

## Research Article

### Look for Efficiency: Real Time PCR Using Cobas 4800 - An Efficient Tool for HPV Screening

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#### Abstract

**Study background:** Invasive cervical cancer is the third most common female cancer worldwide and is related to Human Papillomavirus (HPV) infection. Screening policies are based, in France, on individual initiative (60% coverage): HPV testing is performed after primary Pap smear in case of Atypical Cells of Undetermined Significance (ASCUS). Our objective was to compare 2 different HPV tests on our molecular biology bench dedicated mainly to infectious diseases diagnostic, and integrated to our core clinical laboratory medicine.

**Methods:** In 2012 (group 1), 2414 tests were performed from 128000 pap smears on site with Qiagen technique, that involves Deoxyribonucleic Acid (DNA) denaturation and hybridization with a specific Ribonucleic Acid (RNA) of 13 High Risk (HR) HPV strains, signal amplification and qualitative detection by chemiluminescence. In 2013 (group 2), 2737 samples (125000 smears) were sent to Eylau Unilabs for testing with PCR Cobas-4800, that utilizes a total nucleic acid isolation sample preparation method with real-time Polymerase Chain Reaction (PCR) technology for DNA amplification and detection of 14 HR HPV types in a single analysis.

**Results:** No difference in the positivity frequency was observed between groups 1 and 2 (45.3% vs. 44.2%, NS). With Cobas, HPV-16 was positive in 14.4% (7.5% alone), HPV 18 in 3.9% (1.6%),

and other HPV in 36.0% (27.7% alone). In 232 cases, infection involved more than one HPV.

**Conclusion:** Positivity frequency was comparable in the 2 methods. Moreover, Cobas allows concluding "not contributory", instead of giving a false negative result and offers a great walk towards automation from nucleic acid extraction to detection. Finally, Cobas provides both pooled HR-HPV DNA and individual detection of HPV-16 and 18, responsible for 70% of cervical cancers in France.

**Keywords:** Cervical cancer screening; HPV testing; Molecular biology; Routine screening

#### Introduction

Invasive cervical cancer is the third most common cancer for women worldwide, and the fourth most common cause of cancer deaths, globally [1]. Age standardized incidence and mortality are much higher in developing countries (34.5 and 25.3 p 100 000, respectively in Africa) than in developed ones (4.5-8.3 and 2.1-2.5 p 100 000). This is largely due to a lack of screening in developing countries [1]. In the latest figures produced by World Cancer Research Fund (WCRF) International, cervical cancer was representing 7.9% of all women cancer [2]. In France, cervical cancer has decreased by more than 40% since 1980 but still accounted for about 3,000 new cases per year [3] in 2000, resulting in a thousand deaths. Cervical cancer screening has resulted in a clear decline in cervical cancer incidence and mortality, but women who do not receive recommended screening and follow-up are at increased risk [4], particularly because of absence of treatment of precancerous lesions. This may explain the disproportionately higher cervical cancer incidence and mortality observed in, American Indian and Alaska Native women [5] or in a 'Rest of the World' category, in Australia [6]. In the US, Overall incidence rates for invasive cervical cancer decreased by 54% over the 35 years. Although marked reductions in the overall and race-specific incidence rates of invasive cervical cancer have been achieved, they mask important variation by histologic subtype [7].

Harald zur Hausen's laboratory was the first to demonstrate that genital warts contain Human Papillomavirus (HPV) genomes [8], and to relate this virus to cervical, cancer [9,10]. Among the papilloma virus family, 2 groups can be determined, a High Risk one (HR), resulting in cancer, and a low risk one resulting more often in genital warts, both being sexually transmitted. HR virus result in cell modifications, then malignant transformation, through 2 main signal pathways, involving retinoblastoma protein and P53 protein, and the action of 2 proteins of HPV, E6 and E7 [11].

