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

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Version: 2 Date: 12 April 2007

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

12 Apr. 07

Dear Editor,

I would 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 considered the comments made by the various referees 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 a detailed response to each of the constructive comments of the four referees.

1. Reviewer: Eduardo Berriatua

General

I understand the study aims at investigating the time from birth to 5-6 months of age, when small ruminants raised in the traditional rearing system of Gambia, become infected with Ehrlichia ruminantium, and elucidate whether infections at an early age, which are critical for E. ruminantium endemic stability, depend on vertical and/or tick-borne transmission. The infection state of animals is assessed by PCR and ELISA and results obtained with these techniques are compared.

The questions addressed in the study are important to improve our understanding of the epidemiology of Heartwater in small ruminants which is a major disease in sub-Saharan Africa. The article merits publication however, it needs substantial revision and include a better description of particular study aspects, reorganise some of the information, perform a more complete statistical analysis and improve interpretation of some results.

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

Introduction

The introduction to the study is adequate, but the following needs addressing:

• Between lines 89-91 authors acknowledge problems in the past for specific E. ruminantium serological diagnosis and subsequently imply that this has been overcome with MAP1-B ELISA having greater sensitivity (Se) than older tests. Has this ELISA also improved diagnostic specificity (Sp)?
The MAP1-B ELISA is the most sensitive serological diagnostic assay to detect *E. ruminantium* infection in small ruminant sera; although some reactions still exist with unidentified related ehrlichia species, the assay has the most improved specificity compared to previous serological assays (IFAT, MAP1 ELISA, Competitive ELISA) especially for caprine and ovine sera.

• Last sentence: I would not say that the objective of the study is to “investigating the role of tick transmission in initiating an endemically stable state in indigenous small ruminant exposed to field tick challenge”, because to this you should have monitored tick infection more frequently in a greater number of animals. I would say that your aims were to monitor *E. ruminantium* infection and provide some information to better understand the origin of infection.

The objective of the study has been redefined in the revised manuscript and basically is to monitor the onset (age at first infection) and kinetics of infection; and to compare the performance of the two assays. The study sheds some light on endemic stability it was not designed to proof the relationship between tick transmission and endemic stability.

**Methods**

• It is essential that you give a better description of the traditional extensive rearing system of small ruminants in The Gambia, particularly with regards to place of rearing during the study (from birth to 162 days old), whether indoor or outdoor as this conditions tick exposure, and other aspects such weaning age, feeding habits, type of grazing ground, characteristics of livestock enclosures...

A broader overview of the traditional rearing system is now provided under ‘animals and husbandry system’.

• Lines 110-111. You say study animals comprised 29 lambs…21 kids…13 lambs…10 kids and 4 lambs…This makes 77 animals not 76 as you say before (line 102).

The disparity in the number of animals has been corrected. It is 77 instead of 76.

• How long before had the dams at Kerr Seringe been treated with acaricides for? Could this have prevented or influenced tick and *E. ruminantium* infection in dams and its vertical transmission to newborn animals?. You need to address.

The dams in Kerr Seringe have been treated monthly with acaricide for about 10 months; this would have the effect of significantly reducing tick attachment rate resulting in lowering or disrupting challenge pressure in dams, and consequently vertical transmission of *E. ruminantium* from dam to offspring. This is discussed in the text.
• Were blood samples from newborns always taken after they had ingested colostrum? This is important to interpret PCR and serological results.

All blood samples have been collected after ingestion of colostrum.

• You must give more detail regarding age at first sampling. Saying it was done within 3 days of birth is not quite enough in order to interpret PCR findings because strictly, in 3 days ticks can infect, feed and drop off. Did you check for tick infection before taking blood samples? If you convince the reader that newborns were not exposed to ticks during the first 3 days of life and that your PCR is highly specific, then PCR-positive results strongly suggest vertical transmission of *E. ruminantium* as observed in cattle and this would be a major finding of your study.

Regarding the age at first sampling, a special table indicating the specific age of sampling of each of those animals (0-3 days of age) is now created (Table 3) in the revised manuscript. All animals at first sampling were examined for ticks; this was omitted in the manuscript but is now included.

