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ORIGINAL ARTICLE

Prevalence of high risk human papillomavirus types 16/18 in cytologically abnormal cervical smears in Alexandria, Egypt. A cytological and molecular study

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KEYWORDS

Human papillomavirus;
Polymerase chain reaction;
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Cervical cancer;
Cervical smear

Abstract *Introduction:* In Egypt, cervical cancer ranks as the second most frequent cancer after breast cancer, among women between 15 and 44 years of age. High-risk human papillomavirus (HPV) 16 and 18 detection holds the potential to be used as a tool to detect women, at risk for consequent development of cervical cancer because of their predominance and potentially greater oncogenic nature than other high risk HPV subtypes.

Objective: To determine the prevalence of high-risk HPV 16/18 DNA in women with abnormal cervical cytology.

Subjects and methods: 45 cases were collected from Egyptian women seeking routine gynecologic care. Ten cytologically normal cervical smear cell samples were included in the study as a control to be tested for the presence of HPV 16/18 DNA and were collected from asymptomatic patients having cystorectocele or coming for loop insertion or removal. The 45 specimens were subjected to real-time polymerase chain reaction, using multiplex HPV 16 and 18 PCR kit.

Results: 45 cervical smears were collected in the present study. Cytopathological examination revealed that 5 (11.1%) were ASCUS, 8 (17.8) were LSIL, 5 (11.1%) were HSIL, 1 (2.2%) was squamous cell carcinoma (SCC), 1 (2.2%) was adenocarcinoma and 25 (55.6%) were benign (inflammatory). 20 patients with abnormal cervical cytology and 10 controls were included in the present study. In patients with abnormal cervical cytology, 5 (25%) were ASCUS, 8 (40%) were LSIL, 5

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(25%) were HSIL, and 1 (5%) was SCC and 1 (5%) was adenocarcinoma. Statistical analysis revealed a significant difference between patient and control groups as regards regularity of menstruation where irregular menstruation and higher prevalence of menopausal women, abnormal vaginal bleeding, menorrhagia, vaginal infection, and abnormal cervical appearance were encountered in patients. A statistically significant higher prevalence of married women was found in the control group. There was no significant difference in the distribution of patients and control as regards HPV 16 or HPV 18 in which 20% of patients were HPV 16 positive and 10% of patients were HPV 18 positive compared with none in the control group. 6 were positive either for HPV 16 or 18, while 39 were negative. The HPV 16/18 positive patients had significantly higher age and marital duration when compared with HPV 16/18 negative group. Significantly, most of the HPV 16/18 positive patients were menopause. A significantly higher prevalence of women with cervicitis, contraceptive users and married women was in the HPV 16/18 negative group.

Conclusion: The study generates epidemiological data of prevalence of HPV 16/18 in cytologically abnormal cervical smears in women seeking routine gynecologic care at the outpatient clinics of the Obstetrics and Gynecology Department at El Shatby University. High-risk HPV DNA testing by PCR of cervical samples diagnosed according to the Bethesda 2001 guidelines may benefit the management of patients with abnormal cervical smears, especially among women aged 46 years and older, in menopausal women and in women complaining of PMB. Therefore, HPV DNA testing should be made use of as an adjunct to cervical smears.

1. Introduction

Globally, human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease in both men and women (1). Distinct members of the large family of HPVs are epitheliotropic infecting either cutaneous skin or mucosal epithelia. While most of the infections are benign and transient (2), persistent infection is correlated with the development of cervical and other anogenital cancers (3). In Egypt, cervical cancer ranks as the second most frequent cancer (4) after breast cancer (5), among women between 15 and 44 years of age (4) Asymptomatic genital HPV infection appears to be common and mostly self-limited (6–8)). Exposure can result in no HPV infection, produce a latent HPV infection, or produce an HPV infection correlated with a clinically observable lesion (9). HPV infection begins with entry of the virus into the basal cells of the epithelium (10). Assembly of the viral particles occurs in the nucleus and consequently complete virions are shed from keratinocytes (11,12). It clinically manifests as hyperplastic, hyperkeratotic warts or dysplastic lesions that may go through neoplastic transformation (13).

HPVs are classified into genotypes according to their cervical carcinoma associated risk as high and low (14). The high-risk types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, while the low-risk types are 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP 6108 (15,16). Universally, it is now accepted that nearly all invasive cervical cancers and high grade intraepithelial neoplasias are correlated with the high risk types of HPV (17,18). HPV types 16 and 18 have been established to be the underlying causative agents of more than 50% of cervical pre-cancerous lesions, and more than 70% of cervical cancer cases worldwide (19–21). HPV-16 is more frequently found in squamous cell carcinoma, while HPV-18 is more common in adenocarcinoma (22,23). These two HPV types are classified as human carcinogens according to the International Agency for Research on Cancer (IARC) (24).

The risk factors for HPV viral persistence and development of cervical neoplasia are classified as sexual factors, viral factors and nonviral factors. Sexual factors include the presence of multiple sexual partners (25), at early age sexual intercourse (26) non-use of condoms by partners. Moreover as to sexual activity, age is an important risk factor for HPV infection (27,28). Viral risk factors for HPV that have been studied to date include viral type and variant, viral load, the effect of multiple concurrent HPV infections, and detection of HPV E6 and E7 transcripts (29–40). Nonviral factors include impairment of cell-mediated immunity (41,42), long-term hormonal contraceptive use (43,44), smoking, multiparity (28), coinfection with *herpes simplex virus type 2* (45) or *Chlamydia trachomatis* (46) and nutritional factors (47).

A single cervical cytology test as a cancer screening tool is correlated with a considerable false-negative rate (48), thus more sensitive HPV testing is required, to detect high-grade intraepithelial neoplasia (CIN 2/3) (49,50). At the present time, the National Comprehensive Cancer Network (NCCN) – cervical cancer guidelines propose that HPV DNA testing should complement cervical cancer screening methods, such as regular cervical smears and gynecologic examinations (51).

HPV 16 and 18 genotypes are considered indicators of high risk for cervical cancer, therefore only these two genotypes were tested in our study (52,53).

2. Subjects and methods

45 cases were collected from Egyptian women seeking routine gynecologic care at the outpatient clinics of the Obstetrics and Gynecology Department at El Shatby University Maternity Hospital during the period from July 2010 to May 2011. Two cervical specimens were collected from the endo- and exo-cervices of each woman. The first cervical smear cell sample was collected with a cytobrush for cytological examination. Another cervical sample was taken with a swab for molecular analysis by HPV 16/18 real-time PCR. The inclusion criteria for patient selection included high-risk patients as multipara, women with chronic vaginal infection, post-coital bleeding, postmenopausal bleeding and old age. Exclusion criteria

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included women with a history of hysterectomy or conization and pregnant women (17). Ten cytologically normal cervical smear cell samples were included in the study as a control to be tested for the presence of HPV 16/18 DNA and were collected from asymptomatic patients having cystorectocele or coming for loop insertion or removal.

