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Evaluation of new cytological fixative liquid medium suitability for HPV testing using PCR

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Background. Cervical cancer is a leading cause of cancer deaths in women worldwide. With an introduction of the Papanicolaou (Pap) smear in 1940, the incidence and mortality from cervical cancer decreased since then. To increase cytological testing efficiency, liquid-based cytology (LBC) techniques were discovered. During the last decade many scientists groups published articles about the evaluation of this new technique, comparing it with the conventional Pap smear and trying to determine whether the LBC is efficient enough for HPV detection. The aim of our study was to investigate the suitability of new cytological liquid medium PapSpin for HPV testing using PCR.

Materials and methods. 2495 women were examined during the study, for 160 of women (6.4%) the cytological changes (ASC, SIL and CC) were diagnosed. 160 cervical samples of these women in PapSpin medium (study samples) were tested for HPV using PCR. 10% of women (n = 16) were retested for HPV infection. As control for PapSpin medium the fresh material from cervix in PBS buffer was investigated (control samples).

Results. There was a statistically significant difference between HPV positive study and control samples: there were only 5.6% (9/160) HPV positive specimens in the study samples, whereas in control samples there were 50.0% (8/16) HPV positive specimens ($p < 0.000001$). There was a statistically significant difference in average DNA concentration: it was 6 times higher in control samples than in the study samples (67.5 μg/ml and 11.1 μg/ml, respectively ($p < 0.0001$).

Conclusions. In accordance with our data, PapSpin medium is less suitable for HPV testing using PCR than the fresh material.

Key words: Pap test, liquid based cytology, screening, cervical lesions, HPV DNA

INTRODUCTION

Cervical cancer is one of the most common malignant diseases among women worldwide. Both incidence and mortality from cervical cancer are second, preceded only by breast cancer (1, 2). In some areas of the developing countries, it ranks first (3), while in the developed countries it is the sixth commonest malignant disease (4).

Based upon the epidemiologic evidence, it is maintained that cervical cancer is of viral origin (5). In addition, persistent infection of the cervix with one or more high-risk types of HPV is a necessary factor for the development of cervical cancer (6–8). However, this invasive disease does not develop immediately after the infection. Most often, firstly, precancerous intraepithelial lesions of the cervix are detected (9, 10).

In the 20th century, the incidence and mortality from cervical cancer declined considerably in the developed countries after the introduction of screening with Papanicolaou (Pap) smear and screening programs (11–13). The principle of Pap test is to dye the cervical cells spread and then, fixated on the glass slide using the Papanicolaou method, to study it under a microscope. The gynaecologist uses a spatula or a brush for taking the cytological material, spreads it on the slide and fixates it straight away. The fixated smear is stained using the Papanicolaou method. The cells are stained according to a specific colour-scale allowing the evaluation of their nuclear and cytoplasm components critically. Since its introduction in 1940 by Papanicolaou, Pap smear screening has been widely accepted and used in women screening programmes as an effective method for the prevention of cervical carcinoma.

However, the rate of false-negative smears ranges from 20 to 30% (15), and a high ratio of diagnoses of atypical squamous cells (ASC) have lead to the development of new diagnostic techniques (16). Using liquid-based cytological tests, the clinician does not smear the sample directly onto a glass slide. Instead, the sample is placed in a small bottle containing a fixative solution. In 1996 and 1999, the U. S. Food and Drug Administration (FDA) approved two new liquid-based thin layer cytological smear techniques. These were ThinPrep™ (Cytyc Corporation, Boxborough, MA, USA) and AutoCytePrep™ (now SurePath™)

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(TriaPath Imaging, Burlington NC, USA). Both systems use cells spread in liquid fixative medium to prepare smears (13). In transport container, the fixative liquid both fixates the cells and lyses erythrocytes. The U. S. FDA has approved that after the preparation of ThinPrep thin layer smear the residual material is suitable for HPV testing (17).

