Comparison of Different Assays To Assess Human Papillomavirus (HPV) Type 16- and 18-Specific Antibodies after HPV Infection and Vaccination

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We compared the measurement of human papillomavirus (HPV)-specific serum antibody levels with the virus-like-particle multiplex immunoassay (VLP-MIA), competitive Luminex immunoassay (cLIA), and glutathione S-transferase (GST) L1-based MIA. Using a large panel of serum samples, these assays showed mutually good correlations for both naturally induced and vaccine-derived HPV-specific antibody levels. However, an adaptation of the GST L1-based MIA resulted in an improved correlation with both cLIA and VLP-MIA.

Serological assays that determine human papillomavirus (HPV)-specific antibodies are useful for the evaluation of past HPV infections, as naturally derived antibody concentrations remained relatively constant after HPV infection (1). Monitoring vaccine-derived HPV-specific antibodies can provide information about the immunogenicity of the HPV vaccine, protection in the general population, groups at risk for HPV infections, and possible type replacement. To assess humoral immune responses after HPV vaccination and infection, several serological assays have been developed (2–5). The pseudovirion-based neutralization assay (PBNA) is the “gold standard” and known to provide the most meaningful evaluation of the functional humoral immune response against HPV. However, its use in large seroepidemiological studies or clinical trials is challenging, since these assays are laborious and difficult to perform. In an attempt to overcome the laboriousness of the PBNA, Sehr et al. developed an automated pseudovirion-based HPV neutralization assay. This assay is well suited for high-throughput screening, and validation experiments are in progress (6). Most serological assays available for the determination of HPV-specific antibody responses are alternatives for the PBNA. The most commonly used assay for the determination of vaccine-derived or naturally derived HPV-specific antibodies is the virus-like-particle enzyme-linked immunosorbent assay (VLP-ELISA) (3). The fluorescent multiplex bead-based assays are a good alternative for the ELISA, as they can rapidly detect multiple analytes in a single well with high reproducibility and sensitivity using reduced amounts of serum compared to ELISA. The competitive Luminex immunoassay (cLIA) (7), the virus-like-particle (VLP)-based multiplex immunoassay (VLP-MIA) (8), and the in situ purified glutathione S-transferase L1-based MIA (GST-L1-MIA) (5) have been used for seroprevalence studies in several countries (8–11). In general, comparisons between these serological assays are hampered by the use of different cutoff values and the lack of a good international reference serum.

Here we describe comparison of the cLIA, VLP-MIA, and GST-L1-MIA for the detection of HPV type 16 (HPV16)- and 18-specific IgG antibodies in serum samples of nonvaccinees and individuals vaccinated with 3 doses of the bivalent HPV16/18 Cervarix vaccine (GlaxoSmithKline Biologicals S.A., Rixensart, Belgium).

Serum samples of nonvaccinees were derived from a cross-sectional population-based serosurveillance study in the Netherlands (subset of n = 622) (12) and a cohort study of individuals at risk for HPV infection (subset of n = 80) (13). Serum samples of vaccinees 6 months after they received the third vaccine dose of the bivalent Cervarix vaccine in a 2 + 1 vaccination schedule (vaccination at months 0, 1, and 6) were available from an HPV monitoring cohort study (subset of n = 160) (14). The cLIA, VLP-MIA, and GST-L1-MIA were performed as previously described (5, 7, 8) with an addition to the GST-L1-MIA that median fluorescent intensity (MFI) was converted to Luminex units (LU)/ml using the reference standard as described for the VLP-MIA (8). Serum samples for the serosurveillance study were previously tested in the cLIA (15).

The serum panel used in the VLP-MIA and GST-L1-MIA contained serum samples of the serosurveillance study and both cohort studies of which duplicate results were obtained. Agreement between the assays was estimated using cutoffs previously determined for the cLIA (4) (HPV16, 20 milli-Merk units [mMU], HPV18, 24 mMU) and VLP-MIA (HPV16, 9 LU/ml, and HPV18, 13 LU/ml) (8). Antibody seropositivity in the GST-L1-MIA was assessed using arbitrary cutoffs similar to those for the VLP-MIA for both HPV16 and -18. The degree of agreement between the serological assays was quantified by Cohen’s kappa coefficient (κ).

The cLIA can detect HPV-specific neutralizing antibodies, whereas the VLP-MIA detects the total amount of HPV-specific antibodies. In addition, these assays can distinguish between dif-
different HPV types as well. In the VLP-MIA, 97% and 98% of the results were in agreement with the cLIA for HPV16 (κ = 0.66) and HPV18 (κ = 0.55), respectively. The discordant results were seropositive in the VLP-MIA and seronegative in the cLIA (Table 1). The VLP-MIA measures HPV-specific antibodies directed against neutralizing and nonneutralizing epitopes, which results in a higher percentage of seropositivity than for the cLIA. We showed previously that the VLP-MIA is reproducible and that the reactivity with monoclonal antibodies (MAbs) recognizing conformational epitopes on HPV16 and -18 was type specific (8). In contrast to the VLP-MIA, the cLIA detects high-affinity, neutralizing HPV-specific antibodies by using HPV-specific MAbs directed to a known single conformational epitope that compete with the HPV-specific serum antibodies. Although the detected antibodies in the cLIA have neutralizing capacity, this assay might underestimate the total neutralizing activity of both naturally derived and vaccine-derived HPV-specific antibodies (7).

