PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	POP-Brazil Study Protocol: a nationwide cross-sectional evaluation of the prevalence and genotype distribution of human papillomavirus (HPV) in Brazil
AUTHORS	WENDLAND, ELIANA; Caierao, Juliana; Domingues, Carla; Maranhão, Ana; de Souza, Flávia; Hammes, Luciano; Falavigna, Maicon; Hilgert, Juliana; Hugo, Fernando; Bessel, Marina; Villa, Luisa Lina; Benzaken, Adele

VERSION 1 – REVIEW

REVIEWER	Maarit K Leinonen, MD, PhD, Postdoctoral Researcher Department of Research, Cancer Registry of Norway, Norway
REVIEW RETURNED	10-Jan-2018

GENERAL COMMENTS	This is a study protocol for a planned/ongoing study in Brazil. Authors aim to study HPV prevalence and possible regional and/or personal differences in virus prevalence and HPV types among sexually active young women and men. In my opinion, existing scientific evidence is well described and justifies the conduct of this study. This is well designed study where strengths and limitations are adequately addressed. Furthermore, I would like to acknowledge great efforts that are planned to harmonize and monitor data collection including a pilot study. I have some minor concerns related to clarifications relating to the methods. 1. It is uncertain if the study is at a planning phase or if recruitment is already ongoing. Please specify anticipated length and time period of recruitment. 2. Reference #3 could be replaced with a newer reference on HPV types in cervical lesions, eg. by Guan et al. Int J Cancer 2012. 3. There is an overlap between the primary objective of the study and secondary objectives. To establish HPV prevalence baseline to evaluate future vaccine effectiveness on p.8 L12 is not an independent secondary objective but a primary objective of this study. 4. Study aimed to evaluate HPV prevalence in general population. General population includes pregnant women, women who have recently delivered a baby and also women who have ever had CIN2+ lesion in their life. These are considered ineligible for the study on p.8. I don´t totally agree with exclusions criteria. For instance, pregnancy is an immunosuppressive condition but a possible HPV infection is real, it may only manifest during the pregnancy. However, I understand that there might be national/regional guidelines on ethics and good recruitment praxis.

	Therefore, I would appreciate a few words/sentences why these exclusion criteria were chosen. 4. Please clarify on paragraph "measuremnets" starting on p.9 L42 that which information is collected by personal interview and which information is covered with a standardized questionnaire. 5. "Knowledge about HPV and vaccination" on p.10 L42 also covers questions about screening. 6. Please clarify on p.12 L45-47 "Whenever necessary, real time PCR using the TaqMan system will be performed". In what occasions? 7. In Figure 1 and on p.12 L52 it is uncertain if primary care professionals and participants have access to individual level data in the study platform or some kind of administrative statistics. Please specify, consider possible data protection issues and add this dimension (one more arrow) also in Fig. 1. Also correct became -> become in Footnotes. 8. Study logistics has quite many steps. Is monthly reporting feasible or even needed? I would assume some natural fluctuation from month to month which does not really warrant any action. Maybe every other month or even four times a year would be enough. Of course optimal intensity of reporting is dependent on study human and technical resources. 9. Please add a clarification who are using the private sector in Brazil, i.e. which sociodemoprahpic characteristics are underrepresented in study population. Also, is the 70% coverage (71% on p.5) valid in study target ages or overall? Often people using a private sector are people with higher socioeconomic position but that may not be so distinctive at study target ages. 10. The very last sentence of the study protocol on p.16 underlines and supports the stigma of being an HPV positive. It is nothing to be ashamed of nor represents unhealthy sexual behavior as most people will be infected at some point in their life. I think that it is important to advise HPV positive women to contact the health care in order to explain them what the result mean and allow them to ask questions. But saying that because they are HPV positive, they should learn about healthy sexual habits and STI prevention is wrong. I suggest rephrasing. 11. Is the study supported (p.20 L17) or fully financed by funding sources? Funding is crucial to succesfully conduct this kind of multicentric study. 12. In Figure 2. "Monitoring e resertification". Also, where does "* Amplification of beta-globin" refer to? 13. Why "penile" is a keyword? Population attributable fraction in penile cancer is lower than e.g. in anal cancer. If keyword refers to collection site, it is unprecise still as specimens are collected from penile/scrotal sites. Consider e.g. anogenital.

