FOR YOUR EYES ONLY

REPORT OF THE PILOT STUDY TO VALIDATE HIV ELISA TESTING IN SOUTH AFRICA

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Aim.

To evaluate the possibility that subjects with clinically diagnosed tuberculosis produce antibodies capable of binding to the antigens employed in an HIV antibody test currently in use in South Africa. Detailed aspects of the rationale of the study are to be found in Protocol 7 (Appendix 1).

Strategy.

The work described below was performed at the Department of Virology, Medical University of South Africa (MEDUNSA), Pretoria, between May 7-20, 2003.

The general strategy to determine the specificity of the antibodies being detected by the HIV test was to allow sera to bind to whole cell lysates from two different strains of *Mycobacterium tuberculosis* previously immobilized onto a solid phase support (micro titer wells). Signals obtained after preadsorption of the samples were to be compared to signals from control and untreated samples.

Due to operational considerations, some of the procedures regarding sample preadsorption had to be modified from the original protocol.

1- HIV antibody test: The HIV antibody test used was the Biomérieux '*VIRONOSTIKA HIV-Uni-Form II plus O*', which is a two-step cross linking-type immunosorbent assay that requires the addition of neat serum finally diluted 1:3 with a special sample diluent of unreported composition directly into the assay wells. This test is used in the MEDUNSA clinical virology unit to confirm HIV+ ELISA results from the Abbott AxSym system that is also installed and routinely operated. It differs from most ELISA tests in that it does not involve a secondary antibody to detect bound serum immunoglobulins, nor allow dilution of the sample prior to preadsorption. Therefore, we were required to use whole sera for incubation on the antigen-coated wells.

2- Samples: The original study called for a stratified sample of HIV positive individuals, with a minor proportion of positive values exceeding the positive controls included in the test (high positives), a minor set of low signal positive sera (low positives) and a majority of positive sera around the values of the positive controls supplied with the test (mid-range positives), along with a number of samples from clinically confirmed tuberculosis patients. Due to time and operating constraints, the original sample types could not be obtained, resulting in the testing of samples for which no quantitative data on the HIV antibody status was available and with the common feature of being all in treatment for clinically diagnosed tuberculosis.

We therefore initiated the study with the sample set described below that was collected by Dr. Junaid Ashgar of Prof. Mholongo's Dept. at MEDUNSA from the 5 clinics serving Soshanguve immediately preceding and in the first days of our arrival. It is appropriate at this point to note that we could not have accomplished any work at all without the magnificent assistance of Dr. Junaid Ashgar whose relationship with the nursing sisters was excellent, enabling him to collect the samples in a very compressed time frame. As the pictures in Appendix 2 illustrate, the clinics are not (although one might have expected otherwise) overflowing with HIV/AIDS patients.

Total number of samples.	162	(100%)
HIV antibody positive samples.	99	(61%)
Samples of unknown HIV antibody status.	63	(39%)
Samples with unknown HIV and TB status.	3	
Samples from patients with extrapulmonary TB.	4	

Introductory remarks.

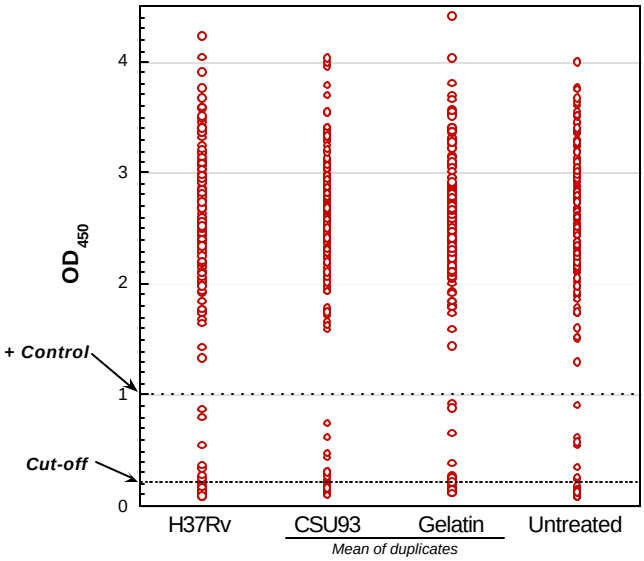
Not fully knowing what to expect in terms of equipment and laboratory space, upon arrival we became acquainted with the resources at our disposal in the Department of Virology of MEDUNSA. These were completely adequate. We also established cordial relations with the various persons in whose laboratory we would be guests, and whose assistance with minor matters of laboratory routine were essential to accomplishing our work.

