Author’s response to reviews

Title: Antibodies against Coxiella burnetii and pregnancy outcome during the 2007-2008 Q fever outbreaks in the Netherlands

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Author’s response to reviews: see over
Dear Ms Nina Titmus,

We thank the editorial team and the two reviewers for assessing our manuscript MS: 3537752084257166, “Q fever infection and pregnancy outcome during the 2007-2008 outbreaks in the Netherlands” of W van der Hoek, JCE Meekelenkamp, ACAP Leenders, N Wijers, DW Notermans and CWPM Hukkelhoven. We have been able to address the comments of the reviewers and have adapted the manuscript accordingly. The manuscript has also been edited by a professional copyediting service (International Science Editing). In this letter we present our point-by-point reply to the comments of the reviewers and a description of changes made.

Reviewer 2 Conall McCaughey

General Comments:
The reported effects of Q fever infection in pregnancy are undoubtedly over-estimated due to various types of selection bias in reported studies. 

We fully agree and this was the rationale to conduct the present study.

This is an important paper, well written and will be of considerable interest. The conclusions are sound. Serological definitions and cut-offs used seem reasonable to me. Inevitably there will always be criticism from individual referees and correspondents regarding these matters in a paper such as this. However there are no internationally agreed definitions and the approach in this paper should be considered to be a pragmatic and mainstream approach.
We are very happy with this assessment. There is indeed a lack of agreed serological definitions and cut-offs in the international literature and we feel that we have taken a pragmatic and reasonable approach.

Discretionary Revisions:
1) To me, a key weakness of this study is that the antenatal booking blood based study design approach is confined to detection of seroconversion in early pregnancy. The authors do acknowledge and discuss this but feel that seroconversion after 12 weeks is unimportant in terms of adverse outcome. I feel that the evidence that later infection in pregnancy is unimportant is sparse. We simply do not know enough about Q fever infection in pregnancy to dismiss this weakness in the study as being unimportant. I feel that the authors should change the discussion to acknowledge that this is an important (but inevitable) weakness. Clearly to have a study design that looks at samples at the end of pregnancy (non routine samples collected specifically for a study) would have been very difficult with considerable logistical problems, very increased cost, ethical implications and many subjects might decline to be involved. However this should be stated and discussed.
We have made changes in the discussion section of the paper (as visible in track changes) to address this concern of the reviewer.
2) On a related theme, I feel it would be useful for the authors to discuss/speculate on the utility and practicality of looking at stored blood spot samples from neonatal testing (Heel prick PKU screening) by IgG. Such samples when eluted can readily be tested by standard serological assays. This would be a useful way of numerating infection in later pregnancy.

Using stored blood samples from the neonatal screening programme (“heel prick”) has been discussed as one of the options during the design of our study. However, the material is not readily available for research purposes and it was not considered feasible to organise the logistics (including the medical ethical review) within a reasonable period of time. Furthermore, as we have also mentioned in the discussion section [ref 23, 24], there is uncertainty about the serological response in newborns.

3) It would be interesting to have the serological data presented a little more thoroughly- what was the range of phase I titres in the past infections? -this is not currently included in the results presented. Where the recent infections tested for phase I antibody & if so what were the results/titres? There is interest in phase I antibody pregnancy and this dataset would be of interest to other workers.

We screened the sera at a dilution of 1:64 and did not do further titrations of positive samples. Among the sera analysed, 30 were positive (≥64) for IgG phase I antibodies. We have now expanded on the serological data by giving results for phase I antibodies. We have now also given the results of reading the slides at the 1:64 dilution, i.e. whether the fluorescence intensity was comparable to the positive control or very intense.

We do have a very large database of serological profiles of Q fever patients that are routinely followed-up in the Jeroen Bosch Hospital after acute Q fever. In the about 100 patients that have a serological profile suspect for chronic Q fever (i.e. IgG I ≥800) there are only two pregnant women. This data is still being analysed and will be reported separately.

4) It might have been useful to apply PCR to the isolated IgM phase II positives to clarify specificity status of these results. The authors might want to mention in the discussion if they feel this would have been useful (presumably this was not done).

PCR was not applied to the samples. In the prospective screening and treatment study that is ongoing, the assessment of the accuracy of the diagnostic tests used for screening (serology vs PCR) is one of the objectives of the study. In that study all serological positive samples are fully titrated and PCR is done on the placentas. We also refer to our response to comment A4 of reviewer 1 regarding the ongoing validation of isolated IgM II positive samples.

5) A couple of sentences explaining the nature (study design) of the large-scale prospective screening and treatment study which started in March 2010 would be useful.

We have added more information on the prospective screening and treatment study at the end of the paper, including the clinical trial number. Results of the study will only be available in 2011 but an article about the study design has been submitted to BMC Womens Health and some details can be found in the publicly accessible clinical trials register.