In many studies, liquid-based cytological preparations were compared with the conventional Pap smears. The results show that the cytological material adequacy and quality was higher in liquid-based preparations, and they are more often suitable for microscopic evaluation when compared with the conventional Pap smear (18–21). According to various data, the rate of the sensitivity of liquid-based thin layer smears ranges from 71% to 95.7%, and the specificity does from 58% to 76.2% (22). In addition, the preparation and examination of the smear require less time (23), while the laboratory efficacy increases with the investigation quality staying good (24).

The biggest disadvantages of these methods are their high cost, extra expenses and time needed for the retraining of cytotechnologists (25, 26). Now there is a less expensive alternative compared to ThinPrep and SurePath techniques. One of them is Shandon PapSpin™ (Thermo Shandon, Pittsburg, Pennsylvania, USA) liquid-based technique. This method does not need any expensive machines, only cytocentrifuge. PapSpin method was compared with the conventional Pap smear, and better quality as well as suitability for the investigation results was evident on PapSpin smears (27, 28).

There was a lot of research conducted over the last few decades on purpose to evaluate the new cytological method and to compare it with the traditional Pap smear (13, 25, 27–29). After the knowledge of HPV influence on the development of cervix cytological lesions, HPV DNA testing was involved into screening programmes as the adjunctive method to cervical cytology (20). However, there are only a small number of articles on the evaluation of liquid cytology medium suitability for HPV testing; the size of the groups investigated was usually too small for making general conclusions.

In 2006, the National Centre of Pathology and the Institute of Oncology of Vilnius University started a new study Evaluation of Effectiveness of Combined Liquid-based PapSpin Cytology and HPV DNA Testing in Cervical Pathology Diagnostics. During the study period a great amount of PapSpin samples were investigated (total number 2495). The aim of this article is to evaluate the suitability of PapSpin fixative liquid medium for HPV testing using PCR method. For this purpose the DNA concentration was measured, and HPV DNA was detected in PapSpin and fresh material specimens.

The practical significance of these data is related with their introduction into the cervical cancer screening programs, involving liquid based cytology and HPV testing in the new HPV vaccine era.

MATERIAL AND METHODS

Subjects and data collection

Women from 30 to 60 years old (not pregnant), who came for prophylactic check-up were invited to take part in this study, in accordance with the selective health check-up for cervical lesions program approved by the Ministry of Health Care. The women who agreed to take part in this study had to sign an agreement. The women were from Vilnius Central, Vilnius Region Central and Vilnius Karoliniškės Outpatient Clinics. They all were comprehensively informed about the tests, possible risks as well as the advantages of the study. The protocol of the study, invitation and a person's agreement form were approved by the Lithuanian Bioethics Committee of the Ministry of Health Care (2006-02-23, No 7).

Cells from cervix transformation zone were used for cytological examination and for human papilloma virus detection. For the cervical specimen, the kit of *Wallach Papette* brush and marked PapSpin bottle with transport PapSpin liquid were used. Before testing, the material was stored in a special container at room temperature (18–20 °C).

2495 women were enrolled in this study. For HPV testing using PCR method, only those PapSpin specimens (study samples) were selected that had cytological diagnosis of pathological lesions: atypical squamous cells (ASC), squamous intraepithelial lesions (SIL) or cervical cancer (CC). Cytological lesions were detected for 160 women (6.4%). The study was not conducted as a case-control study, just 10% of randomly selected women (n = 16) were retested for HPV infection as an internal quality control. As control for PapSpin medium the fresh material from cervix in PBS buffer was investigated (control samples). The sterile brush swabs were used to obtain the cervical samples. The end of the brush with the material was collected in 0.5 ml transport PBS solution, and the end of the brush was chipped off leaving it in the tube. Before testing, all the samples were stored at –20 °C.

HPV testing using DNA amplification method

Polymerase chain reaction was carried out using the consensus human papilloma virus primers to test specimens for HPV DNA. MY09 / MY11 consensus primers were used.

DNA extraction. Before PCR, the material was prepared in a few steps. Before DNA extraction the cytological material from PapSpin liquid was several times washed in PBS buffer (pH = 7.4). Fresh cytological material, collected by the gynaecologist into the tube with PBS, was not additionally prepared. DNA was extracted using column method (SorpoClean™ Genomic DNA Extracion Module, SORPO Diagnostics) according to the manufacturer's protocol.

