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Combining Pooling and Alternative Algorithms in Seroprevalence Studies

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Data from two seroprevalence studies and one comparative study of confirmatory algorithms were used to compare the costs and sensitivities of six algorithms for determining seropositivity to human immunodeficiency virus (HIV). We evaluated confirmatory strategies by using the CBC Recombigen HIV enzyme immunoassay (EIA; Cambridge BioScience, Worcester, Mass.) and immunoblotting followed by radioimmunoprecipitation assay to confirm indeterminate immunoblotting results with and without pooling of samples during screening. The least expensive algorithm was that in which sera were pooled during screening and EIA was used to confirm positive test results. The cost savings associated with this confirmatory test were greater when the prevalence of HIV infection was higher. Savings from pooling of sera for screen testing diminished as HIV prevalence increased. The sensitivity and specificity of EIA with respect to immunoblotting and radioimmunoprecipitation assay were estimated to be 0.9992 and 0.9977, respectively. We found that the implementation of pooling during screening and the use of EIA as the confirmatory test do not affect the statistical reliability of estimates of seropositivity but do result in considerable cost savings.

Accurate prediction of the extent and impact of the human immunodeficiency virus (HIV)-AIDS epidemic requires that proper epidemiological studies be completed. Volunteer studies are exquisitely sensitive to self-selection bias. For this reason, investigators have turned to unlinked seroprevalence surveys in which large numbers of specimens left over from studies that used routinely collected samples are tested after identifying information has been removed. However, such studies are costly because of the large numbers of screening tests needed and the requisite confirmatory assay for screen test-positive samples. Alternative strategies have been developed to reduce the costs of these large serosurveys, including the pooling of samples prior to testing (1, 6, 8) and the use of less expensive tests as the supplemental assay in lieu of the more expensive immunoblotting, immunofluorescence assay, or radioimmunoprecipitation assay (RIPA) (2, 5, 12, 13, 15). In this report, we compare the costs of six algorithms for testing for HIV seropositivity. The following three strategies for confirming screen test-reactive sera were compared: CBC Recombigen HIV enzyme immunoassay (EIA; Cambridge BioScience, Worcester, Mass.), immunoblotting with RIPA to confirm indeterminate immunoblotting results, and immunoblotting with repeat enzyme-linked immunosorbent assay (ELISA) and immunoblottings at a later visit to confirm indeterminate results. These three strategies for confirming repeat screen test-positive samples are illustrated in Fig. 1. Each of the strategies was studied with and without pooling of samples during screening for a total of six algorithms. We demonstrate that the combination of pooling and then the use of the EIA for confirming screen test-reactive samples results in significant cost reductions without impairing the statistical reliability of the study.

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MATERIALS AND METHODS

Study populations. We used data from three studies to compare confirmatory tests and to evaluate the effectiveness of pooling in reducing the costs of screen testing.

In the first study, we compared two confirmatory testing strategies using 2,212 samples received consecutively at the Federal Center for AIDS Laboratories, the Canadian National Reference Laboratory for HIV (9). Sera which were repeatedly positive by ELISA (Genetic Systems Corporation, Seattle, Wash.) were verified as being antibody positive by immunoblotting. Western blots (immunoblots) were performed by using the human T-cell lymphotropic virus type III Western blot kit (Du Pont Co., Wilmington, Del.). Samples which demonstrated antibody reactivity to *env* and *gag* proteins were considered Western blot positive. Samples were negative if no bands were seen on the Western blot and were indeterminate if they failed to meet the criteria of either a positive or a negative sample. Sera which were antibody indeterminate by immunoblotting were then tested by RIPA. Samples reported as immunoblot or RIPA positive were considered to be antibody positive. Concomitantly, all ELISA-positive sera were tested by the EIA according to the manufacturer's instructions. The EIA did not yield any "gray zone" results. Instead, all results were either positive or negative. In the present study, the prevalence of HIV antibody positivity was nearly 50%, because at that time we were testing sera collected from high-risk individuals for specific investigations.

