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Measurement of antibodies to human immunodeficiency virus: an international collaborative study to evaluate WHO reference sera

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Two preparations of human sera, one reactive against human immunodeficiency virus (HIV) and the other unreactive, were evaluated as potential international reference reagents (IRR) in an international collaborative study. Twenty-one laboratories participated and tested these and five other human sera which were found to range from highly reactive to unreactive.

The proposed 'positive' IRR was found to react strongly in all immunoassays and gave all the expected bands in immunoblot systems using HTLV-III, LAV-I or similar virus strains as antigens. The 'unreactive' serum was judged to be negative by ELISA and immunoblots. The end-points determined by ELISAs varied considerably between laboratories, even between those using the same commercial kit. This variation was reduced somewhat when the reactivities of the samples were expressed relative to the proposed IRR.

Human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS) (1, 9, 12), is transmitted primarily through sexual contact or the injection of contaminated blood or blood products such as anti-haemophilic factors (5). Since 1985, the screening of blood donations for anti-HIV has been instituted in many countries in order to minimize the risk of transmission of AIDS via blood transfusions or treatment with blood products. The detection of antibodies to HIV is also of major importance as a relatively simple and rapid determination of the extent and spread of HIV infections (3) and many commercial and 'in house' immunochemical tests are now in use throughout the world. At present, the most commonly used assays are based on enzyme-linked- or radio-immunosorbance, immunofluorescence, immunoblotting, or immunoprecipitation, and variations in the specificities and sensitivities of the techniques reflect inherent differences between the principles of the assays as well as batch-to-batch variations in the preparations of reagents and kits (10, 11). Thus, there is an urgent need for well-characterized reference materials that can be used to define the reliability and sensitivity of the tests, for quality control of batches of kits or reagents, and as common references between laboratories.

This report presents an assessment of two proposed reference preparations of sera, one reactive and the other unreactive to HIV, in a collaborative study involving 21 laboratories in 11 countries.

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MATERIALS AND METHODS

Proposed reference materials

The proposed preparations of antibody-positive and antibody-negative human sera, freeze-dried in sealed glass ampoules, were supplied by Professor K. O. Habermehl (Institute for Clinical and Experimental Virology, Berlin (West)). Each preparation was derived from a single donor; one an asymptomatic carrier of HIV and the other a donor with no known risk factors. Each serum was unreactive when tested for HBsAg antigen by a standard immunoassay and was heated at 56 °C for 1 h before being freeze-dried. When reconstituted as recommended (in 0.2 ml water) these preparations represented concentrations one-twentieth of the original sera; this dilution factor is not included in the calculations presented in this report.

Coded preparations

Seven freeze-dried serum preparations, coded A to G, were supplied to each participant. Preparations A and C were duplicate samples of the reactive proposed reference material, preparation E was the nonreactive proposed reference material. Samples D and F were prepared by the Central Public Health Service Laboratory, Colindale, London. Sample D, derived from a single donor, showed weak reactivity in immunosorbent assays. Sample F, derived from sera pooled from several donors, was highly reactive. Samples B and G, which were NIBSC reference preparations freeze-dried from pooled sera in 1973 and 1967, respectively, were both unreactive.

Design of the study

The study was designed to identify the coded preparations that reacted with HIV antibodies and to ascertain the minimum amount of the reactive samples that could be detected in the methods routinely used by the participants.

The 21 participating laboratories (see Annex) were supplied with duplicate sets of the coded preparations and requested to assay them by the procedures usually employed in their laboratories.

Assay methods

All but three participants assayed the preparations by indirect or competitive ELISA. Nine different commercial kits for ELISA were used: Abbott, Dupont, ENI, Genetic, Organon, Ortho, Pasteur ('ordinary' and 'Rapide'), Travenol and Wellcome. Two laboratories carried out ELISAs using their own 'in house' methods and one included in its series of assays the Abbott 'confirmatory' ELISA, a competitive ELISA based on envelope and core antigens derived from recombinant DNA.

