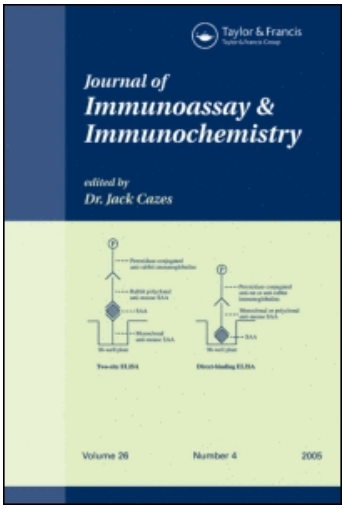

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Rapid Immunoblot Assay (Western Blot) to Detect Specific Antibodies for Human Immunodeficiency Virus, *Schistosoma Mansoni*, and *Taenia Solium* (Cysticercosis)

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To cite this Article Brand, Joy A. and Tsang, Victor C. W.(1989) 'A Rapid Immunoblot Assay (Western Blot) to Detect Specific Antibodies for Human Immunodeficiency Virus, *Schistosoma Mansoni*, and *Taenia Solium* (Cysticercosis)', Journal of Immunoassay and Immunochemistry, 10: 2, 237 — 255
To link to this Article: DOI: 10.1080/01971528908053239
URL: http://dx.doi.org/10.1080/01971528908053239

JOURNAL OF IMMUNOASSAY, 10(2&3), 237-255 (1989)

A RAPID IMMUNOBLOT ASSAY (WESTERN BLOT) TO DETECT SPECIFIC ANTIBODIES FOR HUMAN IMMUNODEFICIENCY VIRUS, *SCHISTOSOMA MANSONI*, AND *TAENIA SOLIUM* (CYSTICERCOSIS).

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ABSTRACT

The introduction of the immunoblot into the repertoire of serodiagnostic assays began with the confirmatory testing of human immunodeficiency virus (HIV) antibodies. The difficulty of producing immunoblot strips and the complexity of the time-consuming serum/conjugate incubation steps deterred more widespread use of the immunoblot.

Using the simple Schleicher & Schuell Accutran™ system, can greatly reduce the complexity for strip production and incubation. By simply increasing reagent concentrations, we can reduce incubation times for sera and conjugates (enzyme-labeled second antibodies) to 5 minutes from the usual 1 hour or overnight incubation times. The resulting RAPID BLOT shows no reduction of sensitivity or specificity.

(KEY WORDS: Rapid Immunoassay)

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INTRODUCTION

The immunoblot is a test that involves separating antigens by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferring the resolved antigens to a nitrocellulose sheet, and identifying disease specific antibodies through their reaction to the resolved antigens. The immunoblot has become a vital diagnostic assay. Since the first experiments in protein blotting conducted by Towbin et al. (1), there were numerous technological advances which increased the number of potential applications for the immunoblot.

Our objective was to greatly decrease the time required to complete the immunoblot assay while maintaining its sensitivity. The newly developed Schleicher & Schuell Accutran products offer a system approach to immunoblot testing. These products were specifically designed to accelerate and systematize the blotting, cutting, washing and incubation steps in immunoassays that involve the screening of multiple samples of filter-bound antigens. This decrease in assay time improves the immunoblot as a rapid screening serodiagnostic assay. This paper describes a rapid immunoblot procedure that decreases the completion time from 3 hours to 30 minutes. Revisions of the immunoblot techniques for Cysticercosis, *S. mansoni*, and HIV were targetted herein, because these three human diseases are routinely diagnosed or confirmed by established immunoblot methods.

MATERIAL AND METHODS

The RAPID-BLOT is conducted in three stages : (a) the antigen mixture is first resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ; (b) the resolved antigens are electrophoretically blotted onto nitrocellulose sheets; and (c) the blotted antigens are then probed with patients' serum specimens. Reactions are visualized by ELISA. Except when otherwise specified, the RAPID-BLOT was performed as follows.

