LABORATORY INVESTIGATION OF LOW POSITIVE AND DISCREPANT HIV SEROLOGY

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In Partial Fulfillment
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of Master of Medicine (Virological Pathology)

By
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Declaration by Lucia Hans

I hereby declare that I have read and am familiar with the current “MMed Part III: Guidelines for Candidates” and have complied with the instructions and stated conditions. The research manuscript, on which this dissertation is based, is my original work and neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. The manuscript is as yet unpublished.

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PART A: PROPOSAL

Title:

LABORATORY INVESTIGATION OF LOW POSITIVE AND DISCREPANT HIV SEROLOGY RESULTS

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INTRODUCTION

In our diagnostic virology laboratory, we test on average 1500 samples for HIV antibody/antigen each month, of which 0.6% produces problematic results. These problematic samples produce either weakly reactive screening and confirmatory results or, discrepant screening and confirmatory results. Both scenarios require additional tests to confirm HIV status thus increasing cost and turnaround time. There is a need to devise an optimal strategy within the laboratory to rapidly and easily manage these samples with minimal additional cost.

The WHO recommends three HIV testing strategies. Strategy I ensures blood transfusion safety while strategies II and III are used for both surveillance and diagnostics in high prevalence and low prevalence areas respectively.[1]The 2010 National antenatal sentinel HIV & syphilis prevalence study reported the South African HIV prevalence as 30.2%.[2] There were 1.8 million new cases of HIV infection in Sub-Saharan Africa in 2011.[3]South Africa (SA) is a high prevalence country and therefore the national HIV testing guideline is based on strategy II. The HIV screening and confirmatory strategy at Groote Schuur is based on these recommendations.

Strategy I requires that only one highly sensitive test be performed and if the result is positive, the blood donation is discarded. Strategy II requires that an initial positive HIV test performed using either a rapid HIV test or ELISA be confirmed with a second rapid test or ELISA. This strategy is recommended when testing asymptomatic patients in areas with a prevalence of greater than 10%; and symptomatic patients in areas with a prevalence of less the 30%.[1] Strategy III requires a third positive test to confirm HIV positivity in low prevalence areas.

On multiple occasions low positive and indeterminate HIV results of unknown significance are obtained. The WHO guidelines do not provide guidance on resolving/clarifying such problematic results. This project aims to characterize these low positive and indeterminate results in order

to determine the scenarios in which false positives not requiring further tests and true positives requiring further tests are more likely to occur. Some of these results are sent out as discrepant but likely false positive results as per national guideline and standard laboratory practice. However poor follow -up and repeat testing of the patients as requested by the laboratory, have made it difficult to determine which results were true false positives. False positive serology results occur when cross reactivity between patient antibody and antigen other than the source antigen occurs. This may occur in cases of immature immune systems, other infections and autoimmune diseases. Published clinical scenarios in which false positive HIV antibody results are produced are pregnancy, hypergammaglobulinemia[4],tuberculosis, vaccination against influenza, receipt of tetanus immune globulin[4] and systemic lupus erythematosis.[5]Reporting false positive results have far-reaching negative repercussions not only on the patient's psyche but also impacts clinical practice and health systems. [6]

It is essential that we gain more insight into determining the significance of low positive HIV results in the different laboratory scenarios as this will guide pathologists in deciding whether more tests are required and, if so, which test will be the best choice. There are three additional tests, viz. the HIV viral load, the p24 immunoassay and the western blot, that can assist in confirming HIV status. These tests differ in assay principle, cost to patient (or the National Department of health) and duration of time required to perform the test. The HIV viral load costs the laboratory R89.76 per sample and the p24 assay costs R205.57 per sample to test. The DOH is charged R 155.65 per p24 and R 293.86 per HIV viral load test performed. An additional test request will add 24 hours to the time taken to produce an authorised result. By determining the most appropriate additional confirmatory test, the cost to patient will be decreased, as unnecessary test requests will be reduced. Turnaround time required to release authorized results will also be reduced. Ultimately the confirmations of low HIV tests will be streamlined allowing the laboratory to function more effectively.

AIM

To establish a validated supplementary HIV testing protocol for the management of low positive HIV serology and discrepant screening / confirmatory HIV serology.

METHODS

The study will be a retrospective laboratory review. A search will be performed on the laboratory tool, the WSDisa database from 01 June 2012 to 31 July 2013 for all HIV screening serology results. The search will include all patients who had HIV screening tests performed at the Groote Schuur Virology laboratory during the period of 01 June 2012 to 15 June 2013. This includes patients who had samples referred to the laboratory from other institutions for resolution of discrepant results.

Inclusion Criteria

- Samples screened using the ARCHITECT (®) HIV Ag/Ab Combo assay with a result of less than 20 S/CO (signal/cut off ratio) and,
- Had an OD result of less than 2.0 when confirmed using the Enzygnost® Anti-HIV 1/2 plus assay

In order to determine the true serological status of these patients, additional tests will be performed on the low positive samples based on the inclusions. The additional tests will be the Abbott RealTime® HIV-1 assay (viral load), the VIDAS®HIV P24 II (p24 antigen) and the BIO-

RAD GS HIV-1 Western Blot. All included samples will have a p24 antigen and HIV viral load test performed. Western blot tests will only be performed on samples with positive Enzygnost confirmatory tests since no readable result can be expected on WB if antibody levels are below the Enzygnost cut-off. The HIV viral load will be performed, as viral RNA is the earliest detectable marker of HIV infection and thus will confirm positivity in patients with low positive serology in early infection where p24 antigen is not detectable yet or has been missed by the ARCHITECT (®) HIV Ag/Ab Combo assay related second window period (see assay description below).[7] The p24 antigen assay will be performed in order to determine the scenarios in which the low positive screening result is a reflection of p24 antigen being produced. The western blot test will determine HIV status in cases of low positive screening and low positive confirmatory tests in order to exclude false positive results because of non-specific cross reactivity.

Assays

ARCHITECT (®) HIV Ag/Ab Combo assay

This is a fourth generation HIV assay that detects the presence of the HIV p24 antigen as well as antibodies to HIV -1 and HIV -2 in human plasma and serum using chemiluminescent technology with the resultant reaction measured in relative light units[8]. A signal to cut-off ratio of equal or greater to one is regarded as positive. A ratio of less than one is negative. We regard samples lower than 20 S/CO as low positive. There is a possibility of a second window period when using the fourth generation assay at a point when the detectable p24 antigen decreases to below the limit of detection of the assay and antibody is not yet detected.

Enzygnost® Anti-HIV 1/2 plus

This is an immunoassay that detects antibodies to antigen of HIV-1 and -2 bound to a microtitre plate. Enzyme bound conjugate binds to the bound antibody. The enzymatic activity of the bound conjugate results in a colour change when a chromogen is added, the intensity of which is proportional to the concentration of antibody present[9]. The results are reported as optic density (O.D.)

Abbott Realtime® HIV-1 assay

The RealTime® HIV-1 assay (Abbott Molecular, Des Plaines, IL) is real time PCR assay that detects HIV viral load in plasma samples. The assay is able to quantify virus over a range of 40 to 10^7 RNA copies per ml.[10]

VIDAS®HIV P24 II

This immunoassay will be used to detect p24 antigen that is detectable in infected blood as early as three weeks post infection. The antigen becomes undetectable as soon as all the p24 form complexes with antibody and therefore is a measure of acute infection. This is a sandwich immunoassay with the antigen measured by fluorescence detection.[11]

BIO-RAD GS HIV-1 Western Blot

This is a qualitative assay that detects and identifies antibodies to HIV in human serum and plasma. HIV-1 specific antibody binds to inactivated HIV antigen fixed onto nitrocellulose sheets. A colour change reaction occurs allowing for the identification of bound antibody.[12] The position and intensity of the bands are compared to reference strips. We will be using WHO criteria to determine results.

STATISTICS

For the sample size calculation, we assume that the information provided by current screening and confirmatory tests alone is most likely able to correctly identify the HIV status of the patients with discrepant or low positive results in 70% cases. With the addition of HIV viral load, p24 and Western Blot tests proposed in this study we would be able to correctly identify the HIV diagnosis 90% of the time. In order to reliably demonstrate this improvement 92 samples are required.

Continuous variables will be summarized in median and interquartile range while categorical variables will be analysed as proportions/percentages with 95% confidence intervals. The sensitivity and specificity of each assay/combination of assays will be assessed and positive and negative predictive values will be determined. Chi-square test will be performed to determine the significance of difference between various testing methods and Receiver Operator Characteristics (ROC) curve analysis will be performed to determine the optimal cut-off for screening HIV ELISA that would predict a true positive result.

ETHICS

This study will take the form of a laboratory review. All patient results and records will be treated as confidential by the investigator. Submission of this study to both the Human Research Ethics Committee and the Departmental Research Committee of Department of Clinical Laboratory Sciences at the University of Cape Town will be made. The study will be performed in accordance with the Declaration of Helsinki.

This investigation will be primarily retrospective and therefore will pose not any risk to the patient. All investigations done contribute to the patient diagnosis and management. Results reported as discordant as per the national guideline may require additional tests in order to

complete the validation. In the event that the new results are contrary to the previously released results, the managing clinician/team will be contacted and amended results will be released, as is standard laboratory practice for any retrospectively discovered erroneous laboratory result.

OUTPUT

This project will be of practical use in the laboratory for the following reasons:

- Decisions made to perform additional tests will be based on laboratory based evidence rather than the pathologist's judgment.
- Teaching registrars and technologists about HIV confirmation of low positive HIV results will be based on laboratory based evidence
- Cost of confirming HIV status and turnaround time required to produce authorized results for low positive HIV screening results will be reduced.

This project will be of value to other virology laboratories and clinicians as it will contribute to the management of low positive HIV results and will therefore be written up for publication.

Word count: 2003

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APPENDIX

ESTIMATED BUDGET (As per cost to laboratory)

TEST	COST (R)	NUMBER	TOTAL (R)
p24	85	20	1700
HIV viral	90	20	1800
Western blot	275	20	5500

PART B: LITERATURE REVIEW

Objectives of literature review

Since the introduction of HIV diagnostic tests 1985, great technical advancement has occurred in this field necessitating the updating of HIV testing algorithms. The objective of this literature review was to track the evolution of diagnostic HIV assays and show how new developments affected the local and international diagnostic HIV screening and confirmatory guidelines.

