Author's response to reviews

Title: Multiple-antigen ELISA for melioidosis - A novel approach to the improved serodiagnosis of melioidosis

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Author's response to reviews: see over
Research article title: Multiple-antigen ELISA for melioidosis - A novel approach to the improved serodiagnosis of melioidosis
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Response to Reviewer 1:

1. How many duplicate readings were made prior to calculating the mean and Standard deviation?

Response: Triplicate readings of each sample were made prior to the calculation of mean and standard deviation. The information has been included in the figure legend.

2. What was the reproducibility of the positives?

Response: The experiment was independently repeated twice and both sets of experiment data revealed the same number of positive samples, thus, the data is highly reproducible. The information has been included in the figure legend.

3. I am unclear as to the difference between the cocktail of antigens used and what is called multiple antigens. This needs clarification. If they are the same then one term only should be used.

Response: We thank the reviewer for the comment and apologise for the confusion. The description of these two experimental components has been rephrased in the Methods section (page 9, line 5 & page 9, line 21).

In brief, as described in the Methods section, the term cocktail of antigens means a single well in the ELISA plate was coated with a mixture of four recombinant proteins (TssD-5, Omp3, Omp7 and smBpF4) whereas for multiple antigen ELISA, the data from individual single antigen ELISAs were reanalysed using the different criteria as described in the Methods section.

For analysis of the single antigen or antigen cocktail ELISA, the cut-off value was calculated from the mean absorbance (plus 2 standard deviations) of healthy controls. Thus, any test sera sample with a mean absorbance greater than this cut-off value was considered positive.

For the multiple-antigen ELISA results, the serum tested was determined to be positive according to the one of the following criteria: (1) any 2 or more antigens specifically react with serum with an absorbance greater than the cut-off value calculated from the mean absorbance (\(A_{405nm}\)) plus 2 standard deviations of healthy controls or (2) any antigen that specifically reacts with serum when the cut-off value was calculated from the mean absorbance (\(A_{405nm}\)) plus 3 standard deviations of healthy controls.
4. Why was the IHA not used as a comparator?

Response:
We obtained the melioidosis sera samples from the collaborating local hospitals. However, the hospitals where melioidosis samples were collected only facilitate culture or IFAT as routine diagnostic methods thus the IHA test was not available as a comparative diagnostic tool at these hospitals.

5. Why were culture positive, IHA negative sera not included? It is well known that up to 50% of culture positive sera will be IHA negative. It would have been useful to see how this antigen combination might have performed.

Response:
We appreciate the comment raised by the reviewer and agree on the importance and value of such samples in the study. However, as mentioned earlier, culture and IFAT were the only routinely used diagnostic tests in the local hospitals where the samples were collected. Hence, due to the limited resource, we are not able to include them in the current study.

Response to Reviewer 2:

1. In the abstract, it is not clear what gold standard was used. IHA appeared at the conclusion of abstract but it was not mentioned earlier and in a good agreement with the other part.

Response:
The melioidosis sera samples used in the current study were collected from local hospitals where culture and IFAT are routinely used as diagnostic tools, but not IHA, thus the gold standard in the current study would be culture and IFAT as mentioned in the abstract. The intension of the current study is to evaluate the four recombinant proteins as potential diagnostic antigens by an ELISA approach and to compare the sensitivity and specificity of ELISA to other commonly used diagnostic methods in the local hospitals, such as IFAT and culture.

2. The objective of the study in the last section of introduction was not clear.

Response: The text has been revised for better clarification (page 6, line 16).

3. The methods need improvement. There is no detail for TssD-5 construction. The author only cited Chieng et al. in preparation which was not useful.

Response: The method section has been revised as recommended with details of TssD-5 construct preparation (page 8, line 6).

4. Page 6: the authors wrote that 4 recombinant proteins are conserved in B. pseudomallei and other Burkholderia species. In page 11, paragraph 2, line 5: the sentence that “this species
specific feature is valuable in endemic regions…” means that TssD-5 was specific to B. pseudomallei. Which one is correct?

