Author's response to reviews

Title: Genotyping of Streptococcus agalactiae (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy

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Author's response to reviews: see over
Sir,

We appreciate the reviewer comments of our manuscript. Their comments have been addressed in the revised manuscript. Hereby, you will find the answers of the comments of both reviewers.

Sincerely

Mario Vaneechoutte
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Reviewer's report
Title: Genotyping of Streptococcus agalactiae (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy
Version: 1 Date: 28 April 2009
Reviewer: Amos Adler
Reviewer's report:
Major Compulsory Revisions:
1. This manuscript is engaging in 2 separate questions— the performance of several culture methods and the heterogeneity of strains, as assessed by serotyping and RAPD. The first question is adequately approached, although it does not add any new data and I doubt that the differences are significant. The second question that the study aims to answer, engage with the strain heterogeneity within a single patient. This question is original, has potential clinical importance and the authors had adequately coped with it. My main concern is the adequacy of RAPD for that purpose. Although RAPD is relatively easy to use, it has some significant limitation compared with other methods (especially sequence-based, such as MLST) in regard with its reproducibility and portability. Hence, it is difficult to use it for understanding the population epidemiology of a species. Hence, I think that a method such as MLST could have been a better choice and would increase significantly the scientific value of the manuscript. However, in my mind it is still acceptable as it is.

A1: We agree that MLST is a superior method for interlaboratory and long term studies. However, for comparing the genotypes per patient or subject, RAPD-analysis is certainly sufficiently reliable to establish similarities or differences between isolates. Moreover, we carried out RAPD on a sequence grade electrophoresis machine (ABI310) and not on agarose gels. This improves reproducibility and makes computer assisted comparison of the fingerprints possible.

I would suggest that the authors will also report the susceptibility differences between the isolates, even only to a small number of antibacterial such as clindamycin and erythromycin, since that may have clinical importance.

A2: Although susceptibility testing was out of the scope of this study, we have now tested both antibiotics for a total of 40 isolates from 8 patients.

We now added the following text to the Results (line 232): “GBS is considered homogenously susceptible to penicilline and amoxycilline. In case of allergy, second choice antibiotics are clindamycin or erythromycin. According to CLSI erythromycine can be tested
with a simple disk test, although this is not done in our routine laboratory. Here we checked 40 isolates from 8 patients and found 26 isolates to be susceptible to both clindamycin and erythromycin, 3 to be clindamycin resistant and 11 to be resistant to both antibiotics. The susceptibility pattern of all strains was homogeneous for six subjects, despite genotypic differences among the isolates, whereas two subjects carried clindamycin resistant strains besides isolates susceptible to both antibiotics.

The following text was added to the Discussion (line 385): “The finding that two out of 8 women carried isolates with different susceptibility indicates that when testing susceptibility for clindamycin and erythromycin, several colonies should be tested, since colonies with different susceptibility may be simultaneously present.”

The following text was added to M&M: “Forty isolates of group B streptococci of 8 pregnant women were tested by disk diffusion for susceptibility to clindamycin and erythromycin. Colonies taken from Trypticase Soy Agar (TSA) + 5% sheep blood (Becton Dickinson) were suspended in 0.5 ml of saline and the inoculum was adjusted to the turbidity of a 0.5 McFarland standard. This suspension was streaked onto TSA + 5% sheep blood to obtain confluent growth, disks were added and the plates were incubated overnight at 37°C with 5% CO₂. Strains were considered resistant to clindamycin and erythromycin when the inhibition zones were less than 15 mm.”

2. ‘Sensitivity and specificity of different culture techniques for the detection of GBS’- since Granada was the most sensitive method, I wonder if they found also non-hemolytic strains on the CNA Agar. If that is the case, it would be interesting to know whether these strains were also pigment-producing on the Granada agar, as these phenotypic traits (hemolysis and pigment production) are genetically linked. If non of the isolates were hemolytic, the authors should comment about the limitations of the Granada agar in detecting these strains.

