

FOLLOW-UP OF HIV WESTERN BLOT INDETERMINATE RESULTS

Ghazi A. Jamjoom, PhD, FRCPath; Jamal Maatouk, MD; Malak Gazal, MBBS; Laila Damanhour, MBBS, MSc; Azza Awliaa, BSc; Nadia Ruwaihi, BSc; Manal Bawazeer, BSc; Huaida Halabi, BSc; Amira Abul Adel, BSc; Asma Abdulla, BSc

Indeterminate results obtained with the Western blot (WB) confirmatory test on HIV enzyme immunoassay (EIA)-positive samples, constituted 15.6% (444/2849) over a 2.5-year period at the referral laboratory for the Western region at the King Fahd General Hospital, Jeddah. Two hundred and fourteen WB-indeterminate samples were followed up by repeat WB testing of subsequent samples from the same patients over a 3-12 month period. One hundred and forty-two samples (66.4%) gave negative results. Sixty-five samples (30%) remained indeterminate. Only seven samples (3.3%) not initially meeting WHO criteria for positivity turned clear-cut positive, with high EIA readings on follow-up. It was discovered initially that a significant proportion of indeterminates was due to low-grade cross-contamination between samples as a result of aerosol backflow during aspiration in the washing procedure. This was eliminated by rinsing the lines between samples, separating samples with high EIA from those with low EIA, and rerunning indeterminate samples. A reduction of indeterminates from 21% to 8.5% subsequently followed. After this improvement, most of the samples that remained indeterminate had low EIA readings, and few bands of mainly anti-gag (p55, p24 or p18) or anti-pol (p51) antibodies, while the few turning positive all had anti-gp160, in addition to anti-p24 or p55. Interestingly, over the last year and a half of the study, 1.4% of the total samples (21/1506) had repeatedly high EIA readings but were negative by WB. In addition, 16 samples (1.1%) were positive for HIV-2. A separate computer-based system for the storage of data was very helpful in ascertaining proper follow-up of indeterminate WB results. *Ann Saudi Med 1997;17(5):518-521.*

Confirmatory testing by Western blot is the method of choice for ascertaining the results of anti-HIV antibody screening positive specimens, as detected by EIA. In the majority of cases the results are either clear-cut positive or negative. Positive samples give many bands, indicating the presence of antibodies to most or all the major viral proteins coded by the env gene (gp160, gp120, gp41), the gag gene (p55, p24, p18), or the pol gene (p66, p51, p31). Negative samples give no bands at all. Occasionally, incomplete patterns of bands corresponding to viral proteins are obtained (e.g., p24 alone, or p55 and p51 only, etc.). Such patterns constitute indeterminate results according to one or all of the guidelines most commonly used for the interpretation of the Western blot (i.e., those of the WHO, American Red Cross, or the US FDA).^{1,2} Indeterminate results may be detected in HIV-negative individuals due to cross-reactive autoantibodies or to antibodies of other unrelated antigens.³⁻⁸ Cross-reactivity to other retroviruses (such as human T-lymphotropic virus [HTLV-1] and bovine or feline

leukemia viruses) has been excluded in several studies as a cause of HIV indeterminates.^{7,9} On the other hand, indeterminate patterns may be obtained from HIV-1 infected patients in the early window period, or later after the disease has progressed^{3,7} and from seroconverting babies of HIV-infected mothers.¹⁰ In addition, HIV-2 infected individuals may also give indeterminate results with HIV-1 specific kits.¹¹ Different proportions of indeterminate results have been reported, usually between 10%-20% but as high as 48%.^{3,12,13} Indeterminate results constitute a diagnostic problem and may be the cause of considerable anxiety to the examinee. Moreover, they cause difficulties to health and immigration authorities and insurance companies, resulting in delays in work permits, unnecessary deportation or denial of insurance coverage. We have analyzed such indeterminate WB results in order to gain a better understanding of their proportion and causes under our local setting, and in order to expedite their follow-up with various referring hospitals or clinics.

Methods

Coded serum samples that were repeatedly reactive for HIV-antibody by EIA were received from several hospitals and health centers in the Western and Southern regions of Saudi Arabia. EIA was repeated on all specimens using Abbott HIV-1/HIV-2, 3rd Generation Plus EIA reagents for

From the Department of Laboratory Medicine, King Fahd General Hospital, Jeddah, Saudi Arabia.

