Author's response to reviews

Title: Detection of human leptospirosis as a cause of acute fever by capture ELISA using a Leptospira interrogans serovar Copenhageni (M20) derived antigen.

Authors:

Enrique Canal (Enrique.Canal@med.navy.mil)
Simon Pollett (spollett@med.usyd.edu.au)
Kristen Heitzinger (heitzk@u.washington.edu)
Michael Gregory (Michael.Gregory@med.navy.mil)
Matthew Kasper (Matthew.Kasper@med.navy.mil)
Eric Halsey (Eric.Halsey@med.navy.mil)
Yocelinda Meza (Yocelinda.Meza@med.navy.mil)
Kalina Campos (kalina_cg@hotmail.com)
Juan Perez (Juan.Perez@med.navy.mil)
Rina Meza (Rina.Meza@med.navy.mil)
Alfredo Guillen (alfredo_guillen@yahoo.com)
Maruja Bernal (Maruja.Bernal@med.navy.mil)
Tadeusz J Kochel (Tad.kochel@med.navy.mil)
Benjamin Espinosa (Benjamin.espinosa@med.navy.mil)
Eric R Hall (Eric.hall@med.navy.mil)
Ryan C Maves (Ryan.Maves@med.navy.mil)

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Author's response to reviews: see over
Dear Sir,

Please find attached our revised manuscript titled ‘Detection of human leptospirosis as a cause of acute fever by capture ELISA using a Leptospira interrogans serovar Copenhageni (M20) derived antigen’.

Our responses to reviewer comments are detailed below. We thank the reviewers for their time and valuable feedback.

We thank you for your ongoing consideration of this manuscript.

Respectfully,

Dr S. Pollett (corresponding author)

MBBS, BMedSci, DTMH

Sydney Institute of Emerging Infections and Biosecurity, University of Sydney, Australia

Phone: 61-0412525637

E-mail: spollett@med.usyd.edu.au.
Responses to reviewer comments:

Reviewer 2:

1. Case definition and selection of the cases followed to be described properly.

We agree that the multiple components of the study methodology may be confusing to the reader and we have now clarified this. As before, case definitions, enrolment criteria and timing of serology performed on the 63 participants are explicitly and comprehensively described in the section titled ‘Setting and Enrolment’ and ‘Measures and Procedures’. In the section ‘Determination of MAC-ELISA optical density cut-off’, we have emphasised that the determination of the Optical Density cut-off was derived from banked leptospirosis positive and negative (by MAT) from previous studies, and are not from the study sample. Likewise, in the section ‘Assessment for MAC-ELISA assay cross-reactivity with other pathogens’ we have emphasised that this was determined using banked archived sera and not from the study sample.

2. How may sera samples are utilized from other infections as controls.

The number of sera from other infections used in the negative control panel has now been specified in the methods section, and the total number of sera used remains in the results section.

3. The specific reason for the use of serovar Copenhageni strain M20 for the antigen preparation for MAC-ELISA. Supportive references are needed in the methods section.

Commercial ELISA IgM assays designed with *L. biflexa* antigen have been validated in this region before, with very poor sensitivity (Russell et al Am J Trop Med Hyg 2003; 69(1):53-57), and we wanted to avoid that species as a source of antigen. The diversity of *Leptospira* serovars in Peru is very broad but we know from previous studies that *L. interrogans* serogroup Icterohaemorrhagiae (the serogroup to which the serovar Copenhageni M20 belongs) is one of the most common serogroups implicated in human disease in this region (Ganoza et al PLoS Med 2006; 3(8):e308) and we believed it was reasonable source of antigen for this assay design. This has now been described in the methods section with supporting reference.

4. Even though strain M20 sonicated fraction was used then why they have utilized the hyperimmune rabbit anti-Leptospira biflexa IgG antibody in the sandwich. It would be more appropriate to use the hyperimmune sera of strain M20.