Literature search strategy

Electronic databases as well as the Google search engine were used to conduct the literature search. The databases were PubMed and Google Scholar. The search terms used were HIV, human immunodeficiency virus, diagnostic tests, antibody antigen, viral load, western blot, discrepant or indeterminate HIV results, false positive, false negative, sensitivity, and specificity. More specific terms e.g. 4th generation ELISA, were used when a more detailed search was required. Articles from 1980 to 2013 were included as the historical perspective of HIV testing was reviewed as part of the manuscript. International and local data were included in the review and the references of citations were studied in order identify additional data. Citations were limited to English language and human studies.

Summary and interpretation of literature, and its implications for the research

HIV diagnostic assays and its associated problems

Antibody Tests

Commercial HIV diagnostic tests first became available in 1985 (Chappel et al. 2009). Four generations of assays have been developed, the first 3 antibody based and the fourth antigen/antibody based (Murphy & Aitken 2011). Chappell et al. (2009) regarded the desire to increase sensitivity and reduce the window period for detection of antibodies as the primary motivation for the upgrade of these assays and also commented on the unacceptably high false positive rate. Naturally the rate of false positives produced is also dependent on the prevalence of the infection in the local area i.e. the lower the prevalence of disease, the lower the positive predictive value and therefore the higher rate of false positives (Kim et al. 2010; WHO 1997). This motivated the confirmation of positive screening results with a highly specific assay. The 1st and 2nd generation tests detected only IgG, the1st generation relying on crude or purified lysate and 2nd using synthetic polypeptide or carefully selected antigen produced by DNA technology (Murphy & Aitken 2011). Third generation assays were introduced in attempt to increase sensitivity and represented a change ELISA principle. These are sandwich assays, the plate well of which is coated with recombinant or synthetic peptides to which anti-HIV IgM and IgG bind. An enzyme linked to an HIV-antigen that binds to both anti-HIV IgG and IgM is added, a colour change reaction follows the addition of a substrate bound to an enzyme, and this shows that both IgM and IgG are detected. According to Daskalakis (2011), the detection of both the IgM and IgG reduces the window period by detecting low levels of antibody produced.

ELISA tests may produce false positive results. Clinical conditions in which this can occur includes recent influenza vaccination, viral infections, autoimmune disease, renal failure and pregnancy and are thought to be caused by cross reacting antibodies (Mahajan et al. 2010; Erickson et al. 2006). Molecular mimicry may also

have a role to play as Bettaieb et al.(1996) demonstrated that there was cross reaction between gp129 and CD61 (platelet gpIIIa).

Antigen/Antibody Assays

The development of the 4th generation assay was again motivated by the desire to increase sensitivity and reduce the window period but in this case by detecting the p24 antigen which is detectable early in infection when not complexed to antibody (Weber et al. 1998).This assay is thus able to detect acute infection as well as established infection by detection of antibodies produced (Brennan et al. 2013). Unfortunately a disadvantage of the assay is that the result produced does not distinguish between the antigen and antibody component, and therefore requires additional steps for confirmation of either acute or established infection i.e. initially a p24 only assay is performed to confirm acute infection; if negative, a 2nd or 3rd generation assay needs to be performed to confirm established infection (Weber et al. 2002).In order to shorten the confirmation strategy, the development of a 5th generation assay should be considered which is able to differentiate between antigen and antibody. Another disadvantage is the potential risk of a second window period. A third of the binding capacity in these assays is reserved for antibody binding so there is the potential for reduced sensitivity of the p24 component (Gürtler et al. 1998; Weber B, Meier T, 2002). In cases where there is an absence of detectable antibody and antigen the result may then be reported as falsely negative. A low positive result may be reported when minimal antibody or antigen is detected, and thereby creating the incorrect suspicion of a false positive result. There has to be a high index of suspicion of primary infection in cases where acute infection is clinically suspected but the 4th generation result is negative, or in the case of low positive results,3rd generation confirmatory assay is negative.

P24 Antigen Assay

Another enzyme immunoassay based test is the p24 monoassay (Fearon 2005). These standalone assays were developed in order to detect acute infections by detecting antigen in the era of antibody only assays (Chappel et al. 2009). Barletta et al. (2004) commented that the p24 assays are relatively insensitive and in some cases detecting as little as 50% of acute infection. They considered the assay of limited clinical utility (Barletta et al. 2004). Indeed, if the aim of performing the assay is to detect acute infection, 50% detection rate is poor. In the early days of 4th generation assay there was still some concern that the p24 component of these assay lacked sensitivity and were therefore regarded as unsuitable to replace the immunoassay. However newer 4th generation versions have shown improved sensitivity and are recommended instead of standalone assay (Ly et al. 2007).

Rapid Tests

Rapid HIV tests were introduced in order to make HIV tests more accessible and thereby identify and treat patients earlier (CDC 2003). Rapid tests are useful as the assays are disposable single use assays, can be stored at temperatures above $4^{o}C$, contain all the necessary reagents required to produce a valid results and are able to do so in less than 30 minutes (Branson 2007). They can therefore be used in a variety of locations including those without refrigeration. The rapid diagnostic assays are able to detect HIV using different sample types namely oral fluid, whole blood, plasma or serum (Daskalakis 2011) These assays are based on lateral flow, agglutination, or most recently, immunochromatographic principles (Constantine & Zink 2005). Situations where these assays are of particular benefit include occupational cases where post exposure prophylaxis can be initiated quickly, and women in labour where prevention of mother to child transmission can be initiated immediately (Constantine & Zink 2005). However these assays also result in new challenges as they are most often performed outside the laboratory environment,

performed by people with minimal laboratory skills, may perform the assay haphazardly as they become used to the simple procedure, and the interpretation of the results are subjective dependent on the means of detection, i.e. colour change or agglutination. This is evidenced by the findings of a study of the voluntary counselling and testing practices in primary health care clinics in South Africa that was performed by the SEAD group (SEAD 2011). They found multiple areas of concern, including inconsistent or absent quality assurance, specimen collection method (no further detail given) and shortened incubation time for result. The subjectivity of interpreting the colour change on the strip of the Determine HIV-1/2/O (Abbott Laboratories, Abbott Park, IL) resulted increased in increased false positive results (Gray et al. 2007). Kilembe et al. (2012) tested a rapid antigen/antigen test but found that only 23%of acute infections were detected.

In 1992 the WHO recommended that countries consider using rapid tests as part of their testing strategy as, according to their weekly epidemiological record, the reliability of these results match or better those of commonly used ELISA and western blot (WHO 1992). This recommendation was reiterated in the 1997 recommendation (WHO 1997). By 2007, the FDA had approved six rapid diagnostic test with both sensitivity and specificity greater 99% thereby supporting the WHO's recommendation of using rapids test instead of ELISA tests (Branson 2007). However there have been findings published that recommend that all rapid tests be confirmed by conventional immunoassays (Fearon 2005). Pavie et al. performed a study published in 2010 which compared the sensitivities of five rapid tests used in hospital outpatient setting and found that assays using oral fluid had less sensitivity than fingerprick whole blood but that serum was more sensitive than the fingerprick whole blood. These findings confirm that these tests need to be validated in local settings.

Western Blot

The western blot assay has long been a mainstay of HIV diagnostic testing, however sensitivity is poor compared to the newer assays. In comparison to the 4th generation assay (2.5 - 3 weeks) and 3rd generation assay (3-4 weeks), the western blot detects antibody at 5 weeks (Daskalakis 2011). The western blot was widely regarded as the gold standard assay for confirmation of the HIV positive screening results (Constantine & Zink 2005). This assay formed part of the first HIV testing recommendations introduced by the CDC in 1997as the confirmation assay following 2 positive enzyme immunoassays (CDC 1997). However the sensitivity of this assay was brought into question as early as 1992, when the WHO recommended that this assay be replaced in the HIV screening strategies as the combination of two different ELISA and/or rapid test was found to have superior sensitivity to the enzyme immunoassay and western blot combination. (WHO 1992; WHO 1997).

Besides relative insensitivity, the other issue facing this assay is that of indeterminate results. Different criteria for the interpretation of the HIV western blot assays have been proposed by various groups (CDC 1989) . Most criteria require reactivity to at least one envelope and one core protein, and any other non-specific bands are interpreted as indeterminate (Constantine & Zink 2005). There are several causes of, and conditions associated with indeterminate western blot results. These include early HIV infection, advanced AIDS, other retroviral infection, autoimmune disorder like systemic lupus erythematosus, kit design issues and assay process (Guan 2007). In an attempt to improve on the unacceptably high indeterminate western blot results, Mahé et al. (2002) published diagnostic criteria for the African context consisting only of the gp160 and p31 bands that correctly classified 96% of samples. The possible production of indeterminate results; the time and labour intensive nature of the assay; the risk of cross-reaction between HIV-1 and -2 (Cárdenas et al. 2013),

and in the face of more accurate assays, this assay should no longer be deemed worthy of the gold standard title.

HIV Viral Load Assays

HIV viral load tests were introduced in the 1990s, initially as a research tool. Monitoring of HIV viral loads has now become routine in clinical practice (Mylonakis et al. 2001). Viral load assessment has been shown to be one of the best predictors of clinical progression of the infection as well as a measure of the effectiveness of antiretroviral drug use (Mendoza & Soriano 2014).In the past use assay was limited to the abovementioned situations (Fearon 2005). However focus has shifted to the role of RNA detection in the diagnosis of acute infection, and in confirmation following positive screening results. Acute HIV infection is defined as the time from HIV acquisition until seroconversion. The initial entry of the virus is followed by an eclipse phase of approximately 10 days when infection is not detectable by current assays (Cohen et al. 2010). HIV RNA is detectable from approximately one and a half weeks following infection by current nucleic acid tests while the current 4th generation ELISA assays are able to detect p24 antigen from two and half to three weeks post infection (Daskalakis 2011). It has been documented Fiebig et al. that the p24 antigen only becomes detectable when the HIV viral load is 10 000 RNA copies/ml (Fiebig et al. 2003). Therefore there is a valuable period of time in which the infected patient is highly viraemic, risking spread of virus, and potentially could be treated but may be missed as the 4th generation screening result will most likely be falsely positive (Cohen et al. 2010). Brennan et al. (2013) recently published a study correlating the p24 sensitivity of ARCHITECT HIV Ag/Ab Combo assay with the RNA viral load. They found that the p24 antigen is detectable once the viral load has reached 58 000 copies/ml. While this study had several limitations, in particular the use of supernatant of HIV infected cultures cells, it does point out that some infections may be missed as a result of assay limitations. Naturally these findings

need confirmation using human samples. The HIV viral load has now been added to the latest CDC screening algorithm with western blot confirmation no longer a feature (CDC 2012; Branson 2010).