The second study was a prevalence study of childbearing women in Quebec. Over a 3-year period, 199,962 heel prick samples were collected as dried blood spots from newborn children and were screened for HIV antibody (7). Eluates which were repeatedly reactive on the screen test were retested by the EIA as well as by immunoblotting, as described above. This population was at low risk for HIV infection; only 125 samples were confirmed to be antibody

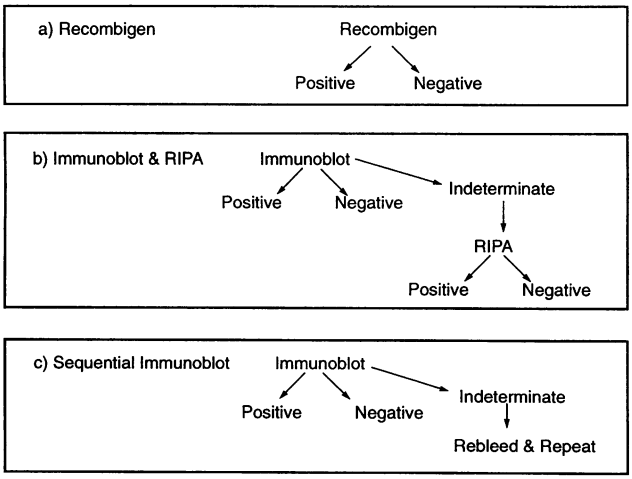

FIG. 1. Flowcharts of confirmatory algorithms.

positive. Data from these two studies were used to estimate the sensitivity and specificity of the EIA relative to immunoblotting and RIPA.

The third study was an ongoing seroprevalence study in British Columbia in which sera left over from a clinical chemistry laboratory were tested for the presence of HIV (14). To date, 40,000 samples from 50 outpatient collecting sites have been tested. Since serum samples were pooled for screen testing in the present study, the data were used to illustrate the benefit of pooling sera and then verifying antibody-positive status by an alternative algorithm. The sera were stored at 4°C before pooling and were then warmed to room temperature on the day that the samples were pooled. Sera were pooled in groups of 10 by adding 200 μl from each sample to a tube with a final volume of 2.0 ml. The pools were mixed by vortexing, and 5.0 μl of the aggregate sample was tested for anti-HIV antibody by using HIV Vironostika (Organon Teknika, Durham, N.C.). With the exception of pooling, the kit was used according to the manufacturer's recommendations. The optical density cutoff used in the present study was that established by the manufacturer's guidelines, but any pool with an optical density reading that was 10% below the cutoff was treated as a reactive sample. All 10 specimens within reactive pools were retested as individual samples. Screen test-reactive sera were then tested by the EIA procedure. Samples which were screen test and EIA reactive were considered to be HIV antibody positive.

Calculation of costs. All costs are given in Canadian dollars. The costs of testing fall into the following two main categories: those of screening tests and those of confirmatory tests. Screening is a two-step process involving the initial testing and repeat testing of reactive samples. The cost of the initial test depends on whether pooling is done. The cost of repeat tests on reactive samples depends on the prevalence of HIV in the population and on the sensitivity and specificity of the screening test.

When sera were not pooled during screening, the initial ELISA cost approximately $1 per sample for labor and $1.18 per sample for materials, for a total cost of $2.18 per sample. When samples were pooled in groups of 10, the cost per sample was approximately $0.55, excluding overhead costs. Each sample that tested positive or that was in a pool which tested positive had to be retested, at a cost of $2.18 per sample. If the probability of an individual specimen testing positive is unrelated to the probability of a positive result among the other samples in the pool, then the probability of a pool testing positive is $1 - (1 - P)^n$, where P is the probability of an individual sample testing positive and n is the size of the pool, for example, 10.