2.1. Sample collection

All cervical smear cell samples obtained were collected from endo- and exo-cervices. Two cervical specimens were obtained from each woman. First cervical scrape smears were obtained by cytobrush for cytological examination (54). Other specimens assigned to molecular studies (HPV 16/18 DNA testing) were collected with a swab (55).

2.2. Sample preparation for cytological examination

The first cervical smear cell sample was preserved in 95% ethyl alcohol then stained by hematoxylin & eosin (H&E) stain for microscopical examination, diagnosis and pathological grading according to Bethesda system 2001 (56).

2.3. HPV detection

Nucleic acids were extracted with the HPV 16 & 18 Real-Time PCR Kit (Liferiver, Shanghai). Assays with PCR to detect HPV 16 and 18 E7 genes were performed with commercially available kit to detect HPV 16 and 18. The Master Mix volume for each reaction was pipetted by taking 35 μl of Reaction Mix (HPV serotype 16 and 18 Reaction Mix) then adding 0.4 μl of Enzyme Mix (DNA polymerase) and then 1 μl of Internal Control ending up with a total of 36.4 μl of Master Mix. The mixture for the PCR reaction included approximately 4 μl of extracted DNA and 36 μl of Master Mix. Thermocycler conditions were initial 1 cycle at 37 °C for 2 min, then 1 cycle denaturation at 94 °C for 2 min, followed by 40 cycles at 93 °C for 15 s and 60 °C for 60 s. The fluorescence was measured at 60 °C; FAM and HEX/VIC/JOE channels.

2.4. Preparation and procedure for transmission electron microscopy (TEM)

The cervical cells were collected from the endo- and exo-cervices with a cytobrush (54). To prevent disruption as a result of the loss of water, the tissue is preserved with a fixative. The obtained specimen was fixed immediately in 4F1G (Formaldehyde, Glutaraldehyde) for 1 day then the specimen was centrifuged to form a pellet. After primary fixation, the tissue was rinsed 3 times each for 15 min with sodium phosphate buffer to eliminate any free unreacted glutaraldehyde. Post fixation in buffered osmium tetroxide (OsO_4) the specimen was then kept in refrigerator for 1.5 h to add contrast and develop membrane sharpness and to stabilize the fine structure to withstand embedding in plastic. After secondary fixation, the specimen was again rinsed 3 times each for 15 min with sodium phosphate buffer to prevent the reaction of osmium tetroxide (OsO_4) with acetone in the dehydration step (57).

Then the specimen was dehydrated by using acetone/water mixture of progressively increasing concentrations starting with 30% till absolute acetone (30%, 50%, 70%, 80%, 90%, 100%) to ensure the complete replacement of buffer and any excess water (58).

Infiltration of dehydrated specimen should be started in 1:1 solution of acetone and embedding the mixture (epoxy resins) overnight. Progressively, the epoxy-solvent ratio is increased until pure epoxy is used (58).

14 drops of hardener were added to 8 ml of warmed embedding mixture shaken for 3 min and then added to the specimen. Embedding is done in a freshly prepared plastic embedding mixture in pre-dried capsules for 1–3 h. Consequently, the tissue, which was primarily hydrated, is solid and stable after embedding (58).

The embedded tissue blocks were polymerized at 60 °C for 2 days then were cured at room temperature for at least one day before attempting to section (59).

For electron microscopy, ultra thin sections about 50 nm were cut by means of LKB ultra microtome and sharp new glass knives. The ultra thin sections were picked on grids and were kept in a petri dish (59). The grids were double stained with uranyl acetate and lead citrate and then examined by the Jeol CX 100 transmission electron microscope (60).

2.5. Statistical analysis

Results were analyzed with the aid of SPSS Software (Statistical Package for the Social Sciences, version 17). Student's *t* test was used for comparison for the normally distributed variables while the Mann–Whitney test was used for comparison of the abnormally distributed variables. Comparison of distribution for the categorical variable was performed using the Chi square test or Yates correction or Fisher Exact test. A p value < 0.05 was considered significant.

3. Results

45 cervical smears were collected in the present study from July 2010 till May 2011 with a mean ± SD of 4.09 ± 2.17 (Table 1).

The cytopathological examination of these smears by H&E according to the Bethesda system 2001 revealed that 5 (11.1%) were ASCUS, 8 (17.8) were LSIL, 5 (11.1%) were HSIL, 1

Table 1 Cervical smears taken during the period from July 2010 to May 2011.

Year	Month	Number of cervical smears
2010	July	2
	August	2
	September	1
	October	7
	November	7
	December	6
2011	January	3
	February	3
	March	3
	April	5
	May	6

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(2.2%) was squamous cell carcinoma (SCC), 1 (2.2%) was adenocarcinoma and 25 (55.6%) were benign (inflammatory).

All the cervical smears for the 10 controls were collected from women having cystorectocele or coming for loop insertion or removal.

In the patient group, the age ranged from 30 to 80 years with a mean of 46.0 ± 11.70 years, while in the control group it ranged from 30 to 52 years with a mean of 39.80 ± 7.59 with no significant difference found between both groups ($p = 0.200$) (Fig. 1).

As regards gravidity, parity and abortion, average in patients were higher than in the control group with no significant difference found between both groups where their median (IQR) in patients was 4 (3), 3.5 (2) and 0.5 (2), respectively while in the control group, their median (IQR) was 3.50 (2), 3 (0) and 0 (1), respectively.

As regards marital age, average in patients were higher than in the control group with no significant difference found between both groups whereby in patients it ranged from 16 to 35 years with a mean ± SD of 22.55 ± 4.807 years while in the control group, it ranged from 18 to 33 years with a mean ± SD of 22.90 ± 5.087.

Table 2 shows the distribution of regularity of menstruation, abnormal vaginal bleeding with its subtypes, postmenopausal bleeding, post-coital bleeding and vaginal infection in the patient group as well as in the control group. A significant difference was found between the two groups as regards regularity of menstruation, where menopausal women were more prevalent in patients (40%) compared to controls (20%) while as to abnormal vaginal bleeding, menorrhagia and vaginal infection, these variables were significantly only present in patients (60, 40 and 100%, respectively).

Table 3 shows the distribution of cervical appearance with its types and dyspareunia in the patient group as well as in the control group. Abnormal cervix was significantly only encountered in the patient group (70%).

As to usage of contraception in any of its forms in the patient group as well as in the control group, no significant difference was found between the two groups (Table 4).

As regards smoking no women in either patient or control group were smokers. Infertility was not encountered in either patient or control group.