Address reprint requests and correspondence to Dr. Jamjoom: Department of Pathology, College of Medicine, King Saud University, P.O. Box 2925, Riyadh 11461, Saudi Arabia.

Accepted for publication 22 June 1997. Received 26 February 1997.

Quantum II (Abbott, North Chicago). The Western blot kit for HIV-1/HIV-2 was obtained from Diagnostic Biotechnology (Singapore), a subsidiary of Genelabs (USA) (HIV Blot 2.2). Interpretation of results was according to WHO criteria (presence of two env bands: gp160, gp120 or gp41, with or without pol bands and gag bands). Indeterminate results were repeated with the same sample and subsequent samples were requested at three and six months. Some samples with intermediate or high EIA or with env bands were followed for up to 12 months. Since 1996, samples with high EIA readings have been tested on a separate plate from those with low EIA. In addition, the step of rinsing the suction apparatus with distilled water after each sample has been added to prevent backflow aerosol contamination.

Results

Table 1 indicates that an average of 15.6% of samples gave indeterminate results. This percentage significantly decreased from the initial 20% in 1994 to 8.3% in 1996 ($P < 0.0001$). After the introduction of the measures to reduce cross-contamination, as described under Methods, the change from indeterminate WB to negative upon repeating of the same sample decreased considerably. HIV-2 infected patients may give indeterminate results with kits intended for detection of HIV-1 only. However, the kits used in the present study incorporated an HIV-2 specific band (gp36) and so could differentiate HIV-2 positive samples. During the period of 1995-1996, 16 samples were diagnosed as positive for HIV-2.

Table 2 summarizes the results of follow-up of 214 indeterminate samples by retesting new samples from the same patients over 3-12 months. The majority of samples remained indeterminate or turned negative. Cross-contamination was probably a significant factor among the samples that turned negative on subsequent testing. Among the samples remaining indeterminate, the reactivity was very common to p55, p24, p51 and p18, although occasionally anti-gp160 reactivity was observed. Among the seven samples that turned definite positive, both gp160 (but not gp120 or gp41) and p24 reactivity were initially present. One sample additionally contained p66 and p51 (pol) reactivity, and could have been interpreted as positive according to the American Red Cross or the US FDA, but not according to WHO, which requires the presence of two envelope bands.

One sample from a baby seroconverted from EIA positive to negative. Another sample with high EIA reading and indeterminate WB (with reactivity to gp160) gave a positive result when tested for p24 antigen.

Twenty-one samples with high EIA readings but negative WB were encountered during the last year and a

TABLE 1. Results of initial confirmatory Western blot testing.

Western blot

	No. of samples	Pos. (%)	Neg. (%)	Indet. (%)
6/'94-5/'95	1417	723 (51)	409 (29)	285 (20)
6/'95-5/'96	804	479 (59.6)	218 (27.1)	107 (13.3)
6/'96-11/'96	628	389 (61.9)	187 (29.8)	52 (8.3)
Total	2849	1591 (55.8)	814 (28.6)	444 (15.6)

TABLE 2. Follow-up of Western blot indeterminates.

	Indet. ↓ neg.	Indet. ↓ indet.	Indet. ↓ pos.
Total indeterminates			
214	142	65	7

Follow-up was for 3-12 months on one or more subsequent samples.

half of the study. We do not know anything about the nature of the cross-reactivity of these samples in the enzyme immunoassay test used. To exclude nonspecific reactivity in EIA (sticky sera), these samples were tested for hepatitis B surface antigen (HBsAg) and hepatitis C antibody (anti-HCV) by EIA (Abbott). All were negative for HBsAg, while one sample was positive for anti-HCV. Finally, 16 samples were strongly reactive with HIV-2 specific gp36 band, and were reconfirmed by a separate HIV-2 Western blot kit (Diagnostic Biotechnology).

Discussion

The Western blot is often offered as a referral service at a few designated centers for the purpose of standardization and surveillance by health authorities. As a result, referral laboratories handle relatively large batches of 20-50 samples in each run. Our results show the necessity of taking appropriate precautions when handling such batches containing a large number of strongly positive samples, together with low-positive or negative samples.

