Journal of Clinical Microbiology

# Use of the Normalized Absorbance Ratio as an Internal Standardization Approach To Minimize Measurement Error in Enzyme-Linked Immunosorbent Assays for Diagnosis of Human Papillomavirus Infection

**Agnihotram V. Ramanakumar, Patricia Thomann, Joao M. Candeias, Silvaneide Ferreira, Luisa L. Villa and Eduardo L. Franco**

***J. Clin. Microbiol.*** **2010, 48(3):791. DOI: 10.1128/JCM.00844-09.**
**Published Ahead of Print 6 January 2010.**

Updated information and services can be found at:
http://jcm.asm.org/content/48/3/791

*These include:*

**REFERENCES** This article cites 19 articles, 13 of which can be accessed free at: http://jcm.asm.org/content/48/3/791#ref-list-1

**CONTENT ALERTS** Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://journals.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://jcm.asm.org/ on September 16, 2013 by UNIVERSIDADE EST.PAULISTA JÃƒÅ¡LIO DE MESQUITA FILHO

JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2010, p. 791–796 Vol. 48, No. 3
0095-1137/10/$12.00 doi:10.1128/JCM.00844-09
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Downloaded from http://jcm.asm.org/ on September 16, 2013 by UNIVERSIDADE EST.PAULISTA JÃƒÅ¡LIO DE MESQUITA FILHO

# Use of the Normalized Absorbance Ratio as an Internal Standardization Approach To Minimize Measurement Error in Enzyme-Linked Immunosorbent Assays for Diagnosis of Human Papillomavirus Infection▽

Agnihotram V. Ramanakumar,[1] Patricia Thomann,[2] Joao M. Candeias,[3] Silvaneide Ferreira,[2] Luisa L. Villa,[2] and Eduardo L. Franco[1]*

*Division of Cancer Epidemiology, McGill University, Montreal, Canada[1]; Ludwig Institute for Cancer Research, São Paulo, Brazil[2]; and Instituto de Biociencias, UNESP, Botucatu, Brazil[3]*

Received 28 April 2009/Returned for modification 1 September 2009/Accepted 28 December 2009

**The serological detection of antibodies against human papillomavirus (HPV) antigens is a useful tool to determine exposure to genital HPV infection and in predicting the risk of infection persistence and associated lesions. Enzyme-linked immunosorbent assays (ELISAs) are commonly used for seroepidemiological studies of HPV infection but are not standardized. Intra- and interassay performance variation is difficult to control, especially in cohort studies that require the testing of specimens over extended periods. We propose the use of normalized absorbance ratios (NARs) as a standardization procedure to control for such variations and minimize measurement error. We compared NAR and ELISA optical density (OD) values for the strength of the correlation between serological results for paired visits 4 months apart and HPV-16 DNA positivity in cervical specimens from a cohort investigation of 2,048 women tested with an ELISA using HPV-16 virus-like particles. NARs were calculated by dividing the mean blank-subtracted (net) ODs by the equivalent values of a control serum pool included in the same plate in triplicate, using different dilutions. Stronger correlations were observed with NAR values than with net ODs at every dilution, with an overall reduction in nonexplained regression variability of 39%. Using logistic regression, the ranges of odds ratios of HPV-16 DNA positivity contrasting upper and lower quintiles at different dilutions and their averages were 4.73 to 5.47 for NARs and 2.78 to 3.28 for net ODs, with corresponding significant improvements in seroreactivity-risk trends across quintiles when NARs were used. The NAR standardization is a simple procedure to reduce measurement error in seroepidemiological studies of HPV infection.**

Serological testing for circulating anti-human papillomavirus (HPV) antibodies has potential clinical utility in measuring cumulative exposure to HPV infection and as a marker of HPV-associated disease, particularly for the anogenital tract (10). Long-term persistent HPV infection tends to yield polyclonal immunoglobulin G (IgG) serological reactivity, which can be measured by using enzyme-linked immunosorbent assay (ELISA) techniques based on type-specific L1-only (7) or L1-plus-L2 virus-like particles (VLPs) as antigens (8, 11). Serological testing for HPV antibodies cannot replace direct HPV DNA measurements or cytological and histological examinations of the target tissue, but it can serve as an adjunctive tool, particularly in molecular epidemiology studies of the natural history of HPV infection and cervical precancerous lesions (3, 16, 17, 19). Thus far, however, HPV serology has not been used in the clinical setting because of a variety of technical and biological limitations (8, 10). Although the specificity of HPV VLP ELISA is reasonably adequate, at over 90%, its sensitivity is relatively low (50% to 60%) (14).

