Determination Of Human Papilloma Virus (HPV) Using Cytomorphology, Immuno cytochemistry and In situ-hybridization In Cervical Smears Of married Women in Khartoum State Sudan 2006

Asma Al Ameer M.Zeen- *et al* *

Referees:

Note: research was accepted as:

Oral presentation : evaluation of some laboratory techniques used in the diagnosis of HPV in cervical smears in the third conference of sudanese society of clinical biology(SSC) 24 -Nov 2008

Poster paper: in the second international conference of applied biotechnology

Abstract:

This descriptive comparative study was aims to measure the sensitivity and specificity of cytological, immunocytochemical and in situ-hybridization detection methods for Human Papilloma Virus (HPV) in cervical smear. Ninety seven cervical

* محاضر- كلية علوم المختبرات الطبية – جامعة شندي

smears were collected from married females with different gynecological complains and twenty one of them were well diagnosed with cervical cancer. In cervical smears , HPV classical cytomorphological changes (Koilocytosis and/or dyskeratosis) were detected in 36% while HPV K1-h 8 antigen was detected in 19% by immunocytochemistry staining method and HPV DNA was detected in 51% by in situ-hybridization technique . HPV DNA was detected in 90% of ladies with cervical cancer smears and in 39% of ladies with other complains smears. Using in situ-hybridization as a gold standard test cytology has a sensitivity of 67.3% and specificity of 85.5%. Immunocytochemistry has a sensitivity of 36.7%, a specificity of 97.7%. This study concludes that cytomorphological screening for HPV in cervical smears is suitable for clinical practical purposes in compare to antigen and DNA detection method.

Keyword: Cytology, immunocytochemistry, insitu–hybridization, Human Papilloma Virus, cervical cancer.

تعيين فيروس الورم الحليمي البشري في مسحات عنق الرحم من نساء متزوجات بولاية الخرطوم بالسودان ٢٠٠٦ باستخدام المورفولوجيا الخلوية، الكيمياء الخلوية المناعية والتهجين في موضعه

ملخص الدراسة

أجريت هذه الدراسة الوصفية المقارنة في الفترة من يوليو٢٠٠٦ الى مايو ٢٠٠٧ بعدة عيادات محولة لأمراض النساء والتوليد بولاية الخرطوم بهدف قياس حساسية ونوعية (خصوصية) طرق الخلويات المتساقطة، الكيمياء الخلوية المناعية والتهجين في الموضع للكشف عن فيروس الورم الحليمي البشري في مسحات عنق الرحم وقد جمعت سبع وتسعون مسحة من عنق الرحم من سيدات متزوجات بشكاوى نسائية مختلفة، وواحد وعشرين منهن شخصن بسرطان عنق الرحم. في المسح الخلوي للمسحات المصبوغة بصبغة بابنيكولا ،وجدت التغيرات التقليدية للفيروس الخلايا المتقعرة و/او المختلة التقرن بنسبة ٣٦٪ و مستضد الفيروس بنسبة ١٩٪ بعد الصبغ المناعي لمسحات عنق الرحم باستخدام الاضداد الاولية وحيدة النسيلة للمستضد HPV clone K1-H 8 بينما وجد الحمض النووي دنا للفيروس بنسبة ٥١٪ باستخدام طريقة التهجين في موضعه بواسطة مسبار واسع الطيف معلم بالبيوتين لدنا الفيروس , وجد دنا الفيروس في ٩٠٪ من مسحات عنق الرحم للنساء المصابات بسرطان عنق الرحم بينما وجد في ٣٩٪فقط من النساء الغير مصابات بعنق الرحم ولديهن شكاوى نسائية اخرى.

باعتبار طريقة التهجين الموضعي طريقة مرجعية كانت حساسية المسح الخلوي للكشف عن الفيروس ٦٧,٣٪ ونوعيتها ٨٥,٥٪ بينما كانت حساسية الكيمياء الخلوية المناعية للكشف عن الفيروس ٣٦,٧٪ ونوعيتها ٩٧,٧٪ .

خلصت هذه الدراسة الى ان المسح الخلوي للفيروس في مسحات عنق الرحم مناسب وعملي للاغراض الطبية وطريقة التهجين في الموضع طريقة بحثية فعالة جدا للكشف عن فيروس الورم الحليمي البشري بينما تقل اهمية طريق الصبغ المناعي للكشف عن الفيروس في مسحات عنق الرحم وذلك لانخفاض حساسيتها.

Introduction:

Cervical cancer is the second leading cause of cancer mortality in women worldwide, causing 240,000 deaths annually of approximately 490,000 cases reported each year, more than 80 percent occur in the developing world, where effective but costly Pap smear screening programs are not in place. Early precancerous changes and early cancers detected by Pap smears are effectively treated and cured with surgical therapy or ablation. In the absence of effective screening, the disease is detected late. Traditional therapeutic options for cervical cancer that have advanced beyond definitive surgical treatment are chemotherapy and radiation therapy, which are associated with many toxicities and do not offer a lasting cure [1].

The risk of cervical cancer is increased by infection with certain types of human papilloma virus (HPV). HPV are small non-enveloped DNA tumor viruses that commonly cause benign papillomas or warts in humans [1,2]. Persistent infection with high-

risk subtypes of human papillomavirus (HPV) is associated with the development of cervical cancer[1]**.** HPV infects epithelial cells, and, after integration in host DNA, the production of oncoproteins, mainly E6 and E7, disrupts natural tumor suppressor pathways and is required for proliferation of cervical carcinoma cell [1,3,4]. Recent studies have shown that HPV-DNA can be found in 99.7% of all cervical carcinomas, with certain types of HPV (16, 18, 45 and 31) being the most frequent [4,5]. Most notably, HPV16 and HPV18 have been shown to be associated with cervical cancer [5]. HPV16 is the prototypic "high-risk" HPV present in more than 50% of cervical carcinomas[3,6]

The prevention of cervical cancer is based on the Papanicolaou smear. Because the majority of cervical cancers are preceded by a cervical precancerous lesion cervical intraepithelial neoplasia (CIN), often by many years, the detection of these precancerous lesions is fundamental to cancer prevention[6] and cytology examination can detect CIN long before any abnormality can be detected[4],

Further more, changes which appears in pap smear are usually associated with active infection of HPV[7], So lack of cervical cytology screening is a significant risk factor for cervical cancer[8], Fewer than 5% of women in developing countries have ever had a Pap screening test [9].

In spite of the success of cytomorphological examination in screening for cervical cancer, major limitations have been recognized, such as the low sensitivity. Therefore, it has been proposed that HPV DNA testing should be used to clarify equivocal Pap smear screening results[5]. The result of HPV testing is useful for managing women whose smears show atypical squamous cells (ASC)[9]. The extreme rarity of HPV-negative cancers reinforces the rationale for HPV testing in addition to, or even instead of, cervical cytology in routine cervical screening[10].