Reviewer 1: Anna Psaroulaki
This is a well written article with importance in its field. The authors clearly state the limitations of the study acknowledging in part previous studies which are building on. The authors grasp the opportunity to assess the possible relationship between Q fever exposure and pregnancy outcome using a significantly large number of samples during the early stages of the Q fever outbreak in the Netherlands. However, there are some major compulsory revisions that need to be addressed in order to decide on acceptance or rejection of this work.

A) Major compulsory revisions
1) Title: “Q fever infection…”. Solely antibody titre values against C. burnetii of #1/64 are not sufficient to diagnose a Q fever infection. In addition to clinical data, laboratory diagnosis of Q fever is based on seroconversion or a fourfold increase in titer which will indicate acute infection. The combination of elevated levels of IgG (>1/200) and IgM (>1/25) to phase II antigens could also indicate a recent infection. On the other hand, high titers of IgG (1/800) and/or IgA (>1/50) to phase I antigen are found in chronic infections. I strongly suggest therefore that the title should be changed to C. burnetii exposure…… or C. burnetii seroprevalence……..
We have changed the title to: “Antibodies against Coxiella burnetii and pregnancy outcome during the 2007-2008 Q fever outbreaks in the Netherlands”, in line with the second suggestion of the reviewer.

2) Methods, second paragraph: Again, recent Q fever infection cannot be defined only by the presence of anti-phase II IgG +IgM antibodies particularly during an outbreak such as the one in Netherlands where there is increased probability of exposure to the bacterium. Since a second sample serum to observe a possible seroconversion was not available, elevated levels of IgG (>1/200 instead 1/64) combined with IgM (>1/64) to phase II antigens could be a more realistic approach to indicate a recent infection.
Our reply under 4.

3) Results, Seroprevalence: The laboratory confirmation of a recent or a past infection which the authors present is weak. “These were combined with 16 sera with an IgM II #1:64 in the group of ‘possible recent infection”. Solely low IgM titres cannot indicate recent infection by C. burnetii. The specific antibody titre values could possibly come as a result to cross reactivity taking therefore into consideration falsely positive samples. The sera therefore that fall into the group of possible recent infection should not be combined with sera with both IgM and IgG antibody titre values falsely increasing the number of seropositive samples.
Our reply under 4.

4) Discussion, fifth paragraph: “A positive IgM against phase II has been considered useful for the diagnosis of acute Q fever for a long time [Hunt et al, 1983]. This sentence is misleading. Indeed, Hunt and his co-workers stated that it is possible in virtually all acute cases, to diagnose Q fever by testing a single convalescent serum. However this should be collected between 2 and 8 weeks after the onset of symptoms. Furthermore, they added that in practice, the best approach, during 1983 I may add, is to test for Q fever complement-fixing antibodies and if that test was positive to then test for phase 2-specific IgM. If that was also positive, “a current (or recent) infection” could be reported. To my experience there is no laboratory today that relies solely in a positive IgM titre, particularly using a cut-off of # 1/64, to diagnose a Q fever positive sample. Even though the authors admit at the contradictory sentence following the
one cited above that “an isolated positive IgM against phase II could be the result of an aspecific reaction”, still they combine the samples with solely IgM II # 1/64 with those with IgM II and IgG II #1/64 in the group of possible recent infection increasing their number of positive samples. In the article by Tissot Dupont the authors observed that the antibody titre cut-off values suggested by the manufacturer resulted in very low specificity of the test producing large number of falsely positive results. In order to effectively increase the specificity of the test the cut-off values had to be increased based on the local baseline to IgM II # 1/256 and IgG II # 1/1024. It has to be further noted that Denmark is considered a low prevalence country concerning Q fever. Thus, in the case of an outbreak such as the one in Netherlands the cut-off values should probably be further increased in order to achieve an acceptable specificity of the test. It is my opinion therefore that the cut-off values used in this study are far too low to be indicative of a positive Q fever sample serum, particularly during an outbreak of such an extent where the probability of exposure to the bacterium is highly increased. Thus, too many falsely positive samples could be included in the study. It would be interesting to assess the change in the number of the positive samples after increasing the cut-off values of the test to the ones used in the study in Denmark. In any case, since prevalence of Q fever varies greatly worldwide the establishment of cut-off values using healthy volunteers and/or pregnant women of a non-endemic area is of the utmost importance in order to tackle the problem of cross-reactivity and falsely Q fever positive samples.

Comments A 1-4 of the reviewer are about the serological methods used and the interpretation of the serological results, and more specifically on the use of IgM II. The cut-off values are too low according to the reviewer and therefore the specificity will be low (hence, many false positives caused by aspecific reactions).

