Author's response to reviews

Title: Spatial distribution and risk factors of Brucellosis in Iberian wild ungulates

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Author's response to reviews: see over
We thank Dr. McGiven for his effort in improving this manuscript. In the following section, we respond point by point to the final issues raised by the reviewer.

1) It is not clear from the paper whether the authors suggest that, especially for swine, that disease in livestock poses a risk to wildlife or whether disease in wildlife poses a threat to livestock. For example in the penultimate sentence in the abstract disease in wild boar is presented as a threat to disease in domestic pigs, whereas the sentence at the start of the second paragraph of the background states that wild animals are often at risk as a consequence of contacts with infected livestock.

We were writing intentionally the manuscript in that way because our results support clearly that Iberian wild ruminants are not a significant reservoir for brucellosis infection (this was stated in lines 383-384 of the Discussion). However, because of the extremely high real and apparent prevalence in wild boar and also in Iberian domestic pigs reared outdoor, the respective role of each species in the wild boar-domestic pig interaction remains unexplained. Obviously, we are not able to respond if the current B. suis infection problem in Spain had the main origin in the wild reservoir (probably the wild ancestor of the actual domestic pigs was infected thousands of years ago), or by the contrary, the current situation of disease in wild boar is due to the high prevalence of the problem in Iberian domestic pigs. In any case, given the extremely high prevalence of the disease in wild boar, we can support our statement (lines 319-320) on the important threat that the infection in this wild species can pose for domestic pigs.

If the authors have clear thoughts and conclusions then these should be presented clearly rather ambiguously which is currently the case within their document.

Response: It is not our intention to prolong indefinitely the debate on this matter again. However, we disagree with your perception on the ambiguity in which our results have been presented. As stated in the Abstract and Discussion, our data support clearly that wild ruminants are not a significant reservoir or threat for brucellosis infection in domestic ruminants in the Iberian Peninsula.

On the contrary case, it has been clearly documented that wild boar is the main reservoir for brucellosis in domestic pigs due to B. suis biovar 2 in Europe (Porcine Brucellosis. Scientific Opinion No. EFSA Q-2008-665. The EFSA Journal, 2009, 1144, 1-112). The prevalence of brucellosis in wild boar has been maintained always uniformly high in most European countries in absence of brucellosis in industrial (closed) pig breeding systems (a generalised situation in Europe). The outbreaks of disease described in the last few years in domestic pigs in France and Central Europe have been produced always when brucellosis free pigs are moved from closed (indoor) to outdoor (open air) breeding systems. In all these outbreaks, the wild boar (by breaking the fences and entering in the open air farms) has been identified as the responsible of transmission in
these breeding systems. However, the epidemiological situation in Spain is clearly different since an important part of the domestic pig farms (the black Iberian pigs raised under extensive breeding systems in the South West of Iberian Peninsula) has been ancestrally reared outdoor. Brucellosis prevalence in these extensively reared farms has been always very high, while brucellosis was inexistent in intensive (indoor) breeding systems. Because of the extremely high prevalence identified also in wild boar, the respective role of each species in the epidemiology of infection remains unexplained. However, independently of this particular situation in extensively reared black Iberian pigs, and as it has been clearly reported in most European countries affected by the problem, wild boar represent the main reservoir of \textit{B. suis} biovar 2 infection for the overall domestic industrial farms. We hope that this apparently ambiguous situation be now better understood by the referee. With the intention of avoiding extending this debate, we have eliminated the term “reservoir” from the different parts of the manuscript dealing with brucellosis in wild ruminants and also in pigs/wild boar. When appropriate, instead of “reservoir” we have stated that wild boar can constitute a “threat” or a “potential hazard” for domestic pigs.

2) In line 436-438 the authors also discuss the spread of brucellosis from domestic pigs to wild boar. I think the manuscript would benefit from more clarity regarding the background hypothesis of disease transmission between wild and domestic species. See above comment. Accordingly, we recommend maintain this part of the text as it was.

See my comment above.

Response: OK. See also the response given.

3) Can the authors clarify what exactly they mean by ‘contact’ in lines 110 & 111 (and line 260 of the results). Do they mean exposure, seroconversion, infection and how do each of these measurements relate to prevalence (or seroprevalence)? In lines 303 & 304 the terms positive iELISA and prevalence are used in consecutive sentences to describe what I believe is the same information. The term serum antibody prevalence is used in the figures. Can the authors clarify their terminology in this regard.

You are right and the term “apparent prevalence” is used in the new manuscript version.
The term contact is still included in the manuscript and it is still not clear what exactly the authors mean by this.

Response: In that related with the serological results, the word “contact” was used as equivalent to “exposure resulting in seroconversion”. We have now used “antibody responses” whenever possible to avoid confusion. However, the word “contact” also appears in text but in other contexts, particularly to describe the degree of interaction between different animal species.