Among sudanese women, breast, cervical and ovarian cancer remained the three most common cancers over period from 1967 to 2004, Breast and Cervical Cancer account for about 50% of all cancers in

Sudanese women . Routine screening for both these cancers has markedly reduced the mortality in the developed world, but in developing countries, which largely lack screening programs, these two cancers remain the primary cause of death due to cancer (combined crude mortality 18.5/100 000). Widespread testing for HPV has reduced the mortality from cervical cancer in developed countries (age-adjusted mortality 4.0/100 000), but in Sudan (12.7/100 000), as in other developing countries (11.2/100 000), cervical cancer remains the major cause of death due to cancer in women [11]. According to WHO reports, the age adjusted incidence rate of cervical cancer in Sudan is 19.02 per 100,000 population [12] and in reports from Wad Medani, cervical cancer account 34% of treated female gynaecological malignancies during 1999- 2005 [13]. A major challenge to treatment of cancer in Sudan, as in most developing countries, is that most patients first present with advanced stage disease. A total of 78% of Sudanese patients have stage III or IV disease (TNM classification) when they first seek

medical treatment (data from Sudan Federal Ministry of Health). In these stages, treatment may often involve multiple modalities, including surgery, radiotherapy, chemotherapy and hormone therapy, and has a markedly diminished chance of success. In addition, cancers like cervical cancer are largely curable if detected early[11]. Therefore, there is an urgent need for better early detection of cancer in Sudan [11,13]; to make treatment more effective, less costly, less invasive, and more accessible and acceptable to patients [13].

In Sudan HPV DNA have been detected in 94% of cervical biopsies in Ebba retrospective studies 2006 using PCR method for HPV DNA detection. 94% of samples harbored high risk and 11.7% low risk HPV genotypes. HPV 16 was found to be the most frequent HPV genotype 82.5 %. HPV 18, 45 and 52 were the second, third and fourth HPV genotypes identified in Sudan. Mixed infections mainly composed of HPV 16, 18, 31, 33, 35, 45, 52, 58, and 68 were observe [12].

There is exist nowadays many different HPV tests that all have their own technical, analytical and clinical properties. The choice for an HPV test for a given purpose should therefore not solely be based on practical considerations, but also on the intention for its use. In particular, when aiming at the detection of clinically relevant HPV infections, it is of utmost importance to use a clinically validated HPV detection assay[14].

The purpose of the current study is to evaluate three laboratory methods used for detection of HPV, all protocols used were manual and are not in need to special lab settings or instruments and can be carried out in any routine microbiology or histopathology labs and their results can be interpreted with bright field microscope but they were varied in their costs and their end targets (cytomorphology of HPV, HPV K1-h 8 antigen and HPV DNA). Further more, we determine burden of this infection among Sudanese women. We wish Results yield of this study would help researchers and planners of cervical screening programs in choose and select a useful laboratory test for HPV screening especially in such low resource country.

Material and method: this descriptive comparative study was conducted in Khartoum state between July -2006 to May-2007.

Cervical smears samples have been collected from married females aged between 15-72 years in the gynaecological referred clinics of Bahrry teaching hospital, Ombada teaching hospital, Al Ahfad family health centre, Al-Daw Hajoj medical insurance center and out patient clinics of Radiation Isotopes Center Khartoum (RICK). Women in referred clinics came from different areas in Khartoum state but in (RICK) hospital new cases of cervical cancer came from over all Sudan. Females with one of the following clinical manifestation were included: cervical cancer, vaginal discharge, postmenopausal bleeding, primary infertility, routine screening, women wanting IUD (intra uterine device), post coital bleeding, recurrent abortion and menstrual disturbance. Pregnant ladies and those with cervical cancer under radiotherapy treatment were excluded because of the privacy of cervical samples, samples have been collected only from females who were refer to make Pap test for medical purposes or opportunistic screening and chooses by gynaecologist but females who were diagnosed with cervical cancer were included for their high probability to carry the HPV virus as documented in the literature ,after informed consent**.**

Sample size: because studies determine sensitivity and specificity are classified as descriptive study of a dichotomous Variable, sample size when calculated in the confidence interval or the level of uncertainty set at 0.2 and confidence level set at 95% was **96. [15]** **(Table 1)**

Sample collection: Cervical smears were collected under supervision of specialized gynaecologist using plastic spatulas under speculum vision. Disposal speculums of different sizes were used for this purpose. The speculum was introduced into female vagina without lubricant gel to avoid it's interfering with sample. Then notched end of the spatula that corresponded to the contour of the cervix was rotated 360. Processed Cervical material spread gently in one direction on 3-6 slides each fixed and processed following manufacturer instruction for immunocytochemistry and in situ hybridization methods. Slides for Papanicolau stain processed as in routine cytopathology labs. Silinized slide were used in the in situ-hybridization protocol.

Staining procedure: Papanicolaou stain: as in routine cytopathology labs ,all staining solution provided from Ral company. (Figure 1(A-B)).

Immunocytochemical (ICC) staining for HPV antigen in cervical samples: the specimens are first incubated Primary Antibody provided with of Dakocytomation (Monoclonal Mouse Anti – human Papillomavirus (HPV) Clone: K1H8 Code M 3528) reacts with a non –conformational internal linear epitope of major capsid protein of HPV-1, which is broadly expressed among the different HPV subtypes. Anti – HPV yields a positive immunostaining to the nuclei of infected cells. Occasionally, the cytoplasm of koilocytic cells was observed to be immunoreactive and it was found to be immunoreactive with paraffin of a formalin-fixed HPV infected tissues which were demonstrated by Southern blot hybridization to be infected with HPV type 6,11,16,18,31,33,42,51, 52,56 and 58. Anti – HPV yields a positive immunostaining to the nuclei of infected cells followed by incubation with the Labeled Polymer (Dakocytomation EnVision System Alkaline Phosphatase (AP) for use with mouse primary Antibody code K1396), staining is completed with 5- 30 min incubation using fast red chromogen which results in a red – colored precipitate at the antigen site . Mayer hematoxylin used as blue nuclear counter stain and Dakocytomation Glycerol mounting medium C0563 an aqueous –based mounting medium

was used for mounting. negative control performed by skipping primary antibody incubation step on one third (circled by DakoCytomation-pen) of the smear.

Positive control: Positive HPV histological sections taken from verrucus skin lesions or cervical carcinoma previously diagnosed by pathologist to be HPV positive showing koilocytotic changes were used as positive control to test solutions efficiency and staining protocol. (Figures 2& 3 (A-C)).

Insituhybridization for HPV DNA detection: DakoCytomation Wide Spectrum HPV Biotinylated DNA Probe Code Y1404 which hybridizes to anogenital human Papillomaviruses (HPV) including types 6,11,16,18,31,33,35,39,45,51, and 52 was used for localizing HPV DNA in cervical smears either nuclear or cytoplasmic. Following hybridization of the biotinylated probe to target sequences on the smear a stringent wash solution containing a blocking agent is used to remove excess bound probe and to block nonspecific binding sites on the tissue which may otherwise react with the detection reagents. Then using the ISH Detection System K0601 which utilizes alkaline phosphatase (AP)-conjugated streptavidin to localize DakoCytomation wide

spectrum HPV biotinylated DNA probes. The streptavidin ¬alkaline phosphatase conjugate is then allowed to react with the biotin groups on the hybridized probe molecules. Finally, the site of hybridization is visualized by the colorimetric reaction of the enzyme conjugate with its substrate, BCIP (5-bromo-4-chloro-3-indolyl phosphate), and the concomitant reduction of NBT(nitro blue tetrazolium). This reaction results in the deposition of an insoluble blue purple product at the site of hybridization. Counter stain with fast red was used for background this step can be excluded and Dakocytomation Glycerol mounting medium C0563 an aqueous –based mounting medium was used for mounting. Positive control (Human DNA) Biotinylated DNA probe Code 1414 which hybridizes to total genomic human DNA provided as fragments of biotinylated double-strand DNA of 500 base pairs or less ,was used first to sure that protocol of ISH is working. (figures 4(A-B)& 5 (A-C))

