Anal human papillomavirus infection: a comparative study of cytology, colposcopy and DNA hybridisation as methods of detection

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Abstract
Objective—To compare anal cytology, colposcopy and DNA hybridisation as methods of detecting anal HPV infection.

Subjects and Design—Patients attending: (1) a genitourinary medicine (GUM) clinic with ano-genital warts; (2) a surgical out-patient department with anal fissure or haemorrhoids were examined for evidence of anal HPV infection.

Results—Considering GUM clinic attenders, 17% (38/225) and 40% (90/225) had perianal or anal canal warts respectively. Colposcopic examination revealed anal acetowhite lesions without warts in 28% (63/225). Cytological evidence of HPV infection was found in 98%, 83%, and 90% of patients with anal canal warts, perianal warts and acetowhite lesions respectively. Anal intraepithelial neoplasia (AIN) was documented in 22% of patients with anal canal warts compared with 6% with perianal warts (p < 0.01). HPV DNA was detected from the anal brushings of 71%, 50%, 32%, and 29% of patients with anal canal warts, perianal warts, acetowhite lesions and a normal anal examination respectively. HPV type 6/11 was detected in the majority of HPV positive samples.

Considering surgical out-patient attenders with no history or signs of anal warts, 25% showed cytological evidence of anal HPV infection and HPV DNA was detected from anal brushings in 3% (2/71).

Conclusion—Anal examination with the colposcope is a useful method for detecting subclinical HPV infection. Anal cytology may prove helpful for detecting AIN, however, since koilocytosis was rarely seen, the specificity of the cytological criteria for anal HPV infection in the absence of AIN is uncertain. DNA analysis of anal brushings proved only moderately sensitive.

Introduction
Genital warts are one of the commonest sexually transmitted diseases in the United Kingdom. The number of new cases seen at departments of genitourinary medicine in England and Wales in the decade 1976 to 1986 rose by a factor of 2.8 in males and 3.6 in females.1 In recent years clinicians have appreciated that, in addition to producing clinically obvious lesions, human papillomavirus (HPV) may result in subclinical infection. Cytology has proved a useful method for detecting subclinical HPV infection of the uterine cervix and the characteristic cellular changes produced by HPV are well documented.2

Colposcopy has the advantage of allowing direct visualisation of HPV infected epithelium. Following topical application of an acetic acid solution the characteristic features of subclinical cervical, vaginal, vulval, perianal and penile HIV infection may be readily identified by the experienced colposcopist.3 Recent reports have documented an association between HPV type 16 infection, anal squamous cell cancer4,5 and anal intraepithelial neoplasia.6-13 Current knowledge, however, of the epidemiology and clinical manifestations of anal HPV infection is limited. Detection of subclinical anal infection by anal cytology has been reported but confirmation of results by other methods, such as DNA hybridisation, has not been performed. This is important as the specificity of cytological changes at sites other than the cervix is uncertain. Although the colposcope has been used to identify anal dysplasia,11 the feasibility of using the colposcope to detect subclini-
These specimens were then snap frozen to the Cytobrush with no clinical abnormalities, from the anal verge and acetowhite epithelium. A further 5% of examined anal brushings from the anal canal and anal verge were collected for subsequent HPV analysis by DNA hybridisation. All subjects underwent naked eye examination of the perianal area and anal canal; examination by colposcope was not performed.

**Subjects and methods**

**Study group**

Patients attending a department of genitourinary medicine with ano-genital warts which were newly diagnosed or untreated in the previous three months were recruited into the study. Basic demographic data were obtained by direct questioning.

Anal cytology was performed on all subjects by blindly inserting a Cytobrush approximately 1\(\frac{1}{2}\) centimetres into the anal canal. This allowed sampling of the anal squamo-columnar junction (transitional zone), an area predisposed to dysplastic change. Material was smeared on to glass microscope slides and immediately fixed in 96% alcohol. All cytology smears were stained by the Papanicolaou method and examined by one observer (GK). The adequacy of anal smears was established by the presence of columnar and/or metaplastic epithelium. Morphological criteria used for establishing the presence of HPV infection were the same as those used in assessing cervical smears. They were based on the presence of koilocytes, keratinisation including parakeratosis and dyskeratosis, binucleation and multinucleation and anucleate squames which all reflect late gene expression of HPV. The assessment of intraepithelial neoplasia was based on the presence and degree of nuclear changes (dyskaryosis).

The perianal area was examined by naked eye and then with the colposcope before and after application of 5% acetic acid solution. Colposcopic findings were recorded and categorised as: (a) normal (no abnormalities detected); (b) condylomata acuminata present; (c) acetowhite epithelium only—no condylomata visible. All subjects then underwent proctoscopy with detailed examination of the anal canal by naked eye and then by means of the colposcope. Clinical findings, before and after application of acetic acid, were recorded as described previously for the perianal area.