Antigens

The RAPID-BLOT was optimized for human immunodeficiency virus (HIV), *Schistosoma mansoni*, and *Taenia solium* (cysticercosis). HIV (NCI-Fredricks, Lot# P2946)[1] was blotted at 0.02 mg/ml, *S. mansoni* adult microsomal antigen (MAMA), prepared from adult worms (2) at 0.05 mg/ml, and *T. solium* (3) at 0.02 mg/ml. All antigens were SDS treated according to the protocol of Tsang et al. (4). Relative molecular-weights of the resolved protein bands on the transferred blot were calibrated against unstained molecular-weight standards and stained with Aurodye (Janssen, Newton, MA). The unstained molecular weight

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[1]Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U. S. Department of Health and Human Services.

standard was composed of a 1:1 mixture of low molecular weight standard (Pharmacia, Piscataway, NJ), and high molecular weight standard (Biorad, Rockville Centre, NY). Prestained protein standards (Bethesda research Labs, Gaithersburg, MD) were used to separate different areas on a single blot.

Antibody source (serum)

A standard serum pool (SMPR Pool) was prepared from 14 Puerto Rican patients with parasitologically confirmed *S. mansoni* infections. A serum pool from healthy persons was used as a negative control for *T. solium* and *S. mansoni* immunoblots. A standard serum pool (TSP) was prepared from patients with biopsy proven *T. solium* infections. HIV positive serum was the CDC reference CAT# VS2151, and the HIV negative serum was the CDC reference CAT# VN2152.

Electrophoresis

All SDS-PAGE chemicals (unless specified otherwise) were from Bio-Rad Laboratories. Our gel system contains a gradient resolving gel (5% to 20%) and a 3% stacking gel. Gels are 130 x 70 x 0.75 mm. Electrophoresis in a discontinuous buffer system was conducted as previously reported (5). Briefly, sample stacking current = 2.5 mA per gel, resolving current = 25 mA per gel. Voltage was allowed to float and limiting wattage during resolution was set at 30 watts per gel.

Immunoblotting

After SDS-PAGE separation, resolved protein bands were electrophoresed onto nitrocellulose sheets (Schleicher & Schuell, Keene, NH) as previously described (5). After blotting, the nitrocellulose is cut into 3mm strips with a strip cutter (Schleicher & Schuell) obtaining 40 strips from a single blot. Strips are stored at -70°C until needed. The processing and washing of all immunoblots were completed with the Accutran™ system (Schleicher & Schuell). The immunoblot procedure was adopted from Tsang et al. (5).

Exposure to serum specimens

All serum dilutions were made in phosphate-buffered saline (PBS = 0.01 M dibasic sodium phosphate/0.01 M monobasic sodium phosphate, 0.15 M sodium chloride, pH 7.2), 0.3% Tween 20, 5.0% nonfat dry milk. We routinely dilute all sera 1:50 but this dilution can be varied to optimize sensitivity. Dilution titrations ranged from 1:4 to 1:65,536. In various experiments, the serum dilutions were allowed to remain in contact with the strip for 5 min, 15 min, 30 min, 60 min, and overnight. Overnight incubations are conducted at 4°C and all other incubations at room temperature. All incubations require continuous gentle agitation on a rocker.

Exposure to indicator antibody/enzyme conjugate

We prepared affinity-purified goat anti-human IgG (heavy and light chain activity) labeled with horseradish peroxidase (POD),

with a 1:3.9 IgG:POD molar ratio. Diluted horseradish peroxidase labeled conjugate was added to each strip after serum incubation and appropriate washing. Both conjugate concentration and time of incubation were separately varied to optimize sensitivity. Conjugate concentration varied from 1:50 to 1:1000 and incubation times varied from 5 min. to 60 min.

Substrate

The substrate for our immunoblot is H_2O_2/3,3'-diaminobenzidine (Sigma, St. Louis, MO). Substrate was added to each strip after conjugate incubation and appropriate washing and was allowed to develop for 10 min. The reaction is stopped by washing ten times with water.

Washing

Proper washing is critical after serum incubation, conjugate incubation, and substrate development. The number of washes and time of each wash were varied to determine optimal washing conditions. It is necessary to wash with PBS containing 0.3% Tween 20 after both serum and conjugate incubations. Tween 20 slightly inhibits POD activity, therefore, it is equally important to wash with PBS alone immediately before adding substrate. After enzyme/substrate interaction, the reaction is stopped by washing ten times with water.

Densitometry

Quantitation of immunoblot bands was accomplished by laser scanning densitometry (Zeineh-Biomed Instruments, Fullerton, CA) of photographs of blots. Densities of all photographs were standardized by the incorporation of a standard grey-scale. Blot strips on the positive prints were scanned, and the relative areas under peaks were quantified. During all scanning sessions the grey-scale was used as reference to adjust the base line and gain of the densitometer to the same levels. All relative peak areas whether considered individually or summed were thus comparable throughout the study. Only diagnostic bands were quantitated as follows: HIV = GP-41 and P-24, *S. mansoni* = GP-30, and Cysticercosis = GP-50, -42/39, -24, -21, -18, -14, and -13. The GP prefix indicates their glycoprotein nature while the numbers are their weight as they appear on SDS-PAGE. Interpretations of individual bands for these three infections were reported previously (5,6,3).