HIV diagnostic algorithms

It must be noted that it is impossible for a single algorithm to the cover all the scenarios that may occur in all settings and patients groups and therefore it is important to take the local setting into account when deciding upon a strategy.(Murphy & Aitken 2011).

WHO Recommendations

In 1992, the WHO released recommendations for the selection and use of HIV antibody tests in its Weekly Epidemiological Record.(WHO 1992) This record clearly sets out the objectives of HIV antibody testing. The objectives were 1) screening of blood and blood and blood product for transfusion safety, 2) the monitoring of HIV infection trends and prevalence for surveillance, 3) diagnosis of HIV testing, and 4) research for various HIV related studies. This document proposes strategies for HIV testing determined by the objectives of the test. By elegantly explaining the role of sensitivity, specificity and prevalence when choosing an assay, one is clearly able to understand how the strategies where decided upon. The three strategies of HIV testing proposed in this document are a) serum tested with one ELISA or rapid assay is considered positive or negative based on the result of this one test, b) all serum is tested with a rapid or ELISA test, and reactive serum is retested using a second ELISA or rapid test based on a different antigen preparation or test principle, and) similar to strategy II except that a third test is required to confirm serum positivity. The positive predictive value of a test will be low in a low prevalence setting and therefore a supplemental test is required to confirm results. These strategies form the

basis of the various international HIV testing algorithms. This document also discussed the relevance of equivocal results, recommending that a second blood sample is required a minimum of two weeks later and retested using the appropriate testing strategy. This is probably the origin of the clinical and laboratory practice of asking for a repeat specimen following the reporting of equivocal or indeterminate results. In my experience, this is not a good strategy as patients in our setting are often lost to follow up and the requested follow-up sample is never sent.

The WHO updated this record in 1997 following the expansion of available antibody tests available (WHO 1997). Minor modifications were made to the three strategies previously recommended. This document clearly states that test combinations should be evaluated in the context where they will be used before wide-scale implementation. The preference for ELISA or rapid tests instead of western blot assay is reiterated with reduced sensitivity and cost being the motivating factors for this choice. Clear recommendations are made for the appropriate testing strategy for the local prevalence of infection. A diagrammatic version of the strategies suggested in the previous record clearly sets out the algorithm for HIV testing.

CDC Recommendations

The initial CDC recommendation for HIV testing considered a sample HIV positive if it had two reactive enzyme immunoassay results and a positive confirmation on western blot (CDC 1993). This recommendation was based on the 99% sensitivity of the enzyme immunoassays (EIAs) and 99% specificity on repeated EIAs in use at that time. The western blot assay had comparable sensitivity and high specificity when strict criteria determining result status was used (CDC 1993). Although the CDC presented revised guidelines in 2006, there was no change in testing strategy recommended but rather an attempt to increase the rate of earlier diagnosis by expanding testing to all health care settings, screening more people, employing

voluntary opt-out testing and removing the requirement for prevention counseling. It was only when the FDA approved the 4th generation assay and more sensitive rapid tests that differentiated between HIV-1 and -2 were developed, that a change in the actual testing strategy was proposed (Ginocchio et al. 2011). The new recommended testing algorithm includes screening all samples with a 4th generation assay or if not available, a 3rd generation assay. If the result is negative it is accepted as negative, but if positive the sample needs to be tested using an point of care assay that differentiates between HIV -1 and HIV-2,viz. the Bio-Rad Multispot® Rapid HIV-1/HIV-2 Test, the only FDA approved test currently (Torian et al. 2011). If that result if negative or indeterminate, an HIV RNA test is required (CDC 2012). These new recommendations no longer include the western blot assay as confirmatory assay and do not include the standalone p24 immunoassay. The emphasis of this algorithm is on high sensitivity at screening so that early and established infections are not missed as the risk of rare false positive results that will be resolved by further testing (CDC 2012). See appendix B for algorithm.

A cost and outcomes study comparing the previous recommended CDC HIV diagnostic algorithm and the newer one (Hutchinson et al. 2013). They found that for specimens that were HIV positive, the lab costs for the newer algorithm were cheaper than the preceding algorithm in general which they attributed to the high cost of the western blot assay recommended in the earlier assay. As the new guidelines recommended 3rd or 4th generation screening assay, the cost of using 4th generation assays was more expensive but detected 10 / 50 000 more infections. Therefore they believed the extra cost may be justified. In the South Africa context, where the prevalence and incidence are higher, use of a 4th generation assay and RNA viral load detection would be justified in terms of cost particularly regarding timeous diagnosis of infection, and of preventing the spread of new infections by means of early diagnosis.. Minimal comment is made by Hutchinson et al. about the

use of the high cost viral load in indeterminate cases. It may be too soon after the introduction of the new recommendations and therefore the sample size is not yet large enough. Perhaps it's too early following its introduction and therefore the sample size is not large enough. In light of this new algorithm, further studies will now be possible to determine the cost-effectives of using RNA tests in the diagnosis of HIV.

UK Recommendations

The Health Protection Agency in the United Kingdom (UK) similarly recommends that samples are screened by a 4th generation assay; positive samples undergo a second test by the same assay or one of equivalent sensitivity. A second sample is requested for final confirmation but no mention of assay type is made. Negative samples are managed according to the clinical history i.e. if symptomatic, repeat in a week; if not regular screening recommended. Indeterminate samples are managed according to local policy (Health Protection Agency 2012). The UK algorithm does not recommend HIV viral load directly but it seems as though there is an opening for it be used under the umbrella of local guidelines. See appendix C for algorithm.

South African Recommendations

The South African National HIV counselling and testing policy guidelines are based the WHO recommendations and include screening with rapid tests and confirmation of a positive result with a second rapid test. Indeterminate results are referred for ELISA at the local laboratory. If that result is positive, the sample is reported as positive and if the result is negative, it is reported as negative (National Department of Health 2010). No provision is made for samples that might be indeterminate or discrepant in a district or academic laboratory setting where 4th generation, p24 immunoassay and HIV viral load testing is available. See appendix D for algorithm.

Identification of gaps or needs for further research

Currently the most sensitive ELISA assay is the 4th generation assay but there is a need for a 5th generation ELISA assay that will be able to differentiate between antigen and antibody detected. This will simplify the strategy for confirmation of infection by showing detecting whether it's an acute or established infection at screening. Once the validity of the new algorithm is assured, it will then be possible to create a protocol for confirmation with an HIV viral load for acute infection and an antibody confirmation assay for the antibody positive samples.

There is a paucity of local publications on the validation of the 4th generation ELISA assays in the South African population. It will be useful to know the rate of false positive and false negative results of these assays, particularly as the prevalence of HIV is the highest in our setting and every attempt needs to be made to detect acute infection in order to stem the tide of new infections. Removal of the potential second window period, that may produce false negative results, is therefore of clinical importance. It will also be useful to obtain an estimate of what the 4th generation ELISA S/CO cut-off is for samples that are more likely to be falsely positive as this will reduce the costs as less unnecessary tests will be performed. A biological window when infection is not detectable will always exist this should be kept in mind. The proposed new research can then be used to make recommendations on strategies to deal with low positive or discrepant serology results, as there is a lack of provision for what should be done in these cases in the South African guidelines.

More publications are necessary on the utility of the standalone p24 assay particularly in terms of sensitivity in our populations and whether performing a HIV

viral load on all the samples that are antibody negative on confirmation will be cost effective and of clinically utility.

Screening for HIV-2 is not part of the South African guidelines and studies are required to determine the prevalence particularly as migration from rest of Africa to South Africa continues. High prevalence of HIV-2 infection is a potential reason for discrepant HIV diagnostic test results as the 4th generation assay is able to detect but not differentiate between the two virus while current molecular method only detect HIV -1. A high prevalence of HIV-2 will motivate for inclusion of HIV-1 and HIV-2 differentiation in the South African national guidelines.

Word count: 3834

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PART C: MANUSCRIPT

Title:

LABORATORY INVESTIGATION OF LOW POSITIVE AND DISCREPANT HIV SEROLOGY RESULTS

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Abstract

Weakly reactive or low positive and discrepant HIV serology results across different platforms are common diagnostic dilemmas in virology laboratories. The HIV viral load, p24 immunoassay and western blot are among tests performed to ascertain the true HIV status of problematic samples, but the lack of local standardised algorithms means that resolving these dilemmas is resource intensive. This study aimed to refine our HIV testing algorithm, through examining the performance of each of the adjunct HIV diagnostic assays in our routine laboratory setting. Our objective was to minimise cost and delay in providing the best possible result to the patient. Of the 48 weakly reactive samples examined, 24 (50%) were found to be true positive. The routine 3rd generation confirmatory assay lacked both sensitivity (72%) and specificity (91%) in this scenario. The p24 assay lacked sensitivity (37%) while the HIV viral load assay showed excellent sensitivity (100%) and specificity (100%). ROC analysis suggests by using low and high cut-offs, one can achieve good sensitivity or specificity respectively, thereby informing the correct subsequent testing strategy. The poor sensitivity of the p24 and the limited utility of western blot assay points toward using HIV viral load as the optimal diagnostic tool when confirming weakly reactive HIV serology.

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Key words: HIV, serology, screening, discrepant, true, positive

AUTHORS' CONTRIBUTIONS

Student: Lucia Hans

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Co-supervisor : Marvin Hsiao

1. Introduction

The diagnostic virology laboratory at Groote Schuur Hospital uses a fourth generation HIV ELISA assay to screen on average 1500 samples for HIV antigen/antibody monthly. Approximately 0.6% of the samples are either weakly reactive or have discrepant confirmatory results. These scenarios are diagnostic dilemmas for both pathologist and clinician and require additional tests to be performed in order to confirm HIV status. The increased turnaround time and cost to the patient or government necessitates an optimised strategy within the laboratory to rapidly and easily manage these samples with minimal additional cost. Current practice involves screening with a 4th generation ELISA assay and then if positive, confirmation with a 3rd generation ELISA assay. The screening 4th generation ELISA and confirmatory 3rd generation can sometimes produce low positive and discrepant results. In this scenario the individual pathologist relies on their experience to determine if an additional test is required and if so, which test to request.