4) Can the authors comment on what impact they feel that the haemolysis of the samples may have had on the performance of the ELISA (given that it presumably was not validated with haemolysed samples) and what is the basis for their opinion.

The degree of haemolysis affects significantly the performance of some classical tests -i.e RBT and CFT-, and also that of some “new” tests (i.e., the FPA). However, in our routine experience with the diagnosis of disease in domestic species, the degree of haemolysis of the samples does not affect significantly the iELISA performance. A recent paper on pig serology also found a very low effect of moderate haemolysis on the ELISA performance (Neumann & Bonistalli 2009, Effect of blood sample handling post-collection on Erysipelothrix rhusiopathiae antibody titres. Veterinary Journal 180: 325–329). In fact, in the wild species in which the diagnostic performance of the culture was relatively high (wild boar), an important proportion of the sera from culture positive animals (and resulting simultaneously positive in the iELISA) was highly haemolysed.

Thank you for this supporting information. I think it would be beneficial to have something like this within the manuscript.

Response: We have now added a sentence in the Discussion, supported by the adequate reference [Neumann & Bonistalli 2009]

5) In line 184-186 the authors describe an assessment of relative specificity. Specificity is the ability of the assay to avoid false positive results and is tested using a non-infected population. However, performing culture on samples from a sero-negative population to see if such samples are culture positive or negative only enables the identification of false negatives or genuine negatives (generously assuming that culture is 100% sensitive). It is not possible to draw a conclusion about specificity with this information. This should also be reflected in the results and discussion section. If the intention is to demonstrate good specificity would it not be preferable to demonstrate that positive results are true and not false positives? However I fully appreciate the difficulty of this given the sensitivity of culture (the ‘gold standard’ for disease confirmation). Is it not
the case that in checking that your negative results are not positive – as you have done – this is more of an investigation of sensitivity and not specificity (i.e checking that you do not have false negatives)?

Sorry but we disagree. We intentionally selected the term “relative specificity” (versus the culture results). According to that, your comments on “true specificity” are not applicable here. Therefore, we are fully convinced that our data are valuable enough to support the good “relative specificity” of the iELISA developed, and we recommend maintaining the text as it was.

I maintain that I am uncomfortable with the use of the word ‘specificity’ as used here. If the authors add the caveat that they are not describing ‘true specificity’ so that they do not unintentionally mislead the reader then this could be a suitable compromise.

Response: With the exception of paragraphs dealing with establishing the iELISA cut-offs with the gold standard populations, the terms “sensitivity” and “specificity” have not been used anymore in the remaining parts of the manuscript. Instead, we have been always using the terms “relative sensitivity” or “relative specificity” (always referred to the culture results or to a combination of both serological and culture results, respectively). In addition, when adequate, we have been also using the term “apparent prevalence”. We hope that the average reader would be now able to understand properly the meaning of all these terms.

6) In the discussion (line 323) the authors state that they have identified that the wild boar are an important reservoirs of B.suis infection for domestic pigs. The directional connection between the two populations has not been shown by this study it has been presumed based on earlier referenced work. The authors have correctly demonstrated that the wild boar population presents a threat or potential reservoir.

OK. See respective comments made above.

I hope this comment means that we are agreed that the work has not proved that the wild boar population is a reservoir of infection for domestic pigs but has proved that it is a potential reservoir and a threat. The distinction may seem slight but I believe it is important that the detail is correct (even in their statement above the authors state ‘the respective role of each species in the wild boar-domestic pig interaction remains unexplained’).

Response: see comments in point one.
7) The authors continue to describe the problems of testing in wildlife populations (up to line 348) none of which are addressed by their work. I therefore question the relevance of including this information.

Here we disagree with your perception. Having in consideration the difficulty in obtaining gold standard sera from wildlife, we proposed an alternative approach based on the use of gold standards from domestic species closely related from the phylogenetic standpoint. Moreover, we consider it relevant to make criticisms to the different publications (most conducted using a non adequate methodology), and we strongly suggest keeping the whole sentence as it was.

I was more referring to the text from lines 324 up to 348. I think this is too much information, not all of which is directly relevant, and can be edited down.

Response: Sorry but we disagree. We consider that the comments made in lines 323-346 are directly related with relevant aspects of the methods used in the paper, being of interest for the adequate comprehension of the published literature data. We will ask you for some help with this subject to avoid additional delays in the publication of this manuscript. One of our main reasons for choosing an on line open access journal was that these journals have not too strict space limitations.

8) In line 352 the combination of serology and bacteriology is described as the ideal approach. I don’t think it is ideal but it may be the most effective.

Probably this is simply a dialectical problem in your conception of the word “effective” and that of our “ideal” approach. We hope that you will agree that, either considered effective or ideal, this approach is the most recommendable from the technical standpoint.

I agree that the approach if the most recommendable, but it is not ideal. The original description from the authors may be due to a dialectical thing but I am in the fortuitous position has having English as my first (and sadly only!) language and the difference between ‘effective’ and ‘ideal’ is real. I would highly recommend that the authors make this simple change.