RESULTS

Serum Incubation Optimization

Serum pools were serially diluted in 4-fold steps from 1:4 through 1:16384 (Fig. 1). The serum dilution series was then incubated with blotted antigen strips for 5 min or 60 min. For all serum titration experiments, conjugate exposure is 5 min at 1:250 dilution. 1:50 serum dilution was determined to be in excess for

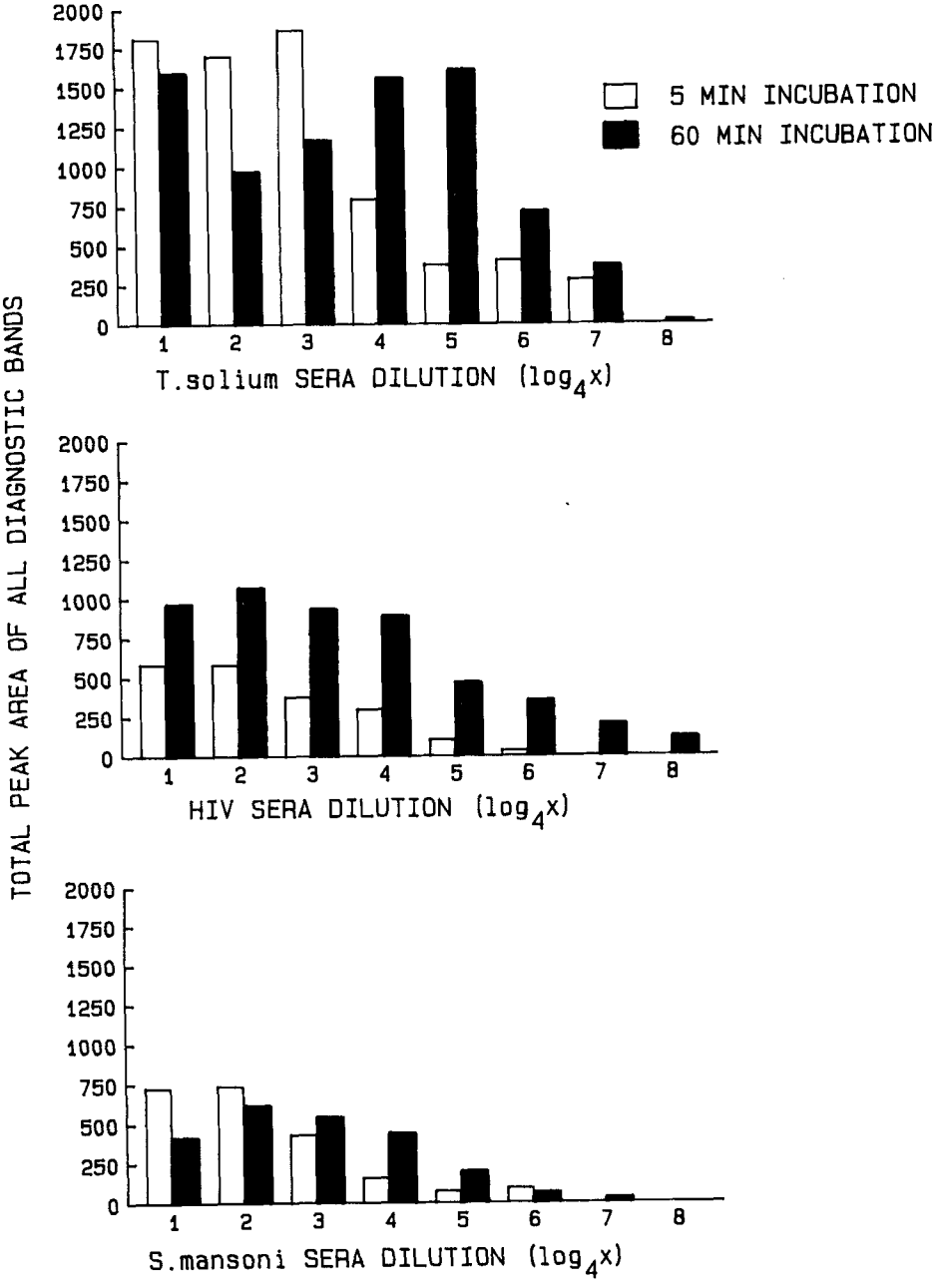

FIGURE 1. Quantitation of immunoblots using serum dilutions in 4-fold steps. Conjugate exposure is 5 min at 1:250 dilution.