Among the main technical issues that affect its clinical utility are the lack of cross-laboratory standardization and reproducibility. Aside from continued improvements in assay design and antigen preparation over the years, antibody measurement relies on determining VLP ELISA optical density (OD) values for serum samples and comparing them against suitable negative and positive controls to ascertain HPV seroreactivity. The crude OD values generated by ELISA are prone to substantial intra- and interassay variability that stems from day-to-day variations in reagent batches, technical performance related to pipetting, reagent preparation, instrument reading, and other laboratory conditions, all of which contribute to measurement errors in ascertaining seropositivity status based on predetermined crude OD values.

Being mindful of the above-described issues and based on our previous experience (9) and the experiences of others (6, 8, 13), we set out to standardize the VLP ELISA protocol that we have used in our long-standing cohort study of HPV infection and cervical neoplasia. We report herein our use of normalized absorbance ratios (NARs) as a technical refinement to control for the sources of variation that can affect the validity of seroreactivity in a study whose laboratory measurements spanned a period of nearly 10 years.

## MATERIALS AND METHODS

**Study design.** The Ludwig-McGill cohort study is a prospective, repeated-measurements investigation of prognostic markers in the natural history of HPV infection and cervical neoplasia. Detailed descriptions of the study design and methods were previously reported (4, 12, 15). In brief, women attending a

* Corresponding author. Mailing address: Department of Oncology, McGill University, 546 Pine Ave. West, Montreal QC H2W1S6, Canada. Phone: (514) 398-6032. Fax: (514) 398-5002. E-mail: eduardo.franco@mcgill.ca.

▽ Published ahead of print on 6 January 2010.

maternal and child health program in a low-income area of Sao Paulo, Brazil, were enrolled between November 1993 and March 1997. A total of 2,528 women in the age range of 18 to 60 years were enrolled in the study, for a response rate of 70.4%. Follow-up visits were scheduled every 4 months in the first year and twice yearly thereafter for up to 10 years. Ecto- and endocervical cell samples were collected at each visit with the Accelon Biosampler (Medscand). Conventional Pap smears were prepared for cervical cytology, and the remainder of the samples was placed into tubes containing Tris-EDTA buffer (pH 7.4) for subsequent HPV DNA testing. Risk factor information was collected via questionnaire-based interviews at most visits. All subjects gave written informed consent, and the protocol was approved by research ethics boards of all participating institutions with renewal on an annual basis.

**HPV DNA testing.** DNA was extracted from cervical cell specimens, purified by spin column chromatography, and tested for the presence of HPV DNA by the MY09/11 and PGMY09/11 PCR protocols, which amplify a conserved 450-bp segment in the L1 HPV gene (1, 5). Typing of amplified products was done by dot blot hybridization with individual oligonucleotide probes and by restriction fragment length polymorphism (RFLP) analysis, which permitted the individual detection of more than 40 HPV genotypes, including HPV-16, whose results are presented here. To verify the specificity of hybridizations, we included more than 30 positive controls of different HPV types for all membranes. To check for the presence of host cell DNA in extracted specimens, we used an additional set of primers (GH20 and PC04) that amplify a segment of the beta-globin gene (1). Appropriate steps were taken to avoid specimen contamination (4).

**HPV-16 serology.** Serum samples were separated from the clotted blood specimens and stored at −20°C until testing. A previously described ELISA technique was used for the semiquantitative measurement of IgG antibodies to HPV-16 VLPs (8) constructed with both the L1 and L2 capsid proteins. Recombinant HPV-16 VLPs prepared with the baculovirus system (7) were kindly provided by John Schiller, Laboratory of Cellular Oncology, U.S. National Institutes of Health. Polystyrene ELISA microtiter plates were coated with 50-μl aliquots of a preparation containing 2 mg of VLPs per 100 ml of phosphate-buffered saline (PBS). Plates were incubated for 1.5 h at 37°C and then washed three times by flooding with calcium- and magnesium-free PBS. Nonspecific reactive sites in the wells were blocked for 2 h at room temperature with PBS containing 0.5% skim milk and 0.1% newborn calf serum (PBS-MNCS) and then washed as described above. Plates were incubated with serum samples diluted 1:10 and 1:50 in PBS-MNCS for 2.5 h at 37°C. Following repeated washings, 50-μl aliquots of a conveniently diluted (by prior block titration) peroxidase-labeled anti-IgG conjugate were added to the wells, and the plates were incubated for 1 h at room temperature. Following an additional washing cycle, a chromogen-substrate mixture (0.1 mg/ml *O*-phenylenediamine and 0.003% hydrogen peroxide in 0.15 M PBS [pH 6.0]) was added to the wells to reveal the bound enzyme. After 45 min, absorbances were read at 490 nm with a colorimetric plate reader. Replicate blank wells that substituted PBS-MNCS for the diluted serum samples were included in all plates and processed as described above for all assay steps.