The cost of confirming samples which are repeatedly screen test reactive can exceed the cost of screen testing of all samples. The numbers of true- and false-positive test results depend on the prevalence of HIV in the population and on the sensitivity and specificity of the screening test. Screen test-positive samples are confirmed with either the immunoblot or the EIA at costs of $48.50 and $3.90 per test, respectively. When the immunoblot is used to confirm screen test-positive results, some samples may have indeterminate results and must be retested by either RIPA, at a cost of $105, or a repeat immunoblot. The cost of materials associated with immunoblotting, EIA, and RIPA are $45.00, $3.00, and $80.00, respectively. The remainder of the costs of performing these tests are for labor.

RESULTS

Sensitivity and specificity. We estimated the sensitivity and specificity of the EIA as a confirmatory test compared with the "gold standard" algorithm of immunoblotting and RIPA using data from studies of high-risk individuals and childbearing women. In the study of high-risk individuals, the sensitivity and specificity of the EIA were calculated to be 99.9 and 99.7%, respectively, as reported previously (3).

Of the 199,962 heel prick specimens collected from newborns, 417 were repeatedly reactive by ELISA and were retested by immunoblotting and EIA. One hundred twenty-five samples were antibody positive by both methods. Fifty-four samples with immunoblotting-indeterminate results were retested by RIPA and were found to be negative, resulting in a total of 292 samples which were antibody negative by both methods. There were no samples with discordant results. In the present study, the EIA was found to have 100% sensitivity and specificity when compared with immunoblotting and RIPA combined.

When results from the two studies were aggregated, 1,294 samples were antibody positive by both methods, 1,330 samples were antibody negative by both methods, 3 samples were positive by the EIA but negative by immunoblotting and RIPA, and 1 sample was negative by the EIA but positive by immunoblotting and RIPA. By using the combined data, the sensitivity and specificity of the EIA with respect to immunoblotting and RIPA were calculated to be 0.9992 and 0.9977, respectively, with corresponding 95% confidence intervals of 0.998 and 1 and 0.995 and 1.

The results from three samples which were found to be positive by EIA but negative by immunoblotting and RIPA may have been due to the imperfect specificity of the immunoblotting technique. If, however, the samples that gave these discordant results were cross-contaminated during processing, as suspected, these results would then represent a worst-case scenario for the performance of the EIA. The discordant specimens could not be retested since they were from foreign Red Cross blood donors.

The sensitivity of the proposed algorithm is the product of the sensitivity of the ELISA and the sensitivity of the EIA. The specificity of the proposed algorithm is 1 − (1 − specificity of ELISA) · (1 − specificity of EIA). The sensitivity and specificity of a screening ELISA confirmed with

TABLE 1. Numbers of expected test results for 100,000 ELISAs[a]

ELISA result	No. of expected test results for the following prevalence of HIV:			
	0.001	0.01	0.03	0.10
True positive	100	1,000	3,000	10,000
Testing positive	300	1,196	3,188	10,160
False positive	200	198	194	180
False negative	0	2	6	20
Indeterminate immunoblot	70	69	68	63

[a] The sensitivity and specificity of the ELISA were both assumed to be 99.8%. Of the false-positive ELISA samples, 34% were assumed to have indeterminate immunoblotting results.

the EIA compared with those of a screening ELISA and then immunoblotting and RIPA were calculated to be 0.997 and 0.999995, respectively.

Costs of alternative algorithms. In order to calculate the costs associated with the various algorithms, it was necessary to estimate the number of samples that would test positive during the screening process and the number of indeterminate results from the immunoblot. The numbers of different types of results depend on the prevalence of HIV infection, the sensitivity and specificity of the screening test, and the percentage of false-positive samples yielding indeterminate immunoblot results. Genetic Systems Corporation reports that the sensitivity and specificity of their ELISA are 99.9% each; however, we assumed a sensitivity and specificity of 99.8% each to be conservative. In the study of high-risk individuals, 402 of the 1,039 specimens with false-positive ELISA results had indeterminate immunoblotting results. Of the 292 specimens with false-positive ELISA results in the study of childbearing women, 54 had indeterminate immunoblotting results. Combining these data, we estimated that 34% of the false-positive samples would have indeterminate immunoblotting results. With these estimates, we calculated the expected test results from 100,000 screening tests for prevalences of HIV infection ranging from 0.001 to 0.10 (Table 1).