As to distribution of marital status and pelvic pain in the patient group as well as in the control group, a significant

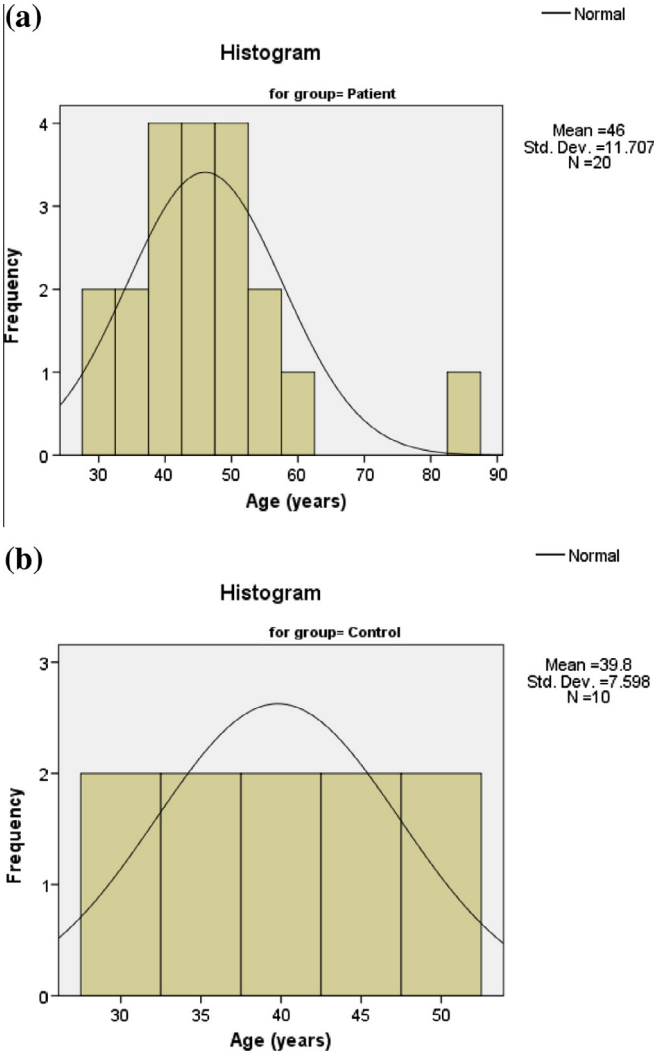

Figure 1 Distribution of patients with abnormal cervical cytology 10 (a) and control group 10 (b) according to age.

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Table 2 Distribution of patients with abnormal cervical cytology and control group according to regularity of menstruation, abnormal vaginal bleeding with its types, postmenopausal bleeding, post-coital bleeding and vaginal infection.

	Patient group (n = 20)		Control group (n = 10)		Test of significance (p value)
	n	%	n	%	
Regularity of menstruation					
Regular	0	0	8	80	X^2_Y = 10.821, p = 0.001*
Irregular	12	60	0	0	
Menopause	8	40	2	20	
Abnormal vaginal bleeding	12	60	0	0	X^2_Y = 13.460, p = 0.000*
Menorrhagia	8	40	0	0	FE, p = 0.005*
Polymenorrhea	2	10	0	0	FE, p = 0.540
Intermenstrual bleeding	2	10	0	0	FE, p = 0.540
Hypomenorrhea	1	5	0	0	FE, p = 1.000
Postmenopausal bleeding	6	30	0	0	FE, p = 0.074
Post-coital bleeding	3	15	0	0	FE, p = 0.532
Vaginal infection	20	100	0	0	FE, p = 0.000*

X^2_Y: Yates corrected Chi square; FE: Fisher Exact; P: probability.
* Significant if <0.05.

Table 3 Distribution of patients with abnormal cervical cytology and control group according to clinically abnormal cervix with its types and dyspareunia.

	Patient group (n = 20)		Control group (n = 10)		Test of significance (p value)
	n	%	n	%	
Abnormal cervix	14	70	0	0	FE, p = 0.000*
Cervical polyp	2	10	0	0	FE, p = 0.540
Cervical ulcer	2	10	0	0	FE, p = 0.540
Cervicitis	7	35	0	0	FE, p = 0.064
Cervical mass	4	20	0	0	FE, p = 0.272
Dyspareunia	5	25	0	0	FE, p = 0.140

FE: Fisher Exact; p: probability.
* Significant if <0.05.

Table 4 Distribution of patients with abnormal cervical cytology and control group according to usage of contraception and its types.

	Patient group (n = 20)		Control group (n = 10)		Test of significance (p value)
	n	%	n	%	
Usage of contraception	14	70	8	80	FE, p = 0.682
Oral contraceptive pills	6	30	3	30	NA
Injectable	5	25	2	20	FE, p = 1.000
IUD	10	50	7	70	FE, p = 0.440

NA: not applicable statistics; FE: Fisher Exact; p: probability (*significant if <0.05).

Table 5 Distribution of patients with abnormal cervical cytology and control group according to types of marital status and pelvic pain.

	Patient group (n = 20)		Control group (n = 10)		Test of significance (p value)
	n	%	n	%	
Marital status					
Married	13	65	10	100	X^2_Y = 6.699, p = 0.035*
Divorced	4	20	0	0	
Widow	3	15	0	0	
Pelvic pain	5	25	6	60	FE, p = 0.108

X^2_Y: Yates corrected Chi square; FE: Fisher Exact; p: probability
* Significant if < 0.05.

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difference was obtained between the two groups as regards marital status where all women in the control group were married compared to 65% in the patient group (Table 5).

Regarding positivity of HPV 16 or 18 PCR, there was no significant difference in the distribution of patients and control as regards PCR 16 or PCR 18 in which 20% of patients were PCR 16 positive and 10% of patients were PCR 18 positive compared with none in the control group.

6 were positive either for PCR16 or PCR 18, while 39 were negative. The positive PCR patients had significantly higher age when compared with the negative PCR group. Fig. 2 shows that in the positive patient group, the age ranged from 46 to 83 years with median (IQR) of 51.50 (13) years, while in the negative group it ranged from 20 to 60 years with median (IQR) of 39.00 (13) years ($p = 0.000$).

As regards gravidity, parity and abortion, average in positive PCR patients were higher than in the negative PCR group with no significant difference found between both groups where their median (IQR) in positive patients was 5 (4), 3.5 (3) and 1.5 (2), respectively while in the negative group, their median (IQR) was 3 (3), 3 (2) and 0 (1), respectively.

Regarding marital age, average in positive patients was higher than in the negative group with no significant difference attained between both groups. In the HPV 16/18 positive patient group, marital age ranged from 18 to 35 years with a mean ± SD of 24.50 ± 6.595 years, while in the HPV 16/18 negative patient group it ranged from 13 to 30 years with a mean ± SD of 21.62 ± 4.766 years.

