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Research article

Human papillomavirus type 16 and 18 viral loads as predictors associated with abnormal cervical cytology among women in Saudi Arabia

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ABSTRACT

The detection of HPV viral DNA is regularly conducted with cervical screening. However, using a molecular marker such as the viral load may serve as a predictor associated with disease detection and progression. The present study aimed to screen for and genotype HPV among women in Saudi Arabia, develop and validate sensitive quantitative polymerase chain reaction (qPCR) assays to detect viral load for the two most common HPV types, namely 16 and 18, and assess whether HPV viral load could be used as a marker for cervical abnormality and disease progression. This study examined 733 specimens (both formalin-fixed paraffin embedded specimens and PAP smear samples) from women who underwent cervical screening. The specimens and samples were processed for DNA extraction and then tested for HPV DNA using nested PCR. Approximately 165 specimens (18%) were positive for HPV. Those specimens were genotyped using a reverse line blotting hybridization assay. The results indicated that the most common HPV types detected were a single infection with HPV 16 (51%) or with HPV 18 (28%) followed by infections with multiple HPV types (~7%). A qPCR TaqMan assay developed and validated in-house was used to determine viral load for HPV genotypes 16 (n = 80) and 18 (n = 45). Viral loads for both HPV types were significantly associated with cervical cytology grade ($P < 0.05$). The odds ratio (OR) for the HPV 16 viral load was high for specimens with cervical cancer (OR, 18.8; 95% CI, 4.3–82.9) or for those with high-grade squamous intraepithelial lesions (OR, 14.7; 95% Cl, 2.43–88.49). For the HPV 18 viral load, the OR was significant only for specimens with cervical cancer (OR, 11.1; 95% Cl, 2.2–54.9). Logistic regression models for HPV 16 and for HPV 18 viral load levels were significant, with higher viral load associated with cervical abnormalities. These findings indicate that viral load is a predictor significantly associated with cytology abnormality in women who are positive for high-risk HPVs and suggest that integrating a viral load test into current clinical screening practices for HPV-positive women is warranted in Saudi Arabia.

1. Introduction

Human papillomavirus (HPV) infection is the leading cause of cervical cancer. Approximately 99% of all cervical cancer cases are caused by some form of HPV infection [1]. There are about 200 HPV types known, with 40 of them sexually transmitted and 13 of them classified as high risk for cancer. Types 16 and 18 are the most frequently detected high-risk types and lead to the most dangerous infections, with HPV 16 predominant in squamous cell tumors and HPV 18 predominate in adenocarcinoma [2]. Hence, two of the most effective approaches for preventing cervical cancer are HPV vaccination and routine cervical screening programs. Unfortunately, both of these programs have not yet been implemented in Saudi Arabia.

Data on HPV infection prevalence in Saudi Arabia is inconsistent; whereas some publications on positive sexually transmitted infections report a percentage as low as 5.8% [3], others report a much higher percentage of 17% [4]. The most commonly detected HPV genotypes in Saudi Arabia are 16 and 18 [5,6].

One of the most commonly used biomarkers for cervical cancer is HPV testing, which is considered a viral marker. However, this test does not

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explicitly measure the progression of the disease. Refining HPV viral markers with specific molecular targets may provide a more informed approach to measure the progression of the disease [7]. In diseases related to viral infections, viral load has been shown to be a promising marker for clinical progression [8, 9, 10]. Although HPV viral load has been showing great potential in predicting cytology abnormalities [11], many studies have reported conflicting results, which may be attributable to their small sample sizes or their use of different types of detection assays [12].

The three main objectives of this retrospective study were to develop and validate an in-house viral load assay for HPV 16 and 18, to gain a greater perspective on the distribution of HPV genotypes in Saudi Arabia, and to determine the importance of viral load as a biomarker for cytology abnormality progression.