Result and discussion:

Cytomorphology of HPV: In the cytological screening of Papanicolau stained cervical smears the most frequent HPV cytomorphological changes were binucleation (38%), Koilocytosis (35%), nuclear variations (34%) and dyskeratosis (20.6%) Figure (6&7). These frequencies were lower than those with W.Gray as he reports the most frequent HPV cytomorphological change as hyperkeratosis 66% followed by Parakeratosis (34%)[16] and higher than that of E. Korobowicz who reported a 15% frequency of Koilocytosis and/or dyskeratosis[17] but going with the study of Cecilia M and college in which multinucleation, binucleation and abnormal mitoses were significantly associated with HPV DNA detection among the histological criteria [18]. Sensitivity percent of cytology using classical cytomorphological changes 67% (Table 2) in this study is similar to that of A.K. Lie using PCR as the gold standard he found a sensitivity of 63% for cytology [19] but much higher than that of Yamamoto who recorded a low sensitivity of 26% for when compare with identification of HPV infection by PCR [20]. The low sensitivity in this study agreed with the fact that the koilocyte alone has low sensitivity for

detection of HPV infection as stated by M. Bibbo [3]. To the contrary specificity in this study 89% was very high compared to that of A.K. Lie (41%) [19] and similar to that of Yamamoto (95%) [20], also agreed with that of S. A. Morse where diagnostically, the koilocyte is an excellent indicator of HPV infection with a high degree of specificity[2]. W. Gray who stated that the most specific criteria for HPV infection in cervical smear is the presence of koilocytosis, dyskeratosis, Parakeratosis and Karyorrhexis and using the presence of two out of these three criteria alone yield a diagnostic specificity of 100%, with a sensitivity of 36% [16]. The presence of non classical HPV cytomorphological changes in samples of this study 49/79 (51%) were strongly significant statistically when compared with HPV DNA presence at same smear (Table 3) This result agreed with M. Bollmann study who found nuclear changes, disorders of keratinisation, abortive koilocytes and 'measles cells' as well as degenerative changes significantly associated with the presence of HPV DNA by PCR[21] and the study done by A D Várnai where there was no “negative cytology ” in HPV positive cases. Even in the absence of cellular atypia, minor non-classic signs of HPV-effect could be detected in the smear, if carefully assessed[22],

also the study by A. Schneider in which a panel of nine non classic criteria were evaluated and when these criteria used in combination, statistically discriminated analysis could correctly identify 84% of the HPV-positive group [23].

Monoclonal Mouse Anti – human Papillomavirus (HPV) Clone: K1H8 antigen detection by Immunocytochemistry: HPV antigen detected in (19%) of the studied smears (figure 8) Low percentage of HPV antigen (19%) positivity in this study may explained by the fact that antibodies are directed against capsid proteins, which are only found in productive[19] but not in latent (non productive) infection as stated by P. Birner& Yamamoto [24,20] Sensitivity and specificity of immunocytochemical staining for HPV antigen using insitu hybridization as golden standard test- were 36.7% and 97.7% respectively (table 2), and this result agree with comparisons of this assay with HPV DNA probes have shown that such polyclonal antibodies are at least 90% specific but not more than 50% sensitive, most likely because, in many cases ,only small amounts of late structural proteins are present [25]. The low sensitivity in this study confirmed by Peter Birner who stated that immunohisto- and -cytochemistry are not suitable for

detection and typing of HPV infection in cervical smears and in paraffin-embedded sections of cervical dysplasia and cancers [24]. S.A.Morse is agree and stated that this method is substantially less sensitive than DNA detection and even routine cytologic examination[3] what appear in (table 2) .

HPV DNA detection by insitu-hybridization : HPV DNA was detected in (51%) of cervical smears (figure 9). This percentages is near In the study done by M. Bollmann HPV DNA was detected by GP5+/6+ and MY09/MYll consensus primer PCR assays 76 of 164 cases (46.3%) had HPV positivity by PCR [21]. in this study results were higher than those of Cecilia M. study in which HPV was detected by ISH in 51/138 (37%) and by Hybride Capture2 test in 66/138 (48%) [20] HPV DNA prevalence among ladies with complains other than cervical cancer was 29/75 (39%) (Table 4). This result was very high in comparison to the study done by Majdi Mansour in Sudan by PCR where HPV DNA was detected only in 7/81(8.6%) of cervical samples [26]. wide range in prevalence is explained by the different nature of participating populations in such studies, and by technical evolutions in the diagnostic tests used. HPV DNA present in 20/22 (90%) of females with cervical cancer, this percent agreed

with H.A.El tahir study in sudan in which 94% of studied fresh tissues from females with cervical cancer [27].

Conclusion: In this study depending on classical cytomorphology of HPV only to detect HPV infection in cervical smears this method is easy to handle and cost effective for clinical practical purposes with high specificity and moderate sensitivity although the gold standard method is insitu hybridization which is suitable for research purposes , especially in low resource areas, Papanicolau stained smear beside its diagnostic value in detection of many gynaecological situation and feasibility in HPV detection, would it be more valuable if non classical HPV cytomorphology putted in consider through cytology screening for HPV changes in cervical smears ?!. any way Immunocytochemical staining method is not suitable for HPV screening due its very low sensitivity although it is highly specific.

Acknowledgment: To **Mrs. Margaret Middltone (Santa Barbara hospital –labs at UK)** for invaluable advices cytology interpretation , my teacher **D. Majdi Mansour , Mr. Mutaz Ibrahim Ali and Mr. Mazen Mabrouk** for training me on laboratory techniques needed in the study . to **Prof. Ahmed**

Mohamed El- Hassan for his invaluable advices ,to **Dr. Jamal El-Emeri** for his support ,to **Dr El Tijani Mussa** for his help in statistical analysis . to team work in out clinic of RICK hospital with aid of **Dr Ahmed Omer A Khalid** and al ahfad family health center.

Reference:

[1] J.B. Liao, **Cancer Issue Viruses and Human Cancer,** Yale J Biol Med, 2006 ; 79 (3-4): 115–122.

[2] M.Bibbo, **Comprehensive Cytopathology** , 1st Ed 1991 ,united state of America: Saunders company.

[3] S.A.Morse, R.C.Ballard. K.K.Holmes and A.A.Moreland, **Atlas of Sexual Transmitted Diseases and AIDS.** 3rd Ed 2003Spain: Mosby.

[4] V.Kumar, R.S .cotran and S. Robbins, **Robbins Basic pathology**, 7th Ed 2004, India: Elsevier.

[5] R. Hamkar, T. M.Azad, M. Mahmoodi, S. Seyedirashti, A. Severini and R. Nategh, **Prevalence Of human Papillomavirus In Meandering Province- Islamic Republic of Iran-,** Eastern Mediterranean Health Journal. 2002. 8 (6): 805–811

[6]- J.Holland. E.frei . **Cancer medicine** . 6th Ed 2003. American Cancer Society . Dc Becker ink..