As to distribution of HPV 16/18 positive and negative patients according to regularity of menstruation, abnormal vaginal bleeding with its types, postmenopausal bleeding, post-coital bleeding and vaginal infection, a significant difference was found between the two groups as regards regularity of menstruation, where most of the positive patients (83.3%) were menopause (Table 6).

As regards distribution of cervical appearance with its types and dyspareunia in the positive patient group as well as in the negative group, cervicitis was significantly higher in the negative HPV 16/18 group (76.9%) (Table 7).

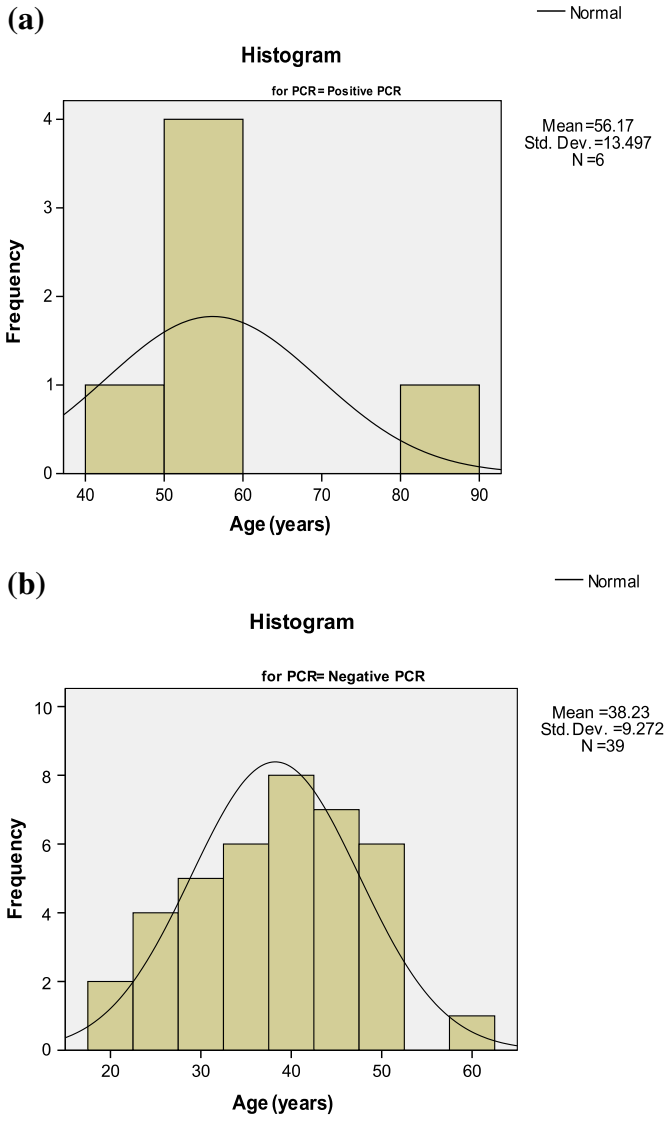

Figure 2 Distribution of HPV16/18 positive 15 (a) and negative 15 (b) patients according to age.

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Table 6 Distribution of HPV 16/18 positive and negative patients according to regularity of menstruation, abnormal vaginal bleeding with its types, postmenopausal bleeding, post-coital bleeding and vaginal infection.

	Positive patient group ($n = 6$)		Negative patient group ($n = 39$)		Test of significance (p value)
	n	%	n	%	
Regularity of menstruation					
Regular	0	0	12	30.80	$X^2_Y = 13.188$, $p = 0.000^*$
Irregular	1	16.7	24	61.50	
Menopause	5	83.3	3	7.70	
Abnormal vaginal bleeding	1	16.7	24	61.5	FE, $p = 0.07$
Menorrhagia	1	16.7	15	38.50	FE, $p = 0.399$
Polymenorrhea	0	0	5	12.80	FE, $p = 1.000$
Intermenstrual bleeding	0	0	8	20.5	FE, $p = 0.572$
Hypomenorrhea	0	0	1	2.6	FE, $p = 1.000$
Postmenopausal bleeding	3	50	3	7.7	FE, $p = 0.024^*$
Post-coital bleeding	1	16.7	4	10.3	FE, $p = 0.529$
Vaginal infection	6	100	39	100	NA

Table 7 Distribution of HPV 16/18 positive and negative patients according to clinically abnormal cervix with its types and dyspareunia.

	Positive patient group ($n = 6$)		Negative patient group ($n = 39$)		Test of significance (p value)
	n	%	n	%	
Abnormal cervix	5	83.3	34	87.2	FE, $p = 1.000$
Cervical polyp	1	16.7	3	7.7	FE, $p = 0.448$
Cervical ulcer	0	0	4	10.3	FE, $p = 1.000$
Cervicitis	2	33.3	30	76.9	FE, $p = 0.049$*
Cervical mass	2	33.3	5	12.8	FE, $p = 0.230$
Dyspareunia	0	0	13	33.3	FE, $p = 0.160$

Table 8 Distribution of HPV 16/18 positive and negative patients according to usage of contraception and its types.

	Positive patient group ($n = 6$)		Negative patient group ($n = 39$)		Test of significance (p value)
	n	%	n	%	
Usage of contraception	2	33.3	30	76.9	FE, $p = 0.049^*$
Oral contraceptive pills	0	0	12	30.8	FE, $p = 0.171$
Injectable	0	0	7	17.9	FE, $p = 0.569$
IUD	2	33.3	23	59.0	FE, $p = 0.383$
Non contraceptive users	4	66.7	9	23.1	FE, $p = 0.049^*$

As to usage of contraception in any of its forms, a significant difference was attained between the positive patient group (33.3%) and the negative group (76.9%) as shown in Table 8.

As regards smoking no women in either positive or negative group were smokers. Infertility was not found in the HPV 16/18 positive patient (0%) compared with 4 (10.3%) in the HPV 16/18 negative patient group.

Regarding marital status and pelvic pain in the positive patient group as well as in the negative group, married women were significantly more prevalent in the negative group (89.8%) than in the positive patient group (33.3%) as shown in Table 9.

As regards cytopathological diagnosis, in the HPV 16/18 positive patient group, 0 (0%) was ASCUS, 2 (33.3%) were LSIL, 2 (33.3%) were HSIL, 1(16.7%) was squamous cell carcinoma, 1(16.7%) was adenocarcinoma and 0% was benign, while in the HPV 16/18 negative patient group 5 (12.8%) were ASCUS, 6 (15.4%) were LSIL, 3 (7.7%) were HSIL, 0 (0%) was squamous cell carcinoma, 0 (0%) was adenocarcinoma and 25 (64.1%) were benign with no significant difference found in the distribution of positive and negative groups.

