

# Decreased neutrophil phagocytosis and killing of bacteria in COVID-19 patients

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## **Research Article**

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# Abstract

A new coronavirus disease was described in December 2019 (COVID-19) in Wuhan City, Hubei Province, China and has reached pandemic status. According to the World Health Organization, the incubation time from being infected to symptom emergence averages 5-6 days for COVID-19 but can be up to 14 days. The mortality rate varies in different countries but is greater in elderly people and in patients with cardiovascular disease, diabetes and chronic respiratory diseases. Patients with chronic respiratory diseases often have reduced neutrophil function. We sought to measure neutrophil phagocytosis and bacterial killing in COVID-19 patients. 30 COVID-19 patients and 9 healthy individuals were recruited from the Masih Daneshvari Hospital (Tehran, Iran) from March-May 2020. Polymorphonuclear (PMN) cells were isolated from whole fresh blood and incubated with green fluorescent protein (GFP) labelled *methicillin-resistant Staphylococcus aureus* and *Pseudomonas aeruginosa*. Phagocytosis was determined by measuring the fluorescence of co-cultures of bacteria and neutrophils and reported as the lag time before exponential growth. The number of viable bacteria was determined after 70 h by the Colony-Forming Unit (CFU). Bacterial phagocytosis of SA ( $22\pm 0.9$  versus  $9.2\pm 0.5$ h,  $p<0.01$ ) and PA ( $12.4\pm 0.6$  versus  $4.5\pm 0.22$ ,  $p<0.01$ ) was significantly reduced in COVID-19 patients compared with healthy control subjects. After 70h there was a significant increase in CFU in COVID-19 subjects compared with healthy control subjects for both SA ( $2.6\pm 0.09 \times 10^8$  versus  $0.8\pm 0.04 \times 10^8$  CFU/ml,  $p<0.001$ ) and PA ( $2.2\pm 0.09 \times 10^9$  versus  $1.0\pm 0.06 \times 10^9$  CFU/ml,  $p<0.001$ ). These results suggest a defect in bacterial clearance by neutrophils in COVID-19 patients.

## Introduction

A new coronavirus disease was initially reported in December 2019 (COVID-19) which resulted in a severe acute respiratory disease.<sup>1</sup> COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which is the seventh member of the coronavirus family to infect humans and has a 76.5% amino acid homology with SARS-CoV with more than 85% of its genome sequence being similar to several bat coronaviruses.<sup>1,2</sup> Coronaviruses are RNA viruses with an extensive range of natural hosts.<sup>3</sup> Based on sequence analysis, SARS-CoV-2 may have originated from bats or living animals from the Wuhan seafood market or its surrounding areas.<sup>4</sup> The development of human-to-human transmission was the final step in the virus cycle that induced the epidemic and finally the pandemic form of disease.<sup>5</sup> COVID-19 disease spreads through the respiratory tract with lymphopenia and cytokine storms. It mainly happened in severe disease and showed the existence of immunological dysregulation in severe disease.<sup>6</sup>

Activation or suppression of the host immune response is often predictive of disease severity.<sup>7</sup> Neutrophils have significant role in the host defense against micro-organisms and in the innate immune response. Phenotypic heterogeneity and functional flexibility by neutrophils showed their significance in the adjustment of immune function.<sup>8</sup> The release of cytokines such as IL-6 and IL-1 $\beta$  promotes the recruitment of neutrophils and cytotoxic T cells into the lungs of COVID-19 patients.<sup>7</sup> Activated

neutrophils release leukotrienes and reactive oxygen species (ROS) that cause pneumocyte and endothelial injury, leading to acute lung injury.<sup>7</sup> Neutrophils act as a powerful guard against bacterial attacks. Neutrophils can destroy bacteria inside the phagolysosome using lysosomal enzymes released from the granules and the production of reactive oxygen species (ROS).<sup>9</sup> During coronavirus infection, neutrophils and monocytes accumulate at the site of infection leading to the release of cytokines and chemokines such as IL-1, IL-6, IL-8, IL-17, IL-21, TNF- $\beta$ , and MCP-1.<sup>3</sup> These cytokines can also activate T lymphocytes and acute viral infections are associated with the abundant influx of neutrophils into the lung tissue followed several days later by a virus - specific CD8<sup>+</sup> T-cell response.<sup>10</sup>

