

MINIREVIEW

Frequency, Causes, and New Challenges of Indeterminate Results in Western Blot Confirmatory Testing for Antibodies to Human Immunodeficiency Virus[∇]

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More than 2 decades of developments in the field have led to a wide variety of test kits available for addressing the different needs of surveillance, diagnosis, and monitoring of human immunodeficiency virus (HIV) infection and antiretroviral therapy (20, 23). These test kits are based on different technologies, including immunoassays and molecular testing. Typically, enzyme-linked immunosorbent assays (ELISAs) and rapid tests for detecting anti-HIV antibodies are used for blood screening and/or surveillance purposes. The former include third-generation ELISAs that utilize the antigen sandwich design for improved sensitivity, while the latter term refers to immunochromatographic tests or other simple antibody tests requiring less than 30 min of assay time. Since the viral capsid (core) p24 protein can be detected earlier than anti-HIV antibodies (93), attempts had also been made to develop a new generation of ELISA capable of simultaneously detecting the p24 antigen and anti-HIV antibodies (34, 52, 76, 101, 103). Significant reductions of the window period were achieved, which is ideal for blood screening and surveillance (102), but users were also advised to be mindful of possible limitations when utilizing such a combination assay (52, 81, 83). With specific panels, a delay in detection by a fourth-generation ELISA was evident compared with a dedicated p24 antigen assay (81). This shortcoming of fourth-generation ELISAs is understandable, because the surface area of the solid phase for detection is constant and limited regardless of whether it is utilized for a single test or combined assays (101). It should also be noted that an additional procedure for the dissociation of immune complexes employed by dedicated p24 antigen assays but not by combined assays was found to facilitate sensitive detection (85). However, this does not mean that using ELISA dedicated for detecting p24 antigen has no shortcomings. The viral p24 protein is a transient marker that can fall to an undetectable level after initial acute infection and only reemerges at the advanced stage of AIDS in patients (93, 107). Hence, tests utilizing this marker are more valuable for situations such as screening blood products from individuals before antibodies are produced, detecting early infections in persons who have been exposed but are seronegative, or mon-

itoring antiviral therapy (20). In this respect, the diagnostic value of a p24 antigen assay is restricted to detecting infections in newborns of HIV-positive mothers or individuals who have had very recent high-risk exposure (59). In fact, dedicated p24 antigen tests add very little to safety when used in blood screening (1), especially in view of the advances made with molecular testing (22). For even earlier detection, nucleic acid tests were often found to have a clear advantage in sensitivity over p24 antigen assays (22, 51, 84) or other immunoassays (66). The nucleic acid test approach utilizes molecular technologies, such as in situ hybridization, reverse transcription-PCR, nucleic acid sequence-based amplification, and branched-chain DNA, for the detection of viral nucleic acid, such as viral RNA or proviral DNA. In particular, by measuring the levels of viral RNA, the number of HIV virions in the blood (viral load) can be established. Therefore, RNA testing is a valuable tool, not only for the early detection of infections, but also (more commonly) for monitoring disease progression and antiretroviral therapy. However, one must be mindful of the fact that the approach using RNA testing has not been readily accepted by the relevant authorities for the purpose of diagnostics (17, 20) until very recently. To date, only one RNA qualitative assay has been approved by the U.S. FDA for use as an aid in the diagnosis of HIV type 1 (HIV-1) infection, including primary or acute infection (<http://www.fda.gov/cber/approvltr/hivhcvgen100406L.htm>). The precaution and exclusion are primarily due to the uncertainty in providing a correct diagnosis under certain circumstances. The shortcomings of HIV RNA testing include variability in general in viral-load measurements, inconsistency in performance when testing different genotypes or clades, and sensitivity to inhibiting substances sometimes present in specimens that may result in false negatives (60, 86). In addition, there were reported cases of false positives (FPs), suggesting that HIV viral-load testing is an inappropriate tool for the diagnosis of acute HIV infection (70). Furthermore, the lack of a universally recognized standard for quantification impedes a valid comparison of results from different assays or verification of equivalence among assays using different amplification technologies (20). In short, the inherent limitations of the procedure make it inappropriate to use the procedure alone as a confirmatory test for the purpose of diagnosis. In this respect, the Western blot immunoassay, since its development in the late 1980s, has been the most commonly accepted confirmatory test. To many, it re-

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mains a gold standard for validating screening test results and provides valuable information for the diagnosis of an HIV infection (20, 23, 57, 87). In contrast to ELISAs or rapid tests that provide results reflecting the reactivities of antibodies to any or all of the antigens indiscriminately for screening purposes, Western blotting or line immunoassays with separated viral proteins immobilized on membrane testing strips generate specific information on the reactivities of antibodies to individual proteins (74). Such specific and detailed information can then be interpreted according to approved criteria set by the relevant authorities for validating the positive results of screening tests and hence ruling out those with false detection (15, 106). However, with reactivity specific to at least two of the proteins p24, gp41, and gp120/gp160 required for interpretation as a positive result, Western blotting from time to time generates reactivity profiles that are not compatible with either a positive or a negative interpretation and hence produces indeterminate results. Although these Western blot-indeterminate (WBi) results do not facilitate a final clinical decision, they often reflect the true serological statuses of the tested subjects, such as having acute HIV-1 or HIV-2 infections or possessing certain interfering factors. Hence, indeterminate results are more acceptable than erroneous HIV antibody-positive results by screening tests because the former indicates the right direction for further evaluation of the patients (63).

