Peer Review File

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Reviewer A

This is a nice paper on co-infections in patients with severe COVID-19.

Comment 1. Recommend adding a table with the definitions used for severe and critical COVID-19 for the reader to review.

Reply 1: We apologize for not providing information on how we defined severe and critical cases. We have now provided this information in a new Table 1.

Changes in the text: The information on severity definition is provided in Table 1.

Comment 2: The data is from April 2020, is there no data available from 2021?

Reply 2: We assume the reviewer is asking whether we have collected samples from 2021. Unfortunately, since there were no more COVID-19 patients admitted to hospital since 2020, we don't have any data from 2021.

Comment 3: Would consider specifying timepoints for laboratory data collection for patients. How often were the labs collected, was there a specified period of time or was it until hospital discharge or death?

Reply 3: We collected respiratory tract and serum samples every other day and the samples were collected until hospital discharge or death.

Changes in the text: This information is now described in methods section (see Page 6, lines 120-121), to read:

"*Sample collection began on 7 February and ended on 8 Aug 2020 (until discharged or death), and was performed once every two days.*"

Comment 4: It is not clear in the results section that case 1 died. It is stated that case 1 was discharged but in table 1 it states case 1 died on day 192 of hospitalization.

Reply 4: We apologize for this mistake. Patient 1 has died after 192 days in hospital.

Changes in the text: We have now corrected the mistake in the context (see Page 10, line 201-204), to read:

"*...while case 1 and 2 showed prolonged infection and were transferred from isolated ICU to ICU on 52th day after the onset. Case 2 eventually recovered and was discharged on 48th day after the onset of disease, while cases 1 and 4 died after 192 and 25 days in hospital, respectively.*"

Re-review:

Comment 1. The revision of Co-infecting pathogens can contribute to inflammatory responses and severe symptoms in COVID-19 has improved the manuscript. There are still some grammatical errors that need addressing.

Reply 1: We apologize for the grammar errors; the manuscript was edited by third-party language polishing service, and the certificate is attached here "EdanzEditingCertificate_103654.pdf ."

Comment 2. At the end of the discussion it seems awkward to end with limitations. Please add a concluding sentence or a short conclusion section.

Reply 2: We agree with the reviewer that the ending is a bit abrupt and therefore add a short conclusion sentence after "Limitation".

Changes in the text: We have added a sentence (see Page 18, line 399-401), to read: "*Despite these limitations, our study was able to demonstrate the complexity of the COVID-19 infectome, the potential interactions of pathogens with the host, and their potential roles in disease progression in severe and critically ill patients.*"

Comment 3. Figure 1, Case 1: Include the death symbol on day of death.

Reply 3: Revised as suggested.

Comment 4. Case 2: the days are out of order, 47…59…48.

Reply 4: We apologize for this mistake, and it is now corrected.

Changes in the text: Correction has been made in Figure 1 and in the context (see Page 10, line 207-208), to read: "*Case 2 eventually recovered and was discharged on 64th day after the onset of disease, while cases 1 and 4 died after 192nd and 25th day in hospital, respectively.*"

Reviewer B

The manuscript "Coinfecting pathogens can contribute to inflammatory responses and severe symptoms in COVID 19" is an informative study which follows the course of SARS-CoV-2 infection, together with that of certain bacterial and fungal pathogens in 4 COVID-19 patients with differential outcomes in an ICU setting. The conclusions of the paper suggest that the changing cytokine profile in such patients,

contributing to the "cytokine storm" which is the major cause for morbidity and mortality in COVID-19 patients, is also contributed to by the co-infections, and not just COVID-19. While this is an important insight into the pathology of COVID-19, and should have significance clinically, especially for the balancing of the immunosuppressive treatment in COVID-19 with combating the co-infections, the conclusions are surely limited by the small number of patients in the study. However, these might be taken more as case studies rather than as an attempt to extrapolate the conclusions to the whole population, and this should be mentioned in the abstract as well as the conclusion of the paper. There are also some contradictory information and statements in the paper which makes some of the conclusions confusing. These and some other major issues need attention and rectification from the authors to make the manuscript acceptable for publication:

Comment 1: It is stated in p10 of results (line 177) that patient 1 was discharged on 57^{th} day after onset of disease and only patient 4 died. However, table 1 shows clinical outcome of patient 1 as death 192 days after onset. These are contradictory information and it is not possible to understand which is correct. This needs to be rectified and the correct information provided.

