High HPV load and sexually transmitted infections increase the risk of abnormal cervical cytology in HIV-infected women in India

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Sexually transmitted infection (STI) is a major public health problem in human immunodeficiency virus (HIV)-infected individuals. This study evaluated the effect of STIs, such as herpes simplex virus type-2 (HSV-2) and Chlamydia trachomatis (CT) on human papillomavirus (HPV) copy number and associated cervical cytological abnormalities in context of HIV infection. Cervical cells from 74 HIV seropositive and 50 seronegative women were examined for HPV, HSV-2 and CT DNA by PCR. HIV infected women had higher HSV-2 (P=0.002) and HPV infection (P=0.001) in cervix. HPV 16 was detected as the most predominant genotype. Combination of HIV and other STIs (HSV-2 and CT) was associated with higher HPV prevalence in cervix (P<0.01). Cervical HPV viral load (VL) was increased in HIV infected subjects co-infected with STIs compared to those with only HPV infection (P=0.008). Women with abnormal cervical cytology had higher HPV copy number/cell compared to those with normal cytology (P<0.001). In conclusion, STIs may not have direct effect on cervical cytological abnormalities, they increase HPV VL that in turn worsen cervical cytological complications in HIV infected women. Therefore, screening of STIs in HIV infected high-risk Indian women may be important to evaluate HPV burden and abnormal cervical dysplasia.

Keywords: Cervix, Chlamydia trachomatis (CT), Herpes simplex virus type-2 (HSV-2), HSV, Human immunodeficiency virus (HIV), Human papillomavirus (HPV), STI, Viral load

Human Immunodeficiency Virus (HIV) is a major global public health issue. World Health Organization (WHO) has estimated 36.7 million HIV positive cases worldwide by the end of 2016 with 1.8 million people becoming newly infected in 2016 alone. Africa has been reported to be the most affected, with 25.6 million cases, almost two thirds of the total global infections3. According to National AIDS Control Organization (NACO), more than 2.1 million people in India are infected with HIV in 20165. HIV infection, apparently has no cure. However, it can be effectively controlled and prevented from transmission by antiretroviral (ARV) drugs. Although highly active antiretroviral therapy (HAART) has declined drastically AIDS related morbidity and mortality, not all people have access to antiretroviral drug due to high cost, particularly in developing counties like India. As a result, India is still suffering from high prevalence of HIV and has the third largest HIV epidemic in the world5. Acquired Immuno-deficiency Syndrome (AIDS), the most advanced stage of HIV infection, which takes 2-15 years to reach, is defined by the development of certain cancers or other severe clinical manifestations. In other words, the immunosuppressive conditions in HIV infected subjects increase susceptibility of acquiring other opportunistic infections (OIs) and developing cancer, particularly cervical3,4.

Cervical cancer is one of the leading causes of cancer deaths worldwide. In 2017, USA reported an estimated 12820 new cases of invasive cervical cancer and death toll of the 4210 due to cervical cancer. It remains the second most common cancer among women, particularly of age group 20-39 years5,7. It is reported to be the leading cause of cancer death among women in sub-Saharan Africa8. In India, cervical cancer has been reported to cause 20.7% fatalities7. Another study reports approximately 132000 new cases and 72000 deaths due to cervical
cancer each year in India accounting for 26% of the 275000 deaths worldwide\(^9\). The alarming situation of cervical cancer underscores the need to improve screening rates and increase the acceptance of and access to human papillomavirus (HPV) vaccination\(^5\). The persistent HPV infection is the major cause of developing cervical cancer and its precursor lesions. Carcinogenic HPV 16, 18, 31, 33, 35, 45, 52 and 58 are associated with more than 90% of all invasive cervical cancer (ICC)\(^10,11\), and hence termed as ‘high risk’ HPV. HIV-infected patients are at higher risk of having HPV\(^12,13\). HPV 16 and 18 have been reported as the most prevalent HPV types in HIV positive patients\(^14\). Studies have shown that individuals living with HIV are more susceptible to acquisition of multiple types of HPV with greater risk of HPV related diseases and are less likely to clear the virus\(^15,16\). Persistent HPV infection results in progression of cytological and histological abnormalities in the epithelial cells of cervix from normal to cervical intraepithelial neoplasia (CIN) to ICC\(^17\). However, occurrence of such abnormalities in cervix and neoplasia are more pronounced in women co-infected with HPV and HIV\(^18,19\). High HPV load also increases the persistence of HPV infection\(^20\) and the risk for development of cervical lesions\(^21\). However, no such data are available from India.

