Author's response to reviews

Title: Longitudinal monitoring of Ehrlichia ruminantium infection in Gambian lambs and kids by pCS20 PCR and MAP1-B ELISA

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Version: 3 Date: 14 June 2007

Author's response to reviews: see over
To The Editor, BMC Infectious Diseases

12 June 07

Dear Editor,

I would once again like to express my appreciation to the reviewers for their invaluable and critical comments on the manuscript entitled ‘Longitudinal monitoring of Ehrlichia ruminantium infection in lambs and kids by pCS20 PCR and MAP1-B ELISA in The Gambia’, MS: 1807674744128690. We have reconsidered their comments and tried to adequately address the concerns of each of them. The queries raised were constructive and addressing them availed us the opportunity to further improve the quality of the manuscript. Herewith we provide response to each of the comments:

Reviewer 1

Reviewers's report
Title: Longitudinal monitoring of Ehrlichia ruminantium infection in lambs and kids by pCS20 PCR and MAP1-B ELISA in The Gambia
Version: 2
Date: 1 May 2007
Reviewer: Theo de Waal

Reviewer's report:
General

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

The authors have answered most of my comments on the manuscript successfully. A few minor issues however are still of concern.

The number of animals still appear to be a problem. The authors indicated the correct number is 77, but on page 5 – line 3 – 76 is still indicated.

That was an oversight and has been corrected

When estimating prevalence of infection (exposure) the period when colostral antibodies would normally be detected should be excluded as this is not true prevalence.

We agree with the reviewer in principle; however, the serological assay in this study did not distinguish between maternally derived antibodies and antibodies derived through exposure. Thus we use prevalence for detectable specific antibodies generally; but based on the results (decay of antibodies) over time in some animals we highlighted the possibility of the presence of maternal antibodies and this has been reported in the manuscript.
My previous comment that ELISA test does not detect infection still stands. This is not adequately dealt with in the text.

We agree that serological do not show the true infection status of an animal and this has been highlighted on page 17 line 17-19.

 Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

 Discretionary Revisions (which the author can choose to ignore)
The manuscript seems a bit long.

 What next?: Accept after minor essential revisions

 Level of interest: An article whose findings are important to those with closely related research interests

 Quality of written English: Acceptable

 Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

 Statistical analysis has been adequately addressed in this manuscript.

 Declaration of competing interests:
I declare that I have no competing interests

Reviewer 2

Reviewer's report

Title: Longitudinal monitoring of Ehrlichia ruminantium infection in lambs and kids by pCS20 PCR and MAP1-B ELISA in The Gambia

Version: 2

Date: 7 May 2007

Reviewer: Suman M Mahan

Reviewer's report:

General

The paper reads a lot clearly and the data is supported by the authors’ conclusions. The Tables as presented also make the data review a lot easier and clear. They show that the two diagnostic assays do not have total agreement and that the PCR is more reliable in detecting E. ruminantium infection and that a one time test is probably not an accurate predictor of infection. They suggest that vertical transmission seems to occur in small ruminants and that is a justified interpretation, but needs further proof to define in utero versus colostral. However increased susceptibility to HW and vertical transmission in lambs and kids may not be an efficient mechanism of resistance as some animals died later on. Endemic stability and its dependence on vertical transmission would mean that these animals should not die of heartwater unless they are exposed to different non-cross protective strains of E. ruminantium.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
I have the following suggestions to improve the paper further.

1. **Suggested Change of Title to:** Longitudinal monitoring of Ehrlichia ruminantium infection in Gambian lambs and kids by pCS20 PCR and MAP1-B ELISA.

This has been addressed in the revised manuscript

2. *The paper now highlights that serology is not definitive proof of heartwater infections hence the sentence Line 16 page 2 should read. Antibodies detected by MAP-1B elisa also varied…. -- there is considerable evidence that even this assay can pick false positives. Due to this and other reasons serology is not a reliable predictor of prevalence of infection at least for heartwater. This is further enforced by the authors statement that whilst PCR showed up +ve animals MAP-1B elisa did not!*

This highlighted in the revised manuscript.

