CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background

The enzyme-linked immunosorbent assays (ELISAs) are the most commonly used techniques for the laboratory diagnosis of HIV infection [1]. First generation ELISAs were licensed over 25 years ago, and the quality of the ELISAs have been continuously improved by the use of recombinant antigens and synthetic peptides in the second generation and the sandwich technique in the third test generation [1, 2].

The UNAIDS/WHO has given recommendations for the use of combined screening assays for the diagnosis and confirmation of HIV infection [3]. Currently, a combination of antibody ELISA [4-7], or simple rapid assays [1] based on different test principles have been used in alternative diagnostic testing strategies for laboratory diagnosis in resource-limited countries. The standard procedure for laboratory diagnosis of HIV infection usually includes screening for virus-specific antibodies using an enzyme-linked immunosorbent assay (ELISA) [8], followed by confirmatory testing of screening positive samples [1]. In developed countries, screening of HIV antibodies was followed by confirmatory testing, most commonly by using Western blot (WB) assay. However, testing strategy using WB is very costly for use in resource-limited countries like Tanzania [9].

Recently, new fourth generation screening assays which permit a simultaneous detection of HIV antigen and antibody have been developed and have reduced the diagnostic window phase between time of HIV infection and laboratory diagnosis on average by four days in comparison to third-generation antibody assays because antibodies to HIV are absent in the very early phase of HIV infection [10-20]. With the p24 antigen combined in assays, HIV infections can be detected from few days to many weeks before antibody seroconversion, and this is useful in routine screening of blood [11, 15, 21].

Enzygnost HIV Integral (Dade Behring, Germany) is among new fourth generation assays that detect HIV p24 antigen and HIV antibodies simultaneously in a single

one test batch [9, 22] and have been licensed since 1997 [13, 15, 20]. The Enzygnost HIV Integral II assay is based on the antigen sandwich format combined with detection of p24 by utilizing murine monoclonal anti- p24 on the solid phase and in the conjugate. The combined assays offer potential benefits of early detection of HIV infection via p24 Ag detection and also improve seroconversion sensitivity at a technical burden and financial cost [11, 23].

A previous testing strategy adopted by the Tanzania National HIV reference laboratory used the Murex HIV Ag/Ab Combination (Abbot Murex) followed by Enzygnost® anti-HIV-1/2 Plus (Dade Behring, Germany) and discordant results between the two ELISAs were resolved by WB analysis [24].

HIV ELISA testing algorithm aims to reduce the number of false positive and false negative results in HIV testing. In any algorithm, each blood sample is tested serially with two Enzyme immunoassays (EIA) tests that should be highly sensitive and have different detection formats. The first screening test is performed on the blood sample, and the second screening test is done as soon as possible after the sample in the first test gives a positive reaction. If the result of the first screening test is negative, the test is reported as negative; if a result of the first screening test is positive, the same sample is tested using the second screening test. If the result of the second test is positive, the test is reported as positive; if the result of the second test is negative, the test is considered as discordant [4, 22, 24] and confirmatory tests using Western blot is conducted. However, use of Western Blot for serological confirmation is expensive, time-consuming and has technical disadvantages, such as lack of standardization in performance, variability in quality of strips and difficulty in interpreting reactions and the presence of indeterminate reactions [25-29]. Therefore, attempts to evaluate performance of Ag/Ab combination HIV ELISAs are important to come up with high specificity and sensitivity that can be obtained without using western blot and at a considerable reduction in costs [5, 24].

Therefore, attempts to improve tests have been an ongoing process so as to reduce number of false results, as occurrences of even a small number of false results by these tests can have profound implications as it can waste resources in discarded

blood units and unnecessary verification of reactive results using more expensive tests. Also false negative results can have serious consequences for blood recipients [1].

Evaluation of ELISA assays for the detection of HIV antigen and antibody focus on performance characteristics such as sensitivity, specificity, PVP and NVP for use in small laboratories with limited facilities. The information is intended for use by those responsible for deciding which tests to use by the potential users of tests so as to enable application of the algorithm and choose the best test for the particular situation. [9]

Evaluation also determines operational factors that include, laboratory infrastructure, access to a reference laboratory, desired characteristics of the test (antigen, antibody), simplicity of test procedure, equipment necessary to perform the test, performance time, shelf-life of the reagents, reasonable costs, long shelf life at ambient temperatures, rapidity of performance, technical skill of laboratory staff, laboratory logistics (continuous supply of kits, stability of electrical source, maintenance of equipment, spare parts, availability of service, etc· long shelf life at ambient temperatures.[9,15]

Evaluation of the assay is required once a new assay is introduced into use. Results that will be obtained are intended for use by health policy-makers, blood banks, and national AIDS prevention programmes. The results may also be evaluated in conjunction with consideration of other factors, such as experience with a given test, availability, cost, service and troubleshooting provided locally by manufacturers, etc., to help select HIV antibody and/or HIV Ag/Ab assays appropriate to local needs.

In Tanzania, evaluation of HIV ELISA has been an ongoing process especially when an assay is withdrawn or a new assay has been introduced. In 2006 evaluation of HIV antibody and antigen/antibody combination ELISAs for use in an alternative confirmatory HIV testing strategy was performed where the performance of two antibody enzyme-linked immunosorbent assays (ELISAs) and

new algorithm was proposed [9]. Likewise in 1999, there was a study that evaluated the accuracy of an alternative confirmatory strategy for detection of antibodies to HIV-1: experience from a regional laboratory in Kagera, Tanzania [30].