2. Materials and methods

2.1. Specimen collection

The use of all specimens for the present study was previously approved by the Research Advisory Council (Ethics Committee) at King Faisal Specialist Hospital and Research Centre (KFSH & RC) (RAC 1005-033). In total, 933 specimens were obtained, 618 from Papanicolaou (Pap) testing using a PreservCyt brush (ThinPrep Pap Test Boxborough, MA, USA), and 316 specimens from archived biopsied, formalin-fixed and paraffin-embedded (FFPE) cervical specimens. The stages of the abnormal cytology were identified using the Bethesda classification system [13].

2.2. Nucleic acid extraction

The DNA extraction for the samples obtained by the Pap test was performed using a Gentra Puregene Cell Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The DNA extraction for the FFPE cervical specimens was conducted using a QIAamp DNA FFPE Tissue kit by following the manufacturer's instructions (Qiagen, Valencia, CA, USA). To confirm the presence of DNA in each sample, primers for the β-globin gene were used.

2.3. HPV PCR detection

Nested PCR was conducted to detect HPV. The primer sets were MY09/MY11 and GP5+/GP6+, which target sequences located within the L1 region of the HPV genome. For positive controls, HeLa or SiHa cells were used; for negative control, UltraPure DNase/RNase–free water was used.

2.4. HPV genotyping by reverse line blotting hybridization

Twenty-three oligonucleotide probes were used in genotyping HPV with a reverse line blotting hybridization assay using a protocol described previously by Al-Ahdal [6].

2.5. Quantitative HPV viral load assays

In-house relative viral load assays (TaqMan) were developed for HPV 16 and HPV 18 genotypes. The assays were performed using a 7500 Fast Real-Time PCR system and software (Applied Biosystems, California, USA).

Genomic template DNA (60–300 ng) obtained from either samples, plasmids, or controls, was amplified using TaqMan Universal PCR Master Mix. Primers and probes were targeting the L1 gene and were custom designed by Applied Biosystems, Warrington, UK Kit Assay #AIY90LA (HPV18) and AIX02E2 (HPV16). The amplification assay was performed in a 20 μL volume containing 10 μL of 2× TaqMan Universal PCR Master Mix, 1 μL of 20× probes and primers mixtures, 1 μL of UltraPure DNase/RNase–free water, and 1 μL of template DNA.

The amplification conditions for assays were as follows: 50 °C for 2 min and 95 °C for 12 min followed by 50 cycles of 95 °C for 15 s and 55 °C for 30 s. All of the probes used had the reporter dye FAM and MGB-NFQ quenchers. The positive control for HPV 16 was a SiHa cervical carcinoma cell line, and the positive control for HPV 18 was a HeLa cell line. The negative control was UltraPure DNase/RNase–free water.

Tenfold plasmid dilutions were used to construct 5-point standard curves for all three target sequences (HPV-16:ATCC: 45113, HPV-18:ATCC:45152D, and the human albumin gene), and the viral load was normalized to the input amount of cellular DNA. All samples were run in triplicate, and the mean values were expressed as the mean of the $\log_{10}$-transformed numbers of HPV copies per microliter. All of the accepted runs met the real-time qPCR experiment MIQE guidelines [14].

2.6. Demographic and clinical statistical analyses

All data collected were stored and analyzed using SAS, version 9.4, and SPSS, version 24, software. The goodness of fit (χ^2) test was used to assess for association between categorical variables. The statistical significance for the viral load with other variables was determined by the Kruskal-Wallis rank test and Mann-Whitney test. Logistic regression and univariate models were assessed to test for association between the viral load and other variables. All P values reported were 2-sided and were considered statistically significant at $P < 0.05$.

3. Results

In total, 933 Specimen were collected. The mean age of the women in this study was 45 years old (range, 11–95 years). The HPV testing results indicated that 165 specimens (18%) were positive and 768 specimens (82%) were negative for the presence of HPV. A descriptive analysis between HPV status and clinical characteristics of the specimens used in the study is provided in Table 1. The HPV positive specimens were genotyped by reverse line blotting. Figure 1 shows the percentages and frequencies of the HPV types detected by reverse line blotting.

For HPV 16, the viral load of 80 specimens was determined. Figure 2 shows the distribution of HPV 16 viral load by cytology grade. For HPV 18, the viral load of 45 specimens was determined. Figure 3 shows the distribution of HPV 18 viral load by cytology grade. Overall, higher viral load in both HPV-16 and HPV-18 was found predominately in specimens that had cervical cancer or HGSIL.

