

Test Reliability Is Critically Important to Molecular Epidemiology: An Example from Studies of Human Papillomavirus Infection and Cervical Neoplasia¹

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Abstract

To demonstrate that it is critically important to achieve excellent test reliability before conducting full-scale molecular epidemiological studies, data were compared from two consecutive case-control studies of human papillomavirus (HPV) infection and cervical intraepithelial neoplasia. The major methodological difference between the two studies was the much greater reliability of the HPV test used in the second study. Although the first study used an assay considered state-of-the-art at that time, mediocre test reliability led to (a) a weakened association between HPV and risk of cervical intraepithelial neoplasia, (b) a weakened association between known risk factors for cervical intraepithelial neoplasia and HPV prevalence, (c) failure to demonstrate that HPV infection explains the known risk factors for cervical intraepithelial neoplasia, and (d) a marked reduction in the estimated proportion of cervical intraepithelial neoplasia attributable to HPV infection. With an improved assay, the second study strongly supported the idea that HPV infection is an intermediate end point explaining the known epidemiology of cervical intraepithelial neoplasia. Based on this experience and supportive theoretical considerations, we recommend that researchers optimize the reliability of innovative assays before application to full-scale molecular epidemiological projects.

Goal of the Article

In collaborative "molecular epidemiology" studies, the importance of measurement error is often undervalued by both laboratory scientists and epidemiologists. To illustrate this point, a comparison is presented of data from two consecutive case-control studies of HPV² infection and cervical intraepithelial neoplasia. The comparison demonstrates how critical measurement error can be in molecular epidemiology, even when other epidemiological concerns have been addressed.

This article is intended for epidemiologists and laboratory scientists who are considering a collaborative molecular epidemiological study, incorporating newly developed, state-of-the-art laboratory assays into full-scale population studies. These collaborative studies are increasingly frequent in cancer research. Epidemiologists are eager to translate the powerful advances in understanding the molecular pathogenesis of cancer into assays that can be used in etiological and screening studies. Many molecular biologists wish to promote the clinical application of their advances.

Successful collaborative studies between molecular biologists and epidemiologists require a careful, joint consideration of methodological priorities, to avoid crushing the project with often competing demands deriving from the two disciplines. For example, should the study rely on more laborious, optimal testing techniques or can more rapid "shortcuts" be used? Should the project staff collect optimal biospecimens (e.g., biopsies) or those types of specimens more acceptable to the patient and clinician? Is it important to complete a large study to achieve good statistical "power" or is it wiser to assure quicker project completion and lower cost?

In our experience, the two most important epidemiological concerns in molecular epidemiological studies are assay reliability and the relatively unbiased selection of controls. This article will demonstrate the critical importance of assay reliability, defined as the ability

of the assay to generate consistent, comparable results when applied to many clinical specimens tested over the course of an epidemiological project, which can take months or even years. The supportive examples will be drawn from two investigations of HPV and cervical intraepithelial neoplasia. The underlying supportive theory of the discussion has been discussed in several previous publications (1-3).

HPV Infection and Cervical Neoplasia

Some background details concerning this area of cancer research are necessary to make sense of the examples. Epidemiological studies have consistently observed associations of sexual factors with risk of cervical cancer and its preinvasive precursor lesion, cervical intraepithelial neoplasia (4, 5). The major sexual risk factors have been shown to be lifetime number of sexual partners and age at first intercourse, with lifetime number of sexual partners being the most important single factor (5). These epidemiological observations have motivated the search for a venereally transmitted causative agent. HPV infection was first suggested to be that central etiological agent by zur Hausen *et al.* (6), who used DNA hybridization methods to detect HPV types 16 and 18 in a small group of cervical cancer specimens. DNA hybridization methods remain the primary means of testing for HPV infection. More than 70 types of HPV have been defined, of which about 20 are found in cervical specimens. It is now generally accepted that most cervical cancer specimens contain DNA of HPV types 16, 18, or a few other types (7). Cervical intraepithelial neoplasia specimens generally contain either the cancer-associated types or other types (e.g., 6, 11, 42) not found in cancers.

The early case series of HPV infection and cervical cancer were small, had informally chosen control specimens, and did not attempt to assess confounding by other covariates (8). However, these early studies used relatively accurate DNA hybridization analyses of tumor biopsies and derived the correct answer that HPV infection is strongly associated with risk of cervical cancer (later extended to intraepithelial neoplasia as well).

