Author’s response to reviews

Title: Development of a multiplex PCR to detect and discriminate porcine circoviruses in clinical specimens

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Author’s response to reviews:

Dear Editor,

On behalf of my co-authors, we thank you very much for giving us an opportunity to revise our manuscript, we appreciate editor and the reviewers very much for their positive and constructive comments and suggestions on our manuscript entitled “Development of a multiplex PCR to detect and discriminate porcine circoviruses in clinical specimens” (ID:INFD-D-18-02099R1).

We have studied reviewer’s comments carefully and have made revision which marked in red in the paper. We have tried our best to revise our manuscript according to the comments. Attached please find the revised version, which we would like to submit for your kind consideration.

We would like to express our great appreciation to you and the reviewers for comments on our paper. Looking forward to hearing from you.

Thank you and best regards.
Your sincerely,

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Responses to reviewers:

Reviewer 1 (Karen M Harmon):

1. The authors describe a multiplexed PCR for the detection of Porcine Circoviruses types 1, 2, and 3. Various assays for these targets are available singly or in combinations of two of the three but nothing for all three. As more PCV subtypes are identified, this is a useful tool, but in this reviewer’s opinion, case must be taken in interpretation of results, especially when clinical significance of the various agents may not be well understood.

Reply: We greatly would like to express our thanks for the confirmation of our work. According to your suggestions, we added an animal experiment to verify the multiplexed PCR assay, and added some interpretation of the case in the results section and discussion section in the MS.

2. Recommended edits:

Line 10: change "special primers" to "primer pairs".

Reply: Thank you for the careful review. We have changed “special primers” to “primer pairs”.

Line 11: change "detected" to "tested".

Reply: We have changed “detected” to “tested”.

Line 15: change "and PCV3" to OR PCV3”.

Reply: We have changed “and PCV3” to “OR PCV3”.

Line 78: How many GenBank sequences of each were used in primer design?

Reply: Thirty six nucleotide sequences were obtained from GenBank for primer design. The sequence number of GenBank are as follows: PCV1 (KX827790.1, KY437725.1, KX827784.1, KX827782.1, KX816645.1, KJ808815.1, KJ680360.1, KJ408799.1, KF732857.1, KC990120.1, KC447455.1, JX566507.1), PCV2 (MG229682.1, MK405697.1, MK820653.1, MK347411.1, MK347407.1, MK347391.1, MK347387.1, MK347360.1, MK347352.1, MK347351.1,
MK542370.1, MH553305.1), and PCV3 (KX898030.1, KX966193.1, KY075988.1, MF069116.1, MF162298.1, MH823220.1, MH683051.1, MH916636.1, MH636589.1, MH548437.1, MH547278.1, MH107163.1).

Line 151: change to "was added to make a total volume of 50 ul per reaction" or similar.

Reply: We have changed as suggested in our MS.

Line 163-164: I assume the PCV1, PCV2 and PCV3 target genes were amplified in the multiplexed reaction. The way it is currently worded is ambiguous in that it may be interpreted that these were run with the individual primer pairs. Please clarify.

Reply: Thanks for your careful review and constructive suggestion. We added the sentence “Each DNA/cDNA of the virus mentioned above was amplified using the three defined primer pairs in a reaction respectively.” in our MS. We hope it will be better.

Line 172: Change "and" to "or"

Reply: We have changed “and” to “or”.

Line 177: change "detected" to "tested"

Reply: We have changed “detected” to “tested”.

Line 205: change "known" to "believed" or similar.

Reply: We have changed “known” to “believed”.

Lines 228 - 229: change to "the sizes of the amplified PCR products..."

Reply: We have changed as suggested in our MS.

Lines 237 and 240: change "Practiced" to "run"

Reply: We have changed “practiced” to “run”.

Line 240:1: change to "conventional PCR instrumentation" (or equipment)

Reply: We have changed it in our MS.

Line 261: change "consumption" to "cost"

Reply: We have changed “consumption” to “cost”.

3. Were any of the specimens tested negative for all 3 of the viruses? From Table 2 it would appear so. A column in this table with number of samples negative for all 3 agents would be
helpful. Also, were any samples from "non-clinical" pigs tested? This would also be useful information to evaluate the presence of these agents in the absence of clinical signs to perhaps understand how to best use this assay.

Reply: Thanks for your careful review. Yes, there are some specimens tested negative for all 3 of the viruses. We added an animal experiment and tested the samples used the assay we developed in our MS. The results showed that it can test the viruses in the samples from "non-clinical" pigs, and indicated that it is adaptable for testing field samples.

4. Given the very scarce evidence of the involvement of PCV1 in pathogenicity (only one paper referenced here suggests a link, and the final statement in that paper is "More research is needed to confirm the pathogenic character of PCV1 for porcine foetuses.") I think this paper needs to be very careful about stating that this multiplex PCR could be used as a diagnostic tool for monitoring PCV1. Perhaps it would be more appropriate to say this multiplex PCR could be useful in determining the presence of the various PCVs, but at this point I think it is premature to say it would be a useful diagnostic tool. There should also be a statement that the significance of detection by PCR must be coupled with the clinical status of the animals being tested, and in the case of PCV1 in particular, additional research should be performed to better explore its role in pathogenesis.

Reply: Thank you for the careful review. About the pathogenic character of PCV1, little research has been done on it, and more research is needed to confirm the pathogenicity of PCV1 in the future. And we added a few words to discuss why it was necessary to know the prevalence, or incidence of this potential pathogenic agent in our MS. We think the assay we developed will be adaptable for testing field samples based on the results of the animal experiment. We explained the significance of detection by PCR in clinical specimens and additional research should be performed to explore its role in pathogenesis of PCV1 in DISCUSSION section as suggested. Thanks for your constructive suggestions again.