The cytopathological examination of the abnormal cervical smears showed ASCUS demonstrated in Fig. 3, LSIL demonstrated in Fig. 4, HSIL demonstrated in Fig. 5 and adenocarcinoma of the cervix uteri demonstrated in Fig. 6.

The Transmission Electron Microscopy (TEM) of a LSIL of the cervix uteri showed koilocytotic cell nucleus with numerous intranuclear viral particles (×15,000) demonstrated in Fig. 7 and higher magnification (×30,000) demonstrated in Fig. 8.

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Table 9 Distribution of HPV 16/18 positive and negative patients according to types of marital status, infertility and pelvic pain.

	Positive patient group (n = 6)		Negative patient group (n = 39)		Test of significance (p value)
	n	%	n	%	
Marital status					
Married	2	33.3	35	89.8	$X^2_Y = 9.751$, $p = 0.002^*$
Divorced	2	33.3	2	5.1	
Widow	2	33.3	2	5.1	
Infertility	1	16.7	4	10.3	FE, $p = 1.000$
Pelvic pain	1	16.7	16	41.0	FE, $p = 0.385$

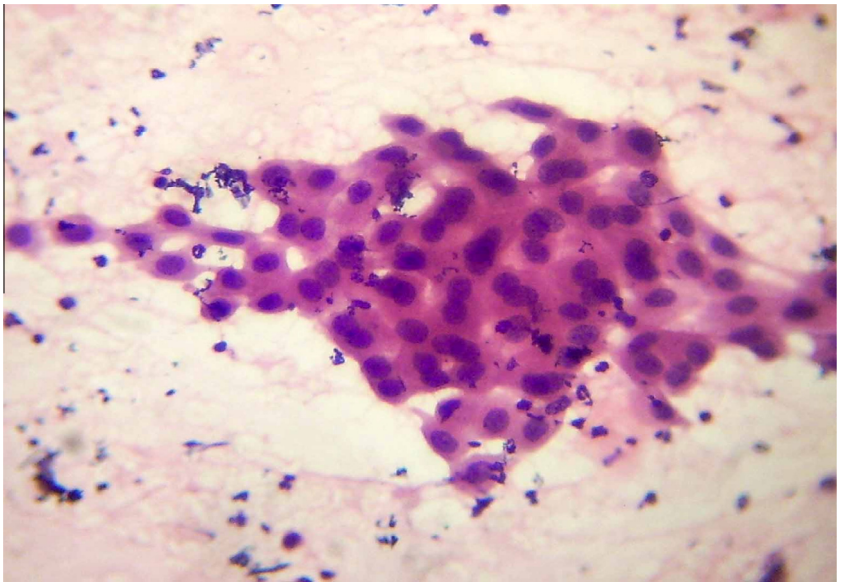

Figure 3 ASCUS of the cervix uteri. Nucleus is 2.5–3× size of intermediate cell nucleus (H&E, ×400).

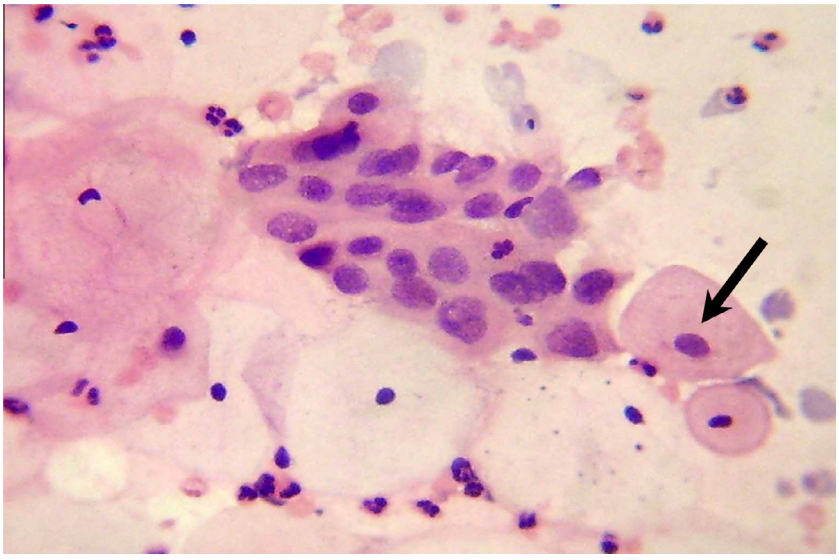

Figure 4 LSIL of the cervix uteri. Hyperchromasia (nuclei darker than those of intermediate cell-seen on right side [arrow]), enlarged cells, slight increase in N/C ratio, no prominent nucleoli. (H&E; ×400).

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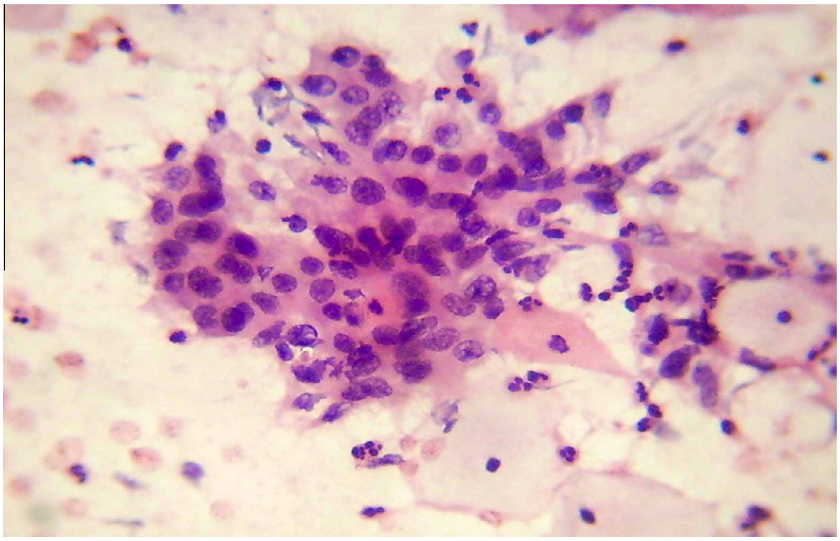

Figure 5 HSIL of the cervix uteri. Cell size is same as squamous metaplastic or parabasal cells; polygonal shape, dense cytoplasm, N/C ratio is 1/3–1/2, Enlarged and hyperchromatic nucleus, irregular (crinkled paper) (H&E; ×400).