[7] A.J.Hazberg, D.S.Raso and J. F. Silver man .**Color Atlas Of Normal Cytology**,1st Ed 1999,New York :Churchill Livingstone.

[8] E.D.Paskett , J.Rushing , R. Jr. D'Agostino, C.Tatum and R.Velez, **Cancer screening behaviors of low-income women: The impact of race,** Women's Health 1997, 3 :203-26.

[9] E.S. Cibas and B.S. Ductman , **Cytology Diagnostic principles and clinical correlates .** 2ed Ed . 2003 .China Elsevier ..

[10] J.M .Walboomers, M.V Jacobs, M.M Manos, F.X Bosch, J.A Kummer, K.V. Shah,P.J.Snijders, J.Peto , C.J.Meijer and N.Munoz. **Human Papillomavirus Is A Necessary Cause Of Invasive Cervical Cancer Worldwide.** J Pathol. 1999 , 189 (1) :1-3

[11] H.M.A.Hamad, **Cancer initiatives in Sudan** .Annals of Oncology, 2006. 17 (8): 32–36.

[12] E.A.Waktola , Msc thesis , **Molecular Characterization of Human .Papilloma Virus from Cervical Precancerous and Cancer Specimens among Ethiopian and Sudanese**

Women. Khartoum university **.**Institute of Endemic Diseases**.** Department of Molecular Biology.2006

[13] M. H Baldo and A.S. Gerais, **Maternal morbidity care in Sudan is substandard**. Sudan Medical Journal 2008 44 (1-3):13-18**.**

[14] P.J.Snijder, D.A. Heideman and C.J.Meijer, **Methods for HPV detection in exfoliated cell and tissue specimens**, APMIS 2010, 118 (6-7): 520–528.

[15] S. B. Hulley, S. R. Cummings, W. S. Browner, D. G. Grady and T. B. Newman. **Designing Clinical Research,** 3rd Ed 2007**.** Lippincott Williams & Wilkins.

[16] W. Gray .**Diagnostic Cytopathology**. 1st Ed 1995 New York: Churchill Livingstone .

[17] E.Korobowicz , A.Kwasniewska and I Georgiades. **The diagnostic value of cytomorphological traits in low and high risk type HPV infections**. Pol J Pathol. 1997; 48 (2):107-12.

[18] C.M. Roteli-Martins , V.A.F. Alves, R.T. Santos, E.Z. Martinez, K. J. Syrjänenand and S.F.M. Derchai**. Value of Morphological Criteria in Diagnosing Cervical HPV Lesions confirmed by *in situ* Hybridization and Hybrid Capture**

Assay. Pathology - Research and Practice 2001, 197(10): 677-682.

[19] A. K. Lie , C. V. Isaksen, S. Skarsvåg and O.A.Haugen, **Human papillomavirus (HPV) in high-grade cervical intraepithelial neoplasia (CIN) detected by morphology and polymerase chain reaction (PCR)—a cytohistologic correlation of 277 cases treated by laser conization.** Cytopathology 1999, 10 (2): 112-121.

[20] L.S.U Yamamoto, V.A.F Alves, M.Y.S Maeda, A. Longattofilho, M.L.Utagawa and J.E.NETO - **A morphological protocol and guide-list on uterine cervix cytology Associated to Papilloma virus infection.** Medicina Tropical de São Paulo, Virology 2004 , 46 (4) :189-193 .

[21] M. Bollmann, A. Bankfalvi, A. Trosic, N. Speich, C. Schmitt and R. Bollmann, **Can we detect cervical human Papillomavirus (HPV) infection by Cytomorphology alone? Diagnostic value of non c1assic cytological signs of HPV effect in minimally abnormal Pap tests** . Cytopathology 2005, 16 (1): 13-21.

[22] Dr. Alinda Dalma Várnai. PhD Thesis. **Combination Of Cytological And Molecular Methods For Improvements In**

Cervical Cancer Prevention. University of Pécs , Faculty of Medicine, Institute of Pathology Pécs .2007

[23] A. Schneider, G. Meinhardt, E. De-Villiers and L.Gissmann, **Sensitivity of the cytologic diagnosis of cervical condyloma in comparison with HPV-DNA hybridization studies.** Diagnostic Cytopathology 2006 3 (3): 250 – 255

[24] P. Birner, B. Bachtiary , B. Dreier , M .Schindl , E. A Joura , G. Breitenecker and G. Oberhuber. **Signal-Amplified Colorimetric In Situ Hybridization for Assessment of Human Papillomavirus Infection in Cervical Lesions.** Modern Pathology 2001, 14 (7) :702– 709.

[25] N.B.Kiviat,L.A.Koutsky . **Human papillomavirus**. In: 6th ed. P.R. Murray, E.J.Baron, M.A.Pfaller, F.C.Tenover, and R.H.Yolken. **Manual of clinical microbiology.** Washington, DC: ASM Press; 1995; p. 1082–1090

[26] M.M Salih, M. M. O. EL Safi, K Hart and K Tobi. **Prevalence of HPV genotypes in cervical smears taken from Sudanese women attending Omdurman Military Hospital**. Third scientific conference of the Sudanese Society of Clinical Biology. 2008. ***Conference proceeding: 42-44.***

[27] H. A. Eltahir, I.F. Elmola, A.M.Elhassan, M. E. Ibrahim1,**Genetic Characterisation of Cervical Cancer among Different Ethnic Groups in Sudan.** First Young Researchers of the Institute of Endemic Diseases Conference ,Conference proceeding ***: 39.***

Table (1)

Sample size for a descriptive study of a dichotomous variable

95% confidence interval

Width of Confidence interval / Expected proportion (P)	**0.10**	**0.15**	**0.20**	**0.25**	**0.30**
0.10	139				
0.15	196	88			
0.20	246	110	62		
0.25	289	128	73	47	
0.30	323	144	81	52	36
0.40	369	164	93	60	41
0.50	384	171	96	62	43

Table2: Sensitivity, specificity and predictive value for immunocyto -chemical staining and classical HPV cytomorphological changes in cervical smears calculated using ISH as golden stander test (n 97)

	positive		Sensitivity %	Specificity%	PPV %	NPV%
	No	%				
HPV classical cytomorphological changes	38	39.1	67.3	89.5	86.8	72.8
HPV antigen immune cytochemical staining	19	19.6	36.7	97.7	94.7	60.2

Table 3: Comparison between HPV DNA positivity in cervical smears by ISH and the presence of HPV non-classical cytomorphological changes in the same smears (n97)

		Insitu-hybridization		
		positive	negative	Total
HPV non classical cytomorphological change	Present	42	17	59
	Absent	7	31	38
Total		49	48	97

P .value is < 0.001

Table 4 : Relation between HPV DNA presence in cervical smears and clinical remarks of study group

	insitu		
	positive	negative	Total
Opportunistic Screening	8	19	27
Vaginal discharge	7	7	14
Cervical Ulcer	3	2	5
Infertility	0	4	4
Abortion	1	0	1
hormonal disturbance	3	7	10
Cancer	20	2	22
postmenopausal bleeding	5	3	8
Postcoital bleeding	0	1	1
PID	2	2	4
Other	0	1	1
Total	49	48	97