REVIEWER	Vanja Kaliterna Public Health Institute of Split and Dalmatia County, Split, Croatia
REVIEW RETURNED	21-Jan-2018

GENERAL COMMENTS	1. Page 5 – instead "HPV infection" put word Brazil (in key words there are HPV prevalence and HPV infection, but there is not Brazil) 2. Page 12 - add - Samples are treated as biohazardous material and all specimens handling is to be performed in biosafety cabinet 3. I suggest that vaccinated persons be included in the exclusion criteria - although a small number of vaccinated will be expected, they will cause unnecessary confusion in the interpretation of the results. 4. Methods should be better described for Protocol (in details) to

	allow the study to be repeated. 5. In my opinion, the greatest objection to this work is in selecting the types of biological samples and selecting the methods of sample processing. All certified tests mainly offer only a cervical sample as a sample type from which the test can be performed. The result can be interpreted only from cervical sample!!! The proposed Linear Array Genotyping Test, according manufacturer recommendations, can detect 37 anogenital HPV DNA genotypes in cervical cells collected in Cobas PCR cell collection media or PreservCyt Solution. The results can be interpreted only in this type of sample in this type of media. Therefore, I consider that other proposed specimens (penile, scrotal and oral) should not be used for HPV detection with the proposed test. Especially this applies to oral samples (mouthwash and gargle) because there are not enough HPV-infected cells in such samples. Due to poor sample selection, a large number of false-negative results can be expected, and consequently the wrong conclusion of the HPV prevalence. I think that this non adequate sampling for proposed method of HPV detection is very large limitation of this study. 6. In fact, the authors themselves have designed whole protocol for women, through objectives, exclusion criteria, outcomes, questions about sexual and reproductive health, and knowledge about Pap test. There are no questions related to men. On the end, I am suggesting that this protocol (POP Brazil Study Protocol: a nationwide cross-sectional evaluation of prevalence and genotype distribution of HPV in Brazil) should be based on women only: the prevalence of HPV and evaluating the most prevalent HPV genotypes in different regions of Brazil and their correlation with social, demographic, economic and behavioural factors. I consider that results of nationwide study about HPV prevalence and genotyping will be very useful in order to establish the impact of vaccination on the distribution of HPV types for any country.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1:

1. It is uncertain if the study is at a planning phase or if recruitment is already ongoing. Please specify anticipated length and time period of recruitment.

Response: and sentence was added to the text, in the recruitment and sample size section (line 120): From January 2017, sexually active women and men from 16 to 25 years old who use the public health system in all…"

2. Reference #3 could be replaced with a newer reference on HPV types in cervical lesions, eg. by Guan et al. Int J Cancer 2012.

Response: Thank you for your suggestion. Guan et al. was included in the text (line 68).

3. There is an overlap between the primary objective of the study and secondary objectives. To establish HPV prevalence baseline to evaluate future vaccine effectiveness on p.8 L12 is not an independent secondary objective but a primary objective of this study.

Response: The sentence was rephrased to make clear the main objective and the secondary objective related to vaccine effectiveness was excluded, as suggested (line 99): "The primary objective of the study is to determine the prevalence of HPV in women and men aged 16-25 in Brazil,

evaluating the most prevalent types and possible differences between regions and risk factors associated with positivity, establishing a baseline vaccine effectiveness evaluation."

4. Study aimed to evaluate HPV prevalence in general population. General population includes pregnant women, women who have recently delivered a baby and also women who have ever had CIN2+ lesion in their life. These are considered ineligible for the study on p.8. I don´t totally agree with exclusions criteria. For instance, pregnancy is an immunosuppressive condition but a possible HPV infection is real, it may only manifest during the pregnancy. However, I understand that there might be national/regional guidelines on ethics and good recruitment praxis. Therefore, I would appreciate a few words/sentences why these exclusion criteria were chosen.

Response: Exclusion criteria were stablished considering clinical situations in which endocervical collection of samples are not recommended, such as pregnancy. Besides, women who have recently delivered a baby and those who ever had CIN2+ lesions present cytological alterations in uterus that may cause a bias to the HPV infection, compared to the general population, justifying their exclusion. It was included a sentence in the text to explain it (line 123): "Because endocervical collection is not recommended during pregnancy in Brazil and/or to avoid selection bias, the following exclusion criteria apply"

4. Please clarify on paragraph "measuremnets" starting on p.9 L42 that which information is collected by personal interview and which information is covered with a standardized questionnaire.

Response: Thanks to take this in your attention. We clarify the sentence in the "Procedures" section to make clear that all information will be obtained by interview (line 188): "All individuals will respond to a standardized interview based on validated instruments…"

5. "Knowledge about HPV and vaccination" on p.10 L42 also covers questions about screening.

