SCREENING FOR HR-HPV AMONGST SUDANESE WOMEN VISITING GYNECOLOGIC CLINIC BY ISH AND PAP.TEST

Sahar Elderdiri GAFAR1
Hussain Gadelkarim AHMED1
Siddig Adam Ahmed HAROUN2
Ehab Mohammedelmadenah MOHAMMED3

1Department of Histopathology and Cytology, Faculty of Medical Laboratory Science, University of Khartoum, Khartoum, Sudan
2Department of Gynecology and Obstetric, Faculty of Medicine, University of Khartoum, Khartoum, Sudan
3Department of Medical Laboratory, College of Health Sciences, Aljouf University, KSA

INTRODUCTION
Cervical cancer is the second cancer among women in Sudan, with more than two-thirds of all women with invasive cervical cancer being diagnosed at an advanced stage [1]. In 2000, cancer in Sudanese hospitals was the third leading cause of death represented 5% of all deaths [2]. Worldwide, about 500,000 new cases of cervical cancer were reported in 2006 and most of these cases have been reported from developing countries in Africa, Central America, and South America where there is lack of screening and early detection programs [3].

Many factors have been proved to contribute to the etiology of cervical cancer with prime suspect Human Papillomavirus (HPV). More than 100 HPV genotypes have been characterized with approximately 40 types infecting the anogenital area. About 13 of these (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66) are strongly associated with the development of cervical cancer and have been designated as the high risk HR-HPV. By contrast HPV 6, 11 and some other genital HPV types are associated with benign genital warts and have been classified as low risk [4].

Worldwide certain high-risk strains of HR-HPV cause nearly 100% of invasive cervical cancer [5]; although most of these high risk strains are identified, but they still need more work in the Sudan. Therefore, the aims of this study was to screen a woman for HR-HPV that are possibly responsible of cervical cancer in the Sudan applying In Situ Hybridization, as well as, to compares the efficacy of conventional cytology in identifying the cytological evidences for the presence of HPV in Pap smear.

BACKGROUND: Human papillomavirus (HPV) is widely accepted as the primary agent involved in the development of squamous intraepithelial neoplasia and cervical carcinoma. Several methods are available for detecting HPV DNA or its cytological evidences (koilocytosis).

OBJECTIVE: To screen for HR-HPV among Sudanese women with gynecologic complains using Pap. Test and In-Situ Hybridization (ISH) methods.

RESULTS: A total of 130 sequential Pap smears determined by cytoplogic examination to be either atypical squamous cells or cells showing koilocytes were tested by ISH HR-HPV. HR-HPV-status was determined in 29/106 (27.3%) samples of cervical scrapes by ISH and in 6/106 (5.7%) by conventional Pap. Test.

CONCLUSION: The overall sensitivity and specificity of Pap test were 20.7% and 98.7% respectively. ISH HPV is more predictive of cervical cytology in patients with non-detectable cervical lesions than is cytological evidence in conventional Pap test.

Key words: HPV, Pap. Smear, ISH, Sudanese

MATERIALS AND METHODS
In this study, a total of 130 sequential cervical Pap smears were investigated from the women attending to the gynecological clinic with clinical complaints. Cytological materials were obtained by standard Szalay cyto-spatula. The obtained materials were used for preparation of two direct smears and immediately fixed in 95% ethyl alcohol, while it were wet. One of the direct smear (for subsequent ISH). Each participant was asked to sign a written ethical consent form during the interview, before the specimen was taken. Ethical consent was obtained from ethical committee of the Faculty Research Board and Hospital.

PAPANICOLAOU METHOD
Ethyl alcohol fixed smears were hydrated in descending concentrations of 95% alcohol through 70% alcohol to distilled water for 2 min in each stage. Then smears were treated with Mayer's Haematoxylin for 5 min, to stain the nuclei, rinsed in 95% ethyl alcohol for 10 second. Then the smears were blued in running tap water for 10 minutes. For cytoplasmic staining the smears were next treated with Orange G6 for 5 min, rinsed in 95% ethyl alcohol for 10 second. Then treated with Eosin EA50 for 5 minutes and again rinsed in 95% alcohol for 10 second and then the smears were dehydrated in absolute alcohol. The smears were then cleared in Xylene and mounted in DPX (Distrene Polystyrene Xylene). All reagents used were from Reactive RAL-Martilac, France and Reidel-Germany.
IN-SITU HYBRIDIZATION METHOD (ISH)

