original article

Human papilloma virus-16/18 cervical infection among women attending a family medical clinic in Riyadh

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BACKGROUND: Prevalence information is lacking on human papillomavirus types 16 and 18 (HPV-16/18) infection in cervical tissues of women residing in Riyadh, Saudi Arabia. In addition, there are no observations on progression to cervical intraepithelial neoplasia (CIN).

SUBJECTS AND METHODS: Pap smear and HPV-16/18 DNA detection by PCR followed by Southern blotting was performed on 120 subjects (Saudi and other Arab nationals) during routine gynecological examination. Some HPV-positive subjects were followed for 4 years, by Pap smear every 6 months and by HPV DNA detection at the end of 4 years.

RESULTS: Overall HPV-16/18 prevalence was 31.6%. HPV-16 prevalence alone was 13.3%, HPV-16 as a mixed infection with HPV-18 was 15%, and all HPV-16 was 28.3%. HPV-18 alone was 3.3%, HPV-18 as a mixed infection with HPV-16 was 15%, and all HPV-18 was 18.3%. Ten subjects had cervical abnormalities with the Pap smear test, six of whom were HPV-16/18 positive, 1 with HPV-16, 1 with HPV-18, and 4 with a mixed infection of HPV-16/18. Of all 23 HPV-16/18-positive subjects, either as individual or mixed infection, followed for 4 years, 7 showed abnormal cytology, 6 at initial examination and 1 during follow-up. Of these 7, 6 reverted to normal without treatment and 1 was treated and became normal after 3 years. None of the subjects progressed to CIN-III.

CONCLUSION: A high prevalence of HPV-16/18 was found, but with a low rate of progression to CIN. A significant association with abnormal cytology was found only in patients with HPV-16/18 mixed infection.

High-risk human papillomavirus (HPV) types 16 and 18 are sexually transmitted and have been strongly implicated in the etiology of cervical intraepithelial neoplasia (CIN).[1] Other high-risk HPV types exist. Women positive for HPV DNA in cervical cells have a higher risk of developing cervical cancer,[2] which accounts for about 10% of newly diagnosed cancers in women worldwide.[3] High-risk HPV DNA has been detected in nearly all cases of cervical carcinomas.[4] In women with abnormal cervical cytology, HPV was reported to have a high prevalence.[5] The advent of polymerase chain reaction (PCR) procedures increased the detection level, and therefore, the estimated prevalence of HPVs in normal or abnormal cytological samples of women.[6] It has been suggested that the molecular identification of HPVs (HPV testing in cervical scrapes) may be very useful for primary screening or secondary triage of patients with certain lesions.[7] However, the technology available to the practicing clinician is still evolving. The detection of cancer-associated types of HPV, particularly HPV-16 and HPV-18, in cervical specimens may predict the presence and future development of CIN. It has been suggested that molecular detection of HPV DNA may be an adjunct or a replacement to current cytological procedures.[8] To our knowledge, the prevalence of high-risk HPVs in women in Saudi Arabia has never been reported and follow-up on HPV-infected women for possible progression to CIN was never conducted. In this study we investigated the prevalence of HPV-16 and HPV-18 in the local population, which consists mostly of Saudi and other Arab women, using PCR and specific primers derived from the ORF E6 region, known with its transforming ability in human keratinocytes. Assessment of HPV-positive

women was carried out for up to 4 years after initial examination.

SUBJECTS AND METHODS

Clinical specimens. During routine gynecological examination in the primary care center of the Department of Family Medicine at King Faisal Specialist Hospital and Research Center, cervical scrapes (both ectocervical and endocervical) from 120 women were obtained with informed consent. Samples were taken by cytobrush for Papanicolaou's (Pap) smear examination and the remainder of the sample was used for this study. Seventy-five of the subjects were of Saudi nationality and 45 were of other Arab nationalities residing in Riyadh (19 Egyptians, 7 Sudanese, 5 Syrians, 3 Palestinians, 3 Jordanians, 3 Yemenis, 2 Libanese, 2 Somalis, and 1 Algerian). All subjects were married women. Due to cultural considerations, they were not asked about sexual partnership, frequency of intercourse, or use of barriers, and HIV testing was not performed. HPV-16/18 DNA detection by PCR, followed by Southern blot hybridization (SBH) for confirmation, was performed on all samples. A Pap smear test was done on all samples at the initial examination and on some HPV-positive patients every six months for up to 4 years, after which HPV-16/18 DNA detection was again performed.

Cell lines and HPV clones. The human cervical carcinoma cell lines CaSki (containing at least 500 copies of HPV-16 per cell) and HeLa (containing at least 10 copies of HPV-18 per cell) and the erythroleukemic cell line K562 were obtained from American Type Culture Collection (Rockville, MD, USA) and grown in a laboratory separate from that for PCR. HPV-16 and -18 DNA cloned separately in pBR 322 plasmid (pHPVs) was a generous gift, kindly provided by Dr. E.-M. de Villiers (Heidelberg, Germany).