P .value is < 0.001

Figure 1-A Pap stained cervical smear show the following HPV

cytomorphological changes: Koilocytes, abortive koilocyte,dyskeratosis,

hyperkeratosis and, binucleation 40X

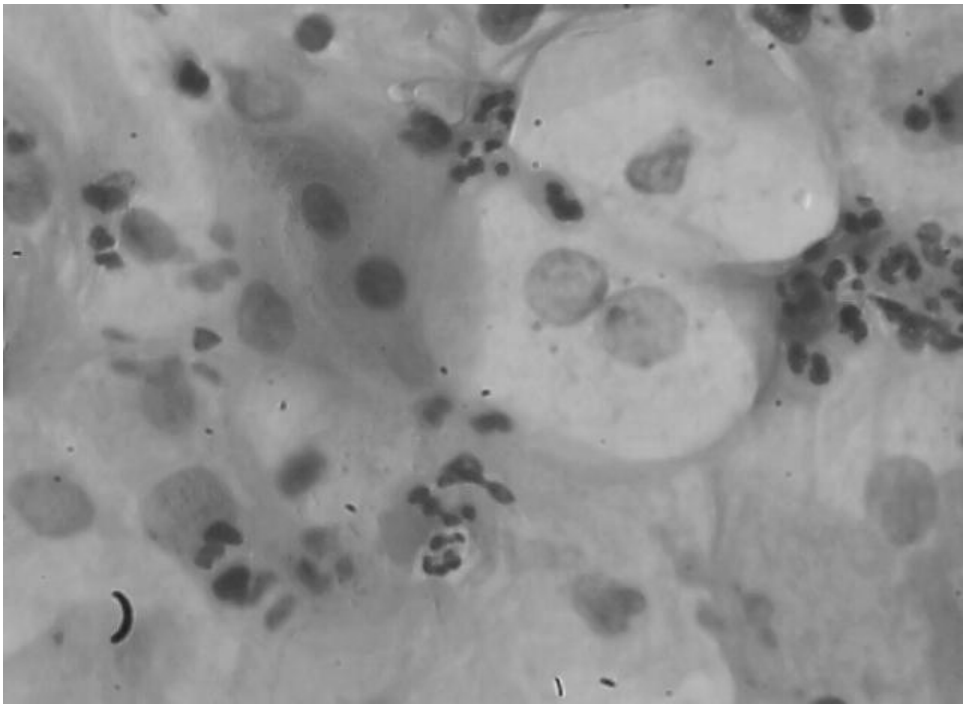

Figure 1-B Pap stained cervical smear show the following HPV

cytomorphological changes: balloon cells, Koilocyte, dyskeratocytes, cytofold 40X

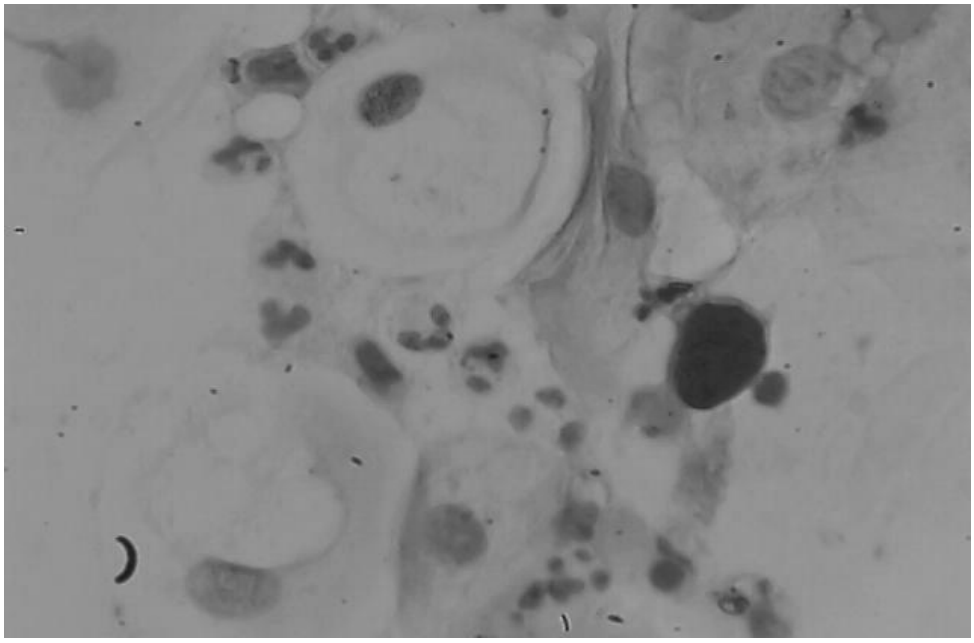

Figure 2 -Positive control tissue (skin verrucas) immunostained:

strong Red signals positive nuclei for HPV antigen 40X

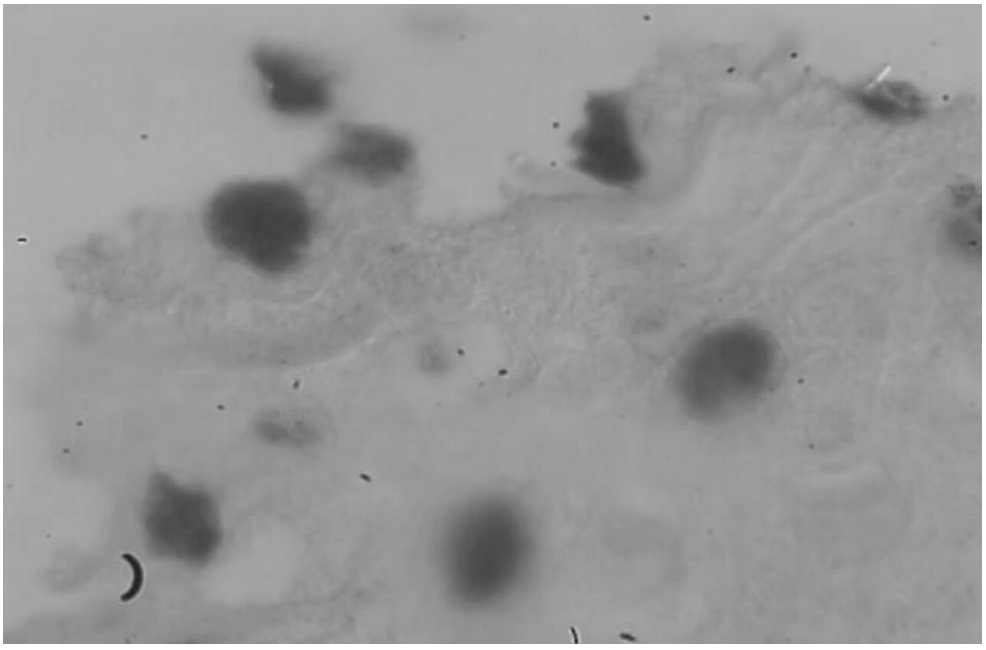

Figure 3- A HPV immunocytochemical staining with (monoclonal antibody HPVclone: K1H8) cervical smears: Positive nuclear signal in abnormal squamous cell 40X