When epidemiologists sought to confirm the results of the early case series in rigorously controlled population studies, it was not possible to rely on HPV testing of biopsies, because of the ethical restriction on biopsying nondiseased women. There followed a several-year period when HPV measurement technology was first adapted to permit the more rapid testing of scant, noninvasively obtained cervical specimens collected at the time of routine gynecological examination by scrape or lavage. Many different combinations of specimen collection and HPV-testing methods were used (9). Interlaboratory comparisons of these varying strategies were generally discouraging, indicating poor assay reliability (10). We conducted our first case-control study of HPV infection and cervical intraepithelial neoplasia during this developmental period, using HPV-testing methods thought to be state-of-the-art at the time but soon proving to be inaccurate (11).

Improved HPV-testing methods were developed and validated after the conclusion of the first project (12, 13). In a second case-control study of HPV infection and cervical intraepithelial neoplasia (14), with a study design virtually identical with the first project but with an improved HPV-testing method, we readdressed the same analytic questions that had motivated the original effort (15). We reasoned

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² The abbreviations used are: HPV, human papillomavirus; Pap, Papanicolaou.

that, if HPV infection is the key venereal agent that explains the association of sexual behavior with risk of cervical intraepithelial neoplasia, the following should be true: (a) HPV infection should be strongly associated with risk of cervical intraepithelial neoplasia; (b) the risk of HPV infection, in turn, should be associated with the same measurements of sexual behavior known to influence the risk of intraepithelial neoplasia; (c) the often-observed associations of the sexual variables with risk of cervical intraepithelial neoplasia should be explained by HPV infection, *i.e.*, statistical adjustment for HPV infection should eliminate the association of intraepithelial neoplasia risk with sexual behavior; and (d) the attributable proportion of cervical intraepithelial neoplasia related to HPV infection should be high, if infection truly is a key intermediate end point on the causal pathway to cervical intraepithelial neoplasia (1). As shown below, the second case-control study confirmed all of these hypotheses (14). Based on a growing body of epidemiological and laboratory evidence, HPV is now widely accepted to be a central etiological factor for cervical neoplasia (16).

The purpose of this article is to show how moderate measurement error during the first study severely limited our ability to study the epidemiology of HPV and cervical intraepithelial neoplasia for each of the four statistical points listed above. The article will show that the epidemiological methods of the two studies were comparable, discuss the repeatability of the HPV measurements used in each study, and contrast the results obtained in the two projects. Finally, a few implications of the comparison will be discussed.

Materials and Methods

We conducted the first case-control study of HPV infection and cervical intraepithelial neoplasia in 1986–1987 at three Washington, DC, area hospitals. The design has been discussed in detail elsewhere (17). In brief, the case-control study was nested within a large cross-sectional screening of 2820 women receiving routine cervical cytological (Pap smear) screening in 13 different obstetrics and gynecology clinics. We successfully recruited 85% of eligible women. The Pap smear diagnosis was used to classify subjects as controls (normal or benign reactive changes, $n = 2517$) or cases (276 subjects with low-grade intraepithelial neoplasia and 27 with high-grade intraepithelial neoplasia were combined for this presentation). To measure HPV infection, a 3-ml cervicovaginal lavage was tested by Southern blot DNA hybridization techniques. Because of the expense of the assay, we tested a sample of the subjects, including 269 (89%) of the cases, and controls matched 2:1 to the cases on age group (± 5 years), race, clinic, and appointment date. We subsequently excluded from the analysis all controls with a medical history of cervical intraepithelial neoplasia or cancer, leaving 400 controls in the analytic data set.

Recruitment for the second case-control study was conducted in 1989–1990 at seven Kaiser-Permanente obstetrics and gynecology clinics in Portland, OR (14). About 22,000 women receiving routine Pap smear screening were recruited, and participation rates among eligible women approached 95%. Again, cervical cytological diagnoses were used to define subjects as cases of intraepithelial neoplasia or controls. For the second case-control study, we tested all of the high-grade cases of intraepithelial neoplasia ($n = 50$), and we selected randomly 450 cases of low-grade intraepithelial neoplasia (72% of the total) to complete a 500-woman case group. For controls, we selected randomly a 500-woman (3%) sample of the 17,824 women in the screening with normal cervical cytological diagnoses and no known medical history of cervical intraepithelial neoplasia or cancer.

