Author’s response to reviews

Title: Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG Respiratory Virus Panel Fast assay, RespiFinder 19 assay and RespiFinder SMART 22 assay

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Version: 3 Date: 28 January 2012

Author’s response to reviews: see over
Submission for publication in *BMC Infectious Diseases*

**Our manuscript MS 1084908583595467; your letter from 15th December 2011**

Dear Dr. Diana Marshall,

Thank you very much for the favorable review of our manuscript “Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG Respiratory Virus Panel Fast assay, RespiFinder 19 assay and RespiFinder SMART 22 assay”.

Enclosed please find the revised manuscript which was modified according to the reviewers` suggestions and a point-to-point response to the reviewers` remarks. We thank the reviewers for their constructive comments and corrections. We gratefully accepted all the comments and corrections, corrected the remarks, added the missing information and worked detailed on the manuscript. All changes made are highlighted in the manuscript (underlined), also further references were added and the reference list was updated.

We hope that the manuscript is now suitable for publication in *BMC Infectious Diseases*.

Yours sincerely,

J. Dreier

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Reviewer 1

Title: Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG Respiratory Virus Panel Fast assay, RespiFinder 19 assay and RespiFinder SMART 22 assay

Version: 2 Date: 30 November 2011
Reviewer: Guus Simons

Reviewer's report:
This is a well written and sound manuscript comparing three multiplex PCR assays for detection of respiratory pathogens. Multiplex PCR assays are gaining more ground in analyzing acute respiratory tract infections. In the article quantified standarized control material in either a mixture of 13 or 4 viruses is used. In addition, two multiplex assays are compared on clinical specimens and hands of time and time of result of the three tests is given.

Minor Essential revisions:

1. The performance of the three multiplex assays was compared to monoplex real time PCR. However, no QPCR data such as threshold cycles values are shown. Strikingly, PCR conditions of up to 50 cycles are used. It is known that ct values > 40 are not relevant at all. It is stated that none of the three multiplex assays was capable of detecting 13 or even 4 viruses at the same time. Ct values of the undiluted, 1:10, 1:100 and 1:1000 are needed for a full comparison.

   Table 1 was splitted (table 1 and 2) according to the suggestion of reviewer 2. Ct values for samples inoculated with 13 viruses (undiluted, 1:10, 1:100 and 1:1000) were given in table 1 and Ct values for samples inoculated 4 viruses (undiluted, 1:1 and 1:100) are given in table 2. We agree with this comment that Ct values >40 are not relevant. The different PCR assays had conditions of up to 50 cycles, however the cut-off value for the decision positive/negative was set to <40 cycles, and none of the analyzed samples had CT values >40. We reduced the specification of conditions to 45 cycles in the Material and Method section, and added the information regarding the cut-off for positivity.

2. To assess the clinical performance only RVP and RespiFinder 19 are compared. No comparison was made with RespiFinder Smart 22 because the analysis of 100 sample was already started before RespiFinder Smart 22 was commercially available. But on page 3 it is stated that the detection rate of RVP and RespiFinder Smart 22 was only 22%? Fewer detections were found........RespiFinder Smart-22: 4%. Please clarify!!

   Page 3: Sorry, this specification was an oversight, we deleted this part “RespiFinder Smart-22: 4%” from the text.

3. Page 3: Conclusions: Multiplex PCR tests have a broad spectrum of pathogens to test at a time, but a lack of sensitivity in comparison to monoplex assays. This conclusion is not correct. In table 2, data obtained with RespiFinder 19 and real time PCR show a very high level of concordance. This indicates that in real life (clinical specimens) RespiFinder 19 is as sensitive as monoplex real time PCR. Moreover, on page 9 it is stated that the viruses that were only detected with RespiFinder 19 showed high cycle threshold points (how high?) in
monoplex PCR methods due to low virus concentrations indicating a high level of sensitivity of the multiplex assay. 

*This statement was revised in accordance to the reviewer’s comments.*

4. Please explain why CMV is tested. In general CMV is not regarded as a respiratory pathogen.

*Page 8 and page 13: TS samples were initially analyzed for bacterial and viral pathogens with our routine diagnostic profile including CMV, Legionella pneumophila, Pneumocystis jirovecii, Mycoplasma pneumoniae and Chlamydophila pneumoniae. The residual material was used for the comparative analysis with the RespiFinder-19 and RVP assay. In order to exclude CMV pneumonia due to reactivation processes, the data was also presented for completeness. CMV is not aWe added this explanation in the manuscript.*

5. Page 2: Background: Therefore, the identification of the causative viruses and bacteria is only feasible using........

