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
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Dear Dr. Alam,
Thank you for the rapid and in-depth review of this manuscript. We acknowledge the effort made by both reviewers and the AE in improving the first version of the manuscript. We have followed most suggestions and provide below a list of answers (indicated in blue color) to the points raised by both reviewers. We hope that with the amendments made the article be now acceptable for publication in BMC.

I look forward to hearing from you. Yours sincerely,

Mariana Boadella
**Answer to the reviewers**

**Reviewer 1:**

**Reviewer's report:**

Wild ruminants

It is important to document that wild ruminants are only spill over and not maintenance host for *Brucella abortus* and *Brucella melitensis*. However, there is no discussion about the source of infection of the few culture positive animals in this manuscript.

This was clearly stated in the document (lines 70-79). The source of infection for wild ruminants is the infected livestock, and this was also discussed with enough detail in the manuscript (lines 394-411).

There is no mention on the molecular fingerprints of those strains as to compare to strains isolated from livestock. This information should be provided in order to understand which are the risk factors for wild ruminants to be infected with *Brucella* sp.

To perform these particular genetic analyses was beyond the objective of our study, and moreover, the number of strains isolated from these species was too low (Table 2) to provide enough information. In any case, we doubt that the information obtained in these molecular studies, other than demonstrating the existence of genetic relationships between the strains isolated from domestic and wild species, could be of some help in identifying the risk factors.

More, the authors should explain why only 1 out of the 31 iELISA positive red deer samples was culture positive: what is the positive predictive value of an positive iELISA in these species?

Here we disagree → Rather than focusing on the predictive value (an epidemiological concept related with the population more than with the real
diagnostic performance of the test) it would be advisable to understand that the sensitivity of the bacteriological tests performed was generally low due to the nature of the samples (from hunted animals) tested for bacteriological analysis. This was discussed in lines 366-369.

Wild boars
It seems out of (reasonable) reach to list 58 additional variables which eventually were not considered to be risk factors. Some of the variable listed are really far fetched and should not have been considered for inclusion in a sound explanatory model.

We explain our statistics → The use of GLM or GLZ in risk factor analysis is not new. In this kind of analyses, it is common to perform a previous screening with a high number of variables, on which a preliminary testing is done, and then to include a selection of them to fit the final model. Only part of these selected variables were included in the final model, and only part of those were actually significant (e.g. variable “Open-air pigs per sq km” is not significant, but included in the regional model, Table 4, Panel B). In this case, we first tested each single variable using single factor generalized models. Only those with p<0.05 were included in the second analytical step, a multiple logistic model.

On the other hand, I am questioning the validity of the findings presented in table 3. Indeed, I have difficulties to understand that brucellosis in wild boar is significantly associated with “road” (for what is means) and with “urban” (for what is means) at the peninsular level. This definitely needs clarification.

The variables “Road” and “Urban” were initially selected by the single factor generalized models. This means that sera from sites with more roads or more urban habitat had a significantly higher probability of testing positive. However, when these variables were tested in the final multivariate model, none was
selected. This means that, when the other significant variables were accounted for, the effect of roads and urban habitats was negligible.

I also have difficulties to understand the association between brucellosis and the abundance of Iberian hares: what does this actually mean?

The variable “Iberian Hare habitat suitability” was statistically significant in the first screening (single factor generalized model) but not selected for the final model. This means that sera from sites with better habitat suitability for Iberian hares had a significantly higher probability of testing positive. However, when this variable was tested in the final multivariate model, it was not selected. This means that, when the other significant variables were accounted for, the effect of Iberian hare habitat suitability (a proxy for Iberian hare abundance) was negligible. In fact, we already stated that “Suitability of Iberian hare habitat, meaning open, flat, sparsely-forested Mediterranean agrosystems, was selected in the first step of the analysis, but not included in the final model. Its weak link with wild boar prevalence may be due to a correlation between Iberian hare habitat suitability and Bio-region 3.”

I also have difficulties to understand that the presence and numbers of open-air raised pigs and pig farms are risk factors. There are 2 main reasons for this:
1. Indeed it is difficult to accept as suggested by the authors that density is not a risk factor within the wild boar population (which we know are infected), (...)

