

Comparison of Peptide Enzyme-Linked Immunosorbent Assay and Radioimmunoprecipitation Assay with In Vitro-Translated Proteins for Detection of Serum Antibodies to Human Papillomavirus Type 16 E6 and E7 Proteins

YEPING SUN,^{1*} KEERTI V. SHAH,¹ MARTIN MÜLLER,² NUBIA MUÑOZ,³ XAVIER F. BOSCH,³
AND RAPHAEL P. VISCIDI⁴

Department of Immunology and Infectious Diseases, The Johns Hopkins University School of Hygiene and Public Health,¹ and Division of Pediatric Infectious Diseases, Department of Pediatrics, The Johns Hopkins University School of Medicine,⁴ Baltimore, Maryland 21205; Deutsches Krebsforschungszentrum, Heidelberg, Germany²; and Unit of Field and Intervention Studies, International Agency for Research on Cancer, Lyon 69372, France³

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Antibodies to human papilloma virus (HPV) type 16 (HPV-16) E6 and E7 proteins in serum are markers for HPV-associated invasive cervical carcinoma. We compared two assays, a radioimmunoprecipitation assay with in vitro-translated HPV-16 E6 and E7 proteins and an enzyme-linked immunosorbent assay (ELISA) with E6 and E7 synthetic peptides, for their abilities to discriminate serologically between patients with invasive cervical cancer and controls. Among the patients, antibody prevalences were higher by the E6 radioimmunoprecipitation assay (55.7%) than by the E6 peptide ELISA (15.5%), but among the controls, they were lower by the radioimmunoprecipitation assay (1.7%) than by the E6 peptide ELISA (5%). For E7, antibody prevalences among the patients were comparable by the radioimmunoprecipitation assay (43%) and the peptide ELISA (41%), but among the controls they were higher by the E7 peptide ELISA (17.4%) than by the radioimmunoprecipitation assay (4.1%). There was good agreement between the E7 radioimmunoprecipitation assay and the E7 peptide ELISA among patients but not among controls. In tests with representative sera, heat denaturation of the translated proteins resulted in a complete loss of reactivity to the E6 protein and a marked decrease in reactivity to the E7 protein. Our study showed that the radioimmunoprecipitation assay discriminates better than the peptide ELISA between patients with invasive cervical cancer and controls and that this is related to the ability of the radioimmunoprecipitation assay to detect conformational epitopes.

Diagnoses of human papillomavirus (HPV) infections and HPV-related cancers have traditionally relied on the demonstration of HPV DNA in clinical samples. Many studies have shown that cells from patients with preinvasive or invasive cervical cancer harbor HPV genomic sequences. HPV type 16 (HPV-16) is the most prevalent HPV type in patients with cervical cancer. Serological assays for HPVs are less advanced, but several studies have found that antibodies to HPV-16 E6 and E7 proteins occur more frequently in patients with cancer than in controls (6, 21). These proteins are oncoproteins, and they are consistently expressed in cervical cancer cells. In the most recent investigations, the presence of antibodies to HPV-16 E6 and E7 proteins was found to be as strong a marker of invasive cervical cancer as the presence of HPV-16 DNA in the uterine cervix (20, 22).

A wide variety of reagents and techniques has been used for the detection of antibodies to E6 and E7 proteins. The earliest studies used bacterially expressed full-length fusion proteins as antigens in Western blot (immunoblot) assays (7, 9, 10, 13). Those tests detected antibodies to linear epitopes, and quantitation of antibody levels was difficult. Subsequently, synthetic peptides representing B-cell epitopes on the proteins were used as antigens in an enzyme-linked immunosorbent assay (ELISA) to detect antibodies (4, 14). These tests measured

antibodies to linear epitopes located on small fragments of the proteins. Most recently, full-length radiolabeled proteins, transcribed and translated in vitro (TT), have been used in a radioimmunoprecipitation assay (RIPA) to identify antibodies (15, 20, 22). This test measures antibodies to both conformational and linear epitopes on the full-length proteins. In view of the diversity of these immunologic assays in different studies, it is not surprising that the antibody prevalence rates varied over a wide range, from 16 to 56% (anti-E6) and 20 to 43% (anti-E7), respectively, in sera from patients with cervical cancer and from 1.7 to 4% and 0 to 15%, respectively, in sera from the corresponding controls.