We agree with this important point and have acknowledged it as a limitation. Commercial *L. interrogans* serovar Copenhageni (M20) rabbit IgG antibody was unavailable for use in this region at the time of the study, hence *Leptospira biflexa* IgG was used instead.
5. What is the concentration of the sonicated antigen used in ELISA. Specify the percentage of Tween 20 used in PBS.

This has now been included in the methods section.

6. Did they perform triplicates of the samples in ELISA? It is not clear that either they have utilized the mean+SD as a cut-off OD in a dilution of 1:400 or used some other measurement. It is mentioned as a titre of ≥1:400. This part should be described properly.

We agree these aspects of the methodology are somewhat complex and we have clarified this in the methods section under ‘Sample testing by IgM MAC-ELISA’. We have also included a figure (see Figure 1) that gives an overview of the methods as other reviewers also requested clarification of these aspects of the methods.

To re-iterate, an optical density was used as a qualitative measurement of IgM. The OD cut-off was determined from banked leptospirosis MAT positive and negative sera (from archived sera, not from the study sample sera). The cut-off was calculated using the receiver operator characteristic (ROC) curve through the Data Statistical Package for the Social Sciences to achieve maximum specificity and sensitivity.

By reading optical density on serial dilutions (from 1:100 to 1:6400) of sera from the study population, a titre was determined in order to give a quantitative estimate of IgM present. This process was performed on acute and convalescent sera.

A positive diagnosis of leptospirosis by MAC ELISA IgM was defined by having a four-fold or greater increase in IgM titre between the acute and convalescent sera, or by an IgM-reactive acute or convalescent sample at a titre of ≥ 1:400.

7. What may be the specific reason for the low specificity of the evaluated MAC-ELISA? If it is only as a rule-out test then what may be the advantage of this ELISA with its complicated procedure rather using simple commercially available dipstick or other ELISA formats.

We thank you for raising these important points about the specificity of the MAC-ELISA.

One potential reason for the limited specificity may be the presence of IgM in patient sera from past *Leptospira* exposure (rather than acute infection). Like many pathogens, *Leptospira* IgM may persist in the host for a prolonged time. This could potentially explain some of the MAT-positive/MAC ELISA-negative results in acute phase sera. This point has been already been raised in the ‘Discussion’ section.

A second potential reason may be cross reaction to other antigens from infections other than leptospirosis. We have demonstrated that some cross-reactivity occurs with certain non-*Leptospira* pathogens. This has also been shown in other studies (Bajani et al Journal of Clinical Microbiology 2003; 41(2):803-9). While those with clinically evident sites of infection where excluded from the study sample, the
exclusion criteria for other infections were not exhaustive and thus other infections may have been present. This point has now been raised in the ‘Discussion’ section.

The poor specificity of this assay is a limitation to its utility. As discussed, some commercial ELISA assays are offer a higher specificity and similar sensitivity. However, other commercial ELISA assays have shown a considerable loss of sensitivity in trade-off for this high specificity (Bajani, M.D., et al., Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. J Clin Microbiol, 2003. 41(2): p. 803-9). Moreover, while simpler commercial ELISA assays are available, their cost may be prohibitive to laboratories in some tropical low-middle income countries which are most affected by leptospirosis. This point has now been raised in this discussion.

8. There is no significance with the incorporation of Table.1 and the heading also very broad. But the table specifies only age & gender composition with the location of the cases. This was not described in the text of the manuscript also. Instead of this table incorporation of a graph for ELISA ODs with their cut-off derived for the confirmed cases (acute, convalescent & both) along with other controls will be of much informative.

We believe that describing key sociodemographic characteristics such as age and sex of the study population is important to determine the generalizability/external validity of the study findings to other study populations. The geographic location of the study subjects is also important to present as the seroprevalence of leptospirosis will likely be higher in locations of high rainfall climate and jungle ecology compared to more arid regions. As positive and negative predictive values are a function of disease prevalence we feel including the locations is thus important. We respectfully request that this table remains in, with a clarification of the geography for each region as requested by other reviewers. We acknowledge the Table 1 title is broad and have suitably adjusted the table title. We have added commentary on the contents of Table 1 in the discussion.