For the purpose of confidentiality most samples are referred under code. Referral laboratories, therefore, do not have access to patient risk factors or clinical information. As a result, proper interpretation of indeterminate WB should be made by the referring physician, who has better access to this information. Referring physicians should thus be aware of the limitations of the WB and the different causes of the indeterminate results, in order to avoid causing unnecessary anxiety to the patients or donors. In the absence of risk factors, WB indeterminate patients with unchanging or a low EIA for six months may be considered uninfected.³ However, risk behavior information may be unreliable and its role in helping the interpretation of laboratory results is not without controversy. Therefore, referral laboratories should make all efforts to resolve any indeterminate results by repeat testing of WB, and by using additional, more sensitive tests.

Several tests have been found to be useful for the clarification of WB indeterminate samples. These include virus culture, radioactive immunoprecipitation (RIPA), line

immunoassay using recombinant viral antigens, immunofluorescence, p24 antigen detection, and the detection of HIV RNA in the serum by the polymerase chain reaction (PCR).^{3,4,7,9,14-16} HIV culture and RIPA are not readily available to most laboratories in developing countries. Detection of p24 antigen lacks sensitivity in the diagnosis of HIV-infected individuals during early seroconversion.¹⁷ Among the remaining procedures, PCR is probably the most sensitive and most commonly applied these days for the purpose of resolving indeterminate WB. Using this technique, several investigators have shown that samples from low-risk people containing reactivity to separate gag proteins (e.g., p24 only, or p55 only), pol proteins (p51) or env proteins do not contain viral RNA in serum or peripheral blood leukocytes.^{5,15,16}

In contrast, individuals at high risk of infection may present with a WB indeterminate pattern early in infection, while having HIV RNA or p24 antigen in their blood.^{15,16} Similarly, late in infection, anti-p24 antibodies may disappear as viremia increases. The resulting pattern, consisting mainly of anti-env proteins, is considered indeterminate according to American Red Cross and FDA criteria, but is positive according to WHO criteria. In situations where patients are first diagnosed late in infection, the WHO criteria is thus probably more applicable. However, it is recommended that a positive result in this case be issued only if the EIA is strongly positive, or if p24 antigenemia is present. Repeat testing by WB and clinical assessment of the patient, as well as consideration of other laboratory parameters (e.g., CD4 count) should be taken into account. It should be emphasized that many indeterminate results can be resolved simply by repeat testing of new samples from the patients with EIA or WB. Otherwise, resorting to a more sensitive test such as PCR may be necessary. PCR may also be used for confirming indeterminate WB results in high-risk patients.

The policy of the Ministry of Health (MOH) regarding donated blood is to discard any EIA repeat-positive units, regardless of their WB results. However, there remains the problem of informing the donors and patients of their test results, which may be resolved by the additional tests outlined above.

The 21 samples we encountered with high positive EIA and negative WB are interesting. As these samples did not react in EIA tests for HBsAg or anti-HCV, it may be deduced that their reactivity is specific for some determinants on the HIV EIA antigens which are absent from the WB lysates. One possibility is bacterial antigens from bacteria used in the manufacture of recombinant antigens for the EIA test. Risk factors for these patients were not known to us, except for one elderly patient who had no known risk factors for HIV, but had macroglobulinemia. Further work is needed to investigate possible reasons for these high-positive EIA-negative WB samples.

In this study, computer-based storage and retrieval of results were essential for the proper follow-up of WB-indeterminants. Such a system is necessary for reference labs handling a large number of specimens. The use of an appropriate coding system for all referring hospitals and clinics is required. The system used was based on a 3-letter prefix to designate the region of the country, followed by a 3-digit patient number. In particular, the same patient number must be maintained for specimens submitted from any one patient, while the sample order may be distinguished by the date or by a suffixed small letter (e.g., ZAN 111-a, ZAN 111-b). Lack of adherence to such a numbering system by some clinics has prevented the follow-up of a large number of patients.

