Author's response to reviews

Title: Genotype distribution of human papillomavirus (HPV) and co-infections in cervical cytologic specimens from two outpatient gynecological clinics in a region of southeast Spain

Authors:

Pablo Conesa-Zamora (pablo.conesa@carm.es)
Asunción Domenech-Peris (asdope@hotmail.com)
Sebastian Ortiz-Reina (sortizr@terra.es)
Joaquin Moya-Biosca (oaquin.moya@carm.es)
Francisco Javier Orantes (orcas30@gmail.com)
Miguel Perez-Guillermo (miguel.perez-guillermo@carm.es)
Marcos Egea-Cortines (marcos.egea@upct.es)

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Author's response to reviews:

First we would like to acknowledge the fast and maybe more important constructive and fair reviews. We would like to state that we have tried to address all the comments of the referees, although in some minor cases we disagree and we have written the corresponding modification. P: stands for Jean-Luc Pretet and A: for authors

Title.
We believe that the study of coinfections is the major contribution of our work. Furthermore since the work we performed was on gynaecological patients from two outpatient clinics, we thought it would be more informative and would put the results in the right perspective if that would also appear. Thus we have modified the title that now is:

Genotype distribution of human papillomavirus (HPV) and coinfections in cervical cytologic specimens from gynaecological patients in a region of southeast Spain.

Dr. Jean Luc Pretet

P: 1- The description of HPV prevalence does not take into account the cytological diagnosis. But it is known that HPV distribution varies according to the lesion type. HPV16 surely represents the most frequently detected type whatever the cytological diagnosis is (1st position in ASC-US, N/B, LSIL, HSIL, cf. Figure 1). In contrast, the prevalence of HPV58 varies from 17% (ASC-US, 3rd position) to 2% in LSIL (8th position). While HPV58 is the second genotype in term of frequency in this series of samples, it is hazardous to conclude that this genotype...
is the second most prevalent type in the region of Murcia.

Is the sample size large enough to detect rare genotypes (less than 5% for example) with a good precision (2 or 3% for example)? This is important to be able to draw sound conclusions regarding type distribution.

A: We do not completely agree with the statements above. Figure 1 was composed of percentages of viral genotypes in different cytological diagnoses. Since both referees coincide in this comment we have modified the text in order to bring the attention to the fact that the population studied stems from females that had attended outpatient gynecological clinic (stated in title, materials and methods and discussion). Furthermore the text has been modified in order to reflect the correspondence between the cytological diagnostics and hpv genotypes. General description of HPV genotype distribution in all specimens has been excluded.

P: 2- The authors have used two different kits to perform their study. They indicated that both tests are DNA chip based test. The reviewer would like to point out that sensitivity of each assay may be different for the detection of specific type of HPV. For example it is documented that Linear array from Roche Diagnostics and InnoLipa from Innogenetics (both based on reverse blot hybridization) are not equivalent in term of sensitivity to identify some HPV types. Thus the authors should not mix results obtained with 2 different kits. Either they do not include results obtained with the HPV GenoArray or present separate results.

A: We have followed the recommendations of both referees and we have taken out the samples performed with Hybribio and included a total of 185 new samples genotyped since the manuscript was submitted, using the Clondiag kit. Thus all samples have been genotyped using the same system. We have modified materials and methods, and all the data throughout the manuscript.

P: 3- The prevalence of HPV infection according to cytology diagnosis is sometimes questionable regarding the literature published so far. For example, HPV positivity > 50% in N/B samples appears really high. The authors nevertheless indicate in their discussion that this rate of infection is within the range of HPV positivity described in two other studies performed in Spain (ref 7 and ref 26). This argument is not acceptable because

(i) Spain is probably one the country with the lowest incidence of HPV infection around the world

(ii) the comparison with the two Spanish studies is not reliable. Indeed, in
reference 7, Gonzales-Bosquet et al. analyzed only abnormal smears (almost 80% of HSIL and LSIL, only 23% in the submitted manuscript) but no normal smears. Interestingly, Gonzales-Bosquet et al. report HPV prevalence consistent with published meta-analyses. In reference 26, de Antonio et al. do not refer to any cytological diagnosis, rendering the comparison with the Conesa-Zamora’s study invalid.