Slides were soaked in 50% alcohol for 30 minutes and then post fixation in 10% neutral buffer formalin for 30 minutes followed by digestion with proteolytic enzyme (S3007; DAKO) at room temperature for 3 minutes. Background quenching was performed by 0.3% H$_{2}$O$_{2}$ in methanol for 5 minutes. After samples preparation, a drop of the GenPoint HPV Biotinylated DNA probe (#Y-1443; Dako) were applied on smears, and were covered with coverslips. The probe and the target DNA was denaturated at 92°C for 5 minutes in an oven. Hybridization was performed in a humid chamber at 37°C overnight. After washing, the GenPoint Kit (#K-0620; DAKO) was used according to the manufacturer's instruction: First, primary Streptavidin-HRP in a dilution of 1:100 was incubated for 15 minutes. After washing, biotin-tyramide solution was incubated for 15 min, followed by the secondary streptavidin-HRP for 15 min. Three to four drops of diluted Diaminobenzidine (DAB) chromogen were applied at room temperature for 5 minutes. The slides were counter stained with hematoxylin, mounted and analyzed with light microscope.

RESULTS

A total of 130 women with gynecologic complaints were participated in this study, their age ranging from 16 to 75 years with a mean age of 33 years. Of the 130 Pap. Smears, abnormal findings were detected among 41/130 (32.3%) patients. Of the 42 patients, 29(69%) were diagnosed cervitis, 1(2.4%) Actinomyces and Chlamydia Trachomatis for each, 5 (12 %) Candida Albicans (Photo 1) and 6 (14.2%) HPV (dyskaryosis), as indicated in Photos 2,3 and Figure 1. However, the infection was found to be statistically significant in this group of patients, (P value 0.003).

Sufficient materials were only available for 106 patients.

Of the 106 patients HR-HPV DNA was detected in 29/106(27.4%) patients (Photos 4,5). Consequently, the risk associated with high risk HPV infection was found to be
Figure 1. Description of abnormal conditions in Pap. Smears

Figure 2. Description of age by HPV, cervitis and other microbial infections

Figure 3. Description of positive HPV with clinical remarks
statistically significant (P value < 0.00). Moreover, of the 29 ISH positive results, 5/29 (17.2%) were detected with cytological evidences of HPV infection (presence of Koilocytes) on Pap smears assessment. Notably, only one smear was detected with Koilocytes but revealed a negative findings in ISH. These results giving the Pap test a specificity of 98.7% and a sensitivity of 20.7%. Nevertheless, of the 130 subjects, only 38/130 (29.2%) have claimed doing regular Pap.test, of whom 13/38 (34.2%) were found positive for HPV.

In regard to the relationship between age and HPV infection, cervitis and other bacterial and parasitic infections, high percentages of positive HPV and infections were identified in age group 31-45 years, constituting, 71%, 55% and 45% for infections, HPV and cervitis, respectively. Moreover, elevated proportions were also identified in age range 16-30 years representing 55%, 41% and 29% for cervix, HPV and infection in this order, as shown in Figure 2.

According to the clinical remarks, HR-HPV were common in women using contraceptive pills 43% followed by women with a history of abortion, underwent routine Pap test, contact bleeding, Fertility problems vaginal discharge and irregular cycle, constituting 43%, 35%, 34%, 28%, 26%, 25%, 21% respectively as indicated in Figure 3.

**DISCUSSION**

Cervical cancer is well known for its high mortality and morbidity worldwide, as well as, its link to HR-HPV etiology was well established. HR-HPV has been implicated in 99.7% of cervical squamous cell cancer cases worldwide [6]. Globally, HPV is typically the most common sexually transmitted infection, although there is significant regional variability in the prevalence of HPV even in regions of close proximity and common ancestry, which may be due to differences in sexual and cultural norms [7]. Although the prevalence of HPV varied from 1.4% to 25.6% in large scale screening worldwide [8], however in the current study the frequency of HR-HPV among women in Sudan, was relatively lower 29 (27.3%), than that reported from only one study from Sudan in this context. The study by Salih, et al. in 2010 [9] has screened 135 samples from Sudanese women using β globin PCR. HPV DNA was detected in 82 (60.7%). However, these variations might be due to differences in the techniques used. This in addition to the fact that they screened both for High and low risk HPV, in contrast we only screened for HR-HPV. Similar results were obtained in other countries,
in which the prevalence of HR-HPV were 33.2% in Benin [10], about 43.2% in Spain [11], and in contrast 4.0% in Algeria, [12]. Although the prevalence of HR-HPV was higher in young than older women were reported [13, 14], in the current study the HR-HPV Infection was higher in middle age, followed by younger age. However, similar findings were reported by Wong, et al. [15]. These variability may be due to differences in sexual and cultural norms [7]. HR-HPV infection most often is transient in younger women and declines with age. With increasing age, the likelihood increases that HPV positivity represents persistent disease and only those who have persistent HR-HPV infection are at risk of cervical cancer [16].

