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BRIEF REPORT

REVISED Human papillomavirus (HPV) detection in vaginal self-samples: evaluation of eNat® as an alternative suspension medium to ThinPrep®PreservCyt® for vaginal swabs

[version 2; peer review: 2 approved]

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Abstract

Human Papillomavirus (HPV) testing on self-collected samples allows for improved coverage rates of cervical cancer (CC) screening programs. ThinPrep®PreservCyt® (HOLOGIC®, USA) medium is widely used for the suspension of cervical and vaginal self-samples. However, this medium is costly, toxic, and flammable, involving special handling procedures which make its use difficult in screening programs, particularly in low- and middle-income countries.

This pilot study aimed to evaluate the analytical performance of eNat® (Copan SpA), an alternative non-alcohol-based suspension medium, compared to ThinPrep®PreservCyt® (HOLOGIC®) for high-risk HPV (hrHPV) detection in vaginal self-collected swabs using three different real-time polymerase chain reaction (RT-PCR) HPV assays: Anyplex™II HPV28 (Seegene, Korea), Papilloplex® High Risk HPV (GeneFirst, UK), and HPV OncoPredict (Hiantis, Italy).

30 women, referred to colposcopy, were enrolled in this observational, prospective pilot study and asked to collect two vaginal self-taken samples, which were suspended in 5 mL of ThinPrep®PreservCyt® or eNat®. Nucleic acids were extracted from 200 µL using Microlab Nimbus platform (Seegene, Korea) and tested with the three different RT-PCR full-genotyping high-risk HPV assays. The HPV results of vaginal samples resuspended in the two different media were compared to those obtained from the reference clinician-collected cervical sample from the same woman.

hrHPV detection in vaginal self-samples suspended in both media demonstrated a substantial agreement with cervical samples with the

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1. **David Hawkes**, VCS Foundation, Carlton South, Australia

2. **Alberto Severini**, University of Manitoba, Winnipeg, Canada

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three assays under-investigation (0.667≤k≤0.796). Moreover, the discordances between vaginal self-samples collected from the same woman were found only in cases of normal cytology or low-grade cytological lesions and were generally related to low hrHPV viral loads as indicated by the quantitative HPV OncoPredict assay (6.24E+02 copies/10,000 cells).
The study's preliminary findings demonstrated a very good agreement between cervical and vaginal self-collected samples suspended in ThinPrep®PreservCyt® and eNat®, suggesting that the latter could represent a good alternative medium in HPV screening programs based on self-collection.

Keywords
Human Papillomavirus (HPV), HPV-testing, vaginal self-collection, ThinPrep®PreservCyt, eNat®



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REVISED **Amendments from Version 1**

We updated the previous version of the manuscript to respond to the requests of the reviewers.

As asked by Prof. David Hawkes, in the results section, we added a table reporting HPV genotypes distribution among cervical and vaginal self-samples suspended in ThinPrep®PreservCyt® and eNat® using the three different evaluated HPV assays (Table 2) to allow a better comparison of the results of the three specimen types with the three different HPV detection kits.

In the discussion section, we also replied to the requests of the second reviewer, Prof. Alberto Severini, underlining that the main limitation of the present study is the restricted sample size which does not allow to perform statistically analysis. The present investigation represents a pilot study aimed to perform a preliminary evaluation of an alternative suspension medium for vaginal-self samples for cervical cancer screening. Finally, in the discussion we underlined that future studies on a larger number of women will be necessary to fully evaluate any potential difference in the performance of HPV testing using eNat® as an alternative suspension medium for vaginal self-specimens.

Any further responses from the reviewers can be found at the end of the article

Plain language summary

Persistent long-term infection with Human Papillomaviruses (HPVs) is associated with the development of cervical carcinoma. Cervical cancer (CC) screening programs based on the detection of HPV can reduce its incidence and mortality. Screening programs, based on HPV testing of self-collected vaginal samples, have been shown to be more acceptable to women and can improve their participation to CC screening programs. ThinPrep®PreservCyt® (HOLOGIC®, USA) solution is widely used for the suspension of vaginal self-samples. However, this solution is costly, toxic, and flammable, requiring special handling procedures, making its use difficult, particularly in low- and middle-income countries. This study aims to evaluate an alternative non-alcohol-based suspension medium, the eNat® (Copan SpA, Italy) for HPV detection in vaginal self-collected samples. This study involved the enrolment of women referred to a gynaecologist for an abnormal Pap test. During their gynaecological visit, women were asked to provide two self-collected vaginal swabs, one to be suspended in the eNat® medium and the other in ThinPrep®PreservCyt® solution, prior to HPV testing. An additional sample, collected by the clinician from the cervix of the same patients, was used as gold-standard reference method to compare HPV results obtained from the two vaginal samples. All samples were tested using three different HPV assays: a commercial kit, and two other HPV tests recently developed as part of a project financed by the European Commission. Preliminary results obtained using all three evaluated HPV tests on vaginal samples suspended in the alternative eNat® medium were comparable to those obtained on vaginal and cervical samples suspended in ThinPrep®PreservCyt® solution. In conclusion, an improved and cost-effective solution for CC screening based on self-collected vaginal samples, suspended in an alternative non-flammable medium, compatible with three different innovative HPV assays, has been evaluated with the aim to favour women's participation, particularly in low- and middle-income countries.