Screening policies are different across countries, from primary Pap smear and HPV testing in case of morphological alteration, to HPV testing as the primary screening, or mixed policies. In France, HPV test is reimbursed by Social Security only in case of Atypical Cells of Undetermined Significance (ASCUS) Pap smear according to Bethesda classification [12], for patients older than 25 years or in case of a post treatment monitoring after a Cervical Intraepithelial Neoplasia (CIN) stage 2-3, according to the French statutory authority ANAES [13] and to the French law [14]. A follow-up was performed

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by the Centre National de Référence des Papillomavirus Humains” (CNR-HPV) to get a better knowledge of the distribution of anomalies and to validate HPV tests [15]. Recommendations were published by the French “Haute Autorité de la Santé-HAS” [16-17].

However, In France, the screening is still imperfectly organized, since its coverage is only of 60%, with 1000 deaths per year [18]. In Paris area, a survey found that 10% of women had never undergone cervical cancer screening testing [19]. Usual smear test is associated to a correct specificity, but a more variable sensitivity [20].

For HPV testing, there are many tests, based on optic density after hybridization or hybridization tests with Polymerase Chain Reaction (PCR) [21]. They may be performed on Deoxyribonucleic Acid (DNA) or Ribonucleic Acid (RNA), but media for sampling and tests providers’ recommendations have to be carefully respected. This is of major importance for the quality of results as for lab accreditation. The objective of this work was to compare the results of 2 different techniques, Digene Hybrid Capture 2 (DGHC2) (Qiagen, Courtabeuf, France) and PCR Cobas 4800 (Roche diagnostic, Meylan, France). The aim was to check whether the new technique would lead to a higher number of detected cases and, possibly, to more false positive and to integrate the test on molecular biology bench organized to manage large flow under automation and multiparametric.

## Material and Methods

Original data come from the same lab. There was a total switch in HPV tests between 2012 and 2013. In 2012 (group 1), 128000 pap smears were performed by gynecologists using Digene media (Qiagen, Hilden, Germany). In 2013 (group 2), 125000 pap smears were performed on liquid phase, using Easyfix (Labonord-VWR Fontenay sous Bois, France 2013) as fixator. Bethesda classification [12] was used in both cases, and only ASCUS samples were checked for HPV according to French rules [16,17]. Our lab is involved in a quality program comparing local results to national ones. The majority of cytology preparations were performed in the same lab (SIPATH-UNILABS), and all ASCUS samples were controlled there, to homogenize the results.

In group 1, 2414 HPV tests were performed on site with Qiagen. This method is considered as a Gold Standard in France. Briefly, the first step consists in a DNA denaturation, followed by hybridization with a specific RNA of 13 High Risk (HR) viral strains (16,18,31,33,35,39,45,51,52,56,58,59,68). Then hybrids are captured on a microplate covered by specific anti DNA-RNA hybrids antibodies coupled to alkaline phosphatase. Reading and interpretation are performed using chemiluminescence for the qualitative detection, and the signal can be amplified 3000 fold. Results are considered positive when above a threshold controlled for each series.

In group 2, 2737 samples were centralized and sent once a week to laboratoire Eylau-Unilabs (Paris, France), which uses PCR-Cobas (Roche Molecular diagnostic). This test utilizes amplification of target DNA by PCR and nucleic acid hybridization for the detection of 14 High-Risk (HR) HPV types in a single analysis. The used primer was the L1 gene region. The test specifically identifies HPV 16 and HPV 18 while concurrently detecting the rest of the HR types (31,33,35,39,45,51,52,56,58,59,66 and 68) globally at clinically relevant infection levels. Specimens are limited to cervical cells collected in Cobas® PCR Cell Collection Media (Roche Molecular Systems, Inc.), PreservCyt® Solution (Cytoc Corp.) and SurePath® Preservative Fluid (not

approved in the US) (BD Diagnostics-TriPath). A « cellularity » test is performed using a measure of beta-globin. Results are considered as positive, negative or not contributory (with no cell, anomaly in preservation or biopsy sample).