It is noteworthy that all animals have been examined for ticks at birth or at the time of first sampling and the period required for transmission of *E. ruminantium* to occur after attachment of an infected tick to a susceptible host is estimated between 27 and 38 hours for nymphs and between 51 and 75 hours for adults; and in the study population some animals sampled right after birth were PCR positive. Diagnostic tests targeting the pCS20 sequence have long been considered to be specific for *E. ruminantium* (Allsopp et al., 1999). Against this background, and also considering the pathogenesis of *E. ruminantium* infection (initial infection of regional lymph nodes etc.), it is highly unlikely that *E. ruminantium* transmitted to neonates at birth through tick bite could appear in the blood stream on the very day or the following day after birth and be detected by PCR.

• Hence, next question is: What is the PCRs Se and Sp?. What is the ELISAs Se?. Move details on ELISAs Sp presently in the results section (page 9, lines 213-15) to materials and methods.

The nested approach significantly improved the sensitivity of the PCR and has shown to detect up to 1 organism in the sample (Faberay et al., 2007 accepted for publication). The assay is so far the most specific for *E. ruminantium* and the specificity of the primers, AB128 and AB129, which we used in the nested round has been determined by Peter et al., 1995. This is indicated in the revised manuscript.

MAP1-B ELISA has a specificity of 98.9 % and 99.4 % for caprine and ovine sera respectively (indicated in methods section of the revised manuscript). These details are now in the materials and methods section in the revised manuscript.

• You need a separate section for epidemiological and statistical analysis (lines 172-179), which is now included under “Indirect MAP-1 ELISA”
A special section on statistical analysis is created in the revised manuscript.

• Comparing ELISA and PCR results using Spearman’s rank correlation is insufficient. I suggest you complete this analysis for example by estimating the Kappa coefficient and degree of agreement between tests.

Kappa statistic has been estimated for all age levels to assess agreement between the two assays and is indicated in Table 4 of the revised manuscript.

Results

• The number of animals in tables 2a and 2b adds up to 62 animals (36 lambs and 26 kids), not 76 or 77 animals as you mention earlier. Are you excluding results from the dead ones? If so, why?

The count of animals includes fatal cases (Table 4) which will give a total of 77 animals. Data in those tables are represented in a different format (by site including mortalities) and the discrepancy (77/76) is corrected in the revised manuscript.

• You say the highest number of PCR-positives was at 99-126 days but in table 2a most animals this age were not tested – please explain.

This was an error, it should have been the ‘highest proportion’ and not ‘highest number of animals’; and the reason for the low number of animals tested is now explained in the revised manuscript. Briefly, batches of some buffycoat extracts on filter paper (for DNA) stored in the freezer were soaked with water due to a freezer failure rendering some samples unsuitable for further analysis. This is explained in the revised manuscript.

• You say almost 19% of animals were PCR-positive when tested shortly after birth and using tables 2a and 2b, the estimate would be almost 18%(11/62).

The proportion of 19% is correct because you did not count animals in the mortality table, which were alive in that time period.

• Did animals that died of Heartwater have clinical signs prior to death and lesions on post mortem examination compatible with the disease? Is the presence of E. ruminantium in tissue alone a definite diagnosis of the cause of the death?

All animals that died of heartwater manifested clinical signs prior to death and showed characteristic post mortem lesions compatible with the disease. The presence of E. ruminantium in brain smears is the gold standard for confirmatory diagnosis of heartwater as the cause of death.
**Discussion**

Discussion is generally comprehensive and it could be improved by adding interpretations to some of the questions that I raise above. Other things are:

- **Line 274.** Specify the test to which animals tested negative and mention here the possibility that some PCR-positives may have been false positives if the PCR test is not 100% specific.

The test is pCS20 PCR and has been specified in the revised manuscript and the concern about specificity is addressed in the revised manuscript.

- **Line 279.** I wouldn’t use “Infection rate” as a synonym for seroprevalence, it would be more appropriate to start with something on the lines of: “Prevalence of infection with E. ruminantium during the study estimated by MAP1-B ELISA, ranged 10-90%.

‘Infection rate’ has been changed to ‘prevalence of infection’ in the revised manuscript.

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

**Results**

- **Apart from previous comments, I find Tables 2a, 2b and 3 very informative, and could be further improved by indicating animal’s location. Moreover, you say in lines 218-19 that these tables “compare” ELISA and PCR tests and they do not, they merely “describe” results. If you wish them to “compare” you could for example add an extra column with kappa coefficients or other alternative comparison statistic.**

The tables have represented in the revised manuscript based on sites/location. A separate table (Table 4) has been created with Kappa values to compare the performance of the two assays.