*The modification was performed according to the reviewers’ suggestion.*

**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:**  
Yes, I am the CEO of PathoFinder the manufacturer of RespiFinder 19 and RespiFinder Smart 22. However, this research was performed independently and without any correspondence or influence by me or any PathoFinder employee.

**Reviewer 2**  
**Title:** Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG Respiratory Virus Panel Fast assay, RespiFinder 19 assay and RespiFinder SMART 22 assay  
**Version:** 2  
**Date:** 29 November 2011  
**Reviewer:** Kanti Pabbaraju  

**Reviewer's report:**  
In this study, three multiplex PCR assays: RespiFinder-19, RespiFinder-SMART- 22 and RVP assays were compared for the detection of a range of respiratory viruses. The authors claim this study as a systematic comparison of sensitivity between the three assays by using quantified standardized control material.

Major compulsory revisions:  
**RESULTS:**  
The study used external plasmid standards for the quantification of virus control material, It would be useful to have a table with the key parameters such as  
- End-point sensitivity  
- Efficiency  
- Dynamic range  
- Specificity
• Reproducibility
For the standard curves used. This would give the reader confidence in the quantification numbers.

We added this information in the methods section (page 16). The specificity of all monoplex-real-time PCR assays was determined by the exclusion of cross-amplification with different bacterial or viral DNAs/RNAs (eight bacteria, seven viruses). The analytical sensitivity was determined to be <10 copies/ml (page 17). We added a table (table 5) showing the key parameters for real-time PCR assays.

The authors have spiked PBS with 13 different quantified viruses simultaneously and used the extract for detection of all the respiratory viruses. They have shown big difference in the detection of these viruses using the three multiplex PCR assays. They mention that all the viruses were picked by real-time PCR. It would be essential for the reader to know how efficiently the 13 viruses were extracted from the sample, this can be determined using the Ct values from real-time PCR assays, since these values are semi-quantitative. Results of the real-time PCR should be included in Table 1.

Ct values were included in table according to the reviewers' suggestion.

Also what is the medical relevance of trying to detect so many targets simultaneously when this current study in addition to others shows that it is uncommon to detect more than 3 viruses simultaneously especially by a multiplex assay. Do you believe that the assay is being appropriately evaluated when it is tested with so many target templates that causes competition between reagents.

We agreed with the reviewers' comment that under real-life conditions a parallel infection with 13 viruses is uncommon up to impossible. This artificial inoculation was only performed to evaluate the performance of the three test systems. For evaluation of real-life conditions, clinical multiple infections were simulated by quadruple inoculation with the three different virus combinations (see comment reviewer 3).

It is mentioned that “The RVP showed the detection of influenza A virus (1.42E+02 copies/ml), RSV (3.94E+01 copies/ml- SHOULD THIS NOT BE 3.94E+02 COPIES/ML) and hMPV (1.78E+02 - SHOULD THIS NOT BE 1.78E+01 COPIES/ML copies/ml) in high dilution ratio (Table 1).

We corrected the concentrations according to the reviewers' suggestion.

For some targets such as Influenza B, PIV 1 and 3 none of the assays detected even the undiluted samples, it would be important for the reader to know how efficiently these were detected by real-time PCR. Also were these targets tested using a higher template concentration to determine the end-point sensitivity for the assays.

CT values of real-time PCR analysis were given in table 1 and 2. A higher template concentration could not be tested due to the absence of available control material.

There was a difference in the detection of pathogens from samples that were eluted in 55ul Vs 100ul only by the Respi-Finder-19 and SMART-22 assays, not the RVP assay even though it is the RVP assay that recommends a 55 ul elution volume. How do the authors interpret these results and what do they propose for their diagnostic algorithm? Also how reproducible was this result?

The manufacturer of the Respi-Finder-19 and SMART-22 assays (PathoFinder) recommend an elution volume of 100 µl, whereas the manufacturer of the RVP assay recommend an elution volume of 55 µl. In our routine diagnostic setting, we used a standard elution volume of 55 µl. Hence, this elution volume was also used for
comparison. As mentioned in the text (page 13) TS samples were initially analyzed for bacterial and viral pathogens and the residual material (DNA/RNA extract) was used for the comparative analysis with the RespiFinder-19 and RVP assay. To avoid false negative results due to a deviation from the manufacturer instructions, we extracted residual material and used an elution volume of 100 µl. This explanation is now given in the Material and Methods section, page 14.