Reply 1: We apologize for this mistake (same one pointed out by reviewer #A in comment 7). Patient 1 has died after 192 days in hospital. Please see our response to Reviewer A.

Comment 2: In lines 194-197 of results, it is stated that *"Importantly, the abundance of these pathogens, measured as reads per million (RPM), showed high correlation with those estimated by an RT-PCR assay (SARS-CoV-2 CT value) and therefore provided reliable means to quantify value) and therefore provided reliable means to quantify pathogen activities ($r= 0.907$, $p<0.001$; Figure S1)."* However, Figure S1

shows correlation between RKM values and Ct values only for SARS-CoV-2 and for none of the other pathogens. This misleading statement should be corrected.

Reply 2: We thank the reviewer for pointing this out. The correlation of Ct and RPM was only estimated for SARS-CoV-2 as evidence that the quantification of meta-transcriptomics approach is reliable. We have revised the text to make this point clearer.

Changes in the text: We have corrected the corresponding statement (see Page 10-11, line 220-223), to read:

"Importantly, the abundance level of SARS-CoV-2, measured as reads per million (RPM), showed a high correlation with those estimated by an RT-PCR assay (SARS-CoV-2 CT value) and therefore provided reliable means to quantify pathogen activity ($r= -0.907$, $p<0.001$; Figure S1)".

Comment 3: Figure 4 is not at all described in the results and I do not understand what the data in Figure 4 represents. Do the data points for the different cytokines/chemokines represent different timepoints from the same patients or data of different patients? This data is important because it is the only one which compares between healthy individuals and patients and it is incredible that the authors do not even describe this data in the results. A similar comparison of inflammatory mediators' levels should be done between the 4 patients individually and the healthy controls.

Reply 3: We apologize for the lack of detailed information on Figure 4 which presents comparisons between data from 4 patients (combined) and the healthy controls. The samples were divided into SARS-CoV-2 active and non-active groups, and the comparisons demonstrate that pathogens other than SARS-CoV-2 can affect inflammatory mediator levels. A paragraph is now added to describe these comparisons. In addition, as requested by the reviewer, we have also provided

separate comparisons of inflammatory mediators' levels between each of the 4 patients and the controls, and these results are presented in Figure S4.

Changes in the text: We have added an additional figure depicting comparisons of inflammatory mediators' levels for each patient (i.e. Figure S4), and have added a new paragraph describing the results from Figure 4 (see Page 13-14, line 283-291), to read:

"To demonstrate that SARS-CoV-2 might not be the only microbial factor that results in high levels of inflammatory mediators we divided the samples from four patients into SARS-CoV-2 (i) non-active group and (ii) active groups, defined on the basis of SARS-CoV-2 RPM levels (smaller or larger than 10^2 RPM). Interestingly, high levels of inflammatory mediators appeared in both actively replicating and non-actively replicating groups (Figure 4A), and similar observations were made for upper and lower respiratory tract samples (measured with mRNA levels, Figure 4B), although it is important to note that the extremely high abundance levels of IL-6 and IL-10 are most likely associated with A. baumannii infections (Figure S4)."

Comment 4: In lines 289-293 it states that *M.odoratus* infection was found in one patient, who died. But in the data the patient 4, who died, does not show any *M. odoratus* infection. This contradiction should be corrected.

Reply 4: We apologize for any confusion. Patient 1 is the one with *M. odoratus* infection and later died. The corresponding description has been corrected.

Changes in the text: We have provided the information that it is case 1 who had *M. odorantus* infection and later died (see Page 15, line 326-329). to read: *"Using this strategy, we identified an opportunistic pathogen – M. odorantus – in case 1 that is not typically included in the screening panels for respiratory pathogens, even though it reached alarmingly high abundance level (>10^5 RPM, or 20.87% of total RNA)."*

Comment 5: An important observation in this study is the lack of correlation between serum levels of inflammatory mediators and mRNA levels of these mediators from URT and LRT. This should be interpreted and whether this really represents an absence of correlation between these parameters, or is an artefact of the estimation processes should be discussed.