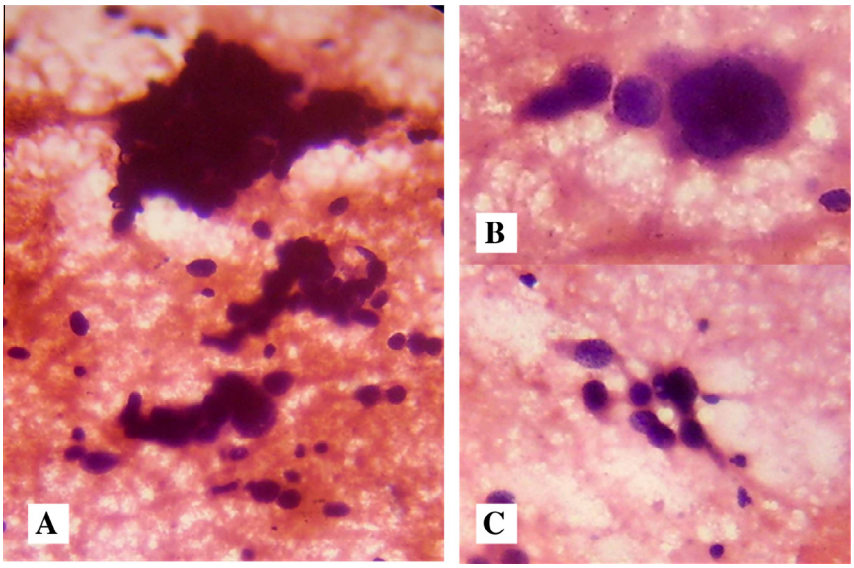

Figure 6 Cervical adenocarcinoma. Cells are pleomorphic, large or small with fluffy cytoplasm, loss of nuclear polarity, true nuclear crowding and clumped chromatin (H&E; A: ×200, B and C: ×400).

4. Discussion

Persistence of oncogenic HPV seems to be essential for the occurrence of cervical neoplasia (17). The malignant transformation activity of HPV-16/18 is well-established. HPV-16/18 DNA testing was chosen for this study because of their predominance and potentially greater oncogenicity than other high-risk HPVs (61). With the arrival of molecular techniques, especially PCR, it is possible to detect these commonly occurring HPV types in cervical scrape smears. The cytologic characteristics of HPV on cervical smear appear to be non-specific (17).

Conventional cervical smear has a limited value in detecting women, anticipated to develop cervical neoplasia. The ALTS study (Atypical squamous cells of undetermined significance – low grade squamous intraepithelial lesion triage study), reached to a conclusion that women with less than cervical intraepithelial neoplasia 2 (CIN 2) status at initial colposcopy stay at risk for subsequent CIN 2+ and that follow-up HPV testing is significantly more sensitive than cytology ($p = 0.015$) for detecting missed prevalent cases. Therefore, HPV testing should be made use of as an adjunct to cervical smears (17).

As persistent infection with high-risk HPV types has been confirmed to be the main contributing factor in the development of cervical cancer, their identification in a cervical smear is essential in estimating a woman's risk of developing cervical cancer, for describing the population in HPV vaccination trials and for monitoring the efficiency of HPV vaccines (62).

The majority of studies done up to now have looked at DNA levels, whereas the study done by Gnanamony et al. in India,

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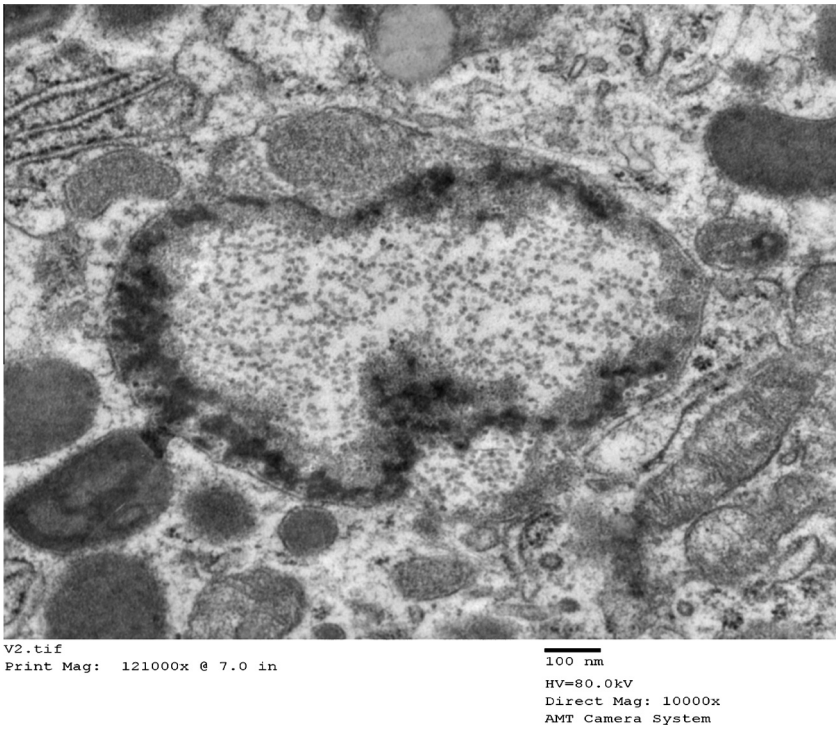

Figure 7 LSIL, Transmission Electron Microscopy (TEM), koilocytotic cell nucleus with numerous intranuclear viral particles (×15,000).

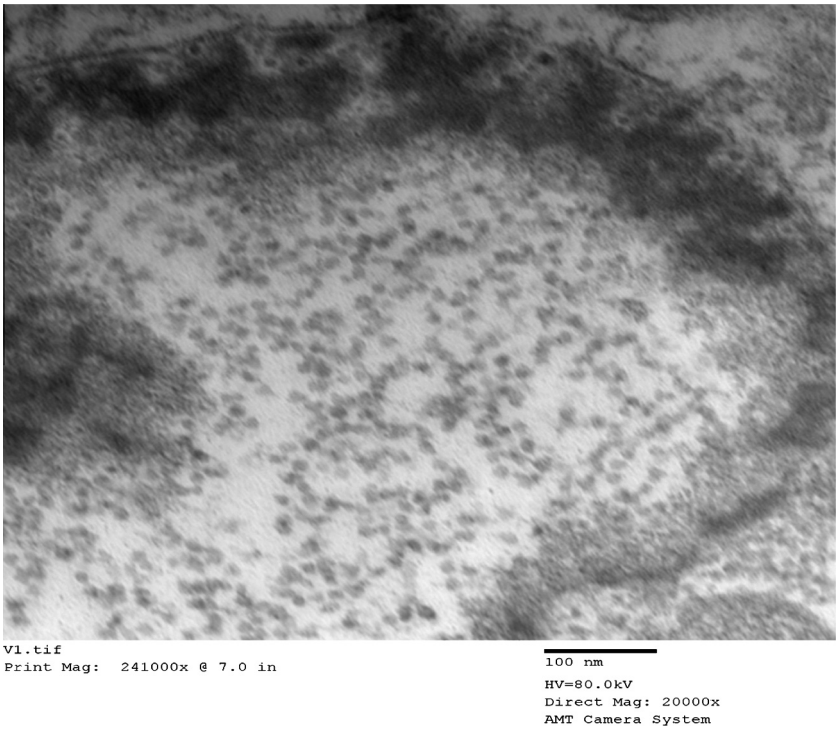

Figure 8 LSIL, TEM, koilocytotic cell nucleus with intranuclear viral particles, higher magnification (×30,000).

showed that active replication, as seen by an increasing mRNA transcript level and not DNA levels, can be a marker of progressing cervical disease (63).

