Serum Antibodies to Human Papillomavirus 16 Proteins in Women from Brazil with Invasive Cervical Carcinoma[1]

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Abstract

Serum samples from 194 cases and 217 controls participating in a case-control study of invasive cervical cancer in Brazil were examined for antibodies to human papillomavirus (HPV) 16 virus-like particles (VLPs) by ELISA. The prevalence of antibody in cases and controls was 47.4 *versus* 24.4% ($P < 0.001$). The prevalence was higher in women who had HPV-16 DNA in the genital tract (54.2%) than in those with other HPVs (36.8%) or no HPVs (44.8%), but the differences were not statistically significant. Among cases and controls, HPV-16 VLP antibodies were associated with a greater number of lifetime sexual partners (χ^2 for trend, $P < 0.001$). Among controls, age was inversely associated with HPV-16 VLP seroreactivity (χ^2 for trend, $P = 0.019$). The sera were previously tested for antibodies to HPV-16 E6 and E7 oncoproteins; there was no correlation between antibody titers to HPV-16 E6 or E7 and VLPs. The HPV-16 serological assays were compared as screening tests for invasive cervical cancer. The sensitivity and specificity estimates were 47.4 and 75.6% for HPV-16 VLP serology, 63.4 and 89.9% for either HPV-16 E6 or E7 serology, and 53.6 and 93.6% for high titers of either HPV-16 E6 or E7 or VLP antibodies. The utility of HPV-16 VLP ELISA as a screening test for invasive cervical cancer is limited by a high seroprevalence in women with probable prior exposure to HVP 16 but without disease.

Introduction

The etiological role of HPV[3] infections in cervical cancer is now well established. The evidence that linked cervical cancers to HPVs is based almost exclusively on analyses of HPV DNA sequences in the uterine cervix. It has been more difficult to identify immunological markers of HPV infections and of HPV-associated invasive cervical cancer. Recently, several investigators have reported that HPV VLPs, prepared by expression of L1 or L1 and L2 genes in baculovirus or vaccinia virus systems, are suitable reagents for the measurement of antibody response to HPV infections in an ELISA (1–3). The antibody response to HPV infections, as measured by ELISA with VLPs, is low titered and type specific but is not detectable in all individuals with documented infections (4–6). In one study, the proportion of infected individuals who develop anti-VLP antibodies varied from 22% in women who had a transient infection to 83% in women who had a persistent infection (7). Higher antibody response is also related to the presence of a higher amount of HPV DNA in the cervix (4, 6).

We have previously reported the results of a case-control study of invasive cervical carcinoma in Brazil in which cervical specimens were examined for HPV DNA and serum specimens were tested for antibodies to HPV-16 E6 and E7 proteins (8). In the present study, we have tested the case and control sera for antibodies to HPV-16 VLPs to relate this serological response to case or control status, HPV type in the cervix, presence of antibodies to E6 and E7 proteins, disease stage, and risk factors for sexually transmitted infections. We also evaluated HPV serology as a screening test for cervical cancer, which could be used in situations in which more sophisticated technology is impractical and cervical specimens are not available.

Materials and Methods

Study Subjects. Serum collection and study design were described in detail in previous reports (8, 9). Briefly, cases were women, age 25–79 years, in Sao Paulo City, with newly diagnosed and histopathologically confirmed invasive cervical cancer and with no previous treatment for the disease. Cases were categorized in clinical stages following the Federation Internationale des Gynaecologistes et Obstetristes classification scheme as stage I ($n = 42$), stage II ($n = 64$), and stage III-IV ($n = 70$). For 18 cases, there was no information on stage of disease. Controls were women recruited from the same hospitals as the cases and were frequency matched to cases in 5-year age groups. Those who were admitted for treatment of a gynecological condition or who had a hysterectomy or conization were ineligible as controls. HPV diagnosis was performed by PCR on samples of exfoliated cells from the ecto- and endo-

cervix collected on gynecological examination, using a combination of general and type-specific primers (10).

Serum specimens were collected at the time of the gynecological examination, stored at $-20°C$, and available for 194 of 199 cases and 217 of 225 controls for this study.