A: The high degree of positivity of N/B is probably due to the fact that female patient came from outpatient gynaecological clinic and therefore they had some type of pathology. As stated above, we have written this in the materials and methods and discussion. We have modified the title to pay attention on this issue and have included also a phrase in the results section.

Gonzalez-Bosquet’s et al. study has only been referenced in the present manuscript when comparing LSIL and HSIL lesions which are included in this article.

As the reviewer points out de Antonio’s et al. study does not referred to cytologic diagnosis or HPV genotypes but the population setting is comparable (outpatient gynaecologic clinic) with ours. In the present version of the manuscript we have only referred to the HPV positivity rate in de Antonio’s series.

P: -116 specimens correspond to samples collected during the follow-up of 42 women. This strategy of sample collection have surely biased the distribution of some HPV types as the probability to find the same HPV from 2 or 3 consecutive samples is likely to be higher than by chance.

A: We have used the first genotyping assay for those cases of follow up, thus we do not sample twice the same person.

P:- The result presentation lacks clarity:
For example, the total number of HPV positive samples (n=251) appears in table 2. This precludes a rapid understanding of table 1 in which the authors indicates that the percentage was calculated from 251 HPV positive samples.
A: This issue has been clarified in the present version of the manuscript.

Editor response

Dear ....
First we would like to acknowledge the fast and maybe more important constructive and fair reviews. We would like to state that we have tried to address all the comments of the referees, although in some minor cases we disagree and we have written the corresponding modification. S: stands for J.Smith; and A: for authors
Title.

We believe that the study of coinfections is the major contribution of our work. Furthermore since the work we performed was on gynaecological patients from two outpatient clinics, we thought it would be more informative and would put the results in the right perspective if that would also appear. Thus we have modified the title that now is:

Genotype distribution of human papillomavirus (HPV) and coinfections in cervical cytologic specimens from gynecological patients in a region of southeast Spain.

A: Both referees claim that HPV distribution changes with each stage of disease. This is correct and reflects the degree of oncogenicity of each genotype.

S: In actuality, the number of HPV types identified in each stage of cervical disease decreases with increasing grade of cervical disease, thus being lower in ICC than in HSIL or than in LSIL.

A: We do not completely agree with the statements above. Figure 1 was composed of percentages of viral genotypes in different cytological diagnoses. Since both referees coincide in this comment we have modified the text in order to bring the attention to the fact that the population studied stems from females that had attended outpatient gynecological clinic (stated in title, materials and methods and discussion). Furthermore the text has been modified in order to reflect the correspondence between the cytological diagnostics and hpv genotypes. General description of HPV genotype distribution in all specimens has been excluded.

S: The decision to include two different assays for HPV detection and combining results needs to be further justified, particularly given that different HPV tests will likely result in different sensitivities for the detection of different individual types.

A: We have followed the recommendations of both referees and we have taken out the samples performed with Hybribio and included a total of 185 new samples genotyped since the manuscript was submitted, using the Clondiag kit. Thus all samples have been genotyped using the same system. We have modified materials and methods, and all the data throughout the manuscript.

S: Please clarify how the data on the follow-up of the 42 women was used in these analyses.

A: We have used the first genotyping assay for those cases of follow up, thus we do not sample twice the same person.

S: Ie the point that low percentage of HPV 18 and the remarkable prevalence of HPV 58 might reduce the effectiveness of the vaccination campaign in this region:
I am concerned about this statement. It is important to differentiate HPV prevalence results found in the population, versus those found in LSIL lesions, versus those found in HSIL and those found in ICC. HPV prophylactic vaccines target the oncogenic HPV types that are most common in ICC. Thus, it is misleading to conclude that if HPV 16 and 18 are not the most common types in HSIL, LSIL or within the population that the vaccine efficacy will be compromised. Based on global review, HPV types 16 and 18 are found to be the most common types in ICC in all geographical regions surveyed.

A: We consider this statement partly correct. It is difficult to know the effect of the vaccines on the HPV population. Our standpoint is that the Region of Murcia could be a good model to study viral evolution and we should wait even for sheer prudence before we make statements about changes in viral population structure. However, we do not conclude that HPV16 and 18 are not the most important, but we do find that HPV 18 has a very low representation in the sample analysed. We have modified the abstract accordingly and we took out the statement about possible lack of effect of the vaccine since it is currently mere speculation.