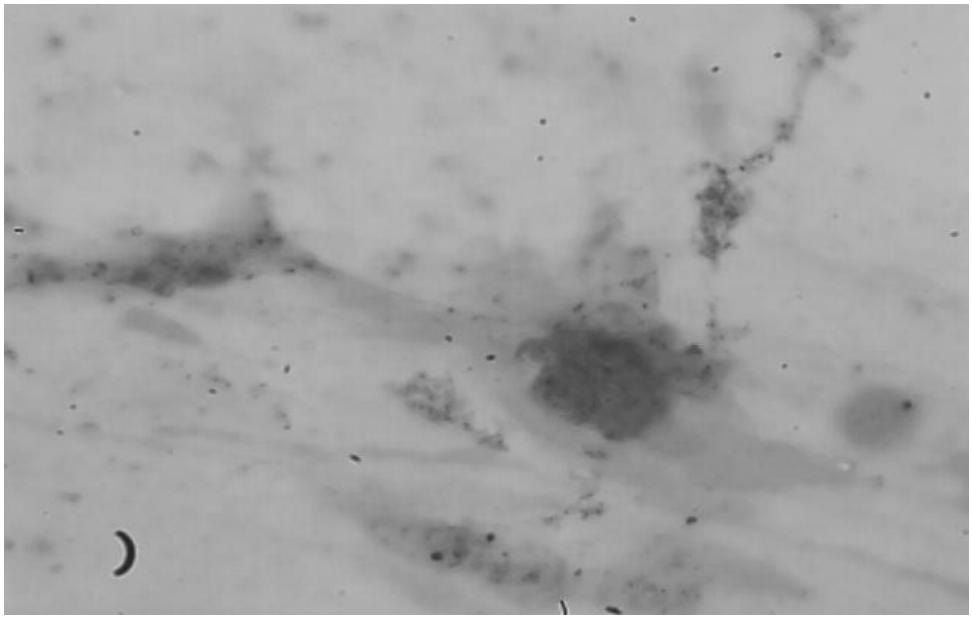

Figure 3- B HPV immunocytochemical staining with (monoclonal antibody HPVclone: K1H8) cervical smears strong Positive cytoplasmic signal indicate episomal form of HPV 40X

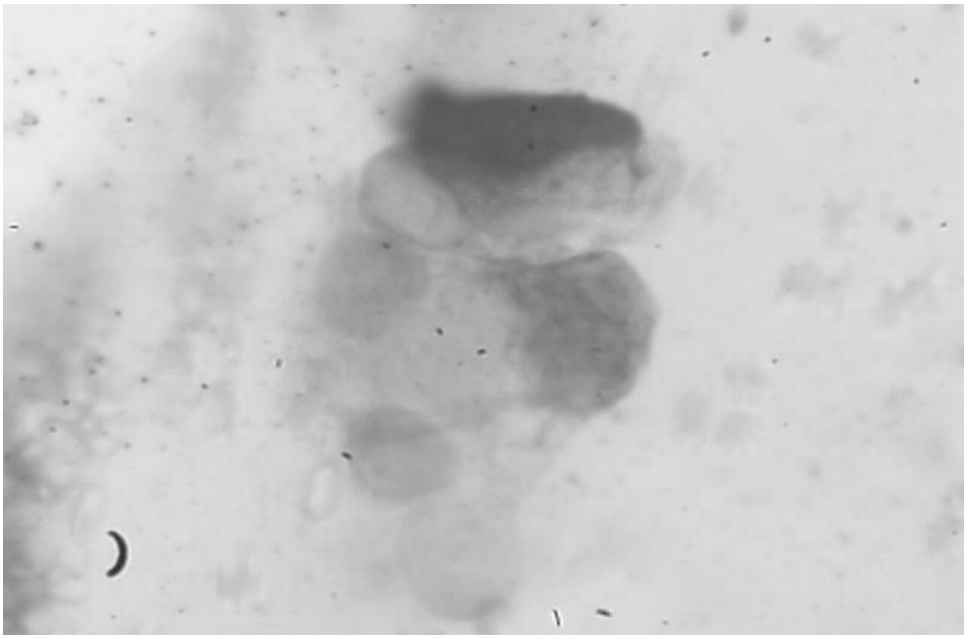

Figure 3- c HPV immunocytochemical staining with (monoclonal antibody

HPVclone: K1H8) cervical smears Positive nuclear and cytoplasmic signals 10X

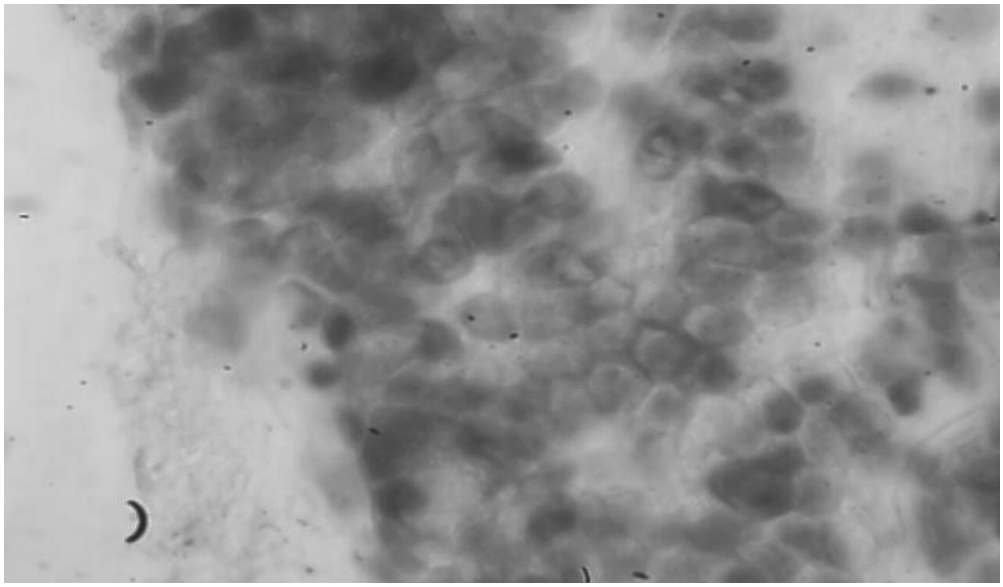

Figure 4-A:Control slides for insitu-hybridization staining :