VLP Production and Purification. The baculovirus construct containing the L1 and L2 genes of HPV-16 was kindly provided by J. Schiller (National Cancer Institute, Bethesda, MD). Sf9 insect cells were grown in a spinner flask to a density of $2 \cdot 10^6$/ml at 27°C in 500 ml of Grace's insect media (Invitrogen, San Diego, CA) with 10% fetal bovine serum, containing gentamicin and Fungizone of recommended concentrations. The cells from a 1-liter culture were infected with recombinant baculoviruses at a multiplicity of infection of ~10 determined by plaque assay. After 1 h of incubation at room temperature, the cells were transferred to a total of 20 square tissue culture plates (245 × 245 mm; Nunc, Naperville, IL) containing 100 ml of Grace's complete media with antibiotics in each plate. The infected cells were allowed to grow for 72 h at 27°C in a high-humidity incubator and harvested by gentle scraping with a pipette and pelleted by centrifugation. The VLPs were purified as described by Kirnbauer *et al*. (1). Successful preparation of VLP was confirmed by electron microscopic examination as well as SDS-PAGE.

ELISA. Sera were tested by ELISA for antibodies against HPV-16 VLP as described (6). Briefly, wells of microtiter plates (Corning, Acton, MA) were coated with 50 μl of purified VLP at a concentration of 5 μg/ml in PBS (pH 7.4) and held overnight at 4°C. Fifty microliters of test sera diluted 1:10 in PBS containing 0.5% nonfat milk were added to each of two wells and incubated for 1 h at 37°C. Specific antibodies were detected with horseradish peroxidase-conjugated recombinant protein G (Zymed Laboratories, San Francisco, CA) diluted 1:10,000 in PBS and 0.05% Tween-20 after 30 min of incubation with freshly prepared 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) and hydrogen peroxide solution (Kirkegaard and Perry, Gaithersburg, MD).

Seroreactivity was recorded as absorbance using a microtiter plate reader (Molecular Devices, Menlo Park, CA). The cutoff absorbance for seropositivity was determined by reference to previously tested negative control serum samples obtained from children and college age women at low risk for HPV infection (6). Sera with $A > 0.183$ were scored positive.

Detection of Antibodies to HPV 16 E6 and E7 Proteins. Antibodies were detected by radioimmunoprecipitation of full-length HPV 16 E6 and E7 proteins prepared by *in vitro* transcription and translation as described previously (8).

Statistical Analysis. The distributions of absorbance values were compared for cases and controls by Mann-Whitney U test (Kruskal-Wallis test for more than two groups). Frequencies were compared by χ^2 test or Fisher's exact test (two-tailed). ORs and 95% confidence limits were calculated as approximations of relative risks using unconditional logistic regression analysis. Relationship between VLP and E6 and E7 antibody reactions among cases and controls were analyzed by Spearman's correlation.

Results

Antibodies to HPV-16 VLP in Cases and Controls. The distribution of A values in cases and controls is shown in Fig. 1. The median A values (and interquartile range) for cases and controls were 0.165 (range, 0.099–0.356) and 0.118 (range, 0.080–0.182), respectively. The difference between the two
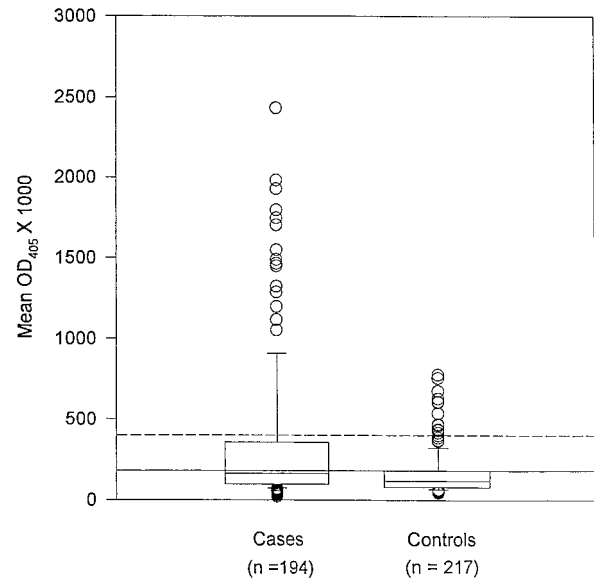

