Original Article

Human papillomaviruses in cervical specimens of women residing in Riyadh, Saudi Arabia: a hospital-based study

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Abstract

Introduction: Certain genotypes of human papillomavirus (HPV) are linked to cervical abnormalities. HPV DNA and genotype prevalence among women residing in Riyadh, Saudi Arabia is investigated in this hospital-based study.

Methodology: Cervical specimens were taken from 519 subjects along with consent and demographic data. DNA was extracted and PCR was performed on all specimens using general primers. Low- and high-risk HPV genotypes were determined by reverse blot hybridization assay using specific probes. SPSS version 17 was used for the data analysis.

Results: Of 519 cervical specimens, 164 (31.6%) were positive for HPV DNA. There was a significant association between HPV positivity and abnormal cytology ($p < 0.00001$). Even though the HPV positivity was relatively high, the squamous intraepithelial lesions were minimal, with one low grade and one high grade case among those HPV DNA-positive specimens. Regardless of single or multiple infections per specimen, HPV-16 was found in 87.8%, followed by HPV-18 in 86%, and HPV-11 in 78.3%.

Conclusions: Amplification technology showed that HPV is common among women in Riyadh, Saudi Arabia, with a strong association between HPV infection and cytological changes. HPV-16 was the most frequent genotype but had a low prevalence of cervical cancer.

Key words: HPV; infection; genotypes; Riyadh, Saudi Arabia

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Introduction

Human papillomavirus (HPV) has been implicated in the etiology of cervical cancer [1]. Developing countries are known to have the vast majority of new cases worldwide [2]. About 130 genotypes of HPV have been identified through sequencing the gene for the major capsid protein, L1. The majority of these genotypes are harmless. However, some are classified as low risk, causing genital warts (HPV-6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81), while others were considered high risk (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) for cervical cancer [3, 4]. HPV-16 and HPV-18 are globally believed to be directly involved in the cervical carcinogenesis process [5-7]. The worldwide HPV positivity rate among women with normal cervical cytology was reported to be 10.4%, even though some East African countries showed a prevalence of 31.6% [8].

Most of the published data about cervical changes among women in Saudi Arabia did not investigate the existence of HPV [9-12]. Aside from a few studies [13-16], there is insufficient information about HPV

prevalence and its involvement in cervical cancer in Saudi Arabia, especially from population-based studies. The prevalence of HPV infection and the HPV genotypes contributing to HPV infection have never been evaluated in the city of Riyadh, the capital of Saudi Arabia, with a population of over four million people. With the advent of bivalent and quadrivalent vaccines and the reported benefits of vaccination [17], it has become essential to investigate the prevalence of HPV, especially in areas where such information is not available. In this hospital-based study, we aimed to establish HPV prevalence and genotype distribution among women living in Riyadh to elucidate the situation for possible future vaccination or triage programs. Furthermore, studying variation of HPV prevalence from one country to another, and even one city to another, is important for epidemiological surveys.

Methodology

Study subjects and specimen collection

Cervical specimens (cytobrush) were taken for HPV detection and Pap examination, with institutionally approved consent forms and demographic data sheets, from 519 normal women (Saudi citizens and non-Saudi legal residents) attending Obeid Specialized Hospital in Riyadh for routine checkups. The age of subjects ranged between 20 and 74 years (37 ± 9). Due to cultural and religious considerations, only married, divorced, and widowed women who were not pregnant were approached. The gynecological examination of the subjects was performed according to the recommendations of the American Cancer Society. Specimens were collected in a sterile tube containing 5 mL of RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 4000 ×g for 15 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of phosphate-buffered saline (PBS) by gentle vortexing and stored at -80°C until used.

HPV DNA detection

DNA was extracted from cells using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. As an internal control, the β-globin gene was used and PCR was performed as described by Camargo *et al*. [18] with modifications. Extracted DNA was amplified to detect the L1 gene of HPV in a nested PCR procedure using MY09/11 and the GP5+/GP6+ primer sets (Table 1) [19,20]. Briefly, a final reaction volume was made of 25 µL containing 200 ng of the extracted DNA (for round 1) and 5 µL of 1:20 diluted round 1 amplicon (for round 2), 12.5 µL of 2X GoTaq Green Master Mix (Promega, Madison, WI, USA), and 1 µM of each primer. Primer GP6 was biotinylated at the 5' end. The cycling conditions included denaturation of the template DNA for 1 cycle of 95°C for 2 minutes, amplification of the target DNA for 35 cycles of 95°C for 1 minute, 55°C for 1 minute (round 1) or 49°C for 1 minute and 30 seconds (round 2) and 72°C for 1 minute, and a final extension for 1 cycle of 72°C for 5 minutes. Positive controls were composed of extracted DNA from SiHa cells (HPV-16) and HeLa cells (HPV-18), while negative controls contained DNA from BC-1 cells (no HPV DNA) and the reagent mixture (no DNA). PCR products, along with molecular weight standards, were subjected to gel electrophoresis and viewed under a UV illuminator. To avoid false positivity, the procedure of Kwok and Higuchi [21] was followed.