Positive dark blue signals nuclear and cytoplasmic tissue (skin verrucas) 40X

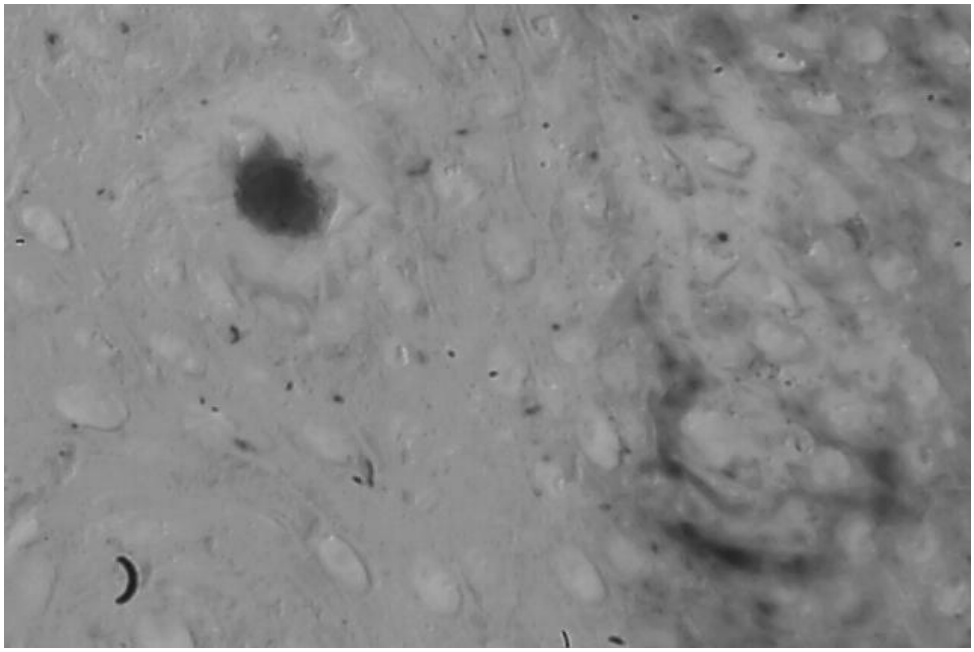

Figure 4-B:Control slides for insitu-hybridization staining :

Negative cells (cytology smear submandibular swell) 40X

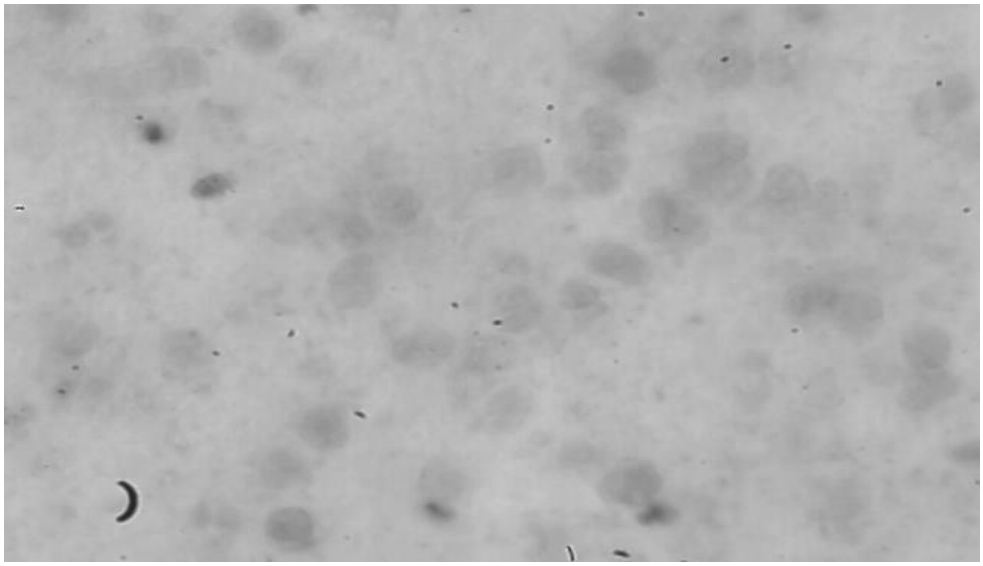

Figure 4-C :Control slides for insitu-hybridization staining : Positive cells nuclear stained with Positive control (Human DNA) Biotinylated DNA probe (pusy cytology smear of submandibular swell) 40X

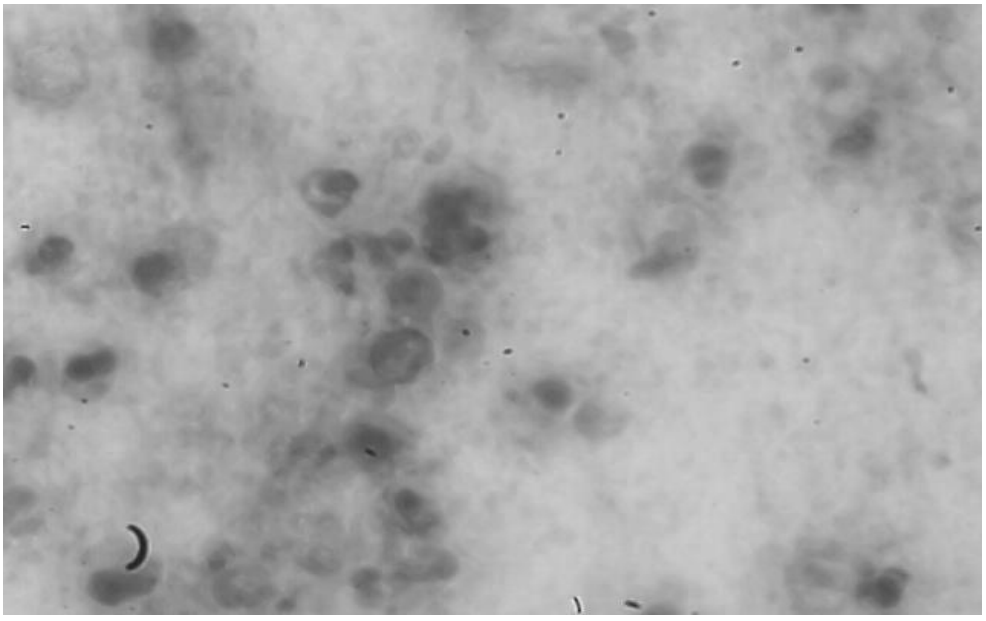

Figure 5 – A HPV insitu-hybridization staining (wide spectrum HPV Biotinylated DNA probe) cervical smears: Photograph (10) positive nuclear blue signals 40X

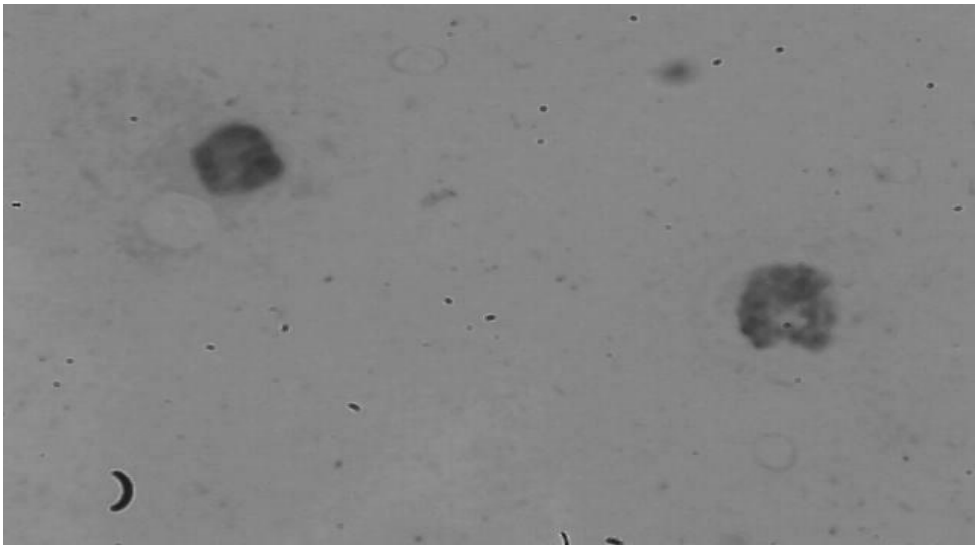

Figure 5 – B HPV insituhybridization staining (wide spectrum HPV Biotinylated DNA probe) cervical smears: positive cytoplasmic and nuclear signals 10X