Fig. 1. Seroreactivity in HPV-16 VLP ELISA of 194 cervical cancer cases and 217 controls. The length of each *box* corresponds to the interquartile range, with the *top* boundary of the box representing the 75th and the *bottom* boundary the 25th percentile. The *horizontal line* in the box indicates the median value. The *small bar* at the end of the *line* extending upward from the box plot shows the 90th percentile. Outlier values are shown as *open circles*. The *solid horizontal line* extending across the graph marks the cutoff for positivity, and the *dotted horizontal line* marks the cutoff for strongly positive sera. When the sensitivity and specificity were calculated using various cutoff A values, similar ORs were obtained for case-control comparisons (0.183 A units [chosen cut point], OR, 2.8; 0.200 A units, OR, 2.5; 0.250 A units, OR, 2.4; 0.300 A units, OR, 2.8; and 0.350 A units, OR, 3.5).

distributions was significant by Mann-Whitney test ($P < 0.001$). Fig. 1 also showed that A values of 0.400 and above were found most frequently among cases; 46 of 194 (23.7%) case sera, and 13 of 217 (6%) control sera were strongly positive ($P < 0.001$). A values >0.776 were found exclusively in case sera, in 27 specimens. The cases and controls were also compared for the proportions of sera that were antibody positive (47.4 *versus* 24.4%; χ^2 test, $P < 0.001$; OR, 2.8; 95% CI, 1.8–4.3). HPV-16 seropositivity was associated with cervical cancer even after controlling for HPV-16 DNA status (OR, 2.3; 95% CI, 1.3–4.2).

Antibodies to HPV-16 VLPs by HPV Diagnosis and Case-Control Status. The HPV-16 VLP antibody prevalence was examined by HPV DNA prevalence in the genital tract as well as by case-control status (Table 1 and Fig. 2). Among cases, the antibody prevalence of 54.2% in HPV-16 DNA-positive cases was higher than that of cases that had other HPVs (36.8%) and cases that had no HPVs (44.8%), but the differences were not statistically significant. Among controls, the antibody prevalence was higher for women positive for other HPVs and the same for women positive for HPV-16 DNA and for HPV DNA-negative women. In case-control comparisons, the prevalence in cases was significantly higher than that in controls for women who had HPV-16 DNA in the genital tract (54.2 *versus* 20%; $P < 0.05$) and for women who had no HPV (44.8 *versus* 20%; $P = 0.004$).

We also examined whether the distribution of A values of antibody-positive sera differed by HPV DNA categories. The median A value of HPV-16-positive cases (0.194) was higher than that of cases with other HPVs (0.139) or no HPVs (0.124),

DNA diagnosis	Cases		Controls	
	No. tested	No. (%) positive	No. tested	No. (%) positive
HPV-16	96	52 (54.2)	10	2 (20)
Other HPV	57	21 (36.8)	22	8 (36.4)
No HPV	29	13 (44.8)	160	32 (20)

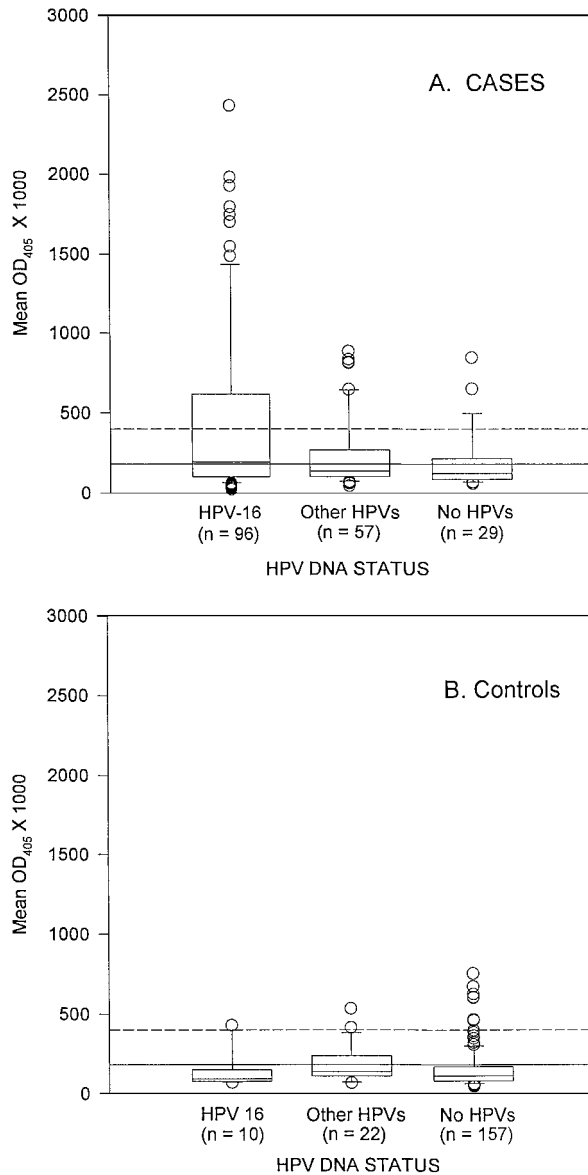[a] HPV DNA diagnosis was not available for 12 cases and 25 controls.