In our investigations, many of the serum specimens were tested for E6 and E7 antibodies by both TT-RIPA and ELISA (15, 22). This provided an opportunity to compare the two tests for their abilities to discriminate serologically patients with invasive cervical cancer from their controls. We report here that, in comparison with ELISA with synthetic peptides, the TT-RIPA detected greater reactivity in sera from patients and lesser reactivity in sera from controls and was therefore the more discriminating test with respect to invasive cervical cancer.

MATERIALS AND METHODS

Serum specimens. The serum donors were participants in a case-control study of cervical cancer in Colombia and Spain (16). Briefly, the case patients were women with pathologically confirmed incident cases of invasive cervical carcinoma. The controls were population based and consisted of women with

* Corresponding author. Mailing address: Department of Immunology and Infectious Diseases, The Johns Hopkins University School of Hygiene and Public Health 615 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 614-1492. Fax: (410) 614-1491.

normal cervical cytologies and were frequency matched to the cases in age distribution and country of residence. The virological diagnoses were performed by ViraPap, PCR, or Southern blot analysis of exfoliated cells from the cervix for both patients and controls (8). In the present study, we compared the TT-RIPA and peptide ELISA results for all sera on which both tests were performed. The serum donors comprised 97 patients with invasive cancer with a virologic diagnosis of HPV-16 and 121 controls (15, 22).

ELISA with synthetic peptides. The identification of immunoreactive regions on the E6 and E7 proteins and the methodology for the peptide ELISA for the detection of antibodies to HPV antigens in serum have been described previously (14, 15). Briefly, four peptides representing two immunoreactive epitopes on E6 (amino acids [aa] 1 to 23 and 8 to 37) and two on E7 (aa 6 to 35 and aa 29 and 52) were synthesized with a Milligen/Biosearch model 9050 peptide synthesizer by the protocols specified by the manufacturer. After purification by high-pressure liquid chromatography, the peptides were coated onto the wells of microtiter plates at the following concentrations: 10 µg of E7 (aa 6 to 38) and 30 µg of E7 (aa 29 to 52) per ml of phosphate-buffered saline (pH 7.2) and 25 µg of E6 (aa 1 to 23) and 10 µg of E6 (aa 8 to 37) per ml of 0.06 M carbonate buffer (pH 9.6). Human sera were tested in duplicate at a 1:25 dilution. The assay was completed with a goat anti-human immunoglobulin G conjugated to horseradish peroxidase (Zymed, Laboratories, San Francisco, Calif.) and 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS) substrates. For each serum sample, the mean absorbance of the buffer wells was subtracted from the mean absorbance of the wells coated with peptide to calculate a net absorbance value. Individual sera were scored as antibody positive or antibody negative for each peptide by using a cutoff value which was calculated as the mean plus 3 standard deviations of the absorbance values of the control sera, after excluding the outliers (15, 22).

TT-RIPA. TT-RIPA was performed as described previously (15, 22). Plasmids containing HPV-16 E6 or E7 open reading frames cloned downstream of a T7 promoter (5, 23) (gifts from Peter Howley) were linearized with *EcoRI* and *PstI* restriction enzymes, respectively. One microgram of plasmid DNA was transcribed in vitro with 10 U of T7 RNA polymerase in the presence of cap analog (5'-7meGpppG) according to the manufacturer's instructions (Stratagene, La Jolla, Calif.). One microgram of the RNAs was used to program a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega, Madison, Wis.) in the presence of [³⁵S]methionine (Amersham, Arlington Heights, Ill.). Immunoprecipitation assays were performed by incubating 5 µl of lysate and 5 µl of human serum in a 200-µl volume of RIPA buffer (10 mM Tris-HCl [pH 8.0, 140 mM NaCl, 0.025% NaN₃, 0.1% Nonidet P-40) for 16 h at 4°C on a rotator at 30 rpm. The, 80 µl of 1:1 slurry of prewashed protein A-Sepharose beads (Sigma, St. Louis, Mo.) suspended in RIPA buffer was added and the mixture was rotated for 3 h at 4°C. After washing twice with 300 µl of RIPA buffer, once with RIPA buffer without Nonidet P-40, and once with 10 mM Tris-HCl (pH 6.8), the beads were resuspended in 100 µl of sodium dodecyl sulfate (SDS) sample buffer containing 60 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, and 100 mM dithiothreitol, and the mixture was boiled for 5 min. After a brief centrifugation, the supernatant was transferred to a scintillation vial containing 10 ml of Aquasol-2 scintillation fluid (Du Pont, Boston, Mass.). The radioactivity was measured as counts per minute in a Beckman LS-230 liquid scintillation counter. The individual serum specimen was scored as seropositive or seronegative by a cutoff value calculated as the