STIs remain as major public health problem in HIV-infected individuals. According to Centers for Disease Control and Prevention (CDC), individuals infected with STIs are 2-5 times more susceptible for acquiring and transmitting HIV. On the other hand, presence of HIV increases the prevalence and virulence of other STIs. Syphilis, herpes, chlamydia and gonorrhea have been shown to be common in HIV-infected individuals\(^4,22\). The co-infection of HIV and other STIs have been observed in association with symptomatic and asymptomatic reproductive tract infections such as genital ulcer and cervicitis\(^23,24\). However, little information is available on the effect of microenvironmental factors in cervix, such as HSV-2, Epstein-Barr Virus (EBV) and CT, on HPV VL and cytological changes in HIV-infected women in India.

In the present study, we tried to determine the prevalence of multiple types of HPV and other STIs, such as HSV-2 and CT in HIV-infected Indian women and evaluate the effect of such co-infections on their cervical cytology.

**Materials and Methods**

**Study subjects**

The study included 124 women (age between 14 and 54 years; mean age = 24.3 years) attending School of Tropical Medicine, a National AIDS Control Organization (NACO) approved referral center of Kolkata, India. They were referred mostly by different state hospitals and some non-government organizations (NGOs) because of suspicion of having high risk behaviour for acquiring HIV infection and/or unexplainable clinical symptoms. The participants (74 HIV positive and 50 HIV negative) in this study were either commercial sex workers or spouses of HIV-infected men. Most (87%) of the women were from low socioeconomic status. All of them provided their informed consent for participating in this study. They were interviewed to obtain relevant socio-demographic information viz. age, ethnic group, marital status, education (number of years of formal education) and number of lifetime partners through structured questionnaire. Procedures followed for this study were in accordance with the ethical standards of the institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

**Polymerase chain reaction (PCR), Pap testing and silver staining**

Cervicovaginal lavages (CVL) were obtained from the women and immediately placed on ice. After centrifugation at 1500 rpm for 10 min in cold, cellular pellets were separated from the CVL supernatant. A part of the cervical cell sample was smeared on glass slides for Pap staining and the rest were stored at −80°C as dry pellets for DNA extraction. The cervical cell pellets were lysed by proteinase K digestion and DNA was extracted by phenol-chloroform and ethanol precipitation method. Initially, a 268 bp fragment of cellular β-globin gene (house-keeping gene) was amplified to test adequacy of the target DNA. HPV DNA was detected by using MY09/11 primers. DNA of HPV positive cases was amplified further with HPV 16 and 18 type-specific primers\(^25,26\). Epstein-Barr virus (EBV), HSV-2, CT DNA was detected using suitable primers\(^27,29\). Cervical smears were assessed by standard Pap staining.
The HPV positive (by MY09/11 primers) samples were retested by a quantitative low stringency PCR using general primer GP5/6 to co-amplify the specific HPV DNA along with DNA sequences from human genome. Varying concentrations of HPV 16 plasmid DNA were used as standards. The PCR products were electrophoresed on 8% polyacrylamide gel and bands were visualized by silver staining. The bands corresponding to HPV and human genome fragments were quantitated by densitometry. The amplicons were diluted to get exact quantification. The logarithm of the ratio between intensities of these two bands is directly proportional to the logarithm of the amount of HPV DNA in individual samples.

**Statistical analysis**

The data were analyzed using STATA (version 13.0) statistical software. Pearson chi-square test, odds ratio and 95% confidence interval was tested to ascertain the association between HIV prevalence and sociodemographic variables. The correlation of HPV load with cytology and HIV infection was analyzed by multivariate logistic regression analysis. Test of proportion was applied for categorical variables to assess any significant difference in the prevalence rate of STIs among HIV seropositive and seronegative women. Yates correction was applied for any cell value <5.

**Results**

Table 1 depicts the sociodemographic variables with respect to HIV serostatus of the women. HIV infection was significantly greater in sexually active young subjects and was most frequent (38/53, 71.7%) among women in the age range 20-29 years. Although, most of the women in this study were Hindu, no significant association between any ethnic group and HIV infection was found. HIV infection varied from 57 to 68% in multiple ethnic groups. Marital status also had no significant impact on HIV infection in this population. Less educated (<10 years) subjects were found to be at significantly higher risk for this viral infection. Compared to the HIV seronegatives, a higher percentage of seropositive women had a history of multiple lifetime partners (20/24, 83.3% vs. 4/24, 16.7). Together, women in sexually active age group, with less education and uncontrolled sexual behavior were found to be at highest risk of contracting HIV.

