

Viral load of human papillomavirus in women with normal and abnormal cervical cytology in Kuwait

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Abstract

Introduction: Human papillomaviruses (HPV) are the most commonly known sexually transmitted agents. Almost all cases of cervical cancer are caused by persistent infection. This study was conducted to ascertain whether there is a difference in HPV load in cervical samples with normal and abnormal cervical cytology reports in Kuwait.

Methodology: HPV-positive abnormal ThinPrep samples (n = 206) and normal ThinPrep samples (n = 120) were taken from women attending gynecology clinics. Real-time PCR was used to measure the viral load for all HPV genotypes.

Results: The median normalized viral load in samples with normal and abnormal cytology reports was 0.86×10^{-7} and 4.66×10^{-7} , respectively (p = 0.001). Median normalized viral load of high-risk (HR), intermediate-risk (IR) and low-risk (LR) HPV was 4.04×10^{-7} , 0.71×10^{-7} and 2.38×10^{-7} , respectively, (p = 0.002).

Conclusions: The findings suggest that, in the absence of a proper screening programme in Kuwait, quantification of HPV viral load could be considered as a surrogate virology test to identify women with abnormal cytology. Further population-based prospective studies are needed to include more women with high-grade and invasive carcinoma reports.

Key words: HPV; real-time PCR quantitative assay; viral load, women with normal and abnormal cervical cytology

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Introduction

Human papillomaviruses (HPV) are the most commonly known sexually transmitted agents [1]. There are more than 100 recognized HPV genotypes, of which approximately 40 have tropism specifically for anogenital mucosa [2]. Based on their epidemiological association with cervical cancer, anogenital HPV genotypes are divided into high-risk (HR) and low-risk (LR) types [3]. Almost all cases of cervical cancer are caused by persistent infection involving 15 genotypes of the HR HPV [4].

In Kuwait, cervical smears are taken routinely and examined when a woman visits a gynecology clinic. However, in view of the absence of a formal screening programme, there are few studies on the results of Papanicolaou (Pap) smears in Kuwait [5,6] and the prevalence of HPV in the female population. One study has shown an increase in HPV infection, especially in Kuwaiti women [7]. Another study has

reported the presence of HPV DNA in 2.4% of normal cervical samples; LR HPV types were found in 71.8% of infected samples, HR types in 32.3%, and intermediate-risk (IR) types in 7% [8]. A more recent study has reported the presence of HPV DNA in 51% of abnormal cervical samples; HPV16 had the highest prevalence (24.3%), followed by HPV11 (13.8%), HPV66 (11.2%), HPV33 (9.9%), HPV53 (9.2%), HPV81 (9.2%), HPV56 (7.9%) and HPV18 (6.6%). HPV prevalence was 86%, 67% and 89% in women with invasive cervical carcinoma (ICC), high-grade squamous intraepithelial lesions (HSIL) and low-grade squamous intraepithelial lesions (LSIL), respectively. HR and LR-HPV genotypes were prevalent in women at the age of 40 and older [9].

This study was conducted to determine the viral load in HPV-infected cervical samples taken from women with normal and abnormal cytology reports using the quantitative real-time polymerase chain

Table 1. Histological reports of 206 women with cytological abnormalities

Histology Report	Histology Information not available	Negative	Nuclear atypia not amounting to CIN	HPV associated changes	CIN I	CIN II	CIN III	Invasive SCC	Invasive ADC
ASC (n = 61)	36	10	10	5	-	-	-	-	-
AGC (n = 18)	7	7	3	-	1	-	-	-	-
LSIL (n = 103)	23	23	20	19	18	-	-	-	-
HSIL (n = 14)	3	3	-	-	3	2	3	-	-
SCC (n = 7)	-	1	-	-	-	-	1	5	-
ADC (n = 3)	-	1	-	-	-	-	-	-	2
Total (n = 206)	69	45	33	24	22	2	4	5	2

ASC: atypical squamous cells, AGC: atypical glandular cells, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, SCC: squamous cell carcinoma, ADC: adenocarcinoma. CIN: cervical intraepithelial neoplasia.

reaction (PCR) technique. The study also investigates the hypothesis that high HPV concentration is associated with abnormal cytology samples. The viral load of HPV was also investigated in samples with low-grade cervical reports and samples with high-grade cervical reports.

Methodology

Study group

This study is a continuation of previous studies performed by Al-Awadhi *et al.* [8,9] and utilizes the same samples used in those studies. The rationale behind the selection of women, sample size, and power for the studies was discussed in those earlier investigations. Study samples were comprised of 326 HPV-positive ThinPrep samples (Cytoc Corporation, Boxborough, MA, USA) taken from women with normal and abnormal cytology reports. Samples were received at the Cytology Laboratory, Mubarak Al-Kabeer Hospital in Kuwait from December 2006 to March 2010. Informed consent was given by the women who participated in the study, which was approved by the Ministry of Health and the Faculty of Medicine Ethics Committee, Kuwait University.