References

1. Consortium for retrovirus serology standardization. Serological diagnosis of human immunodeficiency virus infection by Western blot testing. *JAMA* 1988;260:674-9.
2. Centers for Disease Control. Interpretation and use of the Western blot assay for serodiagnosis of human immunodeficiency virus type 1 infections. *MMWR Morb Mortal Wkly Rep* 1989;38(suppl 7):1-7.
3. Celum CL, Coombs RW, Lafferty W, Inui TS, Louie PH, Gates CA, et al. Indeterminate human immunodeficiency type 1 virus Western blots: seroconversion risk, specificity of supplemental tests, and an algorithm for evaluation. *J Infectious Dis* 1991;164:656-64.
4. Ramirez E, Uribe P, Escanilla D, Sanchez G, Espejo RT. Reactivity patterns and infection status of serum samples with indeterminate Western immunoblot tests for antibody to human immunodeficiency virus type 1. *J Clinical Microbiol* 1992;30:801-5.
5. Jackson JB, Hanson MR, Johnson GM, Spahlinger TG, Plesky HF, Sowman RJ. Long-term follow-up of blood donors with indeterminate human immunodeficiency virus type 1 results on Western blot. *Transfusion* 1995;35:98-102.
6. Barthel HR, Wallace DJ. False-positive human immunodeficiency virus testing in patients with lupus erythematosus. *Semin Arthritis Rheum* 1993;23:1-7.
7. Celum CL, Coombs RW, Jones M, Murphy V, Fisher L, Grant C, et al. Risk factors for repeatedly reactive HIV-1 EIA and indeterminate western blots. A population-based case-control study. *Arch Intern Med* 1994;154:1129-37.
8. Kashala O, Marlink R, Ilunga M, Diese M, Gormus B, Xu K, et al. Infection with human immunodeficiency virus type 1 (HIV-1) and human T-cell lymphotropic viruses among leprosy patients and contacts: correlation between HIV-1 cross-reactivity and antibodies to lipoarabinomannan. *J Infect Dis* 1995;171:502-4.
9. Sherman MP, Dock NL, Ehrlich GD, Sninsky JJ, Brothers C, Gillsdorf J, et al. Evaluation of HIV type 1 western blot indeterminate blood donors for the presence of human or bovine retroviruses. *AIDS Res Hum Retroviruses* 1995;11:409-14.
10. Jackson JB, Ndugwa C, Mmiro F, Kataaha P, Guay L, Dragon EA, Goldforb J, Olness K. Non-isotopic polymerase chain reaction method for the detection of HIV-1 in Ugandan mothers and infants. *AIDS* 1991;5:1463-7.
11. Centers for Disease Control. Update: HIV-2 infection - United States. *MMWR Morb Mortal Wkly Rep* 1989;33:572-80.
12. Kleinman S, Fitzpatrick L, Second K, Wike D. Follow-up testing and notification of anti-HIV Western blot atypical (indeterminate) donors. *Transfusion* 1988;28:280-2.
13. MacDonald KL, Jackson JB, Bowman RJ, Polesky HF, Rhame FS, Balfour HH, et al. Performance characteristics of serologic tests for human immunodeficiency virus type 1 (HIV-1) antibody among Minnesota blood donors: public health and clinical implications. *Ann Intern Med* 1989;110:617-21.

14. Marin MG, Lillo F, Varnier OE, Bresciani B, Molinelli A, Abecasis C, Bonini PA, Albertini A. Detection of HIV-1 proviral sequences in lymphocytes using a qualitative polymerase chain reaction assay. *Eur J Clin Microbiol Infect Dis* 1995;14:621-5.
15. Pane F, Butto S, Gobbo ML, Franco M, Butteroni C, Pastore L, et al. Direct detection of proviral gag segment of human immunodeficiency virus in peripheral blood lymphocytes by colorimetric PCR assay as a clinical laboratory tool applied to different at-risk populations. *J Clin Microbiol* 1995;33:641-7.
16. Sethoe SY, Ling AE, Sng EH, Monteiro EH, Chan RK. PCR as a confirmatory test for human immunodeficiency virus type 1 infection in individuals with indeterminate western blot. *J Clin Microbiol* 1995;33:3034-6.
17. Busch MP, Taylor PE, Lenes BA, Kleinman SH, Stuart M, Stevens CE, et al. Screening of selected male blood donors for p24 antigen of human immunodeficiency virus type-1. *N Eng J Med* 1990;323:1308-12.