As the ELISA OD was calculated from banked sera (and not from sera of the study sample), and because OD is only a component of the definition of ELISA IgM positivity (see Figure 1) and because the primary objective of the study was to assess the performance of the assay in a sample of patients presenting with fever of unknown origin, we feel that including OD in a main results table will be confusing to the reader and respectfully request it is not included in the main results.

9. Throughout the manuscript the species and serovar description is improper. In most of the places it is not as per the nomenclature. The spelling of the species and serovar names are also specified incorrectly in the manuscript in some places. So the authors have to take care of the descriptions properly.

We agree Leptospira nomenclature is an evolving field and inconsistencies do exist in the literature. For this article we used the internationally standardised reference for species and serovar nomenclature from the Taxonomic Subcommittee of the
International Society of Leptospirosis (http://www.med.monash.edu.au/microbiology/staff/adler/ils.html). Could the reviewer kindly please specify in which instances the nomenclature is felt to be incorrect?

We regards to the spelling of certain species/serovars, we indeed note that *L. borgpetersenii* was misspelt and this has been corrected where appropriate. We thank the reviewer for detecting this error.

**Reviewer 1:**

**Major Compulsory Revisions:**
None

**Minor Essential Revisions:**

1. **Materials and Methods, Sample testing by IgM MAC-ELISA** -- after determining OD cut-off for your test, you then determine serum positivity based on dilution of serum testing positive. Why not use OD units as with many other ELISA tests (or simply positive versus negative)? Please provide discussion in M&M or Discussion

We thank the reviewer for raising this important point and have clarified this in the methodology. The determined optical density cut-off is of qualitative use only. The titre reported gives a quantitative measurement of IgM positive, i.e. at what dilution of analyte the OD remains above the cut-off. Quantitation of ELISA IgM, particularly in paired sera, is useful to differentiate acute infection versus past exposure. We have clarified this in the methods.

2. **Materials and Methods, Sample testing by MAT** -- "serogroups" in the pentultimate sentence should be "serovar"

We have corrected this error.

3. **Table 1** -- define "Location". Are these cities, provinces, states, etc.? Also, define units used for "Age" (e.g., years, months)

We have now defined location and units of age in Table 1.

**Discretionary Revisions:**

4. **Abstract, Background** -- delete the last 10 words of the final sentence. You have already stated MAT is the gold standard.

We thank the reviewer for this suggestion.
Reviewer 3:

First of all, it is confused which group of patient were used and for which testing? We suggest to present particular patient’s group with which authors achieved particular result (sensitivity, specificity, leptospirosis confirmation, etc).

Thank you for this valuable feedback. There are multiple components to the study design and we agree it is complex. We have clarified which results were derived from the study sample in the methods section, and have also included a flowchart (Figure 1) which should guide the reader through the methods. Please see below for further elaboration.

Title page should indicate corresponding author that I miss in sent the article.

This has now been added.

The Background is clearly stated. The section ends with a brief aim that is being reported in the article. Some printing errors must be corrected, and I suggest authors to check carefully the text. This section also lacks reference no. 10, please add it.

We have checked for spelling errors and have corrected them. Reference number 10 was indeed not cited and this has been corrected.

Methods and Results are not reported well. Please, check the following comments. Methods:
1. Herein it is not clear which patients groups were included and tested with both IgM MAC-ELISA and MAT?

In the first part of the section authors report “…. patients aged five years or more with fever equal to or greater than 38.0° C for equal to or less than 7 days who presented for medical care …….. were invited to participate”; in the second part of the section authors report: “Patients were ineligible to participate if a clinically identifiable focus of infection was present“; But later on in this section (Assessment for MAC-ELISA assay cross-reactivity with other pathogens) authors report that “Banked sera specimens from patients with evidence of other infections were also used as negative controls” and “were tested by the leptospirosis MAC-ELISA assay to assess for cross-reactivity between leptospirosis and these other pathogens”. So, if patients with determined infections were included for IgM MAC-ELISA validation, authors must state their number as well as number of patients suffering by particular infection different than leptospirosis.