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**Discretionary Revisions (which the author can choose to ignore)**
Results

• It would be interesting to further elaborate on the time dynamics of PCR-positive animals. For example, it is evident that after a PCR-positive results animals were often PCR-negative on subsequent samplings. What was the probability for having a repeated PCR-positive result after being PCR-positive a first time? This is useful information for understanding the pathogenicity of infection the validity of the PCR test.

Regarding the time-dynamics of PCR-positive animals, it is nearly impossible to make any reliable probability estimate of repeated PCR-positive after being PCR-positive for the first time. This will vary significantly between animals and would depend on the variability of individual immune responses (which could depend on the nutritional status, concurrent infections etc.) to control parasitaemia. It is believed that fluctuating rickettsaemia occurs in infected animals as highlighted in the manuscript and no specific pattern has been observed.

Discussion

• Lines 281-85. It is sensible to assume that observed seroprevalence time pattern is the results of maternally derived antibodies. My comment is that it would have been wise to test the dams by ELISA and PCR in the perinatal period as this would have allowed you to correlate dam and offspring results and probably support some of your conclusions. Maybe you would like to comment on this.

Certainly, testing the dams would have made a better correlation and help confirm the presence of maternal antibodies in the neonates. However, the high prevalence *E. ruminantium* antibodies in neonates with no or little exposure to vector transmission coupled with the decay of the antibodies or seroreversion over time (about three months later) appears to be a strong indication of antibodies of maternal origin.

• Line 292: I would appreciate a brief explanation for “a down-regulatory effect on the production of antibodies”.

The immunological basis is not known. However, the phenomenon of down-regulation of antibodies to *E. ruminantium* was first reported in cattle (Semu et al., 2001). It has been observed that antibody responses to *E. ruminantium* in general, and to MAP1-B in particular, in cattle exposed to repeated infective natural tick challenge are down regulated following first exposure to the organisms. Seropositive rates, due to down-regulation in the production of specific antibodies in these animals, despite their continuous and repeated exposure to infective tick bite, were significantly lower than anticipated.

• Lines 295-298. Interesting finding indeed. What about newborns being immunotolerant to the infecting *E. ruminantium* strain and not developing an antibody response? This occurs in ruminants following pestiviral infection (Bovine Viral Diarrhoea and Border Disease) in utero during early pregnancy. May be worth mentioning this.
Immunotolerance is an interesting phenomenon and could be a likely occurrence in traditionally managed small ruminants exposed to field challenge. It could be one of the mechanisms by which vertically infected neonates survive/tolerate the infection without detectable antibodies thereby contributing to the establishment of endemic stability. The possible occurrence and role of this phenomenon is mentioned in the revised manuscript. Many thanks for the constructive comments.

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

Level of interest: An article of importance in its field
Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.
2. Reviewer: Zerai Woldehiwet

General
The aim of the study was to establish the kinetics of infection with E. ruminantium in newborn lambs and kids, which are thought to have a higher degree of resistance than adult sheep and goats, by testing for the presence of specific nucleic acids by PCR and for antibodies by ELISA under field conditions in the Gambia. Blood samples, which were collected on the day of birth or within 3 days after birth and every 1 or 2 week thereafter, were used for PCR or ELISA; the animals were monitored for tick infestation every week.

The main findings of the study were:
 a) tick infestation was first detected 16 weeks after birth  
 b) E. ruminantium was detected by pCS20 PCR in 57 of 76 animals at least once  
 c) Some animals (14 of 73) were PCR-positive as early as 3 day after birth  
 d) The number of PCR-positive animals increased with age  
 e) Specific antibodies were detected by ELISA in most animals (90.2%) one week after birth  
 f) The number of sero-positive animals declined with age  

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

The main conclusions were:
 a) As ticks were not detected until 16 weeks after birth, the detection of E. ruminantium in lambs/kids as young as 3 days old suggests transmission other than tick feeding, including vertical transmission.

Suggestions for improvement

• Unfortunately the authors did not keep any of the lambs/kids under tick free conditions. This would have helped to clarify the possible role of non-tick transmission.

Indeed none of the lambs or kids were kept under tick-free condition as the one of main objectives of the investigation was to determine the onset (age at first infection) of E. ruminantium in field-tick exposed newborn small ruminants.

• Even if the lambs/kids were exposed to ticks, 3 days is too short, as the incubation period following tick transmission could be one week or longer.

