Author's response to reviews

Title: Epidemiology of serotype 19A isolates from invasive pneumococcal disease in German children

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Author's response to reviews: see over
Dear Dr. Manibo,

Please find attached the revised version of our manuscript entitled 'Epidemiology of serotype 19A isolates from invasive pneumococcal disease in German children'.

We have extensively revised the complete text of the paper. We have recalculated and revised all data and calculated statistical significance where needed and advised. We have made all changes suggested by the reviewers, resulting in extensive changes in the text, all tables, and both figures.

We have listed the reviewers comments and our answers below. Also, we have marked the changes we made to the manuscript in yellow.

We feel that the paper has substantially improved, due to the very constructive remarks of all four reviewers. We hope that this revised version of the paper will be eligible for publication.

Sincerely,

Mark van der Linden

**Editor's Requirements:**

**Copyediting:**
After reading through your manuscript, we feel that the quality of written English needs to be improved before the manuscript can be considered further.

We advise you to seek the assistance of a fluent English speaking colleague, or to have a professional editing service correct your language. Please ensure that particular attention is paid to the abstract.

*The paper has been edited by a native English speaker.*

**Abstract:**
Please format your abstract according to the guidelines for authors <http://www.biomedcentral.com/info/ifora/abstracts>. Potential referees will be asked to review the manuscript having seen only the title and abstract, so it is important that these are both informative and concise.

*The Abstract has been revised and formatted according to the guidelines.*
In this paper the authors describe the status of serotype 19A S. pneumoniae isolates collected from children with invasive disease in Germany from 1997 to 2011. The paper gives a clear overview of this important serotype, and analyses trends in prevalence, antibiotic resistance and clonal spread. The study is well-performed and the methodology is appropriate and well justified by the authors. The results are consistent with other recent reports of this serotype. The main strength of this study is that isolates were collected by a National Reference Laboratory and had isolates from all over the country. The presented data are of importance to the scientific community, but there are several points that need to be clarified before publication.

1. The major issue is pneumococcal vaccination. Authors divided the results in two periods, pre-PCV7 and post-PCV7. However, the PCV13 was introduced in Germany in December 2009. Authors should include when PCV7 and PCV13 started in the abstract, methods and results sections, as well as if the PCV10 vaccine is used. Since PCV13 includes serotype 19A it is important to know the role of this vaccine on this serotype in the last period. Do the authors observed any effect of PCV13 vaccination against serotype 19A?
   We agree with the reviewer on this point, and have added data on vaccination in the abstract, methods and result section. We have also discussed the findings in the discussion section. Interestingly enough, the introduction of higher-valent vaccination did not affect the increase in 19A. Probably since it is too early after PCV13 start (Dec. 2009) to see results.

2. The second issue is the “imported clone” CC320. Authors emphasized that 3 of 7 isolates of this clone were collected from children who recently came to Germany from other countries. Were these isolates the first ones of this CC detected? What is the origin of the remaining four CC320 isolates? Since this clone has been identified in other European countries this comment could be included only in the discussion section.
   We thank the reviewer for this interesting point. We looked into this in more detail and came to some interesting findings:
   In total 6 ST320 isolates have been detected among children in Germany, 5 19A (in this paper) and 1 19F. The 19F (MDR) was detected in June 2006, and was the first ST 320 found in Germany. A foreign origin could not be detected. The first 19A ST 320 isolate was from the USA (11.2008), the second from India (03.2009), the third from UK (12.2009). The fourth and fifth could not be traced to foreign origin (01.2010, 05.2010).
   Only very few other CC320 clones with different STs have been detected in Germany including 2432 (1x: 19A, 1x 14MDR), 3259 (19A), 271 (2x 19F, MDR) and 236 (19F, S).
   We have added these findings to the results and discussion sections of the text.

3. Page 13, line 2. CC320 has been identified in other European countries: Poland, Spain, Italy, as you can see at the MLST database.
   We have added this to the text.

4. Table 1. Include PCV13 period.
   We have included that into the table

5. Table 2, % of change. This data could be excluded and include the statistical significance if appropriate.
   We have omitted the % change data and added statistical significance.
6. Table 3. Revise spelling in diagnosis column. Since macrolide and lincosamide resistance are frequently mediated by the same resistance mechanism (ermB), it could be interesting to know the phenotypes of macrolide resistant isolates better than rates of resistance to clarithromycin and clindamycin.