The aim of this study was to evaluate HIV Antigen and Antibody Combination ELISAs for laboratory diagnosis of HIV infection in Dar es Salaam, Tanzania. In the evaluation, Enzygnost HIV Integral II Antibody/ Antigen (Siemens, Germany), Murex HIV antigen/antibody (Abbott Murex, UK) and Vironostika HIV Uniform II antigen/antibody (Biomerieux, the Netherlands) were included, for screening. INNO-LIA HIV I/II immunoblot (Innogenetics, Belgium) was used as a confirmatory test for the discordant results and 5% of reactive serum samples and 4% of nonreactive serum samples**.** The objective were to evaluate the performance and operational characteristics of HIV combination ELISAs as well as to formulate HIV ELISA testing algorithms that could be used as an alternative HIV testing strategy for lab diagnosis of HIV infection**.**

1.1.1 Laboratory diagnosis of HIV infection

In 2007, a study was conducted on diagnosis of HIV-1 infection in children younger than 18 months in the United States, in this study laboratory diagnosis of HIV infection was described [31]. Laboratory-based methods for the diagnosis of HIV-1 infection can be divided into two groups which are immunologic methods assays that detect the antibody response to HIV-1 or the extent to which the immune system has deteriorated as a consequence of HIV-1 infection (this include enzyme immunoassays or enzyme-linked immunosorbent assays (ELISAs) as well as rapid serologic tests and virologic assays that detect HIV-1 genetic material or components of the virus. All laboratory tests used for the diagnosis of HIV-1 infection must be confirmed (i.e. the diagnosis of HIV-1 infection is never based on only a single test result).

1.1.1.1 Detection of HIV antibodies

Enzyme-linked immunosorbent assays (ELISA) is a type of immunologic methods, which detect presence of antibodies in the blood against HIV. ELISAs are less expensive than virologic assays, and they can be readily used for testing large

batches of samples; however their use in batch testing means that, generally, there is a delay of up to one week in obtaining test results, depending on the laboratory system used, and such delays often preclude their use in settings such as labor-and-delivery units.

One of the limitations with the use of antibody assays is that false-negative results can occur among individuals who are HIV-1–infected but have not yet begun to produce antibodies against HIV-1 (i.e., within the first 6 weeks of infection, including those with clinical signs and symptoms of the acute retroviral syndrome) or among individuals in the late stages of HIV-1 infection with concomitant hypogammaglobulinemia.

Secondly, there are no reliable HIV-1–specific antibody assays to differentiate between maternal and autologous antibody in the child because of transplacental transfer of maternal immunoglobulin G (IgG) antibody, children born to HIV-1–infected women are seropositive (even if the child is not HIV-1–infected); therefore, a positive ELISA result in a child younger than 18 months must be confirmed virologically. Also false-positive ELISA results can occur among individuals with immune stimulation (e.g., among those with acute [non–HIV-1] viral infections or autoimmune disorders, among pregnant women, and among recipients of multiple transfusions).

Rapid tests are immunological tests for detection of IgG antibodies against HIV-1
Such assays are based on 1 of 4 principles: particle agglutination, immunodot (dipstick), immunofiltration, or immune chromatography. Rapid HIV-1 antibody tests are comparable to ELISAs in both sensitivity (99.3%–100%) and specificity (98.6%–100%). They require no special instrumentation outside of the test kit, and results can be available in as little as 20 to 30 minutes. Some of these assays are licensed for point-of-care testing so that clinics without extensive laboratory facilities (but that meet recommended standards and have appropriately trained personnel) can perform the tests on-site and report the result to the patient immediately. As with routine ELISAs, confirmation of positive results is necessary,

but confirmation of a negative result is not, and all reactive rapid HIV-1 test results should be confirmed with either a Western blot or an immune-fluorescence assay.

WB assay is typically used to confirm a reactive ELISA or rapid serologic test result as being truly positive. The WB assay detects and visualizes the presence of antibodies against specific HIV-1 proteins (structural and enzymatic). A negative WB assay result indicates that the positive ELISA or rapid-test result represents a false-positive result and that the patient does not have HIV-1 antibodies. A positive WB assay result confirms the presence of HIV-1 antibodies; thus, in individuals older than 18 months, it is diagnostic of HIV-1 infection.

1.1.1.2 Detection of HIV antigens

This is a type of virologic assay that detect the components of the virus (e.g. assays for a viral capsid protein (p24 Antigen), the viral protein p24 exists either bound to anti-p24 antibody or unbound (free) in the bloodstream of HIV-1–infected individuals. In general p24 antigen assays have been used much less frequently than HIV-1 DNA- or RNA-amplification techniques for diagnosis of HIV-1 infection because of the relatively poor sensitivity of p24 antigen assays; however they are more affordable and easier to perform but require specific equipment and trained personnel.

1.1.1.3 Detection of nucleic acids

These are types of virologic assays known as nucleic acid amplification tests (NAATs) that detect HIV nucleic acids such as HIV DNA or HIV RNA), and are the most widely used assays for diagnosis of HIV in children younger than 18 months. Because viral nucleic acid may be present in very small quantities in test samples, NAATs are used to increase the amount of HIV proviral DNA or HIV RNA, or the amount of positive signal, in a test sample.

HIV DNA Assays allow detection of cells that harbour quiescent provirus as well as cells with actively replicating virus. Advantage of these assays is that, they are mostly used assays to date for diagnosis of HIV-1infection in infants and young children; However they are costly, require special equipment, experienced

laboratories, trained personnel, false-positive results if laboratory contamination occurs, Not currently licensed for use in diagnosing HIV-1 infection.

HIV RNA Assay detects plasma (cell-free) viral RNA by using different techniques. Methods of amplification of HIV-1 RNA include target (nucleic acid sequence-based amplification and reverse transcriptase PCR) and signal (branched-chain DNA) amplification techniques. These techniques are widely available, short turnaround time. However they are costly, require special equipment, well established laboratories, trained personnel, low copy numbers (eg, 10 000 copies per mL) may represent false-positive results, Not currently licensed for use in diagnosing HIV-1 infection

1.1.1.4 Virus isolation/ culture

HIV-1 culture involves incubating peripheral blood mononuclear cells (PBMCs) from a patient with in vitro–activated PBMCs from an HIV-1–uninfected volunteer and culturing for up to 6 weeks. The PMBCs are maintained in an activated state susceptible to viral infection and replication. Therefore, high levels of viral replication occur within the target PBMCs if infectious HIV-1 is present. Measurement of p24 antigen in the culture supernatant using ELISA allows assessment of the growth of HIV-1 in the PBMCs. The culture is classified as positive for HIV-1 if a significant amount of p24 antigen is detected and demonstrates significantly increasing concentrations of p24 antigen over subsequent culture supernatant fluid collection time points. Although previously considered the gold standard of HIV-1 diagnostic assays for infants and young children, the disadvantages of viral culture [i.e., that it is labor intensive, time consuming (2–3 weeks required for determination of a positive assay result), costly (requires special laboratory and equipment) and poses a biohazard risk] are now generally considered to outweigh its advantages. HIV culture has limited availability, and its routine use in the clinical care of HIV-1–exposed infants and young children has been supplanted by HIV-1 NAATs.