A3: We picked suspected colonies from GBS, both haemolytic and non haemolytic. But we did not detect non haemolytic GBS colonies which are CAMP positive. We have not further commented on this.

In reality Granada does not detect non-pigmented isolates, and on blood-agar non-hemolytic isolates are difficult to detect. Moreover, as also indicated by the reviewer, non-hemolytic isolates are also carotenoid-negative and will be nonpigmented on Granada. So, nonhemolytic strains may have been missed. According to some, these are mostly nonpathogenic either.

We addressed this as follows: Line 273: “Notably, Granada agar does not detect non-pigmented isolates, and on blood-agar non-hemolytic isolates are difficult to detect. Moreover, as also indicated by the reviewer, non-hemolytic isolates are also carotenoid-negative and will be nonpigmented on Granada. So, nonhemolytic strains may have been missed.”

3. The authors need to discuss the limitations of the study.

A4: Except for possibly missing some rare nonhemolytic isolates (a possible minor shortcoming which is now addressed, see A3), we don’t think that there are important limitations to this study.

Minor Essential Revisions
Abstract:
1. The authors should detail that the indirect culture onto Granada agar was following overnight incubation in LIM.

A5: We wrote: “or ii) indirectly onto Granada agar resp. iii) Columbia CNA agar after overnight incubation in Lim broth” and so this actually states that also Granada agar was after overnight incubation in Lim broth, but we rephrased as follows (line 37): “or
indirectly onto ii) Granada agar resp. iii) Columbia CNA agar, after overnight incubation in Lim broth”.

2. Please fill in the number: “Of these, 19 harbored GBS in both rectum and vagina, only in the vagina and 8 exclusively in the rectum.”

A6: We have added the number ‘9’ (line 41).

Background:
1. Regarding the term ‘neonatal invasive disease’- since there is no neonatal non-invasive disease, I would just write either ‘neonatal infection’.

A7: Several publications use ‘neonatal invasive disease’ (e.g. references 7 and 18), but we agree with the reviewer and have changed throughout the manuscript.

2. Delete: “vast majority of all cases”

A8: This has been replaced by ‘has been shown’ (line 67).

3. The authors should elaborate on the rate of maternal carriage, screening practice and rate of neonatal disease in their own country.

A9: We added following text at line 289: “Our data, are also in correspondence with other results on GBS prevalence in our country. Rectovaginal colonization with group B streptococci in Belgium is 13-25 %. These data are based on different studies carried out by the Belgian reference laboratory for GBS in collaboration with the section of epidemiology of the Scientific Institute for Public Health (ISP-WIV, Brussels) [40]. E.g., Blanckaert et al. [32] compared the results of GBS screening on Granada agar with those obtained using standard Columbia blood agar at two participating centers in Belgium. They reported GBS-positive culture results of 10–30% of pregnant women. The Flemish Study Centre for Perinatal Epidemiology evaluated GBS prevalence in Flanders and found an average colonization rate of 16% among Flemish pregnant women [41].”

Results, tables and figures:
1. The sentence ‘indirectly onto Columbia CNA agar resp. Granada agar after incubation overnight in...’ is not clear.

A10: We have changed as follows at line 178: “or indirectly, by subculturing onto Columbia CNA agar resp. Granada agar, following overnight incubation in LIM broth.”

2. Table 1- please add statistics.

A11: We carried out the McNemar test for correlated percentages to compare the sensitivity of the culture media for the detection of GBS from vaginal and rectal samples.

We added the following text at line 186: “Culture of vaginal specimens by direct plating on Columbia CNA agar was significantly less sensitive than culture in Lim broth with subculture on Granada agar or subculture on Columbia CAN agar (McNemar test, p < 0.0001). Also for the culture of rectal specimens, direct plating onto Columbia CNA agar was significantly less sensitive than culture in Lim broth with subculture on Granada agar (p < 0.0001), which was more sensitive than culture on Lim broth with subculture on Columbia CAN agar (p = 0.0313).”