TABLE 1. Prevalence of antibodies to HPV-16 E6 and E7 among patients with cervical cancer and controls by peptide ELISA and TT-RIPA

Antigen(s)	Assay	% Positive		Odds ratio (95% confidence interval)
		Patients (n = 97)	Controls (n = 121)	
E6	Peptide-ELISA ^a	15.5	5.0	3.5 (2.1-5.8)
	TT-RIPA	55.7	1.7	74.7 (25.2-219.5)
E7	Peptide-ELISA	41.2	17.4	3.3 (2.7-4.0)
	TT-RIPA	43.3	4.1	17.7 (11.1-29.7)
E6 or E7	Peptide-ELISA	46.4	20.7	3.3 (2.8-3.9)
	TT-RIPA	72.2	5.8	42.2 (28.3-62.7)
E6 and E7	Peptide-ELISA	8.3	2.5	3.5 (1.4-8.9)
	TT-RIPA	26.8	0	

^a A serum specimen positive in the ELISA for either or both peptides of the protein was considered positive for the protein.

mean counts per minute plus 3 standard deviations of the counts per minute of the control sera, after excluding the outliers.

RIPA with heat-denatured E6 and E7 proteins. A mixture of 5 µl of rabbit reticulocyte lysate containing radiolabeled target protein (E6 or E7) and 200 µl of RIPA buffer was heated at 85°C for 15 min or 100°C for 5 min and was chilled on ice. Five microliters of serum was added, and the mixture was incubated at 4°C for 12 h. Then, 80 µl of 1:1 slurry of protein A-Sepharose beads suspended in RIPA buffer was added, and the mixture was incubated for another 3 h. The immunoprecipitation assay was then completed as described above, and the immunoprecipitated radiolabeled E6 or E7 protein was detected by SDS-15% polyacrylamide (National Diagnostics, Atlanta, Ga.) gel electrophoresis and autoradiography.

Statistical analysis. The frequencies of seroreactivity were compared by the χ^2 test by using Fisher's exact probability when necessary. The correlations between TT-RIPA (counts per minute) and the peptide ELISA (absorbance value) for seroreactivity to E6 and E7 peptides and proteins were examined by simple linear regression analysis (Pearson's correlation coefficient). Coefficient of agreement (kappa) was used to evaluate the agreement between the TT-RIPA and the ELISA (2, 11).

RESULTS

Comparison of antibody prevalence by TT-RIPA and peptide ELISA. Table 1 summarizes the results of the peptide ELISA and the TT-RIPA for the detection of antibodies to HPV-16 E6 and E7 in serum among patients with invasive cervical carcinoma and controls. The antibody prevalence in patients was higher by TT-RIPA than by ELISA for each of the four antigen categories (E6, E7, E6 or E7, E6 and E7). The most marked difference between the two tests was for the reactivity of sera from patients to E6 (55.7% in TT-RIPA versus 15.5% in ELISA). In contrast to the results with sera from patients, the antibody prevalence in controls was lower by TT-RIPA than by ELISA for all four antigen categories. As a result of this differential reactivity of the sera in the two tests, the odds ratios for seropositivity by TT-RIPA were 5- to 21-fold higher than those for seropositivity by peptide ELISA. HPV-16 DNA was detected in the uterine cervixes of all patients with invasive cervical cancer. Eight control women

TABLE 2. Correlation between peptide ELISA and TT-RIPA

Peptide ELISA result and antigen	TT-RIPA			
	Patients		Controls	
	No. tested	No. (%) RIPA positive	No. tested	No. (%) RIPA positive
Positive				
E6	15	11 (73.3)	7	0
E7	40	32 (80)	21	2 (9.5)
E6 or E7	47	37 (78.7)	25	2 (8.0)
E6 and E7	8	6 (75)	3	0
Negative				
E6	82	43 (52.4)	114	2 (1.8)
E7	57	10 (17.5)	100	3 (3.0)
E6 or E7	50	33 (66.0)	96	5 (5.2)
E6 and E7	89	20 (22.5)	118	0

were positive for HPV DNA, including one for HPV-16. In none of the virus-positive controls were antibodies to HPV-16 E6 or E7 protein detectable by TT-RIPA. By peptide ELISA, antibody to E7 (aa 6 to 35) was found in the HPV-16 DNA-positive control, and antibody to E7 (aa 29 to 52) was found in one control positive for an unknown HPV type.