That is correct, 3 days is short for the manifestation of clinical disease for heartwater

b) The authors rightly concluded that the presence of specific antibodies in >90% of the animals during the first week of life was probably due to maternal antibodies.
Suggestions for improvement

• Unfortunately no information was given regarding the possible association between the detection of nucleic acid in particular period (say week 1) and seropositivity in subsequent periods (say week 2 and 3).

This is addressed in the revised manuscript, notably the *E. ruminantium* antibody dynamics of neonates, which were positive by PCR at day-0 to day-3 after birth over the course of the study period.

• Also it would have been of interest to establish whether the 57 animals which were PCR-positive were also found to be subsequently ELISA-positive. This would have helped to establish whether or not the PCR test was always detecting true positives and to rule out the detection of nucleic acids of other related organisms.

A correlation analysis of the degree of agreement between PCR positivity and MAP1-B ELISA was given. Additionally, *Kappa* coefficient was calculated for each age level to provide a more comprehensive assessment of the agreement between the two assays which also provides indication regarding the possible of the *E. ruminantium* antibodies in most neonates. Moreover, positive serology results determined by MAP1-B ELISA could not be reliably used to validate the specificity of the pCS20 because of concerns of cross-reactions with antibodies to other unidentified ehrlichial species; the reverse though may be true.

c) The resulting atypical antibody response is probably a reflection of vertical transmission.

Regarding vertical transmission, it is important to mention that the experiment was not designed to proof the occurrence of vertical transmission, which will certainly require special transmission experiments. However, our findings strongly suggest that the phenomenon occurs in small ruminants under the traditional husbandry system, which should stimulate further investigation. In this study, all animals have been examined for ticks at birth or at first sampling; secondly, the period required for transmission of *E. ruminantium* to occur after attachment of an infected tick to a susceptible host is estimated between 27 and 38 hours for nymphs and between 51 and 75 hours for adults; and in our study population some animals sampled right after birth were PCR positive (Table 3 in revised manuscript). Diagnostic tests targeting the pCS20 sequence have long been considered to be specific for *E. ruminantium* (Allsopp et al., 1999). Against this background, and also considering the pathogenesis of the infection (initial infection of regional lymph nodes etc.), it is highly unlikely that *E. ruminantium* transmitted to neonates at birth through tick bite could appear in the blood stream on the very day or the following day after birth and be detected by PCR.
Suggestions for improvement

• The issue of vertical transmission is a complex one. Depending on when the transmission occurred, the embryo lamb/kid may be born fully immune, without the presence of the agent, or immunotolerant and persistently infected, without detectable antibodies but with the agent being persistently present in the blood or other tissues. Immunotolerance usually occurs in foetuses infected before the first half of gestation (e.g. in humans infected with rubella virus and in ruminants infected with pestiviruses).

The potential occurrence and role of immunotolerance engendered through vertical transmission discussed in the revised manuscript.

• Unfortunately the authors did not present data to support or to reject the null hypothesis that there was no vertical transmission.

Please refer to comments under c

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

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Discretionary Revisions (which the author can choose to ignore)
Unable to decide on acceptance or rejection until the authors have responded What next?: 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.
Declaration of competing interests:
I declare that I have no competing interests
3. **Reviewer:** Theo de Waal

**General**

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

*Line 64 – more appropriate reference to the reduced susceptibility of animals to *E. ruminantium* – the cited references refers mostly to *Theileria.**

An appropriate reference (Uilenberg, 1990) has been added in the revised manuscript

*Line 73, 87, 172 – format of citation*

The citation have been properly formatted in the revised manuscript

*Line 102 & 110-111 – there seems to be a discrepancy between the number of animals tested; 76 or 77?*

The discrepancy of 76 or 77 animals has been corrected in the revised manuscript. The correct number is 77.

*Line 121 – it is not clearly stated if animals were examined at birth (or within 3 days) for ticks as well*

This clearly an oversight but it is now stated in the revised manuscript under Methods; all newborn animals were examined for ticks at birth or at first sampling.

*Line 123 – reference to preparation and staining of brain smears*

The reference (Purchase, 1945) is cited in the revised manuscript

*Line 140 – indicate 3’ and 5’ end of primer*

The 5´ and 3´ ends of the primers are now indicated

*Line 153 – what about inclusion of negative controls in the PCR assay?*

All PCR reactions included negative controls composed of DNA/RNA free water to rule possible contamination of the samples.
Line 171 – determination of cut off not clear.