DNA Extraction. Cells from cervical scrapes and from cell lines grown to log phase were washed twice in phosphate buffered saline, pH 7.2 (PBS), pelleted, and resuspended in 0.5 mL of lysis solution (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% SDS and 250 mg/mL proteinase K). These preparations were incubated at 37°C overnight and then extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v). Sodium acetate was added to a final concentration of 0.3M and the nucleic acids were precipitated with 2.5 volumes of ice-cold 95% ethanol. The precipitates were resuspended in 1 mL of 10 mM Tris-HCl, pH 7.5, and treated with RNase A (5 mg/mL) at 37°C for 1 hour to remove RNA. The DNA was extracted with chloroform/isoamyl alcohol (24:1, v/v) followed by ethanol precipitation. The resultant DNA was dissolved in sterile distilled water to a final concentration of 1 mg/mL for PCR analysis.

PCR amplification and Southern blot hybridization. Separate reactions were carried out to amplify HPV-16 and HPV-18 target sequences using 1 mg of genomic DNA isolated from cervical cells as template. Cloned pHPVs DNA (2 pg) and cellular DNA from CaSki (100 ng) and HeLa (250 ng) cells were used as HPV-positive controls. Cellular DNA from K562 DNA (1 mg) was used as an HPV-negative control and a PCR reaction mixture with no DNA template serving as a reagent control. As described previously,[9] primers from the E6 ORF and internal probes were custom-synthesized by our core facility and used for this study, based on the methods used by McNicol and Dodd.[10] The reaction mixture contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01 % (w/v) gelatin, 200 mM of each dNTP (dATP, dGTP, dCTP and dTTP), 50 pmol of each primer pair, and 2.5 U of native Taq DNA polymerase in a final volume of 50 mL. All reagents were from Perkin-Elmer. The mixture was overlaid with 50 mL of light mineral oil to prevent evaporation and then incubated for 10 minutes at 94°C for DNA denaturation. Thirty-five cycles of PCR amplification were performed. Each cycle consisted of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 2 minutes. In the last cycle, the extension step was increased to 5 minutes to allow complete formation of duplex DNA. Only positive displacement pipettes were used. Preparation of DNA samples was performed in a room separate from that of PCR analysis. Amplified products (10 mL) were analyzed on 2% agarose gel electrophoresis in TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA pH 8.3), stained with ethidium bromide (1 mg/mL) and photographed under UV light. Southern blot hybridization was carried out with 10 pmol of internal oligonucleotide probes, labeled at the 5'-end with g-32P[ATP]. Hybridization and high stringency washing were carried out at 42°C. Membranes were air-dried and exposed to X-ray films at -70°C.

Statistical methods. We used the chi-square test, Fisher's exact test, or McNemar test for statistical analysis. Exact *P* values are reported. A *P* value less than 0.05 was considered significant.

RESULTS

Of the 120 samples used in the study, 38 (31.6%) were found positive by PCR for HPV-16 or HPV-18. Of the 38 positive specimens, 16 (42.1%) were positive for HPV-16 only, 4 (10.5%) were positive for HPV-18 only, and 18 (47.4%) were positive for a mixed infec-

tion of both HPV-16 and HPV-18 (Table 1). Thus among all subjects of this study, the prevalence of HPV-16 alone was 13.3% (16/120), of HPV-18 alone was 3.3% (4/120), and of both viruses as a mixed infection was 15% (18/120). When found alone or as a mixed infection, the prevalence would, therefore, be 28.3% (34/120) for HPV-16 and 18.3% (22/120) for HPV-18. Table 1 also indicates that females infected with one type of the two viruses under study are more likely to have the other virus (P=0.012). Information that was culturally possible to obtain through the subjects did not reveal any significant association between most characteristics and the HPV prevalence, except that relatively fewer educated subjects showed a relatively higher rate of infection in both the Saudi and other Arab groups (Table 2). Table 3 indicates that there was a significant association with abnormal cytology only in patients with HPV-16/18 mixed infection (P=0.042). It shows that of all subjects, 10 (8.3%) showed minor cytological changes (reactive and reparative changes, inflammatory cellular changes, and low-grade squamous epithelial lesions [LGSL]) in the Pap smear test. Of these 10, only 6 were HPV-infected (1 HPV-16, 1 HPV-18, and 4 HPV-16/18), making the prevalence of HPV 60% (6/10) in those with cytological changes and 5% (6/120) in all subjects. Follow up by Pap smear testing was carried out every 6 months on some of the HPV-positive subjects, who remained positive for HPV DNA after the follw-up period. Seven of the 16 HPV-16-positive (HPV-18-negative) women were followed. One of them showed abnormal cytological changes at the initial exam, but reverted to normal after 2 years. Among the other six who were HPV-16 positive but with normal cytology, a 4 year follow up showed that 5 remained normal and 1 developed LGSL, revealing HPV changes and chronic cervicitis upon colposcopy and cervical biopsy. This patient reverted to normal cytology after 3 years. All 4 subjects who were HPV-18-positive (HPV-16-negative) were followed. One had LGSL at initial examination, developed CIN-II after one year, was treated by laser and reverted back to normal after 3 years. Three were normal for cytological changes at the initial examination and remained normal after a 4-year follow-up period. Twelve of the 18 subjects with HPV-16/18 mixed infection were followed. Four showed minor cytological changes at the initial exam, but reverted to normal after 1-4 years of follow up. Eight were normal for cytological changes at the initial examination and remained normal after a 4-year follow-up period.