The median viral loads for both HPV types stratified by clinical and demographical characteristics are given in Table 2. A Kruskal-Wallis rank test was conducted for each of the assays with other variables. A significant difference in viral load for both HPV types was detected in cytology grade and abnormality: patients with more severe cytology grades had a higher viral load.

A Mann-Whitney test was conducted pairwise for HPV 16 viral load and cytology grade groups. A significant difference was detected between normal and cervical cancer specimens, cervical cancer had higher viral load than normal specimens. likewise, significant difference was detected between HGSIL and normal specimens, with HGSIL having higher viral. The viral load between HGSIL and cervical cancer specimens was also significantly different (Appendix A.1 in the Supplementary Material).

For HPV 18, a Mann-Whitney test assessing the difference in viral load of normal and cervical cancer specimens was significantly different. However, additional tests for the viral loads between HGSIL and normal specimens or between HGSIL and cervical cancer specimens found no significant difference (Appendix A.2 in the Supplementary Material).

Univariate models were used to test whether HPV viral load could be a predictor associated with cytology grade. For HPV 16 viral load and cytology grade, the univariate model was significant ($\chi^2 = 22.8$, $P = 0.0001$). In addition, the odds ratio (OR) was significant when comparing specimens with normal cytology (reference = 1) with cervical cancer (OR

Table 1. Demographic and clinical characteristics by HPV status, with the goodness-of-fit and *t* tests.

Characteristic	HPV Positive n = 165, 17.68% (n) %	HPV Negative n = 768, 82.32% (n) %	Total n = 933 (n) %	χ^2 (*P*)
Age, years				
11–30 (n = 112)	(16) 1.7	(96) 10.4	(112) 11.9	14.3 (0.003)**
31–45 (n = 378)	(63) 6.8	(315) 34.1	(378) 40.9	
46–60 (n = 331)	(54) 5.8	(277) 29.9	(331) 35.8	
>60 (n = 103)	(32) 3.46	(71) 7.68	(103) 11.2	
UNK (n = 9)	NA	(9) 1.4	(9) 1.4	
Mean (Standard Deviation)	44.7 (12.2)	47.6 (13.5)	***t* test = 2.64 *P* = 0 .005***	
Religion				
Muslim (n = 854)	(148)15.9	(706)75.8	(854)91.6	0.97 (0.32)
Non-Muslim (n = 70)	(17) 1.8	(53) 5.7	(70) 7.5	
UNK (n = 9)	NA	(9) 0.1	(9) 0.1	
Nationality				
Saudi (n = 759)	(136) 14.6	(623) 66.8	(759) 81.4	0.15(0.96)
Non-Saudi (168)	(29) 3.1	(145) 15.5	(174) 18.7	
UNK (n = 6)	NA	(6)0.1	(6)0.1	
Marital status				
Married (n = 765)	(125) 13.6	(640) 69.4	(722) 83.2	15.23 (.002)*
Divorced (n = 28)	(7) 0.8	(21) 2.3	(27) 2.9	
Widowed (n = 51)	(19) 2.1	(32) 3.5	(51) 5.5	
Single (n = 78)	(14) 1.5	(64) 6.9	(78) 8.4	
UNK (n = 55)	NA	(55) 5.9	(55) 5.9	
Histology grade				
Normal (n = 635)	(66)7.3	(569) 63.6	(635)68.1	134.9 (<.0001)***
ASCUS (n = 18)	(3)0.3	(15) 1.6	(18) 1.9	
LGSIL (n = 68)	(9)1.0	(59) 6.3	(68) 7.2	
HGSIL (n = 65)	(21) 2.3	(44) 4.7	(65) 6.9	
Cervical Cancer (n = 121)	(64) 20.3	(57) 6.1	(121) 12.9	
UNK (n = 26)	(2.0)0.2	(24)0.3	(26)0.5	

Abbreviations: ASCUS, atypical squamous cells of undetermined significance; HGSIL, high-grade squamous interepithelial lesion; LGSIL, low-grade squamous intraepithelial lesion; UNK, unknown.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

= 18.8; 95% Cl, 4.3–82.9) and with HGSIL (OR = 14.7; 95% Cl, 2.43–88.49) (Appendix B.1 in the Supplementary Material). For the HPV 18 viral load and cytology grade, the univariate model was also significant (χ^2 = 16, P = 0.003), and the OR was significant only for the comparison of normal cytology (reference = 1) and cervical cancer (OR = 11.1; 95% C, 2.2–54.9) (Appendix B.2 in the Supplementary Material).