Currently it takes 6 working hours to produce a positive HIV serology result and the cost to client is R99.36. If additional tests are required, the turnaround time can increase by at least one working day to more than a week dependent on the tests requested; and the cost can increase from an additional R161.88 (p24 test) to as much as R1145.27 (p24 + HIV viral load + western blot). See Table.1 below for costs of tests to the Department of Health (DoH). The specifications of the tests and their role in HIV testing will be described below.

Test	Cost to Department of Health
HIV 4th gen EIA	R 49.68
HIV 3rd gen EIA	R 49.68
HIV p24 EIA	R 161.88
HIV viral load	R 305.61
HIV WB	R 578.42

Table.1 Cost of tests to the Department of Health (Source: NHLS price list 7 February 2014).

In an attempt to streamline HIV diagnostic testing internationally, the WHO in 1997, recommended three HIV screening strategies (WHO 1997). The recommendations are

currently still in place. The choice of strategy is dependent on the objective of the test. Strategy I ensures blood transfusion safety and is recommended in symptomatic patients in settings where the prevalence is greater than 30% while strategies II and III are recommended for both surveillance and diagnostics in high prevalence and low prevalence areas respectively (WHO 1997). South Africa currently follows the strategy II recommendations as it is regarded as high prevalence country. As of mid-2013, HIV prevalence in South Africa was 10.0% with certain sectors of the population having a prevalence as high as 17.4 % (women aged 15-49) (Statisitics South Africa 2013). However the national prevalence was reported as much higher among antenatal women at 24.9% (95% CI: 28.7% -30.2%) in 2009 (National Department of Health 2010).

Strategy I requires that blood donation specimens be discarded after a single positive result is produced using a highly sensitive assay. Strategy III requires a third positive test to confirm HIV positivity in low prevalence areas. Strategy II requires that an initial HIV screen is performed and if positive, confirmed with another rapid HIV test or ELISA. This strategy is recommended for use in countries with greater than 10% prevalence in asymptomatic patients, and equal to or less than 30% in symptomatic patients. The South African recommended serial HIV testing algorithm is based on this strategy (see Appendix D) and hence the screening and confirmatory strategy used at Groote Schuur Hospital is based on these guidelines (National Department of Health 2010). These guidelines make provision for discrepancies between the initial two tests which are usually rapid tests performed at local clinics, by recommending that those samples be sent to laboratories for ELISA tests but does not make provision for discrepancies or indeterminate results following ELISA tests in those laboratories.

The CDC introduced new guidelines in 2010, which recommends screening with a 4th generation assay, followed by discrimination of HIV-1 and -2 for reactive samples, with confirmation of indeterminate results with HIV viral load (Branson & Mermin 2011; CDC

2012). The development of the new algorithm was motivated by the lack of sensitivity of the p24 assays and western blot as well as the increased sensitivity and availability of the HIV viral load assays (Murphy & Aitken 2011).

When cases of low positive or indeterminate results of unknown significance are obtained there isn't any clarity within the WHO guidelines or within the South African testing algorithm on how to manage the situation. The aim of this study was to determine how to differentiate between true and false positives, in order to guide laboratory staff in decision-making. Additionally, the study aimed to determine whether the semiquantative 3rd generation ELISA optic density result had bearing on a true positive result as well as whether any particular test stood out as the optimal test to be used consistently in an algorithm aimed at determining true HIV status.

HIV diagnostic tests are currently serology based tests detecting antibody or antigen and molecular tests detecting viral nucleic acid. These tests were first introduced in 1985 and evolving technologies have led to great advances (Constantine & Zink 2005). In our setting we use a 4th generation HIV ELISA assay which detects both early appearing p24 antigen and antibody, reducing the window period and allowing for detection of early and established infections (Chavez et al. 2011). The assay does not differentiate between the antigen and the antibody (Cohen et al. 2010). There is a rare phenomenon reported for this assay, namely the second window period, during which the p24 antigen is no longer detectable having complexed with antibody and the free antibody is still below the lower of detection of the assay, potentially allowing for false positive results (Meier et al. 2001; Speers et al. 2005). The advantage of using a 3rd generation ELISA as the confirmatory assay is that negative results may indicate an acute infection where only p24 was detected in the 4th generation screening assay. This early infection scenario therefore requires additional confirmation with the p24 mono-immunoassay, or subsequent samples to demonstrate seroconversion. The p24 assay, however, has been documented as having inferior

sensitivity compared to HIV RNA detection, risking false negative results. Therefore the HIV viral load assay, although not licensed for HIV diagnosis, is used to confirm acute cases of HIV when antibody is undetectable and the viral load high (Cohen et al. 2010). Additionally, pathologists use the ability of the assay to detect RNA as a diagnostic test in the diagnostic dilemmas described above. In these cases the qualitative result is interpreted as positive(pos), negative (neg) or indeterminate, dependent on the range of the numerical result (neg being lower than detectable levels, indeterminate being greater than LDL but less the 1000 RNA copies/ml, pos greater or equal to 1000 RNA copies/ml). Naturally the implications of false negative tests are immense as this will result in delayed access to care with the resulting health implications and unwitting spread of disease to partners and children. HIV viral load testing is also used to confirm established infection as this assay is licensed for routine HIV viral load measurement in response to antiretroviral therapy. The previous gold standard for HIV confirmation is the western blot assay which fell out favour because of the superior sensitivity and specificity of the newer screening tests (Murphy & Aitken 2011). This assay is still used in our setting when serology and molecular tests remain inconclusive.

It is the responsibility of the diagnostic laboratory to best determine whether positive results are true as misinterpretation can lead to incorrect clinical management decisions and psychological harm. False positive serology can result when cross reactivity occurs i.e. when non-specific binding occurs between the antigen used in the assay and proteins (other than the required HIV antibody) in the patient's serum (Tate & Ward 2004). The clinical causes of false positive HIV serology include recent influenza vaccination, incidental viral infections, autoimmune disease, renal failure, cystic fibrosis, multiple pregnancies, blood transfusions, liver disease, intravenous drug abuse, haemodialysis, or vaccinations against hepatitis B and rabies (Mahajan et al. 2010; Erickson et al. 2006). It is therefore imperative that clinical history is taken into account when deciding on the validity of a result. The negative repercussions of reporting false positive results on the patient's psyche and health, as well at

the impact on clinical practice and health systems, should not be underestimated. In a study performed by Médecins Sans Frontières, 47/326 (14%) people were found to be erroneously diagnosed and the average time spent on antiretroviral therapy by those misdiagnosed was 484 days (Shanks et al. 2013). The aforementioned study was performed in a resource limited setting therefore, on a programmatic level, the reporting of incorrect results represented a considerable waste of resources and motivated for a change in HIV testing algorithm. False negative 2nd and 3rd generation ELISA results have been attributed to subtype incompatibility, recent infection or in some cases no inherent can be found (Evans et al. 1997; Preiser et al. 2000).

The aim of this study was to establish a validated supplementary HIV testing protocol for the management of low positive HIV screening and discrepant screening / confirmatory HIV serology.

2. Materials and method

2.1 Samples and source population

This study was a descriptive study based on a retrospective cohort of samples. The source of study samples was an archive of recent HIV serology serum samples from the National Health Laboratory Service (NHLS) virology laboratory at Groote Schuur Hospital. A search was performed on the laboratory tool, the Disa*Lab database for all HIV serology results from 01 June 2012 to 31 July 2013. The study was performed at a diagnostic pathology laboratory which provides a HIV diagnostic service for a tertiary academic hospital, and receives referrals from primary and secondary level health care facilities across the Western Cape Province. The referred samples often had preceding equivocal or indeterminate HIV rapid tests or other HIV screening ELISA results. A search of the Groote Schuur Hospital NHLS virology laboratory database for HIV screening serology was performed using the laboratory information system software.

In order to assess a range of weakly reactive result without testing a large number of true positive samples, an OD of 20 was chosen as low positive based on local pathologist experience. Samples were included if they met the inclusion criteria of a 4th generation screening ELISA assay result with a signal/cut off ratio (S/CO) of less than 20; and an optical density (OD) result of less than 2 when confirmed with the 3rd generation confirmatory assay. In order to determine the true status of indeterminate and discrepant results, additional HIV diagnostic tests, namely the p24 immunoassay, HIV viral load and western blot assay were performed on remaining samples (see explanation for determination of gold standard below).

2.1.1 Ethics

Approval for performing this study was obtained from the University of Cape Town Human Research Ethics Committee (HREC REF: 458/2013).

2.2 Assays

The ARCHITECT® HIV Ag/Ab Combo assay, a fourth generation dual sandwich EIA, was the screening assay used in this study to detect and p24 antigen as well as antibodies to HIV-1 and HIV-2 in human plasma or serum (Abbott Diagnostics 2010). Anti-HIV-1/2 antibody and p24 antigen in serum bind to HIV antigen and monoclonal anti-p24 antibody on microparticles. After washing, acridinium-labelled conjugate binds to bound antigen/antibody. A chemiluminescent reaction ensues, the result of which is measured in relative light units and reported as a signal to cut-off ratio (S/CO). A signal to cut-off ratio of greater than or equal to 1 is regarded as positive and a ratio of less than 1 is negative. The range of S/CO results reported by the instrument is 1 to 999.

The confirmatory 3rd generation sandwich ELISA used was Enzygnost® Anti-HIV 1/2 Plus, which detects antibodies to HIV-1 and -2 antigen bound to a microtitre plate (Dade Behring

2004). The enzymatic activity of the bound conjugate results in colour change when a chromogen is added, the intensity of which is proportional to concentration of antibody present the result of which is report in optical density (OD).The maximum optical density result is reported is 3.5, and stronger signals are reported as >3.5. Based on in-house laboratory practice, this result determines, whether reflex p24, western blot or HIV viral load tests will be done.

The Abbott Realtime® HIV-1 assay is a real time PCR assay that detects HIV viral load in plasma samples (Abbott Molecular 2011). The assay is able to quantify virus over a range of 40 to 10^7 RNA copies per ml plasma. Absence of detected virus is reported as lower than detectable (LDL). For the purposes of this study, samples that are greater than LDL but with a result of less 1000 RNA copies/ml were regarded as equivocal and greater than 1000 RNA copies were regarded as positive.

In order to confirm primary HIV infection, the VIDAS®HIV P24 II was used. This sandwich immunoassay detects the p24 antigen by fluorescence (BioMerieux 2010). Samples are regarded as positive if reported as greater than 5 pg/ml. The maximum result reported is 400 pg/ml; stronger signals are reported as >400 pg/ml.