all infections tested. Serum incubation time was varied from 5 min to overnight at this dilution (Fig. 2). For the serum incubation time experiments, conjugate exposure is for 1 hr at 1:1000 dilution (concentration and time used in standard immunoblot).

Conjugate Incubation Optimization

The immunoblot was initially conducted by using conjugate diluted at 1:1000 and varying the conjugate incubation times from 5 min to 60 min (Fig. 3A). There is no significant difference in immunoblot results with conjugate (1:1000 dilution) incubation times of 15, 30, or 60 min (Fig. 3A). Further experiments were conducted to determine the conjugate concentration required to optimize the immunoblot with a conjugate incubation time of 5 min. Conjugate titrations from 1:50 to 1:1000 for both 5 min and 15 min incubation times (Fig. 3, B and C) were used to perform immunoblots. For all conjugate optimization experiments, a 1:50 dilution serum incubation time of 1 hour was used.

Washing Optimization

Immunoblots were performed with varying wash fluid exposure times. After both serum and conjugate incubations, strips were washed with four changes of PBS/Tween with 0, 1, and 5 min exposures (Fig. 4). In experiments with 0 time washings, PBS/Tween fluid was added to strips and immediately aspirated. For the washing experiments, serum was incubated for 5 min at 1:50 and the conjugate for 5 min at 1:250.