Introduction

According to data from GLOBOCAN 2020, a total of 604,127 new cases of cervical cancer (CC) and 341,831 CC related deaths occurred worldwide in 2020. The recent call of action proposed by the World Health Organization (WHO) to eliminate CC sets as one of the goals the screening of 70% women by the age of 35 and again by the age of 45 using high-performance assays, such as human papillomavirus (HPV) testing by 2030[1].

The introduction of HPV testing as an analysis tool offers the possibility of using self-sampling to increase participation in screening programs for CC prevention. HPV testing on self-samples has been reported to be similarly accurate in the detection of cervical precancerous lesions, as well as HPV testing performed on clinician-collected cervical samples. Moreover, self-sampling allows women who, for socio-cultural reasons, do not access gynaecological examination, to take part to screening programs[2–4].

ThinPrep®PreservCyt® (HOLOGIC®, USA) is an alcohol-based solution that serves as transport and liquid preservative for performing liquid-based Pap Smear on cervical samples. Therefore, HPV testing is routinely performed on the same type of sample and, for analogy, vaginal swabs for self-sampling have been usually suspended in ThinPrep®PreservCyt® solution.

Because of its high percentage of methanol, ThinPrep®PreservCyt® is flammable and requires special handling and additional costs for transport. These characteristics of ThinPrep®PreservCyt® make it difficult to introduce its use in self-collection-based CC screening in low to middle income countries. To overcome these problems, a non-alcohol-based medium to suspend self-collected vaginal samples that supports HPV nucleic acid stability and that is suitable for molecular HPV analysis is necessary[5].

eNat® (Copan Italia SpA, Brescia Italy) is a lysing based molecular collection medium already used for nucleic acids amplification assays[6–8]. Moreover, it preserves and stabilizes nucleic acids and desaturates proteins and inactivates microbial agents potentially contained in clinical samples.

This pilot study aims to evaluate the analytical concordance of HPV testing conducted on a physician-collected cervical sample (gold-standard) as compared to that performed on two dry vaginal self-collected samples eluted respectively in ThinPrep®PreservCyt® and eNat®.

Methods

Ethics and consent

This study was approved by The Ethics Committee of the University of Milano-Bicocca, Monza, Italy (Protocol n. 0037320/2017 and 0086409/2018). All participants gave written informed consent prior to participation.

Study design and samples collection

30 women, referred to colposcopy for a recent abnormal Pap smear reported as either low-grade intra-epithelial lesion (LSIL), high-grade intra-epithelial lesions (HSIL), atypical squamous cells of undetermined significance (ASCUS), or atypical glandular cells of undetermined significance (ACGUS), such as ASCH (atypical squamous cells – cannot exclude HSIL) and ACG (atypical glandular cells), were enrolled as part of an ongoing study (from March 2020 to January 2021). Immunocompromised patients, women with autoimmune diseases or any diseases involving the immune system, including HIV infection, with a presumed or confirmed pregnancy, with a diagnosis of any malignancies, or undergoing or having finished a course of chemotherapy during the six months preceding the study were excluded from the study. After signing the written informed consent, women were provided with the vaginal collection devices as well as written instructions illustrating how to perform the self-sampling; medical and nursing staff were also available if further assistance was required by the participating women. All enrolled women autonomously collected two vaginal-self samples using FLOQSwab® 552C.80 device (Copan Italia SpA, Brescia Italy) prior to coloposcopic examination. The two vaginal self-collected samples were numbered to trace the order in which they were collected and kept dry at room temperature until analysis. During the colposcopy examination, a cervical sample was taken by the gynaecologist using an L-shaped Endo/Esocervical FLOQSwab® (Copan Italia SpA, Brescia Italy). Women underwent biopsy and/or conization depending on the colposcopy outcome, according to the local clinical protocol. All specimens were transported to the Laboratory of Clinical Microbiology of the Department of Medicine and Surgery, University of Milano-Bicocca, Italy, where they were processed.

Pre-analytic sample processing

Clinician-collected cervical samples, obtained using the L-shaped swab, were immediately placed in 20 ml of ThinPrep®PreservCyt®. In the laboratory all specimens were vortexed for 30 seconds, and subsequently 1.5 ml aliquots were dispensed in cryovials; one was used for DNA extraction and the others stored at -20°C.