Results.

A. Preadsorption.

Preadsorbption of the serum samples was done on immobilized *Mycobacterium tuberculosis* whole cell lysates of two strains: H37Rv and CSU93, obtained from Colorado State University (See Appendix 3) as described in Protocol 7. As controls, sera were tested without undergoing preadsorption on the solid phase-bound antigens and preadsorbed on an inert protein substrate (gelatin).

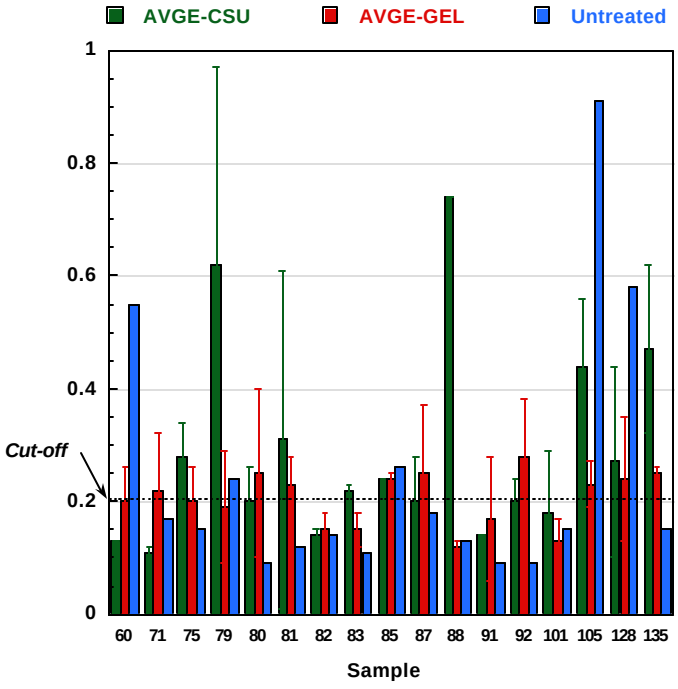
The graph below shows the values of all the HIV antibody (1032) tests done in this study. The ordinate represents optical density values and the abscissa the treatment of the samples.



Samples adsorbed against CSU93 lysate and those adsorbed against gelatin were tested in duplicate and the average is represented, untreated and H37Rv-adsorbed sera were tested singly. The cut-off and positive control values represented are typical and correspond to the experiments in which concordance in the triplicates agreed best (see below). As can be seen, the majority of the untreated values are above 2, beyond the mid-range values around the values of the positive controls, and beyond the linear range of this (or just about any other) spectro-photometric assay (see section D below).

The samples with OD values lower than 2 in the untreated set comprise 26% (42 of 162), those lower than 1 comprise 17% (27 of 162) and those between the cut-off value and the positive control value are only 4% (6 of 162), using the values for untreated samples as reference.

Upon cursory inspection of the raw preadsorption data, it is obvious that no large differences can be gleaned between the groups. That is to say, no significant loss of signal upon preadsorption is apparent. However, upon more detailed inspection of the data generated in duplicate, a large variation between duplicates in a significant number of samples did become obvious. The graph below shows preadsorption and untreated data for a number of samples of previously unknown HIV antibody status.



The blue bars represent the test done on untreated samples, the red are those same samples preadsorbed against an irrelevant protein (gelatin) and the green were preadsorbed against a Mycobacterial antigen preparation.

