Reviewer's report

Title: Dynamics of microbiota during mechanical ventilation in aspiration pneumonia

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Reviewer: Benjamin Wu

Reviewer's report:

Otsuji et al. and authors present an article titled "Dynamics of microbiota during mechanical ventilation in aspiration pneumonia", whereby they demonstrate by using an 16s RNA gene clone library the microbiota dynamics of mechanical ventilation on the lower airway and upper airway of subjects. They show that the act of intubation can have changes to the lung microbiome (via tracheal aspirates), but they also show that there is a depletion of anaerobic bacteria and with the start of IV antibiotics the lower airway microbiome becomes enriched with taxa with an increased potential to be antibiotic resistant.

I have several commendable points to the article: 1) The authors do a very good job with specimen collection and time keeping. If the collection is at the time that authors specify, this is one of the first manuscripts that describe changes to the lung microbiota at certain time points and raises several mechanistic questions to the development of HCAP and VAP. 2) The rigorous clinical data re: reasons for intubation and mechanical ventilation - the authors seem to select their subjects/participants very carefully and captures hopefully a specific group of patients. Finally, 3) The manuscript is overall seems to give specific insight into mechanistically how subjects may develop VAP and I believe that the manuscript has slight overstatements, but overall is coherent and offers a mechanism (e.g., depletion of healthy normal anaerobic microbiota in the airway and possibly a selection pressure of antibiotics on the lower airway microbiota). One important point, but I am unsure where this would go is the trachea width point that the authors bring up - I am not sure what the relevance or the research to back up their statements.

There are several weaknesses, by which are addressed here, but also within the criticisms below. 1) The clonal 16S rRNA library that the authors created and then utilize PCOA and multidimensional visualizations to assess may be short-sighted. Using 16S rRNA microbiome via 16S rRNA gene amplification (Illumina MiSeq) and collection may capture more enrichment as microbiota with less presence in the microbiome (less enriched) may be missing from this analysis. This isn't a criticism of the 16S rRNA library clones that the authors have created, it is pointing out the limitations of their analysis. 2) Small sample size: This is a major criticism which I am not sure if the authors can change, but there is a small n which the authors are making conclusions upon. Most of their discussion is supported by their work, I would just make sure that the small sample size is emphasized in the criticisms/weakness in their discussion. 3) There is a lack of multivariate analysis, there is plenty of clinical data especially with good time keeping, but have the authors controlled for time spent on vent/age/antibiotics/FiO2, length of hospital stay/length of intubation? I have not seen any multivariate analysis - for example, it would be interesting to see if those subjects with Enterococcus in their lower airway started with
a small percentage of Enterococcus. Moreover, if the presumption made by the authors is true, there should be analysis performed by performing a regression between time on the ventilator and %anaerobes. 4) The authors should state that they did not obtain any background samples - I would mention that clearly or if they did and were not able to produce clones, I would mention that as well. 5) Finally, the authors should review their figures - supplemental figure s1 may benefit from non-hierarchical heatmaps. In my opinion, the main title figures tell us very little (especially the PCoA) which do not tell us anything at all. 6) One important time point I would like to see is how long the patients were admitted for prior to intubation.

We will be referring to page numbers on bottom of the page and the leftmost line numbers.

Abstract:

Line 23 "On the other hand, …" It's not clear what the authors are referring to "(A) to (B)". I am sure that the context is provided in the article, but unfortunately it is not clear from just reading the abstract. I would recommend that the authors re-write for clarity and allowing for readers to read the abstract as a stand-alone abstract.

Line 31 "There was a significant trend …" What exactly is the trend that the authors are implying?

Line 31 "It is suggested that …" What or how exactly is it suggested that there is a loss of anaerobic bacteria from the lower respiratory tract? I think that the authors should speak with slightly more specificity in this abstract.

Line 46 "These bacteria are assumed …" I believe that this is an overstatement and should be removed from the abstract.

Background:

Page 3, Line 12 "Several studies indicate that aspiration pneumonia …" While I do not have issue with the sentence preceding this sentence, I have issue with this sentence. I believe that aspiration pneumonia should be considered a distinct entity. I believe that the authors mean to indicate that aspiration/microaspiration is a risk factor/involved in CAP and HCAP. The authors should not conflate aspiration pneumonia and aspiration. I would clarify this sentence as either aspiration or aspiration pneumonia. If the authors did mean aspiration pneumonia, I would recommend that the authors clarify if aspiration pneumonia may actually be misidentified as CAP or HCAP. OR that aspiration is a risk or present in CAP or HCAP (this is what I believe the authors meant, but I am asking the authors to clarify this statement).