The qualitative BIO-RAD GS HIV-1 Western Blot was used to detect and identify antibodies to HIV in human serum and plasma (Biorad 2013). HIV-1 specific antibody binds to HIV antigen fixed onto a nitrocellulose membrane. A colour change reaction allows for the identification of bound antibody. The position and intensity of the bands are compared to the reference strips. The WHO criteria for HIV-1 western blots positivity were used to determine results. The criteria require 2 ENV bands (gp160, gp120, gp41), while POL (p66, p51, p32) and GAG (p55, p40, p24, p17) bands may be present but not required for a positive result. Negative samples do not have bands and indeterminate samples have profiles that do not fit the positive or negative criteria (WHO 1991).

2.3 Test flow

All samples were screened using the 4th generation HIV ELISA assay and confirmed with the 3rd generation ELISA as part of routine test flow. If the confirmatory test was negative, both the p24 assay (routinely) and HIV viral load was performed (as part of the study in which additional tests were performed in order to compare the standard practice of performing p24 only initially; with performing the HIV viral load test which was thought to potentially be an improved practice). If both screening and confirmation results were low positives weakly reactive, an HIV viral load was performed. If the HIV viral load result produced was lower than detectable level of the assay or less than 1000 RNA copies/ml, the sample was tested using the western blot assay (see figure 1). If the result was greater than 1000 copies/ml it was regarded as a positive sample and no further testing was done. If the western blot was positive, the sample was regarded as positive. If the western blot result was negative, the result was considered indeterminate and clinician was asked to send a new sample.

2.3.1 Gold standard for true positives

True positive samples had an HIV viral load ≥ 1000 RNA copies/ml or an HIV viral load < 1000 RNA copies/ml with conclusive western blot, otherwise were regarded as false positive and a second sample requested.

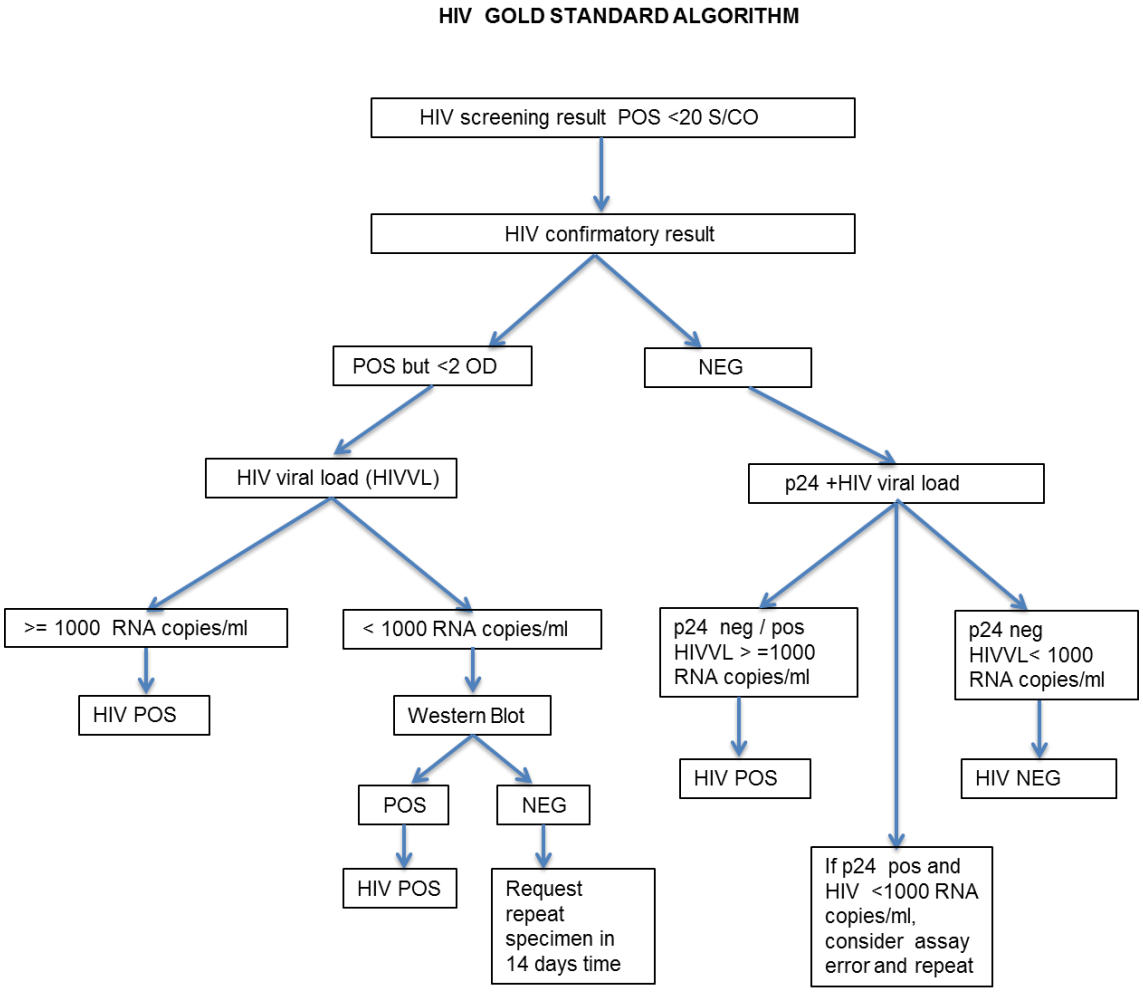

Figure 1. Flow diagram to determine the gold standard for HIV positive samples. Both p24 and the HIV viral load were performed when the HIV confirmatory results was negative because of uncertainty of the correct strategy.

2.4 Interpretation and statistical analysis

Statistical analysis was performed using statistical software Stata (version 11.1, StataCorp, Texas, USA) and Graphpad Prism (version 6.03, San Diego, USA). Continuous variables were summarised using median and interquartile ranges and categorical variables were analysed as proportions/ percentages with 95% confidence intervals with binomial distribution. The sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of the assays were determined using 2X2 tables. Non-parametric Spearman's correlation was used to determine correlation. Receiver Operating Characteristic (ROC) analysis was

performed and the optimal cut-off for the HIV chemiluminescent-based screening assay was determined as a means to predict a true positive result.

3. Results

3.1 Patients

The median age of patients included in the study was 30 years old (IQR: 26-43); 29 (60%) were women; none of the samples were follow-up samples from the same patient as follow-up samples were not available.

3.2 Assay results

Of the 18423 samples screened using the 4th generation ELISA assay, 13213 (71.7%) were negative, 5123 were true positives and 87 were indeterminate or low positive. Forty-eight samples fulfilled the inclusion criteria and were included in the analysis. An additional 39 samples had a S/CO of less than 20 but had insufficient sample left to perform the confirmatory 3rd generation ELISA assay and therefore did not fulfil the inclusion criteria. The 48 samples had all been screened for HIV using the 4th generation ARCHITECT (®) HIV Ag/Ab Combo assay. The median of the semiquantative relative light units produced was 6.92 S/CO (IQR: 2.4-15.31 S/CO). Twenty samples of 48 were positive when tested using Enzygnost® Anti-HIV 1/2 plus. The median O.D. of the samples deemed to be negative was 0.04 and was 1.56 OD for the samples deemed to be true positives. Forty-five samples were tested for HIV RNA using the Abbott Realtime® HIV-1 assay. Twenty two samples had detectable virus and 23 did not have detectable virus. Twenty-four samples were tested for the p24 antigen using the VIDAS®HIV P24 II assay; 22/24 were negative. Six samples had the BIO-RAD GS HIV-1 Western Blot performed, all of which were positive. Twenty-four samples were classified as true positives.

3.3 Statistical Analysis

3.3.1 Sensitivity and specificity

For the 48 samples that were tested, the sensitivity of the 3rd generation HIV confirmatory assay was 72% while the specificity was 91%. The PPV of this assay was 90% and the NPV was 75% in this weakly reactive or discrepant serology population. For the 24 samples that had the p24 test performed, the sensitivity of the assay was 37% and the specificity was 100%. The NPV and PPV of this assay was 100% and 72%, respectively. The sensitivity of the 3rd generation ELISA assay (72%) and that of the p24 assay (37%) compared poorly to HIV viral load when it is used as the gold standard. Only 6 samples were had the western blot test performed as determined by the gold standard criteria. The sensitivity, specificity, PPV and NPV of the western blot assay in this context was 100%.

3.3.2 Correlation

Despite a significant correlation between 4th and 3rd generation ELISA tests (Rho 0.3589, p= 0.0012); and 4th generation and HIV viral load tests (Rho 0.4860, p=0007), when plotting the 4th generation screening value on the x-axis against the 3rd generation confirmatory or against the HIV viral load value on the y-axis, it appears difficult to predict the respective confirmatory 3rd generation and HIV viral load result relative to the screening result (see figure 2 and table 2). Eighty-four percent of samples with confirmatory ELISA OD values above 0.5 had detectable viral loads and there were clearly two subgroups of viral load results reported as either lower than the detectable limit (LDL) or greater than 4 $\log_{10}$(figure 2). The single exception was the result of 3.95 $\log_{10}$. Similar to the screening / confirmatory result comparison, there were two clear subgroups of viral load results reported; either LDL or greater than 4$\log_{10}$. Of the samples that had the p24 antigen test performed and had detectable HIV viral load (range: 4-5 $\log_{10}$), only 28% had a p24 antigen detected. These p24 results ranged between 20 and 30 pg/ml.