1.2 statement of the research problem

Enzygnost® anti-HIV-1/2 Plus that has been used in the previous HIV ELISAs algorithm for more than fifteen years was withdrawn from the market since 2009. A new HIV Antigen/Antibody combination ELISA, Enzygnost HIV Integral II has been introduced and is now available in the market since 2009. However, this has not been evaluated to determine the performance in local settings and the data is lacking.

Importance of the evaluation information is to reduce false positive and false negative results where as the issue of false positives can bring back non ill people into more sophisticated and more expensive tests and this can be a burden to health care systems and anxiety and worry induced into people who have been told that they have tested positive. False negatives results erroneously inform that the results are negative and if the disease is a serious like HIV, and which effective intervention is available, the problem is indeed critical.

HIV ELISA testing algorithm that will reduce number of false positive and false negative results is needed so as to reduce the use of Western blot for serological confirmation as it is expensive, time-consuming and has technical disadvantages, such as lack of standardization in performance, variability in quality of strips and difficulty in interpreting reactions and the presence of indeterminate reactions.

1.3 RATIONALE OF THE STUDY

It is generally recommended to evaluate the assay in the context that will be used for HIV diagnosis of HIV infection. In Tanzania, ELISAs are used in Zonal and National reference laboratories, National Blood Transfusion Centre (NBTS), and Research Institutions. Data on evaluation of the ELISA combination assays is needed to determine their performance in screening the HIV infections from blood donors and also to reduce infections from mother to children. Currently, there is no National HIV ELISA testing algorithm in place. This study will suggest an algorithm that will give a sensitivity and specificity of 100% by comparing the performance characteristics of the assays that will be evaluated in the current study.

1.4 OBJECTIVES

1.4.1 Broad

To evaluate the performance of HIV Antigen/antibody combination ELISA in Tanzania.

1.4.2 Specific

i. To determine the sensitivity of the HIV antigen/antibody combination ELISA.

ii. To determine the specificity of the HIV antigen/antibody combination ELISA.

iii. To determine positive predictive value (PPV) and negative predictive value (NPV) of the HIV antigen/antibody combination ELISA.

iv. To assess the operational characteristics of three different HIV antigen/antibody combination ELISA.

v. To formulate HIV ELISA testing algorithms that could be used as an alternative HIV testing strategy for lab diagnosis of HIV infections

CHAPTER TWO

2.0 METHODOLOGY

2.1 Study design

This was a cross-sectional study design of blood samples from National Blood Transfusion Centre (NBTS), Voluntary Testing and Counselling (VCT), Antenatal Clinic (ANC) and Care and Treatment Centre (CTC) in Muhimbili National Hospital, conducted between October 2011 and January 2012.

2.2 Study settings

The study was conducted in three sites of Muhimbili National Hospital, Antenatal Clinic, Voluntary Counselling and Testing (VCT), Care and Treatment Centre (CTC) and National Blood Transfusion Services (NBTS) Eastern Zone centre located in Dar es Salaam.

2.3 Study population

The study involved blood samples from blood donors at National Blood Transfusion Service (NBTS) in Dar es Salaam, blood samples from attendees at Voluntary Counseling and Testing (VCT) – MNH, attendees at antenatal clinic-MNH and attendees at Care and Treatment Centre (CTC)

2.4 Study duration

The study was conducted between October 2011 and January 2012

2.5 Sample size estimation

$N= Z^2 X p(1-p)/ e^2$, where,

Z= Z score, 1.96

p= prevalence of HIV in blood donors, 6.5% (HIV/AIDS/STI Surveillance report Number 21- Ministry of Health and Social Welfare)

e= margin error, 2%

Therefore; sample size included 600 blood samples.

2.6 Sampling technique

A systematic random sampling was used to collect blood samples between October and November 2011. One hundred and thirty leftover blood samples from pregnant

women attending Antenatal Clinic (ANC), 140 left over blood samples from blood donors and 200 leftover blood samples from HIV positive attendees at Counselling and Testing Centre (CTC) were collected. Leftovers of 130 blood samples from clients attending Voluntary Counselling and Testing (VCT) whose samples had been submitted for HIV testing lab were also collected. In each of the group (ANC, VCT, CTC and NBTS), 10 blood samples were sampled in a manner of selecting every 5^{th} specimen among first 50 blood samples that were collected from the patients daily. The left over blood samples after the routine testing were taken without the patient identifiers. Only the vacutainers that contained more than 5mls of the leftover blood were enrolled.

2.7 Data management

2.7.1 Source of data

At the end of the clinic day, the specimens labelled with numbers were collected from individual clinic. Dispatch book were signed to ensure accountability of the specimens. All specimens collected in a day were recorded in the standardized form (which included date of specimen collection, demographic information of the patients i.e. sex and age, serial number). The HIV serostatus of samples was unknown prior to evaluation. Specimens were taken to National reference laboratory where they were centrifuged and separated. Sera were transferred into the special vials, labelled and were stored in the freezer until the day they were tested.

Data for performance appraisal done by testing laboratory technologists based on the following characteristics in the checklist: number of steps in the test procedure, clarity of kit instructions, kit and reagent packaging and labelling, and ease of performance were also obtained and recorded.

2.7.2 Laboratory investigations

Laboratory screening was performed at National Health Quality Assurance and Laboratory Training Centre (NHQALTC), Dar es Salaam. Each blood sample was tested using the three Antigen/antibody combination based ELISA. Testing on each assay was performed using serum according to manufacturer's instructions of individual test kits. HIV results (optical densities) of individual samples were

recorded automatically by the computer that is directly connected to the ELISA reader. The computer automatically interpreted the positive, negative or inconclusive results. Repeat testing was done on any sample that gave invalid/equivocal results according to manufacturer's interpretation of results. Repeat testing was also recorded. Inno-Lia HIV I/II immunoblot assay was used as a reference method. All samples that were reactive on all or any HIV ELISA assay and 10% of non reactive samples randomly selected were confirmed on the Inno-Lia HIV I/II assay.