Cervical samples were taken from women with a wide spectrum of gynecological complaints attending polyclinics and the colposcopy clinic at the Maternity Hospital. ThinPrep cytological slides were screened, and the adequacy as well as the degree of abnormality was assessed using the criteria set out in The Bethesda System 2001 guidelines [10]. Residual liquid material left from the ThinPrep samples was used for HPV investigation. ThinPrep samples were reported as follows: normal, atypical squamous cells (ASC),

atypical glandular cells (AGC), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), invasive squamous cell carcinoma (SCC), and invasive adenocarcinoma (ADC). Of all 326 samples in the study, 120 had normal reports, 61 had ASC reports, 18 had AGC, 103 had LSIL, 14 had HSIL, seven had SCC and three had ADC reports. All ThinPrep slides were reviewed by two cytologists and a consensus diagnosis was made. The ASC category included ThinPrep slides with atypical squamous cells of undetermined significance (ASC-US) reports (n = 59) and ThinPrep slides with atypical squamous cells that could not exclude HSIL (ASC-H) reports (n = 2). Due to the small number of ASC-H reports, the ASC-US and ASC-H were combined and analyzed as a single group designated ASC. Follow-up information was obtained for women with abnormal cytological reports and the results of a histology-cytology correlation are shown in Table 1. Cervical biopsies were examined by two experienced pathologists.

DNA extraction, real-time PCR and viral load estimation

DNA extracts from HPV-positive samples were subjected to quantification by real-time PCR as described previously [8,9]. Genomic DNA was extracted from the ThinPrep samples and controls stored at -80°C using a NucleoSpin Genomic DNA Tissue kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany) according to the manufacturer's instructions.

The real-time PCR assay was performed using an ABI 7500 real-time PCR (Applied Biosystems, Foster

Table 2. Proportion of women according to cytological diagnosis and their HPV results

Cytology	Age		HPV Genotypes			
	Median	Mean \pm SD Range	HR (n = 197)	IR (n = 10)	LR (n = 119)	All (n = 326)
Normal	40	41.6 \pm 9.6 19 - 67	55 (45.8)*	6 (5.0)	59 (49.1)	120 (36.8)
Abnormal	39	39.1 \pm 10.5 19 - 70	142 (68.9)	4 (2.0)	60 (29.1)	206 (63.2)
ASC	42	40.4 \pm 9.9 22 - 62	37 (60.7)	2 (3.3)	22 (36.0)	61 (29.6)
AGC	41	41.8 \pm 11.2 20 - 59	11 (61.1)	1 (5.6)	6 (33.3)	18 (8.7)
LSIL	36	37.1 \pm 10.9 19 - 70	70 (68.0)	1 (1.0)	32 (31.0)	103 (50.0)
HSIL	38	39.4 \pm 8.1 26 - 55	14 (100.0)	0	0	14 (6.8)
SCC	44.5	44.0 \pm 8.1 33 - 59	7 (100.0)	0	0	7 (3.4)
ADC	52	47.3 \pm 13.6 32 - 58	3 (100.0)	0	0	3 (1.5)
Group 1	39	38.7 \pm 10.7 19 - 70	118 (64.8)	4 (2.2)	60 (33.0)	182 (88.3)
Group 2	42	41.7 \pm 8.9 26 - 59	24 (100.0)	0	0	24 (11.7)

HR: high risk, IR: intermediate risk, LR: low risk, ASC: atypical squamous cells, AGC: atypical glandular cells, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, SCC: squamous cell carcinoma, ADC: adenocarcinoma. Group 1: ASC, AGC and LSIL cases, Group 2: HSIL, SCC and ADC cases. *Figures in parenthesis () give percentages of categories

City, CA, USA) system. The assay was performed on three different plates. The first plate was used to determine the integrity of the target DNA by beta-globin PCR assay, amplifying a target of 268 bp fragment as described previously by Lum and Le Marchand [11]. The second plate was used to screen for the presence of HPV in cervical samples using MY09/11 primers as nucleotide sequences of the HPV L1 Open Reading Frame (ORF) consensus primers [12]. The third plate was used to screen for the presence of HPV infection in cervical scrapes using GP5+/6+ primers as nucleotide sequences of the HPV L1 ORF consensus primers [13].

For absolute quantification, the HPV16 vector (American Type Culture Collection, Manassas, VA, USA) was used at different concentrations to generate a standard curve. The linear range of the assay was from 1×10^1 to 1×10^{11} copies/ μ l. The concentrations of HPV DNA were determined by extrapolation from the standard curve, and then normalized against the concentrations of beta-globin DNA determined by real-time PCR.