To control for the inter- and intra-assay variation in reactivity that is inherent to immunoenzymatic techniques, we included a control human serum pool in triplicate diluted in the same way as the specimens in every ELISA plate. A single batch of this serum pool was prepared beforehand from dozens of blood bank and normal clinical laboratory specimens from female adult donors at the AC Camargo Hospital in Sao Paulo. This serum pool was aliquoted and kept frozen at −20°C. An aliquot from this pool was thawed and processed in the same manner as that used for all study serum samples included in each ELISA run. The same serum pool was used throughout the study.

**Data analysis.** We used two different approaches to measure the reactivity of each sample. First, we calculated the net mean OD value by subtracting the mean blank OD from the mean of ODs for each sample by using values specific for each ELISA plate. This was done separately for the 1:10 and 1:50 dilutions. In the second approach, we computed normalized absorbance ratios (NARs) by further correcting the net mean OD value for each sample and dilution by dividing it by the equivalent mean result (blank corrected) from the serum pool included in the same plate in triplicate. As a consequence, NARs represent multiples of reactivity with respect to that of the serum pool, which presumably reflects the average HPV-16 antibody response from multiple donors of comparable ages. Both estimates (net ODs and NARs) were calculated for each dilution and as an average of the two dilutions, for a total of six values per sample. All estimates of reactivity are interval-scaled variables.

Our statistical analysis approach was intended to assess whether the NAR estimates provided a better expression of anti-HPV-16 seroreactivity than that measured via net OD values under the assumption that by normalizing the net absorbance, i.e., controlling for the within-plate reactivity of the serum pool, one is able to largely eliminate the "composite" noise in the assays due to fluctuations in reactivity caused by different batches of reagents (antigen, conjugate, and chromogen-substrate mixture), polystyrene plates, biases due to drifting accuracy and precision of pipetting, variations in washing efficiency, and other environmental and technical factors that can vary over time. By determining the ratio of the serological reactivity of each specimen to that of the serum pool from the same plate, we aimed to correct for the net influence of these various reaction parameters, thus largely reducing the effect of inter- and intra-assay variations.

Consistent with the procedures described above, we examined the distribution of net ODs for the control serum pool included in all plates for all assay runs to examine the extent of variation in reactivity of this control sample in the real-world setting of a large epidemiological study that spanned several years. We also examined the correlations between the seroreactivities of the serum samples at enrollment (visit 1) and at the first follow-up visit (visit 2, 4 months later) using each of the six estimates described above. Because of the skewness in distributions of OD and NAR values, we used log-transformed data to analyze correlations. We computed both Spearman rank (ρ) and Pearson ($r$) correlation coefficients.

Finally, we assessed the differences in performance between the two types of expression of seroreactivity by examining (i) the agreement between the two expressions of serological anti-HPV-16 IgG response categorized in distribution quintiles of seroreactivity at enrollment and (ii) the association between positivity for HPV-16 DNA in the cervical sample and seroreactivity based on each of the two methods, with values also categorized in quintiles for the six above-mentioned interval-scaled variables. We computed kappa statistics to assess agreement and odds ratios (ORs) and respective 95% confidence intervals (CIs) using unconditional logistic regression to gauge the strength of the associations between HPV-16 seroreactivity and HPV-16 DNA positivity.

## RESULTS

Of the 2,528 women enrolled in the Ludwig-McGill cohort, 2,462 were eligible for follow-up. The sociodemographic characteristics of the cohort were as follows: most women were white (64.4%), the mean age was 32.7 years (median, 32; range, 18 to 59), 19% had attained an educational level beyond high school (22.5% illiterate), and 81.7% were married or living with a common-law partner.

Serological results with the complete HPV-16 L1-plus-L2 capsid antigen were available for 2,048 women and were included in the analysis. For the analysis that compared results for the enrollment (visit 1) and the 4-month follow-up return (visit 2), there were 1,905 pairs of serum samples with evaluable results.

There was considerable variation in net OD values for the control serum pool included in all plates and in all assay runs (Fig. 1). This was evident for both dilutions (1:10 and 1:50). Expectedly, the same influences that affected the net seroreactivity of the control serum pool would have also affected the serological results for cohort participants. As shown in Fig. 2, the NAR correction represents an improvement over the net OD values, judging by the increased strength of the correlation between the seroreactivity measured at the two initial visits, on average 4 months apart. The improved correlation was evident for both dilutions and for the average of the two dilutions, as seen by the tighter distribution of data points using NAR than for the net OD values, which is also reflected in the higher Pearson and Spearman correlation coefficients for all three combinations. The coefficients of determination (Pearson's coefficients squared [$R^2$], representing the extent of variation that is explained by the underlying regression) were 0.389, 0.382, and 0.385 for net ODs and 0.559, 0.577, and 0.624 for NAR values at dilutions of 1:10 and 1:50 and the average of the two, respectively. Using the average dilution as an example, the proportional reduction in variability not explained by the un-