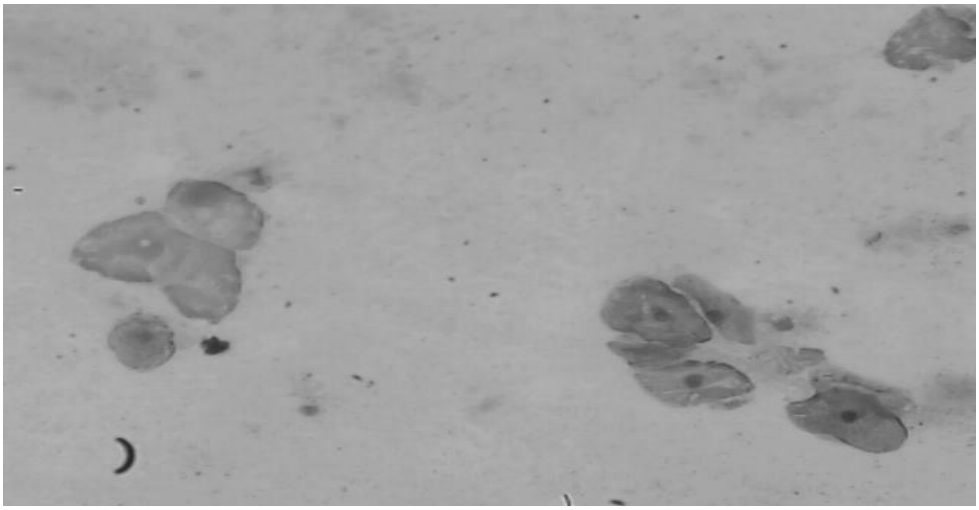

Figure6: frequencies of HPV cytomorphological finding in Pap stained cervical smears (n97)

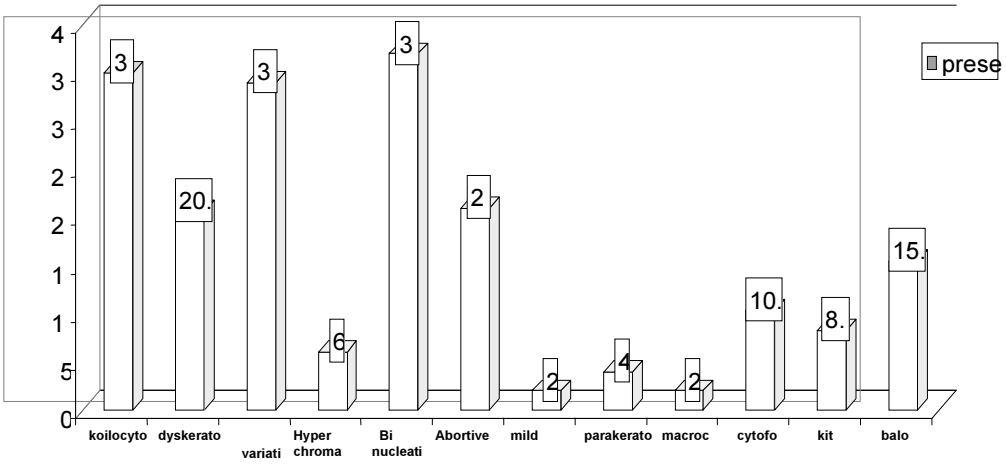

Figure7: Classical cytomorphological changes of HPV in Pap stained cervical smears (n 97)

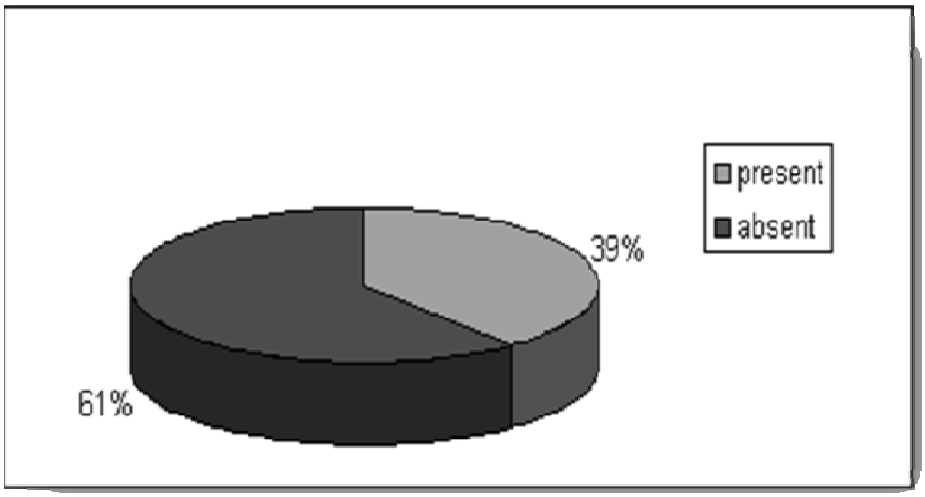

Figure 8: immunocytochemical staining positivity for HPV antigen in cervical smears (n 97)

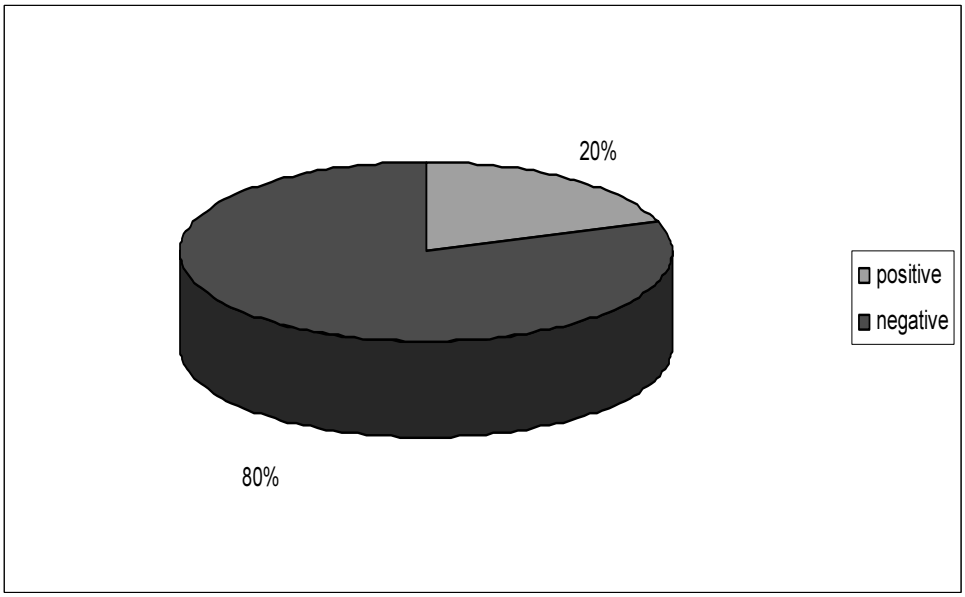

Figure 9: Insitu-hybridization staining findings for HPV DNA in cervical smears (n 97)