In the present study, the age ranged from 46 to 83 years with a median (IQR) of 51.50 (13) years in the positive HPV 16/18 patient group and this result was significantly higher

than the negative HPV 16/18 group which ranged from 20 to 60 years with a median (IQR) of 39.0 (13) years. Lower results were reported in Mexico whereby Velázquez-Márquez et al. reported that women infected with high-risk HPV types, presented an average age of 41.4 years which was insignificantly higher than that of women with low-risk HPV types which was found to be 33.4 years, in the age group ranging from 18 to >55 years (HPV 16 and 18 were identified in 45.9% and 31.1% infected samples, respectively including co-infections) (64). The lower result reported in Mexico may be attributed to that the average age was estimated in relation to high-risk HPV type infection not only of HPV 16 and/or 18.

The mean age in abnormal cervical cytology recorded in Iran by Afrakhteh et al. was 46 years (65) and this was consistent with that reported in this study, whereby the patient group (having abnormal cervical cytology) age ranged from 30 to 83 years with a mean of 46.0 ± 11.70 years, while the control group (having normal cervical cytology) ranged from 30 to 52 years with a mean of 39.80 ± 7.59. However, lower results were noted in the eastern region of Saudi Arabia, Balaha et al. which revealed that the age of women with abnormal cervical smears ranged from 18 to 76 with a mean of 36.5, whereas the age of women with normal cervical smears ranged from 19 to 65 with a mean age of 35.7 (66).

In the current work, in the patient group, gravidity, parity and abortion appeared to be insignificantly higher than those in the control group while same average was noted as regards marital age between patients and controls. In Slovenia, Gavrić-Lovrec et al. noted a lower average as regards gravidity, parity, spontaneous abortions and marital age (1.9 ± 1.1 pregnancies, 1.4 ± 1.0 parturitions, 0.1 ± 0.5 spontaneous abortions and 17.6 ± 1.9 years, respectively) and these lower results may be explained by a higher sample size used in comparison to the present study (67). In the positive HPV 16/18 patient group, gravidity, parity, abortion and marital age were higher than those in the negative HPV 16/18 group, in this work while in Central China, Belinson 2007, reported lower median gravidity, parity, abortion and marital age (3, 2, 0 and 20, respectively) in women with CIN of all grades infected with high-risk HPV infection and these lower results may be attributed to that the average was estimated in relation to high-risk HPV infection not only HPV 16 and/or 18 (68).

A significant difference in the distribution of patients and control was found as regards regularity of menstruation in this study whereby 60% had irregular menstruation and 40% were menopause in the patient group, while in the control group 80% had regular menstruation and 20% were menopause and this denotes that menopausal women are more liable to get CIN. A study in Iran reported by Afrakhteh et al. noted consistent data with this work as it showed that 43.67% of abnormal cervical smears were menopausal (65). However, in Thailand (69) and Turkey (70), lower rates of menopausal women were noted in abnormal cervical cytology which were 24.7% and 23%, respectively. In the current work, in patients with irregular menstruation, 4% were HPV 16/18 positive with statistical significance encountered while no HPV 16/18 positives were found in patients with regular menstruation. Higher rates were encountered in India, whereby Varghese reported that women who had irregular bleeding had a prevalence of 5.7% HPV infection while in those without irregular bleeding, HPV was in 6.1% (71). In the present study, 62.5% of menopausal patients were significantly HPV 16/18 positive. In the United States, Ko et al. reported that 25.3% of peri- and post-menopausal (PMP) women having ASCUS were high-risk HPV positive and this lower rate in comparison to this study may be attributed to that the rate was estimated in ASCUS patients only (72).

The current work revealed a significant difference between patients and control regarding abnormal vaginal bleeding which was present in 60% of patients while not encountered in the control group and all those cases complaining from abnormal vaginal bleeding had abnormal cervical cytology denoting that abnormal cervical cytology is an important cause of abnormal vaginal bleeding. In contrast, in Nigeria, Anorlu et al. noted that in abnormal vaginal bleeding, 1.7% had dyskaryosis, 5.4% had infiltrating carcinoma while 46.6% had normal cervical smears (73). Also, in this work, in women with abnormal vaginal bleeding, 4% were HPV 16/18 positive. However, a higher rate was reported in China, Wang et al. noted that in patients with abnormal vaginal bleeding, 21% were high-risk HPV positive (74).

Furthermore, in this study, 50% of women with postmenopausal bleeding (PMB) were HPV 16/18 positive which was significantly higher when compared with 7.7% in the women without PMB. In India, Varghese recorded that women who had PMB had a prevalence of 15.4% HPV infection which was inconsistent with this study while HPV was in 6% in those without PMB which was close to the rate in the current work (71). A study in Tanzania reported by Mosha et al. revealed that 39% of confirmed cervical cancer cases complained PMB (75) which was close to that noted in this study whereby 30% of patients had PMB compared with none in the control group.

Two case series from United States revealed that post-coital bleeding (PCB) happened in 6% and 10% of 81 and 231 women with cancer cervix, respectively. Yet, 30% of invasive cervical cancer presented with PCB in another case series (76) while in this study, 15% (3/20) of patients complained PCB compared with none in the control group. In this work, HPV 16 or 18 was more prevalent in women with PCB (20%) when compared with the women without PCB (12.5%). Burk et al. in New York, reported that women who experienced PCB, had a statistically insignificant higher tendency toward HPV prevalence as in these women, 31.4% were HPV positive (HPV16 and 18 were the most common types of HPV infection found) (77).

Vaginal infection in this work, was significantly present in 100% of patients while not encountered in the control group and this was consistent with that noted in Tanzania, whereby Mosha et al. reported that all cases of confirmed cervical cancer cases had vaginal discharge. This shows that clinicians should pay attention and at least perform a speculum examination as a primary screening tool in regions with limited resources for screening cancer cervix (75). A high rate of vaginal infection was noted in Pakistan, whereby Khattak et al. recorded that 75% of women with abnormal cervical smears had vaginal discharge (74). On the other hand, in Turkey, 26% of abnormal cervical smears had vaginal discharge (70).

The present study revealed that 70% of patients had significantly abnormal cervix compared with none in the control group and this corresponds with a study in Bangladesh whereby Banik et al. found that about one-third of the patients with an abnormal cervical smear result had a healthy cervix (79).