Reply 5: We agree that the discrepancies in the dynamics of inflammatory mediators needs to be interpreted and discussed thoroughly. We have now new added a paragraph in discussion focusing on the potential mechanisms for this and the possibility of artefact.

Changes in the text: We have incorporated a new paragraph (see Page 17-18, lines 376-391), to read:

"Our results have revealed substantial differences between serum levels of inflammatory mediators and mRNA levels of these mediators from the URT and LRT. One possibility is that these discrepancies are caused by differences in local versus systemic immune responses (49,50). Indeed, a number of mediators are highly expressed in the URT and/or LRT but are absent from the blood, which is expected with many locally confined infections. Nevertheless, our respiratory tract measurements are largely associated with the URT, with the majority of data for LRT missing due to sparse sampling. Therefore, the current respiratory tract data are incomplete and hence cannot be used for conclusive comparisons. Another potential explanation is that discrepancies in inflammatory mediator measurement are due to the differences in the measurement approach used: inflammatory mediators in the serum were measured with a protein assay, while those in the URT and LRT were measured at mRNA level. The latter approach might not have sufficient resolution for differential expression comparisons if the overall expression level for the protein (e.g., IL6, IL-10 and IFN-γ) is low (Figure 3 and Figure S2). Therefore, while our data suggest potential differences of inflammatory mediators in the blood and respiratory

systems, further studies with more complete data points and a consistent analytical method is required to confirm this."

Comment 6: Another important observation is the lack of correlation between pathogen abundance and inflammatory mediators among patients, even those patients which show similar disease course, as the comparison in Figure 5 seems to suggest. This points to heterogeneity in patient responses to co-infection and suggests that such responses vary between patients and co-infecting pathogens. This should be discussed.

Reply 6: We appreciate the reviewer's insightful suggestion and have now incorporated a discussion on heterogeneity in patient responses.

Changes in the text: The corresponding discussion is now added (see Page 16, lines 357-360), to read:

"Furthermore, it is important to note that the dynamics of inflammatory mediators varied substantially among different cases, even for those showing a similar disease course. Since these patients had distinctive "infectomes", it is highly likely that such differences contribute to the highly distinctive inflammatory responses."

Comment 7: It is not stated that which of these co-infections were nosocomial and which might have been already present in the patient and became expressed as a result of immunosuppressive therapy (for example this might happen with HSV). This is a clinically important distinction, especially because nosocomial infections with multidrug resistant bacteria such as A. baumanni is a major threat under ICU settings. That seems to be emphasized with the fact that the only patient who died in the course of the treatment was infected with A. baumanni which caused sepsis. If cellular DNA is available for the patients from the time of hospitalization (disease onset) it would be good if it is evaluated for presence of HSV and CMV in the patient

who showed HSV and CMV coinfection at later stages.

Reply 7: We thank the reviewer for this constructive suggestion. Unfortunately, all samples were collected after patients were admitted to ICU and therefore earlier samples were not available. But we agree with the reviewer that it is important to distinguish nonsocomial infections within our data.

Changes in the text: The corresponding discussion is now added to limitation paragraph (see Page 18, lines 394-397), to read:

"Second, the samples were collected after the patients were admitted to the ICU, such that there was no information on the early phase of the disease. As a result, we cannot determined whether these cases were experiencing nonsocomial infections."

Minor points:

Comment 8: The data about numbers of cases and death from the COVID-19 pandemic in the introduction is very old. This should be updated.

Reply 8: We thank the reviewer for pointing this out. The corresponding information has been updated.

Changes in the text: Changes are made (see Page 3, line 64-65), to read:

"A newly emerged infectious disease, Coronavirus Disease 2019 (COVID-19), caused by a novel coronavirus (SARS-CoV-2) was first reported in December 2019 and has since caused a global pandemic resulting in over 253 million cases and 5 million deaths by November 2021(1-3)."

Comment 9: Fig 1 shows patients as A, B, C, D whereas in the text and in all other figures they are referred to as 1,2,3,4. This inconsistency should be corrected.

Reply 9: Corrected as suggested.

Changes in the text: The case numbers 1/2/3/4 now appear in the figure.

Comment 10: Many references are repeated, eg. 26/31, 27/28.