The correlation between the two tests was examined by calculating the reactivities by TT-RIPA of ELISA-positive and ELISA-negative sera from patients and controls (Table 2). Among the ELISA-positive sera, there was a marked difference between patient and control sera in their positivities by TT-RIPA for the four antigen categories; the positivity by TT-RIPA ranged from 73.3 to 80% for case sera but only from 0 to 9.5% for control sera. Among ELISA-negative sera, the positivity by TT-RIPA was also higher for patient sera (range, 17.5 to 66.0%) than control sera (range, 0 to 5.2%). It is noteworthy that 52.4% of the patient sera but only 1.8% of the control sera that were E6 antibody negative by ELISA were scored as positive by TT-RIPA.

We then examined the agreement between peptide ELISA and TT-RIPA separately for patients and controls using the kappa statistics. Concordant results were obtained for 51.6, 83.5, 92.3, and 81.8% of sera in assays for E6 among patients, E7 among patients, E6 among controls, and E7 among controls, respectively. The corresponding kappa values were 0.09, 0.66, 0.18, and 0.09. The level of agreement between TT-RIPA and peptide ELISA was good only for the E7 assays among patients. The level of agreement between the E7 assays for controls and the E6 assays for patients and controls were no better than those expected by chance alone.

We also examined the correlation between the absorbance values of the peptide ELISA and counts per minute of the TT-RIPA separately for patient and control sera. Sera that were negative by both assays were excluded. Among sera from patients, there was a significant correlation between peptide ELISA and TT-RIPA for seroreactivity to E7 antigens ($n = 50$, $r = 0.7932$, $P = 0.0001$) (Fig. 1) but not to E6 antigens ($n = 58$, $r = 0.0962$, $P = 0.4724$) (data not shown). Among sera from controls, there was a negative correlation between peptide ELISA and TT-RIPA for seroreactivity to the E7 protein ($n = 97$, $r = 0.5011$, $P = 0.0107$) but not to the E6 protein ($n = 112$, $r = 0.4640$, $P = 0.2084$).

We examined the effect of combining the results of TT-RIPA and ELISA for the detection of antibodies in serum. The prevalence of antibodies to HPV-16 by peptide ELISA or TT-RIPA was 77.3% among patients and 24% among controls.

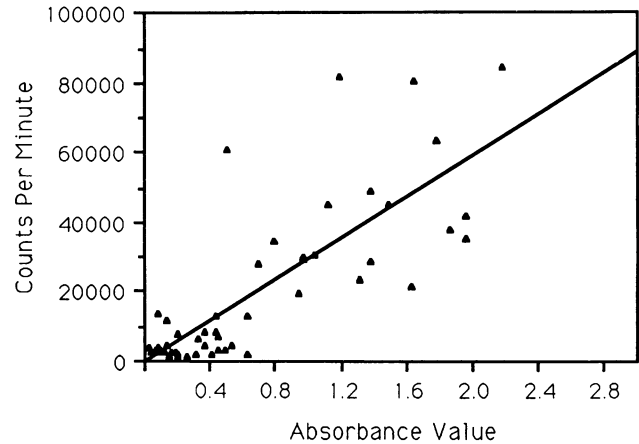


FIG. 1. Correlation between HPV-16 E7 TT-RIPA and HPV-16 E7 peptide ELISA for 50 serum specimens from women with HPV-16-associated invasive cervical cancer. Sera that were negative in both assays were excluded from analysis.

In comparison, the antibody prevalence detected by TT-RIPA alone was 72.2% in patients and 5.8% in controls. Thus, the addition of the results of the peptide ELISA brought about a slight increase in antibody prevalence among patients but a marked increase in antibody prevalence among controls. This increase in antibody prevalence among the controls was largely contributed by reactivity by the E7 peptide ELISA. The antibody prevalence of sera reactive in both the peptide ELISA and the TT-RIPA was 41.2% among patients and 1.7% among controls.