As mentioned by the reviewer, only the two Pathfinder assays showed a difference in the detection. No differences were found in the detection with the RVP assay, although we expected that the dilution of the DNA/RNA extract may result in a reduced analytical sensitivity. We discussed the results obtained with different elution volumes and consequences for diagnostics in more detail in the discussion section (page 12). We did not analyze the reproducibility of these results, but for the example of influenza virus detection or RSV detection by the RespiFinder assays, the different dilutions show concordant results, assuming an informative value.

The second part of Table 1 is difficult to read, this should be split as an additional table.

*This table was split (table 1 and 2) according to the reviewers’ suggestion. Furthermore, CT values of monoplex real-time PCR assays were added to both tables.*

In the results the authors should systematically talk about the comparison of the different targets by the three assays and compare sensitivity for the different targets.

*This section was rewritten and we described the comparison of the three targets in more detail according to the reviewers’ suggestion (page 6ff).*

Table 2 would be more useful if the data was organized by target and the number of positives detected for every target by each of the methods, alternatively this table should be deleted since Figure 1 has the same data.

*This table was reorganized according to the reviewers’ suggestion. Based on splitting of table 1 into two tables, this is now table 3.*

In this sentence “All positive results for respiratory viruses, which were detected with RVP and RespiFinder-19, were confirmed by realtime PCR” do the authors mean that there were no positives detected by either of the methods (RVP or Respifinder 19) that were not confirmed by real-time PCR? This does not come out clearly.

*This sentence was rewritten for clarity according to the reviewers’ suggestion.*

Please comment on ease of use and subjectivity involved in making a positive call for the different assays since these are important factors in a diagnostics lab.

*For this question we replaced figure 2 by table 4 and added further information about test specifications, costs, performance and accomplishment. This table was discussed in the discussion section (page 12). Furthermore, the interpretation of results obtained by the different assays is discussed (page 13).*

**DISCUSSION:**

In the discussion the authors state that “We observed that the focus on the virus detection differs between the three assays, the RVP, the RespiFinder-19 and the RespiFinder-SMART-22 with inoculated samples in different virus concentrations, but the detection limit of all assays was almost the same” Please explain what you mean by “the focus on viral detection differs.” Please explain what you mean by detection limit of all assays was almost the same.”
In some cases the pathogens of the co-infections cause more serious illnesses, e.g. coronavirus RSV or hMPV, than the pathogens of the primary infection, e.g. rhinovirus. How can you determine the primary pathogen and co-infecting virus; please clarify.

*With this study concept we could not differentiate between the primary pathogen and co-infecting viruses. For a differentiation, the clinical course of patients has to be monitored by sampling in scheduled intervals. We added this point in the discussion section (page 12).*

The detection of multiple infections was only possible with RespiFinder-19, please mention that other studies have reported the detection of co-infections by the RVP assay. For routine application sensitivity must balance out time-to-result, Do you believe that the compared assays have a significantly different turn-around-times to impact patient treatment?

*We added a reference reporting the detection of co-infections by the RVP assay according to the reviewers’ suggestion. We further discussed turnaround-times and the resulting impact on patient treatment in the discussion section (page 15).*

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare I have no competing interests.

**Reviewer 3**

**Title:** Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG Respiratory Virus Panel Fast assay, RespiFinder 19 assay and RespiFinder SMART 22 assay

**Version:** 2  **Date:** 9 December 2011

**Reviewer:** Marek Smieja

**Reviewer's report:**

Summary: Dabisch-Ruthe and colleagues examine the analytic sensitivity of three commercial multiplex assays for detection of respiratory viruses. When 13 viruses are all analyzed together, all three assays miss a number of the included viruses, although performance is considerably better using a mix of only four viruses together. The RVP assay detected lower concentrations of influenza, RSV, and rhinovirus, whereas the two RespiFinder assays detected lower concentrations of coronaviruses and adenoviruses. For clinical evaluation, 100 tracheal aspirate specimens from ventilated patients in the Intensive Care Unit are assayed by two or three kits. A large number were found to have parainfluenza or adenovirus by the RespiFinder19 kit, but not by the other kits.