Reply 10: We apologize for the mistake. Repeated references have been removed.

Comment 11: Although the paper is generally well written, there are still some grammatical and spelling mistakes. For eg, in line 295 extend should be extent, in line 312 be triggered should be trigger.

Reply 11: We apologize for the grammatical and spelling mistakes. We have examined the context thoroughly and further grammatical and spelling mistakes have been corrected.

Re-review:

The authors have satisfactorily addressed most of the points raised by me.
Comment 1. There is still confusion about the clinical progression of the 4 cases.
The revised text says:
After treatment, case 3 recovered after 27 days in hospital, while case 1 and 2 showed prolonged infection and were transferred from isolated ICU to ICU on 52th day after the onset. Case 2 eventually recovered and was discharged on 48th day after the onset of disease, while cases 1 and 4 died after 192 and 25 days in hospital, respectively.
It is not clear how Case 2 was shifted to ICU from isolated ICU on 52th day after onset and discharged on 48th day at the same time. This is self-contradictory. Such repeated mistakes reduce confidence in the study.

Reply 1: We apologize for this mistake. The patient is shifted from isolated ICU on 52^{nd} day and discharged on 64^{th} day. This has not been corrected.

Changes in the text: We have now modified this information in Figure 1 and in the context (see Page 10, line 207-208), to read: "*Case 2 eventually recovered and was discharged on 64th day after the onset of disease, while cases 1 and 4 died after 192nd and 25th day in hospital, respectively*."

Comment 2. There are still many grammatical and phraseological errors which should be thoroughly checked and corrected.

Reply 2: We apologize for the grammar errors; the manuscript was edited by third-party language polishing service, and the certificate is attached here "EdanzEditingCertificate_103654.pdf ."

Reviewer C

In this work, the authors investigate the correlation between the total infectome (including SARS-Cov2) and the inflammatory systemic response in 4 severe COVID-19 patients. The authors did find association between some of 8 species and cytokines levels, as well as lung injury indexes. The conclusions were that the pathogens and inflammatory responses are concomitantly critical in causing severe symptoms in the lung (and systemically). The text is well written and informative for the field.

The main limitation is the N and variability within the 4 samples, but understandable for the nature of the data, amount of work, sample collection and involvement of multiple people and institutions. On the other hand, this work opens venue for new investigations that can further improve the understanding of ICU, and disease progression on hospitalized patients with acute lung injures. Considering that most ICUs already treat with antibiotics, antifungal, antivirals and immunosuppressants, it would not improve by a lot the current treatment paradigm but could potentially re-

direct some of the efforts towards a more individualized and effective treatment.
I would like to ask the authors a few questions and make some comments and suggestions.

Comment 1: Was the sample collection and manipulation performed by the same people or did multiple individuals collected and processed the samples?

Reply 1: The samples were collected by different clinicians but with the same collection guidelines, whereas sample processing were carried out by the same person.

Changes in the text: A sentence has been added (see Page 6, line120-127), to read:

"Sample collection began on 7 February and ended on 8 Aug 2020 (until discharged or death), and samples were collected by different clinicians who were provided with the same collection guidelines, performed once every two days. The sampling types included throat swab (TS), BALF, sputum, whole blood, most of which were collected for routine diagnostic purposes. The corresponding healthy control and reagent samples were collected from healthy volunteers and sample collection medium from the hospital. Additional selection criteria for cases involved in the downstream experiments included the availability, timing, and condition of samples."

Comment 1a: Did the individual collection and processing of the samples correlate somehow with the results? And how can one control for nursery contamination of the patients in your experiment?

Reply 1a: No correlation of sample collection personnel and "infectome" was detected. Specifically, each person collected the entire series of samples from the same patients, whose microbial composition and abundance levels changes constantly throughout the course of infection and therefore unlikely to be correlated with the personnel involved. Furthermore, to control for contamination at different stages, the experimental setup in this study included reagent control and healthy controls, which

were collected and processed using the same methods and at the same locations throughout the entire experiment. Nursery contamination can be easily excluded by sequencing the healthy controls, which were collected by the same clinicians.

Comment 1b: This is to understand how much of the phenotype could be due to patient specific (as claimed mainly) versus Method manipulation specific or even Collection specific results?