Nevertheless, WBi results are a real issue in managing testing laboratories and patients for HIV testing, especially at times of high occurrence. When indeterminate results are obtained, they hinder clinical decisions, create more work, take up more resources in laboratories, and cause inappropriate anxiety to patients or even undesirable consequences (73, 87). Although there have been various studies relating to the topic, new circumstances have arisen and new challenges have emerged, particularly with the introduction of fourth-generation ELISAs into the testing scheme. Hence, the intent of the present review is to provide an update to our understanding of this issue so that relevant countermeasures can be developed to overcome the challenges.

CAUSES AND FREQUENCY OF WBi RESULTS

A Western blot for HIV-1 testing typically contains the following proteins: p17, p24, p31, p39, gp41, p51, p55, p66, and gp120/gp160. Most regulatory bodies rely on at least one of the envelope proteins (gp41 and gp120/gp160) with one of the core proteins (p17, p24, and p55) or one of the enzyme proteins (p31, p51, and p66) as the minimum criteria for interpretation of a positive result (15, 106). Incomplete banding profiles of tested strips, showing specific reactivity to any of the viral proteins not compatible with the approved criteria for a positive interpretation, are thus considered indeterminate. WBi results are neither new nor unique to HIV testing (49, 67, 75). In some earlier studies, researchers looked into various cohorts, including HIV initial-screen-positive subjects, healthy volunteers for vaccine trials, healthy blood donors who had negative initial-screen results, and their respective recipients (25, 30, 58, 104). While some of the indeterminate results might be at least partially related to variability in the performance of earlier products (30, 58), others were found to be fairly consistent and reproducible (25). Studying 20 enzyme

immunoassay (EIA)-positive blood donors, Dock et al. (25) obtained consistent antibody reactivity (atypical results of Western blotting) throughout the follow-up period for 19 of the subjects, with only one case of seroconversion for HIV. Further, the study showed that 18 of these subjects were found to have reactivities specific to the core protein on the Western blot and 10 subjects were found with cross-reactivity to human T lymphotropic virus IV (HTLV-IV) (HIV-2) (25). The study thus demonstrated that indeterminate results were due not simply to product defects, but also to factors and conditions attributable to the test subjects. Based on the finding, Dock et al. suggested excluding individuals with EIA-positive results but atypical Western blot findings from the donor pool (25). On the other hand, indeterminate results were also found to exist even in a community with low prevalence with no apparent involvement of any risk factors for HIV (104). A separate study of donors with initial negative screening results revealed that WBi patterns were common in donors and their recipients and suggested that they were not correlated with the presence of HIV-1 or transmission of HIV-1 from donor to recipient (30).

Obviously, our concerns include both the true causes, such as factors/conditions associated with WBi results, and the appropriate approaches for handling the results for various cohorts, especially healthy blood donors from a low-prevalence community. Nuwayhid (64) provided a comprehensive summary of the associated conditions, but an update is overdue. Additional information available since that review should be included in our consideration of how to deal with various situations arising from WBi results. Hence, the attempt here is to include more current information to further clarify all the relevant causes. The following discussion and subdivision of the reported conditions and factors come from a practical perspective, in the hope of providing a clearer picture of the causes so that further appropriate measures can be developed for dealing with WBi (Table 1).

WBi results associated with tested subjects. Although various factors and conditions are recognized as being associated with WBi results in HIV testing, it is important to bear in mind that all atypical Western blots are the result of reactivity or cross-reactivity between the viral proteins and tested specimens. Hence, the serological statuses of test subjects from whom the specimens are obtained ultimately play an essential part in contributing to the indeterminate outcome. When apparent factors associated with assay conditions and the performance of kits can be excluded, WBi results denote to a great extent the immune statuses of the tested subjects, be it a true infection or association with other medical conditions. This was largely demonstrated by various reports correlating WBi results with individuals having true exposure to HIV or other retroviruses (6, 25, 50) or having other medical conditions (96). Even for individuals with no apparent infections, WBi results could still be an indication of subjects having various conditions, including possessing antibodies originating from passive transfer (25).