The same consultant cytopathologist collaborated on both case-control studies; thus, the case definitions were comparable in the two studies. However, the HPV-testing methods were much different. In the second study, we used an improved 10-ml cervicovaginal lavage to collect more uniformly adequate DNA specimens. The specimens were tested by an I.I consensus primer polymerase chain reaction technique, developed by Manos *et al.* (12). This polymerase chain reaction method amplifies DNA from most of the 70 known (and some still unidentified) HPV types, permitting sensitive detection of HPV in minute quantities of clinical specimen. In advance of the study, the

polymerase chain reaction method compared favorably in an interlaboratory experiment (13) to an optimal Southern blot DNA hybridization (not the flawed Southern blot technique used in the first study).

In summary, the major methodological difference between the two projects involved the exposure measurement (HPV test). Other details were quite similar, with two possibly noteworthy exceptions. First, case-control matching was used in the first study alone. However, statistical control for the same factors (age, ethnicity, and clinic) in the second project did not change the conclusions, suggesting that this methodological difference is not likely to be crucial to the point being made here. Second, the population in the first study was younger and poorer than in the Portland project, leading to a higher true HPV prevalence among controls (18, 19) and reduced crude relative risks associating sexual behaviors with risks of HPV infection and cervical intraepithelial neoplasia. This second, more important caveat is discussed further below.

Results

Repeatability of HPV Tests in the Two Projects. Quality control repeat specimens from the first case-control study demonstrated mediocre repeatability of the HPV-testing procedure (Table 1). Concurrently, we observed poor interlaboratory agreement in a comparison of four laboratories (including our study collaborator) performing the same type of HPV test on identical aliquots of specimen DNA (10). There was interlaboratory disagreement regarding the presence of HPV infection in 14 (35%) of the 40 test specimens. Additional disagreements as to HPV type were substantial. We could not directly assess the accuracy of the DNA hybridization method we were using, because there was no reference standard for HPV DNA detection. We inferred, from the poor intra- and interlaboratory repeatability of the method, that the test was producing substantially misclassified and suspect data.

The intralaboratory repeatability of the polymerase chain reaction method used in the second case-control experiment was found to be much better, although it must be noted that masked repeat specimens in Portland were tested in the same batches, while in the DC project, repeat specimens were run in separate batches. This difference biases the repeatability data to apparently better repeatability in Portland. With this caveat, the differences are striking (Table 1). In addition, in the preliminary methodological pretest comparing the method to optimal Southern blot hybridization (the most trusted test for HPV typing), all specimens classified as HPV positive by Southern blot hybridization were also HPV positive by polymerase chain reaction, and agreement regarding HPV type was nearly complete (13). Based on these data, we concluded that the data generated in the second case-control study were likely to be much less misclassified than in the previous study. No direct comparison of the testing methods from the two studies has been performed.

Results from the Two HPV Studies. The comparisons of the two studies' results are shown in Tables 2–4. In Table 2, the odds ratios are shown from each of the two studies for the associations of HPV

Table 1. Intralaboratory repeatability of the HPV tests used in two case-control studies of cervical intraepithelial neoplasia

	Repeat test result		
	Inadequate DNA	HPV negative	HPV positive
Study 1 (1986–1987)			
Original test result			
Inadequate DNA	2	4	0
HPV negative	11	31	5
HPV positive	2	6	10
Study 2 (1989–1990)			
Original test result			
Inadequate DNA	3	2	1
HPV negative	1	21	0
HPV positive	0	0	22

Table 2 Odds ratios associating HPV infection with risk of cervical intraepithelial neoplasia in two case-control studies

	Controls		Cases		Odds ratio (95% confidence interval)
	No.	%	No.	%	
Study 1 (1986-1987)					
HPV negative	279	69.8	127	47.2	1.0
HPV positive	74	18.5	125	46.5	3.7 (2.6-5.3)
Inadequate sample	47	11.8	17	6.3	
Total	400		269		
Study 2 (1989-1990)					
HPV negative	375	75.0	89	17.8	1.0
HPV positive	80	16.0	381	76.2	20.1 (14.4-28.0)
Inadequate sample ^a	45	9.0	30	6.0	
Total	500		500		

^a Most inadequate samples resulted from problems in collection, not testing.