Groups 1 and 2 were compared for positivity percentages by chi-square. Then, genotypes were described in group 2 only, since this was not possible with the technique used in group 1.

## Results

In group 1, 1094 samples out of 2414 (45.3%) were positive compared to 1238 out of 2737 (45.2%) in group 2 ( $p=0.95$ ). Thus, results were comparable in term of positivity. The PCR-Cobas test allowed determining the rate of non-contributory samples, at 1.5% on the primary result, and at 0.6 % after re-checking of all non-contributory samples. This was not possible with hybrid-capture 2, because of the absence of a cellularity control. Genotyping was performed during 14 months, from November 2012 to December 2013. (Table 1) reports the percentages of HPV 16, 18, or others, alone or associated. In total, after exclusion of 17 non-contributory samples, 45.2% of samples were found positive. Among them, HPV 16 was found positive in 14.4% (alone in 7.5%, associated with HPV 18 in 0.8%, and with others in 6.8%). HPV 18 was found positive in 3.9% (1.6% alone, 0.8% associated to HPV 16). Other HPV were found in 36.0% (no associated with HPV 16 and 18 in 27.7%). Finally, in 232 cases, the sample was infected by more than one HPV.

Genotyping result	N	%
HPV 16	204	7,45
HPV 16+18	6	0,22
HPV 16 + other	169	6,17
HPV 16 + 18 + other	16	0,58
HPV 18	44	1,61
HPV 18 + other	41	1,50
HPV Others	758	27,69
Total HPV positive	1238	45,23
HPV negative	1499	54,77
Total contributory	2737	100
Not contributory	17	
Total general	2754	

**Table 1:** PCR/Genotyping results using PCR-Cobas, November 2012-December 2013.

\* Percentages are computed after removing the non contributory patients.

## Discussion

There was thus no significant difference between the results observed with the 2 techniques in term of positivity to HPV. The study involved a large enough number of patients to reach a power of 90% to detect an increase of 5% of positive results (from 45% to 50%). However, we cannot totally eliminate a source of bias, since it has been built as a historical cohort, with a “before vs. after” design. We cannot totally exclude a potential modification in the patient’s characteristics between the 2 periods since there were not available for this study. However, there are also acceptable explanations for this design, since the lab totally switched from one to the other technic, and the choice of the test was then not related to the patient’s characteristics. Moreover, the lab is a reference one in the region, and the number of pap smears was similar in the 2 years, and high (more than 120 000 each year), which reinforces the results validity, even with missing

clinical data. Finally, the absence of difference would have mean, if the tested hypothesis was true (more false positive with PCR), that the percentage of really HPV positive tests would have decreased in period 2, which can be considered improbable. The Easy fix media, used for fixation, may, theoretically, overestimate the number of positive cases, but this was not found in this study, and it appears acceptable for the detection limit, reproducibility and stability [22]. Moreover, it also can be stressed that all ASCUS samples were controlled in SIPATH-UNILABS (Clermont-Ferrand) for better homogenization.

Thus, in this study, amplification by PCR was not related to an increased sensitivity, as some could fear. One limitation is related to the result itself, which is not quantitative, with PCR because of the technique itself, and only concludes that the sample is positive or negative. However, in the previous technique (Qiagen), a result around the positivity threshold often conducted to control the test and to analyze the kinetics, with a question concerning the necessity and/or the pertinence of evaluating the viral load.

One of the strong point of the PCR technique is the presence of a cellularity control, which allows to conclude that a test is “not contributory”, and not to give a false negative result. It is important for the patient’s follow-up not to give a reassuring result, which may conduct to a delayed screening and treatment. On the opposite, a not contributory test will allow to shorten the delay for screening again. On another aspect, it can be discussed that Cobas only tests the L1 region, and not the E6 and E7 regions. However, this can be justified by the fact that this region is apparently much less stable.

