 1% penicillin/streptomycin, 1% L-Glutamine and 10% fetal bovine serum (all from Thermo Fisher Scientific, Waltham, USA). Cells were cultured in humidified atmosphere containing 5% CO_2 at 37°C. For infection, influenza A/PR8/34 (H1N1) was applied to cells at the indicated multiplicity of infection (MOI). After 24 or 48 hours (as indicated), cells were harvested, RNA was isolated, cDNA was synthesized, and qPCR was performed as described below.

Quantitative PCR

RNA from lungs was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized using the iScript cDNA Synthesis Kit #1708890 (BioRad, Hercules, USA). qPCR was performed using the SsoAdvanced SYBR Green Supermix #1725270 (BioRad, Hercules, USA). 18S ribosomal RNA was used as a housekeeping gene. In some uninfected control samples, inflammatory gene expression was undetectably low; these values were therefore excluded from analysis. Primer sequences were as follows:

Species	Gene	Direction	Sequence (5' → 3')
Mouse	18S	Forward	GCAATTATTCCCCATGAACG
Mouse	18S	Reverse	AGGGCCTCACTAAACCATCC
Mouse	Procalcitonin	Forward	CCCTTTCCTGGTTGTCAGCATC
Mouse	Procalcitonin	Reverse	AGCATGCAGGTACTCAGATTCC
Mouse	IL-6	Forward	CCGGAGAGGAGACTTCACAG
Mouse	IL-6	Reverse	CCGGAGAGGAGACTTCACAG
Mouse	IFN-γ	Forward	CACGGCACAGTCATTGAAAG
Mouse	IFN-γ	Reverse	GCTGATGGCCTGATTGTCTT
Human	18S	Forward	GCAATTATTCCCCATGAACG
Human	18S	Reverse	AGGGCCTCACTAAACCATCC
Human	Procalcitonin	Forward	GGAGAGCAGCCCAGCAGACCC
Human	Procalcitonin	Reverse	GTTGGCATTCTGGGGCATGCTAA
Human	IL-6	Forward	CCTTCCAAAGATGGCTGAAA
Human	IL-6	Reverse	CAGGGGTGGTTATTGCATCT
Human	IFN-γ	Forward	CTAATTATTCGGTAACTGACTTGA
Human	IFN-γ	Reverse	ACAGTTCAGCCATCACTTGGA

Supplemental data:

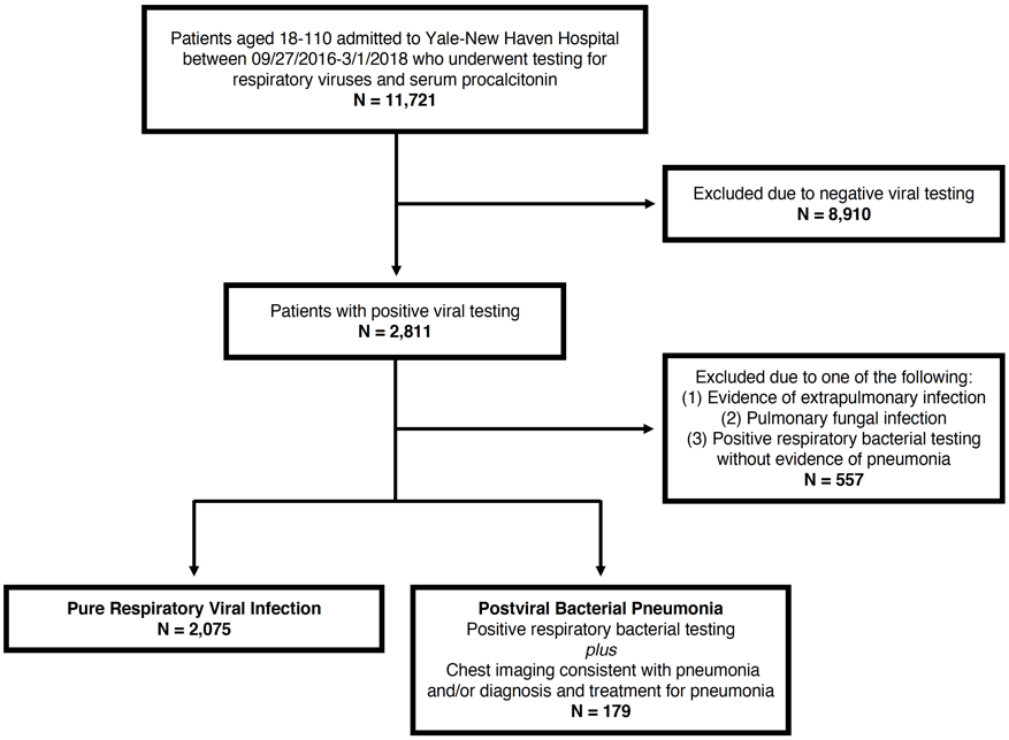

Figure E1. Flowchart depicting clinical study design.