Reviewer 2

GENERAL COMMENTS: This study describes the development and testing of a multiplexed PCR assay for the detection of PCV-1, PCV-2, and PCV-3. The assay would be useful in resource-limited locations that do not have access to real-time PCR capabilities. However, additional information regarding assay design and development/optimization is needed to strengthen the manuscript.
Major comments:

Some additional details about the assay design are needed. Please see MIQE guidelines for publishing real-time PCR assays (PMID: 19246619). While this is guidance for real-time PCR, it contains useful information for PCR-based assays. Specifically:

1. How many sequences were aligned to design the different primer pairs used? Did this alignment and analysis encompass all of the known diversity within the three different virus strains?

Reply: Thanks for the confirmation of our work. Thirty six nucleotide sequences were obtained from GenBank for primer design. The sequences number of GenBank are as follows: PCV1 (KX827790.1, KY437725.1, KX827784.1, KX827782.1, KX816645.1, KJ808815.1, KJ680360.1, KJ408799.1, KF732857.1, KC990120.1, KC447455.1, JX566507.1), PCV2 (MG229682.1, MK405697.1, MK820653.1, MK347411.1, MK347407.1, MK347391.1, MK347387.1, MK347360.1, MK347352.1, MK347351.1, MK542370.1, MH553305.1), and PCV3 (KX898030.1, KX966193.1, KY075988.1, MF069116.1, MF162298.1, MH823220.1, MH683051.1, MH916636.1, MH636589.1, MH548437.1, MH547278.1, MH107163.1), which encompass almost all of the known diversity within the three different virus strains.

2. What kind of in silico analysis was done to confirm the specificity of the designed primers? Was there any significant sequence homology with other viruses that should then be tested?

Reply: Blasting online in NCBI was used to confirm the specificity of the designed primers. And there wasn’t any significant sequence homology with other viruses in this study.

3. What were the gene targets? Table 1 states a target gene, but one is not indicated in the table.

Reply: Thank you for the careful review. We are sorry for missing the information of the gene targets. We added it in Table 1.

4. What concentration of virus was used in the inclusivity and exclusivity testing? It looks like the LODs were done on a mixture of the positive controls. What impact did multiplexing have on the individual assay performance? Were LODs for the individual assays run separately before running them as a multiplex? Was any interference seen when adding all of the primers together?
Reply: Thanks for your careful review. The concentration of virus used in the inclusivity and exclusivity testing was about 105.2TCID50/mL for each strain. Yes, the LODs were done on a mixture of the positive control. The influence of multiple PCR for single PCR mainly embodied in the amplification efficiency of primers. Before the multiple PCR developed, we designed about 10 primers for selecting to use in this study. And after too many times testing, the three primers in our MS were selected to develop this assay. There wasn’t any interference seen when adding all of the primers together.

5. How many replicates were tested for the LODs, inclusivity, exclusivity, and clinical sample testing? How many of the replicates had to test positive for a sample to be considered positive? How many replicates were run at each virus' LOD to demonstrate reproducibility? Do you see differences in performance if you run the dilution series with virus as opposed to a plasmid?

Reply: Three to six replicates were tested for the LODs, inclusivity, exclusivity, and clinical sample testing. At least three replicates had to test positive for a sample to be considered positive. Three to six replicates were run at each virus' LOD to demonstrate reproducibility. There are no differences in performance between the dilution series virus and the plasmid as template after tested using the developed assay.

6. How were the tissues homogenized, and how much tissue was processed? Line 136 says the tissues were diluted 1:5 in PBS and then homogenized? How do you dilute tissues in PBS?

Reply: We are sorry for misunderstanding you here. Each sample including spleen, lung and lymph node, was cut into pieces in a homogenizer, added five times quality of PBS, and then homogenized the tissues using the homogenizer. We have revised in our MS.

7. Line 119: how was cDNA generated, and for which virus was cDNA generated?

Reply: We are sorry for missing this information, and we have added it in our MS.

8. Did you see any differences in viral presence among all of the different tissues you tested? Were there any tissues from the same animal that tested negative with other tissues tested positive?

Reply: Yes, there are some differences in viral presence among all of the different tissues we tested. And there are some tissues from the same animal tested negative while other tissues tested positive. Among the different types of tissues, the highest frequency of PCV1-positive, PCV2-
positive clinical tissues were found in lymph node samples, followed by lung and spleen samples. In contrast, the highest frequency of PCV3-positive clinical tissues was found in lung samples, followed by lymph node and spleen samples (not shown in this paper).

9. Additional information regarding the two different real-time PCR assays is needed.

- For the LOD studies, how does the real-time PCR Cq values compare to the multiplexed PCR results? What Cq value did you get when testing at the multiplex assay LOD?

Reply: About the LOD studies, the real-time PCR Cq values were 22 when testing at the multiplex assay LOD, which indicated that it is a little more sensitive than the multiplex PCR assay.

10. Line 201: Remove "therefore" as the referenced statement does not demonstrate PCV-2 is endemic worldwide.

Reply: We have removed “therefore” as suggested.

ADDITIONAL REQUESTS/SUGGESTIONS:

Minor comments:

- Please change "species" to strains to describe PCV-1, PCV-2, and PCV-3.
- I would remove "rapid" as a descriptor for the assay. Real-time PCR would be rapid compared to a standard PCR followed by running a gel for identification.
- Line 177 and elsewhere: change "detected" to "tested"
- Minor editing would be helpful

Reply: We have revised our MS as suggested. Thanks for your constructive suggestions again.