Effect of heat treatment on seroreactivity in TT-RIPA. In order to determine if the serum reactivity to in vitro-translated E6 and E7 proteins was directed to the conformational epitopes of these proteins, we compared the reactivities of selected serum specimens in the TT-RIPA using heat-denatured and nondenatured proteins. Human sera reacted less strongly with in vitro-translated E7 protein that was heat denatured than with nondenatured E7 protein (Fig. 2). There

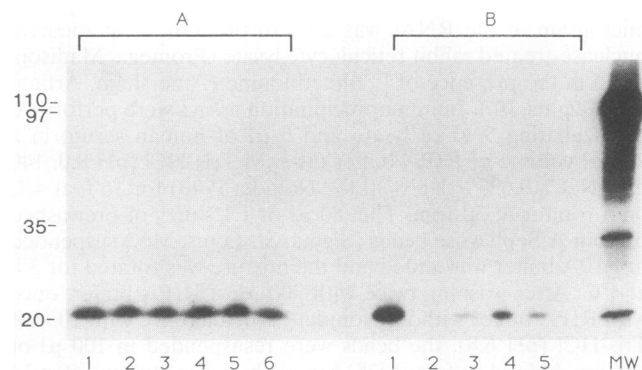


FIG. 2. Comparison of seroreactivity to native and heat-denatured, in vitro-translated HPV-16 E7 protein by TT-RIPA. Rabbit antiserum to HPV-16 E7 fusion protein (lanes 1), E7 TT-RIPA-positive and E7 peptide ELISA-negative human sera (lanes 2 and 3, respectively), and E7 TT-RIPA and E7 peptide ELISA positive human sera (lanes 4 and 5, respectively) were reacted with native in vitro-translated E7 (A) and the same protein after heat denaturation for 15 min at 85°C (B). Lane 6, native in vitro-translated HPV-16 E7 protein; MW, Brome mosaic virus proteins with the indicated molecular weights (in thousands).

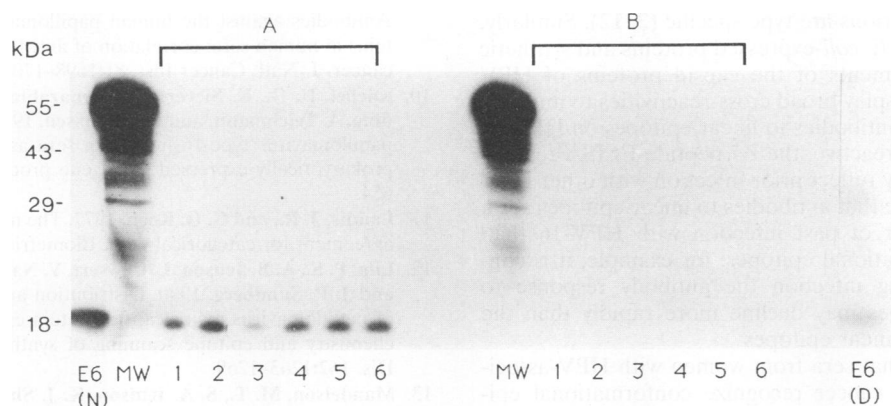


FIG. 3. Comparison of seroreactivity to native and heat-denatured, in vitro-translated HPV-16 E6 protein by TT-RIPA. E6 TT-RIPA-positive and E6 peptide ELISA-negative human sera (lanes 1, 2, and 3), E6 TT-RIPA and E6 peptide ELISA-positive human sera (lanes 4 and 5), and rabbit antiserum to HPV-16 E6 fusion protein (lanes 6) were reacted with native, in vitro-translated HPV-16 E6 (A) and the same protein after heat denaturation for 15 min at 85°C (B). Native and denatured, in vitro-translated E6 protein are shown as E6(N) and E6(D), respectively. MW, proteins made from luciferase mRNA as molecular mass markers.

was less loss of reactivity for sera that were E7 peptide ELISA positive than for sera that were E7 peptide ELISA negative. In contrast to the human sera, a rabbit antiserum specimen raised against an *Escherichia coli*-expressed E7 fusion protein showed increased reactivity to the heat-denatured protein in comparison with that of the nondenatured protein. Human sera that were antibody positive by E6 TT-RIPA alone or by both TT-RIPA and E6 peptide ELISA showed no reactivity to in vitro-translated E6 protein after heat denaturation (Fig. 3). A rabbit antiserum sample that was raised against an *E. coli*-expressed E6 fusion protein also failed to react with heat-denatured E6 protein by TT-RIPA.