Enzygnost HIV Integral II Antibody/ Antigen (Siemens, Germany), was an enzyme linked fluorescent immunoassay based on the simultaneous determination of HIV p24 antigen and HIV specific antibodies. The immunoglobulin's in the test sample reacted with the recombinant proteins/ synthetic peptides of HIV1, HIV2 and HIV1 (subtype O) with which the wells of the micro titration plate were coated. At the same time, any HIV p24 antigens presented in the test sample bound to the monoclonal HIV p24 antibodies were attached to the wells.

After removal of the unbound sample constituents, the specifically bound antibodies were labelled (antigen sandwich) in the second step with biotin conjugated recombinant proteins/ synthetic peptides of HIV1, HIV2 and/ or HIV1 (subtype O). Simultaneously, any bound HIV antigens were labelled by biotin-conjugated monoclonal HIV p24 antibodies (antibody sandwich).

The unbound biotin conjugates were then removed and then, in the third step, conjugate 2 (strep- tavidin/POD) reacted with the biotin conjugates specifically bound to the solid phase. The excess conjugate 2 was then removed and the enzyme activity of the bound conjugate determined (blue reaction). The enzymatic conversion of the chromogen was interrupted by the addition of stopping solution POD (yellow colour reaction) and a first measurement of fluorescence was performed at a wavelength of 450 nm. The colour intensity was proportional to the concentration of HIV p24 antigen and HIV antibodies presented in the sample. At the end of the assay, results were automatically calculated by a reader machine in relation to the standards and printed.

Murex HIV antigen/antibody (Abbott Murex, UK), was based on micro wells coated with synthetic peptide representing an immunodominant region of HIV-1 (O), recombinant protein derived from the envelope proteins of HIV-1 and HIV-2 and an HIV pol protein and monoclonal antibodies raised against p24 of HIV-1. 100 µl of test specimens and control sera were incubated for 60 min in the wells and reactive HIV core and/or antibodies to HIV in the sample or control sera bound to the antibodies and/or antigens on the microwell plates; sample and any excess antibodies were then removed by five buffer washes. In a subsequent step, Conjugate was added to the wells and incubated for 30min which in turn was bound to any reactive HIV core and/or specific antibody already bound to the reagents on the well. Samples which did not contain reactive core antigen or specific antibody would not cause the Conjugate to bind to the well. The conjugate contained recombinant antigens and peptides from HIV-1 group M gp41 and *pol*, HIV-1 group O gp41, HIV-2 gp36, and monoclonal antibodies to HIV-1 p24 conjugated with horseradish peroxidase. Unbound Conjugate was removed away by five buffer washes and the amount of conjugate bound was determined by of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate and hydrogen peroxide added to the wells and incubated for 30min. Wells with bound Conjugate developed a blue green colour which was converted to an orange colour which was read at 450nm after the reaction had been stopped with sulphuric acid. All incubations were carried out at 37°C and addition of each reagent was monitored by a colour change that could be either visually observed or monitored by a spectrophotometer. The simultaneous detection of HIV p24 antigen and HIV antibody in a sample was achieved based on binding of the antigen and an antibody to the solid phase and was proportional to horseradish peroxidase activity. The cut off was calculated as the mean of negative control values plus 0.15. The overall mean must be less than 0.150 for the run to be valid

Vironostika HIV Uniform II antigen/antibody (Biomerieux, the Netherlands), was an ELISA based on a one step ''sandwich'' principle. A mixture of HIV antigens and HIV antibodies coupled to horseradish peroxidise (HRP) served as the conjugate with tetramethylbenzidine (TMB) and peroxidise as the substrate. Upon

completion of the assay, the development of colour indicated the presence of HIV antibody or HIV antigen, while no or low colour development suggested the absence of HIV antibodies or antigen.

Specifically, microelisa wells were coated with HIV-1 gp 160, HIV-1 ANT70 peptide, HIV-2 env peptide (amino acids 592-603) and anti-HIV-1 p24. Each microelisa well contained an HRP-labelled conjugate sphere of the same HIV-antibody/antigen mixture. The specimen diluent, which was added to the wells first, dissolved the conjugate sphere. Then the test sample or appropriate control containing HIV antigen or antibody was incubated in the microelisa wells. If HIV-1 and/or HIV-2 antibody was present in the sample a solid phase antigen/anti-HIV/ enzyme labelled antibody complex was formed. Followed with a wash procedure and incubation with TMB substrate, colour developed which turned yellow when the reaction was stopped with sulphuric acid. If anti-HIV-1, anti-HIV-2, anti-HIV-1 group O and/or HIV antigen was present in the sample, an intense colour developed. However, when the sample was free of anti-HIV and HIV antigen, no or low colour formed with the addition of substrate.

INNO-LIA HIV I/II immunoblot (Innogenetics, Belgium)

The wells of polystyrene microtiter plates were coated with human polyclonal antibodies to HIV. The test sample containing HIV antigen was incubated in a well together with a mixture of biotinylated anti-p24 monoclonal antibodies. After incubation the biotin bound with peroxidase-conjugated streptavidin. Incubation with the peroxidise substrate and chromogen resulted in a blue colour which turned to yellow when sulphuric acid was added. If a sample contained no HIV antigen, the labelled antibody could not bind specifically to the HIV p24 antigen so only a low background colour developed.

Evaluation panels

There were two types of specimen panels included in the evaluation; fresh blood specimens and commercial seroconversion specimens AU PRB945. Seroconversion specimens were used to detect any presence of HIV p24 antigen prior to anti- HIV

seroconversion. It is likely that, assay that were able to detect p24 antigen reacted earlier than assays that detected only antibodies.

2.7.3 Data entry

Data was double entered and checked for accuracy before analysis.