In regard to the clinical remarks high HR-HPV prevalence, was found in women underwent routine Pap testing, and this because HPV infection may asymptomatic, a latent [17], and most frequently detected in a routine Pap test [18]. Similar findings were reported by several studies [19, 20]. HR-HPV in present study was high in women with fertility problems, followed by women with irregular cycle. However, many studies have reported that no significant differences in HR-HPV genotype prevalence in infertile women compared to healthy controls [21, 22]. In contrast in other studies were reported that HPV infection rate of the infertility group was higher than that of the control group [23, 24].

Several studies have reported that induced abortion was independent risk factors for HR-HPV infection [25, 26], we found high risk HPV in women with history of abortion which is similar finding with the study elsewhere [27]. HR-HPV was not detected in women with vaginal fistula. Although, Frisch, et al. [28], reported that HR-HPV was more common among anal fistula cases, but to the best of our knowledge, there is no study investigated the prevalence of HR-HPV among women with vaginal fistula. However further study were needed.

In the recent study HR-HPV was much higher in women with negative Pap smear. Similar results reported by [29, 30]. Only one smear was contain Chlamydia Trachomatis and positive for HR-HPV, the previous findings support the results of Van, et al. [22]; they reported that HPV persistence is associated with concurrent Chlamydia infection and which might increase the host susceptibility to HPV or enhance the effects of HPV.

Several studies reported that conventional Pap smear have lower sensitivity and higher specificity [31, 32, 33]. The limited sensitivity of Pap test due to high susceptibility to intra-individual and inter-individual variability [34]. Therefore, approximately two-thirds of false-negative smears were related to sampling errors, and the remaining third were due to screening and/or interpretable errors, mainly due to the small number of diagnostic cells present in a suboptimal smear [35]. In these studies the sensitivity of conventional Pap smear was 20.7% and specificity 98.7%. Similar findings were reported by [36, 37].

One of the limitations of the present study is the small sample size, as well as, screening for HR-HPV only.

In conclusion: The overall sensitivity and specificity of Pap test were 20.7% and 98.7% respectively. ISH HPV is more predictive of cervical cytology in patients with non-detectable cervical lesions than is cytological evidence in conventional Pap test. Effective triage of patients by HPV analysis using ISH HPV as compared to conventional Pap test has the potential of significant public health impact by reducing needless colposcopies, in addition to unfavorable medical, social, and psychological patient penalty. Pap test is an easy-to-handle method for screening of cervical HPV infection, with very high specificity but with insufficient sensitivity for clinical practice.

Further long term studies with large sample size are required to identify types of high risk HPV to help in introducing of vaccines.

**Legends**

**Photo 1:** Candidaasis in conventional Pap smear. The budding yeast form and pseudohyphae are detected (X40).

**Photo 2:** Koilocytic changes associated with HPV in basal cell perinuclear halo surround by a dense cytoplasmic zone. The nuclei are slightly enlarged with irregular chromatin(x40).

**Photo 3:** Koilocytic changes in intermediate cell associated with HPV, perinuclear halo (Clear zone around the nucleous). The nuclei are slightly enlarged with irregular chromatin. Dorderlein bacilli are present(X40).

**Photo 4:** Pap smear(x40), negative ISH for HR-HPV. The cell and nucous stained by Harris hematoxyline(blue color).

**Photo 5:** Cervical smear (x40).The brown color of (DAB-peroxidase) showing cells containing HPV DNA. Positive ISH for HR-HPV.

**References**


References continues on the next page
References continues from the previous page