Definitely we found no link between wild boar abundance or management and *B. suis* antibody prevalence. This was consistently so at the Peninsular scale (where wild boar management is a proxy for density) and at the regional scale, where 6 different “wild boar variables” were tested. Indeed, in the discussion of the first version, we stated that “In wild boar, contact with several other infectious agents has been linked with density, spatial aggregation or artificial management (e.g. Aujeszky’s disease [58, 59]; Bovine tuberculosis [39]; Porcine circovirus type 2 [55]). However, no relationship between prevalence and wild boar management or
density risk factors has been evidenced in this study. There is no clear explanation for this finding, and further research in this field is needed in order to better identify the factors modulating *B. suis* infection in wild boar.”

Moreover, these findings were also consistent with a prior report from Ruiz-Fons et al. (2006), in which no effects were found in management type (a proxy for density and aggregation) regarding *Brucella* as compared to ADV or PCV2.

(...)

Please consider that farmed pigs have even higher densities and different risk factors than wild boar, even pseudo-farmed wild boar. The variable “Open-air pigs per sq km” was selected by the final model, although its contribution was far lower than the remaining variables. This means that some association exists between open-air pig density and wild boar *Brucella* antibody status, without stating the direction. Unfortunately, information on the brucellosis status of pig farms is not available at a Peninsular scale. The only two Spanish papers addressing this issue were cited in line 442 of the discussion. Probably, swine brucellosis is endemic in open-air reared domestic pigs. We stated that “Three out of the five wild boar estates showing the highest prevalence were open and shared pastures and waterholes with domestic Iberian free-ranging pigs. However, the other two highly infected estates as well as many of the wild boar found infected in our study were living in fenced estates with no possible contact with domestic pigs”. However, recent field data based on wild boar telemetry show the ability of wild boar to undercross fences. Hence, we have modified and shortened these sentences in the new manuscript version.
2. I do agree that association does not mean causation so the question is: who does infect who? In order to clarify, can the author document any case where wild boars were contaminated by open-air pigs? Wild boars are known to sustain B. suis 2 infection. Actually, this is the rule, so this does not need to be discussed as if this is a new finding.

We agree. We cannot document the case and have deleted this part of the discussion in the new version.

Can you please clarify why some variables associated to open-air pig farms are associated with brucellosis in wild boar and other variables associated to open-air pig farms are not (like open-air pig farms in municipality or open-air pig farm per km2)?

In the initial models (first step) we selected an a priori cut-off point of p<0.1. In the final models (second step), cut-off point was of p<0.05; some variables may have been quite close to this cut-off, but only the variable “open-air pigs per sq km” was below 0.05, and hence included in the final model.

Lastly, it is also very difficult to understand that variables like wild boar management, fencing (which fencing is this? Wild boar or open-air pig farm) and wild boar supplemental feeding were not selected. Indeed, these are variables documented to be associated with brucellosis; more the authors refer to this practice to be significantly associated to (bovine) brucellosis in Yellowstone in the US.

We also agree, but these were the results obtained. First of all, it seems that the factors contributing to ruminant brucellosis are different from those contributing to swine brucellosis. As stated in the former version, this kind of association is sometimes found when studying other wild boar infections, but not in the case of brucellosis. Fencing means wild boar hunting estate fencing, as stated in line 100.
However, we agree that this needs to be clarified and we have modified the legend of Table 1.

To summarize, it seems that the statistics may be correct but the epidemiology and the biology of the infection are not! I genuinely do think that confounding risk factors are listed while actual ones were overlooked.

Here we disagree. We honestly tried to include in the analysis all available and possibly influencing variables, and we used a method with no a priori settings to exclude or include any variables. Thus, the variables selected by the final models were those dealing, at least in part, with the factors affecting the probability of a wild boar testing positive. While other (unknown) variables may also have some effect, we firmly believe that the selected ones were the most relevant from the epidemiological point of view.

It would also be very interesting to analyze the trend of the evolution of seroprevalence in wild boars during this long period of time (special and temporal analysis): Rise? Plateau? Cyclic? Indeed this would help in understanding if the management of wild boar is important with regards to brucellosis at the peninsular level. This has indeed a lot of consequences for the competent veterinary authorities in the context of the brucellosis eradication program.