2.7.4 Statistical analysis

The data was analyzed using Epi info 3.5.1 and Microsoft excel software. Sensitivity, specificity, PPV and NPV with 95% CI of each and combined HIV antigen/antibody ELISAs were determined. INNO-LIA HIV I/II immunoblot assay was used as a confirmatory assay for HIV ELISAs. Indeterminate results from immunoblot assay were excluded from the analysis. The formula for calculation of sensitivity, specificity and predictive values is represented diagrammatically in Table 1.

Table 1: Calculation of sensitivity, specificity and predictive values

		True HIV status		
		+	-	
Results of assay under evaluation	+	a True positives	b False positive	a + b
	-	c False negatives	d True negatives	c + d
		a + c	b + d	

Sensitivity= a/(a+c)

Positive predictive value= a/(a+b)

Specificity=d/(b+d)

Negative predictive value=d/(c+d)

Sensitivity

Is the ability of the assay under evaluation to detect correctly sera that contain antibody to HIV (reference assays positive). Thus sensitivity is the number of true positive sera identified by the assay under evaluation as positive (a), divided by the number of sera identified by the reference assays as positive (a+c), expressed as a percentage.

Specificity

Is the ability of the assay under evaluation to detect correctly sera that do not contain antibody to HIV (reference assays negative). Thus specificity is the number of true negative sera identified by the assay under evaluation as negative (d), divided by the number of sera identified by the reference assays as negative (b+d), expressed as a percentage.

Positive Predictive Value (PPV)

The probability that when the test is reactive, the specimen does contain antibody to HIV. This may be calculated in two ways:

1. Using the simple formula a/(a+b) which will give an approximate value.
2. Using the more precise formula which takes the prevalence of HIV in the population taking into account:

$$PPV = \frac{(\text{Prevalance})(\text{Sensitivity})}{(\text{Prevalance})(\text{Sensitivity})+(1-\text{prevalence})(1-\text{specificity})}$$

Negative Predictive Value (NPV)

The probability that when the test is negative, a specimen does not have antibody to HIV. This may be calculated using:

1. The simple formula d/(c+d) which will give an approximate value.
2. The more precise formula which takes the prevalence of HIV in the population into account:

$$NPV = \frac{(1-\text{Prevalance})(\text{Specificity})}{(1-\text{Prevalance})(\text{Specificity})+(\text{prevalence})(1-\text{sensitivity})}$$

Confidence Limit

The 95 % Confidence Limit of the calculated sensitivity and specificity are given in parenthesis and will be calculated using the formula:

$$p \pm 1.96 \sqrt{\frac{P(1-P)}{N}}$$

Where P is the sensitivity or specificity
where N is the number of sera analysed

95% confidence limits are a means of determining whether observed differences in Sensitivity or specificity between assays is significant or not.

Seroconversion sensitivity

The ability of all assays under evaluation to detect early antigen or antibody was assessed using a commercial seroconversion panels.

Criteria for inclusion into the National algorithm

The criteria for inclusion of an individual HIV ELISA assay into the National algorithm were initial sensitivity of 100% and initial specificity of ≥ 99%.

2.7.5 Assessment of operational characteristics

All the three antigen/antibody combination HIV ELISA were evaluated for operational aspects such as storage conditions (2-8 ^{0}C), incubation temperature (37^{0}C), and need of special equipment, number of sera per run, price per kit and the performance appraisal of the assays on technical aspects using 10 technicians that performed the testing assays. These assessments, along with other selected assay characteristics, contributed to an overall appraisal of each assay's suitability for use in the laboratories. To enable comparison between assays, an arbitrary scoring system was used to rate specified assay characteristics as detailed in appendix 4.

2.8 Ethical considerations

Ethical approval was obtained from MUHAS Senate Research and Publication Committee. Permission for blood collection was granted by MNH and NBTS administration.

CHAPTER THREE

3.0 RESULTS

3.1 Demographic characteristics of the samples

Among the study participants 70% (421) were females, Majority of the participants aged between 20 to 30 years where as 59 %(355) are employed and 71 %(424) are married. Other socio-demographic characteristics of the study population are summarised in table 2 below,

Table 2: Socio-demographic characteristics of the study population samples (N=600)

Characteristics	Blood donors n=140 n (%)	Pregnant women n=130 n (%)	VCT n=130 n (%)	CTC n=200 n (%)	Total
Sex					
Male	53 (38)		49 (38)	77 (39)	179 (30)
Female	87 (62)	130(100)	81 (62)	123 (61)	421 (70)
Age groups (yrs)					
< 20	17(12)	3(2)	2(1.5)	-(0)	22(4)
20-30	49(35)	57(44)	68(52.3)	42(21)	216(36)
31-40	43(31)	41(32)	39(30)	69(35)	192(32)
≥ 40	31(22)	29(22)	21(16.2)	89(44)	170(28)
Marital status					
Single	51(36)	38(29)	54(42)	33(17)	176(29)
Married	89(64)	92(71)	76(58)	167(83)	424(71)
Education					
None	3(2)	2(1.5)	7(5)	11(5.5)	23(4)
Primary	103(73)	87(67)	65(50)	110(55)	365(61)
Secondary/higher education	35(25)	41(31.5)	58(45)	79(40)	213(35)
Occupation					
Employed	104(74)	47(36)	58(45)	146(73)	355(59)
Unemployed	11(8)	79(61)	43(33)	51(26)	184(31)
Student	25(18)	4(3)	29(22)	3(1)	54(10)

3.2 Sensitivity, Specificity, PPV and NPV of the Assays evaluated

Among 600 serum samples that were included in the study, 4 samples gave indeterminate results on immunoblot assay (1 positive from ANC attendee and 3 negative from one blood donor and two ANC attendees) and were excluded in the analysis. A total of 209/596 (35.1%) serum samples were confirmed HIV positive. Of these, the prevalence of HIV infection was 2.3% (3/130), 2.3% (3/127), 2.2% (3/139) and 100% (200/200) for VCT clients, ANC attendees, blood donors and CTC patients, respectively.

Three hundred and eighty seven (64.9%) were HIV negative samples. The sensitivity of each assay evaluated was 100% (95% CI; 98.3–100%). All reactive samples were concordantly positive among all the three assays.