Measurement of DNA concentration with BioPhotometer. Concentration of the extracted DNA was measured with BioPhotometer (BioPhotometer, Eppendorf) in the Department of Genetics, Faculty of Natural Sciences, Vilnius University.

DNA amplification using PCR method. PCR was carried out using REDTaq® ReadyMix™. PCR Reaction Mix with $MgCl_2$ (SIGMA, USA) was used to perform the PCR. The composition of the PCR mix: 20 mM Tris-HCl (pH 8.3), with 100 mM KCl, 3 mM $MgCl_2$ 0.002% gelatin, 0.4 mM dNTP mix (dATP, dCTP, dGTP, dTTP), stabilizers, and 0.06 unit/μl of Taq DNA Polymerase. PCR was carried out in thermocycler (BIO-RAD, Germany) starting from the initial denaturation step at 93 °C for 3 min. followed by 40 cycles of denaturation step at 94 °C for 1 min, primer annealing step at 55 °C for 1 min and a chain elongation step at 72 °C for 1 min and 30 s. A final extension for 5 min at 72 °C was used. The amplified PCR products were analysed by electrophoresis in 2% agarosis gel stained by ethidium bromide.

After electrophoresis the products stained by ethidium bromide were analysed in transiluminator (HEROLAB, Germany) using UV light. The results were photographed.

Statistical analysis methods

Microsoft Excel 2003 and EpiCalc 2000 programmes were used for statistical analysis. Student t-Test was used for counting p value. All the data are statistically significant when $p < 0.05$.

RESULTS

Distribution of women according to their age

2495 women were examined during the study. All the women were grouped into six age groups with 5-year interval. There were women aged 30–34 in group I, 35–39 in group II, 40–44 in group III, 45–49 in group IV, 50–54 in group V, and 55–60 in group VI (Table 1).

The average age of women was 42.4 years, the youngest was 30 and the oldest was 60 years old (SD ± 9.0). As it is shown in Table 1, the most active women were 30–34 years old (25.9%), whereas the oldest women (age group VI, 55–60 years old) were less active (10.5%). This difference implies that younger women come for prophylactic check-up more often and they are taking care of their health.

Cytological lesions were detected to 160 women (6.4%) out of 2495. These women were selected for subsequent HPV testing.

The average age of women with cytological lesions selected for HPV testing was 40 years. The youngest woman was 30 years old, while the oldest one was 60 (SD ± 8.78). As the data in Table 2 show, cytological lesions were more often detected for women in age groups I and II. These women formed 3.3% or all the women examined (n = 2495). Hence, cytological lesions are more often detected in women aged 30–39 than in older ones (40 years and more).

Distribution of the investigated women according to cytological lesions

During cytological test the following lesions were detected for 160 women: ASC-US for 42 women (26%), ASC-H (HSIL suspicion) for 6 (4%), AGUS for 3 (2%), LSIL (CIN1) for 51 (32%), HSIL (CINII/CINIII) for 55 (34%) women and 3 women (2%) had various malignant cervix tumours. According to the data, low and high grade squamous intraepithelial lesions were detected most often. These formed 66% of all the cytological findings. 20 LSIL, 25 HSIL and all 3 cancer cases were confirmed by histology.

Comparison of the DNA concentrations in case and control groups

There was a statistically significant difference between the study and controls samples concentrations (11.1 μg/ml and 67.5 μg/ml, respectively, $p < 0.0001$) (Table 3).

The average concentration of extracted DNA from fresh material (control samples) was higher than the average concentration of DNA from PapSpin liquid (case samples). According to these data we assume that using the same DNA extraction technique according to the manufacturer protocol, the cells are lysated and DNA is extracted better from fresh material than from the material with preserving agents (liquid PapSpin medium).

Detection of HPV DNA in study and control samples

Using PCR the DNA was amplified, and the PCR products, after electrophoresis in 2% agarosis gel stained by ethidium bromide, were analysed in transiluminator using UV light.