For HIV infection-related conditions, numerous studies demonstrated beyond doubt that at least a portion of WBi results were from early seroconversion, especially for high-risk individuals, and the incomplete patterns would eventually evolve into the full positive profile, showing great value for

TABLE 1. Causes of and conditions associated with WBi results

Association	Status	Condition	Reference(s)
Subjects	HIV and related infections	Early seroconversion	13, 25, 40, 65, 77
		Advanced AIDS	6, 50
		HIV-2 infection	25, 47
		Abortive infection	31
		Infection with deletion strains	69
		Immunosilent AIDS	46
		Divergent HIV/simian immunodeficiency virus	77
	Other retroviral infection	HTLV infection	25
		CAEV	91
		Other medical condition	Autoimmune diseases (systemic lupus erythematosus, primary Sjogren's syndrome)
	Leprosy		44
	Malignancy		96
	Elevated bilirubin, nucleoproteins, and rheumatoid factor		7
	No infection	HLA	3
		Noninfected children born to infected mothers	11
Recipients of unscreened products		30	
Participant vaccine trial		45, 104	
Donors with consistent WBi results			25, 26, 30, 104
Kit design		Host cell proteins	20, 72, 73
		Interpretation criteria	54, 87
		Optimizations/consistency of kits	30, 58, 105
Assay process		Cross-contamination	39, 62
		Test routine	39
		Equipment	62
		Sample treatment and hemolysis	16, 32, 42

prognosis (13, 25, 40). Indeed, earlier studies had established the associations between atypical Western blot profiles and individuals having incomplete development of antibodies in the acute phase of infection (13, 25, 40), depletion of antibodies in the late stage of AIDS (6, 50), or infection with HIV-2 (25, 47). However, subsequent reports showed that there were more conditions associated with WBi results than previously suggested. Georgoulas et al. (31) reported that WBi in some low-risk blood donors could be an indication of abortive infection, whereas Rhodes et al. (69) offered an explanation for associating the WBi results with infections by deletion strains in identifying long-term survivors who received HIV-positive blood. Furthermore, Kopko et al. (46) documented that there were cases of "immunosilent infection" that would also bring about WBi results. Under the specific circumstances, it is noteworthy that the WBi result obtained for the "immunosilent infection" was due to *env*-related reactivity having only an anti-gp160 band (46) instead of the more commonly occurring anticore (p24) activity (5, 96). Separately, in a very recent report, Schaefer et al. (77) suggested yet another plausible cause for WBi results: individuals having been infected with divergent HIV or simian immunodeficiency virus. Although the study failed to present molecular evidence with all except one of the 70 WBi samples, it did show that the indeterminate result for the particular specimen was due to a recent infection with an HIV phylogenetically close to a recombinant viral strain (77).

Not unexpectedly, some of the WBi results were found to be associated with individuals having infections with HTLV or other retroviruses (25, 35). A recent study determined that there were homologous regions in the surface glycoprotein

sequences of HIV and the corresponding nucleotides of the lentivirus caprine arthritis-encephalitis virus (CAEV) (91). The finding thus revealed the basis for some of the cross-reactivity and suggested that human contact with CAEV could be yet another source of false reactivity and indeterminate results. Although the atypical results of Western blotting obtained in the study were not the WBi results under discussion here in the strict sense, they demonstrated that the cross-reactivity of HIV proteins to associated antibodies against other lentiviruses occurred more broadly than originally revealed (91). Hence, it is not surprising to see the speculation of Hart et al. (35) that schizophrenia, schizophrenia spectrum disorder, and bipolar disorders might be associated with exposure to HIV-related retroviruses, based on the indeterminate results of anti-p24 and -p17 obtained from the subjects studied.

WBi results were also found to be associated with a variety of medical conditions or disorders other than HIV or retrovirus-related infections. Separate studies had established correlation of WBi results with systemic lupus erythematosus, rheumatoid factor, leprosy, post-measles virus infection, elevated bilirubin, polyclonal gammopathies, and hemodialysis (88, 89, 90, 98). However, a study by Urnovitz et al. (96) showed that, in addition to autoimmune diseases, malignancies, urologic disorders, sexually transmitted diseases, and multiple blood transfusions could also be responsible for the occurrence of WBi results. The study included serum samples from individuals having the above-mentioned conditions and requiring medical treatment or hospitalization and revealed a higher frequency of WBi results within the group: 36.7% in contrast to the 9.8% obtained with those from a HIV low-risk group. In

particular, the autoimmune disorders included in the study consisted of rheumatoid arthritis, Sjogren's syndrome, systemic lupus erythematosus, idiopathic thrombocytopenia purpura, myasthenia gravis, multiple sclerosis, and autoimmune hemolytic anemia, whereas the malignancies included chronic lymphocytic leukemia, breast cancer, lung cancer, colon cancer, Hodgkin's disease, and multiple myeloma. The urologic disorders included acute glomerular nephritis, acute tubular necrosis, acute renal failure, chronic renal failure, and nephrotic syndrome (96). Although the study did not pinpoint which of the particular disorders contributed directly to the WBi results, it resembled HIV testing in hospital settings to a great extent and certainly highlighted the significance of other medical disorders in contributing to the WBi results (96). It is noteworthy that the WBi band patterns did not appear at random in that study; they occurred more commonly for the core antigens, particularly p24 (96). Concerning the basis for the cross-reactivity resulting in WBi with the particular cohorts, documented explanations include the following: (i) autoantibodies in systemic lupus erythematosus patients could cross-react with p24 (88, 89), (ii) p24-reactive antibodies in patients with systemic diseases were directed to viral epitopes (43), (iii) anti-HLA sera contained cross-reactive antibodies to HIV viral proteins (3), (iv) mycobacterial cell wall antigens might share common epitopes with HIV (44), and (v) transplantation antigen and/or autoantibody formation in hemodialysis patients could cause abnormal immune reactivity (98).