For the logistic regression analysis, viral load was categorized as *high* for 100 viral copies/μL or more and *low* if it was equal to or lower than 100 copies/μL. The cytology grades were categorized as *normal* and *abnormal*. For HPV 16, the logistic regression model was significant (χ^2 = 18.8, $P < 0.001$), with higher risk of cervical abnormality associated with higher viral load (OR = 14.5; 95% Cl, 3.9–68.4). For HPV 18, the logistic regression model was also significant ($\chi^2 = 9.9$, $P = 0.0002$), with higher risk of cervical abnormality associated with higher viral load (OR = 8.3; 95% Cl, 2.1–33.6) (Appendix B.3 in the Supplementary Material).

When comparing the levels of viral load by HPV type of infection (i.e., single or multiple), the results showed that most of the infections were single. The highest viral loads were found mostly in single infections of HPV 16 or HPV 18.

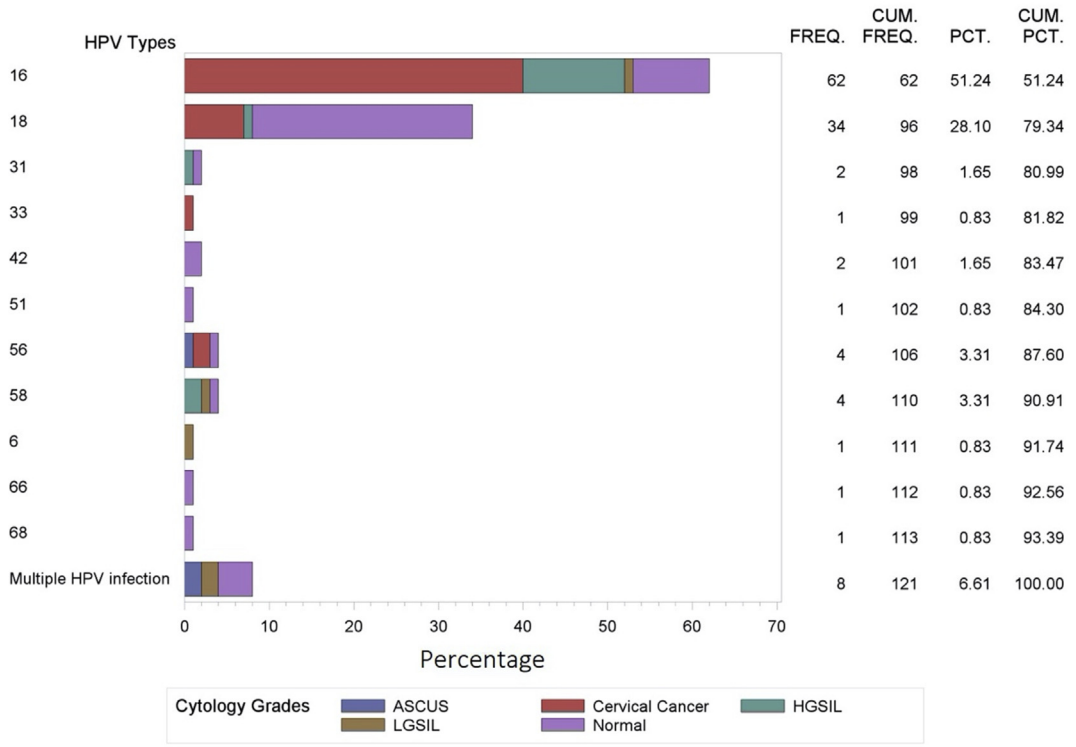
Finally, specimen viral load was furthermore compared with the sampling collection method. Overall, FFPE specimens had higher viral load for both HPV 16 and 18. There was no significant difference between viral load as detected by the Pap test and the FFPE specimens for the HPV 16 assay, whereas a significant difference was detected between the two collection methods for the HPV 18 assay (Appendix C in the Supplementary Material).