HIV infected women are usually at higher risk of having other OIs. To examine how HIV seropositivity alters the prevalence of other STIs in local genit al environment, we assessed the prevalence of HPV, HSV-2 and CT DNA in the cervical cells. EBV was also included in this study as a non-STI (negative control), which was previously detected in cervix of HIV infected patients. The prevalence of HPV genotypes and other STIs detected in cervix of women with respect to their HIV serostatus is summarized in Table 2. HIV positive subjects were at

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of women (n = 124)</th>
<th>HIV+ (n= 74)</th>
<th>P value</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
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<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;19</td>
<td>36</td>
<td>22 (61.1)</td>
<td>0.04</td>
<td>1.09 (0.46-2.60)</td>
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<tr>
<td>20-29</td>
<td>53</td>
<td>38 (71.7)</td>
<td>0.02</td>
<td>2.46 (1.08-5.65)</td>
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<tr>
<td>30-39</td>
<td>28</td>
<td>12 (42.8)</td>
<td>0.03</td>
<td>0.41 (0.16-1.05)</td>
</tr>
<tr>
<td>&gt;40</td>
<td>7</td>
<td>2 (28.5)</td>
<td>0.08</td>
<td>0.25 (0.03-1.54)</td>
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<tr>
<td>Ethnic group</td>
<td></td>
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<td></td>
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<tr>
<td>Hindu</td>
<td>89</td>
<td>51 (57.3)</td>
<td>0.39</td>
<td>0.70 (0.29-1.70)</td>
</tr>
<tr>
<td>Muslim</td>
<td>25</td>
<td>17 (68.0)</td>
<td>0.34</td>
<td>1.57 (0.57-4.41)</td>
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<tr>
<td>Others</td>
<td>10</td>
<td>6 (60.0)</td>
<td>0.98</td>
<td>1.01 (0.24-4.58)</td>
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<tr>
<td>Marital status</td>
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<tr>
<td>Married</td>
<td>93</td>
<td>57 (63.3)</td>
<td>0.52</td>
<td>1.30 (0.53-3.20)</td>
</tr>
<tr>
<td>Unmarried</td>
<td>31</td>
<td>17 (54.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Education (Years*)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>33</td>
<td>28 (84.8)</td>
<td>0.005</td>
<td>5.48 (1.8-17.83)</td>
</tr>
<tr>
<td>10-15</td>
<td>72</td>
<td>42 (58.3)</td>
<td>0.71</td>
<td>0.88 (0.40-1.93)</td>
</tr>
<tr>
<td>&gt;15</td>
<td>19</td>
<td>4 (21.0)</td>
<td>0.0001</td>
<td>0.13 (0.03-0.47)</td>
</tr>
<tr>
<td>Multiple lifetime partners</td>
<td></td>
<td>24</td>
<td>20 (83.3)</td>
<td>0.07</td>
</tr>
<tr>
<td>* Years of formal education</td>
<td></td>
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significantly ($P=0.002$) higher risk of having HSV-2 infections in cervix compared to HIV negatives. Overall, HPV was detected at a significantly ($P=0.001$) higher rate in the HIV positive (49/74, 66.2%) than in the HIV negative women (19/50, 38%). HPV positive samples were further analyzed for two oncogenic viral genotypes, HPV 16 and 18. A greater proportion of HIV infected subjects were positive for HPV 16 (16/49, 32.7%), compared to HIV negative subjects (3/19, 15.8%). In contrast, prevalence of HPV 18 was similar in both HIV positive and HIV negative subjects (15/49, 30.6% vs. 5/19, 26.3%). Dual infections of HPV 16 and 18 were more common among HIV positive patients than HIV negative women. However, HPV types other than HPV 16 and 18 were detected more significantly ($P=0.0007$) in cervix of HIV seronegative patients than among the seropositive women. EBV ($P=0.205$) and CT ($P=0.75$) infections were not significantly associated with HIV infections in this population.

Next, we analyzed whether presence of HIV and/or other STIs (HSV-2 and/or CT) has any relationship with HPV prevalence in cervix. A total of 80 women were positive and 44 women were negative for STIs irrespective of their HIV status. Presence of STIs increased the risk of HPV prevalence irrespective of HIV infection. For instance, 67.5% (54/80) of STI positive women were HPV infected, whereas only 31.8% (14/44) were infected with HPV among STI negative subjects ($P=0.019$). However, highest prevalence (42/53, 71%) of HPV ($P <0.001$) was observed in cervix of patients infected with both HIV and other STIs (Table 3, Fig. 1A). Control women without HIV or other STIs were found to be least susceptible for HPV infection (7/23, 30%). Infection with either HIV or STI is associated with relatively low level of HPV infection (7/21, 33% and 12/27, 44%, respectively) compared to dual positive patients.