Downloaded from http://jcm.asm.org/ on September 16, 2013 by UNIVERSIDADE EST.PAULISTA JÃƒÅ¡LIO DE MESQUITA FILHO

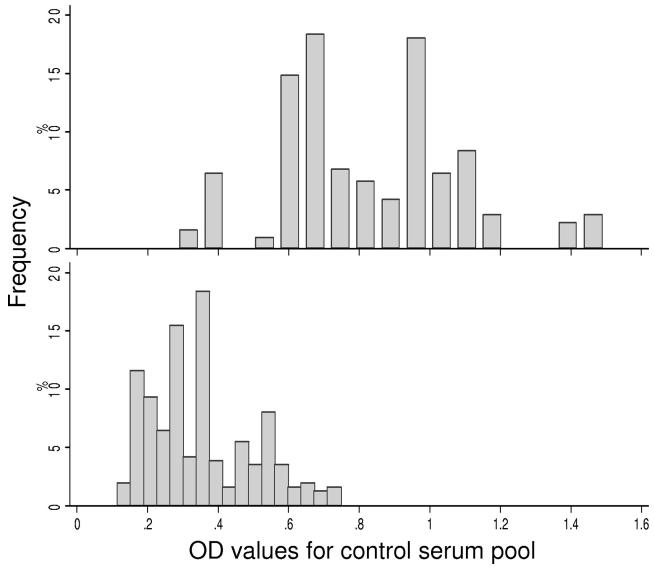


FIG. 1. Distribution of net optical density (OD) values ($n$ = 310) for anti-HPV-16 ELISA retroactivity results obtained with the control serum pool for all assay runs in two dilutions: 1:10 (top) and 1:50 (bottom). The $x$ axis indicates values for individual replicates in each plate, and the $y$ axis shows the proportion among all results.

TABLE 1. Agreement between methods of expressing anti-HPV-16 serological reactivity at enrolment among 2048 women enrolled in the Ludwig-McGill cohort[a]

| Serum dilution | NAR value quintile | Frequency according to net OD value quintile | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 1:10 | 1 | **306** | 88 | 15 | 0 | 0 |
| | 2 | 85 | **185** | 113 | 27 | 0 |
| | 3 | 13 | 98 | **148** | 121 | 29 |
| | 4 | 3 | 29 | 97 | **157** | 124 |
| | 5 | 1 | 11 | 38 | 103 | **257** |
| 1:50 | 1 | **262** | 110 | 30 | 7 | 0 |
| | 2 | 90 | **147** | 126 | 45 | 2 |
| | 3 | 43 | 80 | **132** | 128 | 27 |
| | 4 | 15 | 54 | 84 | **144** | 112 |
| | 5 | 1 | 14 | 39 | 88 | **268** |
| Avg of both | 1 | **297** | 85 | 27 | 0 | 0 |
| | 2 | 90 | **177** | 107 | 36 | 0 |
| | 3 | 14 | 101 | **145** | 123 | 27 |
| | 4 | 8 | 38 | 92 | **172** | 99 |
| | 5 | 0 | 9 | 39 | 81 | **281** |

[a] Results by serum dilution and frequencies based on distribution quintiles. Agreement and kappa statistics by dilution are as follows: 51.4% and 0.3927 for a 1:10 dilution, 46.5% and 0.3317 for a 1:50 dilution, and 52.3% and 0.4043 for the average of both dilutions, respectively. Cells with perfect agreement are shown in boldface type.

derlying regression was thus [(1 – 0.385) − (1 – 0.624)]/(1 – 0.385) or 39% when NAR values were used.

Although NAR and net OD values were correlated with each other at the respective dilutions, there was substantial deviation from perfect agreement when seroreactivity was categorized by distribution quintiles (Table 1). Perfect agreement was in the range of 46.5% to 52.3%, with resulting kappa statistics of 0.3317 to 0.4043, indicating, at best, a fair agreement beyond chance. A marked disagreement (2 or more quintile departures) occurred for 8.1% to 13.5% of the samples (Table 1).