4. Discussion

This retrospective study showed not only that viral load of a cervical specimen with HPV was associated with cytology grade but also that viral load was a predictor significantly associated with cervical abnormality. In 165 cervical specimens positive for HPV infection, viral load was detected in 125, 80 of which were HPV 16 and 45 of which were HPV 18. The rate of positive HPV cases was 18%, which is high compared with those of other studies conducted in Saudi Arabia, and the discrepancies among the results are likely attributable to the biased detection method (amplification vs. hybridization) [4]. One of the most important outcomes of the present study was the development and validation of an in-house qPCR assay for detection of HPV 16 and HPV 18. These newly developed assays were validated with a triple target plasmid to assess sensitivity. Notably, these assays were sufficiently sensitive to detect very low viral copy numbers (0.001 copies/μL) as well as able to detect values as high as 67 million copies/μL.

Several previous studies have argued that a linear relationship between the severity of disease and viral load is associated with specific HPV types [15, 16, 17, 18]. One study reported a significant relationship when the HPV types were 16, 31, 33, 52, and 58, while the association was not significant with HPV types 18, 45, 56, and 59 [19]. The results of the present study found no change in the significance of the association when we compared HPV genotypes 16 and 18.

When assessing for an association between viral load and clinical variables, we found no significant association for either HPV 16 or 18



	FREQ.	CUM. FREQ.	PCT.	CUM. PCT.
16	62	62	51.24	51.24
18	34	96	28.10	79.34
31	2	98	1.65	80.99
33	1	99	0.83	81.82
42	2	101	1.65	83.47
51	1	102	0.83	84.30
56	4	106	3.31	87.60
58	4	110	3.31	90.91
6	1	111	0.83	91.74
66	1	112	0.83	92.56
68	1	113	0.83	93.39
Multiple HPV infection	8	121	6.61	100.00

Figure 1. Distribution of HPV Types detected with reverse line blotting by cytology grade. The most detected type of HPV was 16 (51.2%), followed by 18 (28.1%), and multiple HPV infections (6.6%). For HPV 16, most of the specimens were diagnosed with cervical cancer, followed by high-grade squamous interepithelial lesion (HGSIL), and normal cytology results. For HPV 18, most of the specimen histology results had a normal diagnosis, followed by a cervical cancer diagnosis. Abbreviations: ASCUS, atypical squamous cells of undetermined significance; Cum, cumulative; Freq, frequency; LGSIL, low-grade squamous intraepithelial lesion; PCT, percentage.

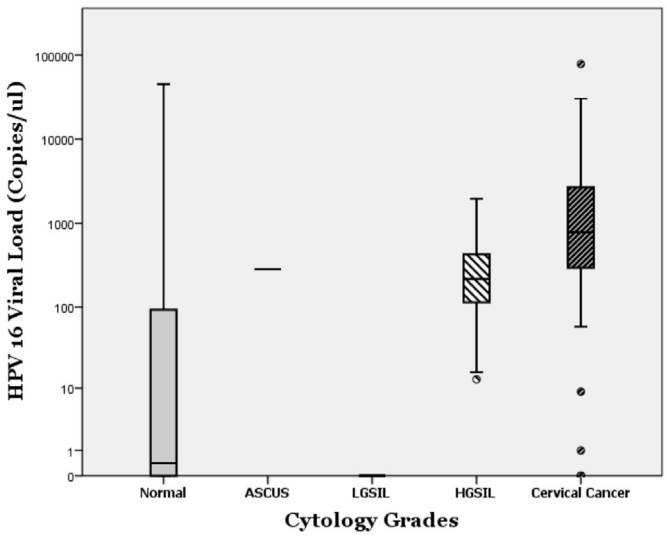


Figure 2. Distribution of viral load for HPV 16 assay by cytology grade. The highest detected viral load was found in cervical cancer specimens (78,514 copies/μL), followed by HGSIL specimens, and lastly normal specimens. There was only one ASCUS specimen and one LGSIL specimen. Data are represented as boxplots, with the bottom and the top of the box representing the first and third quartiles; the band inside the box, the median; the whiskers, the lowest and the highest data points within 1.5 × the interquartile ranges of the first and upper quartiles; and the circles, outliers. Group differences were evaluated by Kruskal-Wallis tests.

