Author's response to reviews

Title: Evaluation of real-time PCR for diagnosis of Bordetella pertussis infection

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Author's response to reviews: see over
Thank you for considering our manuscript for publication in BMC Infectious Disease. The comments from the two reviewers were very helpful. We believe we have been able to respond to the comments, requested changes and critique. We have modified the manuscript accordingly.

**Reviewer SKP in the general section comments that the concluding statement on page 2 regarding lack of impact from *B. holmesii* in Alberta does not address the possibility of changes in epidemiology over time.**

We have re-written this statement as the reviewer is correct that we can only really comment on lack of impact during the time-frame of the study. We undertake periodic analysis of specimens retrospectively to assess any change in *B. holmesii* as discussed in the final part of the manuscript.

**Reviewer SKP in the minor essential revisions section queries the necessity of Table 1 as much of the information repeats previous publications.**

As suggested, we have removed Table 1 in the revised manuscript and included the new sequences for the novel TPR assay in the text instead.

**Reviewer SKP in the minor essential revisions section asks about the novel nature of the TPR assay.**

This may not have been clear in the original text and we have made some minor modifications to clarify. The TPR assay was designed for this study using the TaqMan platform and primer express software. This is the first report of its development and use and the primers and probe are novel. Those with access to the ABI PCR platforms and software should be very familiar with the approach used and we are not aware of any general references to the system beyond the literature from ABI directly that would be relevant here.

**Reviewer SKP in the minor essential revisions section asks that we refer to a publication for the two base pair change between *B. pertussis* and *B. holmesii***

This has been done in the revised manuscript.

**Reviewer KC in the major compulsory revisions asks for re-organization of the abstract to shorten the background and provide more experimental detail as well as correct the punctuation.**

In the modified version of the abstract the suggested changes have been made. We believe this revised abstract better reflects the study and is easier to read. The background has been cut back and the methods, results and conclusion expanded to provide more detail.

**Reviewer KC in the major compulsory revisions asks for clarification on the processing of specimens.**

The Regan-Lowe medium did contain cephalexin which would have prevented isolation of *B. holmesii*. However, only 12% of the specimens in the study were collected in this medium. Also culture was not relied on for identification of *B. holmesii*. The PCR molecular beacon assays which have been evaluated and published previously for
detection and differentiation of *B. pertussis*, *B. parapertussis* and *B. holmesii* (Templeton et al., reference 8 in the manuscript) were utilised in our study for identification of *B. holmesii* in extracted samples. These assays were found to be very sensitive for *B. holmesii* and performed according to the published literature. Thus our interpretation of negative results for *B. holmesii* in our population during the time course of the study seems to be valid. We have amended the text and provided some additional data in the manuscript to clarify our use of these additional assays to address this point (and also to respond to comments below). This was an omission in our original submission in an attempt to reduce the length of the manuscript.

**Reviewer KC in the major compulsory revisions asks for clarification on the potential for different samples volumes analysed by PCR and culture.**
Although some specimens came without a DFA slide pre-prepared (and thus these had to be split three ways) the volume of specimen analysed by PCR and culture was always the same. An extra sentence has been added to the methods section to make this point clearer. Information concerning the number of specimens for which a slide was submitted for DFA is not available but the comparison between methods is still valid.

**Reviewer KC in the major compulsory revisions section identified two mistakes in the primer/probe sequences.**
Based on comments from the other reviewer, this Table has been removed and only the novel primers and probes (for TPR) have been included in the text. Sequences for these have been double checked.

**Reviewer KC in the major compulsory revisions section asked for the Results section to be re-organized for clarity with analytical validation of the IS481 assay to come first.**
This has been done in the revised manuscript.