Spelling in diagnosis column has been revised. All except one have ermB, two not tested, 16 cMLSB, 18 iMcLSb, 1 not tested. We have not added this information to the paper, since we feel that it is not within the scope.

7. Figure 2 could be improved including which variables are referred to left or right axis. We have improved the figure.

Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Needs some language corrections before being published
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests: I declare that I have no competing interests
Reviewer 2: Walter Demczuk

Major Compulsory Revisions:

1) The claim that increased use of cephalosporin is driving the increase of serotype 19A is tenuous at best. The data presented in this manuscript does not support this. On page 12, line 15 the author states “This shows the main burden of serotype 19A is caused by the expansion of existing clones.” This statement seems to contradict the claim of selective pressures of antibiotic use are responsible. Figure 2 describes the percentage of 19A has dramatically increased during 2006/2007, however there is no indication of similarly dramatic increased antibiotic usage at this time, and very little change in DDDs of penicillin and macrolides. 19A levels seem to increase concurrently with antibiotic use and no causal relationship can be established.

We do not claim in this paper that cephalosporin is driving the increase of serotype 19A solely. We merely indicate that it could be one of the factors playing a role. Figure 2 shows that the 19A-increase only starts in 2008-2009 (triangles). Cephalosporin usage, in fact, has been rising from 2002 till 2011. So the increase in use clearly precedes the increase in 19A. We have now calculated that the increase in use between the pre- and post-vaccination period is highly significant. Therefore, it cannot be ruled out that cephalosporin use is a factor driving 19A increase. We realized that our figure was difficult to interpret because of the chosen colours. We have improved the figure to make it clearer. We have also changed the discussion to emphasize that cephalosporin use is only one of the possible factors driving 19A increase.

Minor Essential Revisions:

2) Page 2, line 4: Indicate which pneumococcal vaccine was recommended, I assume it was PCV7.

In Germany there are no recommendation for a specific vaccine. The recommendation states 'pneumococcal conjugate vaccine' only. Obviously in 2006 this was PCV7. However, in 2009 PCV10 (April) and PCV13 (December, replacing PCV7) were also used under this recommendation. Choice of vaccine lies with the parents. Additions have been made to the text (see comment 1, reviewer 1)

3) Page 2, lines 5-11: This section discussing the distribution of clonal complexes is very confusing. I suggest re-wording to create a more concise and summarized account of when the groups of CC’s appeared.

We have completely reworded this section. See remark 2, reviewer 4.

4) Page 2, line 13: This seems to indicate that the increasing use of cephalosporins is the direct cause of the increase in antimicrobial resistance. I do not think this has been positively established yet. Suggest using “has coincided with.”

We agree with the reviewer, and have made the suggested change.

5) Page 3, line 4: Start sentence with words “Ninety three”, not a number. Perhaps combine this sentence with the previous one?

We have changed the sentence, now starting with ‘to dat’. We have also altered the amount of serotypes to 94, which is the current number of known serotypes.
6) Page 5, lines 1-5: The authors describe an active surveillance study in the first sentence, then refer to it as a passive system in the second sentence. Is the second sentence referring to the laboratory-based surveillance? Please clarify this section.
We agree with the reviewer and have corrected this mistake. Our laboratory surveillance system described here is passive.

7) Page 5, line 6: This is the first time “ESPED” is introduced, should indicate what the acronym indicates.
Done

8) Page 6, line 1: Define what the authors consider “multi-drug resistant,” for example “resistant to two or more classes of antimicrobials.”
We have defined MDR in the text.

9) Page 6, lines 16-23: Define PCV13, GKV, WldO, ATC/WHO.
PCV13 has now been explained in the methods section. Other abbreviations have been added to the text.

10) Page 6, line 22: “European Surveillance of Antibiotic Consumption project (ESAC).”
Done

11) Page 8, lines 15-16: Awkward wording of “resistant plus intermediate isolates.” in this sentence can be changed to include the penicillin non-susceptible terminology. On line 16 “NS” is not yet defined.
Wording has been changed, Pen NS has been defined.