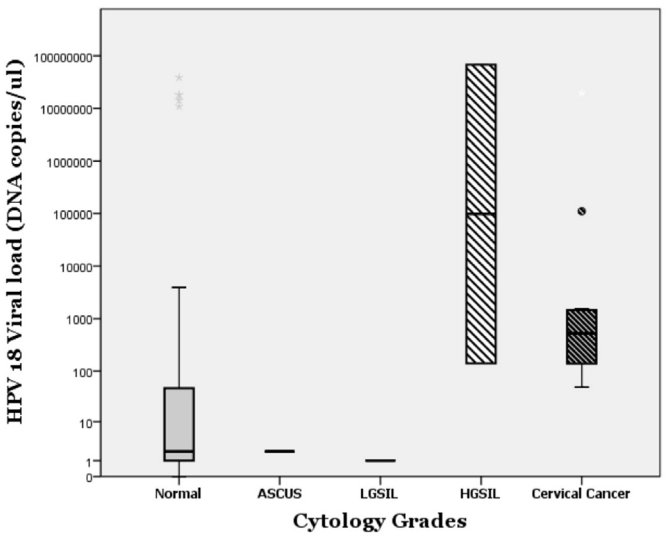


Figure 3. Distribution of viral load for HPV 18 assay by cytology grade. The highest detected viral load was found in HGSIL specimens (67,701,024 copies/μL), followed by cervical cancer specimens, and lastly normal specimens. There was only one ASCUS specimen and one LGSIL specimen. Data are represented as boxplots, with the bottom and the top of the box representing the first and third quartiles; the band inside the box, the median; the whiskers, the lowest and the highest data points within 1.5 × the interquartile ranges of the first and upper quartiles; and the circles, outliers. Group differences were evaluated by Kruskal-Wallis tests.

Table 2. Median viral load stratified by patient demographic and clinical characteristics assessed by the Kruskal-Wallis rank test.

Characteristic	HPV 16 (Total = 80)	HPV 18 (Total = 46)
	Copies/μL (n)	Copies/μL (n)
Age, years		
≤30	540.3 (5)	2 (4)
31–45	202.8 (27)	8 (21)
46–60	292.1 (27)	326 (18)
>60	728.2 (21)	61.84 (3)
Kruskal-Wallis (*P* value)	**(0.092)**	**(0.47)**
Marital status		
Married	350.11 (57)	13 (41)
Divorced	1824.61 (3)	15.37 (2)
Widowed	524.708 (15)	1375.2 (1)
Single	281.68 (5)	16,191,521 (2)
Kruskal-Wallis (*P* value)	**(0.24)**	**(0.186)**
Cytology grade		
Normal	0.73 (14)	2 (31)
ASCUS	0.008 (1)	1.90 (1)
LGSIL	284.39 (1)	1.19 (1)
HGSIL	218.1 (15)	33,850,583 (2)
Cervical cancer	783 (49)	582 (11)
Kruskal-Wallis (*P* value)	**(0.001)** **	**(0.011)** **
Cytology outcome		
Normal	0.72	2
Abnormal	525.9	442.82
MW (*P* value)	**(0.001)** **	**(0.002)** **
Infection Type		
Single	525.9	15.9
Multiple	142.2	1.9
MW (*P* value)	**(0.30)**	**(0.64)**

Abbreviations: ASCUS, atypical squamous cells of undetermined significance; HGSIL, high-grade squamous interepithelial lesion; LGSIL, low-grade squamous intraepithelial lesion; MW, Mann-Whitney; UNK, unknown.
**$P < 0.01$.