Neutrophils are the first line of immune defence against any invading agents and they possess granules that contain many mediators including antimicrobial peptides, proteolytic enzymes, and ROS generated via the action of NADPH oxidase.<sup>11</sup> Neutrophil antiviral mechanisms include viral phagocytosis which leads to the activation of pattern recognition receptors (PRRs) by melanoma differentiation-associated protein 5 (MDA5) and Toll like receptors (TLR-7). Neutrophil activation results in the production of myeloperoxidase (MPO),  $\alpha$ -defensins and the induction of neutrophil extracellular traps (NETs) for trapping the virus and enhances interactions with CD8<sup>+</sup> T cells and NK cells.<sup>11</sup> Infection of neutrophils by influenza A/WSN (H1N1) virus results in the aggregation of neutrophils and macrophages in the lung and phagocytosis of the virus.<sup>12</sup> In addition, Saitoh and colleagues demonstrated that neutrophils are able to capture HIV-1 in-vitro and reduce viral infectivity by the generation of NETs.<sup>13</sup>

Since bacterial co-infection is observed in COVID-19 patients<sup>1</sup>, we hypothesised that neutrophils from these subjects have an attenuated neutrophil function. We aimed to compare the ability of peripheral blood neutrophils from healthy control subjects and COVID-19 patients to phagocytose and kill GFP-labelled bacteria.

## Materials And Methods

### Patients

30 COVID-19 patients (23 PCR positive and 7 PCR negative) with respiratory system involvement as detected by CT and chest X-ray and 9 healthy control subjects (without lungs involvement and negative PCR) were enrolled between March-May 2020 at the Masih Daneshvari Hospital Tehran, Iran. All COVID-19 infected patients were diagnosed based on the World Health Organization (WHO) interim guidance. Patients were confirmed positive for SARS-CoV-2 nucleic acid in the respiratory samples using real-time reverse-transcriptase polymerase-chain-reaction (RT-PCR) or serum specific antibodies and chest imaging. Demographic data is presented in **Table 1**.

### Neutrophils isolation from COVID-19 patients and healthy subjects

Polymorphonuclear (PMN) cells were isolated from whole blood using heparin as described earlier.<sup>14</sup> Briefly, phosphate buffered saline (PBS) was used to dilute blood and the diluted blood put over Ficol-Paque (Sigma Aldrich- Merck Company- Darmstadt, Germany) in sterile conical tube and centrifuged at 760xg, for 20 min in room temperature. The supernatant was removed and the pellet resuspended in lysis buffer (150mM NH<sub>4</sub>CL, 10mM KHCO<sub>3</sub>, 0.1mM EDTA, pH 7.4), re-pelleted and washed with PBS before centrifugation at 350xg, for 5 min, at 4°C. Cell pellets were finally resuspended in HEPESIII buffer (20mM HEPES) supplemented with 0.5% w/v BSA, 1mM CaCl<sub>2</sub>, 5mM glucose and counted.

## Preparation of bacterial strains

The *Methicillin resistance Staphylococcus Aureus* (SA) strain MW2 and *Pseudomonas Aeruginosa* (PA) (gifted by Prof. L. Koenderman, Utrecht University, The Netherlands) were used. These bacteria had been transformed to express green fluorescent protein (GFP).<sup>15</sup> SA was grown in Luria-Bertani (LB) (Sigma-Aldrich, USA) broth with chloramphenicol (10µg/ml) (Sigma-Aldrich) overnight at 37°C and 60xg, until the OD<sub>600nm</sub> reached 0.5. The suspension was centrifuged and the pellet resuspended in HEPES buffer to give an OD<sub>600nm</sub> = 0.5. The bacterial suspension was aliquoted and frozen at -80°C. PA was cultured on LB agar with kanamycin (15µg/ml) and ampicillin (10µg/ml) overnight at 37°C. The next day, one bacterial colony was cultured in LB broth and grown to an OD<sub>600nm</sub> = 0.5. Bacterial Pellets were washed and resuspended in HEPES III and diluted again to OD<sub>600nm</sub> = 0.5. GFP-labeled bacteria were detected using flow cytometry (BD FACSCalibur) and fluorescence was evaluated in the FL1 channel (530nm).