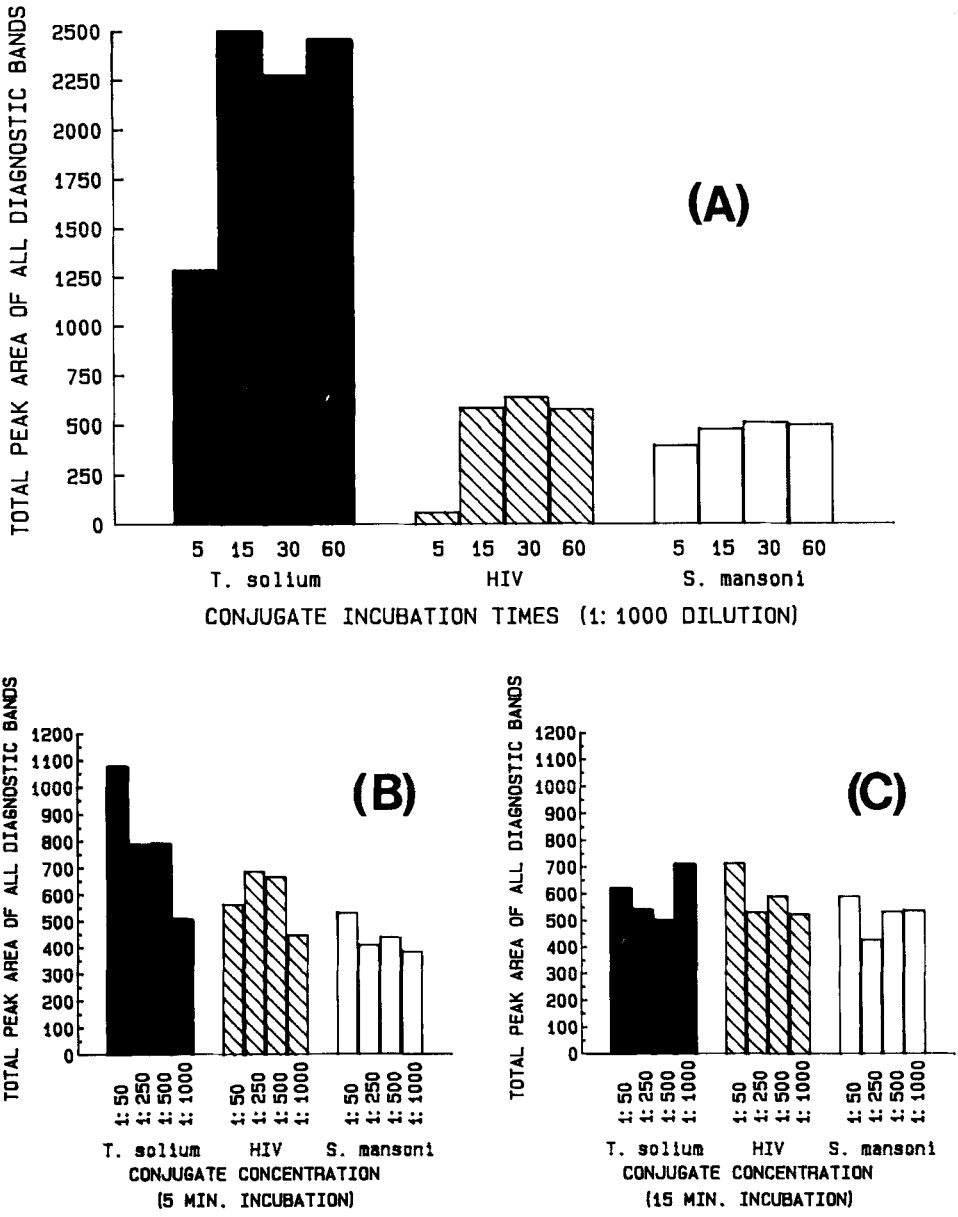

FIGURE 2. Quantitation of immunoblots varying serum incubation times at a 1:50 dilution. Conjugate exposure is 1 hr at 1:1000 dilution.

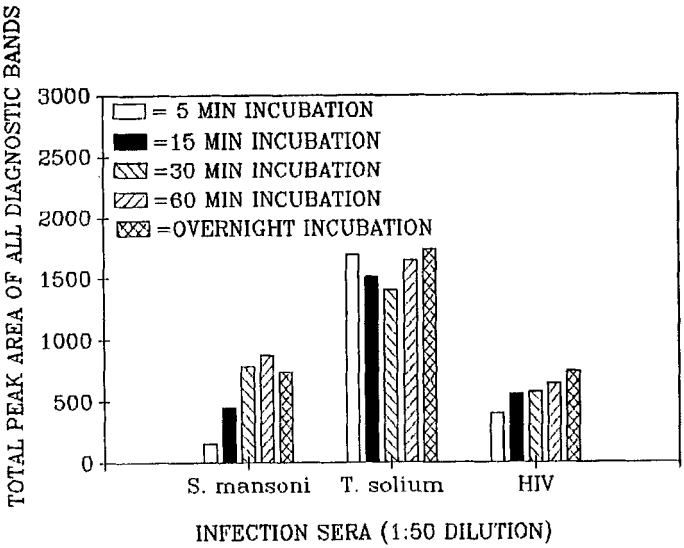

FIGURE 3. Quantitation of immunoblots optimizing conjugate conditions after a 1-hr serum (1:50) incubation.
(A)=Quantitation of immunoblots varying conjugate incubation times at a 1:1000 dilution. (B)=Quantitation of immunoblots using a conjugate titration at 5-min exposure. (C)=Quantitation of immunoblots using a conjugate titration at 15-min exposure.

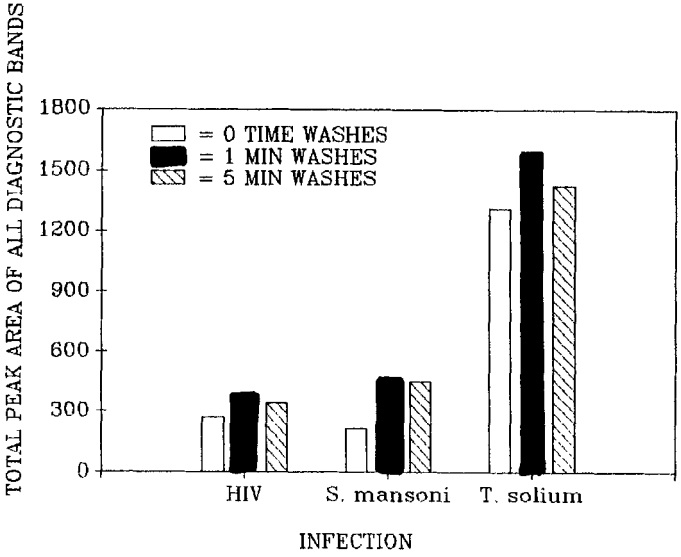

FIGURE 4. Quantitation of immunoblots performed with varying wash incubation times. Serum (1:50) incubation and conjugate (1:250) are each 5 min.