Further brush samples were taken from condylomata and acetowhite areas or, in the case of subjects with no clinical abnormalities, from the anal verge and anal canal. These brush samples, together with the Cytobrush used for obtaining anal cytology, were immediately placed into Eppendorf tubes containing 0.5 ml of phosphate buffered saline (PBS), vortexed to dislodge cellular material and the brush discarded. These specimens were then snap frozen and stored in liquid nitrogen whilst awaiting processing for DNA hybridisation.

Biopsies were taken from some of the subjects with perianal or anal canal acetowhite lesions for histological examination.

**Comparison group**

Patients with an anal fissure or haemorrhoids attending a surgical out-patient department were recruited for comparative study. These patients had no past or present history of abnormal cervical cytology, genital warts or other sexually transmitted disease. Basic demographic data were recorded on all subjects. Anal cytology was performed as described previously and cytobrushings from the anal canal and anal verge collected for subsequent HPV analysis by DNA hybridisation. All subjects underwent naked eye examination of the perianal area and anal canal; examination by colposcope was not performed.

**DNA hybridisation technique**

Anal brush samples previously stored in liquid nitrogen were thawed and centrifuged at 3000 rpm for 6 minutes to obtain a cell pellet. The supernatant PBS was removed with a fine pipette and the cells lysed with 200 \(\mu\)l of 0.4 M sodium hydroxide at 80°C for 10 minutes. Following the lysis procedure the cellular debris was collected at the base of the tube by centrifugation at 3000 \(g\) for 3 minutes. The supernatant containing cellular DNA was loaded on to a nylon membrane and hybridised, according to the method described by McIntyre and Stark, with cloned HPV DNA 32P labelled probes of types 6, 11 and 16. CaSki cells (containing HPV 16 DNA) and purified HPV type 6 and 16 DNA were positive controls, HeLa cells (containing HPV 18 DNA) and C33 cells (a mouse cell line devoid of HPV DNA) were negative controls.

**Results**

The study group comprised 225 subjects—110 females, 64 heterosexual males and 51 homosexual males of mean age 26, 25 and 30 years respectively. Heterosexual males and females reported a mean number of two sexual partners in the previous year and the mean age of first sexual intercourse was 19 years. Anal warts were documented in 128 (57%) subjects. Thirty eight (17%) had perianal warts only and the remaining 90 (40%) subjects had anal canal warts with or without perianal warts. A further 63 (28%) subjects without anal warts had colposcopically visible areas of acetowhite anal epithelium consistent with HPV infection. The findings on anal examination in relation to sex and sexual orientation are shown in table 1. Anal canal warts were found more frequently than perianal warts alone in heterosexual and homosexual males (\(\chi^2\) test
p < 0.001) and was the commonest lesion in this latter subgroup. A total of 33 subjects (15 anal warts; 11 anal acetowhite lesions; 7 normal anal examination) were seen on more than one occasion and had repeat samples taken. The findings on anal cytology (repeats included) in relation to anal clinical findings are shown in table 2. Smears, including repeats, were considered inadequate in 13% (13/102) of subjects with anal canal warts, 15% (6/41) of those with perianal warts only and 22% (16/74) with anal acetowhite lesions only. Considering adequate smears, there was cytological evidence of anal HPV infection in 98% (87/89), 83% (29/35) and 90% (52/58) of the same groups respectively. Thirty four subjects had only genital warts (penis/vulva) and showed no abnormalities on anal examination. Seven underwent a repeat examination after one month which was again normal. Of the adequate anal smears obtained from this colposcopically normal group, 81% (26/32) showed features consistent with HPV infection.

Excluding repeat examinations, the anal smears from 167 subjects showed cytological features of anal HPV infection of which 13% (22/167) showed additional dyskaryotic cells suggesting an underlying anal intraepithelial neoplasia (AIN). Excluding repeat samples, features suggestive of AIN were found in 22% (17/79) of the adequate smears taken from the group with anal canal warts compared to 6% (2/32) of subjects with perianal warts and 4% (2/49) with anal acetowhite lesions (χ² test p < 0.01). Regarding sex and sexual orientation, AIN was reported in 28% (13/47—8 AIN I; 5 AIN II) of the adequate anal smears from homosexual males compared to 6% (5/91—3 AIN I; 2 AIN II) and 7% (4/56—3 AIN I; 1 AIN II) of smears from females and heterosexual males respectively (χ² test p < 0.001). Three homosexual males were HIV antibody positive and three HIV antibody negative. The remaining subjects were of unknown HIV serostatus.

