September 12th 2022

Dear Recommender,

First of all, we would like to thank you and the reviewers for the time spent in reading and analyzing our paper.

As you will see in the marked version of the manuscript, we basically accepted all suggestions made by the reviewers and modified the manuscript accordingly.

In addition, you will find our detailled answers to reviewers to clarify as much as possible some misunderstanding or errors we could have done.

We uploaded the new version of the manuscript in bioRxiv and sent you the document with marked revisions. We are looking for your instructions to move forward with this article.

Sincerely yours,

Damien Meyer

Please find hereafter detailed response to reviewers
Reviewer 1

1) It is unclear to me if the number of goats used for evaluating the humoral response upon immunization with Ape is enough to reach valid conclusions and also I recommend to use statistical analysis to validate the significance of the differences found.

Author's answer: antibody response of several goats vaccinated whether with inactivated or attenuated bacterial vaccine was tested. We only showed one goat for each type of vaccination but in general all animals showed a significant antibody response respectively 3 and 5 weeks post vaccination for goat #614 and #915. We don't try to obtain a quantitative result ; we just wanted to show that there is an antibody response against Ape protein as it is shown in the literature that adhesins are gold vaccine candidates for Chlamydia-like bacteria.

2) determine antibody titers is enough to recommend an immunogen as a vaccine candidate? Or other factors should be also determined?

Author's answer: recommendation of an immunogen as a vaccine candidate could be completed by antigen similarity tests, antigenicity assays, mature epitope density, adhesion probability to assess antigen probability as describe by Zai et al. https://veterinaryresearch.biomedcentral.com/articles/10.1186/s13567-021-00939-5

Minor concerns:

1) Ape was identified as a possible iron transporter, does the intracellular iron concentrations during the infections are known? If is there a correlation with reduction of iron availability and higher expression of the ape gene?

Author's answer: we know from Moumene et al, 2018, that Ehrlichia ruminantium able to sense changes in iron concentrations in the environment and to regulate the expression of

virulence factors accordingly. erxR, virBD, tr1, and map1 genes were upregulated in response to iron starvation; this might have the same effect on ape gene expression.

2) provide more details of the possible advantages of using Ape for vaccines, is it present in all *Ehrlichia ruminantium* strains? Is it sequence conserved?

Author's answer: ERGA_CDS_01230 (Ape) nucleotide sequence has between 86.79 and 99.91% identity when blasted with 12 other strains of E. ruminantium. It seems that the sequence of Ape is quite conserved among Ehrlichia ruminantium. The Ape amino acid sequence strain Gardel has 99.73% homology with Welgevonden strain

3) correct some minor writing mistakes such as

L 28-29 "remain to elucidate." To "remain to be elucidated"

L 431 "against rApe.." remove one dot.

L 475, and L 517 "E. ruminantium , particularly" "(Tiwari et al., 2012) ; Lyme disease "remove spaces.

L 529 "Althoygh"

Author's answer: all corrections suggested have been done

3) scientific names in the references should be in italics.

Author's answer: all corrections suggested have been done

Reviewer 2

The manuscript by Pinarello et al. provides the functional characterization of the transmembrane protein Ape of Ehrlichia ruminantium. The topic is important and the study is an important contribution to the field. Before publication, however, the authors should consider several points referred below.

Introduction

Line 51: Taxonomic names above genus levels do not go in Italics.

Lines 53-58. Sentence too long. Please, split.

Line 62: Remove 'ongoing'

Line 64: Why 'but'? Change to 'and'

Line 82: Change to '…what is observed in other….'

Lines 84-86: This sentence needs revision.

Author's answer: line 51 to 84, all the suggested corrections have been done

Methods

Line 163: What a 'qPCR Sol1' is supposed to mean?

Author's answer: all corrections and detailed were added in the text

Lines 150-169: In these two paragraphs is not clear what is being normalized, the ape gene expression, or the bacterial levels, or both? To avoid confusion and increase clarity, please, separate these two in independent sub-sections with different headings. In addition, the concept of normalization using housekeeping genes has a rationality that is not applied here. What is the validity and limitations of this way of normalizing gene expression? The absence of 'reference gene with a sufficiently stable expression is available for E. ruminantium' does not validate the use of 'normalization… in relation to the number of bacteria present at each stage of development'.