3. **Line 25 change the word “that” with ‘because’**

It has been changed

4. **Line 26: change ‘young animals’ with ‘young small ruminants’ as this will not encompass calves which is otherwise insinuated.**

It has been changed

5. **Introduction: Page 3: Epidemiology in young bovines is better defined than in small ruminants. Hence the statement Line 6 ‘The epidemiology of heartwater in young animals….should read more accurately if ‘young animals’ is replaced with ‘small ruminants’ especially because in calves there is some solid data regarding this subject.**

The replacement has been done in the revised manuscript

6. *The sentence Line 6-7 should be rephrased to read more correctly: In heartwater-endemic areas where extensive husbandry systems exist and tick control is absent or limited, the numbers of Amblyomma ticks is high and animals are subjected to almost continuous tick, and presumably E. ruminantium challenge (1). Given this statement is expected to be true, why then were tick numbers so low in Bansang where one would expect the tick control to be low due to economics of the costs involved?*

It is true that tick attack rate is lower in Bansang area located in the eastern part of The Gambia and the appropriate references (Faburay et al., 2005, 2007) to confirm this have been included in the manuscript (page 16, line 16-18). This low level of *A. variegatum* tick abundance depends on the natural distribution. Secondly, the study coincided with the period of reduced abundance of the vector and we may expect increased rate of infestation towards the end of the rainy season or at early dry season (November – January) the period of peak abundance of *A. variegatum* nymphs

6. Line 12 change ‘of’ to ‘to’ after tick transmission.

It has been changed in the revised manuscript

7. Line 18: Replace West Africa with The Gambia as it is a small part of West Africa.

The replacement has been done in the revised manuscript


The recommendation is considered in the revised manuscript

9. Line 4: There needs to be accuracy of quoted references: pCS20 sequence was first described in the paper by Waghela et al 1991-Ref 12. In this paper and the subsequent paper Mahan et al 1992 J Clin Microbiol. 30(4):981-6, its specificity was demonstrated. In the Mahan et al 1992 paper the first description of the pCS20 PCR assay using AB 128 and AB129 primers was made. Mahan et al 1992 is not quoted in the paper. The Ref 11 quoted compares the pCS20, Map1 and 16s PCR assays/probes for sensitivity of detection of E. ruminantium. This paper confirmed that the pCS20 was the superior target for detection of E. ruminantium DNA. All these reference quotations need to be corrected!

This was an oversight and the appropriate references as indicated have been inserted in the revised manuscript.


It is done in the revised manuscript

11. Page 5: Line 8: In context to specificity for heartwater and MAP-1B elisa, the statement here needs to be rephrased as serology and presence of E. ruminantium infection are not always synonymous, due to false positive detection. I would recommend the following re-phrased sentence: “A recent serological study showed presence of MAP-1B specific antibodies at all three sites. In the presence of A. variegatum ticks we presume that these antibodies are due to exposure to E. ruminantium.” The they have the tick infection data to support their sentence.

The rephrasing has been done in the revised manuscript

12. Page 7 Line 16: Add reference quote Mahan et al 1992 with Ref 13 for pCS20 primers AB 128 AB129. This paper was the first to describe the use of these primers.

The appropriate reference has been added
13. Under PCR methods DNA extraction: were the positive control DNA samples prepared the same way as the test samples i.e. using the modified Plowe extraction method? Ideally this should have been done the same way to ensure the sensitivity of the assay was not affected by the sample preparation method.

Yes, the positive DNA samples were prepared using the same DNA extraction method.


Based on sequence analysis this showed 280 bp; however in reference literature it showed 279 bp and has been changed accordingly in the revised manuscript.

15. Page 11 Line 3 to 9. This whole paragraph needs to be rephrased because the data compares the 3 sites for tick attack rates and none else. According to the data shown, Kaneba has the highest challenge and not low as the other sites are lower. So this section needs rephrasing.

The rephrasing has been done in the revised manuscript

16. Line 23. The sentence starting with Nineteen per cent…is out of place because from quoting Table 2a etc earlier, Table 5 is referred to next without reference to Table 3,4. Either the Tables are renumbered or the sentence is moved to a later time in the section.

This sentence has been shifted to the appropriate place in the results section.

17. Page 12; Line 10. Delete and ‘a carrier infection’ because they have not shown that these animals were able to transmit to ticks. End sentence at persistence of infection!