Response: The title of the section was changed to (line 170): "Knowledge about HPV, vaccination and screening tests"

6. Please clarify on p.12 L45-47 "Whenever necessary, real time PCR using the TaqMan system will be performed". In what occasions?

Response The sentence in the text was altered to clarify the use of real time PCR (line 230) "Real-time PCR using the TaqMan system for HPV type 52 will be performed to confirm the results obtained by the Roche's test as a combination of HPV types 52, 33, 35 and 58."

7. In Figure 1 and on p.12 L52 it is uncertain if primary care professionals and participants have access to individual level data in the study platform or some kind of administrative statistics. Please specify, consider possible data protection issues and add this dimension (one more arrow) also in Fig. 1. Also correct became -> become in Footnotes.

Response: In the page 10 (line 191), we modify the sentences to clarify that all each primary care health professional will be able to see the result of people recruited by them and also add a sentence to make clear participants will be able to see the results in an external webpage. "An online platform for data entry will be used by primary care professionals to include participant data, biological sample information and photographs. The same platform will be used for study process control and to make the results available to primary care health professionals. Participants will have access to an external webpage where they would find information about their results, protected by a password provided during interview. In cases where high-risk HPV infection are detected, participants will be asked to return to primary care units to be informed about the result."

8. Study logistics has quite many steps. Is monthly reporting feasible or even needed? I would assume some natural fluctuation from month to month which does not really warrant any action. Maybe every other month or even four times a year would be enough. Of course optimal intensity of reporting is dependent on study human and technical resources.

Response: Because all interview and sample collection are registered in the web-based platform, monthly reports are, actually, very simple to obtain. Indeed, exporting data from this platform enable

researcher team to have a close control of the number of participants interviewed in each city and even in each primary care unit as well as the number of samples collected. Besides, as a way to keep primary care professionals motivated, the reports include a “Top 5” section, recognizing those who more actively recruit eligible individuals.

9. Please add a clarification who are using the private sector in Brazil, i.e. which sociodemoprahpic characteristics are underrepresented in study population. Also, is the 70% coverage (71% on p.5) valid in study target ages or overall? Often people using a private sector are people with higher socioeconomic position but that may not be so distinctive at study target ages.

Response: The 70% coverage is in overall population. Population who uses private health services in Brazil generally represents those with higher socioeconomic classes (A and B). Most Brazilian people within the study target ages either uses public health system or dependents of older people's private health insurances. We add a sentence in the text to explain that (line 281): “The private section in Brazil is used mainly for classes A and B, and it is important to highlight that for some services as vaccination, the Public Health System covers virtually 100% of the population.”

10. The very last sentence of the study protocol on p.16 underlines and supports the stigma of being an HPV positive. It is nothing to be ashamed of nor represents unhealthy sexual behavior as most people will be infected at some point in their life. I think that it is important to advise HPV positive women to contact the health care in order to explain them what the result mean and allow them to ask questions. But saying that because they are HPV positive, they should learn about healthy sexual habits and STI prevention is wrong. I suggest rephrasing.

Response: We totally agree. The paragraph was re-written to clarify this issue (line 302): “In cases of no infection or infection with low-risk HPV type, participants will be informed of negative results via web access. On the other hand, participants infected with high-risk HPV types will be advised to go to the health care unit for their results, where they will be oriented about the meaning of the result and instructed about follow up according with the national guidelines.”

11. Is the study supported (p.20 L17) or fully financed by funding sources? Funding is crucial to succesfully conduct this kind of multicentric study.

Response: The study is fully financed by Hospital Moinhos de Vento through the Program for Supporting the Institutional Development of the Public Health System (PROADI-SUS), supported by the Ministry of Health of Brazil, with a funding participation of the Pan-American Health Organization. The sentence was rephrased.

12. In Figure 2. "Monitoring e resertification". Also, where does "* Amplification of beta-globin" refer to?

Response: Figure 2 was edited.

13. Why "penile" is a keyword? Population attributable fraction in penile cancer is lower than e.g. in anal cancer. If keyword refers to collection site, it is unprecise still as specimens are collected from penile/scrotal sites. Consider e.g. anogenital.

Response: Keywords were edited.

Reviewer 2:

1. Page 5 – instead "HPV infection" put word Brazil (in key words there are HPV prevalence and HPV infection, but there is not Brazil)

Response: Keyword were edited.

2. Page 12 - add - Samples are treated as biohazardous material and all specimens handling is to be performed in biosafety cabinet

Response: The suggested sentence was included in the text (line 218). “All samples are treated as biohazardous material and all specimens handling is to be performed in biosafety cabinet.”

