Evaluation of urine as a potential alternative sample for the diagnosis of Human Papillomavirus (HPV) related cervical lesions: A pilot study

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Abstract: Background: Despite the high prevalence of both human papillomavirus (HPV) infections and cervical cancer ,the ability to test for HPV infection of the uterine cervix is limited by a lack of an easy sample collection method that does not require gynecological examination**.** This study was done to evaluate urine sample as an alternative easily obtained, non-invasive sample for the diagnosis of HPV cervical lesions. **Patients & Methods:** This study was carried out on 40 female patients who were divided into two groups: **Group I:** comprised 30 female patients with HPV related cervical lesions (flat condyloma) (histologically diagnosed in bunch biopsy). Their ages ranged from 21 to50 years (mean=35.8±12.4). **Group II:** comprised 10 female patients complaining of symptoms or have a colposcopic picture similar to that of flat condyloma but were negative for HPV changes by histological examination. Their ages ranged from 23 to 46years (mean= 32.7±8.2).For the 40 subjects of the study, detection of HPV-DNA in urine samples was done using nested real time PCR. Results: HPV-DNA was detected in urine of 66.7% of HPV pathologically positive cases (group I) and 30% of HPV pathologically negative cases (group II). Detection of HPV DNA in urine samples significantly correlated to the diagnosis by pathological examination (p=0.042).Also HPV-DNA was detected in urine of 75% of age group 20-40 years in group I, and in urine of 100% of the same age group in group II. This rate is higher than the other age groups. However no statistical significance was found. **Conclusion:** Urine being easy non-invasive sample, acceptable by most of people, may allow rapid screening and diagnosis of HPV infection. It can also be valuable in follow up of women with cervical dysplasia. This will allow the diagnosis of HPV infection. without need for pelvic examination and invasive samples either for Pap testing or biopsy taking. Also urine testing for HPV-DNA may be used for epidemiologic purposes especially in population where it may be difficult to obtain genital specimens for religious or cultural reasons or in children or young adolescents especially before vaccination. It can also be valuable in follow up of women with cervical dysplasia.

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Introduction

Human papilloma viruses (HPVs) are DNA tumor viruses which induce epithelial cell proliferation during the course of a productive infection. Cancer of the uterine cervix is still common in the developing world and is responsible for approximately 15%of cancers in females and a lifetime risk of about 3%. *Abdel-All et al., (2007)* reported that five out of six women with cervical cancer are from developing countries, and 80% of them are diagnosed at advanced stages. HPVis the most common cause of cervical cancer and cervical cancer represents the second most common cancer after lung cancer, affecting women of different age groups with a prevalence of about 20% in young sexually active women. (**Rabia Faridi** et al 2011).

Screening for cervical cancer is accomplished by use of a yearly pelvic examination and a Papanicolaou (Pap) smear, which detects abnormal cellular changes or premalignant lesions of the cervical tissue *(Parkin et al., 1999 and Sellors et al., 2000)*.However, the efficacy of the Pap smear as a screening method of cervical cancer has been criticized due to substantial false negative rates and low participation rates. Strategies for preventing cervical cancer in these countries must overcome barriers such as inadequate medical infrastructure and poor rates of participation in screening programs *(Sellors et al., 2000)*. Therefore, a

screening method of cervical cancer bypassing an invasive colposcopic examination would provide a greatly increased chance of participation.

Urine can be contaminated by desquamated HPV infected cervical cells *(Jacobson et al., 2000 and Song et al., 2007).* Molecular biological methods to detect DNA in urine are routinely used to diagnose common sexually transmitted diseases (STDs) such as Chlamydia trachomatis *and* ,Neisseria gonorrhoeae . Also HPV-DNA could be detected with high sensitivity in urine samples. *(Song et al., 2007)*. The HPV types identified in urine samples in most cases represent the same HPV type infecting the cervical epithelium. This suggest that urine may be a practical sample for testing of HPV urogenital infection. *(Jong et al., 2008)*.

The aim of this study :to evaluate the urine as a non invasive, easily collectable sample for the diagnosis of HPV related cervical lesions as an alternative method for the invasive colposcopic examination.