Vaginal self-collected swabs were transported dry to the laboratory. One swab was suspended in 5 ml of ThinPrep®PreservCyt® and the other in 5 ml of eNat®. In order to avoid bias associated with the order of vaginal swab collection, the first vaginal swab collected from 15 women was suspended in ThinPrep®PreservCyt®, while the second in eNat®; for the remaining 15 patients the first specimen was suspended in eNat® and the second in ThinPrep®PreservCyt®. Five aliquots of 1 ml were made from each of the vaginal specimens; one was used immediately for nucleic acids extraction prior to testing and the others stored at -20°C.

Nucleic acids extraction and HPV detection

One aliquot of the cervical and of each of the vaginal samples was extracted using STARMag 96x4 Universal Cartridge Kit (Seegene, Korea) on Microlab Nimbus (Seegene, Korea) platform, a completely automated Liquid Handling Workstation for nucleic acid extraction and polymerase chain reaction (PCR) setup of up to 72 specimens. DNA was extracted from a 200 µL volume of each sample and following extraction eluted in 100 µL of the kit elution buffer, according to the manufacturer's instructions.

Cervical and vaginal specimens were tested for HPV genotypes using three different real-time PCR full-genotyping HPV assays: Anyplex™II HPV28 (Seegene, Korea), Papilloplex® High Risk HPV (GeneFirst, UK) and HPV OncoPredict (Hiantis, Italy).

The first assay can identify 14 hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and 14 Low-risk HPV (lrHPV) types (6, 11, 26, 40, 42, 43, 44, 53, 54, 61, 69, 70, 73, and 82) in two different reaction mixes by means of real-time PCR assays. Papilloplex® High Risk HPV is able to detect and types 14 hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) based on specific melting profiles. HPV OncoPredict assay detects and quantifies 12 hrHPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) and uses C-C chemokine receptor type 5 (CCR5) to detect sample's cellularity both to evaluate sample adequacy and to allow for normalization of viral load.

All three Real-time assays were performed using a CFX96 PCR Thermal Cycler (Bio-Rad, Hercules, USA) according to manufacturers' instructions using 5 µl of template DNA in a total volume of 20 µl for Anyplex™II HPV28. Analysis with Papilloplex® High Risk HPV and HPV OncoPredict was performed using 5 µl of extracted DNA in a total volume of 20 µl.

Statistical analysis

Patients' age was described by median value and interquartile range (IQ, range: IQ1-IQ3). Viral load was expressed as number of viral genome copies (cp)/10,000 cells. Agreement between HPV testing results on different types of samples and different tests was evaluated with the Cohen's kappa (κ) statistics using GraphPad QuickCalcs 2014 software. Agreement was defined as slight (0.00<k<0.20), fair (0.20<k<0.40), moderate (0.41<k<0.60),

substantial (0.61<k<0.80) and almost perfect (0.81<k<1.00) as previously reported[9].

Results

Population analysis

The median age of the 30 women enrolled for this study was 36.5 years (interquartile range (IQ): 29.3–47). Most of the women (23/30; 76.7%) presented cytological alterations: low-grade intra-epithelial lesion (LSIL) was the most frequently detected (14/30; 46.7%) followed by the atypical squamous cells of undetermined significance (ASCUS) in 5/30 (16.7%) women. High-grade intra-epithelial lesions (HSIL) were found in 3/30 (10.0%) women, atypical glandular cells (AGC) in 1/30 (3.0%) woman and 7/30 (23.0%) women had a negative (NEG) Pap smear result.

Following colposcopy examination results, 5/30 (16.7%) women underwent conization: histological result was cervical intra-epithelial neoplasia grade 1 (CIN1) for one woman, cervical intra-epithelial neoplasia grade 2 (CIN2) for two women and cervical intra-epithelial neoplasia grade 3 (CIN3) for two women.

Prevalence of hrHPV in cervical and vaginal samples

17 out of 30 (17/30, 56.6%) cervical samples were found to be hrHPV positive using Anyplex™ HPV28 detection kit, 16/30 (53.3%) with HPV OncoPredict and 15/30 (50%) with Papilloplex® High Risk HPV. Among vaginal self-samples suspended in ThinPrep®PreservCyt®, 20/30 (66.6%), 19/30 (63.3%) and 20/30 (66.6%) were hrHPV positive with each of the three assays, respectively; while among those suspended in eNat® 20/30 (66.6%) were found to be hrHPV positive with all three kits.

Figure 1 shows the distribution of different hrHPV genotypes among cervical and vaginal samples according to the three different HPV assays. HPV16 and HPV31 were the hrHPV types most frequently detected with the three different methods, in cervical and vaginal-self samples suspended in ThinPrep®PreservCyt® and eNat®. None of the enrolled women were found to be positive for HPV33 or HPV35.

hrHPV detection agreement

As reported in Table 1, for all HPV DNA kits, a substantial agreement for the detection of any hrHPV between vaginal swabs eluted in either eNat® or ThinPrep®PreservCyt® media and cervical samples was observed.