Table 1. Prevalence of HPV-16 and HPV-18 in 120 cervical specimens.

		HPV-18		Total
		Positive	Negative	
HPV-16	Positive	18	16	34
	Negative	4	82	86
Total		22	98	120

Positives for both HPV types, 38/120=31.6%. McNemar test: P=0.012

Table 2. Characteristics of 120 subjects.

Characteristic	HPV positive (n=38)		HPV negative (n=82)	
	Saudis	Others	Saudis	Others
Mean age in years (SD)	37 (2.6)	36.3 (1.5)	33.7 (1.4)	36 (1.6)
Mean education level in years (SD)	8.5 (1.4)	11.7 (0.9)	10.5 (0.7)	13.8 (0.4)
Contraceptive use	10/19	10/19	24/56	14/26
Pregnancy	0/19	2/19	1/56	2/26
Menopausal	4/19	0/19	9/56	2/26
Postpartum	2/19	4/19	10/56	3/26

Table 3. Occurrence of HPV DNA by PCR in relation to cytological diagnosis at the initial examination.

	n	Number of women with positive cytology		
		HPV-16[a]	HPV-18[b]	Both[c]
Normal	110	15	3	14
Abnormal	10	1	1	4

a Infection with HPV-16 only. Fisher's exact test: P=0.604
b Infection with HPV-18 only. Fisher's exact test: P=0.297
c Infection with both HPV-16 and HPV-18. Fisher's exact test: P=0.042
d Cytological changes (CIN-I or less) seen in Pap smear tests

DISCUSSION

HPV-16/18 are mainly sexually transmitted and their malignant transformation activity is well-established. Prevalence varies geographically and cannot be accurately evaluated since the disease is subclinical and not reportable, the extent of detection of HPV DNA varies with methods, and the infection sometimes regresses. In this study, the sample represented the population of the capital city in Saudi Arabia, and the results were used to determine the prevalence among inhabitants, which include Saudis citizens and other Arab women who are either working in the country or accompanying their working spouses. HPV-16/18 were selected for

this study because of their predominance and potentially greater oncogenic nature than other high-risk HPVs. The prevalence of HPV in the general population varies from one geographical area to another, is dependent on the type of sample used, and is also dependent on the technique and primers and probes used. Since our interest was to examine HPV-16/18, we selected primers and probes from the area of the virus genome (E6 region) that is implicated in the oncogenic process and known to mediate the degeneration of p53 tumor suppressor protein.[11] Others have used different consensus primers, producing different results. Our finding of 31.6% prevalence of HPV-16/18 in our presumably normal population is rather high compared to recent studies from Columbia,[12] India,[13] Mexico,[14] Lebanon,[15] and Sweden.[16] A past study from England showed a prevalence of 84% of HPV-16 in normal cervical cytology.[17] More recent studies from Greece,[18] the Czech Republic,[19] and the United States[20] revealed that high-risk HPV was found in 41.8%, 55%, and 42.4%, respectively, of women with normal cytology. HPV infection in Riyadh in the examined population is high compared with some countries even though extramarital sexual activities and multiple sexual partnership are religiously and traditionally not acceptable for both men and women in Arab and Muslim countries, although the latter may be the reason for the relatively lower prevalence of HPV compared with other countries]. Another possible explanation for our relatively high prevalence is that our samples were both ectocervical and endocervical. Most other studies used vaginal lavages or one of the two cervical sites. As is the case with most countries,[21] HPV-16 is more prevalent in our population, and the majority of HPV-18 positive cases are actually a mixed infection with HPV-16. Since our study was limited to those two HPV types, other HPV types may have been present and the possible existence of PCR inhibitors in cervical specimens[22] may have contributed to the lower prevalence than in some other countries. Although cervical cancer is considered the second most common neoplasia in women worldwide, records in the Saudi National Cancer Registry rank cervical cancer ninth among female cancers in Saudi Arabia.[23] It is, therefore, not unusual to notice the low rate of progression to CIN in spite of the high prevalence of HPV-16/18. Of all 23 subjects who were HPV-16/18-positive and were followed, 7 showed abnormal cytology, 5 of whom reverted to normal, 1 was treated and reverted to normal and 1 is still under observation but with no progression to CIN-II. This is in agreement with studies[24] that showed approximately 60% of subjects with cervical cytological changes (CIN- I) regress back to normal. Further studies will be conducted to determine the occurrence of HPVs in various types and grades of cervical cancers and among various age groups. The worldwide reports on the importance of HPVs as sexually transmitted diseases and its cause-effect relationship with cervical cancer will necessitate the need for HPV mandatory screeing, particularly for HPV-16 and HPV-18, for which a vaccine is available and already approved by the United States Food and Drug Administration.[25,26]

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