The first thing to note is that the SD on many samples is very high. The second is that in many cases the SD actually crosses the cut-off value, indicating uncertain qualitative outcome (seropositive or negative) for the test were it repeated on the same

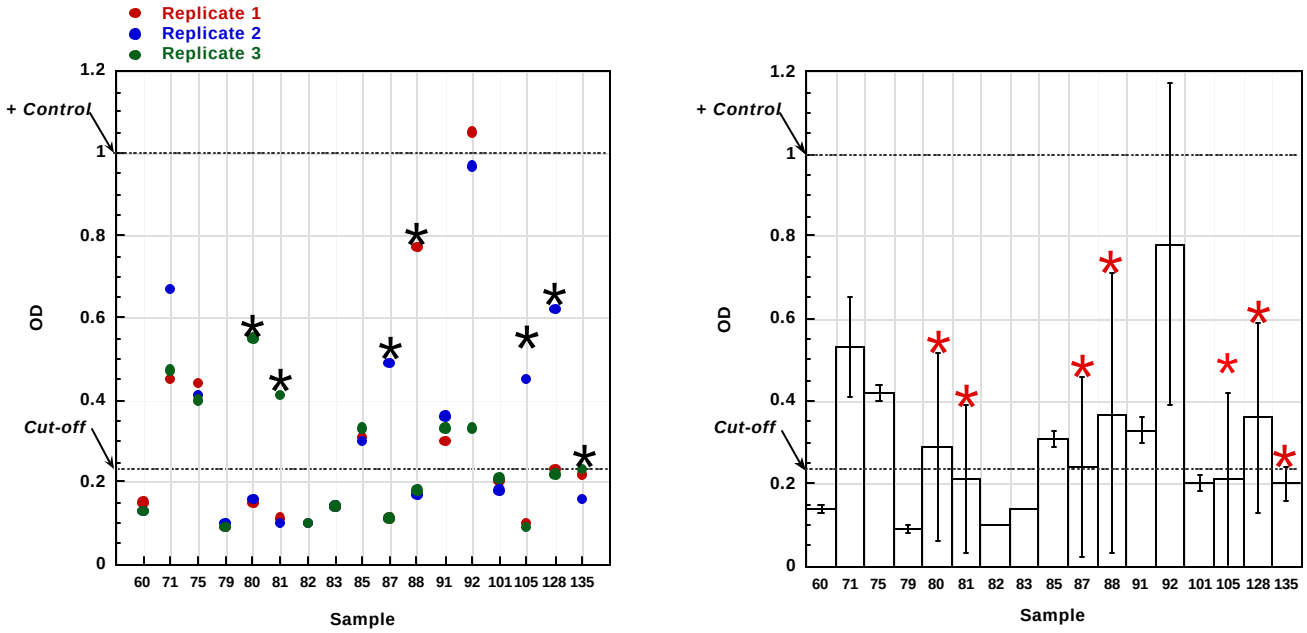
sample. The third is that in some cases, upon preadsorption, some samples score

higher than the untreated and some lower, irrespective of whether an antigenic preparation or an inert protein are used. For the purpose of the preadsorption study, as put forth in the original design, this data is not interpretable. The significant variation between duplicates precludes a quantitative analysis of loss of reactivity, and shows considerable inconsistency in the outcome of the test when performed in replicate.

At this point we had to consider whether the observed variations were due to the manipulation of the samples necessary for preadsorption or, alternatively, whether this variability was inherent to the test itself, even when performed on completely untreated samples in replicate (which had not been done as we had not expected the test to perform so imprecisely).

B. Assay variability.

As the untreated samples where not tested in duplicate, we decided to evaluate whether the source of variation lie in the preadsorption treatments or whether it was due to an inherent variation in the "test itself". To do this, a subset of untreated samples with readings below the positive control value for the assay (which, depending on plate-to-plate variation, is around 1) were re-tested in triplicate. The scatter plot on the left shows the outcome of the three tests for each individual serum sample. The bar graph on the right shows the average of the three determinations with the standard deviation.