The concordance between vaginal self-samples suspended in ThinPrep®PreservCyt® and eNat® was demonstrated to be almost perfect with percentages of agreement of 100.0% (30/30; k=1.000) 100.0% (30/30; k=1.000) and 96.7% (29/30; k=0.927) respectively with Anyplex™II HPV28, Papilloplex® High Risk HPV and OncoPredict.

Data regarding HPV genotypes distribution in each sample is reported in Table 2.

For all HPV DNA tests, no differences in HPV detection rate related to the order of vaginal specimens' collection were observed.

Viral load quantification

HPV OncoPredict quantification kit was used to determine HPV type specific and total viral load for all tested samples.

The mean value of the total normalized viral load in cervical samples was lower than that detected in vaginal samples suspended either in ThinPrep®PreservCyt® or eNat® (2.03E+05 cp/10,000 cells vs 3.26E+05 cp/10,000 cells and 4.59E+05 cp/10,000 cells; respectively). Interestingly, similar viral loads were detected in both vaginal samples irrespective of the suspension medium used. Discordant results in type-specific HPV infection between the two vaginal swabs/woman were associated with either low-grade or negative cytology or with viral loads that were below 6.24E+02 cp/10,000 cells.

Discussion

This pilot study compared the analytical concordance of hrHPV DNA detection in self-collected vaginal swabs resuspended in two different media (eNat® and ThinPrep®PreservCyt®) as compared to that detected in clinician-collected cervical samples.

Tested cervical specimens were found to be hrHPV positive with a percentage ranging from 50% to 56.6% using the three investigated hrHPV detection kits; whilst a range of 63.3% to 66.6% positivity was observed among vaginal swabs. HPV16 and HPV31 were shown to be the most prevalent hrHPV types in both cervical and vaginal samples, as also previously reported[10,11]. The slight variation in the distribution of hrHPV types may be due to the difference in the anatomical sites of samples' collection[12,13], but also to the difference in the total volume used for sample suspension; cervical samples and vaginal specimens were suspended in 20 mL and 5 mL, respectively. The reduction in volume of collection medium may, in fact, improve HPV detection and, at the same time, reduce the costs of HPV screening[5].

In this study, substantial agreement in hrHPV detection was demonstrated between cervical and vaginal self-samples with a concordance rate ranging from 83.3% to 90.0% for the different assays and suspension media; this is in agreement with previous reports[13–16] confirming that self-sampling could be a procedure to improve screening coverage rates. With growing evidence to support this alternative method for sample collection[2,14], the introduction of self-sampling, as a strategy to prevent CC may increase participation of women not attending