*Author's answer: no housekeeping gene can be used in the case of E. ruminantium because none of them shows enough stability. It is know well admitted that normalization wan be done by the number of genomes of the bacteria (*https://doi.org/10.1038/s41467-022-31176-9 *; DOI: 10.1111/j.1574-695X.2011.00901.x; https://doi.org/10.1016/j.mimet.2010.06.013).*
*In our article we thus normalized of the Ape gene expression, using the level of bacterial genome (*pCS20 copy number*) as it is the only way to do it properly for this bacteria. We added the sub sections asked in material and methods.*

Lines 168-169: What '2 others kinetics' were used?

Author's answer: we corrected indicating "2 others biological replicates"

Lines 204-208: Provide further details on the starting material used for the mass spectrometry analysis and on how the protein was processed for the mass spectrometry analysis. Separate this in a new section named 'Mass spectrometry analysis'. Did the identity of the protein was also checked by mass spectrometry?

Author's answer: We explained in material and method that rApe was concentrated and purified by gel filtration, after dialysis, and before mass spectrometry which showed an unique pic at 66kDa. Identity of rApe was also confirmed by Orbitrap analysis. This has been included in material and method as well as in the results part.

Line 205: Add a hyphen between 'MALDI' and 'TOF'

Line 231: Add the isotype fo the anti-O-GlcNAc antibody.

Author's answer: line 205 and 231 corrected accordingly

Lines 217-235: It is not clear here and/or in the legend of figure 3 what was used as ' negative control' in the 'Western Blot for O-GlcNac Glycoprotein detection'. Please, specify it.

Author's answer: the negative control used is BSA. This was added in material and method and also in the legend of figure 3

Line 221: Is it '3.198 kDa'?

Line 251: Add the manufacturer of the 'chondroïtinase' used in the study.

Line 323: Change 'four' to '4'.

Line 313: Please, include the isotype of the secondary antibody used here.

Author's answer: line 221 to 313 : corrections made as asked

Results

Line 351: Not clear in methods and/or here how the 'number of transcripts' was calculated. In any case, this is not what is shown in Figure 1B.

*Author's answer: first we quantified E. ruminantium along the developmental cycle, as represented in figure 1A. Then, we measured Ape gene expression by qRTPCR but those results were normalized using the bacteria genome number (*pCS20 copy number)*. So figure 1B doesn't show number of transcripts but rather the fold change of the normalized transcripts levels, compared to 96hpi*

Lines 353: The term 'E. ruminantium DNA copy number' is confusing. What gene in particular are the authors referring to here? pCS20? If yes, then change to 'E. ruminantium pCS20 copy number'.

Author's answer: Indeed it is the case. Suggestion was taking in account and modification was done

Line 361: Structural results are poorly presented. For example, where is the iron binding structure located? Also, see comment on Figure 2 below.

Author's answer: we modified the figure to better show the iron binding domain

Line 371: These results require further specification. Please, provide images of the acrylamide gel "SDS PAGE" before and after rApe protein purification. Also, how rApe was specifically detected? Anti-O-GlcNAc antibody does not labeled rApe specifically. How the authors did ruled out that the anti-O-GlcNAc antibodies recognized O-glycosylated on a protein different to rApe?

Author's answer: Identity of rApe was confirmed by Orbitrap and blasted with the possible major ferric iron binding protein precursor (Q5FFA9) of E. ruminantium. This has been included in material and method as well as in the results part. Figure 3 shows a band because the anti-O-GlcNAc antibodies recognized the O-glycosylated rApe protein we migrated in that well. We also obtained a band for a recombinant protein of a different size (data not shown), which was expected to be O-glycosylated and which was confirmed by the western blot.

Line 378: The results on E. ruminantium invasion after enzymatic treatment of BAEC with chondroïtinase are not clearly connected to those concerning the functional characterization of rApe. A question that could have been addressed was whether rApe binding (line 392) to BAEC decreases after chondroïtinase treatment of BAEC. This experiment could link (if there

was a link of course) the presence of GAG on the surface of BAEC to the binding of rApe to BAEC. This would have connected the chondroïtinase experiment with the functional characterization of rApe.