Genotyping by reverse line blot hybridization

Twenty-three oligoprobes (C12 aminolink) as described by van der Brule *et al.* [22] were synthesized (Metabion, Germany) as shown in Table 2. A miniblotter for spotting in parallel all different oligoprobes on a carboxyl-coated nylon membrane (Biodyne C 0.45 µm, Pall Corporation, Pensacola, FL, USA) was used. Biotinylated PCR products were pipetted into the parallel channels of the miniblotter in such a way that the channels were perpendicular to the rows of immobilized oligoprobes deposited previously. Subsequently, membranes were incubated with 30 µL of streptavidin-horseradish peroxidase diluted in 63 mL of buffer B (100 mM Tris-HCL, 150 mM NaCl, pH7.5) and 7 mL of blocking agent (0.5g Liquid Block in 10 mL PBS + 10 µL Tween 20) for 30 minutes at room temperature. The fluid was aspirated, the membrane was washed three times for 5 minutes each time with wash buffer B (0.3% Tween 20 in buffer B), and rinsed with buffer B. Detection was performed using the ECL detection liquid kit and the membrane was dried on filter paper for 1 minute. The membrane was exposed to hyperfilm in a dark room for 1 minute, and results were recorded.

Statistical analysis

SPSS version 17 was used for the data analysis. The χ^2 Mantel-Haenszel (M-H) test of independence was used to assess the association between the categorical variables. M-H odds ratio estimates were produced with a 95% confidence interval (CI). Type I error rate was set at 5%.

Table 1. Primer sequence for the L1 gene used for HPV detection by PCR

Round	Primer	Sequence (5' - 3')	Size	Reference
First	MY09	CGT CCM ARRGGAWACTGATC	450 bp	[19]
	MY11	GCMCAGGGWCATAAYAATGG		
Second	GP5+	TTTGTACTGTGGTAGATACTAC	150 bp	[20]
	GP6+	Biotin-GAAAAATAAACTGTAAATCATATTC		

M = A or C; R = A or G; W = A or T; and Y = C or T

Table 2. Sequences of probes used for reverse line blotting to determine HPV genotypes. All with 5'-^{12}C aminolink [22]

HPV genotype	Probe sequence (5′ – 3′)
Low risk	
HPV-6	ATC CGT AAC TAC ATC TTC CAC ATA CAC CAA
HPV-11	ATC TGT GTC TAA ATC TGC TAC ATA CAC TAA
HPV-40	GCT GCC ACA CAG TCC CCC ACA CCA ACC CCA
HPV-42	CTG CAA CAT CTG GTG ATA CAT ATA CAG CTG
HPV-44	GCC ACT ACA CAG TCC CCT CCG TCT ACA TAT
HPV-54	TAC AGC ATC CAC GCA GGA TAG CTT TAA TAA
High risk	
HPV-16	GTC ATT ATG TGC TGC CAT ATC TAC TTC AGA
HPV-18	TGC TTC TAC ACA GTC TCC TGT ACC TGG GCA
HPV-26	AGT ACA TTA TCT GCA GCA TCT GCA TCC ACT
HPV-31	TGT TTG TGC TGC AAT TGC AAA CAG TGA TAC
HPV-33	TTT ATG CAC ACA AGT AAC TAG TGA CAG TAC
HPV-35	GTC TGT GTG TTC TGC TGT GTC TTC TAG TGA
HPV-39	TCT ACC TCT ATA GAG TCT TCC ATA CCT TCT
HPV-45	ACA CAA AAT CCT GTG CCA AGT ACA TAT GAC
HPV-51	AGC ACT GCC ACT GCT GCG GTT TCC CCA ACA
HPV-52	TGC TGA GGT TAA AAA GGA AAG CAC ATA TAA
HPV-53	GTC TAT GTC TAC ATA TAA TTC AAA GCA AAT
HPV-56	GTA CTG CTA CAG AAC AGT TAA GTA AAT ATG
HPV-58	ATT ATG CAC TGA AGT AAC TAA GGA AGG TAC
HPV-59	TCT ACT ACT GCT TCT ATT CCT AAT GTA TAC
HPV-66	TAT TAA TGC AGC TAA AAG CAC ATT AAC TAA
HPV-68	TCT ACT ACT ACT GGA TCA GCT GTA CCA AT
HPV-73	GTG TAG GTA CAC AGG CTA GTA GCT CTA CTA

Table 3. Association between HPV-DNA positivity and cytological examination

HPV-DNA	Pap smear results		Total
	Normal	Abnormal*	
Positive	130	34	164
Negative	325	30	355
Total	**455**	**64**	**519**

P value < 0.00001; Odds ratio (OR) = 2.833 with 95% confidence interval (CI) of 1.665-4.820; *Most cases were ASCUS

