Association of serum and mucosal neutralizing antibodies to human papillomavirus type 16 (HPV-16) with HPV-16 infection and cervical disease

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We investigated neutralizing antibodies to human papillomavirus type 16 (HPV-16) in serum and cervical washes from 84 women with normal cytology or cervical disease. Serum neutralizing antibodies were detected in 78 % of women infected at the cervix with HPV-16, compared with 35 % (P=0.002) of women infected with HPV-16-related types (9 HPV types), 14 % (P<0.0001) of women infected with HPV-16 non-related types and none of HPV-uninfected women. A significant correlation between HPV-16 infection and serum HPV-16-neutralizing antibodies was observed (r=0.97; P=0.032). Cervical neutralizing antibodies were detected in 38 % of women with HPV-16 infection and in 17 % of women infected with the HPV-16-related type HPV-31. Cervical neutralizing antibodies correlated with HPV-16 infection (r=0.95; P=0.08), but not with cervical disease. Serum and cervical HPV-16 antibody responses were not affected significantly by human immunodeficiency virus type 1 infection. In conclusion, serum and cervical HPV-16-neutralizing antibodies were found to correlate with HPV-16 infection, but not with cervical disease.
Correlation of neutralizingAbs with HPV-16 infection

investigation and informed written consent was obtained from all women. Their ages ranged between 22 and 62 years (mean, 35 years). Twenty of the 84 women (24%) were negative for cervical disease [no cervical intraepithelial neoplasia (CIN)] by colposcopy and histology, and either they had cleared their cervical disease (as determined by cytology) by follow-up, or the primary cytological diagnosis was not supported by histology. Twenty-seven of the 84 women (32%) had histologically confirmed CIN 1 and 37 of 84 had CIN 2/3 (44%). Serum samples were collected from the 84 women and cervical washes were available from 43 of 84. DNA was extracted from cervical cells by using a QIAamp DNA mini kit (Qiagen) and HPV typing was performed by using the Roche reverse linear array HPV genotyping assay (kindly supplied by Dr Janet Kornegay, Roche Molecular Systems, Alameda, CA, USA), according to the manufacturers’ instructions. HPV-16 VLPs for ELISA were kindly supplied by MedImmune Inc. HPV-16 antibodies were determined from serum samples diluted 1 : 100 by direct polymer ELISA and from cervical washes diluted 1 : 5 by capture polymer ELISA, as described previously (Marais et al., 2006). Serum antibody responses were considered positive at cut-off points of \( A_{492} = 0.3 \) for IgG and \( A_{492} = 0.039 \) for IgA. Total IgA and IgG levels were determined for cervical samples as described previously (Marais et al., 2001). Cervical HPV-16 IgA and IgG responses were standardized to the total IgA and IgG to account for the fluctuation of antibody levels in cervical secretions during the menstrual cycle according to Rudin et al. (1998). Cervical antibody responses were considered positive at cut-off points of \( A_{492} = 0.05 \) for IgG and \( A_{492} = 0.06 \) for IgA. These were confirmed by viewing scatter-plot analysis of the \( A_{492} \) values obtained for both IgA and IgG (not shown).