OK, with subsequent changes: Proper testing for temporal trends was not carried out in order to avoid pseudo-replication since sampling was opportunistic and heterogeneous among years. Instead, the models were fitted with sampling season as random factor (from 00-01 to 08-09) to control for any time effect. So, it was not correct to display the effect of the hunting season in Table 3. This has been deleted, and we have also deleted from the results and the discussion all references to temporal trends. We agree with the reviewer that this information is relevant for pig management. As suggested, this subject will be studied later on, based on a targeted sampling design.
ELISA
The Protein G ELISA has been established since 15 years in wild boars. Surprisingly, the authors refer to publication 56, in which the test is described but unfortunately without referring to it for the set up of the iELISA.

We were fully aware of the existence of that publication (Godfroid et al., 1994), that we included in the reference list. However, the iELISA used in that publication was based in other previously reported (Limet et al., 1988, Ann. Med. Vet., 132:565-575), and whose main characteristics (antigen, substrate, etc) were fully different to that used in our iELISA. The only common reagent used by Godfroid et al. and us, was the protein G as the conjugate. Therefore, we were not using the same setting up procedures because of the important test differences. In any case, we stress that we established the specificity threshold using also a collection of sera taken from brucellosis free animals, an identical approach to that used in the Godfroid et al. article. It is also relevant to indicate that the criteria used to set up the specificity and sensitivity were fully different. The approach followed by Godfroid et al. for setting up specificity was empiric (based on the selection of the cut-off using the mean OD of the negative controls + 5 SD), and resulted in a 99.7% specificity. In our case, the cut off was established mathematically (ROC analysis) to result in 100% specificity when testing the sera from brucellosis free animals. Moreover, the criteria used by Godfroid et al. to set up the sensitivity (i.e., assessment of analytical sensitivity against the OIEISS standard serum) are largely questionable since: i) the use of only one serum -which was, moreover, of bovine origin (when the objective of the paper was setting up a test to be used in wild boar)-, and ii) more importantly, the assessment of the analytical sensitivity against the OIEISS can never be used as an indicator of the diagnostic sensitivity of a given test. In our case, the diagnostic sensitivity was defined using many sera from Brucellosis infected animals, and belonging to the domestic species more related phylogenetically to each wild species studied.
Can you please explain the rationale to use the rationale to use B. melitensis 16 M LPS (M dominant) to test for antibodies raised against B. suis biovar 2 (A dominant).

This rationale was clearly indicated in lines 352-354 of the Discussion. The classical hypothesis of Wilson and Miles with regards to the existence of two Brucella epitopes (A and M) assessed by agglutination with monospecific antisera is today obsolete. According to nuclear magnetic resonance studies and competition binding assays, it has been demonstrated that the O-chain of smooth brucellae is a homopolymer of N-formyl-perosamine either exclusively in $\alpha$ (1-2) linkages (for example in B. abortus biovar 1) or in $\alpha$ (1-2) plus $\alpha$ (1-3) in a $\geq$ 4:1 proportion (for example 4:1 in B. melitensis biovar 1). In addition to a common epitope (C/Y) shared with Yersinia enterocolitica O:9, these O-chains carry three basic overlapping epitopes: M (present in O-chains with $\alpha$ (1-3) linkages), A (present in O-chains with no $\alpha$ (1-3) linkages), and C (in which the proportion of $\alpha$ (1-2) to $\alpha$ (1-3) linkages is higher than 4:1). This C epitope is common to all types of Brucella O-chains including B. suis, and the most relevant from the diagnostic standpoint (Douglas, J. T., and D. A. Palmer. 1988. Use of monoclonal antibodies to identify the distribution of A and M epitopes on smooth Brucella species. J. Clin. Microbiol. 26:1353–1356.; Rojas, N., et al. 1994. Immunochemical identification of Brucella abortus lipopolysaccharide epitopes. Clin. Diagn. Lab. Immunol. 1:206–213.; Weynants V et al., 1996. Characterization of a Monoclonal Antibody Specific for Brucella Smooth Lipopolysaccharide and Development of a Competitive Enzyme-Linked Immunosorbent Assay To Improve the Serological Diagnosis of Brucellosis. Clin. Diagn. Lab. Immunol., 3: 309–314; Weynants, V. et al. 1997. Characterization of smooth lipopolysaccharides and O polysaccharides of Brucella species by competition binding assays with monoclonal antibodies. Infect. Immun. 65:1939–1943.). For that reason, whenever properly obtained, the whole cellular suspensions or the S-LPS extracts obtained from species containing the A/C epitopes (i.e., B. abortus and B. suis) are perfectly suitable for the diagnosis of infections induced by M/C species (i.e., B. melitensis) and vice versa (Alonso-Urmentea B et al 1998. Evaluation of Lipopolysaccharides and Polysaccharides of Different Epitopic Structures in the Indirect Enzyme-Linked Immunosorbent Assay for Diagnosis of Brucellosis in Small Ruminants and Cattle. Clin. Diagn. Lab. Immunol. 5: 749–75). This explains the successful and widespread use of the RB test (made with B. abortus whole cells as antigen) for the diagnosis of B. melitensis infections in ruminants.
No data on the optimal dilution of sera, according to the species is presented. More, it seems difficult to accept from a practical point of view to have different working dilutions according to the species to be tested.