DISCUSSION

The lack of authentic viral proteins has been a vexing problem for the development of serological assays for HPVs. We developed serological assays for HPV-16 E6 and E7 proteins that use radiolabeled in vitro-translated proteins in an immunoprecipitation reaction. This test has the potential to measure antibodies to conformational as well as linear epitopes. In the present study, we compared peptide ELISA and TT-RIPA for the detection of antibodies to HPV-16 E6 and E7 among patients with invasive cervical cancer and controls.

The TT-RIPA was clearly the more discriminating test between patients and controls because, in comparison with the peptide ELISA, it detected antibodies more frequently in the sera of patients and less frequently in the sera of controls. Among the patients, the most striking finding was the higher level of reactivity of sera in the E6 TT-RIPA (56%) in comparison with that in the E6 peptide ELISA (15%). The reasons for the greater reactivities of sera in the E6 TT-RIPA may include the presence of conformational epitopes or additional linear epitopes on the full-length E6 protein. The peptides in the ELISA span only aa 1 to 37 of the 158 aa in the E6 protein. However, it is unlikely that additional linear epitopes are present on this protein because extensive analysis of overlapping peptides for the entire E6 open reading frame has failed to identify other reactive peptides (4). In addition, Sasagawa et al. (17) reported that very few serum specimens from women with invasive cervical cancer reacted with purified, full-length, *Escherichia coli*-expressed E6 protein by ELISA. Furthermore, our observation that sera failed to react

with in vitro-translated E6 after it was denatured by heat treatment supports the possibility that the epitopes on the E6 protein are primarily conformational. The observation of Stacey et al. (18) that human sera which react with baculovirus expressed full-length E6 protein by immunoprecipitation frequently do not react with the same protein by Western blot analysis also supports this conclusion.

In contrast to the findings for the E6 protein, the proportions of sera from patients reactive in the E7 TT-RIPA and E7 peptide ELISA were comparable, 43 and 41%, respectively. In addition, the agreement between the tests was good and the level of reactivity of sera in the two assays was strongly correlated. These results suggest that the immunodominant epitopes on the E7 protein are primarily linear. This conclusion is consistent with the findings of other investigators who have detected antibodies to E7 in women with cervical cancer using a variety of assays and reagents that would be expected to detect primarily linear epitopes (21). However, the decrease in seroreactivity following heat treatment of the in vitro-translated protein indicates that serum also contains antibodies to conformational epitopes on the E7 protein. Furthermore, sera with equivalent reactivities to the nondenatured protein showed variable reactivities to the denatured protein, suggesting that patients differ in their responses to conformational and linear epitopes. Nindl et al. (16a) have also observed that in vitro-translated proteins contain heat-sensitive epitopes and that antibodies to E6 protein in serum are directed against conformational epitopes.

Among the controls, a greater proportion of sera were reactive in the E7 peptide ELISA (17%) than in the E7 TT-RIPA (4%). In addition, there was poor agreement between the assays and antibody levels in the two tests appeared to be inversely correlated. There are several possible explanations for the reactivity of control sera in the E7 peptide ELISA and not in the E7 TT-RIPA. The ELISA-positive results may include false-positive reactions unrelated to HPV infection. Unlike the case patients, only one control woman was HPV-16 DNA positive by PCR of cervical scrapings. It is also possible that the reactivity in the peptide ELISA was due to a cross-reaction with other HPVs. Antibodies which have been generated against denatured capsid proteins of bovine papillomaviruses have been shown to cross-react with capsid proteins of many different papillomaviruses, whereas antibodies which are

raised against intact virions are type specific (3, 12). Similarly, antisera raised against *E. coli*-expressed proteins and synthetic peptide-encoding fragments of the capsid proteins of HPV have been shown to display broad cross-reactivities to multiple HPV types (1, 19). If antibodies to linear epitopes on HPV-16 E7 peptides are cross-reactive, the E7 peptide ELISA reactivity among controls may reflect prior infection with other HPV types. It is also possible that antibodies to linear epitopes are a more sensitive marker of past infection with HPV-16 than antibodies to conformational epitopes; for example, it is conceivable that following infection the antibody response to conformational epitopes may decline more rapidly than the antibody response to linear epitopes.

Our study showed that sera from women with HPV-associated invasive cervical cancer recognize conformational epitopes on the HPV-16 E6 and E7 proteins. For the serological diagnosis of HPV-associated invasive cervical cancer, assays that are able to detect antibodies to conformational epitopes are superior to assays that detect only linear epitopes.

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