RESEARCH NOTE

Use of real-time PCR to detect human papillomavirus-16 viral loads in vaginal and urine self-sampled specimens

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ABSTRACT

Increasing the accuracy of self-sampling methods to detect oncogenic human papillomavirus (HPV) infection would contribute to the wider application of these approaches. In this study, 120 women were tested for HPV-16 by conventional and quantitative real-time PCR (QRT-PCR) in cervical and self-sampled vaginal and urine specimens. QRT-PCR had a higher detection rate, and the HPV viral load in all three sampling sites correlated with the severity of disease, as determined by histology. The vaginal and urine viral loads correlated with HPV-16 positivity according to both conventional and QRT-PCR, and were proportional to the cervical viral load.

Keywords Detection, human papillomavirus-16, oncogenic human papillomavirus, real-time PCR, self-sampling methods, viral load

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Testing for human papillomavirus (HPV) using molecular methods may be more sensitive than cytology for primary cervical screening [1], and self-sampling could significantly increase compliance with preventive strategies. It has been reported previously that HPV detection results obtained using self-collected vaginal samples are highly concordant with those obtained using physician-collected cervical samples [2], while urine testing is only useful for detecting severe lesions [3]. In order to increase the detection rate, the present study investigated the use of quantitative real-time PCR (QRT-PCR), which is a detection method that is considered to be more sensitive than conventional PCR [4].

A previous large study revealed that the probability of an abnormal Pap test is directly proportional to the HPV viral load [5]. High HPV viral loads have also been associated with an increased risk of developing cervical intra-epithelial neoplasia, even in smears with normal cytology [6–9], and may predict the severity of cervical lesions [10,11] and the presence of occult lesions [12]. These previous studies all used physician-collected cervical specimens, which are currently considered to be the reference standard. Since self-sampling is increasingly being considered to be a more convenient screening method, the present study also investigated whether the viral load in vaginal and urine specimens is associated with more severe lesions, and whether it correlates with results obtained using the conventional cervical HPV test. Only one previous study has measured viral loads in urine samples [13].

Following Institutional Review Board approval, 100 patients positive for HPV-16 (15 cancerous, 36 high-grade and 49 low-grade lesions) were recruited from among women referred to the colposcopy clinic. A further 20 patients with normal cervical cytology were also recruited as controls. A physician-collected cervical sample was taken from all women for HPV testing before they had a colposcopy. Before the pelvic examination, all women collected a self-sampled vaginal specimen by rotating a soft endocervical collection brush four times in the vagina, and also provided a first void urine specimen [2,3].

Initial qualitative detection of HPV-16 was performed using a conventional PCR protocol, while the HPV-16 viral load was determined using QRT-PCR. The primers used were specific for the E1 or E6 genes [2,3,11]. The E6 primer set performed best in conventional PCRs, and the E1 primer set performed best in QRT-PCR; the respective results were used for comparison of

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Table 1. Viral load (copies/μg DNA) in different specimens, grouped according to the histological classification

	Minimum	25%	Median	75%	Maximum
A. Viral load—cervix (p <0.001)					
Low grade (*n* = 49)	2245	6040	26 366	64 775	6 994 554
High grade (*n* = 36)	3077	46 048	123 678	1 199 321	9 182 746
Cancer (*n* = 15)	94 110	210 408	779 344	2 641 284	7 558 141
B. Viral load—vagina (p <0.003)					
Low grade (*n* = 49)	0	5238	16 653	87 332	984 650
High grade (*n* = 36)	0	5105	20 503	257 487	912 387
Cancer (*n* = 15)	13 302	31 484	78 835	188 952	838 045
C. Viral load—urine (p <0.001)					
Low grade (*n* = 49)	0	0	0	13 155	356 478
High grade (*n* = 36)	0	2 945	6 580	40 046	66 444
Cancer (*n* = 15)	0	7640	18 200	42 220	177 530