The cut-off point is made clearer: For each plate, the cut-off value was calculated as two times the percentage positivity of the negative control serum relative to the positive control serum (Van Vliet et al., 1995; Semu et al., 2001).

Line 203 – The 1 tick collected at Kerr Seringe was a nymph (Table 1)

The word nymph has been omitted and is now inserted in the revised manuscript.

Line 247 – ELISA test do not detect infection, only exposure to the organism

It is agreed, the ELISA test does not determine the actual infection status; it only shows exposure to infection. We are mindful of this.

Line 250-253 – It is not possible to make this conclusion as animals were not examined for ticks at birth and not all animals were examined. It has been shown that E. ruminantium can be transmitted by ticks 27-38 hours after attachment (Allsopp et al., 2004. Heartwater. In: Infectious Diseases of Livestock 2nd Edition. Ed Coetzer & Tustin; 504-535. See also statement in line 262-264 indicating high tick abundance at the time of animals being born. Not convinced that these results support the occurrence of vertical transmission.

It was an omission that sampling of ticks at birth was not mentioned; this is now stated in the revised manuscript that all animals were examined for ticks at birth or at the time of first sampling. In an attempt to convince you of the possible occurrence of vertical transmission; first the period required for transmission of E. ruminantium to occur after attachment of an infected tick to a susceptible host is estimated between 27 and 38 hours for nymphs and between 51 and 75 hours for adults (as you rightly mentioned above) and in our study population some animals sampled right after birth were PCR positive (Table 3 in revised manuscript). Secondly, diagnostic tests targeting the pCS20 sequence have long been considered to be specific for E. ruminantium (Allsopp et al., 1999). Against this background, and also considering the pathogenesis of the infection (initial infection of regional lymph nodes before invading the blood circulatory system etc.), it is highly unlikely that E. ruminantium transmitted to neonates at birth through tick bite could appear in the blood stream on the very day or the following day after birth and be detected by PCR. These results indicate that tick transmission could not be the route and strongly suggest that vertical transmission possibly occurs in small ruminants under the traditional husbandry system; however to proof this will require the conduct of special transmission experiments. The study was not designed to proof the occurrence of vertical transmission, we short of stumbled on this interesting finding and should stimulate further investigation.

Furthermore, line 262-264 in the manuscript under review, referred to dams, which had not been treated with acaricides and therefore were exposed to comparatively high tick challenge (reference, Faburay et al., 2007, provided in the revised manuscript) and possibly infectious challenge as well. These dams would remain carriers of infections possibly with sufficient parasitaemia to facilitate vertical transmission of the infection to their offspring.
Only 2 of the animals that died of heartwater were positive at birth but only died at age of 22-49 days – therefore does not prove that cause of death was due to parasites transmitted vertically.

The possibility of the two animals possibly dying from vertically transmitted infection has been removed from the revised manuscript, as it could not be proved that was the case. However, it is argued in the text in the revised manuscript that such possibility should not be entirely discounted in animals that later suffer from immunosuppression due to several factors including haemonchosis, trypanosomosis, starvation etc, all of which are common among small ruminants under the traditional husbandry system.

why would vertical transmission be protective?

Vertical transmission of *E. ruminantium* from dam to offspring at a time of increased tolerance/resistance to infection induces protective immunity in the latter to subsequent infection with antigenically homologous strains of the parasite. Under sustained field tick challenge, these animals maintain their carrier status of infection, a prerequisite for the establishment of the condition of endemic stability among traditionally managed indigenous ruminants.

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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

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

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

'I declare that I have no competing interests'
4. Reviewer: Suman M Mahan

General

The data does NOT define the specific role of tick transmission in initiating an endemically stable state in indigenous small ruminants exposed to field challenge in The Gambia. The supporting data for this statement is that the earliest tick infestation detected was at Keneba during week 16 of observation. However the first mortality (not clear at which site these were recorded), was detected due to heartwater on days 28, 42, 56, 63, 84, 112, (lambs), day 63, 91 (kids) before any ticks were found on these animals. The first tick infestation was during week 16 of life and by inference heartwater mortalities should have only been observed 2-4 weeks after the first record of infestation. Since all the mortalities were before the ticks were noticed on these animals it puts the issue of tick transmission and endemic stability into a cloud of uncertainty. Secondly, and most important parameter in defining endemic stability, should have been to show that the surviving animals were resistant to live virulent challenge. The data presented does not indicate any such challenge with live E. ruminantium, to demonstrate that they had been previously exposed and were resistant to re-infection. Hence, the objective was not achieved. However, the authors did observe some interesting findings which they described by longitudinally monitoring the infection status of the subject animals. I suggest that this become the objective of the study and the paper!!!