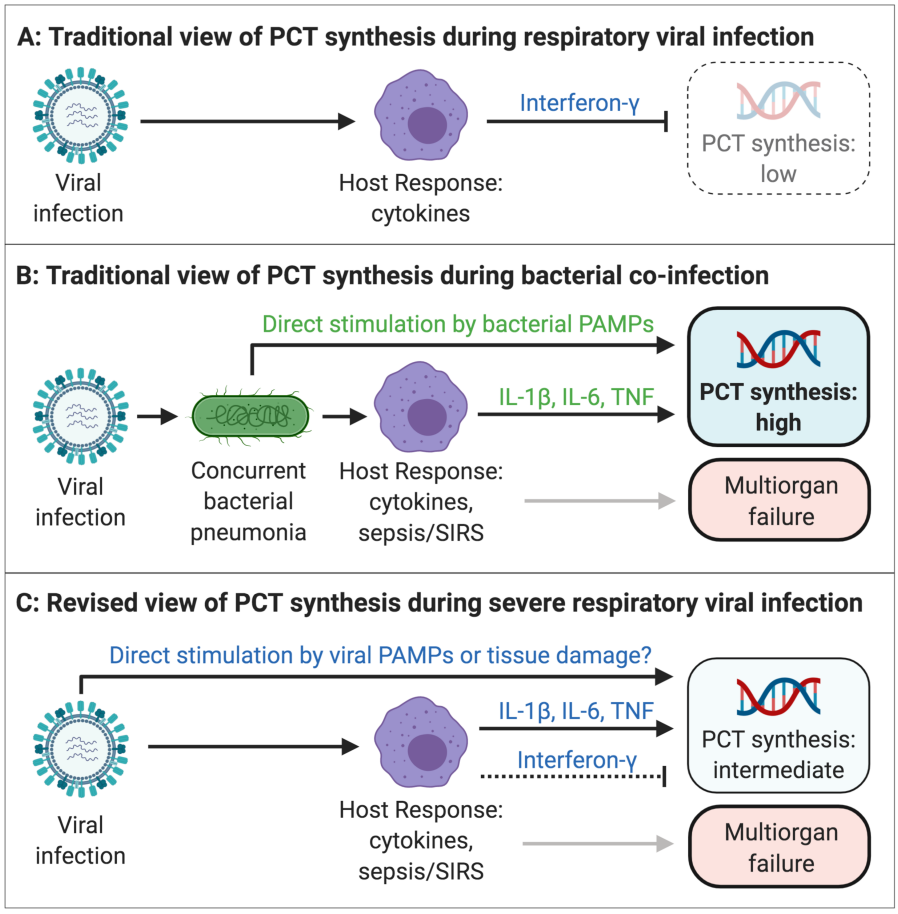

Figure E2. Models of procalcitonin regulation during respiratory viral infection. The conventional view holds that (A) viruses elicit IFN-γ, resulting in suppression of PCT synthesis, and (B) elevated PCT indicates the presence of bacterial co-infection. Bacteria are known to stimulate PCT production via pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide and pro-inflammatory cytokines. C, In the present study we demonstrate that severe respiratory viral infection can elicit an intermediate level of PCT synthesis despite IFN-γ signaling, indicating that interferons are not sufficient to suppress PCT (dotted line). The mechanisms by which viral infection stimulates PCT expression remain unknown.