3. I suggest that vaccinated persons be included in the exclusion criteria - although a small number of vaccinated will be expected, they will cause unnecessary confusion in the interpretation of the results.

Response: Indeed, we expect a small number of vaccinated individuals and the inclusion of those individuals will avoid selection bias and will allow to see the characteristics of such individuals. Those individual (we don’t know the percentage) will be excluded of the prevalence calculation and those data will be interpreted separately.

4. Methods should be better described for Protocol (in details) to allow the study to be repeated.

Response: The More details were included in the Procedures section, to allow replicability and modifications are colored in red.

5. In my opinion, the greatest objection to this work is in selecting the types of biological samples and selecting the methods of sample processing.
All certified tests mainly offer only a cervical sample as a sample type from which the test can be performed. The result can be interpreted only from cervical sample!!!
The proposed Linear Array Genotyping Test, according manufacturer recommendations, can detect 37 anogenital HPV DNA genotypes in cervical cells collected in Cobas PCR cell collection media or PreservCyt Solution. The results can be interpreted only in this type of sample in this type of media. Therefore, I consider that other proposed specimens (penile, scrotal and oral) should not be used for HPV detection with the proposed test.
Especially this applies to oral samples (mouthwash and gargle) because there are not enough HPV-infected cells in such samples. Due to poor sample selection, a large number of false-negative results can be expected, and consequently the wrong conclusion of the HPV prevalence.
I think that this non adequate sampling for proposed method of HPV detection is very large limitation of this study.

Response: We choose the sample procedures and processing techniques according to literature and to permit comparison with previous studies.
The use of Qiagen collection kits and Roche® linear array are already widely described in the literature, either for women and men. The penile sample collection was based on the HIM study (Repp et al. Male Human Papillomavirus Prevalence and Association with Condon Use in Brazil, Mexico, and the United States. The Journal of Infectious Diseases, 2012; 205: 1287-93) and Flores et al (Reliability of sample collection and laboratory testing for HPV detection in men. Journal of Virology Methods, 2008; 149:136-143) who uses the same collection kit and methodology for detection and HPV typing.
Regarding cervical samples, Qiagen collection kits were used in the United Kingdom HPV survey (Howell-Jones et al. Prevalence of human papillomavirus (HPV) infections in sexually active adolescents and young women in England, prior to widespread HPV immunization. Vaccine, 2012; 30: 3867-3875) and in the survey done in US (Dunne et al. Prevalence of HPV types in cervical specimens from an integrated healthcare delivery system: baseline assessment to measure HPV vaccine impact. Cancer Causes Control, 2013; 24: 403-407). Both uses the same Roche® linear array methodology for HPV typing.
For oral HPV, we also choose the same technique of collection and processing of previous studies, to allow further comparison (CDC Laboratory Procedure Manual – Human Papillomavirus from Oral Rinse - https://wwwn.cdc.gov/nchs/data/nhanes/2013-2014/labmethods/ORHPV_H_OHPV_R_MET.pdf; Steinau et al. Prevalence of cervical and oral human papillomavirus infections among US women. J Infect Dis, 2013; 209: 1739-43 and Rollo et al. Prevalence and determinants of oral infection by Human Papillomavirus in HIV-infected and unifected men who have sex with men. PloSOne. https://doi.org/10.1371/journal.pone.0184623, September 14, 2017).

6. In fact, the authors themselves have designed whole protocol for women, through objectives, exclusion criteria, outcomes, questions about sexual and reproductive health, and knowledge about Pap test. There are no questions related to men.

On the end, I am suggesting that this protocol (POP Brazil Study Protocol: a nationwide cross-sectional evaluation of prevalence and genotype distribution of HPV in Brazil) should be based on women only: the prevalence of HPV and evaluating the most prevalent HPV genotypes in different regions of Brazil and their correlation with social, demographic, economic and behavioural factors.
I consider that results of nationwide study about HPV prevalence and genotyping will be very useful in order to establish the impact of vaccination on the distribution of HPV types for any country.

Response: the men were included in the protocol to allow the evaluation of herd effect, very important in terms of disease dissemination and vaccination impact, one of the main objectives of the study. A secondary objective regarding herd effect was added to the text to make this clear.

VERSION 2 – REVIEW

REVIEWER	Maarit K Leinonen, MD, PhD, Postdoctoral Researcher Department of Research, Cancer Registry of Norway, Norway
REVIEW RETURNED	30-Mar-2018

GENERAL COMMENTS	Authors have adequately addressed all of my concerns in this revision. I have no further comments, and I am happy to recommend publishing the protocol.