Author's answer: Chondroitin concentrations to 0.4 and 0.9U/mL resulted in FC of 0.52 and 0.58, respectively, corresponding to a 50% reduction of the number of bacteria compared to the untreated condition, which highlights the role of chondroitin sulfate and dermatan sulfate in adhesion of E. ruminantium to the cell. Since rApe, a E. ruminantium transmembrane protein binds to BAEC, we could expect that a chondroitin treatment of the BAEC before flow cytometry would have decreased fixation of rApe on the BAEC.

Line 385: The authors argued that increased FC with 0.2U/mL 'may be explained by inter-well variability', but the same could apply for decreased FC with 0.4U/mL and 0.9U/mL, i.e., 'decreased FC with 0.4U/mL and 0.9U/mL may be explained by inter-well variability'. Please, add the mean and standard deviation for each experimental condition as well as the statistical analysis. In the legend, it would be valuable to also add the amount of technical and biological replicates included in this experiment.

Author's answer: this assay was done once with two PCR replicates with similar results. Moreover, we didn't present the result obtained with a concentration of 0.25U/mL, done as a biological duplicate and for which a FC of 0.885 and 0.927 for each of the biological replicate was obtained. This FC is below the FC of 1.2 obtained with a concentration of 0.2U/mL

Line 393: Please, use the abbreviated form, E. ruminantium (same for line 410).

Author's answer: modification was done

Line 398: Not clear how this 'auto-fluorescence of the cells' was used as negative control. What happened when the auto-fluorescence of the cells was measured on cells without recombinant protein incubation?

Author's answer: In fact, we measured the fluorescence of the cells without incubation with recombinant protein. So the background signal obtained was considered as negative control, (fluorescence was low) and could easily be compared to the condition with GFP fluorescence of the recombinant protein

Lines 399-400: This statement is not very precise. With 70 µg of rApe, the curve is still increasing and a plateau has not been reached. Please, correct. Two of three more rApe concentrations could have been used to find the saturation.

Author's answer: terms used were changed to 'bending of the curve starts'

Line 414: Was this 'adsorption' of the beads after 30 minutes incubation quantified? If yes, add the quantitative results.

Author's answer: No quantification was done. It was more a qualitative than a quantitative approach

Line 428: If goats were not vaccinated against 'rApe', then how the authors know that the animals 'showed an increase in humoral response against rApe'. Or are the authors referring to the fact that the sera of animals 'vaccinated with an inactivated or attenuated bacterial

vaccine' recognized the rApe in an ELISA assay suggesting an immune response against native Ape? Please, reword or clarify.

Author's answer: rApe is a transmembrane protein of E ruminantium. Since vaccination were performed with inactivated or attenuated E. ruminantium, it is suggested that rApe could have elicited an immune response, which explains the humoral response we showed in this article

Lines 423-433: Please, include the antibody isotype that was measured here.

Author's answer: done

Figures

Figure 1. The 'axis y' shows the 'log 10' (legend, line 732). However, this is not what is written in the 'axis y' of the figure 1A. Please, be consistent.

Author's answer: modification was done

Figure 2. The image shown in Figure 2A is not explicit enough. Please, provide arrows and increase the magnification to show details. What is the difference between the 3D structures shown to the left and the right of panel A.

Figure 3. Not clear in which way this figure shows that 'rApe is an O-glycosylated recombinant protein.' Please, use arrows to show the bands of interest and provide the molecular weight of each protein in the protein ladder.

Author's answer: Figure 3 shows a band because the anti-O-GlcNAc antibodies recognized the O-glycosylations of the rApe protein we migrated in that well. An arrow showing rApe was added.

Figure 6. Not clear in which way this figure shows that 'rApe interacts with cell lysate and membrane fractions'. Please, use arrows to show the bands of interest and provide the molecular weight of each protein in the protein ladder.

Author's answer: on the membrane were transferred after migration the different cell fractions (cytoskeleton, membrane and organelles) and the whole cell lysate. This membrane was then incubated with rApe. The presence of rApe was revealed by the use of an anti GFP HRP antibody. Since bands are seen on the whole cell lysate and the membrane and organelles fraction, it means that rApe could specifically attach to proteic elements which composes the fraction and lysate.

Figure 9. This figure was not cited in the text. Please do so.

Author's answer: This figure was cited Line 472