Table 2. Comparison between results obtained using conventional and QRT-PCR detection with self-sampled specimens (vaginal and urine) and results obtained with cervix specimens from 100 HPV-16 cervical-positive patients with proven abnormal histology (by cone or colposcopically-directed biopsy)

	Cervix PCR-positive, *n*	Vaginal PCR sensitivity, *n* (%) (95% CI)	Urine PCR sensitivity, *n* (%) (95% CI)	Vaginal QRT-PCR sensitivity, *n* (%) (95% CI)	Urine QRT-PCR sensitivity, *n* (%) (95% CI)
Cancer	15	14 (93.3) (68.1–99.8)	13 (86.7) (59.5–98.3)	15 (100) (78.2–100)	14 (93.3) (68.1–99.8)
High grade	36	33 (91.7) (77.5–98.2)	26 (72.2) (54.8–85.8)	35 (97.2) (85.5–99.9)	30 (83.3) (67.2–93.6)
Low grade	49	38 (77.6) (63.4–88.2)	16 (32.7) (19.9–47.5)	43 (87.8) (75.2–95.4)	19 (38.8) (25.2–53.8)
Total positive	100	85 (85.0) (76.5–91.4)	55 (55.0) (47.5–65.0)	93 (93.0) (86.1–97.1)	63 (63.0) (52.8–72.4)

QRT-PCR, quantitative real-time PCR.

the methods. Viral copy numbers in each sample were calculated by comparing the QRT-PCR results with a standard curve for HPV-16, created using ten-fold serial dilutions of a plasmid DNA standard, equivalent to 10^7–10^1 copies of HPV-16 DNA. Viral loads were determined in duplicate, with mean values expressed as HPV copies/μg of total DNA.

In all patients, the viral load values in cervical specimens were higher or equal to those in vaginal specimens, which were higher or equal to those in urine specimens (Table 1). In cancer cases, the viral load in the cervical specimens was considerably higher (median ten-fold) than that in the vaginal specimens, and was even higher (median 42-fold) than that in urine specimens. The viral load difference was less pronounced between high- and low-grade lesions. Furthermore, the median HPV viral load increased significantly in line with the increased severity of cytopathological changes in cervical (p <0.001), vaginal (p <0.003) and urine (p <0.001) specimens (Table 1). Such a correlation has been reported previously only for cervical samples [6–12], but no previous study has tested all three sampling sites simultaneously.

The application of QRT-PCR improved the performance of both self-sampling methods for all histological classes (Table 2). When the performance of QRT-PCR was compared with that of conventional PCR, the vaginal QRT-PCR had the highest sensitivity, followed by conventional vaginal PCR, urine QRT-PCR and conventional urine PCR (Table 2). The kappa measure of agreement with the cervical PCR was 0.625 (standard error (SE) 0.082) for the conventional vaginal PCR, and 0.816 (SE 0.066) for the vaginal QRT-PCR. For the urine specimens, the conventional PCR had a kappa value of 0.289 (SE 0.067), compared with a kappa value of 0.362 (SE 0.059) for the QRT-PCR.

Regression analysis revealed that HPV-16 viral loads in cervical specimens correlated with those in vaginal samples and urine (data not shown). A significant correlation (p <0.05) was also found between increased viral loads in the cervical specimens and enhanced sensitivity of HPV-16 detection using both self-sampling methods. The positive self-sampling specimens correlated with higher cervical viral loads, which are considered to be an ominous prognostic sign [5–9]. Discordant results between positive cervical and negative vaginal and urine samples often showed low viral loads, usually <6000 copies/μg of DNA. Women with low viral loads are more likely to clear their HPV infections than those with higher viral loads [6]. Therefore, from a clinical viewpoint, it would probably be of greater value to refer patients with a high viral load to a specialist. The present data indicate that women at high risk