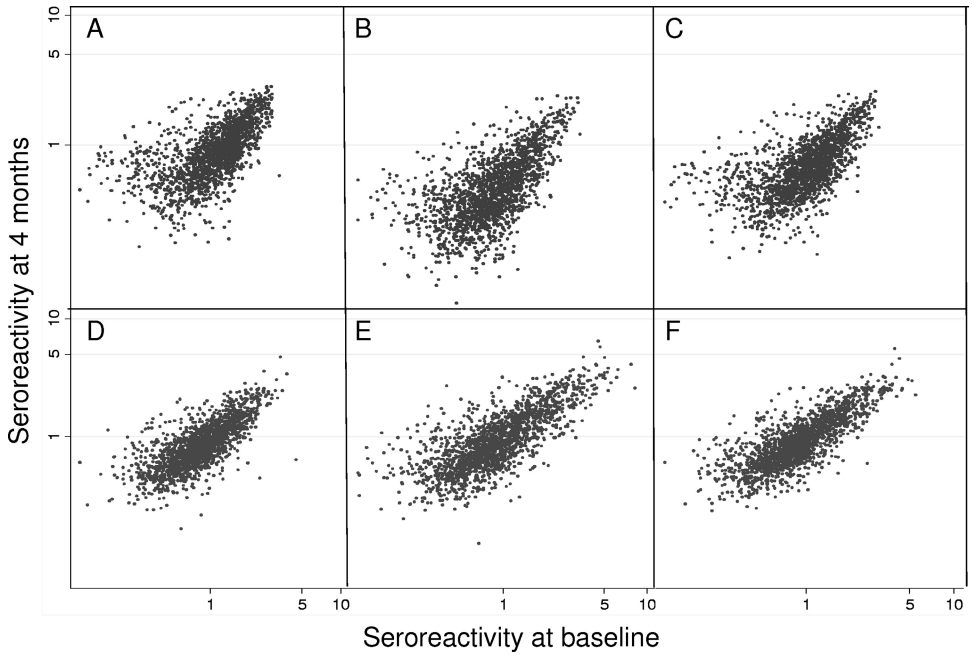


FIG. 2. Correlation between anti-HPV-16 seroreactivities at baseline and at the first follow-up visit at 4 months ($n$ = 1,905 pairs) using net OD (top row) and NAR (bottom row) values. Each series of graphs shows correlations for 1:10 (A, OD; D, NAR) and 1:50 (B, OD; E, NAR) serum dilutions and for the average of the two dilutions (C, OD; F, NAR). Log-transformed values were used to calculate correlation coefficients shown below. Spearman rank and Pearson correlation coefficients for each graph are 0.7082 and 0.6239 (A), 0.6397 and 0.6184 (B), 0.6679 and 0.6207 (C), 0.7616 and 0.7475 (D), 0.7550 and 0.7596 (E), and 0.7877 and 0.7902 (F), respectively.

Downloaded from http://jcm.asm.org/ on September 16, 2013 by UNIVERSIDADE EST.PAULISTA JÃƒÂ¡LIO DE MESQUITA FILHO

TABLE 2. Prevalences and odds ratios of cervical HPV-16 infection (as HPV-16 DNA positivity) at enrollment according to quintiles of anti-HPV-16 serological reactivity among 2,048 women enrolled in the Ludwig-McGill cohort[a]

| Serum dilution and quintile of seroreactivity | NAR value | | | Net OD value | | |
|---|---|---|---|---|---|---|
| | Cutoff value | No. of samples positive for HPV-16 DNA/total no. of samples (%) | OR (95% CI)[b] | Cutoff value | No. of samples positive for HPV-16 DNA/total no. of samples (%) | OR (95% CI)[b] |
| 1:10 | | | | | | |
| 1st | 0.559 | 6/409 (1.5) | 1.0 | 0.439 | 7/408 (1.7) | 1.0 |
| 2nd | 0.779 | 8/410 (2.0) | 1.34 (0.46–3.89) | 0.689 | 4/411 (1.0) | 0.56 (0.16–1.94) |
| 3rd | 0.993 | 8/409 (2.0) | 1.34 (0.46–3.90) | 0.928 | 9/411 (2.2) | 1.28 (0.47–3.47) |
| 4th | 1.310 | 8/410 (2.0) | 1.34 (0.46–3.89) | 1.205 | 18/408 (4.4) | 2.64 (1.09–6.40) |
| 5th | >1.310 | 27/410 (6.6) | 4.73 (1.93–11.59) | >1.205 | 19/410 (4.6) | 2.78 (1.16–6.69) |
| 1:50 | | | | | | |
| 1st | 0.530 | 5/409 (1.2) | 1.0 | 0.189 | 7/411 (1.7) | 1.0 |
| 2nd | 0.744 | 8/410 (2.0) | 1.61 (0.52–4.96) | 0.296 | 7/405 (1.7) | 1.02 (0.35–2.92) |
| 3rd | 1.008 | 9/410 (2.2) | 1.81 (0.60–5.46) | 0.419 | 4/411 (1.0) | 0.57 (0.16–1.95) |
| 4th | 1.502 | 9/409 (2.2) | 1.82 (0.60–5.48) | 0.625 | 17/412 (4.1) | 2.48 (1.02–6.06) |
| 5th | >1.502 | 26/410 (6.3) | 5.47 (2.08–14.39) | >0.625 | 22/409 (5.4) | 3.28 (1.39–7.76) |
| Avg of both[c] | | | | | | |
| 1st | 0.561 | 5/409 (1.2) | 1.0 | 0.331 | 7/409 (1.7) | 1.0 |
| 2nd | 0.766 | 7/410 (1.7) | 1.40 (0.44–4.46) | 0.502 | 6/410 (1.5) | 0.85 (0.23–2.99) |
| 3rd | 0.996 | 10/410 (2.4) | 2.02 (0.68–5.96) | 0.676 | 6/410 (1.5) | 0.85 (0.23–2.99) |
| 4th | 1.428 | 10/409 (2.4) | 2.03 (0.69–5.99) | 0.895 | 16/412 (3.9) | 2.19 (0.83–6.42) |
| 5th | >1.428 | 25/410 (6.1) | 5.25 (1.99–13.84) | >0.895 | 22/407 (5.4) | 3.25 (1.33–9.06) |