Moreover, in the section “Determination of MAC-ELISA optical density cut-off
"authors report about “50 banked Leptospira-negative control sera from healthy subjects from Lima, Peru and 18 banked positive control sera samples from Peruvian patients with known MAT-confirmed”.

Taking together, a lot of participants were tested with IgM MAC-ELISA? So, the section Material and Methods must be supplemented with exactly cited patients’ groups:
- Febrile illness of unknown etiological agents
- Febrile illness caused by brucellosis, bartonellosis, yellow fever, dengue, Oropouche virus, Caraparo virus, Venezuelan Equine Virus, P. vivax, P. falciparum, hepatitis A, hepatitis B, HIV, and Treponema pallidum
- 50 banked Leptospira-negative control sera
- 18 banked positive control sera samples

We thank the reviewer for raising this important issue. We agree that the multiple components of the study methodology may be confusing to the reader, particularly as the ELISA MAC IgM was tested on multiple groups of sera, and we have now clarified this. In response to other reviewer feedback, we have also included a Methods flowchart to guide the reader (Fig. 1).

To re-iterate, the main objective of this study was to assess the performance of this MAC-ELISA assay in a population of patients with fever of unknown aetiology in a developing world setting. Before this objective is answered, we are obligated to present the process by which the assay was designed, including the use of external controls i.e. banked sera (not from the study sample) to determine the ELISA OD cut-off and the cross-reactivity of the assay.

Thus, the stages of the methods are as follows (see Fig 1 and revised methods section):

a) Determination of the ELISA OD cut-off using 18 banked known Leptospira positive control sera samples (determined by MAT) and 50 banked known Leptospira negative control sera samples (determined by MAT). The ELISA OD was calculated using a ROC curve. This was not calculated using sera from the study sample.

b). Determination of the cross-reactivity of the ELISA MAC IgM assay with other pathogens. This cannot be answered using sera from the study sample as presence of another infection was an exclusion criterion. Thus, the ELISA MAC IgM was performed on banked/archived sera from patients with known other infections. In cases where the ELISA MAC IgM was positive in sera of patients with other infections, MAT was performed to exclude Leptospira co-infection.

c). Determination of the sensitivity, specificity, PPV and NPV of the ELISA-MAC assay in the study population, i.e. those presenting with fever of
unknown aetiology and who met study inclusion criteria. This could only be done after determining the ELISA OD cut-off using banked control sera first.

2. It is not clear how authors determined results of IgM MAC-ELISA? In the section “Determination of MAC-ELISA optical density cut-off” authors cited two groups of patients (50 banked Leptospira-negative control sera from healthy subjects from Lima, Peru and 18 banked positive control sera samples from Peruvian patients with known MAT-confirmed leptospirosis) to calculate OD cut-off but later (in Results) they did not report any data of these two patients groups?

Before testing the MAC-ELISA IgM assay on the study sample (those presenting with fever of unknown aetiology) the ELISA OD cut-off had to be determined. This was done using archived control sera, that is: 18 banked known Leptospira positive control sera samples and 50 banked known Leptospira negative control sera samples. The ELISA OD was calculated using a ROC curve. This has been clarified in the methods.

At the same time authors reported (section Sample testing by IgM MAC-ELISA «Positivity of acute and convalescent sera was defined as a reactive IgM at titres of # 1:400 «. So, it is not clear how authors determined results of IgM MAC-ELISA, by OD or by titer? These parts must be written clearly.

We agree this is somewhat complex and have clarified this in the methods section under ‘Sample testing by IgM MAC-ELISA’.