Immunoblots were carried out by 15 laboratories. All except two, whose techniques involved the use of a mouse monoclonal antibody specific for human IgG and labelled with ^{125}I or protein A labelled with ^{125}I, used peroxidase-linked anti-human IgG for the identification of antigen-antibody complexes and eight used biotin-avidin amplification of the enzyme system.

One participant used the Karpas method (8) and one used an assay based on particle agglutination (PA).

Method of analysis

For each test the reactivities of the coded samples A to G and the end-point titres for samples A, C and F were taken to be those recorded by the participants. End-points were defined as the reciprocals of the highest dilutions of the reconstituted original materials *in normal serum* (not the final dilutions in the assay wells) that gave positive responses in the assays.

Potency ratios of C and F were expressed as ratios of their titres to that of A in the same assays.

RESULTS

Classification of sera by reactivity in ELISA and the immunoassays

Samples A, C and F were found reactive in all tests and samples B, E and G were reported as negative in all but one test. The exception was a test based on particle agglutination (PA) in which sample E was judged to be weakly reactive. Sample D was found to be reactive or weakly reactive in 40 of the 48 ELISAs performed, in both the PA and Karpas test and in two of the four fluorescence microscopy (FM) tests (Table 1). In the eight ELISAs in which sample D was identified as unreactive, it had a higher optical density (OD) than the negative control (although not of course as high as the OD of the cut-off limit), and in all but one the ODs were at least twice that of the negative control.

Titration of samples A, C and F

Participants carried out single or duplicate assays for individual manufacturers' ELISAs or by their local methods. Some laboratories used several manufacturers' ELISAs; in particular, laboratory 1 used seven different kits. For each kit, the geometric means of the titres for A, C and F and of the potency

Table 1. Assessment of reactivity of sample D by immunoassays

Assay method	No.of laboratories	No. of assays	No. with stated reactivity		
			+	±	−
ELISA kits:					
Abbott	9	12	9	2	1
Dupont	2	3	3	—	—
ENI	1	2	—	—	2
Genetic	1	2	—	2	—
Organon	3	4	1	1	2
Ortho	2	3	3	—	—
Pasteur[a]	5	8	6	1	1
Travenol	1	1	—	—	1
Wellcome	6	10	8	2	1
ELISA 'in house'	2	3	1	1	1
All ELISAs	18	48	31	9	8
Fluorescence microscopy	4	4	1	1	2
Karpas method	1	1	1	—	—
Particle agglutination	1	2	2	—	—

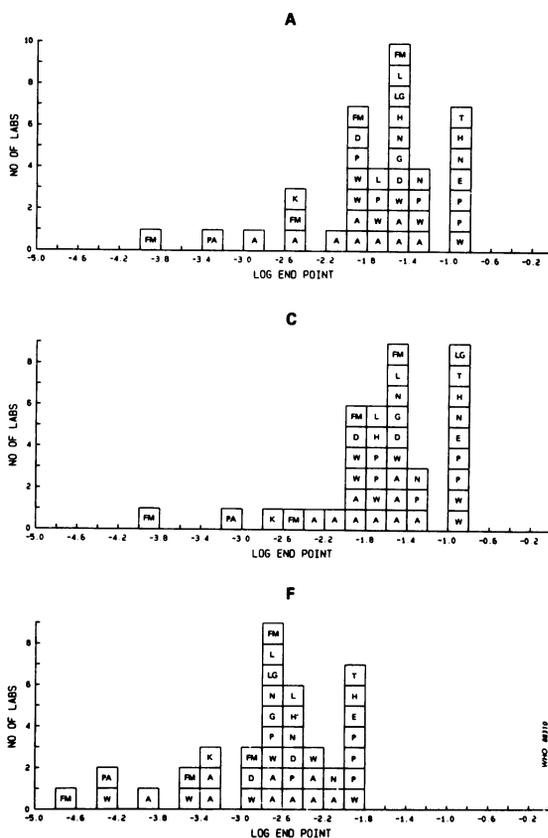[a] Includes assays using the 'Rapide' version.