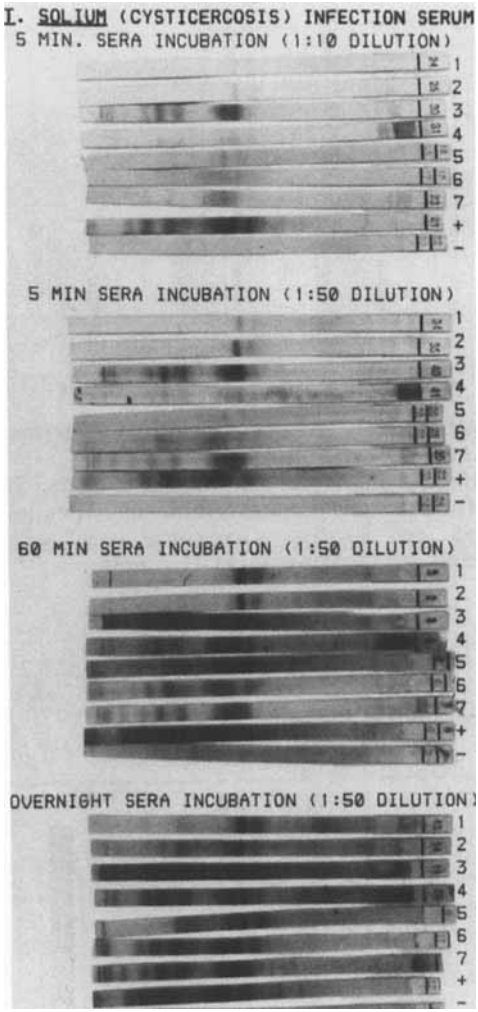

FIGURE 5. Immunoblot strips completed with sera selected based on low specific antibody levels. Serum incubation times are varied from 5 min to overnight. Conjugate (1:250) exposure is 5 min.