For co-infections, it is classic but, again, the ASCUS population is not the same as cancer, and the rates of HPV 16 and 18 are usually lower than in invasive carcinoma. There were co-infections with several HR viruses, which only can be detected only by genotyping.

For future, the relative places of genotyping and of phenotyping are in question. Moreover, viral strains vary across continents and may change with vaccination 16-18 [21]. Also, in genotyping, the notion of primer remains important. Indeed, companies use different primers, some encoding the L1 viral protein, while some others use primers encoding the E6 or E7 protein directly implicated in the transformation process, or testing from messenger RNA [21].

Goldhaber-Fiebert et al., [23] concluded that “For both vaccinated and unvaccinated women, age-based screening by use of HPV DNA testing as a triage test for equivocal results in younger women and as a primary screening test in older women is expected to be more cost-effective than current screening recommendations”. In a large case control prospective cohort, involving 8575 women in the intervention group and 8580 control, Bulkman et al., [24] were able to demonstrate that HPV DNA testing in cervical screening lead to earlier detection of CIN3+ lesions. A similar study [25] on more than 12000 women concluded that the addition of an HPV test to the Pap test to screen women in their mid-30s for cervical cancer reduces the incidence of grade 2 or 3 cervical intraepithelial neoplasia or cancer detected by subsequent screening examinations. In term of public health and epidemiology the French legislation needs to be re-evaluated because HPV positive patients are not reimbursed for known HPV positive patients, except in some regions with a financial involvement of patients. In France, Clavel et al., [26], in a cohort of 7932 women found a much higher sensitivity in detection of HG CIN with HPV testing (100%) than with conventional (68.1%) and liquid-based (87.8%) cytology, together with a slightly higher

specificity. They also found that the assessment of the viral load was not reliable for predicting CIN in normal smears. Their conclusion was in favor to propose HR-HPV testing in primary screening in association with cytology. With conventional cytology it significantly improves the detection of High Grade Squamous Intraepithelial Lesion. There is no screening campaign using HPV test in France, except in some regions, but with a financial involvement of patients, and screening for cervical cancer remains mostly individual [27]. An association combining cytology and HPV test may allow a better screening and could allow to lengthen the time between 2 screenings in case of negativity [24]; These test are complementary in term of results and their association can also help as monitoring tool for patients beyond the screening [24-26].

The Cobas 4800 has been shown an efficient tool compared with Hybrid Capture 2 by Heideman et al., [28]. They found a strong agreement between the cobas 4800 HPV test and HC2 (97.3% and 98.3% for controls and cases, respectively). Similarly, Park et al., [29] demonstrated, with HC2, a sensitivity of 96.6% with a specificity of 89.1% for detecting HR HPVs, while, with Cobas, they were 91.7% and 97.0%, respectively, thus very close. These 2 studies involved a relatively small sample (less than 1000 and 356 respectively). These results allowed us to totally switch from HC2 to Cobas, between 2012 and 2013; and to check them in real life.

## Conclusion

The final objective of screening remains to determine high-risk patients, with the aim of proposing to them an appropriate management according to the disease stage. HPV-DNA analysis by real time PCR on Cobas 4800 is offering a great walk away automation from nucleic acid extraction to detection and provides both pooled high-risk HPV DNA and individual detection of HPV 16 and 18, the two types responsible for nearly 70% of cervical cancer. Thus, on this large routine study, several objectives were verified. The positivity rate was similar, the false negative results were decreased, and the cost was also decreased. Moreover, one very interesting added value of Cobas 4800, in our daily practice, is the possibility of using it for an open panel of molecular tests, like the detection of several sexually transmitted diseases (*chlamydia trachomatis*, *Neisseria gonorrhoea*), and some other cancers, as colorectal (BRAF-V600E mutation), or lung cancer (Epidermal Growth Factor -EGFR- mutation).

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