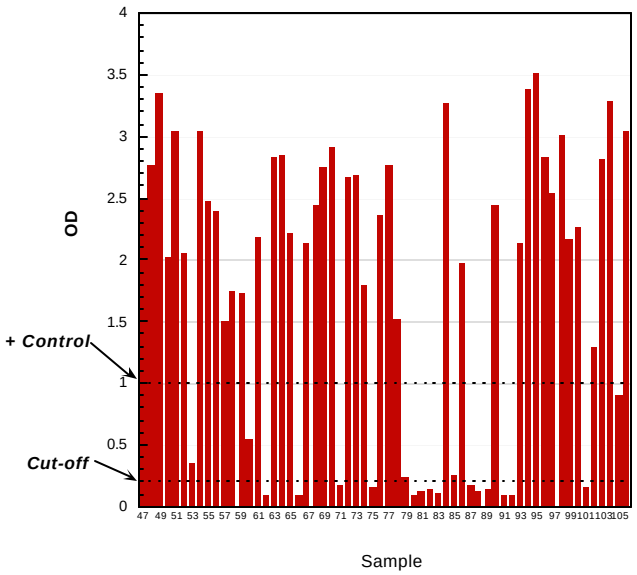
The most important thing to note is that 7 of 17 samples (marked with asterisks) tested yield inconsistent results that in practice means supplying presumably quantitative and accurate information when in fact that is not possible. This situation is worsened because in actual HIV antibody testing samples are never tested even in duplicate.

The second thing to notice is that although not giving inconsistent results (in terms of positivity or negativity, or crossing the cut-off), another 2 samples exhibit very high standard deviations.

These results, completely unexpected given the guidelines supplied by the manufacturer, imply that in order to quantitatively assay the cross-reactivity between presumed anti-HIV antibodies and antigens from other sources (*M. tuberculosis* in this case), we must first optimize the performance of the test to obtain results that are reproducible within acceptable bounds, ideally less than 5% variation from replicate to replicate -- see **Recommendations** (below) for details on how to address these problems experimentally.

C. Results from tuberculosis patients with unknown previous HIV antibody test results.

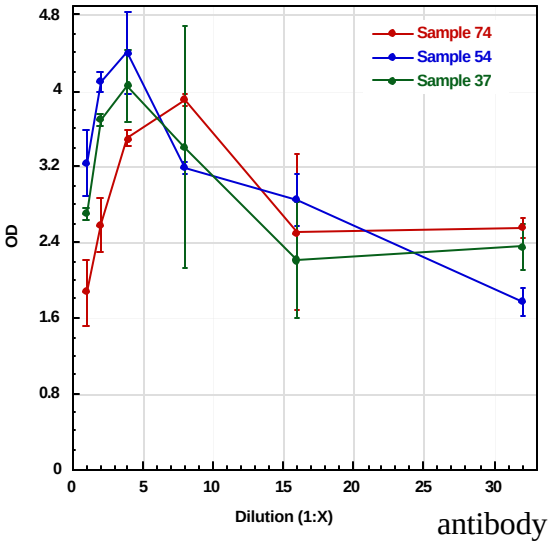
A portion (63 of 162, 39%) of the samples used in this study were obtained from tuberculosis patients who had never been tested for HIV antibodies. The results of the untreated HIV antibody test for 58 of those samples are shown below. Of these, only 14 turned out to be negative (24%), yielding a prevalence of 76% for HIV antibody$^+$ status for a random population of tuberculosis patients. This is considerably higher than the 50% HIV$^+$ status reported by official government sources.



These data are from the original preadsorption experiment and were done singly. A portion of these samples (those with reactivity below the positive control value) were included in the triplicate testing described above.

D. Linear range of the assay.

The non-linear response of the assay, for three samples with OD values of 1.5 or higher is shown in the following graph.



In this experiment, samples with high OD values were serially diluted (1:2, 1:4, 1:8, 1:16 and 1:32) in the sample diluent provided by the manufacturer of the HIV antibody test. The dilutions series were tested in duplicate and the average ± SD for each dilution is plotted against dilution factor.

If the response of the assay was linear, one would expect a drop in OD of half for each successive dilution, which is clearly not the case. It is precisely because of this non-linearity and the variation discussed above that the effect of preadsorption on the outcome of the HIV antibody test could not be measured.

Conclusions and Comment.

The preadsorption experiment. So far, we can draw no conclusions from the preadsorption experiments. As most of the samples are well above the reliable linear range of the spectrophotometer, changes in signal, although dramatic in some cases, are not significant.

For those samples around or below the positive control value (≈ 1), there is too much imprecision in the HIV antibody test itself to draw conclusions of any statistical significance.