It has been deleted

18. Line 23. rephrase to 0-3 days of age carried antibodies to MAP1-B antigen of E. ruminantium.

The rephrasing has been done in the revised manuscript

19. Line 15 the kappa coefficient is different from the Table 4.

The correction has been done in the revised manuscript

20. Discussion. Page 16: Line 15. Insert: Presuming that HW infections were the cause of the sero-positive reactions, the prevalence of E.r infection....

The recommended insertion has been done

21. Page 17: Line 2-4. The MAP 1B negative result in older animals the authors state elsewhere due to maternal abs decline, but here they suggest a down-regulation mechanism, which is possible but probably not the reason this positive to negative observation. This can only
confirmed by tick infection pick ups. Maternal abs wane in older animals and that is probably why the animals became negative by MAP1B elisa. The BVDV persistence argument is acceptable but then they are contradicting their earlier statements. The authors mention that there is an inverse relation of susceptibility to heartwater and here they have a completely different explanation. They need to qualify their statements.

There is a clear distinction between the presence of maternal antibodies and down-regulation of antibodies. The former was in reference to animals (e.g. 1301, 1302, 13081319; Table 2a) that became consistently seronegative after being initially seropositive; whereas the latter referred to animals that showed intermittent seropositivity (4312, 4315, 4337, 4372) and thus being the reason for suggesting the occurrence of upregulatory-downregulatory effect on antibody production. Immunotolerance specifically referred to animals that showed PCR positive but tested seronegative which phenomenon has been explained in detail in the text. It is important to note that these phenomena have only been postulated based on the observations made in this study and that under field conditions we may expect the occurrence of all these scenarios. Importantly, we highlighted them in this paper to stimulate further investigation to elucidate these occurrences.

22. One of things the authors miss to point out is that the higher tick challenge at Kaneba is associated with higher HW death rate at this site and no HW deaths at Bansang with almost no ticks!!

This association has been highlighted (page 13; line 16) in the revised manuscript (please see the results section under mortality.

23. Table 1: Move the key from title and place it under Table with other footnotes

An oversight; the key has been moved

24. Table 2a, 2b, 2c. The authors correctly suggest that vertical transmission of E. ruminantium seems to also occur in lambs and kids as observed in calves. How do they explain that some PCR positive animals in the first 10 days of life still died of HW later on? Neonates are supposed to be resistant and be refractory to infection during early life and this aspect of their susceptibility is supposed to promote endemic stability. Also the animals that died of HW, were they infested with Amblyomma ticks during neonatal life. If this information is available, they should provide it in the paper. I suggest that they add another column to their 3 Tables 2a,b,c to show the tick attack pattern for the respective animals. The earliest HW death was on day 28, was this due to vertical transmission or tick transmission? Tick transmitted infections usually have an incubation period of 14-21 days (max 28 days) in the field, which means that ticks would have had to transmit to this animal immediately after birth! However, they only found ticks at week 16 and that was at Kaneba, not at Kerr Seringe where the death occurred.

We agree with the reviewer regarding such occurrence; however in the field situation we should anticipate different scenarios and therefore the possibility of vertically infected offspring, which due to multiple factors (cited in the text), became immunosuppressed and in time dying from the infectious agent (heartwater, HW) should not be discounted. This has been explained (page 18,
line 22 onwards; and the author`s personal experience on page 19, line 1-6). Furthermore, *A. variegatum* ticks were collected from few animals that died of heartwater at Keneba and Kerr Seringe and this now shown in a new column in Tables 2a, 2b and 2c. I fully agree with the reviewer regarding the incubation period of tick-transmitted infections; and the fact that no ticks were detected on neonates (0-3 days of age, which is reported in manuscript) led us to suggest that a mechanism order than tick transmission played a role. It was suggested this could be through vertical transmission. However, protection of vertically infected young may not be necessarily absolute and as explained above could in time result in death in immunosuppressed animals.

25. Table 4. The data should be separated by the 3 sites to differentiate the tick attack patterns of these sites. They are different enough from the Table 1 data to warrant this. It would further expand the scope of the data. Would the author care to comment if the tick attack data as being usual for these three sites?