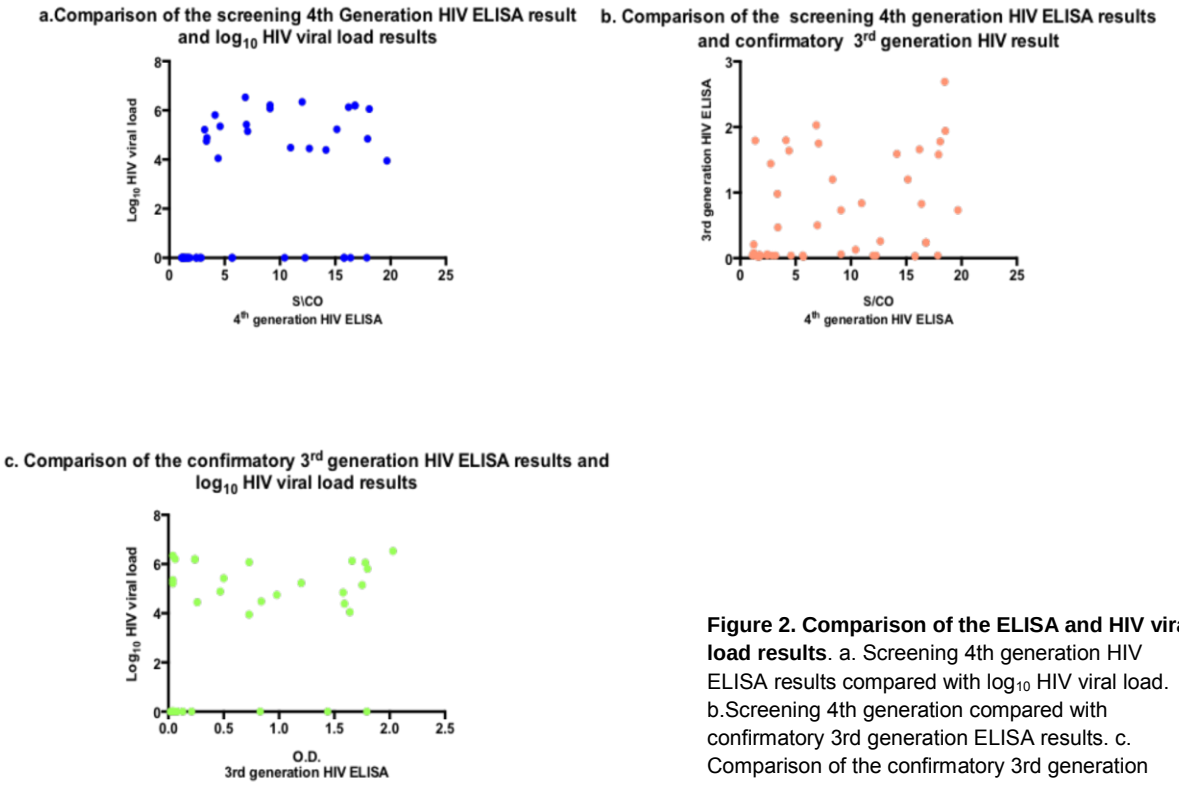

Figure 2. Comparison of the ELISA and HIV viral load results. a. Screening 4th generation HIV ELISA results compared with log_{10} HIV viral load. b.Screening 4th generation compared with confirmatory 3rd generation ELISA results. c. Comparison of the confirmatory 3rd generation ELISA results and log_{10} HIV viral load results.

SPEARMAN r	4th generation screening and 3rd confirmatory test	4th generation screening test and HIV viral load
r	0.3598	0.4860
95% confidence interval	0.07569 to 0.5899	0.2160 to 0.6870
P value		
P (two-tailed)	0.0120	0.0007

Table 2. **Spearman's correlation of the 4th generation screening values and the 3rd generation confirmatory and HIV viral load values**. The 0.3589 and the 0.4860 shows correlation between the between the 4th generation screening results and the 3rd generation confirmatory and HIV viral load, respectively. (Data generated in Graphpad Prism version 6.03, San Diego, USA)

3.3.3. ROC analysis

Results from all the assays underwent Receiver Operating Characteristic (ROC) analysis. The most significant result was that of the 4th generation HIV ELISA screening test. As shown in figure 3 and table 3, samples with a S/CO ratio of greater than 3.19 should be considered as true positive in this sample set, as according to the ROC analysis, using this value as the a cut-off of 3.19 will result in the most correctly classified results (83%). The area under the curve was 0.8035.The specificity at this cut-off was poor at 65%. By using a higher cut-off, the specificity can be improved but at the cost of sensitivity (see supplementary data for full table). At a cut-off of 17.93, the specificity is 100% but the sensitivity is 20%.

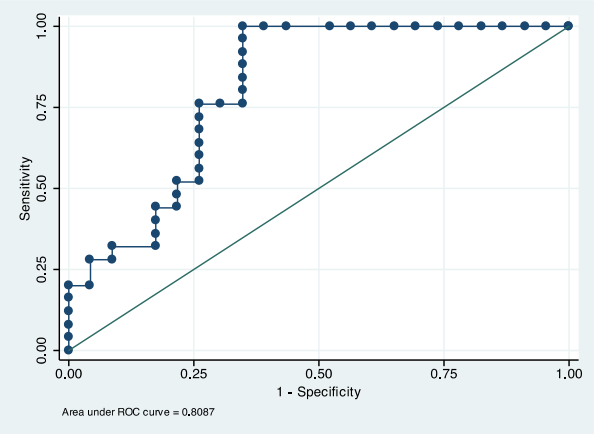

Figure 3. The diagrammatic representation of the ROC curve analysis for the HIV 4th generation ELISA screening test.

Table 3.Receiver Operating Characteristic (ROC) analysis for the 4th generation HIV ELISA screening test used in the diagnosis of HIV infection. The cut-off point of 3.19 was shown to correctly classify the highest number of results. Sensitivity was 100% at this point but specificity was 65.22%. A cut point of 17.93 results in specificity of 100% but the sensitivity was only 56%. See appendix for full table.

Cut-off point	Sensitivity	Specificity	Correctly Classified
(>= 1.1)	100.00%	0.00%	52.08%
(>= 3.19)	100.00%	65.22%	83.33%
(>= 17.93)	20.00%	100.00%	58.33%
(> 19.68)	0.00%	100.00%	47.92%

4. Discussion

The 4th generation HIV ELISA used in this study lacked specificity when looking at this dataset of samples with S/CO <20, and therefore confirms WHO guidelines that confirming positive screening results with a highly specific set of assays will be valuable. For this sample group, the 3rd generation HIV confirmatory assay had better specificity (91%) than sensitivity (72%), as is required of a confirmatory assay. The 91% specificity reflects the two false positive tests in the dataset. The positive predictive value of this confirmatory assay was 90% which was an indication that the majority of cases detected as positive were truly positive. However, according to the NPV, only 75% of samples were correctly classified as negative. The p24 assay lacked sensitivity but the positive predictive value of 100% confirmed that a positive result in this study is truly positive. The lack of sensitivity raises the question of whether the p24 assay should be used as a screening tool for acute HIV infection in this setting. The HIV viral load assay proved to be the best option for confirmation of problematic serology in this laboratory as the sensitivity, specificity, PPV and NPV were ideal. Therefore, if a sample in this laboratory has low positive screening and confirmation results, or discrepant screening and confirmatory serology, these results deem the HIV viral load as the best option to most likely to confirm HIV status in problematic cases.

A major finding of the study was that screening value of 3.19 S/CO was the cut-off that determined whether the result was truly positive or not. In this sample set of low positive screening tests, any value above this cut-off was more likely to be correctly classified as a true positive in 83% of cases but would still require additional tests to confirm positivity. The area under the curve of 0.8 suggests that this test has good discriminatory value as a diagnostic test and therefore can be used as a screening test. However, the small sample size included in this study probably does not allow for these findings to be transferred to all laboratories using these assays. It does, however, allow for recommendations that can advise staff in this laboratory when faced with difficulty while interpreting HIV serology

results. The ROC's main contribution is to show that each laboratory needs to determine its own "greyzone" in order to guide further testing.

The ROC result for suspecting true positive 4th generation ELISA screening results was quite low and this will certainly impact the threshold at which pathologists will request additional tests, particularly the viral load, in order to determine true HIV status.

The HIV viral load assay was the best additional confirmatory assay with excellent sensitivity and specificity, but this was probably due to the pre-determined criteria of a true positive result. Furthermore, as mentioned in the results, there was a clear distinction between negative and positive viral loads and the positive viral load results were high. However using the HIV viral load test as a confirmatory test will be of limited diagnostic value in patients who are elite controllers as they will not have virus detectable by current assays (Bello et al. 2009).

The p24 lacked sensitivity at 37% thereby confirming previous publications of low sensitivity (Daar et al. 1999; Barletta et al. 2004; Pascual et al. 2002). Daar et al documented the sensitivity of p24 antigen at 88.7%. Barletta et al. quoted that the antigen was detected in only 50% of acute infections, while Pascual et al. produced positive results in only 65% of samples with viral loads between 1000-10000 RNA copies/ml. Our study detected only 28% of samples with viral loads above 4 $\log_{10}$.This low sensitivity, in addition to the negative predictive value of 72%, supports additional the additional HIV viral load requests in cases where the result is negative, and acute infection is still suspected. Therefore the question is whether the p24 assay should be still be performed or whether the cost of doing both assays for a number of cases will be cheaper than doing the viral loads for confirmation for all low positive and discrepant results.

In our study 87/ 18423 total samples required additional tests In a simple costing calculation it was assumed that all samples will have a screening and a confirmatory assay performed,

and if discrepant, a p24 and an HIV viral test would follow. The p24 test and HIV viral load costs the client R161.88 and R305.61, respectively (see table 1). By doing both the client will be charged an additional R467.49. By doing just the HIV viral load the client will save R161.88 per patient. Therefore, based on this study, confirming the HIV status of uncertain samples (after screening and confirmation ELISAs) with the HIV viral load will be a cost saving measure in our laboratory The p24 takes less time to perform than the HIV viral load, but it is the current laboratory practice to batch these samples in order to save cost of assay controls. Therefore the turnaround time of the p24 may be longer the than the HIV viral load which, although the assay takes longer to perform, is performed every day. However, it is possible to perform the assay for just one sample in urgent cases which cannot be done for HIV viral load. In the future, when faster and single feed viral load platforms become available, performing urgent viral loads should probably no longer be problematic. An assay that would be able to distinguish between antigen and antibody would assist in solving this issue as well. This will potentially be an upgrade of the current 4th generation assay to a 5th generation ELISA, i.e. an assay that detects both antigen and antibody with differentiation. Weakly reactive HIV screening results may be true positives. The low positive 4th generation HIV ELISA results may reflect a variation of the so-called “second window period” that has been detected in this assay class reflecting a time point when p24 antigen has declined but the antibody is still below the level of detection of the assay.The detection of acute HIV is dependent on the sensitivity of the p24 component and for the ARCHITECT (®) HIV Ag/Ab Combo it has been shown to detect p24 at 58 000 RNA copies/ml (Brennan et al. 2013).This may be the explanation for high viral loads associated with low screening values. Patients may be undiagnosed HIV elite controllers with positive serology and undetectable HIV viral loads (Vermeulen et al. 2013). Some elite controllers have been shown to mimic newly infected individuals with low antibody responses and poor avidity (Bello et al. 2009). Patients infected with HIV-2 may be mistakenly diagnosed as having false positive serology as current HIV viral load assays in use in South Africa detect only HIV-1. A high index of

suspicion must be held for this possibility in cases where persistently positive HIV serology is accompanied by persistently negative viral load results.