## Neutrophil phagocytosis and colony forming units (CFU) counting

Measurements of GFP fluorescence intensity were determined with the FLUOstar Optima (BMG Labtech, Ortenberg, Germany) with bottom optics as described previously.<sup>16</sup> Isolated neutrophils were co-cultured with PA and SA in 96-well imaging plates (black, clear bottom; Corning Life Sciences, Tewksbury, MA, USA). Briefly, 5×10<sup>6</sup> cell/ml and human pooled serum (Sigma-Aldrich) with HEPES III buffer was incubated with 2×10<sup>8</sup> CFU/ml of bacteria. The plate was placed in the Fluostar at 37°C with constant shaking (150 rpm) for 70 hours (interval shaking time was every 20 min) as previously described.<sup>17</sup> GFP fluorescence was measured every 20 minutes (excitation 485nm/emission 520nm). For CFU counting, bacterial suspensions after 70h exposure with cells in FLUOstar plate were removed from plates and then 20µl of the suspensions diluted in serum and cultured on UTI-Agar plates (HiCrome™-HIMEDIA) overnight at 37°C. In this CFU test colony-forming units test bacterial suspensions from 6 out of 30 COVID-19 patients and 4 out of 9 healthy people were evaluated.

## Statistical analysis

All analysis were performed in triplicate, and all experiments were repeated up to five times. Results are presented as mean  $\pm$  SEM. Statistical tests (Kruskal-Wallis) were analyzed using GraphPad Prism (version 8). Results were considered statistically significant when  $p \leq 0.05$ ;  $p \leq 0.01$ ;  $p \leq 0.001$ ;  $p \leq 0.0001$ .

## Results

### Neutrophil phagocytosis

The presence of GFP-labelled bacteria was used to assess peripheral blood neutrophil phagocytosis. Lag time is the time before the bacterial population starts exponential growth in a new environment<sup>18</sup> and in these experiments reflects relative neutrophil phagocytosis function. Neutrophils from COVID-19 patients were significantly less efficient at phagocytosis of GFP-SA ( $22 \pm 0.9$  versus  $9.2 \pm 0.5$ h,  $p < 0.01$ ) (**Fig 1A**) and GFP-PA ( $12.4 \pm 0.6$  versus  $4.5 \pm 0.22$ ,  $p < 0.01$ ) (**Fig 1B**) than cells from healthy control subjects. There was no increase in lag time in the absence of neutrophils (**Fig 1A & B**).

### Bacterial growth

After 70h culture with neutrophils from COVID-19 patients, samples showed an increase in GFP-SA growth ( $2.6 \pm 0.09 \times 10^8$  CFU/ml) compared with neutrophils from healthy control subjects ( $0.8 \pm 0.04 \times 10^8$  CFU/ml) (**Fig 2A**). This indicated that a greater number of GFP-SA were alive in the cultures with COVID-19 patient cells. In the absence of any neutrophils, GFP-SA numbers reached  $2.8 \pm 0.03 \times 10^8$  CFU/ml (**Fig 2A**). GFP-PA growth with neutrophils from healthy subjects reached  $1.0 \pm 0.06 \times 10^9$  CFU/ml compared to  $2.2 \pm 0.09 \times 10^9$  CFU/ml with neutrophils from COVID-19 patients (**Fig 2B**). The growth seen with COVID-19 patient cells was similar to that seen in the absence of any cells ( $2.4 \pm 0.09 \times 10^9$  CFU/ml) (**Fig 2A & B**).

## Discussion

This pilot study demonstrated a decreased phagocytic capacity of neutrophils isolated from the systemic circulation of COVID-19 patients in comparison with control healthy subjects against both gram positive and gram negative bacteria. Importantly, both SA and PA infection are associated with patients whose immune systems are suppressed.<sup>19,20</sup> Neutrophil phagocytosis is important in reducing bacterial growth<sup>15</sup> and this is supported by our limited data examining SA and PA CFUs. A decreased lag time until bacterial outgrowth occurs also reflects a reduced antibacterial capacity of the neutrophils.<sup>16</sup> Overall, the reduced neutrophil phagocytosis of PA and SA in COVID-19 patients compared to healthy subjects enables greater bacterial colonization. This reduced innate immune function of neutrophils may also impact on viral clearance if defects in anti-viral capacity occur. Neutrophils phagocytosis is most efficient in the presence of opsonins such as specific immunoglobulin (Ig)G and IgM. IgG or IgM bound to the

microbial surface is recognized by C1q which activates the classical complement pathway. PMNs express receptors for IgG (FcγRI, FcγRII, and FcγRIII) and opsonic complement molecules C3b and iC3b (CR1, CR3, and CR4).<sup>21</sup> The improvement seen in COVID-19 patients with intravenous immunoglobulins<sup>22</sup> or potentially by reducing complement activation<sup>23</sup> suggests that the reduced phagocytosis and killing seen in COVID-19 patients may result from lower levels of immunoglobulins in these subjects.