We agree with the referee and the objective of the research is redefined and limited to monitoring E. ruminantium infection in newborn lambs and kids, and to compare the performance of the two assays. However, possible role of tick transmission in initiating endemic stability could be postulated based on the findings of the study. All newborn animals were examined for ticks at birth/time of first sampling (this has been omitted in the manuscript under review but now included in the revised one); and the earliest period of detectable tick infestation was at week 16 of age and mortalities due to heartwater occurred before infestation by the vector. Additionally, pCS20 sequences were detected in neonates on or immediately after birth (day 0) to day 3 (Table 3 of the revised manuscript); the period required for transmission of E. ruminantium to occur after attachment of an infected tick to a susceptible host is estimated between 27 and 38 hours for nymphs and between 51 and 75 hours for adults, and it is highly unlikely that E. ruminantium transmitted to neonates at birth through tick bite could appear in the blood stream on the very of or the following day after birth and detected by PCR. And the fact that mortalities occurred before any detectable tick infestation, we agree with the referee that the role of tick transmission is uncertain and supports our suggestion/conclusion that animals got infected vertically which in time resulted in mortality due to possible immunosuppressive factors such as haemonchosis, trypanosomosis, starvation etc., all of which are common under the traditional husbandry system. It is postulated that vertical transmission of E. ruminantium may play an important role in initiating endemic stability by ensuring wider dam to offspring transmission. However, in agreement with the referee, to confirm that endemic stability occurs within the population would require a virulent live challenge of the surviving animals, which could be a subject of another investigation and is outside the objective of the present study.
This paper presents data regarding *E. ruminantium* infection dynamics of neonates-lambs and kids in an endemic heartwater region of The Gambia. The data addresses some key areas that are undefined in the epidemiology of heartwater in these animal species and for this region. Understanding the epidemiology of infection in neonates would facilitate better control strategies for heartwater. The data presented suggests that lambs and kids are probably exposed to *E. ruminantium* infection in early life either through in-utero transmission (vertical) or via colostrum or ticks and also suggests an inverse relationship of age with susceptibility to heartwater. They select 3 sites in the Gambia but do not clarify their proximity to each other and their heartwater endemic history. That would be of value if that data is available. In addition, one location seems to have a high tick challenge whereas the other two were of lower challenge. The authors’ conclusion that serology and pCS20 PCR assay combined defines the heartwater status of a population is only correct if serology is specific for heartwater. There is compelling evidence that the MAP-1B ELISA detects antibodies in animals where known vectors of heartwater do not exist. Hence, this conclusion needs to be modified to put more emphasis on detection of the organism supported by biological data of infection through transmission or clinical evidence. It is unfortunate that tick infection acquisition followed by transmission experiments were not conducted on the neonates which would have provided the confirmatory evidence to conclude and confirm the KOCH’s postulates.

The proximity of the selected sites to each other is now included and a brief history of their heartwater endemic status provided. Regarding the specificity of the MAP1-B ELISA; despite reported detection of false positives, it is highly likely that positive MAP1-B results from heartwater endemic areas, particularly if the animals are also pCS20 positive, could be a strong indication of exposure to *E. ruminantium*. Thus the use of both assays in conjunction may improve the results of heartwater epidemiology; although this could be further enhanced when supported by biological data of infection through transmission or clinical evidence; this aspect is now emphasized.

1. There are some areas of the data that are extrapolated to conclude the infection status of these lambs and kids which are not reliable. In particular, the use of serology and the indirect MAP 1B ELISA data to conclude that exposure to heartwater- *E. ruminantium* infection is present based on sero-positive results. Despite the fact that the assay has been shown to have high sensitivity for detection of anti-*E.ruminantium* antibody responses in sheep, it is the specificity of the assay that is of concern. Although it is documented that this assay is the most specific assay for detection of antibodies to *E. ruminantium* infection, there are numerous papers which demonstrate that false positive reactions are still detected and these could be due to an unknown agent that cross-reacts with *E. ruminantium*. The level of false +ve is variable from region to region and there is no data that defines this. At best one can only state that the sero-positive reactions in the presence of the tick vector *Amblyomma variegatum* or another vector species of this genus may be heartwater related. Since the animals were kept in an extensive farming system, their exposure to other serologically cross-reacting agents can not be discounted. Hence the conclusions based on the serology should be TONED DOWN. In particular in the
Introduction and on page 5 of M+M under Study sites lines106-107. Emphasis on PCR+ve and clinical signs, postmortem brain smears should be made to state with certainty that the infection is due to heartwater/E. ruminantium infection.