Reply 1b: As mentioned in **reply #20**, contamination from methods or environments are effectively ruled out by reagent controls as well as the observation that the "infectome" of each patient is highly dynamic in nature. Importantly, none of the pathogens detected correlated with sample collectors or other environmental factors.

Comment 2: The results of patient 4 suggest the spike of inflammatory cytokines in very high levels in the blood as well as in the upper respiratory tract only 2 days before death. With this result, do you think that the cytokine storm syndrome seen was a consequence of or the cause of death?

Reply 2: We thank the reviewer for the instructive comment. While our data might not be enough to support this, we speculate that death is associated with high inflammatory cytokine levels triggered by secondary infection of *Acinetobacter baumannii*.

Comment 3: The figure 4 result is badly discussed in the results session. I would suggest the authors to explore that in more detail. For example, Figure 4A shows that most cytokines and chemokines are not different between active and non-active phases. But on line 294, the authors say that the results confirmed that cytokine storm contributed directly to the acute lung injury. How come in some patients the same cytokines and at same amounts do not contribute for that outcome?

Reply 3: Please refer to our Reply #10 (Reviewer B). Figure 4 has now been thoroughly described and discussed (Page 17, lines 376-391). Figure 4 shows that high levels of cytokine and chemokines are observed in both active and non-active phases. The intention for these comparisons is to demonstrate that high level of cytokine might be associated with pathogens other than SARS-CoV-2, although SARS-CoV-2 is the one who trigger the initial storm. However, we do not infer the relationship between "cytokine storm" and lung injury, and this is examined later with correlation analyses (see page 14 line 296-299): *"In both cases 1 and 2, the Murray score was significantly correlated with CPIS and several inflammatory mediators in the blood (i.e., IL-1Ra, IL-6, IL-18, TNF-α and IP-10) (Figure 5), suggesting that pathogens and inflammatory responses are critical in causing severe symptoms in the lungs. Conversely, SARS-CoV-2 was significantly correlated with Murray score in case 1 ($r=0.636$, $p<0.05$) but not case 2 ($r=0.485$, $p=0.067$)."*

Comment 4: In the discussion session, the authors mention the superiority of the meta-transcriptomic approach over the qPCRs, which I agree. But are there limitations to the meta-transcriptomic approach we should know of?

Reply 4: Thank you for pointing this out. As you note, meta-transcriptomics simultaneously detect and quantify all types of pathogens within the sample and is therefore a powerful approach in the diagnosis of complicated infections(1,2). Nevertheless, the cost, turnaround time, regulatory considerations also remain major hurdles for the routine implementation of clinical mNGS in patient care settings.

Changes in the text: A sentence has been added to reflect the limitation of meta-transcriptomics approach (see Page 15, line323-326), to read:

"In contrast, our meta-transcriptomics approach, although more expensive and time-consuming in practice, reveals all potential pathogens actively expressing RNA molecules within the host throughout the course of the disease (40-42)."

Comment 5: Could the cytokine level increase be eliminating non-pathogenic microbiota and then helping to select for the growth of pathogenic ones?

Reply 5: We agree with the reviewer that during the infection the microbiome at respiratory tract changed significantly such that “Infectome” often replaces commensal microbiome as the dominant component. However, our data here are not adequate to infer a causal relationship of cytokine level and changes in respiratory microbiome. This is an important direction of future research.

Comment 6: On Figure 2, the scales are so different between the patients, especially for the case 4 CRP. Is that a normal variation? Or is it comparable between all cases? This patient particularly seems to be already in Multi-Organ-Failure process when the material was collected and assessed.

Reply 6: We apologize for this variation; the scale is now unified for four patients. The plasma concentration of C-reactiveprotein (CRP) in healthy person is <10mg/L. As an acute-phase protein, the plasma concentration of CRP deviates by at least 25% during inflammatory disorders. The highest concentrations of CRP are found in serum, with some bacterial infections increasing levels up to 1,000-fold. In this study, the expression of CRP in the 4 cases was increased during pathogen active phase and inflammatory disorders.

Changes in the figure: The ordinate scale was unified. See Figure 2.