The production of pseudovirions and the neutralization assay were performed essentially as described by Pastrana et al. (2004). Serum or cervical wash was diluted (twofold dilutions) and pseudovirion stock was used at a 1 : 20 000 dilution. We used H16V5 monoclonal antibody (provided by Neil Christensen, Penn State Milton S. Hershey Medical Center, PA, USA) as a positive control and neutralizing buffer as a negative control. For detection of SEAP in supernatants, 125 \( \mu l \) supernatant was transferred into 96-well non-treated tissue-culture plates and clarified by centrifuging at 1000 \( g \) for 5 min. Detection of SEAP in 15 \( \mu l \) clarified supernatant was performed by using a Great EscAPe SEAP detection kit (BD Biosciences) according to the manufacturer’s instructions. After the addition of substrate, plates were incubated for 20 min at room temperature and read on a Digene DML 2000 microplate luminometer. Positivity was defined as the highest dilution of serum that reduced SEAP activity by \( \geq 50\% \) in comparison to the reactivity in the wells that received pseudovirions, but no monoclonal antibody/serum/cervical wash. Any serum antibody titres of \( <1:5 \) dilution were taken to be negative, as it has been reported that low serum dilutions (from 1 : 10 to 1 : 40) might display non-specific inhibition of pseudovirus activity (Pastrana et al., 2004). Cervical antibody titres of \( <1:5 \) dilution were taken to be negative. Statistical analyses were performed by using a paired \( t \)-test (GraphPad Prism); a Mann–Whitney \( U \)-test was applied for independent sample comparisons and a \( \chi^2 \) test was applied for prevalence comparisons. Spearman’s correlation coefficient (\( r_s \)) was calculated by using a non-parametric correlation (GraphPad Prism). \( P \) values of \( \leq 0.05 \) were considered statistically significant.

To determine whether the detected HPV-16 antibodies correlated with active HPV-16 infection, women were stratified according to those who had cervical HPV-16 infections (27 of 84, 32%), those infected with HPV-16-related types (29 species; 26 of 84, 31%), those infected with other HPV types (22 of 84, 26%) and those with no detectable HPV infection (nine of 84, 11%). Serum HPV-16-neutralizing antibodies were detected in 78% (21 of 27) of women infected with HPV-16, compared with 35% (nine of 26; \( P=0.002 \)) of women infected with HPV-16-related types, 14% (three of 22; \( P<0.0001, \chi^2 \) test) of women infected with other types and no HPV-negative women (Table 1). Women infected with HPV-16-related types and those infected with other HPV types showed no significant difference in serum neutralizing-antibody prevalence (\( P=0.09; \chi^2 \) test). A significant correlation between HPV-16 infection and serum HPV-16-neutralizing antibodies was observed (\( r_s=0.9; P=0.032 \)). There was a statistically significant difference in the magnitude of the serum neutralizing-antibody responses, as determined by the intensity of the relative light unit reading, in HPV-16-infected women compared with women infected with HPV-16-related types (\( P=0.026, \) Mann–Whitney \( U \)-test), as well as compared with those infected with other HPV types not related to HPV-16 (\( P=0.002; \) Fig. 1). There was no statistically significant difference between women infected with HPV-16-related types and women infected with other HPV types (\( P=0.193 \)). The high prevalence (78%) of serum HPV-16-neutralizing antibodies in HPV-16-infected women in this study was similar to that reported by Pastrana et al. (2004). The presence of HPV-16-neutralizing antibodies in women infected with HPV-16-related types possibly indicates cross-neutralizing activity between HPV-16-related types. HPV-33 (an HPV-16-related type) has been shown to share neutralizing epitopes with HPV-16 (White et al., 1998); also, there is evidence that vaccination with HPV-16/18 vaccine shows cross-protection against HPV-31 (an HPV-16-related type) (Harper et al., 2006). The detection of antibodies in HPV-16-uninfected women could indicate earlier cervical HPV-16 infection or HPV-16 infection at another site (Marais et al., 2001). The stability of antibodies may decrease over time. Viscidi et al. (2005) reported that ten women who were HPV-16-seropositive at the baseline, but HPV-16 DNA-negative at the cervix, were not protected from HPV-16 reinfection during the follow-up period, suggesting a change in the ability of the
antibodies to neutralize the virus over time, and thus the failure to neutralize the virus (Viscidi et al., 2005).

To investigate whether the detected HPV-16 antibodies correlated with cervical disease status, the women were stratified according to CIN grade (CIN 1, CIN 2/3 or no CIN, based on colposcopy and histology). Serum HPV-16-neutralizing antibody prevalence was observed to be high in women with normal cytology (45%, nine of 20) compared with women with CIN 2/3 (41%, 15 of 37), but the differences were not statistically significant (Table 1). Thus, neutralizing antibodies did not correlate with cervical disease severity. The magnitude of neutralizing-antibody titres also showed no correlation with disease severity (data not shown). The high serum HPV-16-neutralizing antibody prevalence observed in women with cervical disease suggests that HPV-16-specific neutralizing antibodies did not correlate with a better cervical disease outcome. This was in contrast to a report by Kawana et al. (2002), who showed that HPV-16-neutralizing antibodies were associated with CIN regression and were influenced by HPV viral load.