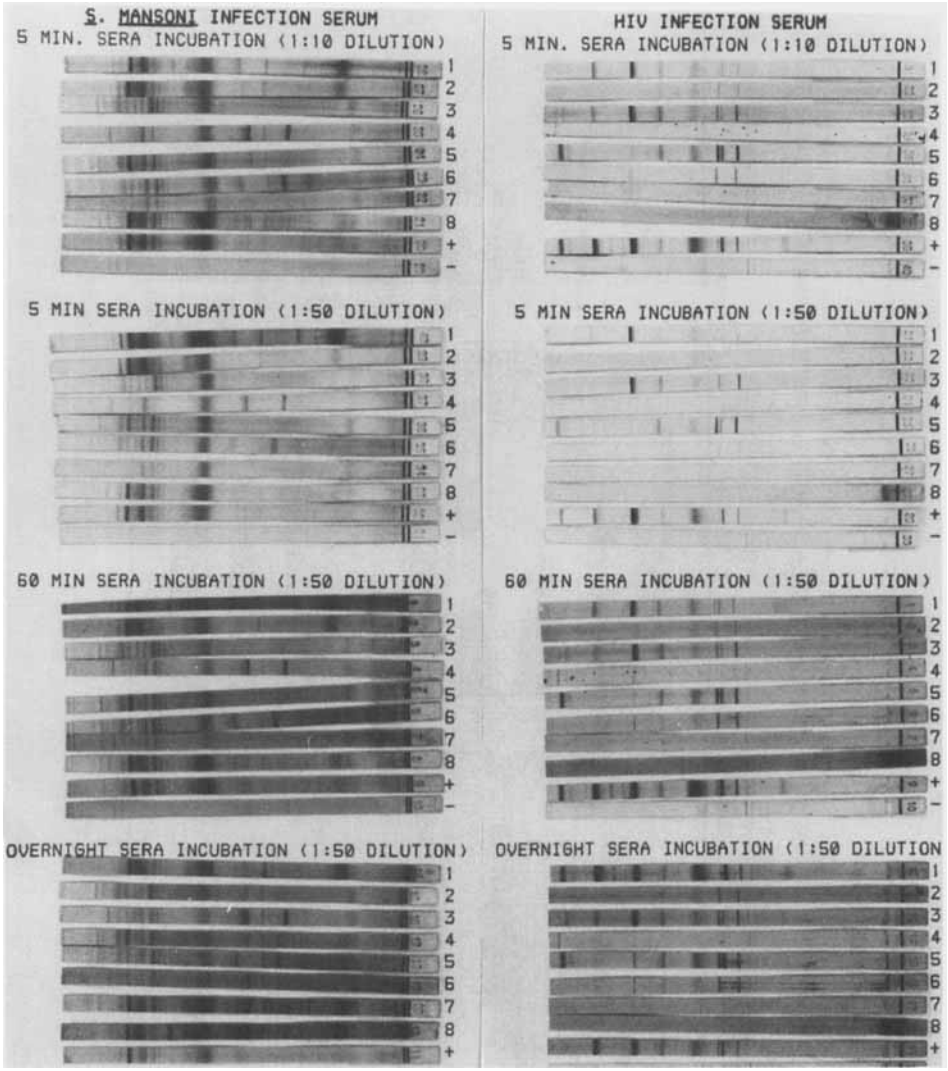

FIGURE 5, continued.

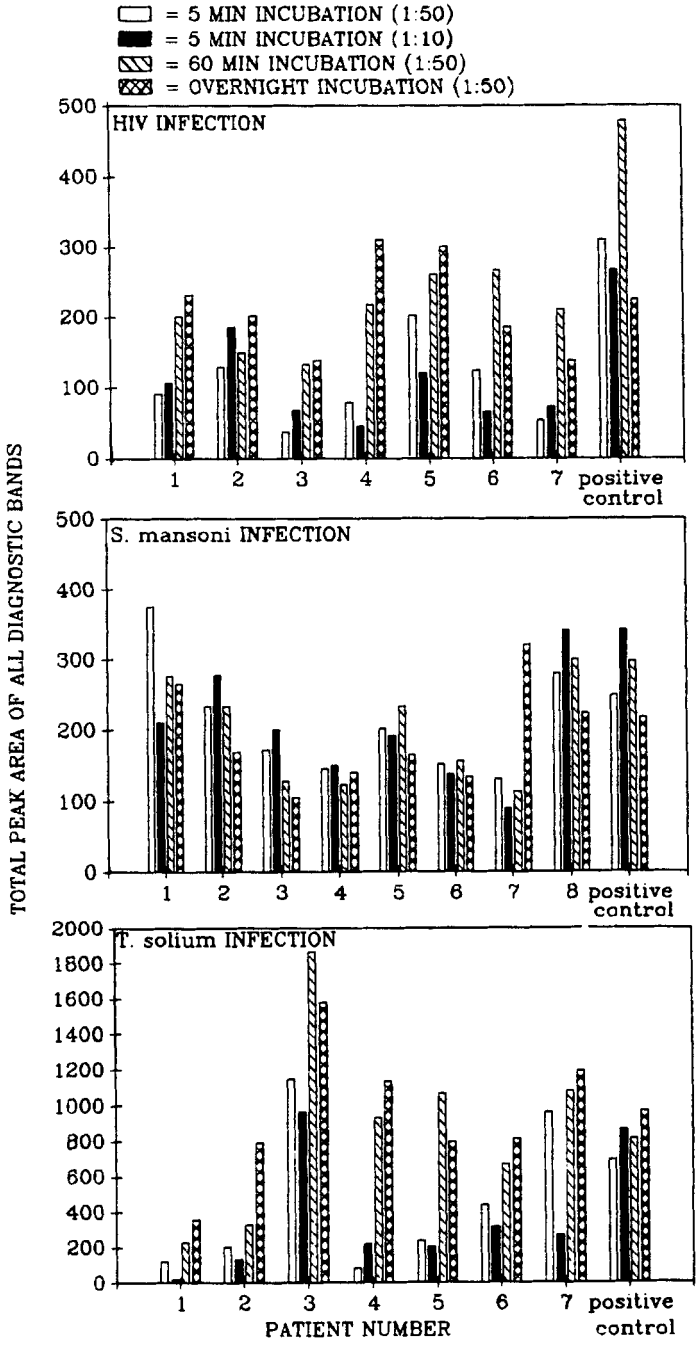

FIGURE 6. Quantitation of immunoblots completed with sera based on low specific antibody levels.