Table 3 Odds ratios associating HPV infection and lifetime number of sexual partners in the control groups of two studies of cervical intraepithelial neoplasia

Lifetime no. of sexual partners	No. of subjects	% HPV positive	Odds ratio (95% confidence interval)
Study 1 (1986-1987)			
1	64	10.9	1.0
2	53	20.8	2.1 (0.8-6.0)
3-4	81	25.9	2.8 (1.1-7.2)
5-9	71	25.4	2.8 (1.1-7.2)
10+	75	18.7	1.9 (0.7-5.0)
Study 2 (1989-1990)			
1	107	2.8	1.0
2	55	16.4	6.8 (1.8-26.2)
3-5	109	22.0	9.8 (2.8-33.6)
6-9	66	28.8	14.0 (4.0-49.7)
10+	68	25.0	11.6 (3.2-41.2)

infection with risk of intraepithelial neoplasia. The odds ratios were 1 order of magnitude higher in the second study. In particular, many case subjects in the first study were HPV negative, even those with high-grade cervical neoplasia (almost certainly in error, based on subsequent work; 20). HPV type-specific comparisons, not shown in Table 2, were also dramatic. In the first study, for example, the odds ratio associating all intraepithelial neoplasia with the two major cancer-associated HPV types (16 and 18) was 8.4 (3.0-22.9), and the estimate of risk was paradoxically lower in high-grade compared to low-grade disease. In the second study, the odds ratio for HPV 16/18 and all intraepithelial neoplasia was 50.1 (27.4-93.2), increasing to 167.3 (55.8-501.7) when restricted to high-grade intraepithelial neoplasia.

Table 3 shows the associations observed in the two studies between HPV infection and lifetime number of sexual partners, the main sexual risk factor for cervical intraepithelial neoplasia. Only a weak association of HPV infection with sexual behavior was seen in the first study, in accordance with three other large data sets published at approximately the same time that also suffered from testing error (3). No other risk factors for HPV infection were found. In contrast, the expected strong association of sexual behavior and HPV infection was convincingly demonstrated in the second project, confirming the results of another recent analysis using the same polymerase chain reaction test method (21). In the second study, moreover, we observed several additional univariate risk factors for HPV infection, including low socioeconomic status, oral contraceptive use, and young age. These factors are established univariate predictors of risk for cervical intraepithelial neoplasia.

We were able recently to test about 700 specimens from another sample of the DC control population, using the newer HPV method (19). This reexamination of the DC population has demonstrated a strong association of HPV infection and number of recent sexual

partners, which was not noted in the first study. The apparent association of lifetime number of partners and HPV positivity is truly weaker in the higher-risk DC population than in Portland, perhaps related to the higher HPV prevalence in the DC population (*i.e.*, in this instance, testing error was not the only cause of the differing results). Testing has also confirmed, however, that the original study failed completely to detect some other, important correlates of HPV infection (19), particularly a strong decrease in HPV positivity with increasing age.

In Table 4, the effects of adjustment for HPV infection are shown, for the associations of lifetime number of sexual partners and risk of cervical intraepithelial neoplasia. In the second study alone, we were able to demonstrate that HPV infection is likely to be the causal intermediate end point explaining the long-observed association of sexual behavior with risk of intraepithelial neoplasia. The other univariate predictors of risk of cervical intraepithelial neoplasia found in the data set (smoking, low educational status, age, and oral contraceptive use) were also explained by adjustment for HPV infection in the second study only.

The attributable proportions of cervical intraepithelial neoplasia explainable by HPV infection were 36.2% for the first study and 77.1% for the second. The attributable proportions were calculated according to the formula:

Attributable proportion

$$= \% \text{ HPV positivity among cases} \times 1 - \frac{1}{\text{Relative risk}}$$

The conclusions drawn from the two studies, based on these statistics, would clearly be different. In the first study HPV infection would appear to be a risk factor of some importance, but not the central etiological agent. Based on the second study, a central, causal etiological role appears more likely. Of note, the prevalence of HPV infection in cases and the attributable risk increased even further, to near 90%, when misclassification of the disease end point was reduced by expert pathology panel reviewers unaware of the HPV infection status (14).