We consider that the role of LSIL and HSIL as precursor of ICC is consistent and accepted by the scientific community. Therefore the study of HPV genotypes in well-established precursor lesions would help to discern the distribution of genotypes in advanced stages of the disease. This is one of the reasons why HPV genotyping in LSIL clinical practise has important clinical implications to discern the possible course of this lesion. The HPV genotype distribution varies worldwide, not only in general population but also in ICC cases.

S: Please add a reference concerning the statement that there are more than 200 HPV types..is this referring to humans or other species?

A: If referred to human they are about one hundred. There is controversy in terms of what is considered a variant and a new species and we decided to write “more than a hundred” leaving the two citations, which have phylogenetic reconstructions clinically relevant.

S: Please add a caveat ie the 20-30% prevalence of multiple infections—results are clearly dependent upon the HPV typing assay used.

A: This consideration have been included in this version of the manuscript

S: Also, please include a caveat that the data on HPV vaccine cross-protection has not necessarily proven to be clinically relevant. I would thus put caveats on this
when cross-protection is referred to in the text.

A: This consideration have been included in this version of the manuscript.

S: Ie the statement, “the effectiveness of HPV vaccines will depend upon the particular HPV genotype/distribution of the region”. Please see comments above under the abstract section. This statement seems to presume that certain types will replace others (niche hypothesis), although this has not been proven and may be purely theoretical. This should be make clear in the introduction and/or methods section.

A: We have modified the introduction, and stated that the vaccine could have an impact on the genotype distribution. We have also commented on the clinical relevance of the cross protection of the vaccine as suggested.

What we wanted to say is that HPV vaccines would be more effective in terms of high prevalence of genotypes against which the vaccines provide protection. This not necessary imply that the absence of certain genotype must by replaced by another.

S: The figures on the overall prevalence of HPV found among all women included in this study are not particularly useful, given that these data are dependent upon the proportion of women with HSIL, LSIL and normal diagnoses. I would suggest, thus, present the HPV results by each stage of cervical disease (normal, LSIL, HSIL), rather than combined figures.

A: This suggestion has been taking into account and the percentages for each HPV genotype have been referred to each cytologic diagnoses.

S: I would suggest eliminating the data on the 14 women with undetermined cytological status. Given that this is not a representative population based sample, these data do not add much.

A: We have excluded these specimens from our series.

S: Please include an analysis of A5 and A9 limited to women with normal cytology.

Please provide the data on the prevalence on the most common HPV types as single type infections. This is particularly important for low risk type HPV 6, which in most cases will be accompanied with another high-risk type.

A: We have included this consideration in the manuscript (see figure 2 and table 3)

S: Please add a reference concerning the lower sensitivity of HPV 53 in currently
available diagnostic tests.

A: We cite three references in the original manuscript. Amongst them, the most widely used is the Hybrid capture II. Its sensitivity is cited (Reference Poljak et al.).

S: Line 262, it is difficult to compare the prevalence of multiple infections in Munoz et al (all invasive cervical cancer cases) with that of the present study, which has different grades of cervical disease less severe than ICC.

A: We do not establish any type of comparison between our study an those of Muñoz et al . We cite that work because it establishes the oncogenic potential of the different HPV genotypes. In order to clarify that point we edited the phrase.

S: Line 307-310, the statements about HPV vaccine cross protection here seem a stretch. If my understanding is correct, cross protection is likely due to closer phylogenetic grouping, rather than competition between different HPV subtypes.

A: Cross protection should be the result of protein epitopes shared by the different genotypes and recognized by antibodies raised by the vaccinated person. Although this should be correlated with phylogenetic relations it may not always be the case. As far as we understand, phylogenetic algorithms use conserved DNA or aminoacids to build the trees. They do not take into account three-dimensional epitopes. We do not state that cross protection is only due phylogenetic grouping, and as suggested by Woodman et al, studies of coinfection on vaccinated populations should shed light about competition or synergy. We have cited again that work (see reference) to state this point.

S: Table 1, unclear what the others category. Please add data on negative HPV results and missing results, if applicable.

A: ”Other/s category” has been removed from both tables (in the present version tables 1 and 3)

We consider that showing negative results would complicate table 1 interpretation. This data can easily been calculated by substracting No. cases minus HPV positivity n.

“Coinfection” has been defined in table 3