The HIV confirmatory assay had two false positive results as reflected by the specificity of 91%. As discussed earlier in the manuscript, there are a multitude of clinical conditions that can result in the production of false positive HIV serology results. In my experience, false positive results usually have low ODs and are western blot and HIV viral load negative. They can be differentiated from acute infection where the viral load is high

It is questionable whether performing the western blot assay added value. The sample size was small. The western blot assay was only performed in cases where there are antibodies in the sample, i.e. the 3rd generation confirmatory assay is weakly reactive. Therefore it was not of use in cases where acute infection was suspected based on the screening and confirmatory results (56% of our dataset). However, there are western blot seroconversion profiles that are based on the total number of positive bands present but these bands do not make up a full banding pattern and therefore do not give a clear positive result (Hecht et all, 2011). Performing the assay requires training, and with multiple manual steps being present, it is quite time-consuming. The results are that are produced are sometimes difficult to interpret and are reported as indeterminate in those cases (Cárdenas et al. 2013). The aim of the study was to streamline the process of troubleshooting difficult results by developing a validated HIV testing protocol. The aforementioned factors limit the use of the western blot in a potential algorithm. However, it may still have role when all other tests are inconclusive, antibodies have been detected in the samples, and one has easy access to a laboratory performing the assay.

The quantitative HIV DNA assay was not included in this study as the assays used in this study and in routine HIV screening are performed on serum or plasma samples. The package insert of the HIV DNA test states that the sample type of choice is whole blood. The purpose of the test is for early infant testing (SOP GPL2192) and therefore not within the age

group of patients at whom the algorithm is aimed. The assay also, in pathologists' experience, lacks sensitivity which may have compromised the aim of the study.

Comparing the quantitative HIV viral load assay with semiquantative assays may be regarded as limitation but it is acceptable to compare the quantitative HIV viral load with semiquantative diagnostic tests when it is being interpreted as a semiquantative diagnostic test.

The result of this type of analysis may be altered in different sample sets. This study may be regarded as a "pilot" study. A prospective study with a larger sample size and sufficient sample volume required to perform all the assays assessed in this study may produce a different cut-off value. Performing this analysis in a community with a different HIV prevalence may affect the outcome of ROC analysis as well i.e. if the prevalence was lower, the ROC cut-off for the 4th generation EIA may be higher.

It is a limitation of this study that the history of each patient was not reviewed but this was a laboratory-based study and therefore beyond its scope. By reviewing the history, it would have been possible to exclude the abovementioned clinical causes. It would also have been useful to know the history when determining true positives as it would have been possible to exclude samples from known HIV positive patients erroneously sent for serology, resulting in misleading serology and HIV viral load results. It is acceptable to compare the quantitative HIV viral load with semiquantative diagnostic tests when it is being interpreted as a semiquantative diagnostic test.

It is imperative that clinicians are closely involved with interpretation and management of difficult HIV serology results. Consultation with clinicians will result in pathologists having access to more in-depth histories than is written on most clinical request forms thereby allowing them to make more informed decisions when analysing results. The importance of sending new specimens for additional tests, particularly in cases where sample volumes are

initially insufficient for extra tests, or allowing for a certain time period to elapse after initial tests are inconclusive to allow potential development of conclusive results, needs to be clearly conveyed to clinicians in order to negate potential poor follow-up and thereby allow patients access to treatment as soon as possible. Unfortunately South Africa has a low doctor to patient ratio, 0.77 per 1000 as compared to the UK and Australia where the ratio is 2.3 and 2.47 per 1000 respectively (B Strachan et al. 2011). Seeing many patients and therefore not being able to remember individual patients in order to follow up results could possibly a reason for poor responses to requests for additional samples.

5. Conclusion

This study illustrates the importance of validation of assays with samples from the local population in each individual laboratory. Cost of the assay, the number of samples requiring confirmation, the HIV prevalence and subtype composition in the local population, staff skill and turnaround time should all be determining factors when recommending assay for a particular laboratory setting. We have found that that using the HIV viral load as confirmatory assay for discrepant and weakly reactive or low positive serology samples is the best strategy in our laboratory as the results are reliable and it is economically viable. The results of this study concur with the new CDC guidelines in suggesting the HIV viral is the optimal assay to confirm HIV infection in problematic diagnostic cases.

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PART D: APPENDICES

Appendix A: Full Received Operating Analysis table
Appendix B: CDC HIV diagnostic algorithm
Appendix C: HPA diagnostic algorithm
Appendix D: South African recommended serial HIV testing algorithm
Appendix E: Ethics approval
Appendix F: Journal of Virological Methods author information pack

Appendix A: Full Received Operating Analysis table

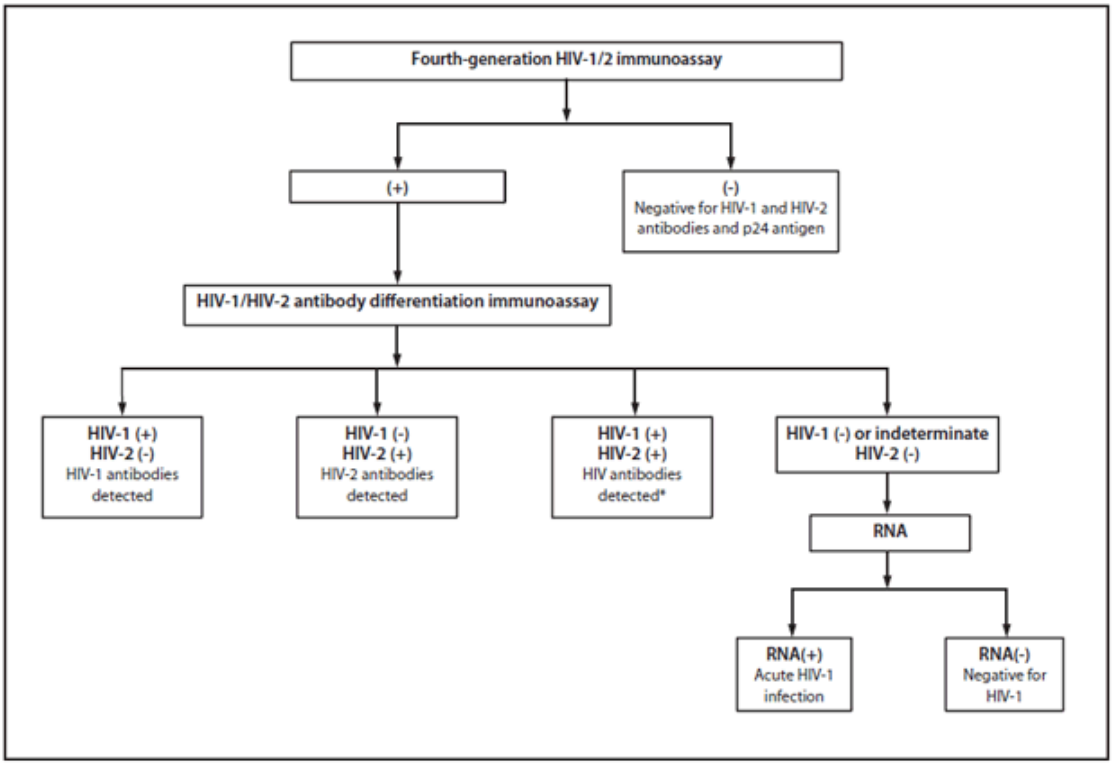
Table. Full Receiver Operating Characteristic (ROC) analysis for the 4th generation HIV ELISA screening test used in the diagnosis of HIV in this study.

Detailed report of Sensitivity and Specificity

Cutpoint	Sensitivity	Specificity	Correctly Classified	LR+	LR-
(>= 1.1)	100.00%	0.00%	52.08%	1.0000	
(>= 1.14)	100.00%	4.35%	54.17%	1.0455	0.0000
(>= 1.21)	100.00%	8.70%	56.25%	1.0952	0.0000
(>= 1.22)	100.00%	13.04%	58.33%	1.1500	0.0000
(>= 1.27)	100.00%	17.39%	60.42%	1.2105	0.0000
(>= 1.36)	100.00%	21.74%	62.50%	1.2778	0.0000
(>= 1.47)	100.00%	26.09%	64.58%	1.3529	0.0000
(>= 1.5)	100.00%	30.43%	66.67%	1.4375	0.0000
(>= 1.68)	100.00%	34.78%	68.75%	1.5333	0.0000
(>= 1.72)	100.00%	39.13%	70.83%	1.6429	0.0000
(>= 1.86)	100.00%	43.48%	72.92%	1.7692	0.0000
(>= 2.44)	100.00%	47.83%	75.00%	1.9167	0.0000
(>= 2.76)	100.00%	56.52%	79.17%	2.3000	0.0000
(>= 2.88)	100.00%	60.87%	81.25%	2.5556	0.0000
(>= 3.19)	100.00%	65.22%	83.33%	2.8750	0.0000
(>= 3.36)	96.00%	65.22%	81.25%	2.7600	0.0613
(>= 3.39)	92.00%	65.22%	79.17%	2.6450	0.1227
(>= 4.13)	88.00%	65.22%	77.08%	2.5300	0.1840
(>= 4.41)	84.00%	65.22%	75.00%	2.4150	0.2453
(>= 4.59)	80.00%	65.22%	72.92%	2.3000	0.3067
(>= 5.66)	76.00%	65.22%	70.83%	2.1850	0.3680
(>= 5.7)	76.00%	69.57%	72.92%	2.4971	0.3450
(>= 6.87)	76.00%	73.91%	75.00%	2.9133	0.3247
(>= 6.97)	72.00%	73.91%	72.92%	2.7600	0.3788
(>= 7.07)	68.00%	73.91%	70.83%	2.6067	0.4329
(>= 8.34)	64.00%	73.91%	68.75%	2.4533	0.4871
(>= 9.1)	60.00%	73.91%	66.67%	2.3000	0.5412
(>= 9.11)	56.00%	73.91%	64.58%	2.1467	0.5953
(>= 10.43)	52.00%	73.91%	62.50%	1.9933	0.6494
(>= 10.97)	52.00%	78.26%	64.58%	2.3920	0.6133
(>= 12.01)	48.00%	78.26%	62.50%	2.2080	0.6644
(>= 12.27)	44.00%	78.26%	60.42%	2.0240	0.7156
(>= 12.66)	44.00%	82.61%	62.50%	2.5300	0.6779
(>= 14.16)	40.00%	82.61%	60.42%	2.3000	0.7263
(>= 15.15)	36.00%	82.61%	58.33%	2.0700	0.7747
(>= 15.8)	32.00%	82.61%	56.25%	1.8400	0.8232
(>= 16.2)	32.00%	91.30%	60.42%	3.6800	0.7448
(>= 16.38)	28.00%	91.30%	58.33%	3.2200	0.7886
(>= 16.78)	28.00%	95.65%	60.42%	6.4400	0.7527
(>= 17.86)	20.00%	95.65%	56.25%	4.6000	0.8364
(>= 17.93)	20.00%	100.00%	58.33%		0.8000
(>= 18.08)	16.00%	100.00%	56.25%		0.8400
(>= 18.48)	12.00%	100.00%	54.17%		0.8800
(>= 18.54)	8.00%	100.00%	52.08%		0.9200
(>= 19.68)	4.00%	100.00%	50.00%		0.9600
(> 19.68)	0.00%	100.00%	47.92%		1.0000