Fig. 1. Frequency distributions of the end-point dilutions obtained for samples A, C and F.
Each square denotes an estimate from one test; the letters in the squares refer to the type of assay. A, D, E, G, H, N, P, T and W denote the following commercial kits of ELISA: A, Abbott; D, Dupont; E, ENI; G, Genetic; H, Ortho; N, Organon; P, Pasteur; T, Travenol; and W, Wellcome. L and LG denote 'in house' versions of ELISA.
Letters FM, K, and PA denote the following methods other than ELISA: FM, fluorescence microscopy; K, Karpas; and PA, particle agglutination test.

ratios for C and F obtained by individual laboratories were calculated. The frequency distributions of these values are shown in Fig. 1 and 2. One of the participants using the Abbott kit obtained much higher titres for A and F than did the other participants using both this and other kits. Further, its titres for C were tenfold and 100-fold lower than those for A. The results from this participant were, therefore, considered atypical and excluded from the subsequent analyses.

The laboratory mean titres varied considerably over about a 20-fold range. There were, however, no obvious differences between the titres from different kits: for instance, the ranges of titres for Abbott, Pasteur and Wellcome overlapped with each other.

The laboratory mean potency ratios of C, a coded duplicate of A, were mostly unity. All but one laboratory (mentioned above) found the titres of A and C to be within one dilution step (Fig. 1) although sometimes these dilution steps were as large as 5- and 10-fold. The laboratory mean potency ratios for F were more variable than those for C. However, the potency ratios were less variable than the titres (Fig. 1 and 2). One laboratory's results gave a potency ratio, based on a single assay, of 256, tenfold higher than the other estimates, and had the highest titre for F (16 384).

This titre and potency ratio were, therefore, considered atypical and excluded from the subsequent analysis.

Differences between the laboratories' estimations of titres were found to be significant by analyses of variance, even between those using kits from the same manufacturer. Expressing the reactivities of samples C and F relative to A showed that the differences between laboratories using the same commercial kit were no longer statistically significant. However, there were still significant differences between the potency ratios from different tests. For

example, the overall mean potency ratio, i.e., the geometric mean of the laboratory mean potencies, for Wellcome was 22, about three times higher than those for Abbott and Pasteur kits (6 and 7, respectively). The overall mean potency ratios for the other kits fell within this range.

Immunoblots

Fifteen participants tested samples A to G by immunoblot techniques. The results are given in Table 2. The use of control antigens ('mock' anti-

C

gens) was reported from only two laboratories. Faint reactive bands in the regions of relative molecular mass (M_r) 24×10^3 and 64×10^3 were detected consistently by one participant using 'mock' antigen from H9 cells; more information is required on this important aspect of the assays.

The relative molecular masses recorded in Table 2 are those assigned by the individual participants. For convenience of presentation, bands reported within narrow ranges of M_r are not differentiated and are classified in groups (e.g., $(32–34) \times 10^3$ and $(110–160) \times 10^3$).

Samples A and C were duplicates of the proposed reactive reference serum. As was hoped, they produced identical results for each immunoblot system and are considered together. One participant reported on the detection of antibodies to peptides p24 and gp41 only. All participants, except one who reported a weak reaction in the p65 region for sample E, and another who observed a reaction to p24 antigen in sample G, detected no antibodies to HIV in samples B, E and G. Of the positive samples, F reacted most strongly in all immunoblots but, except for less frequent detection of antibodies to the envelope antigens gp110-p160 in A and C than in F, samples A, C and F were qualitatively identical.

All participants detected antibodies to p24, gp41 and p53/55 in A, C and F, three reported no antibodies to p17/18, and only one reported no antibodies to p65.