[a] Results by serum dilution and method of expressing seroreactivity.
[b] Odds ratios and 95% confidence intervals determined by unconditional logistic regression.
[c] Average NAR and average OD values calculated by taking the average of results at 1:10 and 1:50 dilutions.

The above-described analyses did not take into account whether the apparent improvement in quantifying serological reactivity via the NAR values translated into a greater validity with respect to the correlation with the presence of HPV-16 infection in the cervix. Table 2 shows the latter associations for all six combinations of methods for measuring seroreactivity and serum dilutions. There were 57 HPV-16-positive subjects at enrollment when we restricted the cohort to the 2,048 subjects who had evaluable serological results. The strength of the correlations with HPV-16 DNA detection was also increased with the use of the NAR method for all combinations. There was an improved risk stratification using NAR compared with the net OD values both in terms of the risk gradient and in terms of the consistency of the associations by quintile of seroreactivity. The differences in regression slopes were statistically significant for all 3 comparisons (Table 2) and favored stronger dose-response trends for NAR values (range of slopes, 0.746 to 0.915) than for net ODs (range of slopes, 0.564 to 0.602).

## DISCUSSION

While many studies have employed ELISA techniques for the detection of specific HPV antibodies in epidemiological studies, only a few have focused on assay validation and optimization (6, 8, 13). We expand on this topic by reporting the advantages of using a control serum pool to derive NAR values that control for the inherent variation in ELISA performance in a molecular epidemiological study in which serological testing was performed in many different runs over a period of several months. The relevant classes of serum antibodies against HPV capsids are IgG and IgA, as demonstrated by their correlation with the presence of type-specific HPV DNA (18). The IgA response is not long-lasting, as it correlates with recent and lifetime sexual partners of younger women (18), but it may help to predict the clearance of HPV infections (2). In contrast, IgG is more representative of past exposures. We demonstrated only the advantages of the NAR approach for IgG antibodies, but it is plausible to assume that the simple internal standardization procedure that we proposed would be valid for serological studies of any class of antibodies or isotypes of specific classes.

Because of its intrinsic property of correcting for the fluctuations in ELISA seroreactivity readouts, NAR values represented a more valid measurement of serological reactivity to anti-HPV-16 IgG antibodies than net OD values. We based this conclusion on two sets of findings: (i) that NARs were more strongly correlated for paired serum specimens taken 4 months apart than net ODs and (ii) that the strength and dose-response of the relationship between seroreactivity and the detection of cervical HPV-16 DNA were greater and more evident with NARs than with net ODs. These findings were reproduced irrespective of the serum dilution.

Although NAR values are arbitrary, unitless measurements, they are internally standardized. As ratios, they represent a multiple of seroreactivity with respect to the benchmark serum pool. Whether or not the latter is composed of subjects free of past HPV exposure is irrelevant. The precaution that we took of including samples from female blood bank donors and samples from female outpatients with normal blood chemistries, all taken from adult women, in the serum pool was intended simply to obtain a standard that would perform in the middle of the seroreactivity range that the HPV-16 capsid ELISA is supposed to detect.