The reviewer mentions the paper by Tissot-Dupont in this respect, but we assume that this is by mistake and that she refers to the paper by Villumsen et al. (our reference 22). That paper indeed recommends an IgM II cut-off of ≥256, as we have also mentioned in the discussion section of the paper. The focus of the study by Villumsen et al. in Denmark, a low prevalence country, was to increase the specificity of the cut-off value, hence, reducing the number of false positives. In fact, the study has an almost exclusive focus on specificity with the message that the cut-off should be based on local conditions. However, we argue that the choice of cut-off point, when there are no definite rules, must also depend on the reasons for performing the test (Giesecke J, Modern Infectious Disease Epidemiology, Arnold, London, second edition, 2002, page 192). This implies that different cut-offs could be used in the same area for individual patient diagnosis, for seroprevalence studies, and for serological screening. The underlying question at stake for studies on C. burnetii seropositivity and pregnancy outcome is whether a screening programme to detect asymptomatic cases is needed. Raising the cut-off considerably as suggested would result in very few women with aspecific reactions being labelled as (false) positive. However, a number of women with recent infection but relatively low titres would be missed. We feel that the chosen cut-off of ≥64 is an acceptable compromise between sensitivity and specificity of the screening test. Obviously, when applied in a real screening programme, confirmation of recent C. burnetii infection and decision to treat or not would depend on additional laboratory tests (serology, PCR) and patient characteristics. Furthermore, we believe that a high specificity is especially important for low prevalence situations (such as Denmark), more than for high prevalence situations (such as in part of the Netherlands). In a situation of a low pre-test
likelihood the rate of false-positive results will be higher and thus the positive predictive value will be lower. The cutoff of 1:64 is a compromise of the manufacturers instructions, which sets the cutoff at 1:16 if both phase I and II IgG are positive and 1:256 for a solitary phase II IgG.

We acknowledge (as also mentioned by reviewer 2) that any choice of test and cut-off can be criticised. We are currently analysing the very large serological and PCR database of Q fever patients that is available in the Jeroen Bosch Hospital. In one of the research activities the sensitivity and specificity of a solitary IgM II result will be determined. Preliminary results indicate that an isolated positive IgM II as determined by IFA is a clear indication of a recent (PCR-confirmed) C. burnetii infection.

We challenge the notion that no laboratory relies on a positive IgM II ≥64. For example from Australia: Field et al. in J Clin Microbiol. 2000;38: 1645-7: “A positive IgM result was defined as having an end point titer of ≥48.” And: from Germany: Frangoulidis et al. in Ann N Y Acad Sci. 2006;1078:561-2: “IgM Phase II test results were considered positive when titers of 1:64 or higher were seen.” In the Netherlands an isolated positive IgM II in a patient with suspected Q fever must be notified by the medical microbiology laboratory to the municipal health service. The municipal health service will notify the case in the national infectious diseases surveillance database only after they have contacted the patient or the treating physician and confirmed that there is a compatible clinical presentation with at least fever, or pneumonia, or hepatitis.

B) Minor essential revisions
1) Abstract, Conclusion: It is not advisable to compare the results of this study with the 4 studies cited in the article since 3 of them (Carcopino et al 2007, Denman et al 2009 and Diaz et al 2001) are supported on confirmed Q fever cases based on seroconversion and PCR.
We have removed the comparison with the 4 studies from the abstract and from the conclusion.

2) Methods, first paragraph: “Exposure (infection with C. burnetii during pregnancy)...” Exposure to the bacterium does not necessarily mean or lead to infection. Storage conditions of the sample sera should be mentioned in order to assess the quality of the samples. It is known, for example, that storage of sera at 4°C for prolonged periods of time will negatively affect the nature of the possibly present antibodies. This phenomenon can lead to the presentation of different antibody titre values from the same sample over time. In this case, where just a cut-off of #1/64 is used for determining exposure to C. burnetii even a single dilution change could affect the result.
We used ‘exposure’ in terms of the epidemiological exposure-outcome relation. We have adapted the text to avoid confusion. We have now included information on the storage conditions of the sample sera.

3) Methods, Sample size calculation: The authors assume that the prevalence of antibodies against C. burnetii among pregnant women in the high-risk area would be around 10%. Further information is needed to demonstrate how this assumption came up.
We have added information on a seroprevalence study that was done in 2007 and on which we based the 10% estimate with a reference to the source.

C) Discretionary Revisions
1) Results, Pregnancy outcome, paragraph 2: The authors state that the mean birth weight of the babies was not significantly associated with positive serology. It would be interesting to check whether this parameter, among other adverse pregnancy outcomes, is affected when taking into consideration sample sera only from 2008 which is actually the first year of the outbreak. An additional appealing study would be the comparison of the data collected concerning pregnancy outcomes in the Q fever endemic region with a non endemic one in the Netherlands. We thank the reviewer for these suggestions. In fact, even more interesting would be to repeat the analysis for 2009 at the peak of the epidemic. However, information on pregnancy outcome for 2009 pregnancies will only be available from the Netherlands Perinatal Registry (PRN) in late 2010. In line with the second suggestion, we have initiated a study with the PRN to look at regional differences in pregnancy outcome.

We hope you will find the adapted manuscript suitable for publication in your journal,

Sincerely yours,

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