Appendix B: CDC HIV diagnostic algorithm

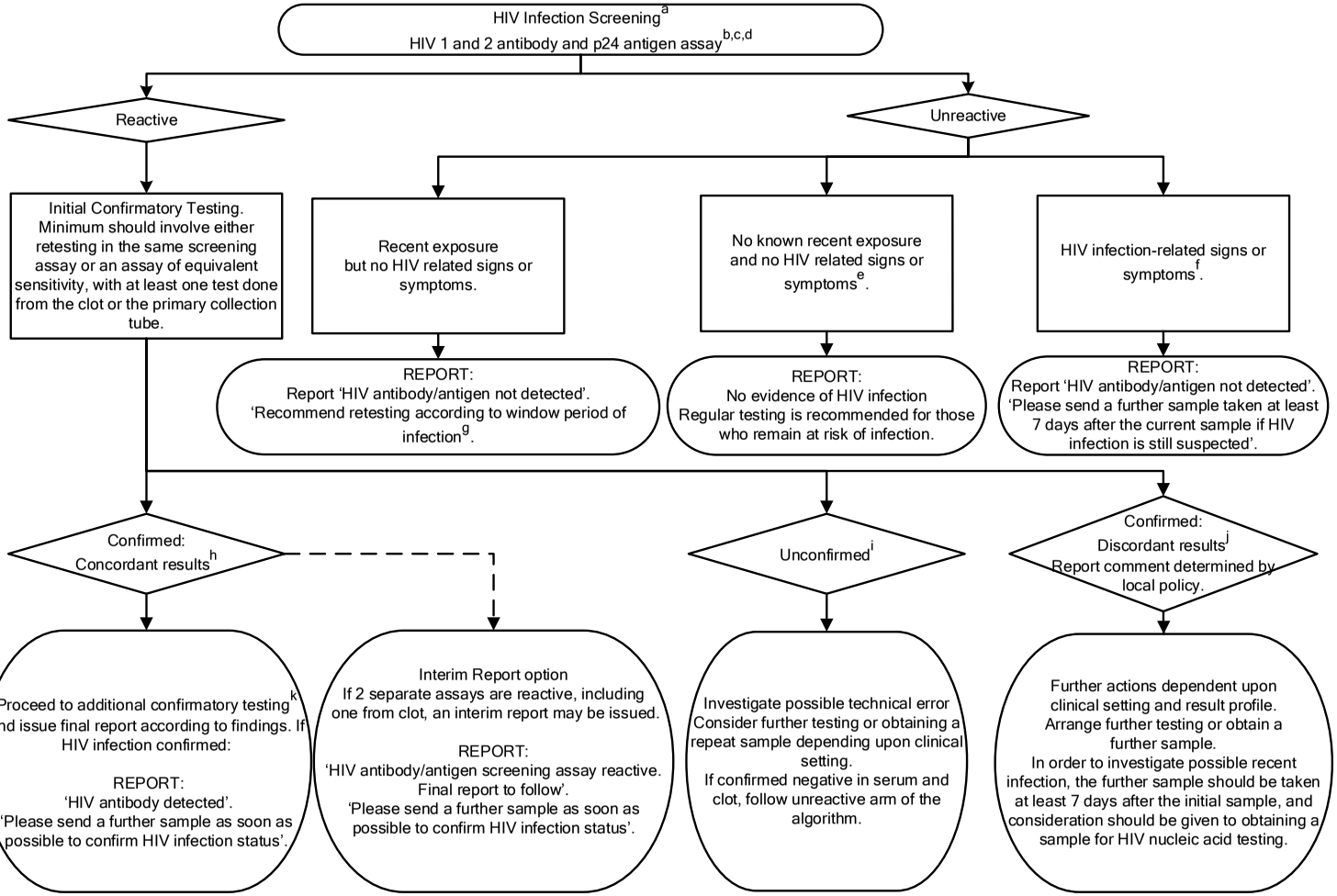
Source: Centers for Disease Control and Prevention (CDC). Detection of acute HIV infection in two evaluations of a new HIV diagnostic testing algorithm - United States, 2011-2013. MMWR Morb Mortal Wkly Rep 2013;62:489-94.



Appendix C: HPA diagnostic algorithm

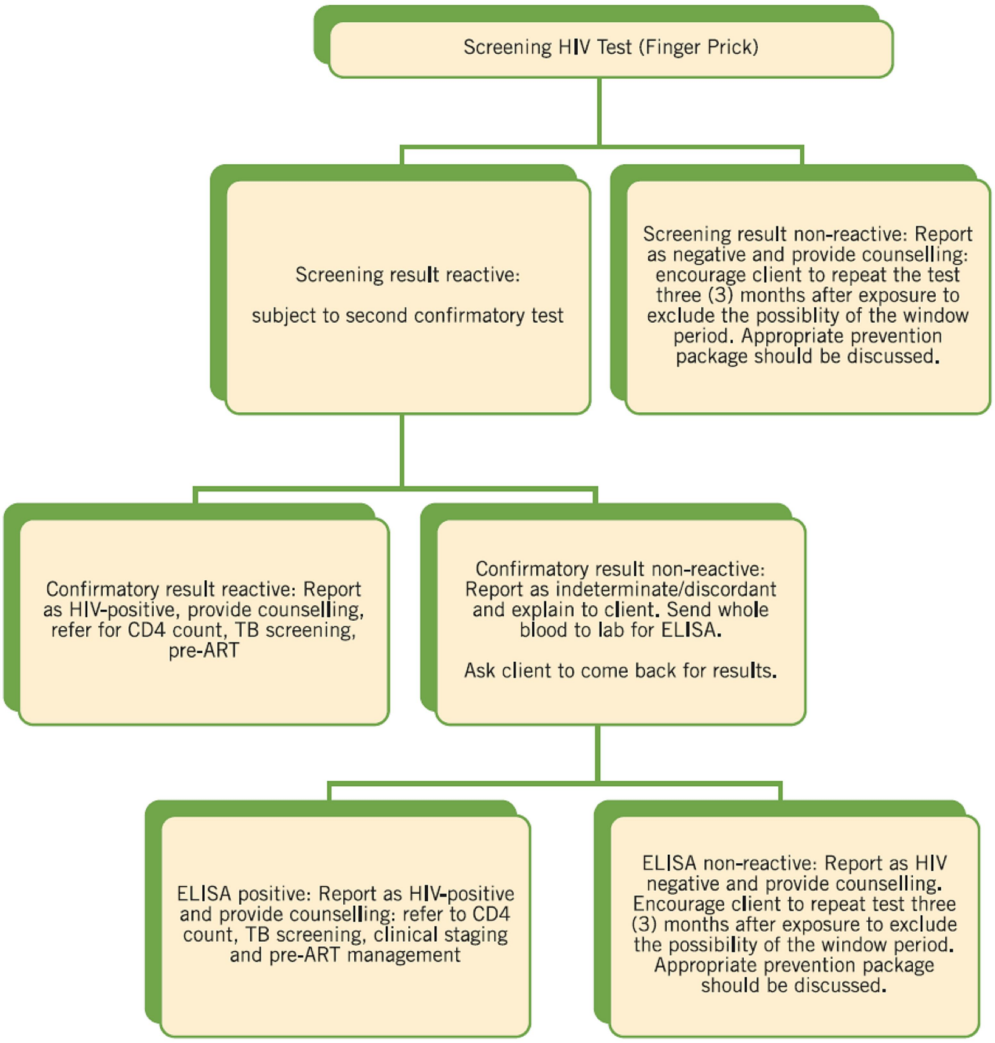
Source: Health Protection Agency. (2012). Anti-HIV Screening. UK Standards for Microbiology Investigations. V 11 Issue 3.1.

Anti-HIV Screening[1-7]



Appendix D: South African recommended serial HIV testing algorithm

Source: National Department of Health, 2010. National HIV Counselling and Testing (HCT). Page 23.



Appendix E: Ethics approval

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Human Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
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e-mail: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

31 July 2013

HREC REF: 458/2013

Dr L Hans
c/o Dr S Korsman
Clinical Virology
C18, NGSH

Dear Dr Hans

PROJECT TITLE: LABORATORY INVESTIGATION OF LOW POSITIVE AND DISCREPANT HIV SEROLOGY RESULTS

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year till the 15th August 2014

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/research/humanethics/forms)

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC. REF in all your correspondence.

Yours sincerely

pp TuBurgess

PROFESSOR M BLOCKMAN
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This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

s.thomas

Appendix F: Journal of Virological Methods author information pack

JOURNAL OF VIROLOGICAL METHODS

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

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The Journal of Virological Methods publishes original papers and invited reviews covering techniques on all aspects of **virology**. These include methods for studying the morphology, assembly, replication, composition, function and physiochemical properties of **viruses** and their components; the **purification** of viruses and their components; **cultivation**; properties of **viral antigens**, production of **antibodies**, and techniques for studying the **immune response** to virions, viral subunits, and components; the detection and identification of viruses and **viral infections**; **assay** of viruses and **viral infectivity** and the investigation of **transmission** and **pathogenicity**; and methods for investigating the **suppression** or **inhibition** of viral growth.

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