HPV sequencing

Samples showing positive amplification for the presence of HPV by GP5+/GP6+ and/or MY09/MY11 were subjected to DNA sequencing to establish the

HPV type identity, as previously described [8,9]. The samples were analyzed using Sequencing Analysis software version 3.7 (Applied Biosystems, Foster City, CA, USA). The HPV sequence alignment was performed with sequences presented in the GenBank database using BLASTn software (<http://www.ncbi.nlm.nih.gov/blast/html>) and the HPV Database maintained by the Theoretical Biology and Biophysics laboratory at Nebraska Center for Virology (<http://ncv.unl.edu/Angelettilab/HPV/Database.html>).

Method of analysis

Data management and analysis were performed using Statistical Package for Social Sciences (SPSS version 17.0; IBM, SPSS Inc., Chicago, USA). Epidemiological classification of HPV types was carried out according to Muñoz *et al.* [14], and Al-Awadhi *et al.* [8]. HR-HPV genotypes included HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV53, HPV56, HPV58, HPV59, HPV66, HPV68, HPV73 HPV82 and HPV97. IR-HPV genotypes included HPV62, HPV67, HPV83, HPV84 and HPV87. LR-HPV genotypes included HPV6, HPV11, HPV43, HPV54, HPV61, HPV82, HPV74, HPV81, HPV90, HPV102 and HPV106.

The continuous variables (*i.e.*, age and viral load) were examined for normality of data using the

Table 3. Normalized viral load analysis of different cytology groups

Cytology Report	Number of Women	Median (IQ)*	P – value
Normal	120	0.86 (0.38 – 5.25)	0.001 ^{&}
Abnormal	206	4.66 (2.22 – 8.34)	
ASC	61	5.01 (2.54 – 8.34)	0.391**
AGC	18	5.94 (3.35 – 8.37)	
LSIL	103	4.46 (1.97 – 8.74)	
HSIL	14	3.79 (2.13 – 8.15)	
SCC	7	2.48 (1.85 – 39.20)	
ADC	3	2.13 (1.09 – 3.24)	
Group One	182	4.69 (2.26 – 8.34)	0.306 ^{&}
Group Two	24	2.88 (1.91 – 8.36)	
HR	197	4.04 (1.81 – 7.75)	0.002**
IR	10	0.71 (0.34 – 5.31)	
LR	119	2.38 (0.54 – 7.99)	

*Median= value $\times 10^{-7}$, IQ: Inter-Quartile, ASC: atypical squamous cells, AGC: atypical glandular cells, LSIL: low-grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, SCC: squamous cell carcinoma, ADC: adenocarcinoma. Group 1: ASC, AGC and LSIL cases, Group 2: HSIL, SCC and ADC cases. HR: high risk, IR: intermediate risk, LR: low risk, [&]Mann-Whitney U Test was used, ** Kruskal-Wallis test was used.

Kolmogrov-Smirnov test and descriptive statistics were presented as median and inter-quartiles. Non-parametric Mann-Whitney U or Kruskal-Wallis tests were used to test the differences in normalized viral load between cytology groups. A two-tailed probability value ($p < 0.05$) was considered statistically significant.

Results

A total of 326 HPV-infected women with normal and abnormal cytological reports were investigated. The age, cytological findings, and risk type of HPV infection of the study population are shown in Table 2.

The outcome of normalized viral load in samples with normal and abnormal cytology reports is shown in Table 3. Analysis of the results showed that the median normalized viral load in samples with normal cytology reports was significantly lower ($p = 0.001$) than the normalized viral load in samples with

abnormal cytology reports. The normalized viral load was compared in samples with abnormal cytology reports including ASC, AGC, LSIL, HSIL, SCC and ADC; however, the results did not show any statistically significant difference. The normalized viral load was also assessed in two groups of samples. Group One included samples with ASC, AGC and LSIL cytology reports, and Group Two included samples with HSIL, SCC and ADC reports. Similarly, no significant viral load differences were observed between Group One and Group Two (Table 3).

The median normalized viral load of HR-HPV, IR-HPV and LR-HPV was 4.04×10^{-7} , 0.71×10^{-7} and 2.38×10^{-7} , respectively, ($p = 0.002$). On further analysis, a significant difference was found between the normalized viral load of HR-HPV and IR-HPV ($p = 0.031$). A statistical significant difference was also noticed between HR-HPV and LR-HPV ($p = 0.003$)

Discussion

This study was conducted to assess the HPV viral load in women with normal and abnormal cervical reports in Kuwait. The outcome of the study shows that the amount of HPV DNA in cervical scrapes is an important parameter to distinguish between cervical scrapes with normal and abnormal cervical cytology. This finding is in agreement with the observations of Schmitz *et al.* [15] and van Duin *et al.* [16], who showed that the viral load of HPV significantly differs in women with normal and abnormal cytology reports.