HPV-16-neutralizing antibodies in cervical washes of 43 women were assessed and 19% (eight of 43) had detectable cervical neutralizing antibodies. Six of eight who had detectable cervical neutralizing antibodies were infected with HPV-16 and two of eight were infected with HPV-16-related types. None of women infected with HPV-16 non-related types and HPV-negative women had cervical neutralizing antibodies (Table 2). The correlation of HPV-16 infection and cervical neutralizing antibodies was positive ($r_s = 0.95$), but not statistically significant ($P = 0.08$). Cervical neutralizing antibodies were detected in 39% (seven of 18) of women with CIN 2/3, 8% (one of 12) of women with normal cytology and no women with CIN 1. Cervical neutralizing antibodies did not correlate with cervical disease severity ($r_s = 0.5; P = 1$), but tended to increase with increasing CIN grade. Importantly, cervical neutralizing antibodies also showed a clear association with cervical HPV-16 infection or with infection with closely associated HPV-16 types, and were not found in women infected with HPV-16 non-related types or who were HPV-negative. Cervical HPV-16-neutralizing antibody prevalence was high in women with high-grade cervical disease, indicating that cervical HPV-16-specific neutralizing antibodies did not correlate with a better cervical disease

![Fig. 1](image-url)

**Fig. 1.** Magnitude of serum HPV-16-neutralizing antibodies in women infected with HPV-16, HPV-16-related types ($\geq 9$ species), HPV-16 non-related types and HPV-negative women. HPV-16-neutralizing antibody-negative samples were given a zero value. There was a statistically significant difference between women infected with HPV-16 and HPV-16-related types ($P = 0.026$, Mann–Whitney U-test) and between women infected with HPV-16 and other HPV-16 types not related to HPV-16 ($P = 0.002$, Mann–Whitney U-test).
neutralizing antibodies was found to decline with age, even when the aged 22–30-year-old women and further to 50% (two of four) in those aged 31–39 years, decreasing to 80% (12 of 15) in 31–39-year-old women, and neutralizing antibodies were detected in 47% (20 of 43) of serum and 19% (eight of 43) of cervical samples. The prevalence of serum antibodies was examined further with age, from 55% in the younger women to 48% in those aged 31–40 years (22), 31–39 years (24 of 56; P = 0.006). Systemic and local cervical neutralizing-antibody responses were compared, but found not to correlate significantly within individuals (r = -0.1; P = 0.36; paired t-test).

The prevalence of serum antibodies was examined further according to the age of the participants: 22–30 years (n = 22), 31–39 years (n = 46) and ≥40 years (n = 16). Neutralizing antibodies declined gradually in prevalence with age, from 55% in the younger women to 48% in those aged 31–39 years and 25% in women aged ≥40 years; these prevalences were not found to be statistically significant. It was postulated that this decline in prevalence could be associated with the decreasing prevalence of cervical HPV-16 infection with age, from 36.4% in the 22–30-year-old women to 33% in the women aged 31–40 years and 25% in the older women (≥40 years). Analysis of serum neutralizing-antibody prevalence with age was then restricted to HPV-16-infected women, and a prevalence of 88% (seven of eight) was found in 22–30-year-old women, decreasing to 80% (12 of 15) in 31–39-year-old women and further to 50% (two of four) in those aged ≥40 years. So, the prevalence of neutralizing antibodies was found to decline with age, even when the analysis was limited to women that were cervically HPV-16-infected, supporting the above theory. We reported previously (Marais et al., 1997) that serum IgG antibody prevalence decreases with age. According to our previous study (Marais et al., 1997), older women shed fewer HPV particles than younger women, leading to lower expression of structural proteins (HPV L1/L2) to elicit antibodies and thus, possibly, to lower levels of neutralizing antibodies. Different production of hormones at various ages may also influence antibody responses (Marais et al., 1997; Sonnex, 1998).