Table E1. Viral etiologies in patients with bacterial co-infection

Virus	Bacterial co-infection (*n* = 179)
Influenza	47 (26.1%)
Rhinovirus	69 (38.3%)
Respiratory syncytial virus	21 (11.7%)
Human metapneumovirus	19 (10.6%)
Parainfluenza	15 (8.3%)
Adenovirus	4 (2.2%)
Cytomegalovirus	4 (2.2%)
Herpes simplex virus	1 (0.6%)

Table E2. Specificity of procalcitonin for clinical conditions at various thresholds

Clinical condition	Specificity (%) for condition at procalcitonin threshold of *x*					
	x=0.1	*x*=0.25	*x*=0.5	*x*=1	*x*=2	*x*=5
Bacterial co-infection	43.7	71.7	81.6	87.9	93.5	97.1
Renal insufficiency (Cr > 1.5)	**49.3**	**77.8**	**86.4**	**91.1**	**95.1**	**97.5**
Death	42.5	70.9	81.0	87.4	93.2	96.7
Renal failure requiring dialysis	43.1	**72.0**	**82.0**	**88.0**	93.4	96.9
Cardiovascular shock	43.2	**71.8**	**82.2**	**88.6**	**94.0**	**97.3**
Respiratory failure requiring PPV	43.3	**71.9**	**82.0**	**88.2**	**94.0**	**97.3**
Fever (temperature > 37.9°C)	**50.7**	**78.3**	**87.1**	**91.9**	**96.3**	**98.8**
ICU admission	**46.0**	**75.2**	**84.8**	**90.5**	**95.6**	**97.9**

Bolded: Specificity values greater than that for bacterial co-infection.
Abbreviations: creatinine (Cr); intensive care unit (ICU); positive pressure ventilation (PPV).

Table E3. Sensitivity of procalcitonin for clinical conditions at various thresholds

Clinical condition	Sensitivity (%) for condition at procalcitonin threshold of *x*					
	x=0.1	*x*=0.25	*x*=0.5	*x*=1	*x*=2	*x*=5
Bacterial co-infection	81.0	52.5	35.2	24.0	14.5	7.8
Renal insufficiency (Cr > 1.5)	**93.6**	**80.9**	**55.3**	**34.0**	**19.1**	4.3
Death	**91.4**	**65.0**	**46.4**	**31.0**	**16.9**	6.9
Renal failure requiring dialysis	**86.2**	**73.3**	**54.1**	**33.9**	**16.5**	6.4
Cardiovascular shock	**81.3**	**61.9**	**50.0**	**38.8**	**24.6**	**11.9**
Respiratory failure requiring PPV	**82.6**	**62.3**	**47.1**	**32.6**	**23.2**	**11.6**
Fever (temperature > 37.9°C)	70.3	41.7	28.9	19.7	11.7	6.0
ICU admission	74.1	49.9	**36.2**	**26.1**	**17.0**	7.7

Bolded: Sensitivity values greater than that for bacterial co-infection.
Abbreviations: creatinine (Cr); intensive care unit (ICU); positive pressure ventilation (PPV).

Table E4. Clinical history of patients with pure viral respiratory infection and procalcitonin > 20 ng/ml