Table 3 summarizes results of initial and repeat specificity of individual HIV ELISAs. The Enzygnost HIV Integral II and Murex HIV Ag/Ab had specificity of 100% (95% CI; 99.1-100%) followed by Vironostika assays. Initially, Vironostika yielded a specificity of 99.2% (Table 3). Three samples were false positive on Vironostika Ag/Ab ELISA. On repeat testing specificity of Vironostika increased to 99.5% as shown in table 3.

Table 3: Specificity of HIV ELISAs at initial and repeat testing

Assay	Specificity (n=387)			
	Initial testing		Repeat testing	
	Non reactive	%(95%CI)	Non reactive	%(95%CI)
Enzygnost HIV Integral II	387	100(99.1-100)	-	-
Murex HIV antigen/antibody	387	100(99.1-100))		
Vironostika HIV Uniform II Antigen/antibody	384	99.2(97.4-99.7)	385	99.5(98.2-99.9)

The mean (optical density (OD)/cut-off (CO)) ratios of samples which were confirmed to be HIV positive and of those which were false positive are summarized in Table 4. The mean OD/CO values of HIV positive samples on all

the combination assays were above 10. Samples which gave false positive reactions on Vironostika had lower mean OD/CO values compared to the HIV positive samples.

Table4: Distribution of reactivity's Optical density (OD)/Cut off (CO) ratio by assay

Assay	Confirmed HIV positive			HIV false positives		
	N	Mean (range)	95% CI	n	Mean (range)	95% CI
Enzygnost HIV Integral II	209	13.7(5.3-31.0)	13.1-14.5	-	-	-
Murex HIV Ag-Ab	209	12.2 (5.2-16.0)	12.0-12.4	-	-	-
Vironostika HIV Uniform II Ag-Ab	209	15.5(12.1-9.0)	15.4-15.7	3	5.7 (1.3-16.5)	-1.6-12.80

The positive predictive value (PPV) for the Enzygnost HIV Integral II and Murex HIV Ag-Ab were 100% (95%CI; 98.3- 100%) and 98.5% (95%CI; 95.28-99.49%) for Vironostika HIV Uniform II Ag-Ab. After repeat testing PPV for Vironostika HIV Uniform II was 99% (95%CI; 96.6-99.9%). The negative predictive value (NPV) was 100% (95%CI; 99.06-100) for all the assays.

Results of testing of a seroconversion panel are summarized in table 5. Enzygnost HIV Integral II detected HIV infection seven days since first bleed whereas Murex detected first bleed in day 13 and Vironostika detected first bled in day 15. The 15 and 20 days samples were reactive on all three Ag/Ab combination assays.

Table 5: Results on HIV-1 Seroconversion panel AU PRB945

Panel member ID #	Days since 1st bleed	Specimen absorbance/ cut off ratio			Band pattern
		Enzygnost	Murex	Vironostika	Inno-Lia
PRB945-01	0	0.6	0.4	0.5	No bands
PRB945-02	3	0.7	0.5	0.5	No bands
PRB945-03	7	1.2	0.7	0.6	No bands
PRB945-04	13	10.7	6.3	0.9	No bands
PRB945-05	15	16.7	13.6	7.9	Indeterminate
PRB945-06	20	20.7	15	13.7	Gp41, p24

Ratios ≥ 1.0 are considered reactive.

The specificity of various combinations of ELISAs in HIV testing is shown in Table 6. Initial testing using Murex antigen/antibody assay or Vironostika Antigen/antibody assay followed by testing of reactive samples on the Enzygnost Antigen/antibody assay gave specificity of 100%. Initial testing by Vironostika Antigen/antibody assay followed by Murex Antigen/antibody assay gave 100% specificity.

Table6: Specificity of combinations of ELISAs in HIV testing

Assay	Specificity (n=387)	
	Non reactive	% (95% CI)
Murex Ag/Ab + Enzygnost HIV Integral II	387	100 (99.1-100)
Vironostika Ag/Ab + Enzygnost HIV Integral II	387	100 (99.1-100)
Vironostika Ag/Ab + Murex Ag/Ab	387	100 (99.1-100)
Enzygnost HIV Integral II+ Murex Ag/Ab	387	100 (99.1-100)
Enzygnost HIV Integral II+ Vironostika Ag/Ab	385	99.5(98.2-99.9)
Murex Ag/Ab+ Vironostika Ag/Ab	385	99.5(98.2-99.9)

Table 7: Performance characteristics of assays for the specific categories of the study participants

	Study participants							
	ANC		VCT		NBTS		HIV/AIDS clients	
	Sensitivity n(%)95%CI	Specificity n(%) 95%CI	Sensitivity n(%)95%CI	Specificity n(%)95%CI	Sensitivity n(%)95%CI	Specificity n(%)95%CI	Sensitivity n(%)95%CI	Specificity n(%)95%CI
Vironostika	3/3 (100) 29.2-100	122/124 (100) 94.3-99.8	3/3 (100) 29.2-100	127/127 (100) 97./-100	3/3 (100) 29.2-100	136/136 (100) 97.3-100	3/3 (100) 29.2-100	
Murex	3/3 (100) 29.2-100	124/124 (100) 97.1-100	3/3 (100) 29.2-100	127/127 (100) 97./-100	3/3 (100) 29.2-100	136/136 (100) 97.3-100	3/3 (100) 29.2-100	
Enzygnost	3/3 (100) 29.2-100	124/124 (100) 97.1-100	3/3 (100) 29.2-100	127/127 (100) 97./-100	3/3 (100) 29.2-100	136/136 (100) 97.3-100	3/3 (100) 29.2-100	

3.3 Operational characteristics of the HIV antigen/antibody ELISAs evaluated

All the three antigen/antibody combination HIV ELISA were evaluated for operational aspects. Storage conditions (2-8 ^{0}C); incubation temperature (37^{0}C) and need of special equipment were all the same for all the three Ag/Ab combination assays. Both Enzygnost HIV Integral II and Vironostika carry 1-92 sera per run. The price for individual assay for the local vendors available in the country for the combination assays is as summarized in table 8 below