It is now well-established that HPV viral load increases with increasing disease severity [17]. Other studies have related other HR-HPV genotypes such as HPV16, HPV18, HPV31, HPV33 and HPV45, to high-grade cervical abnormality [18].

The current study also shows that the amount of HPV DNA in a cervical scrape might be an important parameter to distinguish between HR-HPV infections that are of clinical relevance and LR and IR-HPV genotypes. This finding is in agreement with those of Snijders *et al.* [19], who showed that the viral load of HR-HPV genotypes is significantly higher than that of LR-HPV genotypes.

This study finds no significant difference in the viral load between Group One (women with ASC, AGC and LSIL reports) and Group Two (women with HSIL, SCC and ADC reports), an association that was previously shown by Lillo *et al.* [20] and Swan *et al.* [18]. In 2012, Origoni *et al.* [21,22] triaged women with ASCUS cytology reports using HPV viral load. They showed that women with CIN II and CIN III in histology had high HPV viral load and they also showed that in these women the viral load of HR-HPV was significantly higher than that found in LR-HPV. One explanation for the difference between our findings and the observations of other investigators [18,20-22] is the small number of women in Group Two in the current study. A second explanation could be that in this study, as is the case of most studies that have measured viral load, integration status was not investigated [23], yet in most high-grade cervical abnormalities, the prevalence of integrated forms of HPV increases and the integration itself is followed by a decrease in viral load [24]. A third possible explanation is that in women with high-grade cervical abnormality, the viral load is high, and low-grade cervical abnormality could also be present at the same time, but only the most severe histological abnormality is reported [25]. The acquisition of new HPV types is associated with changes both in viral load and with the development of new CIN lesions; therefore, measures

of association might be unreliable in studies that rely on a single baseline measurement of exposure [26].

In the current study, real-time PCR was used for the detection and quantitative analysis of HPV because of the advantages of the assay over the conventional method. The target in real-time PCR is detected in real time from the sealed PCR plate and there is no post-PCR processing required; therefore, the risk of false-positive results due to amplicon carryover is substantially decreased compared to that of conventional PCR [27]. In addition, most real-time PCR formats offer the option of applying a melting curve analysis so that the amplicon can be distinguished from a nonspecific product or primer-dimers. The performance of real-time PCR assays, therefore, has increased sensitivity and reliability of the reported results over those obtained by conventional PCR. Real-time PCR assays can be designed to allow the detection and simultaneous quantification of the PCR products over a wide dynamic range of quantitative linearity, which is an important requirement of many diagnostic applications. Because real-time PCR assays are amenable to automation, most of these detection assays are run and completed in the clinical laboratory setting in a shorter time than that required for conventional PCR, thus providing a more rapid test cycle turnaround time [27]. In this study, using the quantitative HPV PCR method, the substantial overlap of viral load values among women with and without cytology abnormality and among HR and LR-HPV genotypes, especially in the range of high viral loads, made it impossible to choose cut-off values for high-grade cytological abnormalities and HR-HPV [28].

Cut-off points defined by real-time PCR methods depend on the PCR method used, and might vary between laboratories using the same PCR method. In this study, two sets of primers were used for HPV screening (*i.e.*, GP5+/6+ and MY09/11), whereas in most published studies a GP5+/6+ set of primers was used [28]. The literature is inconsistent in reconciling HPV viral load with a quantifiable standard. Some report viral load as a function of total input cells, whereas others report viral load as a function of abnormal input cells or as a function of abnormal input cells with suitable DNA quality [16,29]. In this study, HPV viral load was related to the amount of input human DNA, as judged by the concentration of a housekeeping gene such as beta-globin, and expressed as normalized viral load. According to Hart *et al.* [29], high viral loads may be produced in severe disease, rather than being the cause of severe disease. This

suggestion is based on the fact that viral load values are an average of infected and uninfected cells. Also, the viral DNA may be integrated, disrupted, or deleted from the probe target site. Cut-off points might also depend on the screened population, as younger women may show increased viral loads in productive infections [30].

Conclusion

In conclusion, our data show that, in Kuwait, the HPV viral load in women with normal cervixes is significantly lower than that in women with abnormal cervixes and the viral load of HR-HPV genotypes is significantly higher than that of other genotypes. Two viral load parameters may be useful in identifying women at increased risk of developing cervical lesions in a normal population. It may be useful to monitor disease recurrence, and to examine the effect of widespread vaccination on HPV type prevalence in the future. Further population-based prospective studies are needed to eliminate the drawbacks of our study and to include more women with HSIL and invasive carcinoma reports.

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