To re-iterate, an optical density was used as a qualitative measurement of IgM. The OD cut-off was determined from banked Leptospira MAT positive and negative control sera (from archived sera, not from the study sample). The cut-off was calculated using the receiver operator characteristic (ROC) curve through the Data Statistical Package for the Social Sciences to achieve maximum specificity and sensitivity.

By reading optical density on serial dilutions (from 1:100 to 1:6400) of sera from the study population, a titre was determined in order to give a quantitative estimate of IgM present. This process was performed on acute and convalescent sera.

We used a quantitation of ELISA IgM to better differentiate true, acute infection from remote past exposure. A positive diagnosis of leptospirosis by MAC ELISA IgM was defined by having a four-fold or greater increase in IgM titre between the acute and convalescent sera, or by an IgM-reactive acute or convalescent sample at a titre of ≥ 1:400.

3. In section “Sample testing by MAT:" authors did not clarify which group of patients was tested? All four groups: 1. Febrile illness of unknown etiological agents; 2. Febrile illness caused by other pathogens, 3. 50 banked
Leptospira-negative control sera; and 4. 18 banked positive control sera samples?

We thank you for raising this important point. We have clarified this in methods. To re-iterate, MAT was performed on the following groups of sera:

a). Patients with febrile illness of unknown aetiology, i.e. the study sample

b) When the ELISA MAC IgM was positive on the control sera from patients with known other infections. This was performed to exclude *Leptospira* co-infection.

c) The 50 banked *Leptospira* negative control sera. These were determined to be *Leptospira* negative by MAT.

d) The 18 banked positive control sera, These were determined to be *Leptospira* positive by MAT.

4. In section “Sample testing by MAT:« L. borgpetersenii is written incorrectly (borgEpetersenii). Please, correct.

This has been corrected.

Results
In this section, results of testing of all four groups of patients must be reported. Herein, it is not clear where authors find 63 participants and from which group were they? Section Results lacks raw results of all testing. The same is for Table 1, is not correct because it is not known which patients are these 63 reported here?

We thank you for raising this important point. We have clarified the results so the reader may more easily determine which results correspond to which group of sera. Table 2 presents the sensitivity, specificity; PPV and NPV of the ELISA-MAC IgM assay compared to MAT in the study population and answers the study objective. In the text, we have now also presented the raw data used to determine the sensitivity and specificity, i.e. what number of patients with undifferentiated fever were positive by MAT and by ELISA IgM

Sera were tested in acute and convalescent phase. Can authors report results of particular testing? Did authors find any difference between acute and convalescent results? Can authors report these findings? Authors cannot report sensitivity, specificity, PPV, and NPV without previously reporting raw results of all testing, and detailed calculation of particular subject. The same is for Table 2. In this part of Results, the HIV positive patient appeared. So, results of testing of all four patient groups are necessary.
Thank you for raising this important point. We agree presenting the raw data is important and have now presented the raw data used to derive the sensitivity, specificity, PPV and NPV in the main text of the results. This may now be found in the results text.

We had presented the results of the ELISA MAC IgM in banked sera from other infections, including the HIV positive patient. We had indeed already presented the results of the acute, convalescent and paired sera and compared the sensitivity and specificities in each phase. This is a core part of the discussion. We have clarified which results refer to the study population and which refer to the controls with other infections.

Tables
Tables must be without abbreviations (MAC, MAT. M20, CI, etc)

We thank the reviewer for this feedback. We have removed abbreviations where possible or included explanatory footnotes where table space limitations absolutely required abbreviation.

Discussion
Regarding previously comments for Material and Methods and Results. Discussion can be valuated after cited all missing data. It is not clear how many patients with febrile illness were not diagnosed as leptospirosis, and rested negative either by MAT and IgM MAC-ELISA?

Thank you. We have now included these results.

References
References are not cited well (comma, bold/italic letters, “et all”, etc). I suggest authors to look at Instruction for authors.

We thank the reviewer for this feedback and agree correct formatting of references is important.