Response: Sorry for not being aware -unfortunately, our English is not as good as we would like- on the important differences between “ideal” and “effective”. We have now used “most recommendable”, with the hope that this will be satisfactory.

9) Could the authors describe which IgG isotypes protein G detects here please.

OK. G (ie, IgG) was the immunoglobulin isotype concerned, as described in the text.
The isotypes for IgG are IgG1, IgG2a, IgG2b, IgG3 and IgG4. Does protein G bind them all? I ask as the use of a specific anti-IgG1 conjugate is frequently preferred for bovine ELISAs.

Response: Sorry but we do not understand the interest of your question, and their final consequences on the results obtained. Protein G binds preferentially to the Fc portion of IgG immunoglobulins, then detecting all subtypes of the IgG isotype in most animal species (Harlow, E. and Lane, D. eds. (1988). Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory, N.Y., 617 - 618.). Moreover, it also binds to the Fab region, then increasing the ability of detecting these different IgG subtypes (in fact, this reagent is used for purification of IgG1 fragments).

Finally, we have no specific comment on your preference in using anti-IgG1 conjugates for bovine ELISAs, and we are unaware of the technical basis for this preference. When the objective is to obtain the maximal diagnostic performance, and because of the ability for detecting all relevant immunoglobulin isotypes, we would be probably recommending the use of a polyclonal anti-bovine IgG (H+L specificity) conjugate. In any case and as it is clearly indicated in the manuscript, specific conjugates raised against the immunoglobulin isotypes of wild ruminants and wild boar are not commercially available.

10) If this study showed that recovery of bacteria from seropositive animals was similar or better than in similar studies performed elsewhere in the EU (lines 364-365) then why do the authors talk of there being a relatively high number of ELISA pos, culture negative samples?

We considered this comment relevant to assess that our bacteriological methods were at least as good as those reported elsewhere, but also to clarify that the discrepancy is not a problem due to a lack of specificity of the iELISA used, but due to a lack of sensitivity of the bacteriological tests used. Provided that adequate culture media be used (as it was the case here), the quality (generally poor) of the samples used in this kind of studies can be the main explanation of the relatively low performance of bacteriology versus the serology.

I agree with the comments here but feel the manuscript would benefit from this degree of clarity. At the moment the manuscript is slightly ambiguous in describing the relationship between their results and prior data.

Response: We are somewhat surprised by this comment, since we copied almost literally (lines 330-345) the response given to you. We have included also a comment in
the discussion (lines 335-336) indicating that our bacteriological results are comparable to those obtained in similar studies in the EU.

11) I don’t think that the data described in lines 376-383 is adequate to conclude that the iELISA is good enough. I believe that it probably is good enough but my belief is based more on the data from the domestic species and the binding properties of protein G. Can the authors cite any other wildlife studies were protein G has been successfully used as a conjugate in ELISA?

Adequate references were presented in the text (see refs 51 and 52), and we do not consider necessary to include additional ones. However, there are several other references in the literature proving that this reagent is suitable for serological studies in wildlife. As an example, some of us have published recently studies using this reagent for the successful diagnosis of infections by Mycobacteria in both deer and wild boar: Reyes-García et al., 2008. Large scale ELISA testing of spanish red deer for paratuberculosis. Veterinary Immunology and Immunopathology, 124:75-81; Aurtenetxe O et al, 2008. Development and validation of an ELISA for antibodies against *M. bovis* in european wild boar. BMC Veterinary Research 4: 43.

It would be helpful (and recommended) to indicate in the manuscript that references 51 and 52 make use of protein G as this would support their statements. Currently the only way to know this is to be familiar with the methodology of the work or read the references in detail.

Response: We have now tried to better clarify this in the manuscript, including references in which this reagent was used for diagnosing brucellosis and other pathogens in wildlife.

12) The authors also conclude that wild boar present a reservoir of infection for the outdoor domestic pig population. Their study demonstrates that the infection in wild boars are a potential threat/hazard but the mechanism and direction of disease transmission has not been proven here and thus it is not possible to conclude (without supporting references) that the wild boar population is a reservoir of infection (see earlier comments).

As commented above, we disagree with you. Having in consideration the extremely high prevalence of disease in wild boar, any industrial pig activity in open air (out door) breeding systems, as well as small scale open air breeding systems (backyard farms) have a high probability to contact with wild boar, with the ensuing risk of transmission of infection to domestic pigs.
Yes, there is a risk and a threat but until the directional link (hence the analogy to a reservoir) has been categorically proven it is not correct to describe either population as a reservoir of infection to the other.

Response: See our comments above. We have avoided using the term reservoir when adequate.

Final notes:

Ethics – This manuscript included no experimental research requiring authorization by ethical committees, and all samples used were obtained from hunted (by third persons) animals in the wild.

Style – We hope that the revised manuscript conforms to the journal style.