Concern over extrapolating the results of the MAP1-B ELISA with respect to its specificity has been addressed. This is against the background of detection of false positives by the assay in heartwater-free areas (Kakono et al., 2003; Mahan et al., 1998). This is highlighted in the description of the characteristic/performance of the assay.

Another important aspect to remember is that it is widely believed that antibody is not protective against heartwater and proposing the sero-positive state (soon after birth) as the resistant state and the sero-reversion state (older age), resulting in increased susceptibility should also be toned down. The elements in colostrum that are protective have not been defined in any target species. Nevertheless, there is interesting data from the serology, especially the sero-reversion after the initial positive state which at best represents a typical physiological phenomenon of transfer and decaying of passive antibody.

The non-protective role of antibodies against heartwater is remembered and therefore our proposition/conclusion with respect to increased susceptibility of field-exposed newborn animals has been made based on frequency of mortality due to the disease as indicated by survival analysis (Figure 1).

The pCS20 probe is in fact of low sensitivity for detection of carrier animals, but the pCS20 PCR assay has a higher sensitivity and can detect carrier animals. This needs to be corrected in line 81+82.

This was an error and has been rectified in the revised manuscript (Introduction part).

Line 89-91: The MAP-1B ELISA’s increased sensitivity to detect antibodies that react with E. ruminantium MAP1 in sheep or goats is not an inherent property of the assay but due to the fact that the kinetics of development of antibody responses in sheep and goats to E. ruminantium is different from that in cattle where sero-reversion occurs. Sheep and goats respond to heartwater infections by developing a prolonged antibody response, which is unique. This should be clarified in the text.

The sensitivity of MAP1-B ELISA to detect E. ruminantium antibodies in small ruminant sera in relation to persistence of antibodies in these species is described in the INTRODUCTION section of the revised manuscript.
3. The Abstract and the text refer to 76 new born animals being monitored from birth to day 162. However, the data set shown in the Tables 2a and 2b add up to 62. (36 lambs and 26 kids). In Section M+M under Animals lines 110+111 the animals total 77. This issue is very distracting as the percentages and proportions are being described I believe on the total being 76 whereas the data set only shows 62. The authors should clarify. If this is an error it is inexcusable.

In addition, the data presented as lambs and kids separately is also confusing due to the fact that it obscures the comparison of the selected three sites especially since they seem to have a difference in tick attack rates. Hence, I strongly advise that the data be presented for each of the three sites separately and that in fact it could make a very nice comparison of the kids and lambs that exist at the sites Keneba and Bansang. This would also allow for the mortality data comparison per site and animal species in the same Table. A better correlation of PCR +ve animals, survival or mortality and tick infestation data may shed light on whether the in-utero infection has an effect on the fate of the infection after the neonatal resistance expires. Did the authors detect E. ruminantium by brain smear on brain samples of the animals that died during the study? They indicate that they did PCR on the brain. The gold standard of heartwater diagnosis is a positive brain smear. I invite a comment from the authors regarding this comment. PCR detects the organism DNA but these could be dead or alive...the brain smear would demonstrate the infection in the brain capillaries.

The correct number is 77. The 62 animals counted did not include mortalities in Table 4 and if they are considered the numbers certainly add up. Careful reanalysis of data some discrepancies which are corrected in the revised manuscript; this happened due to direct data transfer from one programme to the other! The initial data set was entered in Excel and later copied and pasted in SAS (statistical programme) to deduce percentages/proportions. However, blank data points in Excel are read as 0 (zero) in SAS statistical programme and are counted in the count of results and will give you wrong proportions. The SAS license expired and the transferred data in Stata (used for correlation analysis) was used to deduce the correct proportions (Table 5).

The data is now presented by site including mortalities which certainly makes a nice comparison and interpretation of the data. The gold standard of heartwater diagnosis is a positive brain smear. This is the method that we used generally to confirm mortality due to heartwater; the exception was for 3 cases (because of deteriorated condition of the brain samples resulting from poor storage) and we had to use the pCS20 PCR on brain tissue DNA extract as an aid in confirming the cause of death. In addition to the positive PCR results, these animals manifested clinical signs prior to death and pathological changes highly suggestive of heartwater.