**Reviewer KC in the major compulsory revisions section asked for the information in the Venn diagram in Figure 2 to be replaced by expanded text.**
As the alternative reviewer did not make this suggestion and the consensus view of the authors is that the use of a Venn diagram helps in interpretation of the results, we have left this Figure in the modified manuscript. The text for the manuscript is already complex because of the multiple assays used and so we would prefer not to take this Figure out. The loss of Table 1 has reduced the overall manuscript length.

**Reviewer KC in the major compulsory revisions section asked for details on specificity checks undertaken and interpretation of PCR positive results which were DFA/culture negative**
The specificity of the newly designed TPR assay is inherent in the design and extensive use of sequence alignments for closely related organisms. The assay did not cross-react with *B. holmesii*, *B. bronchiseptica* or *B. parapertussis* and details on this have been added to the revised manuscript (and are provided in Figure 6). Although an extensive range of other related organisms were not tested in this assay, this was not considered necessary because it is used only as a confirmatory assay for specimens already identified
as positive in the IS481 HP assay. The latter assay has been extensively evaluated for cross-reaction with a wide range of other bacteria (Bordetella spp. And others) with only the widely reported pick up of B. holmesii being identified as a clinical problem (e.g. Reischl et al. 2001, reference 7 in the manuscript). B. hinzii does occasionally cause respiratory infection and sepsis but is not picked up by assays targeting IS481 (J. Clin Micro. 43: 30-35). We do not have access to the latter organism to check this lack of cross-reaction experimentally for the two different IS481 assays we have used in his study but a false positive reaction with this organism would not be anticipated based on available sequence data. We can find no evidence of B. avium as a human pathogen but, again, based on available sequence data we would not expect a problem with cross-reaction with this target.

The problem of confirmation of B. pertussis in the 28 IS481 HP PCR positive results is difficult to resolve completely. Although the assay is slightly less sensitive, the IS481 MB assay confirmed the positive result in 26/28 of these cases. Although targeted to the same insertion sequence, this assay is from a different region which helps to eliminate PCR product contamination as a source of false positive reactions. Although 12% of specimens were collected in transport media which would have inhibited culture of B. holmesii our study did not rely on culture for confirmation. If B. holmesii was present in these specimens we would have obtained a positive result in both the IS481 MB and IS1001 MB assays. The latter assay gave positive results for only two samples from which B. parapertussis was isolated.

Reviewer KC in the major compulsory revisions section asked for more information on the results for the 1001 MB assay.
The relevant results section has been expanded slightly to clarify that 95 specimens (68 IS481 positive and 29 IS481 negative) were tested in the 1001 MB assay with identification of 2 B. parapertussis positives that were confirmed by culture. This study was not intended for identification of parapertussis and this would still be undertaken by culture in our laboratory if specifically requested by the submitter.

Reviewer KC in the major compulsory revisions section asked for clarification of how IS481 positive/TPR negative results would be reported.
Modifications have been made to the final paragraph to make our current laboratory algorithm clear. Based on our study data (and publications) we report all IS481 PCR positive results and have provided educational material to physicians on assay interpretation. We ask them to contact us in case of unexpected results (e.g. where results do not fit with the clinical presentation or outcome) for on-going evaluation of our algorithm. TPR, IS481 MB and 1001MB assays are only undertaken retrospectively for our continued QC and monitoring of potential pick up of B. holmesii or other related organisms. We do not currently offer molecular based assays for B. parapertussis.

Reviewer KC minor compulsory revisions
These minor points have been addressed as requested.

Reviewer KC discretionary revisions
We did not feel we had anything further to add on the clinical relevance of *B. holmesii* from our study but this is clearly an interesting topic.

In our current laboratory algorithm we still undertake culture for *B. parapertussis* where clinically indicated (and requested). *B. parapertussis* is not reportable to Public Health in Alberta, there is no vaccine for prevention, prophylaxis is not used for contacts and there is no evidence that treatment has an impact on course of the disease. Thus in our Province we cannot justify the expense of molecular approaches to fill the diagnostic gap in this area, although this may be a future expansion we will consider should resources become available.