Additional results

Essex and colleagues included in their study an investigation of the reactivity of samples A to G in immunoblots in which the recently isolated strain HTLV-IV was used as antigen. No reactions with any
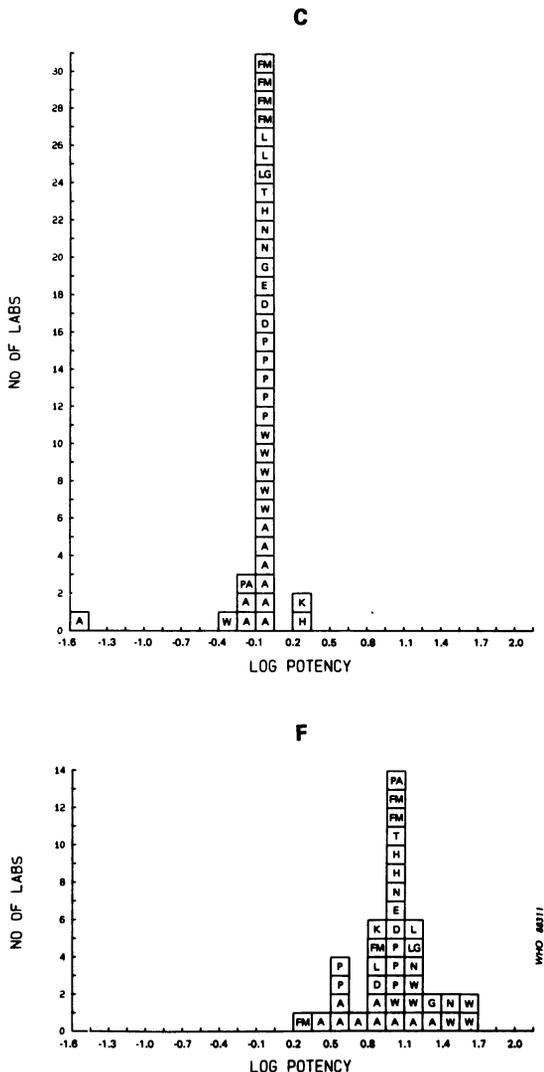
F

Fig. 2. Frequency distributions of the potency ratios of samples C and F in terms of A obtained from individual tests. (See legend in Fig. 1 for description of the numbers and letters in the squares, etc.).

Table 2. *Detection of anti-HIV by immunoblot[a]*

Peptide or glycopeptide (approximate relative molecular mass $\times 10^3$)	Reactive bands/total reports		
	Sample A (C)	Sample D	Sample F
17, 18	11/14	6/14	13/14
24	15/15	13/15	15/15
32, 34	12/14	11/14	11/14
38, 39	9/13	5/14	10/13
41	15/15	6/15	15/15
53, 55	11/14	12/24	14/14
65	13/14	13/14	13/14
110, 120, 160	9/13	7/13	12/13

[a] All participants did not report the presence or absence of each peptide or glycopeptide.

viral antigens were reported for Western blots and only two reactive regions, gp160 for sample A and p24 for sample F, were detected in radioimmuno-precipitation using ^{35}S-labelled HTLV-IV.

DISCUSSION

ELISA kits from nine manufacturers were used in this study. The sensitivities of the various kits were assessed by comparing end-point titres for the highly reactive samples A and F and by whether or not a weakly reactive serum was found 'positive' in the tests. This sensitivity varied between laboratories, even between those using the same commercial kit. Expressing the reactivity of sample F relative to A reduced the variation between tests and resulted in agreement between the laboratories using the same kit. Nevertheless there were still consistent differences between the kits in the comparison of the reactivity of A and F. This possibly reflected the differences in specificities of the ELISA systems.

Overall, immunoblots revealed reactions of sera A (C) and F to all the expected HIV antigens. The presence of antibodies to p24, gp41 and p55 is considered by most workers an important indication of infection with HIV (6, 11, 13). However, for reproducible results the source of antigens, the standardization of electroblotting procedures, and the provision of 'control' antigens require careful attention (6); variations of these factors may well explain the differences shown in Table 2.