There was a statistically significant difference between the infection with HPV of study and control samples: there were only 5.6% (9/160) HPV positive study samples, whereas the number of HPV positive control samples was as high as 50.0% (8/16) ($p < 0.000001$) (Table 4). It is interesting to note, that all

Table 1. **Distribution of all the study women according to their age**

Age group	Age	Number of women (n)	%
I	30–34	645	25.9
II	35–39	412	16.5
III	40–44	374	15.0
IV	45–49	451	18.1
V	50–54	351	14.1
VI	55–60	262	10.5

Table 2. **Distribution of women with cytological lesions by age**

Age group	Age	Number of women (n)	%
I	30–34	52	33.0
II	35–39	35	22.0
III	40–44	18	11.0
IV	45–49	31	19.0
V	50–54	14	9.0
VI	55–60	10	6.0

Table 3. **Comparison of DNA concentration in study and controls samples**

	DNA concentration, mean (μg/ml)	DNA concentration, min (μg/ml)	DNA concentration, max (μg/ml)	± SD
Study samples (n = 160)	11.1	1.0	58.0	20.1
Control samples (n = 16)	67.5	2.0	201.0	45.5

One sided p value $p < 0.0001$

Table 4. **HPV detection in study and control samples**

	Study samples (n = 160)		Control samples (n = 16)		p
	n	%	n	%	
HPV positive	**9**	**5.6**	**8**	**50.0**	P < 0.000001
HPV negative	151	94.4	8	50.0	

the HPV positive control samples were HPV negative in PapSpin medium. 3 of HPV positive control samples were diagnosed as LSIL, 3 as HSIL and 2 as ASC-H. These data allow us to presume that fresh material is more suitable for HPV DNA extraction and detection than the fixating PapSpin liquid material: HPV DNA was detected 8.9 times more frequently in random controls that in study samples.

DISCUSIONS

Our work was one of a few studies in which the suitability of PapSpin liquid medium for HPV testing was evaluated. The big amount of samples was investigated in our study (the total number of PapSpin samples was 2495). 160 PapSpin liquid samples with cytological lesions were tested for HPV. 10% of these samples (16 fresh materials as the internal quality control for PapSpin samples) were retested for HPV infection. According to our data, only 5.6% PapSpin samples were positive for HPV DNA; on the contrary, 50.0% of fresh samples were positive for human papilloma virus ($p < 0.000001$). It must be noted that 3 of HPV positive control samples were diagnosed as LSIL, 3 as HSIL, and 2 as ASC-H. According to our previous study (17, 30), the number of women with different cytological changes infected by HPV ranged from 46.7% (women with LSIL) to 79.3% (women with HSIL).

During the study DNA concentration of all the samples was measured. These data show that the average DNA concentration in study samples (PapSpin fixating liquid) was 11.1 μg/ml (min 1 μg/ml, max 58 μg/ml, SD ± 20.1), whereas the average DNA concentration in control samples was 6 times higher – 67.5 μg/ml (min 2 μg/ml, max 201 μg/ml, SD ± 45.5) ($p < 0.0001$). In our opinion, the preserving materials in PapSpin liquid could have had an impact on such results, i. e. that the extracted DNA concentration was lower. Our results show that PapSpin medium is less suitable for HPV DNA detection using PCR. According to these data, we need further studies based on the modifications of DNA extraction and PCR protocols, or other methods for HPV DNA detection have to be applied.

To date, there are only a few articles available on the evaluation of the suitability of PapSpin liquid medium for HPV testing (27, 28). Other authors (19, 25) used ThinPrep, Surepath methods and their fixative liquids or EasyFix® fixative liquid. HPV was tested using Hybrid Capture II (HCII) or PCR methods. HCII sensitivity was 86.4% (95% PI: 76.5–99.1%, $p < 0.001$) and the specificity was 39.4% (95% PI: 31.2–48.1%, $p < 0.001$). There was no difference in HPV detection using HCII or PCR methods (Kappa test 0.89 ($p < 0.001$) (25).