Sorry but the optimal serum dilutions for each species were clearly described in the manuscript (see lines 151-155). The use of different conditions according to the different animal species involved is a logical procedure and a very common situation in most serological tests for brucellosis (i.e., need of different complement inactivation temperatures in CFT, need for different serum dilutions and different cut-offs in most iELISAs and cELISAs, etc).

Why do the authors use ABTS, which is known not to be the best and most user-friendly chromogen?

This is a personal opinion of the referee. In our hands and that of many other researchers, ABTS is a suitable substrate to be used in the iELISAs for brucellosis. In fact, we have performed some comparative studies with the most commonly used substrates, being the ABTS the most suitable one (Marin CM et al. 1989. Comparison of three serological tests for B. ovis infection of rams using different antigenic extracts. Veterinary Record 125: 504-508).

There is no information provided for positive controls (degree of positivity in quantitative tests). So, the interpretation grid does not mean a lot to the reader. By defining different cut-offs, it is expected that a lot of confusion may be generated. Indeed, if one will test at the same time some wild goats, deer and wild boars (for example), the serum dilution will be 1/100 for the first species and 1/50 for the other species while the cut-off will be 50% of the positive control for the first 2 species and 50% of the positive control for the last one, not mentioning that one has to make sure that the correspondent positive control is correctly chosen.

Some of the questions concerning the serum dilution and the variable test conditions according the different animal species considered have been already
commented above. With respect to the positive controls, this information is detailed in lines 169-176. It is important to clarify (as stated in lines 323-330) that no tests have been adequately validated for use in wild animals. Moreover, for the purpose of this study, the degree of positivity of these sera in other quantitative tests is irrelevant, and the only valuable data is that the selected sera used as controls for validation were belonging to adequate gold standard populations. When taking this in consideration, the interpretation of the figure shown in the paper should be clearly understood by the reader.

No information is provided regarding results in other serological tests. This would help to compare and evaluate the iELISA. Indeed, if results are redundant with other tests then one is allowed to question the added value of this iELISA.

This comment has been responded above. If this comment is referred to the sera from the gold standard populations, this information is not necessary for the purpose of our study, as indicated above. By contrast, if the comment is referred to the use of other tests in the wild populations tested, this lacks of sense. As indicated above and in lines 323-330, the diagnostic sensitivity and specificity of the different serological tests have been not adequately determined for use in wild animals. Moreover, it was not the objective of our study to determine the apparent prevalence of brucellosis in wild ungulates using several diagnostic tests, but to develop and adequately validate a suitable serological test for this purpose. Having in consideration that no gold standard serum populations from wildlife were available, the approach followed for that validation procedure was based on the results obtained with sera taken from brucellosis infected and brucellosis free animals from the domestic species more closely related from the phylogenetic stand point.