The variability of the HIV antibody test. The variability observed warrants no extensive commentary save that, considering the importance for the life of the patient, the outcome of the test is of unacceptable precision, at least when done following the manufacturer's recommendations. The excessive variability in replicate testing of the samples can be addressed experimentally by adding additional steps in the procedure, beyond those claimed sufficient by the manufacturer. These might include clarification of the sera (by centrifugation) and possibly de-lipidization. As most of the samples gathered for this study fall out of the linear range of the spectrophotometer (where significant drops in antibody levels, if occurring, can be meaningfully measured), it may be necessary to normalize the samples by dilution prior to preadsorption to values in the vicinity of the positive control supplied by the manufacturer of the test kits.

The study must be made more rigorous by the inclusion of HIV antibody tests from other manufacturers, besides the one used in this study, currently in use in South Africa. The reasons are three: First, there may be other tests that perform, in terms of reproducibility,

better than the one evaluated here; second, that the same questions of specificity addressed in this study are applicable to other HIV antibody tests; finally, that the congruence of the test must be addressed not only internally (*intra*-assay variability, as we have done here), but also by comparing the same serum samples in different tests (*inter*-assay variability).

The prevalence. Of the TB+ samples collected, 63 were of unknown HIV antibody status. When tested, a very high prevalence of HIV antibody positives (nearing 80%) was found. This is much higher than that generally acknowledged (about 50%). Keeping in mind that the testing was done singly (as is common for medical purposes), this high prevalence may well reflect deficiencies in the assay. How many of these, especially the ones with low to mid-positive values, would score negative if re-tested? Also, it is suggestive of, although by no means demonstrating, a lack of specificity *viz* the antibody repertoire of at least some tuberculosis patients.

Linearity of the HIV antibody test. Although it could be argued that the high signals elicited by the majority of those samples that score positive are a proof of the high sensitivity of the test, in the absence of validation (that is, an alternative gold standard) this claim would have to be proven. If the test is indeed specific in the detection of anti HIV antibodies, it should remain so upon dilution of the samples to values around that of the positive controls. In fact, the positive controls themselves must be tested by preadsorption.

Recommendations.

In anticipation of continuing this work, just prior to leaving, in addition to communicating our gratitude to Prof. Lecatsas for his and his staff's considerable hospitality, we also inquired if he would be willing to host Dr. Stock for an extended stay in order to carry out properly the studies outlined above. He was quite forthcoming in his willingness to do so.

The experiments that we propose below, to be undertaken during a sabbatical period by Dr. Stock, can be categorized as follows.

The first part will address the questions of calibration, and intra and inter-asssay variability of tests commonly in use. Realistically this can be limited to 4: The automated Abbott AxSym system, the Biomerieux test used in the pilot study, the Sanofi-Pasteur kit with which we have some data from Mexico, and one additional test to be designated by the Ministry of Health of the 7-9 different tests currently in use in South Africa.

In these first experiments (expected to take 6 to 8 weeks) 100 HIV+ serum samples of at least 1 ml each are required. The TB status of the patients who donate their blood is not important. It is quite possible that we already have sufficient serum samples stored at MEDUNSA for this. Briefly these experiments would include sample treatments (centrifugal clarification and de-lipidization), use of different diluents and blocking

agents, and importantly different strategies for antigen immobilization and preadsorption, as well as a stringent quantization of the minimal attainable intra-assay variation for each test. Approximately 10-15, 96-well test plates of each type will be needed as well as an equivalent number of AxSym matrix modules. Importantly, these studies will also examine the question of whether there is any relationship between the outcome of these tests and total serum antibody levels, as has been reported in numerous scientific papers, although not for Africa. We will do so by measuring directly the total serum immunoglobulins by means of a commercially available, quantitative test.

The second set of experiments (which will take the remainder of the stay) will address the actual questions of the reliability of the tests in regard to specific detection of anti-HIV antibodies as set forth in Protocol 7.

These studies will utilize the best performing assay found in the work outlined above, and will require an additional 500 HIV+ samples and perhaps as many as 75 test plates (corresponding to roughly 7000 individual tests).

They will include a variety of antigenic preparations including, but not necessarily limited to, serveral Mycobacterial species, *Entamoeba*, adenovirus, HTLV, and *Pneumocystis*, for pre-test adsorption.