PATIENTS and Methods:

The present study was carried out on 40 female patients who were divided into two groups: **Group I:** comprised 30 female patients with HPV related cervical lesions (flat condyloma). Their ages ranged from 21 to 50 years (mean= 35.8 ± 12.4).These patients were selected among women attending the early cancer detection unit (Cytodiagnostic unit) in the Gynaecology and Obstetrics departments, Ain Shams University hospitals from May (2008) till December (2009). Diagnosis of each of these selected HPV infected patients was done by colposcopic examination done by expert staff members of the Gynaecology and Obstetrics Departments, Ain Shams University Hospitals. The following signs were recorded: blood vessel appearance, leukoplakia, punctation and mosaic appearance. Detection of acetowhite areas was done by local application of acetic acid to the suspected regions. The Schiller iodine test was done by application of iodine to the cervical mucosa; abnormal cells (e.g. CIN) do not absorb the reagent and do not stain. A bunch biopsy from the suspected lesions was taken and examined histologically for changes pathognomonic for HPV infection (koilocytic atypia).

Group II: comprised 10 female patients complaining of symptoms or have a colposcopic picture similar to that of flat condyloma but were negative for HPV changes by histological examination. Their ages ranged from 23 to 46 years (mean= 32.7 ± 8.2).

From each patient of both groups the following data were recorded: Personal history including: name, age, residence, occupation, marital status, parity, and number of marriages. The presenting symptoms of the patient were also recorded. Also, history of risk factors for HPV infection including smoking, immune deficiency condition (e.g diabetes mellitus, receiving immunosuppressive therapy, and recurrent infections), regular vaginal douches, history of contraception, other sexually transmitted diseases and symptoms of the partner were included.

Exclusion Criteria: Each patient having current bleeding or using vaginal douches or sexual intercourse 24 hours before the collection of specimen was excluded from the study.

Sample Collection and Processing From the 40 subjects of the present study, 7-10 ml of first voided urine were collected in clean universal containers and were stored at -20ºC till examined. Each urine sample was centrifuged in an Eppendorf at 3000 rpm for 20 minutes. The supernatant was then discarded and the deposit was suspended and then washed three times with 1 ml of phosphate buffer saline (PBS). **The obtained cell pellets were subjected to** Extraction of the DNA using the generation DNA purification capture column kit (Gentra,USA).

DNA amplification and detection: was done by Light Cycler 2.0 System (Roche, Germany) for DNA amplification using SYBR Green I dye as detection format.

Nested real time Polymerase Chain Reaction (PCR) for the detection of HPV-DNA was done by using two sets of HPV amplification primers (Pharmacia, Biotech, Sweden Denmark), MY09/MY11 primer set (primary PCR) *(Manos and Wright, 1999)* and the GP5+/GP6+ primer set (secondary PCR) *(Husman et al., 1995)* ***Positive control (HPV-16 DNA):***was kindly provided in the form of DNA cloned in pBR322 plasmid; by Professor Ethel-Michele de Villiers, Virology Department, Heidelberg University (Germany). ***Negative control*** was DEPC treated water .The software of the Light Cycler 2.0 System was adjusted to perform the following protocol*s (Fuessel Haws et al.,2004)*. *first PCR*: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute then annealing at 55°C for 1 minute 72 ◦C for 1 min. This was followed by a final extension at 72 ◦C for 10 min, and then storage at 4 ◦C,. **second PCR**: initial denaturation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 45 seconds then annealing at 48°C for 4 seconds, 38 ◦C for 30 seconds, 42° for 5 seconds, 66° for 5

seconds and 71° for 1.5 minutes. This was followed by a final extension of 10 min at 72 ◦C, and then storage at 4 ◦C.. Melting Curve for amplicon analysis was done (denaturation at 95°C, annealing at 65°C for 10 s, melting at 95°C).The amplification curves, melting curves and melting peaks were recorded by the instrument. A sample was considered positive when giving a melting peak at 78º as that was given by the positive control.

Statistical analysis: Analysis of data was done by IBM computer using statistical program for social science (SPSS) version 12. Quantitative variables were expressed as mean ± standard deviation (SD). Descriptive statistics: Mean, standard deviation, minimum, maximum and range of numerical data and frequency and percentage of non-numerical data. Student's t test was used to test the difference about mean values of some parameters (for continuous variables).Correlation (Pearson correlation coefficient r) assessing strength and direction of the linear relationship between two variables. Chi-Square test χ^2 was used to test the difference between variable distribution (for categorical data). Results were considered significant when *p* value was ≤ 0.05.