12) Page 10, line 7: Remove orphaned right parenthesis.
Done

13) Page 11, line 7: Indicate what the units for “average 6.4 isolates” are. Is it per year?
Per pneumococcal season. Added.

14) Page 11, lines 20-22: This is a very tenuous claim, since the data presented does not clearly support this. According to figure 2, although a slight increase in cephalosporin use occurs concurrently with the increase of 19A, the use of penicillin and macrolides has remained constant over time.
We have calculated the statistical significance of cephalosporin and azithromycin use before and after vaccination, and found a significant increase in both antibiotics. Therefore, we think that the claim that this could be one of the factors driving the increase in 19A is justified. See answer to remark 1.

15) Page 12, line 1: It is not clear what this sentence is related to. In begins “Second, when..” but there was on “Firstly..” previous to this sentence.
Changed.

16) Page 13, line 16: It is not clear if the increase stated is in overall IPD incidence, or MDR 19A only?
Added: in serotype 19A IPD isolates

17) Page 19, table 3: Title on lower part of table should read “Post-vaccination?”
Done.
18) Page 21, Figure 2: It is not clear what the authors are attempting to represent in this figure. Although the percentage of 19A has dramatically increased during 2009/2010, there is no indication of increased antibiotic usage at this time, and very little change in DDDs. It refutes/disproves the suggestion that increased usage of antibiotics is in some way linked to increased 19A, since 19A levels increased first (or concurrently), then a slight increase in antibiotic use followed. The figure can be simplified by removing the PCV doses line that is redundant since the text describes PCV introduction in 2006.

We have improved this figure to make it more clear. What we want to present here is the possible correlations between antibiotic usage, introduction of PCV and percentage of serotype 19A isolates in IPD. The PCV data in this graph are important because they show that introduction of PCV was very fast (which is not the case for all countries). As described in our answer to remark 1, we have found that the increase in use of cephalosporin in the post-vaccination period is highly statistically significant, and precedes the increase in serotype 19A.

Discretionary Revisions:
19) Page 3, line 1: This is an awkward sentence. Perhaps “Streptococcus pneumoniae is a major cause of infectious disease globally, especially in children.”
Reworded

20) Page 5, line 8: Is there any geographical bias? For example, a higher rate of sampling in certain regions?
There was no geographical bias, and isolates were sent in from all over Germany. A sentence was added to the text.

21) Page 9, line 15: Suggest “The increased proportion of MDR isolates were mainly associated with increased proportions of CC230.”
Text has been changed as suggested.

22) Page 12, lines 2-5: Discuss what is the significance/interpretation of these differences in CCs before and after the PCV?
We agree with the reviewer, and have amended the discussion.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
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 interest.
Reviewer 3: Lena Sechanova

Comments to the Author

The manuscript (MS) by van der Linden et al. addresses prevalence and clonal composition of serotype 19A among invasive S. pneumoniae isolates from children in Germany prior and after to the onset of PCV vaccination in this country. Additionally, PCV7 uptake and antibiotic usage in Germany as possible factors driving the changes in epidemiology of serotype 19A strains were evaluated. In most countries after PCV7 introduction 19A serotype is of particular concern among non-vaccine serotypes which predominate in post-vaccine period. Methods used in the MS are comprehensive and up-to-date including antimicrobial susceptibility testing by microbroth dilution, serotyping and MLST genotyping of large number (158) of serotype 19A S. pneumoniae isolates.

The two main findings of the MS are: 1) a serotype 19A prevalence similar to previously reported rates from other countries after PCV vaccination, and 2) interesting new data on clonal distribution of serotype 19A among invasive S. pneumoniae isolates before and after PCV vaccine introduction in Germany and an association with antibiotic uses and vaccine uptake in the community.

Specific comments:
1) Page 8: Results, second paragraph. “Fourty-four 19A isolates (28%) …” It is generally accepted that the percentage have to appears as 0.0. This should be stated everywhere in the rest of the text - Results and Tables.
   Done

2) P 8, Results, third paragraph, “…54 (9 resistant, 43 intermediate, 34.1%) …” but the total sum in parenthesis is 52 (please clarify).
   Mistake corrected: 9 + 45

3) P 9, Results, seventh paragraph. “The increased proportion MDR isolates and was mainly…” ‘and’ must be omitted (technical error)
   Done

4) P 10, Results, eighth paragraph. “(penicillins, macrolides (including clindamycin excluding azithromycin)…” Data on antibiotic use of azithromycin was given already in this paragraph. The text is suggested to be penicillins, clindamycin, tetracyclines etc…
   We have completely revised this part of the text.