4. M+M Line 117-121. The authors state that they collected blood +/- EDTA on the day the animals were born or within 3 days of birth. Since they attempt to make a distinction between in-utero infections or via colostrum, they should show the data in that manner to demonstrate how many animals actually were PCR+ve at birth and how many were +ve within 3 days which would be either an in-utero route of infection or via colostrum. Interestingly the authors show no serology data for 9 kids for level 1, they should explain why this was the case?? Also they should separate the serology data for those at birth and within 3 days of birth, they show level 1 which is
day 0-10 after birth. The suggested data presentation should be improved to make more definitive statements.

We attempted to make a distinction between possible in utero infection and tick transmission and data has been represented in that format (Table 3 of the revised manuscript). The reasons for absence of data (serology and PCR) for a number of animals are now explained in the text under Results section (pCS20 PCR and Indirect MAP1-B ELISA): Most of the animals were not tested because some batches of buffycoat extracts on filter paper (for DNA) stored in the freezer were soaked with water due to a freezer failure rendering some samples unsuitable for further analysis. Absence of serological data for a number of animals was due principally to erasure of ink labels on the serum cryotubes making them unidentifiable; those samples were consequently excluded from further analysis.

5. Did the authors validate their nested PCR assay in any way?, if so they should indicate this or refer to other publications if published.

This test has been evaluated/validated and has been accepted for publication and currently in press. The appropriate reference (Faburay et al., 2007) is cited in the relevant section of the manuscript.

6. Line 203 insert the word nymph after 1.

The word nymph has been inserted in the revised manuscript.

7. According to my calculations, there were 15 animals that remained negative by PCR not 19!!!

The 19 PCR negative animals included mortality cases, which you probably omitted thus being the reason for the 15; however this has been made clearer in the revised manuscript.

8. Line 210-211 the highest # of +ve animals were in level 5 days 78-98 not days 99-126. In fact during days 99-126 at least 50% or more of the lambs were not tested (nd I presume that it means “not done”)! There isno explanation for nd and why. This should be provided. Table 3: line 212: 6 of 9 were PCR +ve in Kaneba, these are not evident in the Table 3 and it should also be indicated in the text that Kaneba is where the tick attack rate was highest/severest and that has an implication that in higher challenge there will be more transmission to neonates, from mother to neonate. What happened to the 21 kids at Kaneba, what was there fate??

I am sorry about this. It was supposed to be the ‘highest proportion’ and not the ‘highest number’ as previously indicated in the manuscript. The reason for non-availability of data for the lambs is explained above and is in the revised manuscript. All mortalities are now evident in the revised manuscript (Table 2a, 2b, 2c) including the fate of all study animals shown under the status column.
9. Line 213: the sentence is ambiguous and refers to the MAP 1B ELISA “perform satisfactorily”
What does this actually mean, each endemic or non-endemic site is different???

The statement has been reworded to exclude any possible ambiguity; this is made clear in the
introduction section of the revised manuscript showing the characteristic of MAP1-B ELISA with
respect to sensitivity and specificity..

10. Line 223: The authors should indicate the reason for animal 2317 & 2318 “nd” for levels 1-3
serology
how do they know they were +ve during these times in context of what they present here?

The reason for ‘nd’ has been explained above; the result being presented was the correlation
between the PCR and ELISA at a particular time point (level 6 and 7) and indeed animals 2317
and 2318 for level 6-7 both tested seronegative while they were PCR negative; although both
animals were seropositive for level 4 (Table 2c). This has been reworded to make the
interpretation clearer.

CONCLUSION AND RECOMMENDATION: This paper has some very interesting data on
heartwater epidemiology in lambs and kids from birth to day 162. However, what their objectives
were, were not met. See first paragraph for details. The paper’s data is not well organized; the
numbers of animals do not tally. Hence the conclusions are questionable. The data presented has
the potential of being a good account of infection dynamics in neonate sheep and goats. This
paper needs major revision in relation to the above mentioned criticisms before it can be accepted
for publication. The authors should respond by making the suggested changes and re-submit after
the major revisions, for re-evaluation changing their objectives and pointing out their findings to
these objectives.

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

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

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Discretionary Revisions (which the author can choose to ignore)

I 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

Bonto Faburay