Reviewer's report

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

Version: 2 Date: 23 December 2009

Reviewer: John McGiven

Reviewer's report:

In the following document I have added my comments to the authors comments about my original comments. I hope this makes sense.

Reviewer 2: Reviewer's report:

In general I think this is a very interesting and worthy study that should definitely be published. Brucellosis in wildlife is a problem of increasing relevance and data relating to this is valuable. I think that the methods are appropriate as is most of the analysis. However I do think that some of the conclusions are too speculative and should be toned down and some of the analysis requires minor modification. My detailed comments are described below. The paper is also long and could benefit from being a little more concise.

We have tried to shorten the new version of the manuscript. Some analyses have been clarified (see answers to reviewer 1), and we have toned down the conclusions regarding wild ruminants.

OK

Both the abstract and the introduction make no mention of the work aiming to study transmission of brucellosis yet conclusions about this (i.e. using the term reservoir of infection) are drawn from the results.

This has now been modified in the Abstract section of the new manuscript.

OK

The term prevalence is frequently mentioned. It should be stated clearly in the paper that in this instance the term is synonymous with seroprevalence as the precise infection status of each animal studied is not know. If the seroprevalence and prevalence are not equal then the concomitant reduction in statistical confidence (as a result of using seroprevalence to calculate prevalence) should be described in the paper.

Thank you. This is only partially true since many bacteriological isolations were made, at least in wild boar. However, you are right and your comment has been considered. We have made amendments considering that the real prevalence is that determined by bacteriological tests, while the apparent prevalence is evidenced by serological tests. This “apparent prevalence” term rather than “prevalence” or “seroprevalence” has been used in the new manuscript version.

OK
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.

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.

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.

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.

In line 173 determination of the optimal cut-off is described. Do the authors mean optimal conditions? This would certainly fit better with the following sentence

Done. Thank you for this comment. The sentence has been amended.

The cut-off appears to have been selected by ROC analysis as described in lines 176-177. The authors also state in the results (line 250) that the cut-offs were selected to maximise resolution (diff between min infected and max non-infected). This resolution occurs irrespective of the cut-off (placing the cut-off anywhere will not change the resolution) and is due to optimisation of other parameters.

You are right and the sentences were confusing. The optimal conditions (in our hands, the serum dilution and incubation times are usually the most relevant factors) were selected to result in the maximal resolution (i.e., the maximal distance in OD units among the highest negative control serum and the lowest positive control serum). Then, the cut-off was selected to result in 100% diagnostic specificity (and, simultaneously, the maximal diagnostic sensitivity) using the ROC analysis. This obviously has no relationship with the resolution. The corresponding sentences have been now amended in the new version.

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.

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.

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)? 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.

In lines 302-307 the relationship between age, sex and prevalence is described. Age was modelled as a discrete continuous variable and the results show a significant relationship with prevalence. Yet only the sex-age interaction is discussed (421-426) & the age prevalence relationship does not look linear in figure 3. Could the authors comment on this? Could the authors also show what statistic is displayed by the error bars in figure 3.

Thank you for this important comment. As shown in Figure 3, prevalence was similar among age classes, except for adults (age class 4), showing a higher prevalence. This suggests that this age class, the most active one in reproduction, has a higher risk of contact with Brucella than the younger age classes. The manuscript has been now amended by adding the following: “Prevalence observed among adult wild boar was higher than among younger age classes, as expected by the higher participation in reproduction by adults [62].” Error bars were calculated with the adjusted Wald method, as stated in methods.

OK

In lines 311-315 the final regional scale model is described. It would be helpful to the reader if this was more clearly identified as the model created using data from the smaller geographical scale within the Ciudad Real province (bio-region 3).

Thank you again. We modified the text adding in the material and methods that “In the smaller geographical scale model (Ciudad Real province, Bio-region 3).”

OK

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’).

I do not agree with the authors that classical tests such as RBT and CFT should be used (in preference) for wildlife testing (lines 325-329) as these have also not been validated in wildlife.

We fully agree with the case of the CFT (that probably needs of different test conditions according the different animal species considered), and the respective amendment has been made in the new version. However, we disagree in the case of the RBT, which can be considered as a “universal” diagnostic test for all animal species including humans, in which no particular conditions (i.e., temperature of inactivation, dilution of serum, etc) are required for the different animal species tested. Obviously, this classical test has never been submitted to a proper validation study using adequate gold standard populations from all wild animal species, but it has been the object of plenty of validation studies in the domestic species more closely related from the phylogenetic stand point.

OK

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 stand point. 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.

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.

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.

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.

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 spanich 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.

The ELISA was validated using sera from domestic species but the appropriateness of this to translate data interpretation with respect to wildlife was
not discussed in any detail.

We disagree with this comment. In lines 253-257 we present data showing the adequate relative specificity of the iELISA developed with respect to the culture results. Moreover, an important part of the Discussion (lines 358-382) was dedicated to discuss with enough detail these aspects.

OK

In the paragraph beginning line 384 I think that the authors should begin with the data from species where large samples sizes have been tested and draw the conclusions from that (that wild ruminants are not a reservoir of infection) and then discuss the data from the species with smaller sample sizes. This data indicates that the same conclusions are true for these species but the small sample size does not allow the conclusion to be conclusive.

OK. We have modified the whole sentence.

OK

Line 443 states that this study shows that domestic and wild pigs share the same B. suis bv 2 infection. Only wild boar were tested in this study so I think that the sentence is missing a reference to previous work on domestic pigs.

Sorry, we missed including reference 25 (now 24). This reference has been now included and the whole sentence modified.

OK

On line 496 the authors state that the from this work they can conclude that the B. suis biovar infection has probably arrived at a ceiling. I think this is too speculative. It is an interesting hypothesis but cannot be concluded from this work.

We agree. In fact, “season” was included as a random variable to control for sampling season effects. This variable was not properly analyzed in this study, since sampling was not uniform through sites and seasons. Accordingly, all references to temporal trends have been deleted in the new version. See also responses to reviewer 1.

OK

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.

**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.

**Declaration of competing interests:**

'I declare that I have no competing interests’