As the prevalence of human immunodeficiency virus type 1 (HIV-1) infection in these women was high, it was important to determine the impact of HIV-1 seropositivity on HPV-16 antibody responses. Twenty-seven of 84 (32%) women in the study were HIV-1-seropositive. Serum neutralizing-antibody responses were not significantly higher in HIV-positive women than in HIV-negative women (44%, 12 of 27 versus 38%, 21 of 56; P = 0.54). Serum IgG responses were significantly higher in HIV-positive women than in HIV-negative women (67%, 18 of 27 versus 43%, 24 of 56; P = 0.04). No significant differences were observed between HIV-positive and HIV-negative women when they were stratified according to cervical HPV infection or cervical disease status. There appeared to be a decreased capacity in HIV-positive women to elicit neutralizing antibodies at the cervix, whereas this was not so for IgG detected by ELISA. It must be noted that the small number of HIV-positive women reduced the power of statistical comparison. It has been reported previously that HIV-positive women have a higher prevalence of serum IgG than HIV-negative women; HPV viral load or viral replication in HIV-positive women may enhance the induction of HPV antibodies (Viscidi et al., 2003).

This study examined the prevalence of cervical and serum HPV-16-neutralizing antibodies in women with cervical

<table>
<thead>
<tr>
<th>Test group</th>
<th>HPV-16-neutralizing antibodies</th>
<th>HPV-16 IgG</th>
<th>HPV-16 IgA</th>
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<tr>
<td></td>
<td>No.* (% )</td>
<td>P value†</td>
<td>No.* (% )</td>
</tr>
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<td>HPV-16</td>
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<td>ref</td>
<td>9/16 (56)</td>
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<td>7/12 (58)</td>
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<td>0.035‡</td>
<td>6/10 (60)</td>
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<td>HPV-negative</td>
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<td>0.120</td>
<td>6/10 (60)</td>
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<tr>
<td>Normal cytology</td>
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<td>ref</td>
<td>5/12 (42)</td>
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<td>CIN 1</td>
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<td>0.23</td>
<td>8/13 (62)</td>
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<tr>
<td>CIN 2/3</td>
<td>7/18 (39)</td>
<td>0.07</td>
<td>12/18 (67)</td>
</tr>
</tbody>
</table>

*Number of women positive for response/total number of participants tested.
†Compared with HPV-16 or normal cytology (ref) by χ² test. Statistically significant P values are shown in bold.
‡Fisher’s exact test.
disease and normal cytology; the prevalences of neutralizing antibodies were also compared with binding HPV-16 IgG and IgA antibodies detected by ELISA. This is the first reported study to compare local cervical and serum neutralizing antibodies detected by the HPV-16 pseudovirus-based neutralization assay. The present study showed that the HPV neutralization assay was a more genotype-specific serological test than the VLP-based ELISA, as the prevalence of serum neutralizing antibodies was significantly higher in women with HPV-16 cervical infection than in women not infected with HPV-16. Whilst serum and cervical HPV-16 neutralizing antibodies were associated with HPV-16 infection, they could not be correlated with cervical disease regression. Serum HPV-16 IgG was associated with HPV infection and cervical disease status ($r_s=0.84$ and 1, respectively). Cervical HPV-16 IgG antibodies correlated positively with cervical disease severity ($r_s=1$), whilst with HPV-16 infection, the opposite was observed ($r_s=-0.95$). Serum HPV-16 IgA responses correlated with HPV-16 infection ($r_s=0.74$), but not with cervical disease severity. Cervical HPV-16 IgA responses were not associated with HPV-16 infection or with cervical disease status.

The neutralization assay will be important in assessing protective HPV humoral responses in natural-history studies and in individuals vaccinated with HPV vaccines. Further information on HPV-neutralizing antibody responses in South African women should be gleaned from longitudinal studies currently under way.

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References


