Author's response to reviews

Title: Sequential multiplex PCR assay for determining capsular serotypes of colonizing S. pneumoniae

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Author's response to reviews: see over
Point to point response to editor and reviewer comments.

Editor comment:
In order to prevent confusion, the authors also need to make it clear that the primers used in the multiplex PCRs have already been published before but not developed in this study.

This has been clearly stated in the material and method (line 129-130).

Reviewer's 1 report
- Major Compulsory Revisions

Critically, the methods used by the authors are not described in sufficient detail. How was the DNA for the PCR reaction purified? In what conditions were the PCR reactions performed? What was the DNA polymerase used and in how many units per reaction? What was the total volume of the reaction and the magnesium chloride concentration? What was the cycling program used and how many cycles were performed? This is critical information for anyone trying to use the method described by the authors.

As mentioned in the MS lines 136-137, the DNA extraction and PCR methods were identical to those described in the paper published by Pai et al. To answer to the reviewer’s comment, we summarized the critical points of these methods lines 137-143.

- Minor Essential Revisions

1) Lines 72-73. The authors state that 91 serotypes are currently recognized, when in fact 93 distinct serotypes are now recognized. The recent identification of serotypes 6D and 11E brought the total number to 93. The authors should correct this sentence and cite the appropriate references.

This has been modified accordingly (line 73) and related references have been added (Jin, JID 2009 and Calix, JID 2010).

2) Page 5. The authors state that 362 S. pneumoniae isolates were available, but only 332 were tested by PCR. When doing the calculations it seems that the 30 non-typable isolates by the Quellung reaction were excluded, but if this was so it should be clearly stated in line 120.

This has been clarified line 123.

3) Lines 220-221 "As a correlate, our new scheme can be widely applied". Can the authors clarify the intended meaning?
This sentence has been clarified lines 225-228. “This assay could be used worldwide since serotype distribution of colonizing isolates is quite similar between different geographic locations, the most frequent serogroups being 6, 14, 19 and 23 [7]. Other colonizing serogroups frequently isolated from children are 3, 4, 9, 11, 13, 15, 18 and 33 [7].”

4) Line 242. The authors quote references 27 and 30. I believe reference 30 is not appropriate in this context. Maybe the authors meant reference 26 instead?

This is indeed an error and has been corrected (line 249).

5) Lines 248-252. This is an interesting speculation and would constitute a true novel finding. Do the authors have specific data on this?

We observed that the current primer pairs could not serotype around one quarter of the Belgian serotype 23 (line 253-255). At this stage, we can only state that one hypothesis might be that sequence variation in the target gene could explain this limitation. A complete sequencing of the capsular locus (10 to 30 Kb) would be necessary to answer this hypothesis.

6) I have not exhaustively checked the references, but references 26 and 28 are incorrectly formatted - in reference 26 the year of publication is not indicated and in reference 28 no mention of year, volume or page numbers is indicated. The authors should revise reference formatting.

The references have been checked and reformatted.

**Reviewer’s 2 report:**

1. The methods of the carriage study that yielded the Sp strains should be briefly described, to allow the reader judging on whether this was a representative sample of the age group, the number of colonies analysed per participant, and whether multiple carriage was detected.

This information has been added lines 109-111. “Briefly, nasopharyngeal aspirates were collected three times during the school years, the presence of 4 potential pathogens were analysed (S. pneumoniae, S. aureus, H. influenzae and M. catarrhalis).”

2. Part of the result section (approximately lines 134 to 163) appear to be a mix of methods, results and discussion. Maybe the manuscript could gain on clarity by re-structuring this section.

We believe that for clarity reasons, it might be better to briefly
explain the objective of each result as well as their main conclusion.

A technical question is, why only 332 isolates were included in the study. I understand that this is because 30 non-typable isolates were excluded, but I guess that it may have been interesting to see how specific PCR testing is for these non-typable carriage isolates. Maybe this can at least be addressed in one sentence.

This has been tested. The PCR of 23 of the 30 NTP isolates give no amplification at all (even for the CPS positive control). Five of them showed a positive CPS control but no amplification for any of the serotype specific primer pairs. Finally, the last 2 were positive for serotype 33F but the result of a secondary Quellung identification (performed in the National Reference Center) confirmed their identification as NTP. These data would therefore need some complementary analysis before a clear picture can be obtained. For clarity reason and as the aim of this technical advance paper was to confirm the reliability of the PCR method compared to the gold standard Quellung technique, we prefer to keep these data out the present MS. However, to answer to the reviewer comment, we added (lines 124-125) that “The vast majority of these isolates did not show any amplification for the cpsA internal positive control (data not shown)”

All data appear well reported, the discussion and conclusion balanced. Limitations are listed.

The theoretic geographic limitation of one algorithm should be discussed a bit more. Although the main serotypes in carriage seem to be similar across the world, as the author write, the minor serotypes may vary between countries and continents, as disease serotypes vary a lot. This is important for recommending that laboratories in Africa could simply adapt the presented algorithm or whether preliminary studies are required.

This is indeed an important point that has been clarified line 225-228 (see comment 3 from reviewer 1). The importance of the necessary validation in other geographical locations is stressed out lines 257-259) ‘This demonstrates the need to validate the PCR-based method in different populations and locations to assess primers accuracy and specificity within a given serotype.’

In the discussion, I would suggest addressing the need for ongoing control with Quellung technique of PCR non-typable isolates, to assure that no emerging serotype is missed in carriage studies.

Thanks for this comment. This information has been added line 277-278. ‘Moreover, isolates that are non-typable by PCR should be monitored with Quellung technique to assess for the emergence of
variants in pneumococcal serotypes.’

Minor corrections The year of publication is missing for some of the references.

The references have been checked and reformatted.

Figure 2: the cumulative percentage is missing after PCR 4.

The value of 84.2 has been added.

Table 1: column “aim of the study”, section “no plating” should be “PCR-based serotyping directly on clinical specimens”, not “directly on clinical isolates”.

This has been modified accordingly.