Unlike the above-mentioned associations, there were no immediate explanations for WBi results from individuals among healthy blood donors in a low-prevalence community who had no apparent infection. Although there were observations that associated WBi with recipients of unscreened blood products with no apparent seroconversions or infections (12, 30, 48) or loss of antibodies acquired from HIV-infected mothers in seroreverted children (11), none of these examples could readily explain some of the persistent indeterminate results in low-prevalence cohorts. Various long-term follow-up studies revealed no evidence of HIV infection of healthy individuals who had persistent WBi results and hence concluded that WBi results were exceedingly common in testing low-risk donors (26, 30, 37, 38, 41). The CDC has recognized the existence of WBi results in low-prevalence cohorts and has implemented guidelines for designating those individuals negative when no risk factors were involved and no changes in immunoreactivity profiles were obtained with follow-up testing (17). While the recommendations address the practical issues, they fail to address the fact that we lack an answer to the fundamental question of what causes the phenomenon. In this respect, the identification of homologies between HIV type O *env* sequences and 14 different human chromosomes (96) is noteworthy, as is the fact that sera from healthy individuals often contain antibodies to the interspecies antigens of mammalian type C retroviruses (97). It may not be unreasonable to hypothesize the involvement of endogenous retroelements, and further studies in the area may prove rewarding.

WBi results contributed by test kits. It is much less ambiguous that WBi results could also be due to batch-to-batch variation of test kits, especially when the reproducibility of

results has been in doubt (58). Similarly, the performance of kits should be questioned if a sudden change in the frequency of WBi results occurs with no other apparent alterations in the testing procedures or cohorts (62). However, under such circumstances, it is critical to determine whether the WBi results obtained are due to inherent shortcomings of kits or erroneous assay handling (see below), because the manifestations of the two often resemble one another greatly. The inherent shortcomings of test kits could be derived from quality-related issues in material usage, in optimization, or in production of the assay. For example, if nonviral proteins derived from the host cells (in which the virus was cultured) were not removed adequately, some cross-reactivity could result when antibodies reactive to the proteins are present (20, 73). These nonviral proteins could contribute to the WBi result or add to the confusion in interpretation of the results (20). On the other hand, the criteria employed for interpretation of testing results could also affect the ultimate conclusion drawn. One example of this nature was from a report from the 1980s, when an ELISA-seropositive sample was apparently confirmed as positive (subsequently verified as FP) even though it had reactivity only to a p52 protein by Western blotting (73). Obviously, such a result would be interpreted as indeterminate by today's criteria. In a study by Mahe et al. (54), the frequency of WBi results was significantly reduced by modifying the interpretation criteria, even though the assumption on which the modification was based appeared debatable. Furthermore, Syed Iqbal et al. (87) were able to show that applying different criteria recommended by the respective authorities was one of the driving factors determining the frequency of the WBi results for at least one of the commercial kits tested.

WBi results due to assay processes. Other, less frequent, and sometimes overlooked causes for WBi results were related to assay processes. As demonstrated by Ngan et al. (62), the increase of WBi results from 10.5% to 12.2% could easily be attributed to the quality of the product, but careful observations led to the discovery of cross-contamination due to carryover of positive sera from adjacent wells of the tray used for the semiautomated machine. Other, apparently trivial but actually significant factors contributing to the occurrence of WBi, such as sample and reagent dilution, incubation time, and incomplete washing or incubation at temperatures greater than 30°C, were highlighted by manufacturers, with corresponding guidelines for troubleshooting. Sometimes, it was much easier to overlook than to detect inadequate washing, especially when automation was involved, unless a total failure became apparent. In this respect, it is particularly relevant to include the findings of Jamjoom et al. (39) in our discussion. With the introduction of testing samples with high optical density and low optical density separately and additional steps for rinsing the suction apparatus after each sample to prevent backflow aerosol contamination, a significant reduction of indeterminate results from the initial 20% in 1994 to 8.3% in 1996 was achieved (39). Obviously, only careful analyses including all relevant factors would enable effective determination of whether problems with a specific incident were due to inherent shortcomings of kits or assay processes.