5) P 12, Discussion, second paragraph. “The increasing use of cephalosporins and perhaps of azithromycin…” The use of azithromycin in Germany seems to be much more in the post-PCV vaccination period (5% vs. 63%, given in Results, eighth paragraph). Thus, the use of macrolides also favored the selection of multiresistant 19A clones (in my opinion that is one of the possible reasons for an increased rate of MDR 19A isolates after vaccine introduction in Germany).
   We have calculated the statistical significance of the increase in AB use before and after vaccination. We found that increase in both cephalosporin and azithromycin use was significant. We have adjusted the text in the results and discussion sections to include these findings.
6) P 12, Discussion, third paragraph. “Six belonged to CC320 (ST320 (4) and ST2432).” The number of ST320 is 5 (technical error - please clarify).
Done: 5 +1

7) P 15, References. In reference numbers: 9, 14, and 16 the year of publication is omitted. Also reference No.25 is incomplete.
Corrected

8) P 17, Table 1. The title is not clear enough and is suggested to be “Incidence and antibiotic resistance of serotype 19A S. pneumoniae invasive isolates among German children from 1997 to 2011.
Disagree, since we do not present incidences, only reported cases.

9) P 17, Table 1. Additionaly, in Table 1 a period 2010/2011 is included, but in the sections MM (first paragraph) and in Results (first paragraph) you refer the study period from 1997 – 2009? Please clarify.
Mistake was corrected

10) P 18, Table 2. It seems that the numbers of deferent STs in CC 199 (30) and CC230 (18) in the post-vaccination period are not correct. Please, check the numbers of STs in the parentheses.
Numbers were corrected

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: I do not have any financial competing interests in relation to this paper and there are no other potential conflicts of interest. 'I declare that I have no competing interests.

Reviewer 4: Shahin Oftadeh

The article is overall good and worth publishing, although it needs some editing before being published as it is too wordy and has lots of repetition of results in text, tables and discussion. Here is some of my comments/suggestions on different parts of article (all category 2 or 3).

Abstract:
1. It should be stated how many serotype 19A isolates were studied. Also it is not clear that CCs referred to are all serotype 19A.
Number of 19A isolates studied was added. See next point.

2. I suggest line 5 of abstract to be changed to: “….in 2010/11. Eight Clonal Complex (CC) and groups accounted for….all serotype 19A isolates……respectively. While three CCs…..after vaccination, four CCs and several STs first appeared after vaccination…….. could be traced to recent imports from the USA, UK and India.”
Paragraph was rephrased as suggested.
3. Delete the sentence: “Six more…. ….after vaccination” (abstract should only highlight the most important data).
   Done

Introduction:
4. Introduction is a bit too long and only a single paragraph. It needs editing into more logical sequence and concise points.
   We have edited the introduction.

Materials and methods
5. Spell out ESPED
   Done

6. Explain briefly what is meant by “capture recapture”
   We have added an extra sentence to the text. The capture-recapture system is explained in detail in reference 15.

7. Use “approximately”: instead of “roughly about”
   Done

Susceptibility testing
8. Specify the breakpoints for susceptibility interpretation to avoid confusion. (What are the breakpoints in the current CLSI criteria?)
   Done

9. Specify the definition of MDR.
   Done

Antibiotic and vaccine use
10 The last sentence (page 6): (the data were expressed….……) is not clear and is hard to understand! What does “…day using ATC/WHO” mean?
   We have explained the abbreviation in this section and improved the wording.

Results
11. There are lots of unnecessary repetitions of data in tables -select the most important parts to include in the text. Remove detailed data from the text where you have referred to tables and instead just interpret the figures in your text as increased or decreased- significantly or not significantly….so on.
   We have altered the results section and taken out unnecessary repetitions.