Fig. 2.   Seroreactivity in HPV-16 VLP ELISA of cervical cancer cases (*A*) and controls (*B*) by HPV DNA diagnosis. See legend to Fig. 1 for explanation of box plot diagrams.

but the differences failed to reach statistical significance (Kruskal-Wallis ANOVA on ranks, *P* = 0.057). However, as shown in Fig. 2, high *A* values (*A* ≥ 0.400) were found most frequently in the HPV-16 DNA-positive cases. It is noteworthy that among the 32 antibody-positive controls that were negative

for any HPV DNA by PCR, only 7 had high antibody titers to VLP.

VLP Antibody Response in Women with Selected Risk Factors for Cervical Cancer.   Cases and controls were examined for the relationship of VLP antibody prevalence and selected risk factors for cervical cancer (Table 2). In both groups, antibody prevalence increased with increasing number of lifetime sexual partners (χ^2 for trend, *P* < 0.001). Age at first intercourse was not related to HPV-16 seropositivity among cases, but a nonsignificant inverse trend was observed among controls. Antibody prevalence was not significantly different in different age groups of cases. Among the controls, age was significantly inversely associated with HPV-16 seropositivity (χ^2 for trend, *P* = 0.019). There was no association between HPV-16 VLP seropositivity and stage of disease or histological diagnosis (data not shown).

Comparison of Antibodies to HPV-16 VLPs and HPV-16 E6 and E7 as Markers of Invasive Cervical Cancer.   The case and control sera were previously tested for antibodies to HPV-16 oncoproteins E6 and E7 (8). Antibody prevalence to E6, E7, VLPs, and various combinations of antibodies to the three proteins and the corresponding ORs for cervical cancer in relation to a positive serological test are shown in Table 3. One hundred fifty-six of 194 (80.4%) cases and 68 of 217 (31.3%) controls had antibodies to at least one of the three HPV antigens. Twenty of 194 (10.3%) cases and 1 of 217 (0.5%) controls had antibodies to all three HPV antigens. The individual antibody response that was most strongly associated with cervical cancer was that to E6. A little more than half of the cases (54%) and only 6% of controls had antibody to E6.

The correlation between antibody responses to HPV-16 VLP and HPV-16 E6 and E7 proteins was examined by Spearman's correlation separately in cases and controls. No correlation between the antibody titers to E6 or E7 and VLPs was found in either cases or controls (data not shown). Additionally, case sera testing positive and those testing negative in the VLP ELISA demonstrated similar frequencies of positivity in the E6 (47.6 and 52.4%, respectively) and E7 (49.2 and 50.8%, respectively) assays.

HPV Serology as a Screening Test for Cervical Cancer.   The serological assays were also evaluated as screening tests for invasive cervical cancer (Table 4). HPV-16 VLP serology had a sensitivity of 47.4% and a specificity of 75.6%, whereas HPV-16 E6 and E7 serology had a sensitivity of 63.4% and a specificity of 89.9%. If reactivity to any HPV-16 protein was considered indicative of the presence of cervical cancer, serology detected 80.2% of cases, but the specificity of the combined assays was low (68.7%). By adjusting the cutoff in each assay, it was possible to achieve a sensitivity of 53.6% and a specificity of 93.6% for high titers to at least one of the three antigens.