In addition, it is also noteworthy that the CDC considered specimen preparation another relevant factor and in fact cau-

TABLE 2. Frequency of WBi results with different cohorts from different territories

Country or region	Cohort	Population total (n)	No. screen positive (no. negative)	No. of WBi results (%)	Reference
United States	Blood donor	500,000	1,100	160 (14.5)	80
United States	Blood donor	3,270,000	2,660	1,207 (45.4)	27
Peru	General population	1,363	35	13 (37.1)	19
Chile	Donor and risk group	600,000	4,956	409 (8.3)	68
India	Blood donor	39,784	44	5 (11.4)	92
Nigeria	Blood donor	500	186	55 (29.6)	28
Ethiopia	Volunteers ^a	12,124	1,437 (1,475) ^c	91 (6.3)/31 (2.1) ^{c,e}	57
Brazil	Rejected donor	NA ^b	210 ^d	6 (2.9)	4
Central Africa	High-risk group	1,998	1,065	367 (34.5)	5
United States	High-risk group	NA	371	123 (33.2)	96
United States	Other disorders	NA	199	73 (36.7)	96
Saudi Arabia	Hospital/health center cohorts	NA	2,849	444 (15.6)	39
Brazil	Pregnant women	9,786	105	11 (10.5)	21
Cameroon	Pregnant women	859	163	13 (8.0)	33

^a Participants in Ethio-Netherlands AIDS Research Project.

^b NA, not available.

^c Only 1,475 of the 10,687 screened negative were included in Western blot testing.

^d Selected cohort; no association was made with the initial population screened.

^e The two values are number of WBi results among those who screened positive/number of WBi results among those who screened negative, with percentages for each given parenthetically.

tioned users about the effects of heat inactivation of samples prior to antibody testing for HIV (16). However, the interference of conventional heat treatment at 56°C for 30 min (32) appeared to be a much less significant factor, at least with kits of recent manufacture (99).

Another matter of concern relating to processes is the impact of the algorithms employed in testing. However, this important issue warrants a section of its own and is discussed separately below.

Frequency of WBi results. Understandably, the frequency of WBi results varied from kit to kit, from territory to territory, from population to population, and from algorithm to algorithm. In a very recent study, Syed Iqbal et al. (87) were able to associate the different frequencies of WBi results at 7% to 25.6% with different kits (even though the main focus of the study was on the impacts of criteria on result interpretation). However, Urnovitz et al. (96) demonstrated quite clearly that different cohorts would yield different numbers of WBi results, with indeterminate detection at 9.8%, 33.2%, and 36.7%, respectively, for the HIV low-risk cohort, the HIV high-risk cohort, and the cohort with other medical conditions. It appeared that the frequency was somewhat associated with the risk of HIV infection and other medical conditions, predominantly autoimmune-related disorders (96). However, such differences appeared less obvious in a brief examination of other publications covering different territories and cohorts. As shown in Table 2, there were great variations in the frequency of occurrence of WBi results, from 6% to almost 50%, even within the same cohort of blood donors. Although those data were not generated within one study and hence are not strictly comparable, they do provide some indication of the complexity of the issue. It appears highly debatable that the frequency of WBi results could be simply attributed to any one of the factors discussed above or to the differences among the cohorts. In this respect, it is important to recognize the findings by Dodd and Stramer that the number of WBi results was proportional to the number of samples tested rather than the prevalence of infection, based on their studies involving 3.27 million subjects

(27). In fact, they suggested that the use of a primary screening test with poor specificity would be the ultimate cause of WBi results (27). This was very strongly supported by the subsequent findings of Prince and Gross (67), who illustrated the impact of initial screening on the efficiency of Western blot testing for HTLV and the importance of adherence to the recommended algorithm for overcoming a similar WBi problem. Specific to HIV testing, a clear demonstration was provided in the study by Huang et al. (36). The screen algorithm comprising repeat testing with one ELISA instead of the ELISA in combination with a more specific agglutination test generated not only more FPs (143 versus 8), but also more WBi results (88 [36.5%] versus 2 [10%]) with the same testing cohort of 281 samples (36). Separately, it is also noteworthy that 13 WBi results were fully resolved when an additional screen assay was introduced into the testing algorithm prior to the Western blot confirmation (33).

On the other hand, the frequency of bands for individual viral proteins among the WBi patterns appeared not to be as random an event as indicated above. Notably, the band for reactivity to p24 viral protein was the most frequently occurring band among all the WBi patterns regardless of cohorts or study settings (5, 10, 96). Studying 84 blood donors with repeated indeterminate results, Carneiro-Proietti et al. (10) obtained a frequency for the anti-p24 band of 91.6%. Although this was found at a more moderate level of 74% in a separate study of Urnovitz et al. (96), the anti-p24 band remained predominant among all the other antiviral markers, with the next highest reaching only 16% for an HIV-1 low-risk group. Similarly, the frequency of the anti-p24 band was at a lower level of 60% among the WBi patterns obtained with an HIV-1 high-risk group, but it was again the most frequently occurring marker, with the next most frequent reaching the rate of 21.9%. In contrast, the anti-gp120/gp160 bands appeared much less frequently in general, and occurrences were more likely to be associated with the HIV-1 high-risk group (96) or with individuals having silent infection (46).