This may be a good suggestion; however, Table 4 has been introduced based on the recommendation of one of the reviewers and is specifically for comparative statistical analysis of the agreement between the two assays, pCS20 PCR and MAP1-B ELISA (regardless of tick infestation status), at various age levels. It indeed appears to adequately show the comparative performance of the two assays by comparative analysis at various age levels using the *kappa* coefficients. We sincerely thank the reviewer for his insightful and constructive comments, which resulted in significantly improving the quality of the manuscript.

Given that the authors will make these changes this paper can be accepted for publication.

Discretionary Revisions (which the author can choose to ignore)

**What next?:** Accept after minor essential revisions

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

We would like to assure you that the statistical analysis has been adequately and extensively addressed for this study.
Reviewer 3

Reviewer's report
Title: Longitudinal monitoring of Ehrlichia ruminantium infection in lambs and kids by pCS20 PCR and MAP1-B ELISA in The Gambia
Version: 2
Date: 25 April 2007
Reviewer: Eduardo Berriatua

Reviewer's report:
General
Authors have answered adequately the main questions raised and made the necessary changes and I have nothing else to add.

Endemic stability is an epidemiological state of a given population characterized by high levels of infection but low levels of clinical disease. The two main prerequisites for endemic stability are:

Reviewer 4

Reviewer's report
Title: Longitudinal monitoring of Ehrlichia ruminantium infection in lambs and kids by pCS20 PCR and MAP1-B ELISA in The Gambia
Version: 2
Date: 2 May 2007
Reviewer: Zerai Woldehiwet

Reviewer's report:
General

Endemic stability is an epidemiological state of a given population characterized by high levels of infection but low levels of clinical disease. The two main prerequisites for endemic stability are:
1. The infectious agent is more likely to cause severe disease in older animals than younger animals; this is a well-known feature of several tick-borne diseases of ruminants including heartwater.
2. In animals which have recovered from primary infection, the probability of secondary infections causing severe disease is reduced.

The main aim of the present paper seems to be to establish whether lambs and kids contribute to endemic stability by acquiring infection of Ehrlichia ruminantium by vertical transmission or by tick transmission during the first days and weeks of life. The authors have made a good effort at addressing most of the main issues raised and the paper is now very much improved. However, I don't think the major objectives of establishing the role of vertical and tick-borne transmission have been met, essentially because the design of the study was not suitable. The study design did not allow for the definitive differentiation of animals that acquired infection in utero and those that developed post-natal infection (per os by consuming milk containing infected cells or through tick bite). The study contains interesting data on the epidemiology of heartwater in lambs and kids in the Gambia but the paper needs to be rewritten to reflect the findings. I suggest that the authors amend their objectives and conclusion regarding vertical transmission to reflect their data.

Abstract: Lines 3-5 (which indicate the main objectives) and lines 24 to 27, which indicate the conclusions

For the purpose of clarity we hereby restate the objective of the present study: To monitor the onset (age at first infection) and kinetics of E. ruminantium infection in extensively managed newborn lambs and kids at three sites in The Gambia where heartwater is known to occur using pCS20 PCR; and also compared the performance of the pCS20 PCR and indirect MAP1-B ELISA in detecting heartwater infection in small ruminants (please see Abstract and Introduction). The Abstract: Line 3-5 has been rephrased in the revised manuscript to make the objectives of the study more succinct. What we concluded was that our data derived from the monitoring suggest that vertical transmission may contribute to the establishment of endemic stability of heartwater in traditionally managed small ruminants. We acknowledge that to establish or confirm this would certainly require a specifically designed experimental setup including tick transmission experiments. This is a subject of another investigation and the findings of our study should stimulate further research.

Conclusion
Page19, lines 9 to 17. Although the cited papers do show evidence of vertical transmission, the data of the present study is not strong enough for the statement in lines 9 to 17 to be the main conclusion.

The conclusion has been rephrased/modified to reflect more objectively our data and objectives. We hope this will address your concerns. We thank the reviewer for his insightful and constructive comments.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
Discretionary Revisions (which the author can choose to ignore)

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

We would like to assure you that the statistical analysis has been adequately and extensively addressed for this study.

We hope that all queries of the referees have been adequately addressed and sincerely hope that the amended manuscript will now be acceptable for publication in BMC Infectious Diseases.

Thank you very much for your consideration,

Yours sincerely

Dr. Bonto Faburay