Discussion

First, to mention the limitations of the comparison, the data would be even more convincing if we had been able to retest the exact specimens from the first study using the newer HPV test. Some of the differences in results could be explained by differences in the two study populations. Specifically, the Portland population is at much lower risk than the DC population for cervical neoplasia, and the

Table 4 Effect of adjustment for HPV infection on the association between lifetime number of sexual partners and risk of cervical intraepithelial neoplasia

Lifetime no. of sexual partners	Cases	Controls	Crude odds ratio (95% confidence interval)	Adjusted odds ratio ^a (95% confidence interval)
Study 1 (1986-1987) ^b				
1	25	69	1.0	1.0
2	47	61	2.1 (1.2-3.9)	2.2 (1.2-4.0)
3-4	69	90	2.1 (1.2-3.7)	2.0 (1.1-3.6)
5-9	71	79	2.5 (1.4-4.3)	2.4 (1.3-4.3)
10+	48	89	1.5 (0.8-2.7)	1.5 (0.8-2.8)
Study 2 (1989-1990) ^a				
1	40	113	1.0	1.0
2	34	58	1.7 (0.9-2.9)	1.0 (0.5-1.9)
3-5	127	116	3.1 (2.0-4.8)	1.1 (0.6-1.9)
6-9	116	70	4.7 (2.9-7.5)	1.5 (0.9-2.7)
10+	116	74	4.4 (2.8-7.0)	1.6 (0.9-2.8)

^a Adjusted for HPV DNA detection.

^b Excludes subjects with missing questionnaire or HPV data.

Portland women report fewer average lifetime numbers of sexual partners. This difference in populations is not negligible but is unlikely to explain the major divergence of the two studies. The recent testing of specimens from previously untested controls in the Washington, DC, population, using the new HPV method, demonstrated epidemiological associations previously missed there. Moreover, a recent study confirmed the central role of HPV infection in explaining sexual risk factors for cervical neoplasia, in both a low-risk (like Portland) and a high-risk (like the Washington, DC) population (22).

Although consecutive studies can never be compared with absolute certainty of comparability, the data strongly indicate the importance of test reliability in molecular epidemiological studies. This point has been proven more broadly by the entire field of HPV epidemiology, which has made progress in parallel with advances in HPV-testing methods (20). Thus, it is worthwhile to discuss a few of the possible implications of the comparison presented here.

First, the comparison demonstrated that even moderate amounts of misclassification of dichotomous variables, a category that includes many biomarkers and screening tests, can dramatically affect epidemiological associations (3, 23). What true relative risks underlie the odds ratios of 1.5 and 2.0 that we epidemiologists routinely find using other assays (or even questionnaires) that may prove as inaccurate as the HPV test used in the first study?

Epidemiologists might do well to focus on measurement error as a primary issue when they critique ground-breaking clinical studies that use state-of-the-art measurement techniques. We epidemiologists tend to criticize early clinical studies by listing a set of predictable complaints: the case and control groups are often convenience samples leading to possible bias, the studies are too small to generate stable risk estimates, there is usually no control for possible confounding factors, and the use of statistical tests of significance may be absent or naive. These criticisms may be accurate but may also be relatively unimportant compared to measurement error, which is a common problem with newly developed, highly technical assays. In the field of HPV infection and cervical cancer, the early clinical experiments were more correct (in addition to faster and less expensive) than the first generation of carefully performed epidemiological studies, because the small clinical studies were able to use accurate measurements. It is hard to imagine any subtle selection bias or naive use of statistics that would produce conclusions as wrong as those we generated in our first case-control study of HPV infection and cervical intraepithelial neoplasia, despite its large size, methodological rigor, and cost.

Epidemiologists working with data from laboratory assays must study the sources and extent of error in those assays. It is difficult for an epidemiologist to question laboratory scientists who wish to collaborate as to whether their assays are indeed measuring what they expect with adequate repeatability and accuracy to generate correct results. The quality of assays is considered by most laboratory collaborators (and many epidemiologists) to be exclusively a laboratory issue. Nonetheless, exposure measurements should be validated in epidemiological pretests (sometimes called "transitional studies") before the start of a major epidemiological project. Quality control repeats performed as a project progresses can be used to document problems in measurement but cannot prevent the kind of major failure we experienced in our first case-control study of HPV infection and cervical intraepithelial neoplasia. In that project, the mediocre repeatability of quality control samples convinced us to change to a new testing method after 25% of specimens had been tested. Unfortunately, when the second method (the one presented here) failed as well, we were left with inadequate amounts of residual specimen to start again, and a multiyear effort was fundamentally wasted. Epidemiologists wishing to avoid such a lesson can spend the time and

resources to assess their exposure measurements ahead of the main analytic project.

Even the most sophisticated assay cannot escape from biological variability and error. Therefore, we must make sure that our measurement techniques are as trustworthy as possible. In addition, it is essential to choose a fundamentally unbiased group of controls. Other concerns, such as subtler biases of design, statistical power calculations, and proper analytic methods, are more minor issues in molecular epidemiology, which focuses typically on detecting new associations with high relative risks.

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