STANDARD AND ALTERNATIVE ALGORITHMS FOR CONFIRMATION OF HIV INFECTION AND THEIR CONSEQUENCES

Standard testing algorithm. The current standard testing algorithm for diagnosis of HIV infection can be traced back to practices in the mid- to late 1980s and was derived from the CDC guidelines (14, 17). The algorithm requires a sequence of tests starting with repeatedly reactive EIA and a subsequent confirmation of Western blotting (14). Consequently, a sample can be considered positive for HIV only if it tests initially positive by an EIA and subsequently positive by a repeat test in duplicate with the same EIA and is confirmed positive by a supplemental test, such as Western blotting or, less frequently, immunofluorescence assay (17). The algorithm was therefore designed to reduce the chance of an FP, and accordingly, the purpose of Western blotting was to achieve the objective of ruling out FP results. Inherently, the interpretation guideline for Western blots was focused very much on the identification of positive results with relatively little consideration of indeterminate results. Nevertheless, the algorithm itself, with the serial tests prior to confirmation, when adhered to closely could reduce the number of FPs derived from errors of operational origin. While the indeterminate result was posed as an issue for clarification, there was also concern about the cost-effectiveness of the algorithm. Consequently, alternative strategies for HIV testing were proposed and adopted by UNAIDS and WHO (94, 95, 107).

Alternative algorithms. Based on the updated guideline of UNAIDS/WHO (107), three alternative testing algorithms were recommended in dealing with different scenarios for HIV testing. These algorithms, designated strategies I, II, and III, were designed to eliminate the use of confirmatory tests, such as Western blotting, and hence reduce the testing costs for resource-poor countries. In particular, strategy I (requiring only one test) is intended for use in diagnostic testing in populations with an HIV prevalence of $>30\%$ among persons with clinical signs and symptoms of HIV infection and in blood screening and surveillance testing in populations with an HIV prevalence of $>10\%$. Strategy II (requiring two tests) is a moderate approach for use in surveillance testing in populations with an HIV prevalence of $\leq 10\%$ and in diagnostic testing in populations with a prevalence of $\leq 30\%$ among persons with clinical signs and symptoms of HIV infection or $>10\%$ among asymptomatic persons. The most stringent approach is strategy III (requiring three tests utilizing different antigens and/or different test principles) for diagnostic testing in populations with an HIV prevalence of $\leq 10\%$ among asymptomatic persons.

While these approaches might have proven cost-effective in some resource-lacking countries, with different cohorts, or even with different types of specimens (24, 33, 56, 82), the impacts of the recommendations on diagnostic and detection outcomes are beyond immediate comprehension. In pursuing the recommended strategies, various combinations of test kits were reviewed and ultimately adopted in routine HIV testing. Notably, the approaches included using combinations such as ELISA and Western blotting, ELISA and rapid test, or rapid test and rapid test either consecutively or simultaneously. Nonetheless, all combinations were found to have inherent

shortcomings (33, 56, 63, 71, 82). As discovered by Ngan et al., (63) in using three rapid tests for confirmation of 2,256 referral samples, almost half of those positive (28 of 61) by all three tests proved to have indeterminate results by Western blotting. In this case, the uncertainty of WBi results was surely more acceptable than FP results, as the WBi results indicated the right direction for further evaluation (63). Notably, this was not an isolated incident and was further supported by various findings of recent studies (33, 82). In a study involving over 800 subjects, Granade et al. (33) designed different approaches of both serial testing (two consecutive tests) and parallel testing (two simultaneous tests) for their alternative algorithms, using a third test for resolving any discordance. Although they were able to avoid any uncertainty of WBi results with their serial and parallel testing, both approaches produced FP results (33). Similarly, in yet another study of 3,500 samples, the rapid tests under the strategy III approach produced 33 FPs out of the 507 samples identified (82). These examples amply illustrated the impacts of the algorithms utilized on the detection outcomes. Indeed, even the most stringent strategy III for confirmation was not trouble free and hence should be treated with prudence when followed (63). The dilemma, at least in theory, appeared to be whether one wants to face the uncertainty of WBi or "certainty" with a proportion of FP results, in addition to the cost-effectiveness consideration. In reality, alternative algorithms did not automatically lead to an indeterminate-free situation. Using two rapid tests in serial testing following the strategy II approach, Rouet et al. actually produced 113 indeterminate cases out of the 1,293 initial-positive cases, which could be resolved only by additional tests using a supplemental ELISA (71). In fact, an earlier study justifying the cost-effectiveness of alternative algorithms managed to avoid the complication only by excluding the group of samples with WBi results from its data analysis (56).