except for the associations with cytology grades and abnormality. HPV 16 viral load was highest among divorced women who were older than 60 years of age with a single infection and who had cervical cancer. By contrast, HPV 18 viral load was highest among single women between 45 and 60 years of age with a single infection and who had HGSIL grade cytology. In an interesting study, HPV viral load was assessed after surgical removal of the cervical tumor, and the researchers found that viral load was associated with disease progression but not with cytology grade or age [20]. However, another study found that viral load was a significant predictor associated with the progression of cervical cancer in patients with multiple infection [11]. In our population, only 8 patients had multiple HPV infection. Six of those patients had low viral load and were older than 35 years, whereas two of them were in their twenties and had high viral load.

Remarkably, none of our cases positive for multiple HPV infection had cervical cancer or HGSIL lesions. High viral load was mostly found in patients infected with a single HPV type. This finding agrees with numerous previous studies [21]. Multiple infection is commonly associated with lower severity of disease, but when viral load increases with multiple infection, the risk of severe disease doubles [22]. We could not assess this finding further because our sample size for this clinical group was too small.

The distribution of viral loads for HPV 16 and 18 was different: overall, HPV 16 had a higher median viral load (426.9 copies/μL) than HPV 18 (24 copies/μL). A few studies have reported a linear correlation of cervical abnormality and viral load for HPV 16, but linearity was not observed for HPV 18 [23]. In the present study, we found similar results, that is, HPV 16 was mostly correlated with cytology grade, whereas HPV 18 was imbalanced across cytology grade.

The two most well-known genera of HPV are α-9, which includes type 16, and α-7, which includes type 18 [24]. HPV 16 and its genus α-9 are commonly associated with high replicative activity and found in a wide range of cytology grades. By contrast, HPV 18 and its genus α-7 are associated with the development of cytology grade, but they usually show lower replicative activity and a narrower range of cytology grades [25].

One of the biggest differences between HPV 16 and HPV 18 might involve their integration into human cells. HPV 16 has the ability to start cell replication during its episomal state, which increases viral load even when the virus is not fully integrated into human cells. By contrast, HPV 18 can only start replicating after it is fully integrated, which may explain its lower viral load compared with that of HPV 16 [26]. Although the process of viral integration and replication may explain the changes in viral load, other studies have proposed that HPV 18 may use completely different pathways. One study has found that variation in the E2 binding sites of HPV 16 and HPV 18 cause differences in their affinities, which may lead to a change in the replication process and the modulation of carcinogenicity. However, the specific difference in carcinogenicity modulation between these two types is not yet clear and further investigation is required [27].

Our study has a few limitations that should be considered when interpreting our results. First, all our specimens came from one region of Saudi Arabia, which may not reflect the distribution of HPV genotypes across Saudi Arabia. Another limitation was the use of the reverse line

blotting genotyping assay, which detects only 23 genotypes; thus, some of the multiple infection cases may have been missed. We plan to use HPV genotyping assays that detect other genotypes in the future. Our additional future directions include designing viral load assays for other HPV types and investigating molecular levels of HPV genera α-9 and α-7.

In conclusion, the present study made use of cutting-edge technology to measure viral load of HPV among women in Saudi Arabia. We successfully developed and validated sensitive assays to detect viral loads for HPV 16 and 18 genotypes. Those tests showed that viral load of HPV was a predictor significantly associated with cytology abnormality. Our results also indicated that measuring viral load is warranted for women in Saudi Arabia who are positive for high-risk HPV types. The results of such tests may inform and thus improve management of cervical cancer cases driven by HPV infection.

Declarations

Author contribution statement

F. S. Alhamlan: conceived and designed the experiments; performed the experiments; contributed reagents, materials, analysis tools or data; wrote the paper.

D. Obeid: conceived and designed the experiments; wrote the paper.

S. ALmatrrouk, H. Khayat, A. Tulbah: performed the experiments.

T. Al-Muammer, I. Albadawi: performed the experiments; analyzed and interpreted the data.

M. Al-Ahdal: conceived and designed the experiments; performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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