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This indicates that cervical cancer screening, based only on clinical impression and visual examination, can predict to a high degree the presence of cervical disease. 12.8% of women with abnormal cervix were HPV 16/18 positive in this work. A close rate was reported in India, whereby Dasari reported that HPV lesions accounted for 20.5% of the unhealthy cervix or grossly abnormal cervical cases (80).

Significantly, the present work revealed that 6.3% of women with cervicitis were HPV 16/18 positive while 30.8% of women without cervicitis were HPV 16/18 positive. A higher rate was revealed in Shanxi, China, whereby Gao et al. reported that in patients with chronic cervicitis, HPV 16 was in 35.7% while HPV 18 was in 10.7% (81).

In this study, 6.3% of contraceptive users were significantly HPV 16/18 positive while 30.8% of non-contraceptive users were HPV 16/18 positive denoting contraception as a protective factor. In Slovenia, Gavrić-Lovrec et al. reported that HPV 16/18 infection was equally prevalent in contraception users and non-users 67) while in Central Italy, Ripabelli et al. reported that in contraceptive users, 25.4% were high-risk HPV positive while in non contracepive users, 21.2% were high-risk HPV positive (82). In Pakistan, Khattak et al. revealed that 50% of women having abnormal cervical smears used contraception where 38% of these patients used oral contraceptive pills (OCP) (78). Higher usage of contraception was recorded in the patient group in the current work, whereby 70% used contraception in any of its forms, compared with 80% in the control group with equal prevalence of OCP usage (30%) in both groups. Close rates were reported in Iran, whereby Afrakhteh et al. noted that 20.8% of abnormal cervical smears used OCP (65).

Inconsistent data were noted in Washington by Negrini et al., which revealed that 94% of the HPV 16/18 positive women detected had used OCPs, compared to 62% of the women not infected with HPV and reported that all HPV 16/18 positive women having cervical neoplasia had a history of OCP use (83) while in the present work, OCPs were not used in the positive HPV 16/18 patient group compared with 30.8% in the negative HPV 16/18 group.

In this work, none of the patients, controls, HPV 16/18 positives or negatives were smokers. In contrast, Afrakhteh et al. reported that 7% of abnormal cervical smears were smokers, in Iran.(65) In Washington, Xi et al. reported that in a population of women referred to a minor cytologic abnormality, higher HPV 16 and 18 DNA load was related with status as a current, but not former smoker (84).

The rate of married women in the patient group (65%) was significantly lower than the rate in the control group (100%) in this work denoting marriage as a protective factor. Inconsistent data were reported in Nigeria, whereby Audu et al. reported that 25/26 CIN cases were married (85) and a high rate of married women was noted by Mosha et al. in Tanzania, whereby 81.5% of confirmed cervical cancer cases were married (75). In the current work, 5.4% of married women were HPV 16/18 positive while 50% of divorced and widow women were HPV 16/18 positive with significant difference found. Camargo et al. revealed that in married women, a higher rate was estimated where 50.5% were HPV positive while in separate women, 44.2% were HPV positive while in widows, 61.3% were HPV positive in Colombia (86).

Infertility was not encountered in patients, controls, HPV 16/18 positives but was found to be in 10.3% of the HPV 16/18 negative group in the current study. In contrast, Audu et al. noted that 2/26 CIN cases had infertility in Nigeria (85). In the United States, Henneberg et al. reported that previous reports revealed that 31–70% of spontaneous aborted cases were positive for either HPV 16 or 18 where HPV 16 was found to decrease blastocyst formation whereas HPV 18 inhibited the blastocyst hatching process (87).

In Tanzania, Mosha et al. reported that 52.5% and 99.5% of confirmed cervical cancer cases had dyspareunia and pelvic pain, respectively (75) which were higher rates in comparison to this study, whereby dyspareunia and pelvic pain were present in 25% of patients compared with 0% and 60%, respectively, in the control group and these higher rates may be attributed to that these rates were estimated in relation to cervical cancer cases only. Besides, in the current work, dyspareunia and pelvic pain were present in 0% and 16.7% of positive HPV 16/18 patients respectively, compared with 33.3% and 41% of negative HPV 16/18 group, respectively.

No corresponding data were found in Iran, whereby Afrakhteh et al. reported that among abnormal cervical smears, ASCUS was 53.18%, LSIL was 17.73%, HSIL was 10.75% and squamous cell carcinoma (SCC) was 17.08% (65). Also, inconsistent results were reported by Banik et al. who found that in abnormal cervical smears, 2.15% were ASCUS, 77.7% were LSIL and 14.39% were HSIL in Bangladesh (79). However, this study revealed that in the patient group, 25% were ASCUS, 40% were LSIL, 25% were HSIL, 5% were SCC and 5% were adenocarcinoma and these data were close to those noted in Thailand by Yotwimonwat et al. who revealed that in patients with abnormal cervical smears, 20.92% were ASCUS, 20.00% were LSIL, 34.68% were HSIL and 12.29% were SCC (69).

In the present work, in the positive HPV 16/18 patient group, 0% was ASCUS, 33.3% were LSIL, 33.3% were HSIL, 16.7% were SCC, 16.7% were adenocarcinoma and 0% was benign, while in the negative HPV 16/18 group 12.8% were ASCUS, 15.4% were LSIL, 7.7% were HSIL, 0% was SCC, 0% was adenocarcinoma and 64.1% were benign and these data were corresponding to those reported in Washington whereby Negrini et al. reported that HPV 16 and 18 were associated with both LSIL and HSIL, whereas all other HPV types combined were associated only with LSIL (83). This result shows that positive HPV 16/18 PCR was more correlated with HSIL and SCC and that women infected with HPV 16/18 DNA are more prone to progress to advanced stages of cervical disease.

Sharifah et al. revealed that HPV 16 was identified in 23.7% abnormal cervical smears whereas HPV 18 was detected in 2/38 abnormal cervical smears while HPV was not detected in all normal cervical smears in Malaysia (88) and these data were consistent with those recorded by this study whereby 20% of patients were HPV 16 positive and 10% of patients were HPV 18 positive compared with none in the control group with no significant difference found between both groups. In Slovenia, Gavrić-Lovrec et al. reported that HPV 16/18 infection was present in almost half of patients with CIN (67). However, in Iran, Safaei et al. noted that HPV 16 was found in 2% of cytologically negative Pap smears while no HPV 18 was identified (89).

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5. Conclusion

Abnormal cervical cytology was more found in irregularly menstruating and menopausal women, women with vaginal infection and in women having abnormal cervix necessitating pap smears for such category. High-risk HPV DNA testing by PCR of cervical samples diagnosed according to the Bethesda 2001 guidelines may benefit the management of patients with abnormal cervical smears, especially among women aged 46 years and older, in menopausal women and in women complaining of PMB. Therefore, HPV DNA testing should be made use of as an adjunct to pap smears.

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