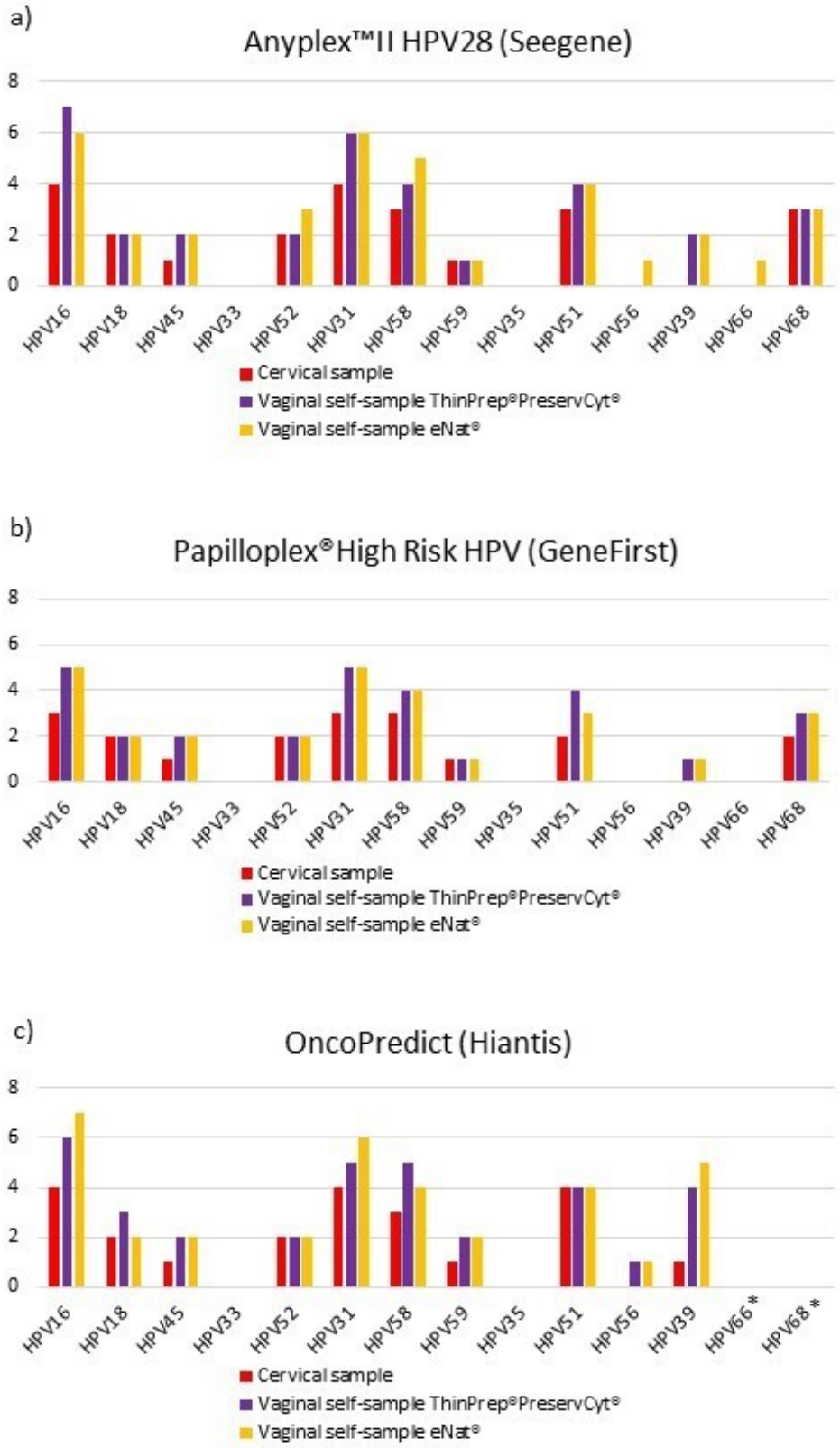


Figure 1. a) Prevalence of high-risk human papillomavirus (hrHPV) infections with Anyplex™II HPV28 (Seegene); b) Prevalence of hrHPV infections with Papilloplex® High Risk HPV (GeneFirst); c) Prevalence of hrHPV infections with HPV OncoPredict (Hiantis). *HPV Oncopredict assay does not detect HPV 66 and HPV68 genotypes.

Table 1. Agreement between clinician collected cervical samples and vaginal self-samples suspended in ThinPrep®PreservCyt® and eNat®.

	Anyplex™II HPV28 (Seegene)		Papilloplex® High Risk HPV (GeneFirst)		HPV OncoPredict (Hiantis)	
	Vaginal self-samples ThinPrep®PreservCyt®	Vaginal self-samples eNat®	Vaginal self-samples ThinPrep®PreservCyt®	Vaginal self-samples eNat®	Vaginal self-samples ThinPrep®PreservCyt®	Vaginal self-samples eNat®
	% concordance; Cohen k	% concordance; Cohen k	% concordance; Cohen k	% concordance; Cohen k	% concordance; Cohen k	% concordance; Cohen k
Clinician-collected cervical samples	90.0%; k=0.791	90.0%; k=0.791	83.3%; k=0.667	83.3%; k=0.667	90.0%; k=0.796	86.7%; k= 0.727

organized prevention programs, and may also be a useful alternative to perform screening in low to middle income countries where CC is still widespread [GLOBOCAN, 2020]. However, the higher costs associated with the use of ThinPrep®PreservCyt® together with its flammable nature may delay its use in self-samples-based CC screening, particularly in low-resource settings that would benefit the most from this cancer prevention strategy. Badman and colleagues had investigated four non-volatile transport media as potential alternatives to ThinPrep®PreservCyt for HPV screening by using HPV-infected cell lines[5]. As eNat® is not flammable, is able to inactivate infectious agents present in the sample and stabilizes nucleic acids in samples stored at room temperature[6,17]. Moreover, it is already routinely used as a medium for molecular HPV detection[8,18] and it could represents a valid alternative to ThinPrep®PreservCyt®. In addition, this study demonstrated an almost perfect agreement between vaginal swabs suspended in ThinPrep®PreservCyt® and eNat® using the three different diagnostic assays. Data obtained using the quantitative HPV OncoPredict detection assay indicated that the discordances in HPV detection observed between the two vaginal swabs collected by each participating woman were related to the low viral load observed in the discordant samples (below 6.24E+02 cp/10,000 cells). Discrepant results had been previously reported in samples with low HPV viral load[18]. Moreover, all the observed discordances were observed in patients with low-grade or negative cytology.