Comment 7: On figure 3, could the authors indicate with an arrow, or vertical line, or else, the time specific interventions were administered, as shown on figure 1? For example, it would be informative to understand the ups and downs on the inflammatory markers and how they relate to when antivirals, antifungal, antibiotics or immunosuppressants were administered around day 34. This is important, because

if could explain better the differences in all cases, as well as instruct the field on what can happen after specific interventions.

Reply 7: We agree that mark treatment measures at the corresponding point in figure 3 is helpful.

Changes in the figure: The corresponding information is now added to figure 3.

Comment7a: Similarly, on figure 5A and 5B, these cases received IFN as a treatment, and the consequence of the IFN is of utmost importance to determine the upregulation of many of the measured factors. To know when the intervention happened it is important to clarify specific effects to the infectome instead of the intervention.

Reply 7a: We thank the reviewer for pointing this out. Case 1 and case 2 received interferon treatment since the day of admission to ICU. Case 1 continued to use until 44 days after onset (SARS-CoV-2 negative), and case 2 continued to use until 25 days after onset (SARS-CoV-2 positive). It is therefore difficult to examine such effect because interferon treatment lasted whenever the patients are under critical conditions.

Changes in the figure: Information on interferon treatment is now added to figures 1, 2 and 3.

Comment 7b: Could the convalescent plasma that cases 1 and 2 receive impacted the figure 5 conclusions?

Reply 7b: We thank the reviewer for pointing this out. Convalescent plasma treatment reduces many parameters, which is added to figure 5.

Changes in the figure: The effect of convalescent plasma on patient is now added to figure 5.

Comment 8: Figure 4 would benefit from using median and interquartile range instead of mean, as outliers are pulling the entire group when most patients in many of the graphs are not actually high.

Reply 8: Corrected as suggested.

Changes in the figure: in Figure 4, median and interquartile range has been used.

Comment 9: On figure 4B, could the authors explain the variation shown on IL-1a and IL-18 on healthy controls.

Reply 9: We thank the reviewer for pointing this out. Such variation is expected with healthy controls because other studies have shown that the expression levels of serum IL-1α and IL-18 in healthy population are between 0-150pg/mL and 0-500pg/mL (4).

References

1. Fischer N, Indenbirken D, Meyer T, et al. Evaluation of Unbiased Next-Generation Sequencing of RNA (RNA-seq) as a Diagnostic Method in Influenza Virus-Positive Respiratory Samples. Journal of Clinical Microbiology 2015;53:2238-50.

2. Wilson MR, Naccache SN, Samayoa E, et al. Actionable Diagnosis of Neuroleptospirosis by Next-Generation Sequencing. New England Journal of Medicine 2014;370:2408-17.

3. Rajendran K, Krishnasamy N, Rangarajan J, et al. Convalescent plasma transfusion for the treatment of COVID-19: Systematic review. Journal of Medical Virology 2020;92:1475-83.

4. Lin E, Vincent FB, Sahhar J, et al. Analysis of serum interleukin(IL)-1 alpha, IL-1 beta and IL-18 in patients with systemic sclerosis. Clinical & Translational Immunology 2019;8.

Re-review:

Comment 1. The responses to my inquiries were satisfactory and the overall manuscript has improved. Still some typos, for example "As a result, we cannot determine(d) whether these cases were experiencing no(n)socomial infections". So another proof-reading is suggested.

Reply 1: We apologize for the grammar errors; the manuscript was edited by third-party language polishing service, and the certificate is attached here "EdanzEditingCertificate_103654.pdf ."

Comment 2. I did not find clearly in the text the descriptions of the lines depicting the interventions on Figure 3. Or discussion of the impact. I find those particularly relevant to the fields of critical and urgent care. But it is up to the authors to comment those or not.

Reply 2: We agree with the reviewer that intervention is relevant for changes in the inflammatory mediator levels. And here we have demonstrated, using our data (Figure 5B), that the use of convalescent plasma can reduce a number of inflammatory mediator, alleviate the patient from "cytokine storms". And relevant text has been added to the result section.

Changes in the text: We have now added relevant description this information between Page 14, line 302-305, to read: "*In addition to "infectomes", the inflammatory mediator levels were also likely to reflect clinical intervention. For*

example, a negative correlation was identified between the use of convalescent plasma usage and IL-1β, TNF-α, IL-10, amongst others (Figure 5B)."