Table 8: Prices for HIV ELISAs

Assays (960tests)	**Price in USD.**
Vironostika Ag/Ab	1,219
Murex Ag/Ab	2,382
Enzygnost HIV Integral II	4685
Vironostika Ag/Ab + Murex Ag/Ab	3,600
Vironostika Ag/Ab + Enzygnost HIV Integral II	5904
Murex Ag/Ab + Enzygnost HIV Integral II	7067

Table 9 summarize performance appraisal of HIV ELISA assays by the testing laboratory technologists,

Table 9: Performance appraisal of HIV ELISAs

Name of the assay	**Enzygnost HIV Integral II**	**Murex HIV Ag/Ab**	**Vironostika HIV Uni-Form II Ag/Ab**
Number of steps in the test procedure	Longer	Fairly	Fairly
Technical complexity	Very easy	Very easy	Very easy
Clarity of the kit instructions	Good	Good	Good
Kit, reagent packaging and labelling	Good	Good	Needs improvement
Ease of interpretation of the results	Very easy	Very easy	Very easy
Duration/ time of testing	Too long	Fairly	Fairly

CHAPTER FOUR

4.0 DISCUSSION

It is important to evaluate performance of HIV Antigen/antibody combination based HIV ELISA continuously to ensure the correct diagnosis of HIV infection especially when the new HIV ELISAs have been developed and introduced in the market. The fourth generation HIV ELISAs are made in a way that HIV p24 antigen is combined with anti-HIV-1 and anti-HIV-2 for early diagnosis of HIV infection. Enzygnost HIV Integral II is among the fourth generation ELISA that was introduced in Tanzania in 2009. In Tanzania ELISA methods are used in Zonal and reference laboratories, National Blood Transfusion Centre (NBTS), Research Institutions. The present evaluation determined the performance characteristic of the three HIV Antigen/antibody ELISA.

The study findings showed that all the three combination ELISAs had 100% sensitivity. A previous study conducted in Germany to evaluate the Enzygnost Integral showed 100% sensitivity for the detection of antibodies to HIV-1, groups M and O, and HIV-2 [11]**.** The antigen /antibody ELISA are designed to be highly sensitive to enable detection of HIV infection as this will provide appropriate and accurate results that will enable distinction between people with infection and those without infection in a health care setup. In another evaluation conducted in UK using five cases detectable only by 4th generation assays, Murex Ag/Ab exhibited sensitivity of 100% which is in agreement with the finding of the present study [33]

In the present study, Enzygnost exhibited the specificity of 100% which is in contrast of the findings from the previous evaluation done in Germany using 4002 unselected blood donors, where the specificity of Enzygnost Integral II was found to be 99.78% initially and 99.80% after retesting, [11]. Based on evaluation of 388 samples, the specificity for Murex Ag-Ab combination assay (100%) was higher as compared to a specificity of 99.4% on a previous study conducted in Tanzania in 2006 [9]. In another evaluation which used 9,289 samples [20], Murex showed a specificity of 99.7% which is lower than the specificity in the present study. The specificity of the Vironostika was found to be lower (99.2% at initial testing and 99.5% at repeat testing) compare to the specificity of Enzygnost Integral II and

Murex. In another evaluation, the specificity for the Vironostika was 98.9% at initial testing and 99.4% at repeat testing [9] which is lower as compared to the present study. One serum that gave negative result after repeat testing increased specificity of Vironostika Ag-Ab to 99.5%. There is a possibility that the initial false positive results obtained when testing these specimens on Vironostika was due to technical error. After repeat testing on the Vironostika, specificity was still lower compared to the Enzygnost HIV Integral II and Murex Ag-Ab; both of the lower specificities were contributed to the false positives results, the false positive results may have been contributed by cross reactions in the sera. This is similar to the previous study conducted in Tanzania [9].

In the present study, samples that were confirmed HIV positive had high mean OD: CO ratio on all the three assays (13.7, 12.2 and 15.2 in Enzygnost, Murex and Vironostika respectively) compared to samples that gave false positive results reaction on Vironostika assays (5.7). Recent studies have shown that samples which are repeatedly reactive in sequential antibody screening assays but which are WB negative should be interpreted with caution regarding their HIV serostatus because some HIV-1 antibody assays have been found to be reactive earlier in the infection process than WB [2, 34]. It is therefore important to take special precautions in those samples showing low OD: CO values

In the present study, the Enzygnost HIV Integral II assay detected HIV infection seven days since first bleed, compared to the Murex and Vironostika Ag-Ab ELISAs which were able to detect infection on thirteenth day after infection. This is similar to comparative evaluations of the fourth generation HIV screening assays performed on the seroconversion panels which have shown that the fourth generation HIV screening assays reduced the diagnostic window of HIV infection by an average of seven days and that they permit an earlier diagnosis of HIV infection by detecting p24 antigen which may be present in samples from individuals with recent HIV infection prior to seroconversion [10, 11, 13-15, 17-19, 35]. It is important to utilize Antigen/antibody combination based ELISA especially in public health arena, where secondary prevention programs involve early disease detection and intervention. Results from the present study indicated

that detection is 7–15 days earlier with the new combined antigen/antibody HIV ELISA, as this will prevent transfusing infected blood from donated blood and also will reduce infections from mother to child. The gold standard that was used could not detect any bands early during seroconversion because it has limited ability to detect antibodies during early HIV infection. Western blot assay can detect antibody at least 3 weeks from the initial HIV infection.

It is necessary to utilize a combination of HIV antigen-antibody assays of HIV infection before transfusion and for diagnosis of HIV in individuals suspected of infection [30]. Even if screening will involve combination of Ag-Ab assays, there is still a need to have a test that will resolve the discordant results because the two assays can lead to false positive or false negative results [9, 30]. Although both Murex and Enzygnost had 100% sensitivity, 100% specificity and 100% PPV of 100%, the study suggests Murex to be used as first screening assay followed by testing reactive samples on Enzygnost due to high cost of Enzygnost. Lower specificity of Vironostika suggests either screening with Vironostika followed by testing reactive samples on Enzygnost or Murex. Samples giving repeatedly discordant results on the Ag/Ab combination assays to be tested with Western blot, however for resource limited country like Tanzania, use of western blot are very costly; hence it is suggested to collect another sample for retesting.