The mean hrHPV viral load for self-collected vaginal swabs, eluted in ThinPrep®PreservCyt® and for those suspended in eNat®, was higher (3.26E+05 cp/10,000 cells and 4.59E+05 cp/10,000 cells; respectively) than that observed in cervical samples (2.03E+05 cp/10,000 cells). The higher HPV viral load detected in vaginal samples may be associated to the differences in the suspension volume of the media used for cervical and vaginal specimens (20 mL vs 5 mL). Viral load normalization based on the samples' human cellularity should have taken into account the differences associated with suspension volumes. Statistical analysis on a larger number of samples will help to elucidate differences in normalized viral loads between the vaginal and cervical samples.

The main limitation of this pilot study is the restricted number of women enrolled. Future studies including a greater number of samples will be necessary to fully evaluate any potential difference in performance of HPV testing using this alternative suspension medium.

Conclusion

In conclusion, this study demonstrated that vaginal self-sampling is a good alternative to cervical swab if the sample is collected in either ThinPrep®PreservCyt® or eNat®, with the second medium allowing viral inactivation and providing a good strategy to further reduce costs.

Previous studies compared different devices for vaginal self-sampling considering cost, simplicity of use and accuracy in HPV detection. FLOQSwab® appeared to be the best option because of its performances, cost and the possibility to transport samples dry[18,19]. To our knowledge, this is the first study that compares eNat® to ThinPrep®PreservCyt® for the suspension of vaginal samples. Future studies including a greater number of clinical samples and other alternative suspension media are necessary to better evaluate the best solution for vaginal self-samples testing.

Table 2. HPV genotypes distribution among cervical and vaginal self-samples suspended in ThinPrep®PreservCyt® and eNat® using the 3 different evaluated HPV assays.

			Anyplex™II HPV28 (Seegene)			HPV OncoPredict (Hiantis)			Papilloplex® High Risk HPV (GeneFirst)		
Sample number	**Cytology**	**Biopsy**	**Clinician-collected cervical samples**	**Vaginal self-samples ThinPrep® PreservCyt**	**Vaginal self-samples eNat®**	**Clinician-collected cervical samples**	**Vaginal self-samples ThinPrep® PreservCyt**	**Vaginal self-samples eNat®**	**Clinician-collected cervical samples**	**Vaginal self-samples ThinPrep® PreservCyt**	**Vaginal self-samples eNat®**
MO241	ASCUS		16, 31, 51, 68	16, 31, 51, 68	16, 31, 51, 68	16, 31, 51	16, 31, 51, 39	16, 31, 51, 39	51, 68	51, 68	51, 68
MO243	LSIL		59	16, 59	59	51, 59	59	59	59	59	59
MO244	LSIL		68	31, 68	31, 68	39	39	31, 39	68	68	68
MO192 T12	NEG		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO245	LSIL		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO186 T12	ASCUS		31	31	31	31	31	31	31	31	31
MO180 T18	LSIL		51	45, 51	45, 51	51	45, 51	45, 51	NEG	45, 51	45, 51
MO246	LSIL		51	51	51	51	51	51	51	51	51
MO247	HSIL	CIN 3	16	16	16	16	16	16	16	16	16
MO034 T30	LSIL		NEG	NEG	NEG	NEG	NEG	NEG	NEG	31	31
MO254	ASCUS		45, 52	45, 52	45, 52	45, 52	45, 52	45, 52	45, 52	45, 52	45, 52
MO043 T30	LSIL		31, 58	16, 31, 39, 58	16, 31, 39, 58	31, 58	16, 31, 39, 58, 59	16, 31, 39, 58, 59	31, 58	16, 31, 39, 58	16, 31, 39, 58
MO228 T12	NEG	CIN 1	NEG	16	16, 58, 66	NEG	16, 58	16	NEG	16	16
MO255	LSIL	CIN 2	16	16, 51	16, 51	16	16, 51	16, 51	16	16, 51	16
MO235 T6	LSIL		16	16	16	16	16, 18	16	16	16	16
MO269	ASCUS		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO270	ASCUS		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO230 T12	LSIL		52	31, 52	31, 52	52	31, 52	31, 52	52	31, 52	31, 52
MO222 T12	LSIL		68	68	52, 68	NEG	39	16, 39	NEG	68	68
MO248	AGC		NEG	58	56, 58	NEG	56, 58	56, 58	NEG	58	58
MO249	LSIL		18	18	18	18	18	18	18	18	18
MO250	HSIL	CIN2	31, 58	31, 58	31, 58	31, 58	31, 58	31, 58	31, 58	31, 58	31, 58
MO229 T6	NEG		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO251	LSIL		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO252	LSIL		18	18	18	18	18	18	18	18	18
MO149 T24	NEG		NEG	39	39	NEG	NEG	39	NEG	NEG	NEG
MO253	HSIL	CIN 3	58	58	58	58	58	58	58	58	58
MO256	NEG		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO210 T12	NEG		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
MO258	NEG		NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Data availability

Zenodo: Human papillomavirus (HPV) detection in vaginal self-samples: evaluation of eNat® as an alternative suspension medium to ThinPrep®PreservCyt® for vaginal swabs. https://doi.org/10.5281/zenodo.6077699[20]

This project contains the following files:

- HPV positivity on cervical and.csv
- hrHPV viral load.csv
- Legend HPV positivity on cervical and vaginal self-samples with different HPV real-time assay.docx
- Legend hrHPV viral load.docx

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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Writing – review & editing: Clementina Elvezia Cocuzza, Rosario Musumeci, Tarig Dafa'alla, Winnie Wu, Santina Castriciano, Paolo Romano.