Major Compulsory Revisions

1. The authors’ objectives for conducting this study are not adequately described. Was the objective to compare the analytic sensitivity for each individual virus with each of the three commercial multiplex assays, and therefore to have some comparison for which viruses are
best detected with which assay? Or was the purpose simply to determine the relative sensitivity for mixtures of 4-13 viruses in the same sample? Those are two very different questions. I see limited utility in attempting to detect 13 infections in the same sample (we have seen up to 6 infections simultaneously in a child), although having done this, it is appropriate to briefly report it without over-interpreting the information. Thus, a brief paragraph summarizing that all multiplex assays performed terribly when tested with this highly artificial mocked experiment is adequate, and may lead to some new thinking on how interference between targets may affect detection. Generally we are most interested in whether we can reliably detect influenza and RSV (which are the only two viruses for which we currently have effective treatment and prevention), and it is interesting that some of the kits detected these viruses even in the presence of 13 viruses in total.

We agreed with the reviewers’s comment that under real-life conditions a parallel infection with 13 viruses is uncommon up to impossible. This artificial inoculation was only performed to evaluate the performance of the three test systems. Clinical multiple infections were simulated by quadruple inoculation with the three different virus combinations. The aims of the present study were as follows: (a) the quantification of commercially available qualitative control material for (b) the evaluation of the performance and sensitivity of the three multiplex assays RespiFinder-19, RespiFinder-SMART-22 and RVP fast assay and (c) the applicability and performance of these multiplex PCR assays in a routine setting. Furthermore, this is the first screening study determining the incidence of infections with respiratory pathogens in a mechanically ventilated patient cohort using multiplex PCR assays. We modified the relevant section in the text and described our study aims in more detail (also referring to comment 2). Furthermore, we added a paragraph regarding the general limitations of multiplex PCR assays (discussion section, page 11).

2. If the primary purpose was to compare assays for analytical sensitivity for each virus, these should be done separately for each virus and not initially combined. Statistical analysis should then be used to appropriately determine whether increased analytical sensitivity has been demonstrated or not. I would suggest repeating the experiments with individual viruses, in replicates of at least 3 for each dilution, and using probit regression or other appropriate statistical models to prove a difference in detected concentrations. If the primary purpose is NOT to compare the individual viruses, but only to examine their interaction within multiply infected mocked specimens, then the authors need to be much clearer in describing this as their purpose, and more open in the limited clinical relevance of such experiments. Even here, unless an adequate number of replicates are done, no statistically significantly difference will be observed. Hence, all of the reported differences could simply be due to chance.

We agreed with the reviewers’s comments. The aim of this study was not the initial determination of the analytical sensitivity of each assay, because up to now no quantified control material is commercially available for a systematic determination of single virus LODs. Therefore, our first approach was at least the quantification of commercially available qualitative control material on the basis of copy standards. In a second approach, we determined the performance and the range of assay sensitivity. Unless samples were not analyzed in an adequate number of replicates to perform statistical analysis, our approach allow the comparison of the performance and the range of sensitivity of the three multiplex assays by analysis of inoculated samples with quantified virus material. Reported differences may be due to chance regarding a single consideration of individual results, however a tendency towards difference in lower or higher detection efficiencies by the different assays is observable. We added this statement in the discussion section (page 15, study limitations).
3. The authors provide virtually no description of their patients. Usually nasopharyngeal swabs or aspirates, and not tracheal aspirates (TA) or broncho-alveolar lavage specimens, are the preferred specimen for detection of respiratory tract viruses. Were NPS or NPA samples collected? Were the TA samples collected for clinical diagnostic purposes? TA samples may be used as a supplement, but there is much less published on their clinical utility. We also need to know who these patients were—were these all children, were they severely immunocompromised? Was there an outbreak of adenovirus or parainfluenza within the hospital or ICU? I have seen only a small number of patients end up on a ventilator as a result of these infections, usually these are co-infections with important bacterial pathogens. A more rigorous clinical evaluation would require consecutive or randomly sampled patients.

We added the information about patients in the material and methods section according to the reviewers’ suggestion (page 13). Tracheal secretion (TS) samples (n = 100) were collected from mechanically ventilated non-immunocompromised patients after bypass, heart or lung operations, who were suspected of atypical pneumonia during postoperative monitoring (material and methods, page 13). Patients ended up on a ventilator due to their severe previous disease, the suspicion for an atypical pneumonias primary arised under mechanical ventilation after X-ray examination. As mentioned by the reviewer, these infections are usually co-infections with bacterial pathogens. Therefore, the main suspected pathogens are bacteria or fungi and our standard diagnostic profile includes Legionella pneumophila, Pneumocytis jirovecii, Mycoplasma pneumonias and Chlamydothilia pneumonias. For bacteria, the recommended material for diagnostic purposes is tracheal secretion or broncho-alveolar lavage. For this reason, nasopharyngeal swabs, nasal swabs or nasopharyngeal aspirates were not collected from this patient cohort. This study comprised screening of a specific patient cohort to determine the incidence of infections with respiratory pathogens.