3. Table 2 needs a much more detailed legend, explaining the abbreviations.

A12: We explained the abbreviations used in the column headings and we added some explanation on the serotype designations.
Discussion:

1. The discussion regarding the different culture methods is too long and should be shorten.

**A13: We have omitted the following lines (line 273):** “Identification of suspected GBS colonies is mandatory when Columbia CNA agar is used and, when there are few colonies or when GBS is mixed with other microflora, subculture is required prior to identification. The use of Granada agar provides a faster answer and reduces laboratory personnel and reagents costs.”

2. ‘Epidemiology’ - it would be interesting to know (either in the discussion or the background section) whether there is previous data from Belgium and what the screening practices in Belgium are.

**A14: We have addressed this now at line 289:** "Our data, are also in correspondence with other results on GBS prevalence in our country. Rectovaginal colonization with group B streptococci in Belgium is 13-25 %. These data are based on different studies carried out by the Belgian reference laboratory for GBS in collaboration with the section of epidemiology of the Scientific Institute for Public Health (ISP-WIV, Brussels) [40]. E.g., Blanckaert et al. [32] compared the results of GBS screening on Granada agar with those obtained using standard Columbia blood agar at two participating centers in Belgium. They reported GBS-positive culture results of 10–30% of pregnant women. The Flemish Study Centre for Perinatal Epidemiology evaluated GBS prevalence in Flanders and found an average colonization rate of 16% among Flemish pregnant women [41].”

3. ‘Serological and genotypic diversity among GBS isolates’ - the sentence “Unfortunately, only one isolate was genotyped per visit and the observed genotypic diversity in our study indicates that the turnover of 8.3% might be an overestimation, caused by picking colonies belonging to different genotypes, both present at both sampling moments, but sampled only once, due to randomness of sampling.” Is too long and obscure and needs to be revised.

**A15: We changed to (line 323):** “Unfortunately, only one isolate was genotyped per visit. Considering the genotypic diversity per subject, as observed in our study, a turnover of 8.3% might be an overestimation. When the same subject, carrying e.g. genotypes a, b and c, is sampled at two different moments, and whereby on each occasion only one colony is picked, the detection of a different genotype may be interpreted as turn over, but it may be that a genotype a strain has picked at the first visit but a genotype b or c strain at the second visit.”

4. The paragraph ‘General serotype distribution’ is too long and should be minimized, as it adds no significant new knowledge.

**A16: We have omitted the following lines (line 383):** “A Portuguese study [13] indicated that isolates with serotypes III and V accounted for 44% of all colonization isolates, showed a prevalence of serotype Ia and III isolates in early onset disease (resp. 31 and 29% of 42 EOD isolates) and of serotype III isolates in late onset disease (64% of 22 LOD isolates), together 69% of all invasive isolates. A Swedish study reported that 62% of the invasive isolates were serotype III [44]. Also a Zimbabwean study of 241 GBS isolates, comprising 124 carrier isolates from pregnant women, revealed comparable data, i.e. 47.7% serotype III isolates, 23.2% serotype V and 17% serotype Ia [16].”

5. The paragraphs ‘Serological and genotypic diversity among GBS isolates’ and ‘Correlation between serotyping and genotyping’ should be combined and altogether shorten.

**A17: We have merged the two sections and have omitted the following texts:**
1. “It is generally agreed that the discriminatory power of genotyping exceeds that of serotyping, if only because only 13 serotypes [3] have been established thus far and because some strains are nontypeable.”

2. “Bohnsack et al. [22] state that not only each serotype usually contains several different genetic lineages but that each genetic lineage may contain several different serotypes. Also, Bisharat et al. [7] found several serotypes per MLST lineage for most of the MLST lineages.”

3. “On the other hand, several studies reported a high degree of congruence between genotyping and serotyping [3, 12]. e.g., Perez-Ruiz et al. [12] found two pairs of women with serotype III strains, also indistinguishable by SmalI restriction digestion and one cluster of three women with serotype Ia strains which were also genotypically indistinguishable. “

4. “Limansky et al. [19] indicated that RAPD was particularly useful for documenting vertical transmission of a given clone of S. agalactiae, as well as for documenting relapse versus reinfection, and suggested clonal relatedness between unrelated S. agalactiae isolates to be associated with some invasive infections.