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Open Peer Review

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Version 2

Reviewer Report 05 August 2022

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✔ **Alberto Severini**
Department of Medical Microbiology and Infectious Diseases, Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada

The authors have addressed my minor comments satisfactorily and I have no more concerns. I recommend that the manuscript be accepted for indexing without further revisions.

Thank you for giving me the opportunity to review this manuscript.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 01 August 2022

https://doi.org/10.21956/openreseurope.16153.r29754

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✔ **David Hawkes**
VCS Foundation, Carlton South, Australia

Thank you to the authors for their responses. I am satisfied with these responses to my questions. The only other item I would raise is more for their own interest. The data for M0244 and M0222T12 showed that the PapilloCheck and Seegene assay produced hPV68 results in 5/6 and 6/6 specimens, however, the GeneFirst OncoPredict assay returned a HPV39 positive result for 5/6 specimens. Considering how genetically close the HPV68 and HPV39 HPV types are I might

suggest a discussion with the manufacturers of that assay identifying a potential source of off-target responses (HPV39 positive result) in the presence of HPV68 (it would require more follow-up to determine whether this observation was for HPV68a and/or HPV68b).

Competing Interests: S Castriciano on behalf of Copan has undertaken research (including funding) with VCS Foundation and a co-authored presentation with the reviewer is being presented at EUROGIN 2022. Copan has also donated stock to VCS Foundation for a self-collection validation study, but they have had not input into the protocol design or outcomes.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 12 April 2022

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Alberto Severini
[1] Department of Medical Microbiology and Infectious Diseases, Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada
[2] Department of Medical Microbiology and Infectious Diseases, Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada

Giubbi *et al.* have compared the detection of HPV in self-collected samples transported in PreservCyt® and in Copan eNat® medium. The results show no difference in sensitivity between the two transport media. These are useful results, which demonstrate the utility of the aqueous eNat® medium for self-collected HPV specimens.
Minor comments:

1. The weakness of this study is the low number of women (n=30). The study does not have the power to detect small differences in performance that may matter when hundreds of thousands of women are tested. The authors should point this out in their discussion.

2. The authors compare the HPV viral loads detected in various types of specimens and conclude that vaginal specimens have a lower viral load. However, there is no statistical analysis to support this conclusion and it is possible that the small differences observed fall within the expected experimental variance.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular diagnostics, HPV molecular epidemiology, Vaccine preventable diseases

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Jun 2022

Clementina COCUZZA

The authors would like to thank Prof. Alberto Severini for his review and comments. (Reviewer comments in italics)

Giubbi et al. have compared the detection of HPV in self-collected samples transported in PreservCyt® and in Copan eNat® medium. The results show no difference in sensitivity between the two transport media. These are useful results, which demonstrate the utility of the aqueous eNat® medium for self-collected HPV specimens.

Minor comments:

- *The weakness of this study is the low number of women (n=30). The study does not have the power to detect small differences in performance that may matter when hundreds of thousands of women are tested. The authors should point this out in their discussion.*

Reply: We agree with the reviewer that the main limitation of this study is the number of women enrolled. As reported in the text of the manuscript, this is intended to be a pilot study aiming to perform a preliminary evaluation of a possible alternative suspension medium for vaginal-self samples. This eNAT medium by Copan has as intended use the resuspension of clinical samples for other molecular tests, such as microbiome analysis, and studies on its use for this purpose have also been previously published. However future studies on a larger number of women will be necessary to better evaluate the performance

of eNAT in vaginal sample resuspension for HPV testing in order to evaluate if any difference in high risk HPV detection is related to the suspension medium.

- *The authors compare the HPV viral loads detected in various types of specimens and conclude that vaginal specimens have a lower viral load. However, there is no statistical analysis to support this conclusion and it is possible that the small differences observed fall within the expected experimental variance.*

Reply: Due to the restricted number of samples analysed no statistical analysis was performed. However, vaginal self-samples suspended in both suspension media demonstrated to have a higher viral load than cervical specimens. As reported in the text, this finding may be partially related to the different suspension volume (5 ml vs 20 ml) used for the two different sample types, but this finding needs to be confirmed by a larger study which would also allow to determine whether the difference in viral load between cervical and vaginal samples is statistically significant.

Competing Interests: No competing interests were disclosed.