Downloaded from http://jcm.asm.org/ on September 16, 2013 by UNIVERSIDADE EST.PAULISTA JÃƒÅ¡LIO DE MESQUITA FILHO

It had not been our intention to define a benchmark for no past HPV exposure so that a threshold of seropositivity could be defined. Deciding on whether or not a woman has been exposed to HPV-16 would have required relying on the history of sexual partners, testing for HPV-16 DNA in multiple mucosal sites in addition to the cervix, and/or applying an age restriction to minimize the opportunity for sexual exposure. While none of these criteria, either alone or in combination, would provide a guarantee of no past exposure, they would introduce circularity in the definition of seroreactivity, because our ultimate goal was to apply serology to study concurrent and subsequent risks of HPV-16 infection and its associated lesions in our cohort. Like net ODs, NARs represent a continuous, interval-scaled variable that can be subsequently examined for optimal cut points to define seroreactivity based on predetermined statistical quantiles or via a correlation with viral and clinical end points. This can be achieved by computing relative risks or ORs or by constructing receiver operating characteristic curves for preselected end points. Moreover, because they are interval scaled, NAR values can be used quantitatively or ordinally, with the advantage that the methodological "noise" that happens in multiple assay runs has been corrected for.

There was considerable variation in net OD values for the control serum pool at both dilutions. It could be argued that one could circumvent the need for the NAR correction by simply placing limits on the permissible range of net ODs yielded by this serum pool and then rejecting assay runs in which these limits had been exceeded. This precaution would have the intended effect of controlling for fluctuations in reagent chemistry, technical skill, and instrumentation but would have required the definition of what constitutes a permissible range beforehand. Such a quality assurance indicator would have to be defined arbitrarily, since accepted deviations in net ODs are not available in the HPV serology literature and would not be easy to harmonize across platforms because of the diversity in the technical performance of VLP ELISAs. Furthermore, in prospective cohort studies with multiple specimens collected over time, such a quality assurance safeguard, even if it could be implemented, would prove costly in the long run. We believe that the internal standardization approach that we described here, or any similar correcting maneuver, provides the necessary quality assurance while obviating the need for wasting reagent and technical time resources in rejected runs. The finding that the internal standardization used to produce NAR values results in improved performance with less measurement error can be seen in the magnitudes of the association with viral end points and in the improved concordance in seroreactivity between shortly spaced sampling visits.

The method that we describe contributes to reducing the fluctuation in assay performance, which is beneficial for seroepidemiological studies of natural immunity, postvaccination surveillance, or the future risk of new or recurrent HPV infections and associated cervical lesions. Although admittedly a technical improvement to assist HPV seroepidemiological studies, VLP serology remains inadequate as a cervical cancer screening tool to assist clinicians in patient management decisions.

In conclusion, as a prelude for conducting comprehensive analyses of the serological data in the Ludwig-McGill cohort study, we defined seroreactivity using a simple internally standardized approach. We believe that this method may be of use in minimizing errors in measuring the humoral antibody response to HPV capsids and may provide a cost-effective alternative to costly quality control safeguards that are required for large epidemiological surveys or cohort studies.

## ACKNOWLEDGMENTS

We are indebted to José Carlos Prado, Maria C. Costa, and Joao S. Sobrinho for performing the HPV DNA assays and to Maria L. Baggio and Lenice Galan for specimen collection and patient management.

Financial support was provided by the Ludwig Institute for Cancer Research (intramural grant to L.L.V. and E.L.F.), the U.S. National Cancer Institute (grant CA70269 to E.L.F.), and the Canadian Institutes of Health Research (operating grant 49396 and team grant 83320 to E.L.F.).