Since high HPV VL has been associated with development of cervical neoplasia, we assessed whether HIV and STI individually or in combination could have an effect on HPV VL. HPV positive samples were classified according to viral copies/cell as follows: <1 to 10 copies/cell as low, 11-100 as moderate and >100 as high. Table 3 shows the distribution of HPV VL (low to high) in subjects with and without HIV and other STIs. Multiple logistic regression analyses showed that HIV or STIs individually had no significant effect ($P=0.1417$ and 0.2049, respectively) on HPV VL; however, women with combined infections with HIV and other STIs were more prone ($P=0.0079$) to have high HPV VL (Table 3 and Fig. 1B). For instance, most of the women with HIV and other STIs had HPV VL in the higher range of 10-100 or >100 copies/cell. HPV copy number was found to be in the lower range (1-10 copies/cell) in patients with HSV-2 and/or CT in absence of HIV. The mean copy number of HPV was highest (~313 viral copies/cell) among patients with both HIV and other STIs and patients having none of the infections had lowest mean HPV copy number (~8 viral copies/cell) (Fig. 1B). These results suggest that HIV along with other STIs increase HPV prevalence as well as HPV copy number/cell.

To examine whether HIV infection concomitant with HPV infection has any role on cytological abnormalities in cervix, the cervical cells were tested...
by PAP staining. Fig. 2 (A-D) represents some of these cytological abnormalities characterizing rounded cells, eccentric nuclei, nuclear enlargement, multi-nucleation and perinuclear halo known as koilocytosis. HPV infection was significantly associated with abnormal cytology, that includes presence of atypical squamous cells of undetermined significance (ASCUS), cervical dysplasia ($P=0.001$). Among women with abnormal cytology, 67.5% (25/37) were HPV positive and 18.9% (7/37) were HPV negative; whereas in the normal cytology group, 27.6% (24/87) were HPV positive and 13.8% (12/87) were HPV negative (Table 4). Similarly, significant correlation was found between HIV infection and cytological abnormalities ($P=0.027$). Although presence and absence of HIV were comparable in women with normal cytology (52.9%; 46/87 and 47.1%; 41/87, respectively), most of the patients with abnormal cytology were HIV positive (75.7%; 28/37) compared to HIV negative (24.3%; 9/37). Dual infection of HIV and HPV further increased the risk of abnormal cytology (Table 4). Presence of either HSV-2 or CT or both in cervix did not have significant effect on cervical cytological abnormalities.

To determine the relationship of HPV VL with abnormal cervical cytology, we analyzed abnormal cytology with respect to mean copy number of HPV (Fig. 3A). Women with abnormal cervical cytology had higher HPV copy number/cell compared to those with normal cytology (mean copy number/cell 482.2 vs. 39.6). HIVinfected patients with cytological abnormality had the highest HPV load. Women with abnormal Pap smears had mostly moderate to high HPV copy numbers, whereas those with normal cytology had largely low copy numbers and very few of them had moderate or high viral burden (Table 4). Finally, to assess whether HPV types have any specific role in determining the cervical cytology status, we analyzed the distribution of HPV types in both cytological normal and abnormal groups (Fig. 3B). No significant association between any of the HPV types and abnormal cytology was observed. Together, HPV VL is one of the determining factors regulating the risk of cervical cytological abnormalities.

### Discussion

This is the first study that investigates the role of cervical microenvironmental factors, such as HPV, HSV-2 and CT in developing cytological abnormalities in HIVinfected women in India. In doing so, we first analyzed risk factors of HIV infection in Indian women. We found that women in the sexually active age, especially between 20 and 29 years, were at the highest risk for HIV infection. The women in this study were from high risk group for acquiring HIV, as they were either sex workers or spouses of HIVinfected patients and from low...
socioeconomic group. Lack of socioeconomic resources comprising of education, occupation and income is linked to practice of riskier health behavior leading to contraction of HIV. Our study supports and extends similar results reported in India and other countries. Various factors, such as less education, poverty, social circumstances and the increased reach of internet in the rural areas of India force young women to become sexually active at young age. In our study population, the risk of acquiring HIV infection increased further when these women became commercial sex workers or spouse of an HIV infected person having multiple partners. As a result, most of the women with multiple partners were HIV positive.