To summarize, this system is not practical, may easily lead to errors in dilutions, misinterpretation and last but not least is not validated yet. It is virtually impossible to validate serological tests according to the gold standard in wildlife species.
Therefore comparison with established tests or other techniques like latent class analysis are recommended.

We fully disagree with this interpretation and the negative criticisms made by this referee, and we support this disagreement in the explanations given above, and in some others that we will make below. The last part of the comment is particularly inadequate also. First, the quality of the serum samples obtained in this kind of studies (hunted animals with haemolysed samples) does not allow the application of all (the referee means classical?) serological tests. Second, as indicated in lines 323-330, no tests have been adequately validated for use in wild animals, and hence a suitable and technically adequate comparison is not possible. Finally, latent class analyses have been postulated as a potential solution to establishing the cut-off thresholds of diagnostic immunoassays, when there is an absence of gold standard, as it is the case here. However, these studies can not be applied in absence of data with tests previously validated, as it was the case in our study. Moreover, because these statistical models are complex, extreme care has to be taken to conduct and describe the sampling from the target population(s) selected (not possible here), the characteristics of other tests included in the analysis (not possible here), and the appropriate choice of model and the estimation methods based on peer-reviewed literature (few or no scientifically adequate publications were available at least for some of the animal species considered). In addition, the assumption concerning the same family of distributions in "negative" and "positive" samples is not always appropriate in these studies. Even when these analyses clearly result in two subpopulations, one can never assume that this identifies "infected" or "healthy" animals with enough scientific value. The latent class analysis is strictly based on quantitative data and inherits all diagnostic uncertainty associated with them. In summary, this analysis should be always considered as an exploratory tool, and moreover, could not have been used in our study.

Yersinia enterocolitica O:9 (YO9)
Yo9 is known to be a major problem in domestic pigs (cross reactivity in serological tests). To which extend is the problem prevalent in wild boar?

Here we agree. Yes, this is an important problem in both pigs and domestic ruminants and the cause of important practical problems in the routine diagnosis of brucellosis. However, we do not know if *Y. enterocolitica* O:9 is also a problem in wild boar in Spain, and this was beyond the objective of our study. According to some preliminary studies (*Godfroid J et al., 1994. Brucella suis biotype 2 infection of wild boars (Sus scrofa) in Belgium. Annales de Medecine Veterinaire, 138:263-268*) the problem was not detected in a study involving 141 wild boar, but the bacteriological methods used could have not been fully adequate. In other studies, however, the existence of the problem in wild boar has been reported in Switzerland (*Fredriksson-Ahomaa M et al., 2009, Prevalence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild boars in Switzerland. Int. J. Food Microbiology, 135: 199-122*), but the existence of this problem in Spain is unknown. In any case, the relevance of this issue was discussed in lines 343-345.

Is an infection with YO9 not a likely explanation for the high number of iELISA+/culture–animals?

No. See above comments on the lower sensitivity of the culture rather than a lack of specificity, as the most probable explanation of this discrepancy.

Information related to YO9 is needed in order to hypothesise as the authors do (line 338-341).

We disagree. We do not consider this necessary, since the adequate comments were made on the Discussion (see lines 343-345).

General comment
This manuscript is too long but also imprecise and incomplete.
We have shortened the new version of the manuscript.

Some important issues have to be addressed whereas some redundant and known information do not deserve to be published again in order to recommend this manuscript for publication. I would recommend to re-writing the manuscript as a short communication highlighting the following:

1. Different wildlife species may be infected with Brucella sp in Spain.
2. Wild ruminants are not sustaining Brucella abortus or B. melitensis infection, while wild boars do sustain B. suis biovar 2 infection and may be a reservoir for open-air pigs.
3. B. suis biovar 2 type A,B and C are found in Spain
4. Can any recomendation be made to the competent veterinary authorities to manage brucellosis in wild boars according to the identified risk factors?

Again we agree. We have shortened the revised version of the manuscript, treating to highlight all these issues.
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.

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.

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.

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.

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.

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.
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.

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.

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).”

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 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.

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.
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.

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.

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 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.

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.

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.

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.

On line 496 the authors state that 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.

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.