Reviewer Report 24 March 2022

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David Hawkes
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The study but Giubbi *et al* is interesting and makes a good step forward for the development of a consistent protocol for self-collection for the purpose of PV-based cervical screening. There are a few minor comments for consideration:

- There may be differences in the samples stored at -20C depending on whether they were in Hologic PreservCyt or Copan eNAT. PreservCyt is alcohol based and generally does not freeze at -20C whereas it would be likely that eNAT would freeze. A freeze thaw cycle is likely to only have been experienced by the samples in eNat which may affect the sample, a freeze thaw cycle may break down DNA or alternatively it may lyse cellular material freeing more DNA . The authors need to address this issue.

- The authors should include a table showing the HPV results per sample (e.g. simply whether the same was positive for HPV or negative for each assay). These data are referred to in the results section and in Table 1. These data would show whether there were any trends in samples (e.g. if the 15 GeneFirst positive samples were also positive on the Seegene and OncoPredict assays - or whether the differences observed were associated with particular HPV types). This would also identify if there were any trends with comparison between the three specimen types.

- The authors need to address that there appears to be a trend (unclear whether it is significant) for lower sensitivity across all sample types for the GeneFirst assay. The Seegene assay has been clinically validated and could be utilised as a comparator assay for the purpose of determining sensitivity and specificity for the two emerging assays.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: Retrospectively realised that S Castriciano is an author. S Castriciano on behalf of Copan has undertaken research (including funding) with VCS Foundation and a co-authored presentation with the reviewer is being presented at EUROGIN 2022. Copan has also donated stock to VCS Foundation for a self-collection validation study, but they have had not input into the protocol design or outcomes.

Reviewer Expertise: HPV diagnostics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Apr 2022

Clementina COCUZZA

The authors would like to thank Prof. David Hawkes for his review and comments. Below we have included the response to his comments for consideration:

- *There may be differences in the samples stored at -20C depending on whether they were in Hologic PreservCyt or Copan eNAT. PreservCyt is alcohol based and generally does not freeze at -20C whereas it would be likely that eNAT would freeze. A freeze thaw cycle is likely to only have been experienced by the samples in eNat which may affect the sample, a freeze thaw cycle may break down DNA or alternatively it may lyse cellular material freeing*

more DNA. The authors need to address this issue.

Reply: We thank Prof. David Hawkes for this consideration related to the preanalytical process. As indicated in the "Method" section, in the paragraph on "Pre-analytical sample processing", it was stated that processing of cervical samples was performed as follows: "In the laboratory all specimens were vortexed for 30 seconds, and subsequently 1.5 ml aliquots were dispensed in cryovials; one was used for DNA extraction and the others stored at -20°C." Similarly, vaginal samples were processed as follows: "Five aliquots of 1 ml were made from each of the vaginal specimens; one was used for nucleic acids extraction and the others stored at -20°C." All samples were therefore analyzed fresh and nucleic acid extraction performed on a sample aliquot prior to freezing and therefore results were not affected by a freeze thaw cycle.

- *The authors should include a table showing the HPV results per sample (e.g. simply whether the same was positive for HPV or negative for each assay). These data are referred to in the results section and in Table 1. These data would show whether there were any trends in samples (e.g. if the 15 GeneFirst positive samples were also positive on the Seegene and OncoPredict assays - or whether the differences observed were associated with particular HPV types). This would also identify if there were any trends with comparison between the three specimen types.*

Reply: File csv reporting all results with all investigated assays for each study sample is available at the link: https://doi.org/10.5281/zenodo.6077699. In particular, you will find the results of the 3 different assays for all samples in the file entitled "HPV positivity on cervical and vaginal samples.cvs and relative file including "Legend of HPV positivity on cervical and vaginal samples.docx". The Open Research Europe journal requires all the data to be open access but limits the number of tables/figures. *However in reply to* Prof. David Hawkes request we include an additional summary table reporting all the HPV results obtained from the analyzed samples. This table can be seen here.

- *The authors need to address that there appears to be a trend (unclear whether it is significant) for lower sensitivity across all sample types for the GeneFirst assay. The Seegene assay has been clinically validated and could be utilized as a comparator assay for the purpose of determining sensitivity and specificity for the two emerging assays.*

Reply: We agree with Prof. David Hawkes comment regarding the lower HPV positivity reported from GeneFirst test compared to the other two. We have reported these results in the manuscript. As you can see in the above table the discordances of HPV positivity in cervical sample are present in two samples (MO180 T18 and MO222 T12). Both samples were collected from women with a cytology result of low-grade cervical dysplasia who did not require a biopsy to be performed at colposcopy based on the clinical findings. Regarding vaginal self-samples, the discrepancy in the HPV detection were shown in only one sample (MO149 T24). In this case the associated cytology was negative for the presence of any cervical alterations.

Competing Interests: No competing interests were disclosed.