HIV seropositivity was associated with increased prevalence of multiple sexually transmitted pathogens in cervix due to similarity in transmission processes. In this study, HSV-2 was detected significantly ($P=0.002$) at higher rate in the HIV seropositive subjects compared to seronegative subjects. Most of our participants had HIV infected sexual partners who had more probability to transmit STIs due to increased shedding of such STIs in their genital secretions. We detected a higher prevalence (32%) of EBV in the HIV infected women than earlier studies reporting 10 to 21%. The prevalence of CT was similar in both the HIV positive and negative groups (20.2 and 18.0%, respectively). Slightly higher (10%) rate of CT infection in HIV infected women has been reported compared to HIV uninfected women (6.6%) in Africa.

In comparing high risk HPV types in HIV infected and uninfected women, we found that HPV 16 was more prevalent in HIV infected women than uninfected women whereas prevalence rate of HPV 18 was very similar in both groups. This is consistent with a previous study where HPV 16 and 18 were detected as the most common genotypes in HIV infected and uninfected subjects and prevalence of both the HPV types were similar in HIV positive and negative patients. Although we found higher rate of double infection of HPV 16 and 18 (26.5%) in HIV positive women compared to that in HIV negative women (10.5%), this difference was not statistically significant. Prevalence of concomitant infection of multiple HPV genotypes in HIV infected women varied considerably in the literature. While a study by Lupi has reported 23% co-infections, Carcino et al. reported as high as 45% co-infections with multiples HPV types. Such variation in data may be attributed to different methodologies used for HPV detection, different study population, country where the study was done and the number of subjects studied.

HPV infection has been reported to be strongly associated with abnormal cervical cytology. Globally, HPV has been implicated in 99.7% of cervical carcinoma. We detected HPV DNA in 93.9% of women with abnormal cytology. In contrast, only 40.2% of patients with normal cervical cytology were found to be HPV positive. An earlier study detected high risk HPV types in 25% of women with normal cervical cytology and in more than 80% of women with abnormal cytology. High risk HPVs especially HPV 16 and HPV 18 increase the risk of cytological abnormalities in cervix and were found to be more common in high-grade squamous intraepithelial lesions (HSIL). In our study subjects, the HIV prevalence in women having abnormal cervical smears was significantly ($P <0.01$) higher (75.7%) than those with normal cervical smears (51.2%). Different studies have shown that HIV infected women have higher rate of CIN than HIV negative patients. It has been shown that incidence of CIN is significantly higher in patients with CD4 count <200 cell/mm$^3$, a measure of HIV disease progression.

We analyzed HPV viral burden using PCR technique to correlate with cervical cytology in the presence of HIV/other STIs. Our data showed that HPV DNA load had a correlation with cervical cytology independent of HIV and other infection status. Subjects with abnormal cervical cytology had higher HPV VL compared to those with normal cytology. The association of higher HPV burden with high-grade cervical lesions has been demonstrated by several investigators. However, our study is possibly the first to report an association of high HPV load with abnormal cytology in Indian women in context of HIV infection. Most of the earlier studies have shown increasing cervical lesions to be associated with higher HPV VL. Measurement of HPV 16 viral burden could quantitate only a portion of the HPV infected samples and the effect of other oncogenic HPV types might not be ascertained. However, our quantitative method showed overall
HPV viral burden irrespective of the HPV types present in the samples. HIV infection is directly related to higher HPV burden. Such increased load in HIV positive women could be due to lack of clearance and input of larger number of HPV infected cells. Large number of HIV infected women in our study with abnormal cytology had high viral burden. Weissenborn et al. also showed elevation of HPV VL in HIV infected women with severe cervical dysplasia. Thus, higher HPV VL among HIV positive women with abnormal cervical cytology in our study raises the possibility that HPV load may be used as a biomarker for the risk of progression of cervical disease amongst HIV positive women.

Presence of other cervical STIs along with HIV increased HPV VL further, while STIs alone had no direct effect on the HPV VL. This increase of HPV burden in patients with HIV and other STIs could be due to increased HIV replication in response to infection with other STIs. STI-induced immune-suppression has been proposed to augment susceptibility to HIV infection or progression of HIV disease, which in turn increases the HPV load. Thus, presence of STIs may have an indirect effect on HPV VL.

Conclusion

Overall, our results indicate that HIV infection is significantly associated with the prevalence of high risk HPV types and HSV-2 in cervix. Furthermore, presence of HIV and other STIs enhance HPV VL in cervix. Such high HPV VL in HIV seropositive women increases the risk of cervical cytological abnormalities. Controlling STIs would be a simple and relatively inexpensive public health measure, especially in underdeveloped or developing countries to slow the HIV epidemic, and hence reduce the HPV burden and thereby lower cervical cancer incidences.

References


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