This study comprised the first screening for respiratory viruses in our hospital, however an accumulation of adenovirus or parainfluenza infections in a station or a chronological correlation was not observed.

4. The authors conclude that the RespiFinder 19 was more sensitive. This may be true for parainfluenza and adenovirus, but would be unlikely to be the case for influenza or RSV. Alternate explanations include non-specificity of the RespiFinder, contamination, or. These should be discussed.

This statement refers to testing of clinical samples. We added this information for clarity. In our opinion, a non-specificity of the RespiFinder could be excluded, because virus detection was confirmed by monoplex real-time PCR. Furthermore, we could exclude contamination of samples. However, the detection of incidental but non-causal virus was discussed in the discussion section (page 12-13) according to the reviewers’ suggestion.

5. The methods should be described before the results. I believe that some of the BMC instructions to the author specify that methods should be at the end, but this is a bizarre placement and certainly most readers expect to see methods before results.

We agree with the reviewers’ criticism but as mentioned by himself, BMC Infectious disease specify this section sequence. Therefore, we remain to the current section sequence.
6. The tables should be altered. The top of table 1 is of marginal interest, and might be better summarized in text. The bottom of Table 1, showing the four multiple infections per sample, is really the main result and needs to be expanded and reformatted in a clear manner.

   *Table 1 was splitted into table 1 (upper part) and table 2 (lower part). CT values were added to both tables according to the comments of reviewer 2. Table was reformatted for clarity and results were described in the results section in more detail (page 6).*

**Minor Essential Revisions**

1. All instances of the word “sensitivity” refer to analytical sensitivity, not clinical sensitivity, and therefore the more correct term is analytical sensitivity.

   *The modification was performed according to the reviewers’ suggestion.*

2. Words such as “impossible”, “not possible”, “systematic comparison” and “clinical usability” are used incorrectly and should be replaced. Without examining every virus in replicates and at various dilutions, and without including every commercial respiratory virus multiplex available (Luminex, Seegene, Resplex etc), this cannot be described as a systematic comparison.

   *The relevant sentences or chapters were rewritten according to the reviewers’ suggestion (impossible: page 4, page 10, page 11; not possible: page 2, page 6; systematic comparison: page 9, page 4, page 6; clinical usability: page 3).*

3. The virus combinations described under results, page 6-7 (which should be described under methods instead) appear incorrect, with 4, 3 [panel 2] and 5 [panel 3] virus mixes described. Elsewhere, 4 viruses per mix are described. This should be corrected.

   *The combination of virus panels were corrected throughout the manuscript, panels were as follows: Panel 1: INF-A H1N1, RSV-A, HRV and Adv; Panel 2: INF-A H1N1, INF-A H3N2, RSV-A, PIV-2; Panel 3: CoV OC43, CoV 229E, Adv, HRV.*

4. Reference 23 appears to be an abstract, but there is a 2011 citation of the same title. This reference should be updated from the 2009 citation to the 2011 citation.

   *The reference was updated according to the reviewers’ suggestion, now reference 9.*

5. Legionella is included on page 8 for the viral results; clearly this is not a virus.

   *This section was rewritten according to the reviewers’ comment.*

**Discretionary Revisions**

Table 2 and Figure 2 are of marginal benefit and could be eliminated.

*Due to the comments of reviewer 2, table 2 (now table 3) was not deleted. We deleted figure 2 but based on the comment of reviewer 2 “Please comment on ease of use and subjectivity...” we added a table (table 4) including the content of figure 2 and additional information.*

**Level of interest:** An article of limited interest

**Quality of written English:** Needs some language corrections before being published

**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.
List of changes made:
- Material and Methods: The description of monoplex real-time PCR assay was shifted behind the description for the RespiFinder 19 and SMART-22.
- The specification of concentrations was corrected throughout the manuscript.
- Grammatical errors were corrected.
- The enumeration of viruses included in the different assays was deleted in the methods section of the abstract, furthermore the conclusion section of the abstract was extended.
- The discussion section was partly reorganized.
- Two references were added and the reference list was updated.