could be identified accurately using QRT-PCR in conjunction with self-sampled specimens (vagina or urine) as an alternative to cytological screening and/or cervical viral load measurement [14]. Qualitative real-time (RT)-PCR protocols for HPV typing are also available and have high sensitivity. In this context, a two-step approach, involving HPV typing by qualitative RT-PCR and measurement of type-specific viral loads only in high-risk HPV-positive self-sampled specimens, would probably be more cost-effective. As the costs of both qualitative and QRT-PCRs for HPV-16 are expected to gradually reduce, and given their higher analytical sensitivity, implementation of the more convenient self-sampling strategy is possible. However, clinical viral load thresholds should be defined and the methods used should be validated in larger populations and standardised with control panels [15].

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REFERENCES

1. Cuzick J, Mayrand MH, Ronco G, Snijders P, Wardle J. New dimensions in cervical cancer screening. *Vaccine* 2006; **24** (suppl 3): S90–S97.
2. Daponte A, Pournaras S, Mademtzis I *et al.* Evaluation of HPV 16 PCR detection in self - compared with clinician-collected samples in women referred for colposcopy. *Gynecol Oncol* 2006; **103**: 463–466.
3. Daponte A, Pournaras S, Mademtzis I *et al.* Evaluation of high-risk human papillomavirus types: PCR detection in paired urine and cervical samples of women with abnormal cytology. *J Clin Virol* 2006; **36**: 189–193.
4. Seth R, Rippin J, Guo L, Jenkins D. Detection and quantitation of HPV gene expression using real-time PCR. *Methods Mol Med* 2005; **119**: 61–72.
5. Bigras G, de Marval F. The probability for a Pap test to be abnormal is directly proportional to HPV viral load: results from a Swiss study comparing HPV testing and liquid-based cytology to detect cervical cancer precursors in 13 842 women. *Br J Cancer* 2005; **93**: 575–581.
6. Dalstein V, Riethemuller D, Pretet JL *et al.* Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. *Int J Cancer* 2003; **106**: 396–403.
7. van Duin M, Snijders PJ, Schrijnemakers HF *et al.* Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. *Int J Cancer* 2002; **98**: 590–595.
8. Josefsson AM, Magnusson PK, Ylitalo N *et al.* Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. *Lancet* 2000; **355**: 2189–2192.
9. Moberg M, Gustavsson I, Gyllensten U. Type-specific associations of human papillomavirus load with risk of developing cervical carcinoma in situ. *Int J Cancer* 2004; **112**: 854–859.
10. Ho GY, Burk RD, Klein S *et al.* Persistent genital human papillomavirus infection as a risk factor for persistent cervical dysplasia. *J Natl Cancer Inst* 1995; **87**: 1365–1371.
11. Oikonomou P, Mademtzis I, Messinis IE, Tsezou A. Quantitative determination of human telomerase reverse transcriptase messenger RNA expression in premalignant cervical lesions and correlation with human papillomavirus load. *Hum Pathol* 2006; **37**: 135–142.
12. Sun CA, Lai HC, Chang CC, Neih S, Yu CP, Chu TY. The significance of human papillomavirus viral load in prediction of histological severity and size of squamous intraepithelial lesions of uterine cervix. *Gynecol Oncol* 2001; **83**: 95–99.
13. Payan C, Ducancelle A, Aboubaker MH *et al.* Human papillomavirus quantification in urine and cervical samples by using the Mx4000 and LightCycler general real-time PCR systems. *J Clin Microbiol* 2007; **45**: 897–901.
14. Pretet JL, Dalstein VD, Monnier-Benoit S, Delpeut S, Mougin C. High risk HPV load estimated by Hybrid Capture II correlates with HPV16 load measured by real-time PCR in cervical smears of HPV16-infected women. *J Clin Virol* 2004; **31**: 140–147.
15. Quint WG, Pagliusi SR, Lelie N *et al.* Results of the first World Health Organization international collaborative study of detection of human papillomavirus DNA. *J Clin Microbiol* 2006; **44**: 571–579.