The results for HTLV-IV confirm earlier findings (2, 4, 7) and emphasize the urgent need for information on the responses of immunoassays to sera from AIDS patients from different geographical areas and for characterization of the genetic and immunological differences between viral isolates. The proposed reference preparation, A, reacted strongly in all ELISAs and related immunoassays and reacted with all the major HIV antigens in immunoblots. Its use will depend on individual requirements but it may be of value as a qualitative check on the specificity of the assays, to calibrate positive controls included in kits and other assays in arbitrary units, to calibrate detection limits (cut-off) in arbitrary units (as done for HBsAg), and to calibrate immunoblots, particularly for defining the optimal amounts of antigen and for determining the relative mobilities of the major peptides and glycopeptides from HIV. Because the unreactive preparation (E), reconstituted in 0.2 ml water as recommended, represents diluted serum, its use as a reference material will be limited.

The WHO Expert Committee on Biological Standardization reviewed the report of this collaborative study in December 1986 and agreed that preparations A and E would be of value as reference preparations for, respectively, positive and negative anti-HIV sera.

The preparations, under code numbers 86/6302 (reactive) and 86/6238 (unreactive) are available from the Director, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts. EN6 3QG, England.

RÉSUMÉ

TITRAGE DES ANTICORPS DIRIGÉS CONTRE LE VIRUS DE L'IMMUNODÉFICIENCE HUMAINE: ÉTUDE COLLECTIVE INTERNATIONALE POUR L'ÉVALUATION DE SÉRUMS DE RÉFÉRENCE OMS

Deux préparations de sérum humain, l'une réagissant avec le virus de l'immunodéficience humaine (VIH) et l'autre inactive, ont fait l'objet d'une étude collective à laquelle ont participé 21 laboratoires de 11 pays.

Ces préparations ont été fournies, sous forme lyophilisée en ampoules de verre scellées, par le Professeur Habermehl (Institut de virologie clinique et expérimentale, Berlin-Ouest). Chaque sérum provenait d'un donneur unique; l'un d'eux était un porteur de VIH asymptomatique et l'autre ne présentait aucun facteur de risque.

L'étude était conçue de façon à identifier, parmi sept préparations codées, celles qui contenaient des anticorps anti-VIH et, dans le cas des échantillons positifs, à déterminer les quantités minimales détectables par les méthodes employées habituellement par les participants.

Pour ces essais, la plupart des participants ont choisi une méthode ELISA indirecte ou par compétition, et ils ont utilisé 9 nécessaires d'analyse commerciaux différents. Quinze laboratoires ont effectué des immunotransferts en utilisant principalement l'IgG antihumaine liée à la péroxi-dase ou l'amplification du système enzymatique par la biotine-avidine pour identifier les complexes antigènes–anticorps.

L'échantillon proposé comme réactif de référence "positif" a réagi fortement dans tous les immunotitrages et a donné toutes les bandes attendues dans les systèmes d'immunotransfert. La sensibilité des divers essais, mesurée par comparaison des titres de fin de dosage, a varié d'un laboratoire à l'autre, même lorsque ceux-ci utilisaient le même nécessaire d'analyse. Cette variation entre laboratoires a pu être réduite en exprimant les résultats en fonction de l'activité de l'échantillon positif proposé.

Les noms et adresses des laboratoires participants sont indiqués dans l'annexe 1 (page 202).

Les réactifs de référence proposés, qui portent les numéros de code 86/6302 (actif) et 86/6238 (inactif), peuvent être obtenus sur demande adressée au Directeur du National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts. EN6 3QG, Angleterre.

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Annex 1

Participating laboratories

National HIV Reference Laboratory, Fairfield Hospital, Fairfield, Victoria, Australia

Red Cross Blood Transfusion Service, Adelaide, Australia

Laboratory Centre for Disease Control, Ottawa, Ontario, Canada

Laboratoire National de la Santé, Departement de Controle des Vaccins à Virus et des Produits Derivés du Sang, Paris, France

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