Weynand and colleagues (27) evaluated PapSpin liquid effectiveness for HPV DNA detection. For this purpose they carried out PCR with wide spectrum primers, but they tested only 22 specimens. 7 LISL and 5 out of 6 ASC-US specimens were HPV positive. The remaining 9 specimens without cytological lesions were HPV negative. Unfortunately, a very small number of specimens were tested, and there was no statistical analysis presented in the article. The assumption of doubtful PapSpin liquid suitability for HPV testing can be made. In the article by Rosenthal and colleagues (28), there were no numbers or statistics of HPV tested specimens given. In addition, none of these papers mentioned any control material used.

CONCLUSIONS

In accordance with our data, the PapSpin medium is less suitable for HPV testing than the fresh material. In the study samples (cervical cells collected in PapSpin medium) HPV DNA was detected with 8.9 times lower frequency in comparison to the fresh control samples (cervical cells collected in PBS buffer). Statistically six times more significant difference in the DNA concentration measures was detected: lower concentration was measured in PapSpin samples after DNA extraction.

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NAUJOS SKYSTOS CITOLOGINĖS FIKSUOJANČIOS TERPĖS TINKAMUMO ŽPV TYRIMAMS PGR METODU ĮVERTINIMAS

S a n t r a u k a

Įvadas. Pasaulyje gimdos kaklelio vėžys užima vieną pirmųjų vietų moterų mirtingumo nuo vėžio struktūroje. Nuo 1940 m., kai buvo pradėtas daryti Papapincolaou (Pap) tepinėlis, sergamumas ir mirtingumas dėl gimdos kaklelio vėžio labai sumažėjo. Siekiant padidinti citologinio tyrimo efektyvumą buvo pasiūlytos skystų citologinių terpių preparatų gamybos technologijos. Pastaraisiais metais daugelis mokslininkų grupių atliko tyrimus ir įvertino šios naujos technologijos efektyvumą lygindami ją su tradiciniu Pap tepinėliu, taip pat vertino skystų citologinių terpių tinkamumą ŽPV DNR identifikavimui molekulinės biologijos metodais. Mūsų darbo tikslas – įvertinti naujos skystos terpės PapSpin tinkamumą ŽPV tyrimui PGR metodu.

Tyrimo medžiaga ir metodai. Į tyrimą įtrauktos 2495 moterys, 160 iš jų (6,4%) nustatyti citologiniai pokyčiai (ASC, SIL ar vėžys). Naudojant ŽPV DNR nustatantį PGR metodą, buvo ištirta 160 moterų gimdos kaklelio citologinių mėginių PapSpin terpėje (tiriamieji mėginiai). 10% moterų (n = 16) buvo pakartotinai ištirtos dėl ŽPV infekuotumo. Tikrinant PapSpin terpę, atsitiktiniu būdu atrinktų moterų gimdos kaklelio mėginiai buvo surinkti į PBS buferį (kontroliniai mėginiai).

Rezultatai. Nustatytas statistiškai reikšmingas skirtumas tarp tiriamosios ir kontrolinės grupės mėginių: tiriamuosiuose mėginiuose ŽPV identifikuotas vos 5,6% (9 iš 160), tuo tarpu kontroliniuose mėginiuose aptiktas net 50,0% (8 iš 16 mėginių) ($p < 0,000001$). Tiriamųjų DNR išskyrimo kokybei įvertinti biofotometru buvo matuota DNR koncentracija. Kontroliniuose mėginiuose nustatyta 6 kartus didesnė vidutinė DNR koncentracija negu tiriamuosiuose mėginiuose (atitinkamai 67,5 μg/ml ir 11,1 μg/ml) ($p < 0,0001$).

Išvados. Darbo rezultatai rodo, kad skystos terpės PapSpin mėginiai yra mažiau tinkami ŽPV DNR tyrimams PGR metodu nei šviežios medžiagos mėginiai.

Raktažodžiai: Pap testas, skystų terpių citologija, citologinė atranka, ikivėžiniai gimdos kaklelio pokyčiai, ŽPV DNR