Neutrophils compose 60% of the leukocyte population present in blood and are the most important phagocytic cells. These cells can defend the host against bacterial infection. Neutrophils kill gram-positive bacteria such as SA using a combination of bombardment with reactive oxygen species (an event called metabolic burst) and the induction of antimicrobial peptides (AMPs) and several enzymes.<sup>24</sup> PA is a Gram-negative opportunistic pathogen capable of infecting humans with compromised natural defenses and causing severe pulmonary disease. PA can facilitate adhesion, modulate or disrupt host cell pathways, and target the extracellular matrix. This bacterium be able to form biofilms that protect bacteria from antibiotics and the host immune system.<sup>25</sup> Inflammation as a result of PA infection is mediated by neutrophils either directly by the release of chemoattractant factors or indirectly through stimulation of other cell types such as epithelial cells. The presence of neutrophils in PA infections helps mediate bacterial killing through phagocytosis, NET formation as well as the release of neutrophil microvesicles.<sup>26</sup> However, co-infection can harm the host immune system by the development of antibacterial intolerance.<sup>27</sup> In viral pneumonia, bacterial co-infection is a key factor driving mortality.<sup>28</sup> Furthermore, 94.2% of COVID-19 patients are co-infected with further pathogens including 9 viruses, 11 bacteria and 4 fungi with bacterial co-infection being the predominant pathogens in all COVID-19 patients.<sup>1</sup> In this study we show a reduced capacity of neutrophils COVID-19 patients to phagocytose and kill two important pathogenic bacteria. A limitation of this study is the lack of analysis of neutrophils from patients with different COVID-19 severity and the restriction to SA and PA. SA and PA are key pathogenic organisms in lung disease and may be important in the pathophysiology of severe COVID-19.

## Declarations

### Conflict of Interest Statement

The authors declare that there are no conflicts of interest

### Ethics approval

The Ethics Committee of the Masih Daneshvari Hospital approved the study and all subjects gave written informed consent.

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## Table

**Table.1.** Demographic information of all COVID-19 participants (PCR +ve and -ve)

	Gender	AGE	PCR	IL-6	CRP	ESR	LDH	Fer	Lymph	IgG	IgM
Patient 1	F	21	+	19.9	-	21	943	118	4	-	-
Patient 2	F	61	+	15.5	29	78	1034	1327	8.7	13.5	10.2
Patient 3	F	58	+	8	19	69	981	288	25	14.3	1.74
Patient 4	F	69	+	14	25	25	977	-	6.6	-	-
Patient 5	F	43	+	8.1	65	95	531	320	-	-	-
Patient 6	M	64	+	12	14	36	384	-	-	-	-
Patient 7	M	71	+	7.9	18	61	245	25	4.3	-	-
Patient 8	M	81	+	28.8	39	104	450	>2000	7	-	-
Patient 9	F	60	+	2.5	37	36	521	285	18	14.83	7.96
Patient 10	F	56	+	9.4	107	35	578		24	14.1	10.1
Patient 11	F	44	+	2.8	62	50	832	466	8	-	-
Patient 12	M	61	+	9.4	-	46	-	347	-	-	-
Patient 13	M	33	+	6.2	107	25	831	1463	18	-	-
Patient 14	F	59	+	13.1	76	45	580	1463	8	-	-
Patient 15	F	28	+	12	47	78	1034	234	12	-	-
Patient 16	M	69	+	13.9	14	36	974	-	58	0.31	1.39
Patient 17	M	71	+	7.5	53	28	521	548	4	13.53	3.38
Patient 18	F	23	+	7.3	-	65	439	132	15	-	-