12. Page 8, first paragraph: I think isolate number should be reported from the year that your study starts with (1997-98); although the numbers slightly drops in 1998-99.
   Sentence was rephrased clarify the minimum and maximum amount of isolates collected in a single season.

13. Page 8, first paragraph: Check the average number of isolates/year (3122total/14years=223).
   All numbers were recalculated and mistakes corrected.
14. Page 8, first paragraph, line 6: Add percentage of proportion (10%) during the post vaccination…
   *Done*

15. Page 8, second paragraph: How were Pen R CSF isolates distributed over time after immunisation was introduced? Was it more in 2006-11?
   *We thank the reviewer for this suggestion. We have assessed the data and found that indeed the rate of PenR in CSF post vaccination is substantially higher. We have altered the text to include this point.*
   
   1997-2006: 19 CSF, 5 PenR, 26.3%. 2006-2011: 25 CSF, 10 PenR, 40.0%. *We have added this information.*

16. Line 5 of Paragraph 3- page 8: add: …increased moderately (but not significantly)…
   *Added*

17. Paragraph 3 page 8: I suggest remove all the percentages in parenthesis (which are in table 1) to avoid repetition Ex: 28 vs 38%, NS; 44.8 vs 48.5 %...)
   *We have removed all the percentages.*

18. The second last paragraph Page 9: summarise the paragraph to: eight CCs or groups accounted for ….before and after vaccination respectively. Three CCs and 7 other STs were not detected After vaccine introduction. Clonal complexes 81…. (Table2). [spell out CC in the beginning of the sentence]
   *Made suggested changes*

19. The last paragraph Page 9: Change the first sentence to: The increased proportion of MDR isolates was mainly due to STs belonging to CC230 and CC320.
   *Done*

20. The last paragraph Page 9, line 3 &5: You don’t need to repeat the MIC categories here if you include them in the methods.
   *Done*

21. The last paragraph Page 9 line 6: Remove the STs from the second last sentence: ST 276 (2)….and so on as they are in the table.
   *Done*

22. Page 10 paragraph 2: As azithromycin is a macrolide - and in the next sentence you are comparing antibiotic classes - it should be included in macrolide class, not separate.
   *We have changed the listed antibiotic in figure 2. We now list azithromycin separately from the other macrolides+clindamycin. This shows the increase in azithromycin use, and also the relatively low fraction of all macrolides represented by azithromycin.*

23. Use “the number of marketed doses of PCV7” instead of “the amount of…” Also in discussion where you are referring to dose of vaccin use: “number of…”
   *Changed*

Discussion

24. A major limitation of this study is that referral of isolates was incomplete. Were those referred representative? Have referral patterns (e.g. from different labs) changed over time? It is surprising that overall numbers of referred isolates have not fallen since 2006- it suggests a
higher proportion being referred (and potentially biased towards antibiotic resistance). This needs to be discussed in this section.

Even though our referral of isolates is incomplete, the data are representative for Germany. There is no regional bias, and referral patterns have been constant for years. Using our capture-recapture incidence calculations, we determined that for about 50% of all IPD cases a sample is sent to the National reference Center. In fact, overall numbers have fallen since 2006 (2005-2006: 292, 2010-2011: 234). Moreover, it should be noted that these numbers include all cases in children under 16 years of age, of which still many are unvaccinated. Numbers of children under 2 years of age have fallen more sharply: 2005-2006: 154, 2010-2011: 100. We have added a paragraph on the limitations to the discussion.

25. Repetition of results in discussion is unnecessary (first paragraphs page 11 and 12). Also some of the numbers in page 12 don’t match with the table – check which are correct.

We have taken out the repetition of results from the discussion. All numbers throughout the entire paper have been rechecked and corrected.

26. Page 13 second paragraph: …”a niche” instead of “an empty niche”

Altered

Tables and figures
27. Table 1: I suggest include the percentage of resistance for each antibiotic

Done

28. Table 2: you have typed camas (,) instead of decimal pointing % column (check the text for this typo as well).

Done

29. Table 3: you have typed pre-vaccination in both sections the second one should be post-vaccination.

Done

30. Table 3: you have typed camas (,) instead of decimal point for PEN MICs Tetracycline and cephalosporin column colour don’t have enough contrast, I suggest you change one of them unless it will be colour printed.

Done

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: I declare that I have no competing interests