Page 3, Line 23 "Culture methods are used for the surveying …" The anaerobic bacteria are fastidious. The tracheal aspirate that is with oral bacteria may be deleterious as well.

Page 3, Line 37 "In some cases, antibiotic treatment …" I am having difficulty making the logical leap between aspiration pneumonia and multi-drug resistant organisms. I believe that I understand that the authors want to connect the two, but is there evidence that oral carriage of
antibiotic resistant markers are of concern? As far as I know, nasal carriage of MRSA increases risk of bacteremia, but it is unclear how oral bacteria/flora is connected to drug-resistance. This seems arbitrarily connected to the research focus of the article, and I caution leaving it in as it is misleading to the reader. (as far as I know, 16s rRNA gene marker identification cannot identify bacterial resistance to antibiotics per se, but can possibly aid in the recognition of bacteria that MAY have increased resistance).

Page 3, Line 55 "In cases of aspiration pneumonia, …" Please provide a hypothesis statement for the readers. I am not sure what the authors want to focus on, however I believe that without a hypothesis statement, the research article runs the risk of being descriptive.

Page 4, Line 6, "To develop a treatment strategy for patients with aspiration pneumonia, …" Not sure what this study would add to the already well-developed treatment strategy that the authors are discussion. I would expand upon this idea.

Methods

Page 4, Line 38 "Inclusion criteria" Was there an inclusion of how long a patient can be admitted prior to recruitment in the study? Was there conclusive diagnosis of aspiration? How was the diagnosis of aspiration pneumonia made?

Sample handing is missing from the methods section. Issues such as 1) were samples spun down? 2) were samples evaluated for cross contamination? 3) Why were samples stored at 4C and left no more than 7 days? (wouldn't the authors keep them colder at -20C)? 4) was the volume of samples recovered? Moreover, regarding lower airway samples, did the authors use the cell-free or cell-associated bacteria component? (please see Dickson 2014 Microbiome "Cell-associated bacteria in the human lung microbiome").

Page 5, Line ### - the authors do not describe any background control sampling, while the oral/saliva microbiome is likely very abundant, it is also good practice to amplify samples for control including: sampling fluid, sampling tools. For example, what we tend to do is take sampling fluid (e.g., sterile saline) on the day of the procedure for control. Others have collected a sterile flush through instruments such as bronchoscope/endoscopy tools in order to understand the possibility of collected bacterial DNA on instruments and to ensure that attribution from the background microbiome can be accounted for in the samples.

Technical controls can include the 16S rRNA of the elution buffer by which the bacterial DNA was collected. I would like to see this data from the authors (for a citation please see Salter et al. BMC Biology 2014).

Results

Page 7, Line 27 "The remaining 22 subjects …" One important time point that the authors did not collect, but is of interest is the time of admission to recruitment into the study. There is some evidence that admission to the hospital system can impact upper airway microbiome. I would recommend that the authors list the time of admission into the hospital as another in their table. I
would also include medications, especially immune modulating medications. I would include it both for possible biases introduced on delay into recruitment, but also to do analysis to see if anyone's baseline microbiome is impacted by early vs. late recruitment into the study and/or early/late recruitment due to respiratory failure.

Figure 1 I would line up the # of cases and at each time point in the flow/time course figure. The section provided by the authors is slightly difficult to understand and should be re-written for improved clarity.

Page 8, table 1 Characteristics of the subjects. Some other information I would think important for the authors: that would make the study more interesting:

1) Mortality and mortality related to pneumonia/infection

2) Length of stay in ICU, mechanical ventilation, and hospital

3) Medications: Vasopressors, antibiotics utilized per patient, culture data (e.g., positive culture, identification of organism)

4) Co-morbidities

5) Is the primary diagnosis the diagnosis that resulted in respiratory failure? This is not clear from the table

I would work on expanding some more information for the table, I believe the requested data are clinically relevant and would be of interest for other readers.

Page 9, Line 12 "The composition of clones assigned to each genus …" I am not sure why the table is split between the orange/white groupings on the left. I would like to see a non-hierarchical evaluation (e.g., using a distance matrix of the authors choice) of these taxa including grouping by both all samples and by time point (please see heat maps in Segal et al. 2016 Nature Microbiology).

1) We would like to know if samples are more similar to patients (they will cluster closer w/ the patients) or if clinical events such as intubation will make patients more similar to each other (collapse of taxa etc.)