Patient 19	M	44	+	2.8	23	-	793	1572	-	-	-
Patient 20	M	46	+	13.6	61	36	599	1613	23	-	-
Patient 21	M	63	+	11.7	-	56	569	1888	5.1	-	-
Patient 22	M	66	+	-	56	15	1119	>2000	20	-	-
Patient 23	M	63	+	11.2	38	-	438	827	15	16.22	1.87
Patient 24	F	39	-	-	-	-	-	-	-	581	95
Patient 25	M	83	-	-	28	31	175	-	11.1	0.47	0.25
Patient 26	M		-	-	24	30	1835	-	14	0.27	0.63
Patient 27	M	75	-	-	-	32	-	-	12	-	-
Patient 28	M	81	-	7.8	34	31	429	-	19.7	0.86	0.19
Patient 29	M	81	-	19.5	-	32	245	276	15.6	0.49	0.6
Patient 30	M	59	-	8.1	11	30	363	131	29.9	-	-
Control 1	F	34	-	3	12	6	321	26	34	-	-
Control 2	M	35	-	4	9	2	222	44	32	-	-
Control 3	M	37	-	5	8.9	7	311	23	27	-	-
Control 4	F	37	-	2	6.8	1	289	56	21	-	-
Control 5	F	38	-	3	13	11	267	76	38	-	-
Control 6	M	36	-	4.2	7.3	4	378	55	25	-	-
Control 7	M	40	-	3.1	8	7	376	66	26	-	-
Control 8	F	38	-	1.1	11	8	289	47	31	-	-