The problem appeared to be that the different algorithms/strategies were intended for the respective purposes, i.e., transfusion/transplant safety surveillance, surveillance diagnosis, and, ultimately, confirmatory diagnosis of HIV infection. However, the outcome of utilizing different algorithms/strategies could hardly be treated in accordance with the defined intents. It is understandable that users of strategy I, II, or III would find it equally hard to accept any uncertain or erroneous outcome, even though different strategies are known to produce different consequences. Hence, in reality, a blood-screening facility adopting an algorithm using one screening assay followed by one supplemental Western blot test would find it hard to deal with WBi results even if such WBi results could be resolved by implementing one additional screening test. In this respect, the finding of Granade et al. that 13 of the indeterminates generated by the one screening assay and one Western blot combination approach were fully resolved by the two screening assays with one Western blot option is interesting (33). Although there were additional variables in terms of the kits used, this example nevertheless illustrated the different consequences using a strategy II approach and the standard algorithm. The finding of Granade et al. thus demonstrated the limitation of Western blotting, and users should be reminded of the fact that this supplemental test was not designed for screening (20). Indeed, additional WBi samples could be generated from a

screened negative population if the cohort was also tested by Western blotting (57).

NEW CHALLENGES AND FURTHER CONSIDERATIONS

Fourth-generation ELISA and WBi. Abundant literature on fourth-generation ELISAs exists, but relatively less was known about the impact on the incidence of WBi or overall testing with the introduction of this technology into the HIV testing algorithms (34, 52, 53, 76, 100, 101, 103). Most of the studies appeared to have centered on the performance of the technology in terms of closing the detection window, and hence, little attention was given to the possible consequences of the addition of the technology to the existing test algorithms. In theory, the potential for nonspecific reactivity with fourth-generation ELISAs should be higher than that of the third generation because the former combine two different test principles in one assay (101). In practice, it appeared inconsistent and debatable whether fourth-generation ELISAs were indeed more inclined to have nonspecificity issues than their third-generation counterparts. While Weber et al. (101) reported improved performance of the fourth generation over the third, Ly et al. (52) demonstrated exactly the opposite, with a better specificity of 99.86% for the third-generation ELISA in contrast to the 99.51% for the fourth-generation test. Although the difference between the two percentages might appear small, in reality, it was equivalent to an addition of over 100 cases of FP with 29,657 cases tested during a 9-month survey in a cohort with a positive prevalence rate of 1.52% (52). In fact, of the 613 positives identified by the fourth-generation ELISA, 143 (23.3%) were found to be FP, in contrast to 42 FP (8.5%) out of the 495 positives identified by the third-generation ELISA (52). Hence, it is not far-fetched to say that an increased rate of WBi could also be associated with using the fourth-generation ELISA, even though none has so far been reported with existing studies focused on evaluation. In reality, an increase in the incidence of WBi seen in customer feedback, especially those with only anti-p24 reactivity, were more often than not associated with the use of fourth-generation ELISAs as the primary test (unpublished data). This appeared explainable, at least in theory, by the fact that only fourth-generation ELISAs had the capacity to detect additional samples with reactivities specific to or associated with viral p24 antigen. Nevertheless, as pointed out by Dodd and Stramer (27), the performance of a primary specificity test was directly proportional to the number of WBi results obtained.

Although WHO and the CDC have acknowledged the existence of the fourth-generation ELISA, neither appeared to have taken the unique requirements of the technology into consideration in the latest update on recommendations for their testing algorithms (18, 107). It is obvious that the simultaneous detection of antigen and antibody by fourth-generation ELISAs will result in identification of samples containing either antibody, antigen, or both. Consequently, confirmation of reactivity generated by these ELISAs would require a special algorithm consisting of two supplemental tests different from those approaches currently recommended. In particular, an antibody test, such as Western blotting or immunoblotting, is needed for verification of the anti-HIV portion of the reactivity, whereas an antigen test or a nucleic acid-based assay is

needed for the p24 antigen portion of the reactivity (52). Consequently, Brust et al. (8) proposed a confirmatory strategy that repeat positives produced by fourth-generation ELISAs should be confirmed in parallel with an antigen-testing and an antibody-testing approach, with each route consisting of a screen test and a supplemental test. This algorithm would keep the residual risk to an absolute minimum, but the excessive use of tests is also obvious. Based on the data of Ly et al. (52) obtained from real laboratory settings, it appeared more cost-effective to use an antibody test for the overall confirmation, since over 95% of the reactivity by the fourth-generation ELISA was anti-HIV related and verifiable by Western blotting. However, a further supplemental test using either a dedicated p24 antigen test or a nucleic acid assay appeared unavoidable for verification of the reactivity related to p24 antigen. Correspondingly, WBi results generated by a fourth-generation ELISA as a primary test in low-prevalence cohorts should be additionally tested with an antigen test or molecularly based test prior to the recommendation of follow-up sampling, unless other factors, such as clinical evidence, pointed to the possibility of seroconversion.

Differentiation of true infection from nonspecific reaction.