Table 2 Prevalence of antibodies to HPV-16 VLPs among cases and controls by age, numbers of lifetime sexual partners, and age at first intercourse

Risk factor	Cases			Controls		
	No. tested	No. (%) positive	OR[a]	No. tested	No. (%) positive	OR[a]
Age (yr)						
26–43	47	25 (58)	1.0 (ref)	54	19 (35)	1.0 (ref)
44–50	52	24 (46)	0.8	49	13 (27)	0.7
51–60	45	25 (56)	1.1	53	12 (23)	0.5
61–78	50	18 (36)	0.5	61	9 (15)	0.3
		χ^2 for trend, p = 0.18			χ^2 for trend, p = 0.011	
No. sex partners						
0				7	1 (14)	1.0 (ref)
1	103	35 (34)	1.0 (ref)	141	22 (16)	1.1
2	45	26 (58)	2.7	38	16 (42)	4.4
3	16	9 (56)	2.5	19	7 (37)	3.5
≥4	30	22 (73)	5.3	12	7 (58)	8.4
		χ^2 for trend, p < 0.001			χ^2 for trend, p < 0.001	
Age, 1st intercourse						
<14	27	15 (56)	1.0 (ref)	13	6 (41)	1.0 (ref)
15–19	104	51 (49)	0.8	94	24 (25)	0.4
≥20 or virgin	63	26 (41)	0.6	110	23 (21)	0.3
		χ^2 for trend, p = 0.18			χ^2 for trend, p = 0.08	

[a] ORs are crude.

Table 3 Antibody prevalence (%) among cases and controls and associated ORs for invasive cervical cancer by different serological tests for HPV-16

Antibody to	Cases (%), n = 194	Controls (%), n = 217	OR[a] (95% CI)
HPV-16 VLP	92 (47.4)	53 (24.4)	2.8 (1.8–4.3)
HPV-16 E6	105 (54.1)	13 (6.0)	18.5 (9.5–36.6)
HPV-16 E7	59 (30.4)	10 (4.6)	9.1 (4.3–19.6)
E6 or E7	123 (63.4)	22 (10.1)	15.4 (9.8–27.0)
E6 and E7	41 (21.1)	1 (0.5)	57.9 (8.4–1145)
E6 or E7 or VLP	156 (80.4)	68 (31.3)	9.0 (5.6–14.6)
E6 and E7 and VLP	20 (10.3)	1 (0.5)	24.8 (3.5–502)

[a] ORs are crude.

Table 4 Comparison of HPV-16 serological assays as screening tests for invasive cervical cancer

Assay	Sensitivity	Specificity
HPV-16 VLP antibodies	47.4	75.6
HPV-16 E6 or E7 antibodies[a]	63.4	89.9
HPV-16 VLP or E6 or E7 antibodies	80.2	68.7
HPV-16 VLP or E6 or E7 antibodies (high titers)[b]	53.6	93.6

[a] These data were reported previously (8).
[b] For E6 and E7 antibodies ≥6000 cpm, for VLP antibodies ≥A 0.400.

Discussion

In a previous study we examined serum antibodies to HPV-16 E6 and E7 as markers of invasive cervical cancer (8). In this study we tested the same serum samples for antibodies to HPV-16 VLPs, and we assessed VLP antibodies and various combinations of antibodies to the three proteins as markers of invasive cervical cancer. The difference between cervical cancer cases and controls in seroreactivity to HPV-16 VLPs was highly significant comparing either distributions of *A* values or antibody prevalence, indicating that VLP antibodies are a marker of cervical cancer. The seroprevalence among cases of ~50% is very similar to that reported in several studies of cervical cancer around the world, where the prevalence has ranged from 35 to 59% (11–14). Although the highest seroprevalence was observed among women who had HPV-16 DNA in the genital tract (54.3%), a high seroprevalence was also seen in women who had other HPVs (38.6%) or no HPVs (44.8%) in the genital tract. The most plausible explanation for the high seroprevalence in women without a current HPV-16 infection is that serological markers reflect a history of HPV exposure, and women with cervical cancer associated with HPV types other than 16 commonly have been exposed to HPV-16 in the past. Cross-reactivity to L1 protein of other HPV types seems unlikely, because studies using sera raised against a particular VLP type have demonstrated no or minimal reactivity in ELISA with VLPs of other, even closely related, types

(15). Even for women whose cancer was presumed to be associated with HPV-16, based on DNA assays of cervical scrapes, the proportion of women with antibodies to HPV-16 VLPs was low (54.2%). Because HPV capsid protein expression is almost never detected in invasive cancers, the absence of immune stimulation by capsid protein may lead to a decline in antibody titer with time. Consistent with this interpretation, we observed a significant decline in antibody titer with age among control women. However, the same trend was not observed among case women; thus additional factors probably contribute to the low seroprevalence among cancer cases. One possibility is that antibody titers in these women declined due to an immunosuppressive effect of cervical cancer.