REVIEWER	Vanja Kaliterna Public Health Institute of Split and Dalmatia County,Croatia
REVIEW RETURNED	02-Apr-2018

GENERAL COMMENTS	1. Although a small numbers of vaccinated individuals are expected, so there is no need that vaccinated persons be included in the study. I still suggest that vaccinated persons be included in the exclusion criteria. 2. In my opinion, the greatest objection to this work is in selecting the types of biological samples and selecting the methods of sample processing - This was not adopted in the corrections. All certified tests mainly offer only a cervical sample as a sample type from which the test can be performed. The result can be interpreted only from cervical sample!!! The proposed Linear Array Genotyping Test, according manufacturer recommendations, can detect 37 anogenital HPV DNA genotypes in cervical cells collected in Cobas PCR cell collection media or PreservCyt Solution. The results can be interpreted only in this type of sample in this type of media. Therefore, I consider that other proposed specimens (penile, scrotal and oral) should not be used for HPV detection with the proposed test. Due to poor sample selection (with low amount of HPV-infected cells in such samples), a large number of false-negative results can be expected, and consequently the wrong conclusion of the HPV prevalence. In my opinion, this non adequate sampling for proposed method of HPV detection is very large limitation of this study. 3. One tip to the end - it might be better to use the universal ThinPrep media (20 ml) instead of Digene Collection Kit (1 ml), because from 1 ml of media is very difficult to aliquot part of the sample to be maintained at -80 as a backup.

VERSION 2 – AUTHOR RESPONSE

Reviewer: 2

1. Although a small numbers of vaccinated individuals are expected, so there is no need that vaccinated persons be included in the study. I still suggest that vaccinated persons be included in the exclusion criteria.

Response: Vaccinated individuals will be excluded of the prevalence calculation and those data will be interpreted separately. These individuals will be included in the study because it will allow answer a question of interest of the Ministry of Health of Brazil: knowing how many people in this age range is already vaccinated. We believe that the interest of different stakeholders need to be take in account in researches of public health interest. Additionally, we will have preliminary data to compare the prevalence of HPV among vaccinated and non-vaccinated individuals, in a sensitive analysis.

2. In my opinion, the greatest objection to this work is in selecting the types of biological samples and selecting the methods of sample processing - This was not adopted in the corrections.

All certified tests mainly offer only a cervical sample as a sample type from which the test can be performed. The result can be interpreted only from cervical sample!!!

The proposed Linear Array Genotyping Test, according manufacturer recommendations, can detect 37 anogenital HPV DNA genotypes in cervical cells collected in Cobas PCR cell collection media or PreservCyt Solution. The results can be interpreted only in this type of sample in this type of media.

Therefore, I consider that other proposed specimens (penile, scrotal and oral) should not be used for HPV detection with the proposed test.

Due to poor sample selection (with low amount of HPV-infected cells in such samples), a large number of false-negative results can be expected, and consequently the wrong conclusion of the HPV prevalence.

In my opinion, this non adequate sampling for proposed method of HPV detection is very large limitation of this study.

Response: We are grateful for the careful review of our manuscript. However, we have to disagree with the reviewer whose comments may be appropriate for clinically relevant assays but do not apply to our study. In fact, there are several publications on the prevalence of HPV using the Roche's Linear Array HPV assay in epidemiological studies. Among them, surveys in the United States of America performed with cervical smears (Khan et al., J Natl Cancer Inst. 2005;97:1072-1079) and oral samples (Beachler et al., J Infect Dis 2013; 208:330-339). Moreover, in Canada, the prevalence of HPV in both anal and cervical scrapings from men and women was obtained by the same assay employed in our study (Coutlée et al., J Clin Microbiol 2006; 44:1998-2006). As an additional example, Sudenga et al. (Eur Urol 2016, 69(1):166-173) determined the prevalence of HPV in male genital samples from three countries, collected with STM collection medium (Qiagen, formerly Digene) and tested with the Roche's Linear Array HPV test, exactly the same as in our study. Moreover we, the same medium and genotyping technique is used by CDC to collect oral and penile samples (protocols in annex). Finally, the WHO document in annex also reassure the use of linear array with the STM medium. Therefore, we are convinced that our protocol is perfectly suitable to define the prevalence of HPV in samples collected from the genitals and oral cavity of both women and men and should be published as submitted.

3. One tip to the end - it might be better to use the universal ThinPrep media (20 ml) instead of Digene Collection Kit (1 ml), because from 1 ml of media is very difficult to aliquot part of the sample to be maintained at -80 as a backup.

Response: 500µg of the material is stored in -80 after centrifugation. We did not have any technical issue with this process until know.