2) We would also like to know of co-occurring bacteria in the tracheal/oral flora travel together, for example in lung microbiome it is recognized that Streptococcus, Prevotella, and Veillonella can be found in the lower airway.

3) There may be limitations to the heatmaps, as the authors use a clone library and not a true microbiome matrix, but I would like to see unsupervised analysis to understand the data.

Page 10, Table 2 - this table is a slight convoluted, I would bold the data where the culture results matched the dominant phylotype at point B. Although, this doesn't say much, especially when the culture data is effective at clinically diagnosing a potential pathogen. What is interesting is when the data is mismatched, there should be a distinction made (possibly re-organizing the table).
Page 11, Line 1
I would discuss the PCoA plot before speaking about the individual components - move this section behind the discussion of the plot. Also, the PCOA plot (figure 2) likely is very non-descriptive as the authors excluded lower abundant phylotypes/clones - these may have been interesting to add back as they may make the distinctions between the saliva and trachea more apparent. Based on supplemental figure 1 of the clones, it is clear that the PCOA plot was likely not going to find any differences between the saliva and the tracheal aspirates - the grouping looks too similar.

One recommendation is to do a Procrustes analysis of the individuals - it is clear at time C that individuals start to resemble each other, but it would be interesting to see if there is any distinction between individual and their samples. Some predictions:
1) Subjects that change the greatest on Procrustes may have significant culture findings
2) Subjects that change the least on Procrustes may be the most "protected" (best clinical outcomes), etc.

Page 11, Line 23 "Dynamics of microbiota…" Could the authors define which bacteria are anaerobes? I reviewed the methods section in the paper and the appendix, but neither has the information, I would write it into the methods for reference and mention it again here.

Page 11 Line 57 "It is noteworthy that most anaerobes .." Some data presented in abstract form has shown that hyperoxia can change the lower microbiome. Which has relevance to subjects who receive mechanical ventilation - did the authors do a regression on the %percentage of anaerobes and the FiO2% of the subjects? (Prediction higher FiO2 lower anaerobe percentage), also the authors can also do a regression on time on vent and percentage of anaerobes while controlling for FiO2%.


Discussion:

Page 12 Line 14, I do believe there has been studies that examined the lung microbiome / tracheal aspirates of patient who are mechanically ventilated, the authors should be commended on the work they did regarding the timing of their samples. The study is novel as it longitudinally assessed the changes to the microbiome. The changes to the microbiome however still needs to be fully assessed - one simple assessment that the authors have not performed was individual changes (e.g., persistence of similar microbiota signals throughout all time points or changes). The authors should also do multivariate analysis with various clinical data which they collected, but did not use this data in their analysis.

Page 12 Line 60, "This phenomenon supports…” I am not sure how the presumption that anaerobes are major pathogens, they may be major players in the lung microbiota during intubation and disappear when the subjects get intubated. Is it plausible that the other way of thinking about this event is that with the disruption of a normal lung microbiome/microbiota, the
lungs become more vulnerable to lower airway infections? It is difficult to interpret the data unless there are mechanistic experiments, so the authors must entertain all assumptions.

Page 13, Line 20 "so it was possible that the cuff of the endotracheal tube was not adequately adhered to the trachea." I would hope that ventilatory technicians are checking cuff pressure and checking for cuff leak. Unless there's another proposal that possibly positional changes impacted the way that the cuff isolated the tracheal. Moreover, it is also well described that intubation does not stop aspiration.

Page 13, Line 43 "After the administration of antibiotics, Enterobacter asbriae, E. cloacae, Corynebacterium propinquum, C. acoolens, Pseudomonas aeruginosa, Klebsiella pneumoniae…” I find this fascinating. There's been some studies that have found that antibiotic application has reduced metrics of microbiomes (decreased alpha diversity), but the authors show that the application of antibiotics increase the taxa which are associated with antibiotic resistant infections, thus this is one of the most interesting conclusions of their study and should be emphasized. One important comparator which is missing are those subjects who are intubated but not given antibiotics - do they go on to develop changes in their lung microbiome? It is not the focus of their study, but would be interesting for them to emphasize (as a potential next step to study).

Page 14, Line 1 - the authors should have a comprehensive accounting of the strengths and weaknesses of their study - if background samples are unavailable then they should say so.

Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

Yes

Does the work include the necessary controls?
If not, please specify which controls are required in your comments to the authors.

No

Are the conclusions drawn adequately supported by the data shown?
If not, please explain in your comments to the authors.

Yes

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If an additional statistical review is recommended, please specify what aspects require further assessment in your comments to the editors.

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