The OR of 2.8 for the association of antibodies to HPV-16 VLPs and cervical cancer was less than that for antibodies to E6 (OR, 18.5) or E7 (OR, 9.1). The weaker association observed for antibody responses to VLPs can be accounted for largely by the higher seroprevalence to VLPs compared with E6 and E7 proteins, among controls. An antibody response to VLPs among controls is consistent with numerous studies showing that VLP antibodies are markers of HPV infection (4–6, 16–23). Among cases, irrespective of their HPV DNA status, there was no correlation between seroreactivity to HPV-16 VLPs and HPV-16 oncoproteins. The reason for this is not clear, but it may reflect differences in the processes responsible for antibody responses to VLPs and to oncoproteins. E6 and E7 antibodies are known to be associated almost exclusively with invasive cervical cancer and to correlate with tumor burden. In contrast, antibodies to VLPs appear after infection and are

probably related to the duration of vegetative DNA replication and expression of capsid proteins. Because the two processes are temporally disparate, it is not surprising that the antibody responses they induce are not correlated. A longitudinal study would be required to address the temporal relationship between antibody responses to different HPV proteins and stage of HPV infection.

The analysis of HPV-16 VLP antibodies in relation to risk factors for cervical cancer showed that seroprevalence markedly increased with an increasing number of lifetime sexual partners. Among controls, seroprevalence increased from 16% in subjects with one partner to 58% in those with more than four partners, and among cases, it increased from 34% in subjects with one partner to 73% in subjects with four or more partners. This pattern has been a consistent finding in epidemiological studies using VLP-based ELISA and would be expected for a sexually transmitted infectious agent (6, 16, 20, 23). The trend was seen in cases irrespective of the presence or absence of HPV-16 infection detected by PCR (data not shown). This observation implies that multiple sex partners and, consequently, the opportunity for multiple exposures to HPV-16 is a determinant of HPV-16 VLP seroreactivity independent of a persistent HPV-16 infection. A possible explanation for this is that repeated exposure to HPV-16 capsid proteins after a transient infection with HPV-16 further stimulates antibody production.

The use of molecular diagnostic techniques, particularly HPV DNA assays, in cervical cancer screening programs is currently under active consideration. Assays for HPV DNA are attractive screening tests because most, if not all, cervical cancers contain HPV genomes. Because HPV infections occur primarily in young, sexually active women, the specificity of PCR in older women at risk for cervical cancer is high. We compared three serological assays as screening tests for invasive cervical cancer. Nearly half of the cervical cancer cases were detected by HPV-16 VLP serology (47.4%), and almost two-thirds were detected by HPV-16 E6 and E7 serology (63.4%). The specificity of HPV 16 VLP serology was poor for a screening test (75.6%), and that of HPV-16 E6 and E7 serology was fair (89.9%). The optimal performance of serological assays was achieved by a combination of high titers of antibody to any of the three proteins (sensitivity, 53.6%; specificity, 93.6%). One caveat to the use of E6 and E7 serology for screening is that E6 and E7 antibodies are markers of invasive cervical cancer but not of high-grade squamous intraepithelial lesions (24). Therefore, serological testing for E6 and E7 antibodies would not identify women with preinvasive disease. Although the HPV-16 VLP ELISA had a sensitivity of only ~50%, testing for reactivity to VLPs of other oncogenic types would most likely increase the sensitivity of VLP serology. However, the specificity of VLP serology would be low, resulting in many false-positive results if the test were used for cancer screening. An effective and commonly used strategy in this situation is to combine a sensitive screening test with a highly specific confirmatory test. This strategy has been used successfully for HIV diagnosis by using ELISA for screening and Western blot for confirmation (25). VLP serology could, for example, be combined with a confirmatory PCR. Given the limited value of cytology in underdeveloped countries and the difficulties in obtaining cervical smears, it is worthwhile to consider the value of serological assays in cervical cancer screening programs in these regions.

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