The cLIA and VLP-MIA are based on VLPs consisting of the L1 major capsid protein of HPV that are expressed in yeast *Saccharomyces cerevisiae* and in a baculovirus expression vector system, respectively (3, 16). However, the development of VLPs for research purposes is time-consuming and complicated, and therefore only a limited number of VLP types is currently available. This hampers the use of VLP-based assays for the measurement of HPV-specific antibodies because VLPs are not commercially available. For the measurement of HPV antibodies, there is value in a low-cost assay that is easy to perform and reproducible, does not require VLPs, and compares favorably in performance with VLP-based assays. The GST-L1-MIA allows the determination of HPV-specific antibodies to a large number of HPV types, as the viral L1 proteins expressed as glutathione S-transferase fusion proteins are easily produced (11, 17). However, the L1 fusion proteins might contain fewer conformational epitopes and more linear epitopes, due to some denaturation of the L1 proteins. The comparison between the GST-L1-MIA and cLIA resulted in an overall agreement of 64% for both HPV16 and -18 (Table 1); however, κ values were poor (κ = 0.09 for HPV16 and κ = 0.03 for HPV18). Discordant results were GST-L1-MIA seropositive and cLIA seronegative. A similar overall percentage of agreement was found in the comparison of the GST-L1-MIA and VLP-MIA for both HPV16 (70%; κ = 0.41) and HPV18 (65%; κ = 0.33) (Fig. 1A and B). When evaluated separately, the vaccine sera showed a higher percentage of agreement for HPV16 (85%; κ = 0.37) and HPV18 (82%; κ = 0.18) than did the sera of naturally infected individuals between the VLP-MIA and the GST-L1-MIA (Table 1). The GST-L1-MIA and adapted GST-L1-MIA showed similar percentages of agreement with the VLP-MIA using only the vaccine sera.

We showed that the GST-L1-MIA is appropriate for measuring vaccine responses. However, the nonspecific background in this assay results in a loss of specificity and subsequently less good correlations with antibody levels close to or below the cutoff values. Waterboer et al. noted that human sera might contain antibodies that directly bind to the Luminex microspheres, resulting in nonspecific background (18). They found that preincubation with polyvinyl alcohol, polyvinylpyrrolidone, and a proprietary reagent (Super ChemiBlock; CBS-K) reduced this nonspecific background. In an attempt to reduce this background even further, we adjusted the GST-L1-MIA by purifying the GST L1 proteins from the bacterial lysates using a commercially available kit based on glutathione-coupled magnetic beads (MagneGST protein purification system; Promega, WI). After elution of the GST L1 proteins from the magnetic beads, the GST L1 proteins were subsequently dialyzed twice against storage buffer (1× phosphate-buffered saline [PBS], 0.01% Tween20, 0.25 M NaCL) using a 20-kDa dialyzing frame (20,000-molecular-weight cutoff [MWCO] Slide-A-Lyzer dialysis cassette; Thermo Scientific).

This purified fraction contained a much higher percentage of GST L1 proteins than the bacterial lysates (Fig. 2). The coupling of this purified fraction to the glutathione-casein-conjugated microspheres instead of the bacterial lysates decreased the nonspecific background and increased the overall agreement with the VLP-MIA for HPV16 (88%; κ = 0.71) and HPV18 (83%; κ = 0.59). Although the overall agreement with the cLIA also increased for HPV16 (89%) and HPV18 (86%), the kappa values were below moderate (κ = 0.30 and κ = 0.10 for HPV16 and -18, respectively) (Fig. 1C and D and Table 1).

In conclusion, a high agreement of seropositivity was observed

### Table 1: Comparison between the VLP-MIA, cLIA, GST-L1-MIA, and adapted GST-L1-MIA

| Assay | Type of results | HPV16 | HPV18 | HPV16 | HPV18 | HPV16 | HPV18 |
|-------|----------------|-------|-------|-------|-------|-------|-------|
|       |                | Total | NI    | VAC   | Total | NI    | VAC   |
| cLIA  |                | No.   | %     | No.   | %     | No.   | %     |
|       | In agreement   | 622   | 76%   | NA    | 602   | 97%   | NA    |
|       | Discordant     | 20    | 3%    | NA    | 11    | 2%    | NA    |
| GST-L1 MIA | Total | 862 | 100% | 702 | 160 | 622 | 160 |
|       | In agreement   | 602   | 70%   | 466   | 66   | 136   | 85   |
|       | Discordant     | 260   | 30%   | 236   | 34   | 24    | 15   |
| Adapated GST-L1 MIA | Total | 862 | 100% | 702 | 160 | 622 | 160 |
|       | In agreement   | 760   | 88%   | 615   | 88   | 145   | 91   |
|       | Discordant     | 102   | 12%   | 87    | 12   | 15    | 9    |

*The total number of serum samples tested in each assay is shown. For each comparison, the number of samples tested and agreeing or discordant percentages are displayed. Serum samples tested in the VLP-MIA are also separated for sera of HPV-negative and naturally infected individuals (NI) and sera of HPV-vaccinated individuals (VAC). NA, not applicable.*
between the VLP-MIA and cLIA. Using purified GST L1 proteins in the GST-L1-MIA, we could increase the overall agreement between the VLP-MIA and GST-L1-MIA possibly due to a decrease in nonspecific background in the GST-L1-MIA. Although these serological assays have different assay characteristics, they can all be used for the determination of vaccine-derived HPV-specific antibodies. For the detection of naturally derived HPV specific antibodies, the cLIA and VLP-MIA are good surrogates for the PBNA. The use of an international reference serum, containing high levels of IgG and IgA antibody against several HPV types, and quality control serum panels could facilitate comparisons between these serological assays.

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