Results

As regards the demographic and clinical data recorded, None of the patients was smoker, had other STD or reported symptoms in her partner.Most of the patients of the present study were married for once. They were all multiparous. Regarding the risk factors for HPV infections 46.7% of group I and 60% of group II did not use contraception, and 20% of group I and 10% of group II had diabetes mellitus. The most prevalent symptoms among group I and II was the presence of vaginal discharge (50% and 40% respectively). The most common colposcopic findings were the appearance of acetowhite areas in 60% of group I and 70% of group II; followed by positive Schiller iodine test in 50% of both group I and group II. This was followed by blood vessel congestion representing 50% and 40% in group I and II respectively. The curves in figures (1 and 2) represent the results of the detection of HPV-DNA in tested urine samples of the present study as recorded by the Light Cycler instrument.

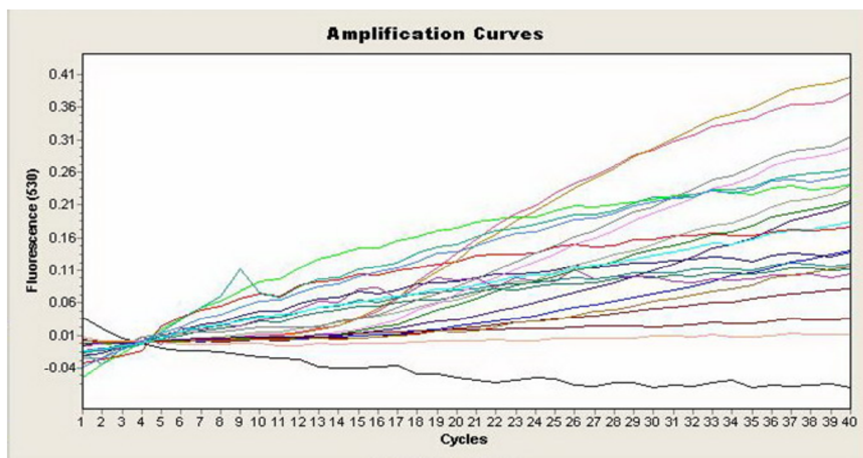

Figure (1) :The amplification curves of different cases of the study.

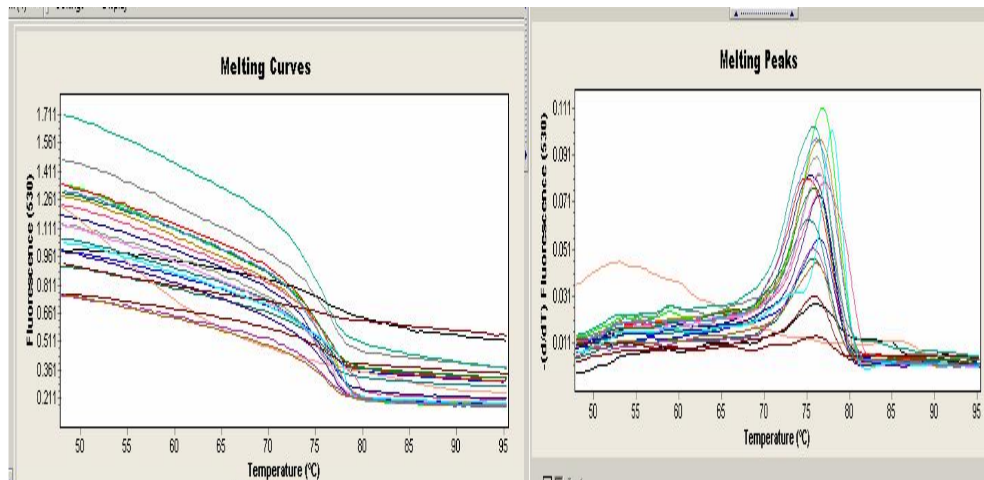

Figure (2) The melting curves and melting peaks of different cases of the study

HPV-DNA was detected in urine of 66.7% of HPV pathologically positive cases (group I) and 30% of HPV pathologically negative cases (group II)(Fig. 3).By comparison between the two methods for diagnosis of HPV infection using Chi square test, it was found that detection of HPV-DNA in urine samples significantly correlated to the results of pathological examination (p = 0.042).The sensitivity of HPV-DNA detection in urine by PCR compared to pathological diagnosis was 66.67% and specificity was 70%. Positive predictive value was calculated as 86.9% and negative predictive value was 41.2%.