Results

A total of 519 cervical specimens were examined for the β-globin housekeeping gene by PCR. All specimens were positive and each gave a 260 bp fragment. They were all tested for HPV DNA by nested PCR. All PCR experiments were repeated twice (or three times when necessary) to ensure reproducibility and correctness. Of the 519 specimens, 164 (31.6%) were found to be positive for HPV DNA and 355 (68.4%) were found to be negative (Table 3). Of 164 HPV DNA-positive specimens, 34 specimens (20.7%) showed cytological abnormalities by the Pap smear test. Out of the 355 specimens that were HPV DNA negative, 30 specimens (8.5%) showed cytological abnormalities by the Pap smear test. The difference was significant using the chi-squared test (p value < 0.00001) (Table 3), as the M-H odds ratio was 2.833. This means that the odds of abnormal Pap smear results among the HPV DNA positives was almost threefold the odds of abnormal Pap smear results among the HPV DNA negative samples. Of the 64 Pap-abnormal specimens, regardless of HPV positivity, 59 were ASCUS (atypical cells of undetermined significance), 3 (1 HPV-positive) were LGSIL (low grade squamous intraepithelial lesion) and 2 (1 HPV-positive) were HGSIL (high grade squamous intraepithelial lesion).

HPV genotyping analysis by reverse line blotting assay on HPV DNA-positive specimens (n = 164) showed different genotypes in either a single-type infection or a multiple-type infection in the same specimen. Some specimens contained a single genotype while others contained up to six genotypes. Table 4 shows that HPV-16 was the most common among samples, comprising 144/164 specimens (87.8%), followed by HPV-18 (141/164, 86%) and HPV-11 (121/164, 78.3%).

Discussion

HPV causes persistent viral infections that are generally benign. Sometimes it proceeds to carcinogenesis if the infection was caused by a high-risk HPV genotype. Most cases of cervical cancer are started by a genital HPV infection that developed over the years to squamous intraepithelial lesions. With the advent of molecular technologies, epidemiological studies revealed the extent of such an infection in various countries and among various clinical and ethnic groups [23]. These studies also showed the extent of involvement of HPV in cervical neoplasia. Recently, there has been more emphasis to study HPV prevalence because cervical cancer has been reported to be the second most prevalent cancer among women worldwide.

In Saudi Arabia, there have been no studies showing the extent of the prevalence of this virus in the community, perhaps because cervical cancer is not frequently seen in the country. Unlike the rest of the world, cervical cancer is considered locally the eleventh most prevalent cancer among women [24]. In addition, a high prevalence of HPV is not expected because HPV is considered to be a sexually transmitted disease and Saudi society is known to be a conservative one when it comes to adult sexuality, which is supposed to exist only after matrimony. However, a previous study described a relatively high prevalence of high-risk HPV infection among women living in Riyadh [14], and recent studies in Saudi Arabia confirmed the prevalence of HPV-16 and other high-risk HPVs in cervical cancer tissues [15,16, 23]. The low prevalence in Saudi Arabia reported in other studies compared to our results is probably due to the use of kits, which depends on the principle of hybridization. We used double amplification (nested PCR) to obtain maximum detection. We followed the universal rules for PCR work as described by Kwok

Table 4. HPV genotypes found in the HPV-DNA positive cases (n = 164) as single or multiple infections in each specimen

HPV genotype*	Number of specimens	%
Low-risk HPVs		
6	3	1.8
11	121	73.8
42		
High-risk HPVs		
16	144	87.8
18	141	86.0
31	4	2.4
33	9	5.5
35	2	1.2
58	3	1.8

*HPV genotypes tested but not found were 40, 44, and 54 (low risk) as well as 26, 39, 45, 51, 52, 53, 56, 59, 66, 68, and 73 (high risk)

and Higuchi [21], with physical separation of pre-PCR, amplification, and post-PCR experiments. The very recent study of Bondagji *et al.* [25] used hybridization methods (Hybrid Capture 2 – HC2) to describe HPV prevalence in the western region of Saudi Arabia, and found high-risk HPVs at a rate of 5.6%. It is well known that hybridization methods have lower detection capability compared to PCR and nested PCR since they will identify the exact amount of DNA available in the sample without amplification. However, when the amplification procedure was used in another recent study, the prevalence rose to 43%, with HPV-16 being the most encountered strain [26].

Our study showed a relatively high prevalence of HPV infection among women residing in Riyadh, Saudi Arabia. For traditional reasons, only married, divorced, and widowed women were enrolled in our study. All of them visited the clinic of a single hospital in Riyadh for routine gynecological examination. We used technology that has been described extensively in the literature and performed the PCR experiments for a minimum of two times on each specimen. Reasons for this prevalence are unknown thus far, but it is though that HPV is acquired from males who usually have more mobility and freedom in terms of travel and general movement. The strong association between HPV infection and abnormal cytology is expected since virtually all studies from all over the world have reported the same finding. In this general screening, we found only a few LGSIL and HGSIL cases. As is the situation in Kuwait, viral load and higher numbers of samples might reveal a situation with a different picture [27]. We are in the process of starting a prospective study to measure E6/E7 mRNA [28] in fresh cervical specimens and then follow those positive cases to explore the progress to cervical carcinoma. We also would suggest that screening and triage of older women should be implemented soon, as planned in Morocco [29] and as performed in the developed world [30].

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