Biopsies were taken of 46 anal acetowhite lesions from 34 subjects. Eighty five per cent (39/46) showed classical histological features of HPV infection, with additional evidence of AIN in three cases.

Excluding repeat samples (table 3), HPV DNA was found in 71% (64/90), 50% (19/38) and 32% (20/63) of the anal brushings taken from subjects with anal canal warts, perianal warts and acetowhite lesions respectively. In total HPV DNA was found in 54% (103/191) of colposcopically detected anal HPV lesions (warts/acetowhite). HPV DNA was detected in 29% (10/34) of brushings taken from subjects with a normal anal examination. No statistically significant association was found between HPV type and the clinical appearance of anal lesion. As shown in table 3, the predominant HPV type detected in all lesions was type 6/11.

Excluding inadequate smears (table 4), HPV DNA was found in 52% (75/145) of all patients with cytologically detected anal HPV infection (without AIN) and in 82% (18/22) of patients with cytological evidence of AIN in association with HPV infection. HPV DNA was found in 37% (7/19) of those with negative cytology. HPV type 6/11 was detected in the majority of DNA positive samples, as shown in table 4.

The “normal” comparison group comprised 71 subjects (39 females and 32 heterosexual males) of mean age 34 years, with a mean number of one sexual partner in the previous year and a mean age of first sexual intercourse of 18 years. None had evidence of anal condylomata on examination. Anal cytology was inadequate in 14 cases. Of the remaining 57 adequate smears, 25% (14/57) showed cytological changes consistent with HPV infection. HPV DNA was found in the anal brushings from two subjects (one hybridised with the type 6/11 HPV DNA probe, the other with type 16).

### Table 1 Anal clinical findings in relation to sex/sexual orientation

<table>
<thead>
<tr>
<th></th>
<th>Normal anal examination</th>
<th>Anal acetowhite lesions</th>
<th>Anal canal warts</th>
<th>Perianal warts only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>21 (19%)</td>
<td>35 (31%)</td>
<td>27 (25%)</td>
<td>27 (25%)</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>13 (20%)</td>
<td>25 (39%)</td>
<td>21 (33%)</td>
<td>5 (8%)</td>
</tr>
<tr>
<td>Homosexual</td>
<td>0</td>
<td>3 (6%)</td>
<td>42 (82%)</td>
<td>6 (12%)</td>
</tr>
</tbody>
</table>

*Repeat samples included; inadequate samples excluded.
†Anal intraepithelial neoplasia.
Table 3   HPV DNA type and anal clinical findings

<table>
<thead>
<tr>
<th>HPV DNA Type</th>
<th>Normal anal examination</th>
<th>Anal acetowhite lesions</th>
<th>Anal canal warts</th>
<th>Perianal warts only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>24</td>
<td>43</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>Type 6/11</td>
<td>7 (70%)</td>
<td>17 (85%)</td>
<td>54 (84%)</td>
<td>17 (89%)</td>
</tr>
<tr>
<td>Type 16</td>
<td>3 (30%)</td>
<td>0 (0%)</td>
<td>4 (6%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>Type 6/11 + 16</td>
<td>0 (0%)</td>
<td>3 (15%)</td>
<td>6 (9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>63</td>
<td>90</td>
<td>38</td>
</tr>
</tbody>
</table>

Numbers excluding repeat samples—percentages relate to DNA positive samples.

Discussion

Subclinical HPV infection has been well documented for the cervix, vagina and vulva but only more recently has attention focused on the anus and anal canal. In the present study, patients with anal warts were subdivided into those with perianal warts only and those with anal canal warts with or without perianal warts. This is an important distinction when comparing findings on anal cytology, which was obtained by gently brushing the anal canal. Our observation that anal canal warts were more common than perianal warts alone in homosexual and heterosexual males is of interest and difficult to explain in the heterosexual male. Details of duration of infection were not recorded and hence there is a possibility that heterosexual men may have had warts for a longer period of time thus increasing the chance of spread to the anal canal. Another possible explanation concerns selection bias. Clinic doctors were asked to refer all patients with a new episode of warts to the research clinic; however, if only those heterosexual men with more extensive anal warts were referred this would inevitably lead to a bias towards patients with anal canal lesions. Further studies are required to clarify this point.

Although detection of HPV infection by means of anal cytology has been previously described, sensitivity has been disappointing. Haye et al found cytological evidence of infection in only 42% of patients with anal warts and Frazer et al reported positive cytology in 70% of patients, results much lower than our detection rates of 98% and 83% for patients with anal canal and perianal warts respectively. Since we used comparable cytological criteria for diagnosing HPV infection, our higher detection rates may be methodological, possibly the result of using a brush to sample the anal canal rather than a wooden spatula or cotton wool swab.