Age/ gender	Viral etiology	Max PCT	Organ Dysfunction	Pure viral infection	Reason(s) for exclusion of bacterial pneumonia or clinical vignette of patients deemed indeterminate for co-infection (bold)
60/M	Human metapneumovirus	234.1	No	Yes	Final diagnosis of sialodenitis in a patient with acute myelogenous leukemia.
90/F	Rhinovirus	27.6	Yes	Yes	Multiple negative lower respiratory cultures including bronchoscopy.
42/M	Rhinovirus	38.7	Yes	Yes	Course consistent with diffuse alveolar hemorrhage due to rapid clearing of radiographic opacities in a patient with granulomatosis with polyangiitis.
62/M	Rhinovirus	21.3	Yes	Yes	Negative lower respiratory cultures and final diagnosis of viral pneumonia and pulmonary edema.
83/F	Influenza	22.3	Yes	Yes	Multiple negative respiratory cultures.
65/M	Influenza	23.0	Yes	Yes	Final diagnosis of influenza and volume overload. Imaging more consistent with edema than infection.
42/M	Influenza	28.8	Yes	Yes	Final diagnosis of sepsis due to influenza; antibiotics were withheld.
23/F	Influenza	20.5	No	Yes	Final diagnosis of vaso-occlusive crisis in the setting of influenza; antibiotics were withheld.
74/M	Influenza	35.4	Yes	Yes	Negative lower respiratory cultures.
75/M	Influenza	38.5	No	Yes	Consolidation absent on imaging, negative lower respiratory culture, and final diagnosis of influenza.
44/F	Influenza	40.7	Yes	Yes	Negative lower respiratory cultures and final diagnosis of influenza.
68/M	Influenza	20.6	No	Yes	Consolidation absent on imaging and negative lower respiratory cultures.
85/M	Influenza	20.3	Yes	Yes	Negative lower respiratory cultures and final diagnosis of influenza.
92/M	Influenza	144.3	No	Yes	Consolidation absent on imaging, negative lower respiratory cultures, and final diagnosis of influenza.
45/F	Influenza	161.4	No	Yes	Consolidation absent on imaging and final diagnosis of influenza.
64/F	Influenza	35.3	Yes	Yes	Consolidation absent on imaging and final diagnosis of influenza.
78/M	Influenza	22.7	Yes	Yes	Consolidation absent on imaging and final diagnosis of influenza.
65/M	**Influenza**	**200.0**	Yes	**Indeterminate**	**Hazy multifocal opacities were present. Respiratory culture was not ordered. Antibiotics were given. Final diagnosis was COPD exacerbation due to influenza, but there was concern for possible superimposed bacterial pneumonia. Ultimately, the decision to treat with antibiotics was such was based on the extremely elevated PCT and the patient's immunosuppressed status (patient was taking prednisone and mycophenolate for kidney transplant).**
90/F	**Influenza**	**75.8**	Yes	**Indeterminate**	**Chest X-ray showed a chronic retrocardiac opacity. Respiratory cultures were not ordered. Antibiotics were given. Discharge diagnosis included bacterial pneumonia, but the patient had no fever or respiratory symptoms.**
87/F	**Influenza**	**192.9**	Yes	**Indeterminate**	**Bilateral opacities were considered to be consistent with pneumonia or edema. A respiratory culture was drawn, but rejected for poor quality. Antibiotics were given. Final diagnosis was influenza complicated by ARDS, stress cardiomyopathy, and pulmonary edema, but bacterial superinfection could not be ruled out.**

Abbreviations: acute respiratory distress syndrome (ARDS); chronic obstructive pulmonary disease (COPD); procalcitonin (PCT)

Table E5. Tabulated results of murine and cellular experiments

Experiment	Figure	Description	Fold change (Influenza / PBS control)	Fold change (compared to day 0 value)	P value	Significance
Murine Lethal Influenza (200 PFU)	2B	Weight Loss Day 1	0.99		0.8852	ns
		Weight Loss Day 2	0.98		0.2657	ns
		Weight Loss Day 3	0.85		<0.0001	****
		Weight Loss Day 4	0.79		<0.0001	****
		Weight Loss Day 5	0.75		<0.0001	****
		Weight Loss Day 6	0.73		<0.0001	****
		O2 Saturation	0.88		<0.0001	****
	2C	Lactate	4.59		0.0008	***
		Creatinine	1.66		0.0015	**
		BAL Protein	16.34		<0.0001	****
		BAL WBC	10.13		<0.0001	****
	2D	PCT/18S RNA	2.64		<0.0001	****
		IL-6/18S RNA	87.39		0.0283	*
		IFNg/18S RNA	100.00		0.0448	*
		Plasma IFNg	4.42		0.0037	**
Murine Sublethal Influenza (10 PFU)	3B	PCT/18S RNA Day 0 v Day 3		4.58	0.0005	***
		PCT/18S RNA Day 0 v Day 7		7.06	<0.0001	****
		PCT/18S RNA Day 0 v Day 16		2.96	0.0564	ns
	3C	IL-6/18S RNA Day 0 v Day 3		109.77	0.003	**
		IL-6/18S RNA Day 0 v Day 7		107.17	<0.0001	****
		IL-6/18S RNA Day 0 v Day 16		122.40	0.007	**
		IFNg/18S RNA Day 0 v Day 3		12.63	0.0005	***
		IFNg/18S RNA Day 0 v Day 7		168.07	<0.0001	****
		IFNg/18S RNA Day 0 v Day 16		80.61	0.007	**
In vitro (MOI 10)	4A	PCT/18S RNA	2.57		0.0094	**
		IL-6/18S RNA	15.82		0.0014	**
		IFNg/18S RNA	3.84		0.0019	**

Abbreviations: interferon-gamma (IFNg); multiplicity of infection (MOI); non-significant (ns); plaque forming units (PFU); phosphate buffered saline (PBS); procalcitonin (PCT); ribonucleic acid (RNA).