Using the expected test results from Table 1 and the costs of each test as described in detail above, we calculated the cost (per sample) of the different algorithms for a range of prevalences of HIV infection (Table 2). The least-expensive strategy was found to be pooling of the samples for screening and use of the EIA to confirm screen test-positive test results. The costs of confirming screen test-positive samples by immunoblotting and RIPA and with sequential immunoblotting were virtually identical. The cost savings associated with the use of the EIA as a confirmatory test were greater for higher prevalences. Substantial savings resulted from pooling of samples for screen testing when the prevalence of HIV was low; however, these savings diminished as the prevalence of HIV increased. As the prevalence of HIV infection approached 10%, the savings associated with pool sizes of 10 became small. The use of smaller pool sizes would be more cost-effective when the prevalence is high.

Pooling. Of the 4,009 pools tested to date in the ongoing seroprevalence study in British Columbia, 349 yielded positive antibody results. These 349 pools contained 489 individual serum specimens which tested positive when the members of the initially reactive pools were tested separately. Each of the pools of sera which tested positive contained at least one positive sample when the 10 samples were assayed individually, indicating that the pooling did not adversely affect the specificity of the Organon Teknika test kit. Given that 489 of 40,090 samples tested were antibody positive, we would have expected 463 pools to test positive according to the formula presented in Materials and Methods. Fewer pools than expected tested positive, however, as a result of the clustering of positive results at one of the laboratory sites.

TABLE 2. Costs of alternative algorithms per sample

Algorithm	Cost with the following prevalence of HIV[a]:			
	0.001	0.01	0.03	0.10
No pooling, EIA	2.19	2.23	2.30	2.58
No pooling, immunoblotting and RIPA	2.40	2.83	3.80	7.17
No pooling, sequential immunoblotting	2.39	2.83	3.79	7.17
Pooling, EIA	0.63	0.84	1.28	2.38
Pooling, immunoblotting and RIPA	0.83	1.45	2.77	6.98
Pooling, sequential immunoblotting	0.83	1.45	2.77	6.97

[a] Costs are in Canadian dollars. An ELISA was assumed to cost $2.18 per sample when pooling was not used and $0.55 per sample when samples were pooled in groups of 10. The costs of the confirmatory assays were assumed to be as follows: EIA, $3.90; immunoblotting, $48.50; RIPA, $105.

Positive predictive value. The positive predictive value, i.e., the probability that the results of the proposed algorithm agree with those of the gold standard, is of considerable interest. This probability depends on the sensitivity and specificity of the test and on the prevalence of the disease in the population tested. We calculated the positive predictive value of the proposed algorithm for a range of prevalences and specificities. Since the sensitivity has the least influence on the positive predictive value, we left this constant at 0.997. The results in Table 3 show that a positive test result from the proposed algorithm is highly predictive of HIV disease for a range of prevalences and specificities and has no appreciable impact on estimates derived from large seroprevalence studies. In the worst case, in which the positive predictive value is 0.91, the seroprevalence derived from the survey would overestimate the gold standard infection by only about 10%.

DISCUSSION

We have not discussed the indirect immunofluorescence test in this report because this procedure is not used exten-

TABLE 3. Positive predictive values of ELISA and EIA

Specificity of algorithm	Positive predictive value with the following prevalence of HIV[a]:			
	0.0001	0.001	0.01	0.1
0.999991	0.91	0.991	0.9991	0.99991
0.999993	0.93	0.993	0.9993	0.99993
0.999995	0.95	0.995	0.9995	0.99995
0.999997	0.97	0.997	0.9997	0.99997
0.999999	0.99	0.999	0.9999	0.99999

[a] The sensitivity of the proposed algorithm was assumed to be 0.997. Positive predictive value = sensitivity · prevalence/[sensitivity · prevalence + (1 − specificity) · (1 − prevalence)].

sively in Canada. That test is less expensive than immunoblotting and RIPA and acceptable performance has been reported for the immunofluorescence test.