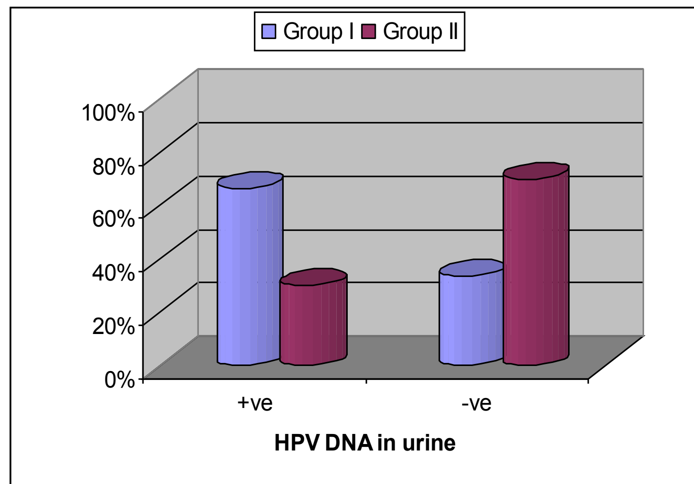

Figure (3): Detection of HPV-DNA in urine by PCR among the patients of the present study.

HPV-DNA was detected in urine of 75% of age group 20-40 years. This rate was higher than the other age groups. However no statistical significance was found.

By comparing patients +ve for HPV-DNA in urine and those –ve for HPV-DNA in urine regarding different parameters: all cases of group I with positive HPV-DNA in urine were married. No much difference between +ve and –ve cases as regards taking contraception, parity (all of them were multigravida), risk factors, symptoms and signs except for punctuation was recorded in 50% of +ve cases and only in 20% of the –ve cases. All cases of group II with positive HPV-DNA in urine were married. No much difference between both groups in other parameters except for the mosaic appearance which was recorded more in +ve cases (33.3%) ,while in only(14.3%) of –ve cases . Vaginal douches and leukoplakia were negative in both groups. However no statistical significance was found for all the parameters .

Discussion

In the present study the first voided urine samples were collected from each patient of both groups for the detection of HPV-DNA by nested PCR. Collection of urine has the advantage that it is noninvasive and is readily accepted in clinical practice. The other advantage is a simultaneous detection of other common sexually transmitted diseases that affect the cervix, such as Chlamydia trachomatis and Neisseria gonorrhoeae infections *(Song et al., 2007)*.This is in contrast to the pathological examination used routinely for the diagnosis of HPV infection which requires the expertise of appropriately qualified and suitably equipped personnel and is relatively expensive and less readily accessible in developing countries *(Stanczuk et al., 2003)*.Therefore, a reliable screening method which is noninvasive and comfortable would increase the participation rate. Perhaps the use of urine sample for the detection of HPV could provide a preliminary screening for cervical cancer and thus circumvent the need for an annual Pap smear for women who are negative for HPV *(Jacobson et al., 2000 and Prusty et al., 2005)*. *Jacobson et al., (2000)* stated that urine HPV test would identify a majority of women with CIN even though substantial proportion of the women without CIN would also be positive for high risk HPVs. On the other hand, a negative urine HPV assay for high risk HPVs would strongly indicate normal cervical cytology.

In the present study, to remove the inhibitory substances from urine samples ,the urine samples were frozen at -20ºC. After thawing the specimens were diluted in PBS then centrifuged. Wash in PBS was repeated three times. Also prior to extraction the pellet was boiled in PBS(*Stanczuk et al., 2003 ,Brinkman et al., 2004* and *Prusty et al., 2005).* **In the present study** detection of HPV-DNA in urine was done by using a real time nested PCR protocol using degenerate broad spectrum primers; MY09 and MY11. The nested PCR was done using the GP5+ and GP6+. This protocol is a commonly used protocol that is documented to detect a broad spectrum of HPV types *(Jacobos et al., 1997 and Van den Brule et al., 2002 and Golijow et al., 2005)*. The rate of detection of HPV DNA in urine was compared with the pathological examination of cervical bunch biopsies for both group I and II. HPV-DNA was detected in urine of 66.67% of HPV pathologically positive cases (group I) and 30% of HPV pathologically negative cases (group II). It was found that the detection of HPV-DNA in urine samples was significantly correlated to the results of pathological examination.

These results agreed with those of study done by *Jacobson et al., (2000)*, who compared the prevalence of HPV detection in urine and cervical samples obtained from sexually active young women. They found higher prevalence of HPV in cervical samples. The sensitivity of the urine PCR assay for detecting cervical HPV was 82%. The concordance between the two samples for specific HPV types was 40%. The authors explained the higher prevalence of HPV in cervix to be due to the fact that HPV in urine largely represents passive carryover of HPV infected cells from the genital tract. They suggested that urine testing for HPV DNA might prove to be valuable only in certain circumstances, e.g. in epidemiologic surveys in population where it may be difficult to obtain genital specimens for religious or cultural reasons and in monitoring of women with cervical dysplasia. *Also Stanczuk et al., (2003)* investigated the presence of HPVs in urine and cervical swab samples collected from a group of women presented with invasive cervical cancer. They reported that the sensitivity of HPV-DNA detection in urine was 73.5%. The type specific concordance between cervical and urine was 79%.