Since we will now be working in the linear range of the assay, the technical difficulties that possibly precluded the detection of lowered signals in the pilot study will no longer be present, and the important issue of the actual specificity of the HIV ELISA test can be properly addressed. In addition to the preadsorption procedures outlined in Protocol 7, we will also immobilize the preadsorbing antigens to cyanogen bromide-activated Sepharose beads, significantly increasing the amount of antibody that can be specifically removed prior to HIV ELISA testing. As calculated from stoichiometric considerations, the amount of antigen that can be bound on ELISA wells is in the nanogram range, whereas using covalent immobilization onto resin, we can increase the amount of antigen in contact with the serum sample to the microgram range.

Logistical and Material Considerations.

Samples: In the future it will be necessary for Dr. Ashgar to not only collect an additional sample set (described above) but to centrifuge the blood samples in their collection tubes, and transfer and freeze the sera prior to Dr. Stock's arrival and in coordination with him. Prof. Lecatsas will facilitate his working in the clinical virology unit along with one of the medical technologists to perform this simple operation perhaps two or three times per week for the time it will take to prepare all the other details. These samples should represent a variety of common clinical conditions such as pregnancy, amoebal dysentery, adeno viral infections, tuberculosis, and hypertension, among others.

HIV antibody tests: Since one of the tests to be examined is the automated Abbott AxSym system that can only be operated by specially trained personnel, we will require some time to be devoted to our test-runs by one of the medical technologists qualified to

operate the system in the laboratory in which we conducted the pilot study and in which we intend to work in the future.

Material requirements, in addition to the HIV tests enumerated above:

Micro centrifuge tubes (1.5 ml), approximately 10,000
Conical centrifuge tubes (15 ml), approximately 1000
Blood collection tubes (Vacutainer), approximately 1000
Serological pipettes (5 and 10 ml), several hundred
Micropipettes (10, 20, 200 and 1000 µl), one of each
ELISA plates (NUNC), 50-75
Cyanogen bromide-activated Sepharose, a few grams
Micro centrifuge, one
Multi-channel pipette (200 µl), one
Pipette tips (10, 200 and 1000 µl), total 20,000
Common reagents such as buffer components
Racks for 1.5 and 15 ml tubes, 20, 100 tube racks
Rubber gloves, 5 boxes
pH meter
Protein quantitation system (BCA-Pierce)
Immunoglobulin detection kits, 10, 96-well ELISA plates

The total approximate cost for all the material above is less than $20,000.00 US

Timetable.

The work proposed above is both technically complex and labor-intensive. Many thousand HIV antibody tests will be performed on variously-treated samples, and large amounts of numerical data will be generated. The time frame in which this will be done is therefore extremely sensitive to delays in administrative procedure, since this is an integrated system of experiments hard, if not impossible, to perform if any of the materials required are not available when needed. Additionally, it would be ideal if Dr. Stock could obtain a qualified technologist for 4 hours a day.

The work in South Africa will compose a part of the sabbatical year Dr. Stock will enjoy in 2004. He is already committed to some interesting but unrelated research in Senegal, and is willing to optimize his use of time by traveling between Pretoria and Dakar as necessary.

Once accomplished, these studies will represent the first scientifically sound validation of HIV antibody testing ever performed in Africa.

Acknowledgements.

We would like to thank: Dr. Nono Simelela for her perfect facilitation of all the logistical aspects of our pilot study; Dr. Junaid Ashgar who made the scientific aspects of the study

possible by his dedicated efforts at sample collection; Prof. Lecastas for his cordial and hospitable treatment, and finally, the medical technologists of the clinical laboratory, Rose, Stefina and Ethel, who educated us on how to perform the ELISA test we were investigating, and on the common practices in their laboratory, with good humor and grace.

Appendices:

1. Protocol 7
2. Photographs of the clinics (4 on one page with description only, no comment)
3. Documentation regarding authenticity etc of TB antigens
4. The experiment, photograph of samples post-adsorption and pre-testing and photograph of the apparatus.
5. Raw data, samples of machine printouts and our working templates
6. Raw data in Excel format