Response: TssD-5 is unique to B. pseudomallei while others are highly conserved in B. pseudomallei as well as in other Burkholderia spp. The text has been revised for clarification (page 6, line 9).

5. Page 7: the author compared ELISA with culture and IFAT. As these two methods use different diagnostic approaches, I would suggest that the data should be analyzed separately. Please give more detail for IFAT and describe the method to interpret positive result and give information for the specificity of the test. I wonder if it is a good gold standard method.

Response: The results section (page 11, line 2) and Table 3 have been revised to include the separate analysis of the ELISA derived data based on culture-positive or IFAT positive sera samples.


6. Table 2: described the detail of samples. It should be referred from “Human sera samples” in methods section.

Response: The text has been revised as recommended.

7. Page 7: there should be a centrifugation step for the preparation of lysate. Please describe the detail.

Response: The methods section has been revised as requested (page 7, line 24).

8. Page 8: please add why the authors use a single serum dilution at 1:1600. How many repeats measurement were performed?

Response: The dilution of 1:1600 was previously determined to be optimal with minimal background and is routinely used in our laboratory. Triplicate readings of each serum sample were measured in each independent experiment. The description was added in the methods section for clarification.

9. Page 9: the comparison between ethnic groups appeared in the first section of result. It should be mentioned in the objective and methods.

Response: The information presented in the results section was only to provide the demographics of the sampling as the number of samples for individual ethnic groups is
too small for any meaningful comparison in terms of disease susceptibility. We have included a brief reference to this in the methods section.

10. Page 9: In the evaluation of recombinant proteins as serodiagnostic reagents: I do not understand why the human sera were tested against the *B. pseudomallei* lysate prior to performing with the recombinant proteins. The data of crude lysate looks promising as the following sentence is “all 68 melioidosis patients sera reacted with this lysate but non-melioidosis did not”. Please clarify why the author studied recombinant proteins despite having ELISA using the lysate.

Response:
Prior to testing with recombinant proteins, we tested all melioidosis sera samples with *B. pseudomallei* lysate to validate and confirm the positivity of the samples based on the prior testing (IFAT or bacterial culture) performed at the hospitals. Whilst we acknowledge that bacterial lysate is a good antigen to analyse in the development of an ELISA-based diagnostic assay, nevertheless this would require large-scale growth of the bacteria to produce sufficient quantities of lysate. Hence, the objective of the current study was to analyse the potential of these recombinant antigens to replace the use of *B. pseudomallei* lysate in an attempt to minimise the growth and handling of large quantities of bacteria which may pose health risks to laboratory personnel.

11. Page 11: Is Omp3 and OmpA are the same antigen? Please clarify.

Response: They are the same proteins and the text has been revised for clarification as recommended.

12. Page 11, paragraph 2: Please give more detail about the format of assay (e.g. immunochromatography) for sensitivity and specificity of TssD-5 of previous report.

Response:
TssD-5 was previously determined to be immuno-reactive with a small cohort (n = 5) of pooled melioidosis sera by western analysis (Chieng et al *in preparation*). Our study is the first to evaluate the immuno-reactivity of TssD-5 with a larger number of samples and assess its diagnostic potential by an ELISA approach.

13. Have the author analyzed the cocktail antigens or multi-antigen of only 2 antigens (TssD-5 and Omp-3)? When the multi-antigen approach was used, please provide results which combination gave the best performance. Does it really need all four antigens in the multi-antigen ELISA?

Response:
The percentage of sensitivity (number of positive/number of total) was calculated for different combinations of 2-4 antigens and it ranged from 38.2% (2 antigens) to 88.2% (4 antigens). The best combination of 3 antigens (TssD-5, Omp3 and smBpF4) gave 82.3% sensitivity, which is still lower than the combination of all four antigens, thus we propose the use of all 4 antigens will maximize the detection sensitivity (88.2% sensitivity) in multi-antigen ELISA format.