## REFERENCES

1. **Bauer, H. M., Y. Ting, C. E. Greer, J. C. Chambers, C. J. Tashiro, J. Chimera, A. Reingold, and M. M. Manos.** 1991. Genital human papillomavirus infection in female university students as determined by a PCR-based method. JAMA **265:**2809–2810.
2. **Bontkes, H. J., T. D. de Gruijl, J. M. Walboomers, J. T. Schiller, J. Dillner, T. J. Helmerhorst, R. H. Verheijen, R. J. Scheper, and C. J. Meijer.** 1999. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. II. Systemic but not local IgA responses correlate with clearance of HPV-16. J. Gen. Virol. **80**(Pt. 2)**:**409–417.
3. **Carter, J. J., L. A. Koutsky, J. P. Hughes, S. K. Lee, J. Kuypers, N. Kiviat, and D. A. Galloway.** 2000. Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection. J. Infect. Dis. **181:**1911–1919.
4. **Franco, E. L., L. Villa, T. Rohan, et al.** 1999. Design and methods of the Ludwig-McGill longitudinal study of the natural history of human papillomavirus infection and cervical neoplasia in Brazil. Rev. Panam. Salud Publica **6:**223–233.
5. **Gravitt, P. E., C. L. Peyton, T. Q. Alessi, C. M. Wheeler, F. Coutlée, A. Hildesheim, M. H. Schiffman, D. R. Scott, and R. J. Apple.** 2000. Improved amplification of genital human papillomaviruses. J. Clin. Microbiol. **38:**357–361.
6. **Karem, K. L., A. C. Poon, C. Bierl, R. Nisenbaum, and E. Unger.** 2002. Optimization of a human papillomavirus-specific enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **9:**577–582.
7. **Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Dürst, L. Gissmann, D. R. Lowy, and J. T. Schiller.** 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. J. Virol. **67:**6929–6936.
8. **Kirnbauer, R., N. L. Hubbert, C. M. Wheeler, T. M. Becker, D. R. Lowy, and J. T. Schiller.** 1994. A virus like particle enzyme linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. J. Natl. Cancer Inst. **86:**494–499.
9. **Nonnenmacher, B., J. Pintos, M. C. Bozzetti, I. Mielzinska-Lohnas, A. T. Lorincz, N. Ikuta, G. Schwartsmann, L. L. Villa, J. T. Schiller, and E. Franco.** 2003. Epidemiologic correlates of antibody response to human papillomavirus among women at low risk of cervical cancer. Int. J. STD AIDS **14:**258–265.
10. **Palefsky, J. M.** 1995. Serologic detection of human papillomavirus-related anogenital disease: new opportunities and challenges. J. Natl. Cancer Inst. **87:**437–440.
11. **Petter, A., K. Heim, M. Guger, A. C.-K. Nig, N. Christensen, M. Sarcletti, U. Wieland, H. Pfister, R. Zangerle, and R. Höpfl.** 2000. Specific serum IgG, IgM and IgA antibodies to human papillomavirus types 6, 11, 16, 18 and 31 virus-like particles in human immunodeficiency virus-seropositive women. J. Gen. Virol. **81**(Pt. 3)**:**701–708.
12. **Schlecht, N. F., S. Kulaga, J. Robitaille, S. Ferreira, M. Santos, R. A. Miyamura, E. Duarte-Franco, T. E. Rohan, A. Ferenczy, L. L. Villa, and E. L. Franco.** 2001. Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia. JAMA **286:**3106–3114.
13. **Studentsov, Y. Y., M. Schiffman, H. D. Strickler, G. Y. Ho, Y. Y. Pang, J. Schiller, R. Herrero, and R. D. Burk.** 2002. Enhanced enzyme-linked immunosorbent assay for detection of antibodies to virus-like particles of human papillomavirus. J. Clin. Microbiol. **40:**1755–1760.
14. **Tabrizi, S. N., I. H. Frazer, and S. M. Garland.** 2006. Serologic response to human papillomavirus 16 among Australian women with high-grade cervical intraepithelial neoplasia. Int. J. Gynecol. Cancer **16:**1032–1035.
15. **Trottier, H., S. Mahmud, J. C. Prado, J. S. Sobrinho, M. C. Costa, T. E. Rohan, L. L. Villa, and E. L. Franco.** 2008. Type-specific duration of human papillomavirus infection: implications for human papillomavirus screening and vaccination. J. Infect. Dis. **197:**1436–1447.
16. **van Doornum, G., M. Prins, A. Andersson-Ellström, and J. Dillner.** 1998. Immunoglobulin A, G, and M responses to L1 and L2 capsids of human

Downloaded from http://jcm.asm.org/ on September 16, 2013 by UNIVERSIDADE EST.PAULISTA JÃƒÂ¡LIO DE MESQUITA FILHO

papillomavirus types 6, 11, 16, 18, and 33 L1 after newly acquired infection. Sex. Transm. Infect. **74:**354–360.
17. **Wang, S. S., M. Schiffman, R. Herrero, J. Carreon, A. Hildesheim, A. C. Rodriguez, M. C. Bratti, M. E. Sherman, J. Morales, D. Guillen, M. Alfaro, B. Clayman, R. D. Burk, and R. P. Viscidi.** 2004. Determinants of human papillomavirus 16 serological conversion and persistence in a population-based cohort of 10 000 women in Costa Rica. Br. J. Cancer **91:**1269–1274.
18. **Wang, Z. H., L. Kjellberg, H. Abdalla, F. Wiklund, C. Eklund, P. Knekt, M. Lehtinen, I. Kallings, P. Lenner, G. Hallmans, C. G. Mählck, G. Wadell, and J. Schiller.** 2000. Type specificity and significance of different isotypes of serum antibodies to human papillomavirus capsids. J. Infect. Dis. **181:**456–462.
19. **Wideroff, L., M. H. Schiffman, B. Nonnenmacher, N. Hubbert, R. Kirnbauer, C. E. Greer, D. Lowy, A. T. Lorincz, M. M. Manos, A. G. Glass, et al.** 1995. Evaluation of seroreactivity to human papillomavirus type 16 virus-like particles in an incident case-control study of cervical neoplasia. J. Infect. Dis. **172:**1425–1430.

Downloaded from http://jcm.asm.org/ on September 16, 2013 by UNIVERSIDADE EST.PAULISTA JÃƒÅ¡LIO DE MESQUITA FILHO