The prime concern with WBi result is obviously the derived uncertainty in decision making for diagnosis, and hence, the ultimate objective in managing WBi results is to enable the differentiation of a true infection from nonspecific reactions. As is understood, anti-HIV antibodies, once developed, persist for a lifetime (107). Furthermore, as reviewed above, incomplete Western blot profiles for individuals having a true HIV infection would be associated only with a primary infection at the time of seroconversion or at the late stage of AIDS. Consequently, our main focus for handling WBi results is on how to identify any true case of seroconversion among all the indeterminate, rather than with monitoring the progression of the disease, as the latter involves no decision making relating to diagnosis. With this focus in mind, it is critical to understand the dynamics of various viral or antiviral markers against the time course in individuals after an exposure to HIV. Furthermore, it is essential that we recognize the subtle difference between the "window period" and "seroconversion." While the former refers to the duration from exposure to the time when respective (viral or antiviral) markers first become detectable, the latter denotes the process of development of antibodies to a specific pathogen. In this respect, there is no existing terminology covering the interval immediately after the window period but prior to the full manifestation of all anti-HIV markers by Western blotting, which in fact is most relevant to our discussion of WBi. Perhaps it is appropriate that we define the period as the "WBi interval," as it is associated only with the Western blot technology and no equivalent progression exists for all viral markers, such as RNA or p24 antigen. Based on the model established by Fiebig et al. (29) using plasma samples from donors, the window periods for HIV RNA, p24 antigen, and anti-HIV antibody are 12 days, 17 days, and 22 days, respectively. Although any baseline established would obviously depend very much on the sensitivity of the kits used for the construction, the finding of Fiebig et al. was not a significant deviation from that of a previous model by Busch and Satten (9). The two exceptions included in the study by Busch and Satten (with a >6-month window period) were

associated with health care workers with needle stick-acquired infections. This is not the most common route of HIV exposure, and hence, application of these results should be treated with caution. Nevertheless, our concern with managing the WBi results is much less relevant with the window period but more so with the "WBi interval." This is because, with a WBi result, the status of a tested individual would have already progressed beyond the window period and to the late stage of seroconversion—the "WBi interval"—if a true HIV exposure occurred earlier. In view of this, the proposal by Fiebig et al. (29) of six laboratory stages of primary HIV infection is most applicable to our discussion here. The six stages are as follows: stage I, RNA assay reactive only; stage II, RNA assay and p24 antigen assay positive; stage III, RNA, p24 antigen assay, and EIA positive but Western blot negative; stage IV, like stage III, but with WBi results; stage V, like stage IV but with WB positive lacking p31 reactivity; stage VI, like stage V but with full Western blot reactivity, including a p31 band. It is noteworthy that the projected duration for each of the stages I to IV is brief, lasting on average only 3 to 5 days (29). Based on these findings, two important features emerged. First, although the window period for WB could be as long as 22 days, once initiated, the progression (the "WBi interval" covering the period of stage IV) to a full profile took no longer than 8 days for true cases of infection. Second, during this "WBi interval," when Western blotting was indeterminate, reactivity detectable by a p24 antigen assay was reduced to a relatively low level but that detectable by an RNA assay was not. In fact, the HIV RNA level peaked at stage III but was sustained at a detectable level well beyond stage VI (29, 61). Some long-range follow-up studies also showed that high levels of HIV RNA were detectable in plasma prior to seropositivity and were maintained thereafter beyond 30 months in both vertically infected infants and heterosexually infected adults (55, 79). Therefore, it is adequate to conclude that although repeat antibody test results can remain negative for months after exposure with some extreme cases, no true case of infection with WBi lasts for weeks without being accompanied by at least one other detectable viral marker. This was indeed supported by a separate study involving 436 patients with primary infection, while none of the 13 cases with WBi results was found to be undetectable by the HIV RNA test (22). Conversely, no true case of seroconversion was evident in follow-up studies after a confirmation of negative results by PCR methods among the 26 samples from 23 individuals having screened positive but with WBi results (78). Consequently, it is possible to use molecular testing for an early resolution of WBi results as an alternative to the current approach with a follow-up test after 1 month (17). It is appropriate to propose that a WBi result can be treated as negative if it is not accompanied by positive detection using a molecular test, in particular, an RNA test with primer sets covering HIV-1/2/O and meeting the sensitivity of today's standard. This is particularly relevant with WBi samples derived from a test algorithm using fourth-generation ELISAs as a primary test for healthy blood donors without any risk factors involved. If the reactivity of a positive result by a fourth-generation ELISA accompanied by a WBi result

cannot be confirmed by another antibody-only test, it is more likely that it will be verifiable by an antigen test or molecularly based test if the reactivity was due to a true infection in the first place. In other words, a negative outcome is indicated if the results were not supported by, or accompanied by, a positive result by an antigen test or a molecularly based assay, especially an RNA assay, with a performance sensitivity meeting today's standard.

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