We and others (2, 5, 12, 13, 15, 16) have demonstrated that alternatives to the standard algorithm of confirming screen test-positive samples by immunoblotting exist. The data presented here illustrate that the implementation of the alternative algorithm does not adversely affect the statistical reliability of estimates of seropositivity. In addition, we found that considerable cost savings accrue. For example, when the prevalence is 0.01 and when samples are pooled for screen testing and EIA is used as the confirmatory test, the materials for testing 100,000 serum specimens cost $84,000. This value is in contrast to a cost of $283,000 for screening samples individually and for confirming positive samples by immunoblotting and RIPA. Although the cost benefit of pooling is substantial, aggregating sera into pools of 10 does not result in a cost savings of 90%. There are considerable overhead costs associated with constructing the pools; these include the costs associated with increased technologist effort, the need for additional record keeping, expenditures on extra laboratory supplies, and interference with normal laboratory routines.

A considerable portion of the cost associated with pooling of sera for screen testing results from the need to test samples individually from each pool which tests positive. In large seroprevalence studies, it may be possible to minimize the number of pools testing positive by pooling samples thought to be at higher risk of seropositivity separately. As shown in the British Columbia seroprevalence study (14), in which seroprevalence varied by geographic area, pooling of samples by laboratory site resulted in many fewer pools testing positive than would have been expected from the overall estimated prevalence.

There may be limitations of pooling sera prior to testing. In a study of samples received in a national reference laboratory, typing by using a human genetic marker, the variable number of tandem repeats, showed that 1% of the samples were mislabeled (11). Other studies have found similar rates of mislabeling (3). Pooling may increase the rate of misidentification because of the increased handling of samples by laboratory staff. In addition, the considerable manipulations required to construct the pools may result in the occasional omission of a sample, and thus a decrease in the sensitivity of the proposed algorithm. Another concern is the cross-contamination of samples as a result of poor laboratory practices as observed in several large seroprevalence studies (10). Laboratory practices which can result in the cross-contamination of samples include the repeated use of the same pipette tip and the too vigorous shaking of racks of open tubes of sera. Lastly, since seroprevalence studies often use residual samples from routine laboratory testing, the integrity of the sera may not be retained. Errors such as those described here could occur with pooling protocols if sufficient detail is not followed in either the laboratory that collects the samples or the laboratory performing the HIV antibody assays. For these reasons, laboratories which use pooling for cost saving purposes must have strong quality assurance programs in place prior to embarking on this strategy.

Pooling may also result in decreased sensitivity and specificity of the proposed algorithm for reasons other than laboratory procedures. Sensitivity may be decreased if antibody-positive samples with low reactivity in the EIA are undetected in pools because the optical density of the 10×-diluted antibody-positive sample falls below the established gray zone of the screening test (10% below the cutoff of the EIA). Specificity could be adversely affected if nonspecific interactions between individual samples during pooling cause false-positive results. Such interactions were not found to occur in the present study, however. In addition, the use of the EIA as the confirmatory assay eliminates the well-known difficulty of the relatively low specificity of immunoblotting (4).

Despite the concerns that we have raised here, we recommend the use of pooling during the screening of samples for HIV antibody and the use of the Recombigen EIA as the confirmatory assay. The potential loss in sensitivity because of the low levels of reactivity of the samples and the potential loss of specificity because of nonspecific interactions between samples is minimal compared with the loss in these measures because of poor laboratory practices. We feel that with proper implementation of strict laboratory practices, such errors can be eliminated and that the possibility of additional errors resulting from the pooling process can be minimized.

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