Control	M	29	-	1	19	4	311	58	36	-	-
9											

# Figures

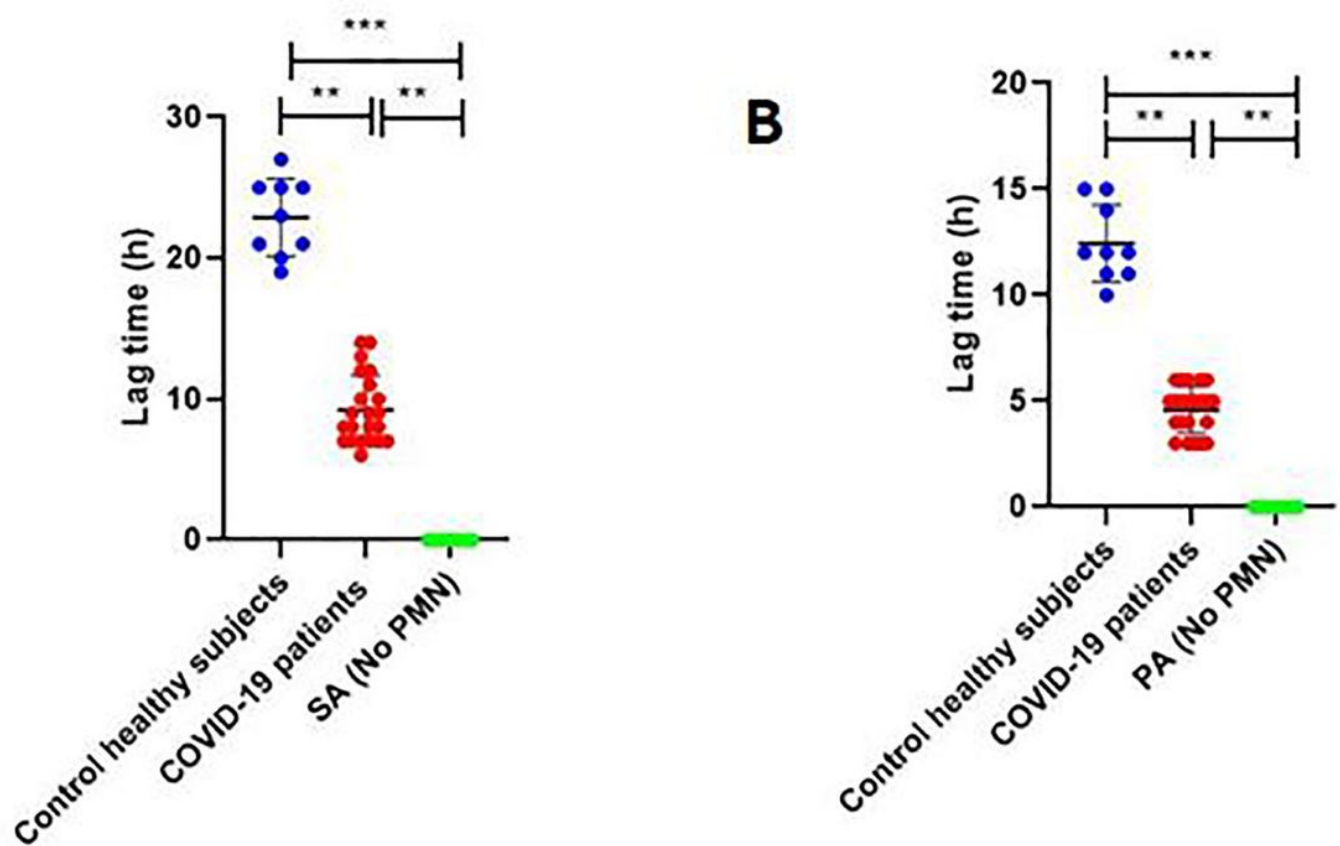
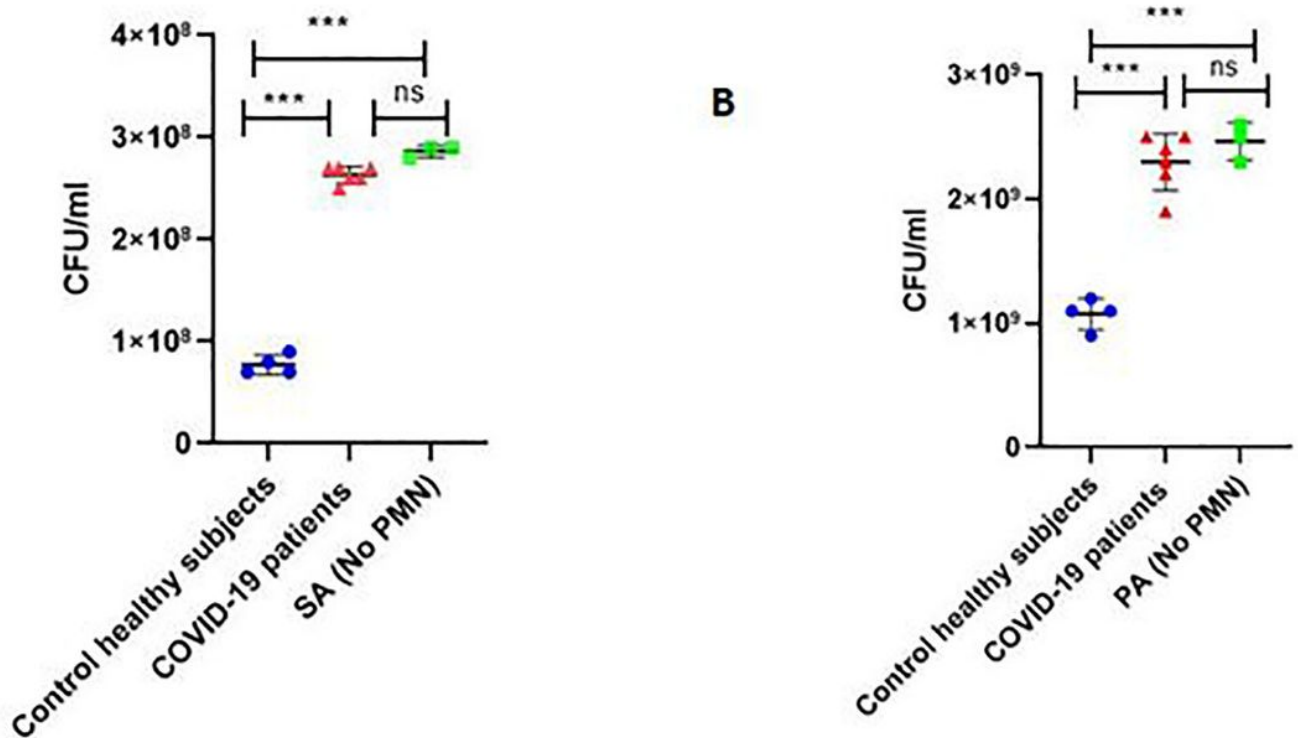


Figure 1

Lag time of green fluorescent protein GFP-labelled methicillin-resistant Staphylococcus aureus (SA)(A) and Pseudomonas aeruginosa (PA)(B) in COVID-19 patients and healthy subjects. The bacterial GFP-signal was recorded every 20 min by FLOUstar Optima. Results are presented as individual patient results with bars as mean ± SEM. \*\*p<0.01, \*\*\*p<0.001. PMN - polymorphonuclear cells



**Figure 2**

Growth of green fluorescent protein (GFP)-labelled methicillin-resistant *Staphylococcus aureus* (SA)(A) and *Pseudomonas aeruginosa* (PA)(B) in COVID-19 patients and healthy subjects. Results are presented as colony-forming units (CFUs/ml) for each individual patient with bars as mean ± SEM. \*\*\* $p < 0.001$ , ns – not significant. PMN - polymorphonuclear cells