5. “The same group [19] found 30 distinct amplification profiles among 52 unrelated S. agalactiae isolates assigned to nine groups by serotyping (including 3 nontypeable strains). most belonging to serotype III,”

6. “and 60 unrelated strains isolated from carriers and found results to be congruent with those obtained with multilocus enzyme electrophoresis.”

6. The authors need to discuss the limitations of the study.

A18: This remark was adressed under remark 3 (same question of reviewer).

Methods:

1. There is no need for a detailed description of the sampling process.

A19: We choose to retain this section, since a recent study (Kim et al. 2009) has shown that e.g. for the vagina there are different regions with different vaginal microfloras and that the results depend on how the samples are taken, e.g. scraping the vaginal wall yields other species composition than swabbing.


2. I wonder how you can perform a CAMP test on non-hemolytic GBS colonies.

A20: First, we did not isolated nonhemolytic colonies in this study. However, in ongoing studies we already have one isolate which is nonhemolytic, Granada without pigment, but positive in CAMP test and in the recently commercialized Chromagar (Biomerieux).

3. The paragraph ‘Identification of the isolates as Streptococcus agalactiae’ should come before ‘DNA extraction…’.

A21: This has been changed accordingly.

Level of interest: An article of importance in its field

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

A: We have checked the revised manuscript with the online language correction service of ErrNet and have changed the manuscript accordingly.

Statistical review: Yes, and I have assessed the statistics in my report.
Reviewer's report
Title: Genotyping of Streptococcus agalactiae (group B streptococci) isolated from vaginal and rectal swabs of women at 35-37 weeks of pregnancy
Version: 1 Date: 28 May 2009
Reviewer: Kathy Agnew
Reviewer's report:
Comment #1 Methods section should come before results & conclusions
A22: We have changed accordingly.

Comment #2 Suggest putting percentages next to actual number 24 (72%) rather than a separate sentence with the percentages. (p 5,9)
A23: We wrote (line 181): “A total of 36 out of 150 pregnant women studied (24%)”, so we do not understand this question on ‘72%’, because it is 24%.

Comment #3 Abstract: results- missing value for # found only in the vagina
A24: This was corrected by adding ‘9’ (line 41).

Comment #4 Abstract: Suggest re-write of sentence that starts: 18 of the 19 subjects.. unsure what is being stated here.
A25: This is about the following sentence(s): “Eighteen of the 19 subjects with GBS at both sites had at least one vaginal and one rectal isolate with the same genotype. Only two subjects were found to carry strains with the same genotype, although the serotype of both of these strains was different. “.
Maybe the problem of the reviewer is that she thinks that the second sentence also relates to the first one. We have changed order of the sentences to avoid this confusion (line 47): “Only two subjects were found to carry strains with the same genotype, although the serotype of both of these strains was different. Eighteen of the 19 subjects with GBS at both sites had at least one vaginal and one rectal isolate with the same genotype.”

Comment #5 Abstract: some brief method description would be helpful
A26: We briefly described the essence of the methodology at the start of the results, but have now changed this to the Methods section of the abstract (line 36): “Streptococcus agalactiae was cultured separately from both rectum and vagina, for a total of 150 pregnant women, i) directly onto Columbia CNA agar, or indirectly onto ii) Granada agar resp. iii) Columbia CNA agar, after overnight incubation in Lim broth.”

Comment #6
p 6. suggest some background as to why authors chose to compare by presence of L crispatus
A26: We are not sure we understand the question here. But we think the reviewer refers to our statement: “The presence of L. crispatus vaginally did not seem to protect against vaginal S. agalactiae colonization,”, which we have now tried to clarify as follows (line 225): “The presence of L. crispatus, generally accepted to confer vaginal colonisation resistance to pathogenic organisms ([26, 27], did not seem to protect against vaginal S. agalactiae colonization,“

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 declare that I have no competing interests