Difficult Diagnostic Cases

To more accurately test the performance of the RAPID-BLOT, several sera were selected based on their low specific antibody levels that produced weak reactions in the standard immunoblot (Fig. 5 and 6). Serum incubation times ranged from 5 min to overnight in this experiment. For the 5-min serum incubation, both 1:10 and 1:50 serum dilutions were used. A serum dilution of 1:50 was used for the 60 min and overnight incubation. For the 1-hour and overnight serum incubations, the conjugate (1:1000 dilution) reaction time was 1 hour. For the 5 min serum incubations, on the other hand, conjugate was diluted at 1:250, and the reaction time was 5 min.

DISCUSSION

For the immunoblot to be more widely accepted as a rapid serodiagnostic assay, the strip production must be simplified, and the serum/conjugate incubation steps must be shortened. Our data show that the latter can be accomplished without reduction of sensitivity or specificity.

Increasing antibody concentrations while decreasing time achieves the same result as decreasing antibody while increasing time (Fig. 1). With *S. mansoni* and *T. solium* antibodies, using serum concentrations of 1:64 to 1:256, respectively, compensated for the shortening of incubation from 60 to 5 min. However, this compensation is not achieved with HIV, possibly due

to low antibody levels in the CDC reference serum. Our data indicate (Fig. 1) that a 1:50 serum dilution and a 1:250 conjugate dilution each for 5 min incubations caused very little reduction in sensitivity or specificity. There is some decrease in peak density at a 1:50 serum dilution at 5 min incubation (Fig. 2); however, by increasing conjugate concentration we can overcome the decrease of peak density at a 1:50 serum dilution.

A decrease in total peak area was observed only with 5 min incubation time when conjugate was used at 1:1000; no difference was seen with longer incubation times (Fig. 3B). This reduction in density can also be negated by increasing conjugate concentration (Fig 4). There is no significant difference with the 1:50, 1:250, or 1:500 conjugate dilutions at 5-min or 15-min conjugate incubations for any of the infections. Therefore, a conjugate dilution of 1:500 can be used in a 5-min incubation time with no reduction in the total peak area for the three infections tested. In general, to determine the optimal conjugate concentration, a titration series was performed. A 2-fold concentrational excess which produced a plateau level of signal was chosen as the optimum. Thus, if a plateau was reached at 1:500, then a dilution of 1:250 was chosen as the optimum.

In addition to shortening the serum and conjugate incubation times to 5 min, it is also desirable to decrease washing times. Incubation times for each wash can be decreased from 5 min to 1 min without any decrease in total peak area (Fig. 4). The binding of

antibody to antigen is generally considered a reversible reaction, albeit one with a high association constant. This reversibility may explain the density decrease in the washes with zero wash exposure time, which is not seen with the 1 and 5 min washes. Antibody, conjugate, or antibody/conjugate complex that detaches will have time to reequilibrate and reattach during the 1- and 5-min incubations. This presumably did not have time to occur with zero wash exposure time blots.

When compared to the the 1 hour or overnight incubations, RAPID-BLOT missed no positive serum specimens (Figs. 5 and 6). The serum specimens chosen for these experiments were selected because their low specific antibody levels made them problematic serodiagnostic cases. In HIV, where sensitivity is critical, the sensitivity of immunoblot must not be diminished with shorter incubation times. Figs. 5 and 6 show this not to be a problem. Although certain sera with low antibody levels decreased in quantitative band intensity, they remained positive when tested by the RAPID-BLOT.

Again, revisions of the immunoblot techniques for Cysticercosis, *S. mansoni*, and HIV were targeting herein, because these three human diseases are routinely diagnosed or confirmed by established immunoblot methods.

The optimal conditions for the Rapid Blot are as follows:

1) Each 3mm blotted nitrocellulose strip is incubated for 5 min with 0.5 ml of serum (diluted 1:50 with PBS/Tween/milk).

2) Wash 4 times, each with 1 ml of PBS/Tw heated to 56°C.

3) Incubate each strip with 0.5 ml of conjugate (1:250 in PBS/Tw) for 5 min.

4) Wash 3 times, each with 1 ml of PBS/Tw followed by 2 washes with PBS alone.

5) Develop each strip using 0.5 ml of DAB/H_2O_2 for 10 min then wash 10 times with H_2O to stop reaction.

All details omitted in the above summary concerning reagent concentration, optimization, etc. are included in the methods section. It is critical that strips be rocking during all incubations and washing.

We have shown that by simply increasing reagent concentrations we can decrease the total assay time for the immunoblot from 3 hours to 30 min, without reduction of sensitivity or specificity. By developing a RAPID-BLOT, we have hopefully facilitated the widespread use of the immunoblot as a serodiagnostic assay.

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