On the other hand, a lower sensitivity of urine testing for HPV DNA was reported by *Sellors et al., (2000)* who detected HPV-DNA in the urine of 44.8% of cases compared to (62.1%) in self collected vulvar swabs, (86.2%) in self collected vaginal swabs and 98.3% in cervical brush samples collected by the physicians. However in the same study, they reported that urine is the most acceptable sample by the women, as 98.4% of the studied women found urine sample acceptable compared to 92.9% for vulvar sampling and 88.2% for vaginal sampling. They recommended further evaluation of self collection of samples as a screening test for cervical cancer prevention programs.

Besides, other studies showed higher sensitivities of urine testing than the present study; of these *Prusty et al., (2005)* who reported a 100% agreement of results on HPV type and positivity between urine and cervical scrapes or biopsies. Thus they suggested that the use of self-collected urine in the form of dry paper smear for easy storage and transport and a single tube-processing opens up a new noninvasive approach for largescale population screening of HPV infection and risk assessment for follow-up management of women and monitoring prophylactic/therapeutic HPV vaccination of young-adolescents or infants in whom use of invasive approaches is not only difficult but also ethically as well as socially

unacceptable. In another study conducted by *Payan et al., (2007),* the concordance for HPV DNA between cervical and urine specimens was excellent (93%). Thus, they concluded that urine samples will allow mass screening of patients with HPV infection.

In the present study it was found that among the 10 patients of group II who were **negative for H**PV by pathological examination; 30% were positive for HPV-DNA in urine samples by PCR. *Vossler et al., (1995)* detected HPV-DNA in 3 (50%) of the women whose cytologic or histologic results were negative. Similarly, *Jong et al., (2008)* detected HPV in urine samples of 81.5% of 27 HIV infected women, compared to 51.5% from their cervical smears. The higher HPV prevalence in urine samples was due to the use of SPF10 for HPV-DNA detection. This method is a very sensitive method that could be well used in paraffin as well as in urine. They also added that urine is a homogeneous material that is simple to collect in contrast to cervical smear samples.

The foregoing studies showed that the use of urine sample for the detection of HPV-DNA could be a considerable method and the difference in the rate of detection could be due to the difference of the methods used.

In the present study detection of HPV-DNA in urine was compared to the pathological diagnosis. The results revealed that the sensitivity of HPV-DNA detection by PCR in urine samples was 66.67% and specificity was 70%. Positive predictive value for the test was 86.9% and negative predictive value was 41.2%. However, **false** positive and false negative results of cervical pathology were reported by some authors. This can be considered as another factor in the evaluation of using urine samples.Many studies done by *Salvia et al., 2004, and Adams et al., 2006* indicated the importance of the presence of other test with histological examination to avoid the false results.

In the present study **different data** were recorded from each patient including personal data, history of risk factors for infection, also the presenting symptoms and colposcopic signs of the patients. In an attempt to draw clinical criteria of the positive HPV-DNA cases diagnosed by examination of urine samples, the following was observed: In group I: HPV-DNA was detected in urine of 75% of age group 20-40 years. This rate is higher than the other age groups. All positive HPV-DNA in urine cases were married. No much difference between positive and negative cases as regards taking contraception, parity (most of them are multigravida), risk factors, symptoms and signs except for punctuation .In group II: HPV-DNA was detected in urine of 100% of age group 20-40 years. All cases positive for HPV-DNA urine were married. No much difference between positive and negative cases in other parameters except for the mosaic appearance which was recorded more in positive cases.However, no statistical significant difference was detected, and these criteria were not discussed by the other authors before.

CONCLUSION

The results raise "the real possibility that self-testing for HPV urogenital infection, using urine, will one day give low- income countries an opportunity to implement cost-effective, practical, and 'women-friendly' cervical cancer screening programs. Urine as an easy non-invasive sample ,acceptable by most of people, will allow the diagnosis of HPV infection without need for pelvic examination and invasive samples either for Pap testing or biopsy taking. Also urine testing for HPV-DNA may be used for epidemiologic purposes especially in population where it may be difficult to obtain genital specimens for religious or cultural reasons or in children or young adolescents especially before vaccination.It can also be valuable in follow up of women with cervical dysplasia.

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