Previous studies have reported positive anal cytology in 33% and 58% of patients without anal warts. Although this has been considered indicative of subclinical infection, the possibility that this may merely reflect poor cytological specificity has not been addressed. We suspect both factors may be relevant. Subclinical HPV infection may be identified as areas of epithelial whitening following the application of an acetic acid solution. Using this technique we have documented colposcopically visible subclinical anal HPV infection in 28% of our study group. Ninety per cent of these subjects demonstrated positive anal cytology. However, 81% of study group subjects with a colposcopically normal anal examination and 25% of our “normal” comparison group also yielded positive anal cytology. Since HPV DNA was detected in only 65% of patients with anal warts (perianal + anal canal) and 32% with acetowhite lesions our DNA hybridisation results do not help to resolve the question of anal cytology specificity. Our DNA hybridisation technique appears less sensitive than the in situ hybridisation method described in a previous study in which HPV DNA was found in 87% of anal warts. However, our findings are comparable to the results of in situ filter hybridisation which documented HPV DNA in 62% of genital warts. Our use of samples obtained by brushing lesions may, in some cases, have provided insufficient material for adequate DNA extraction and subsequent hybridisation. In addition, the sensitivity of DNA hybridisation will be influenced by the HPV genome copy per cell. It is worth noting that of the 34 study group patients with a colposcopically normal anal examination, 29% had HPV DNA detected.

In the present study the sensitivities of anal cytology and DNA hybridisation with respect to anal canal warts were found to be 98% and 71% respectively. The sensitivity of cytology with respect to DNA hybridisation was 93%. As mentioned previously, cytological specificity cannot be ascertained from the current data. Although koilocytosis is considered pathognomonic of HPV infection this was rarely seen in anal smears, the most commonly encountered cellular changes being parakeratosis and

Table 4   A comparison of anal cytology and HPV DNA findings

<table>
<thead>
<tr>
<th>HPV DNA Type</th>
<th>Negative 12</th>
<th>HPV infection 70</th>
<th>AIN 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 6/11</td>
<td>6 (86%)</td>
<td>63 (84%)</td>
<td>16 (89%)</td>
</tr>
<tr>
<td>Type 16</td>
<td>1 (14%)</td>
<td>6 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>Type 6/11 + 16</td>
<td>0</td>
<td>6 (8%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>145</td>
<td>22</td>
</tr>
</tbody>
</table>

Repeat samples and inadequate cytologies excluded. Percentages relate to DNA positive samples.
binucleation. This observation is in agreement with a previous study.48 Future studies using more sensitive methods of DNA detection, such as the polymerase chain reaction, together with histological examination may provide more accurate information by which to evaluate the specificity of cellular changes other than koilocytosis for detecting HPV infection.

It is important to note that although subclinical anal HPV infection may demonstrate typical colposcopic features, less typical lesions require biopsy for histological confirmation. Fifteen per cent of biopsied lesions in the present study failed to show histological features of HPV infection, a finding in keeping with previous studies of subclinical penile HPV infection.21

Cytological features suggestive of anal intraepithelial neoplasia (AIN) of grades I to II were found in a significantly greater number of homosexual males (28% of adequate smears) compared with heterosexual males (7%) and females (6%). No smears showed severe dyskaryosis suggesting AIN III. The reported prevalence of cytologically diagnosed AIN amongst homosexual males has ranged from 3%7 to 39%6 and a more recent study has documented AIN histologically in 30% of anal warts in a group of homosexual and heterosexual men.16 Although AIN has been documented in patients without anal warts,8,18 we found only one patient with AIN in whom there were no clinical features of HPV infection; 86% had anal warts and 9% had colposcopic evidence of subclinical infection. HPV type 6/11 was found in all DNA positive samples from subjects with AIN, in two cases associated with HPV 16. This is in keeping with recent studies which have documented a high prevalence of HPV 6/11 in AIN I and II.11,13 In addition, we have documented HPV 6/11 in 93% of the DNA positive brushings from anal canal and perianal warts, a finding in agreement with previous studies.10,12,13 Of some interest was our finding HPV type 6/11 in all DNA positive acetonewhite lesions, a result which contrasts with a previous study reporting a high prevalence of HPV types 16/18 in subclinical lesions.22

In conclusion, we have found that anal examination with the coloscope is a useful method for detecting subclinical HPV infection. Anal cytology may prove helpful as a screening method for detecting anal intraepithelial neoplasia; however, the specificity of the cytological criteria for anal HPV infection in the absence of AIN requires further study. Although DNA analysis of anal brushings proved only moderately sensitive, this does provide a non-invasive method of detecting and typing for HPV and therefore warrants further assessment, possibly in conjunction with DNA amplification techniques such as the polymerase chain reaction.23

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