Performance characteristic of the assays for the specific categories of the study participants showed that a combination comprising of Vironostika as a first test followed by Murex or Enzygnost as a second test would be suitable for VCT, Blood donors, ANC and HIV/AIDS patients taking into account the element of cost. All the assays have sensitivity of 100%, any of the three can be used as the first assay although Enzygnost HIV Integral II is very expensive compared to Vironostika and Murex

All the kits have same operational characteristic in terms of Storage conditions (2-8 ^{0}C), incubation temperature (37^{0}C) and need of special equipment. When comparing the cost of the kits per these assays, Murex and Vironostika are preferable to Enzygnost as Enzygnost is expensive compare to Murex and

Vironostika, however, both Enzygnost HIV Integral II and Vironostika carry 1-92 sera per run while Murex carry 1-90 sera per run. Performance appraisal revealed highest score for easiness for all the assays; however duration of time of testing and number of steps for Enzygnost HIV Integral II were reported to be longer compared to the other two assays evaluated.

Based on performance characteristics, all the assays qualified for inclusion in the National algorithm. Seroconversion results revealed better performance for Enzygnost as it detected HIV infection seven days earlier than Murex and Vironostika; however operational characteristics support Murex and Vironostika to be preferable to Enzygnost

4.1 LIMITATIONS OF THE STUDY

The present study used only one panel of seroconversion panel (AU PRB 945). Different assays perform differently when using a variety of seroconversion panels. It would have been preferred to use various seroconversion panels to broaden the evaluation on the performance of these assays. This assay could not differentiate if the reactive samples were HIV 1 or HIV 2, as this would have been able to find out the distribution of these two strains within the population of Tanzania.

CHAPTER FIVE

5.0 CONCLUSION

Conclusively, initial testing using either Vironostika or Murex HIV antigen/antibody combination ELISA followed by testing of reactive samples on the Enzygnost HIV Integral II gave sensitivity, specificity, PPV and NPV of 100% with reduced window period.

Based on the current evaluation all the assays have good performance characteristics and all the assays qualified for inclusion in the National algorithm. Regarding on operational characteristics, an element of cost was considered when recommending for the algorithms to be used. The recommended algorithms are Vironostika Ag/Ab as initial testing followed by testing reactive samples on Murex Ag/Ab; Vironostika Ag/Ab as initial testing followed by testing reactive samples on Enzygnost HIV Integral II; and Murex as initial testing followed by testing reactive samples on Enzygnost HIV Integral II

5.1 RECOMMENDATIONS

This evaluation showed that all the three assays have good performance characteristics and operational characteristics. Taking on account the element of cost, the study suggests a combination comprising of Vironostika Ag/Ab as initial testing followed by testing reactive samples on Murex Ag/Ab or Enzygnost HIV Integral II to be used as an alternative confirmatory testing strategy with an advantage of reduced diagnostic window period for screening of donated blood at the National and Zonal blood transfusion centres and in lab diagnosis of HIV infection at the Regional, National and Zonal Hospital Laboratories.

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APPENDICES

Appendix 1: Time plan (Gants chart)

ACTIVITIES	Jun-11	Jul-11	Aug-11	Sep-11	Oct-11	Nov-11	Dec-11	Jan-12	Feb-12	Mar-12	Apr-12	May-12	Jun-12	Jul-12
Proposal development														
Submission of final Proposal														
Ethical clearance														
Preparation for field work														
Data collection														
Data Analysis														
Dissertation writing														
Preparation for defense														
Defense of dissertation														

Appendix 3: GUIDE FORM- FOR DATA COLLECTION

Data collection form for Enzygnost HIV Integral II/ Murex HIV Ag/Ab/ Vironostika HIV II Ag/Ab/

	Test results for ELISAs	**Western blot**	**Results**	**True +ve/True -ve**
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
582				

Test results for HIV ELISAs

(N= 600)				
	Characteristics	**Data**		
	Assay	**Non reactive**	**Reactive**	**Indeterminate**
1	Inno Lia (Confirmatory test)			
2	Enzygnost HIV Integral II			
3	Murex HIV Ag/Ab			
4	Vironostika HIV ii Ag/Ab			

Appendix 4: Data collection form- performance appraisal

Operational characteristics of all Assays under evaluation				
Name of the Institution..... Date.....				
NAME	**Score**	**Enzygnost HIV Integral II**	**Murex HIV Ag/Ab Combination**	**Vironostika HIV Uni- Form II Ag/Ab**
Number of steps in the test procedure - 1-2 - 3-5 - >5	 6 3 1			
Technical complexity -Excellent -Very easy -Fairly easy -Complex	 4 3 2 1			
Clarity of the kit instructions -Good -Needs improvement	 2 1			
Kit and reagent packaging and labelling -Good -needs improvement	 2 1			
Ease of interpretation of the results -Unambiguous -Very easy -Fairly easy -Difficult	 4 3 2 1			
Duration/ time of testing -Too short -Fairly -Too long	 3 2 1			
Total(out of possible 21)				
Comments on the kit				

Appendix 5: Dummy tables

Objective 1: Sensitivity and specificity of HIV ELISAs by initial reactivity

SENSITIVITY					
	Assay	Sensitivity (Reactive samples/Positive confirmed samples by W.B)			
		Initial testing		Repeat testing	
		Reactive(n)	Sensitivity% (95% CI)	HIV False positive(n)	Sensitivity % (95%CI)
1	Enzygnost HIV Integral II				
2	Murex HIV Ag/Ab				
3	Vironostika HIV II Ag/Ab				
SPECIFICITY					
	Assay	Specificity (Non-reactive samples/Negative confirmed samples)			
		Initial testing	Repeat testing		
		Non reactive(n)	Specificity % (95% CI)	Non reactive(n)	Specificity % (95%CI)
1	Enzygnost HIV Integral II				
2	Murex HIV Ag/Ab				
3	Vironostika HIV II Ag/Ab				