African Journal of Pharmacology and Therapeutics Vol. 4 No. 3 Pages 101-111, 2015
Open Access to full text available at http://journals.uonbi.ac.ke/ajpt

Research Article

Development of immunoassays for detection of Human Immunodeficiency Virus based on Consensus *env* gp41 Immunodominant Region Peptide from HIV-1 infections in Kenya

James Kimotho[a,*], Zipporah Ng'ang'a [b], Edna Nyairo [a], Missiani Ochwoto [a], Nicholas Nzioka [a], Francis Ogolla [a], and Michael Kiptoo [a]

[a] *Kenya Medical Research Institute, Kenya*
[b] *Institute of Tropical Medicine, Jomo Kenyatta University of Agriculture and Technology, Thika, Kenya*

* **Corresponding author:** Production Department, Kenya Medical Research Institute, P. O. Box 54840–00200, Nairobi, Kenya; **Tel**: +254-20-272254; **Fax**: +254-20-2720030; **Email**: jhkimotho@kemri.org

Background: Human Immunodeficiency Virus (HIV) is characterized by high rates of genetic variability *in vivo* that could affect the performance of the HIV antibody-based detection kits.

Objective*:* This study aimed at developing immunoassays for HIV based on Consensus *env* gp41 Immunodominant region (IDR) from HIV infections in Kenya.

Methods*:* HIV RNA was extracted from 91 samples collected from 5 regional blood transfusion centers in Kenya. The RNA was reverse transcribed, sequenced in the *env* gp41-Immunodominant Region (IDR) and the Consensus sequence generated used to synthesize corresponding peptide. The Global HIV *env*gp41-IDR Consensus peptide was obtained from the literature and also synthesized. The two peptides were used to separately develop HIV immunoassays based on Enzyme-linked Immunosorbent Assay (ELISA) and Lateral Flow Assay (LFA) platforms and the performance of developed assays was evaluated. The same HIV *env* gp41 IDR peptides were used to develop ELISA-based immunoassays for determination HIV Incidence / Recency.

Results*:* The study did not find significant difference between the performance of the immunoassays that were developed with Consensus *env* gp41-IDR peptide (Kenya) and those developed using Consensus *env* gp41-IDR peptide (Global). However, the study found a significant difference between the performance of HIV ELISA for HIV Incidence testing that was developed with Consensus *env*gp41-IDR peptide (Kenya) and that which was developed using Consensus *env*gp41-IDR peptide (Global) with the former displaying superior performance.

Conclusions*:* The developed immunoassays demonstrated that both Consensus *env* gp41-IDR peptides (Kenya and Global) could be used to develop HIV immunoassays but Consensus *env* gp41-IDR peptide (Kenya) could be more suitable for development of HIV Incidence assays in Kenya.

Keywords: HIV, Consensus sequence, *env* gp41-Immunodominant Region, Immunoassays

Received: June, 2015
Published: September, 2015

ISSN 2303-9841

1. Introduction

HIV infections continue to be a great menace to the lives of many people globally with 35 million living with HIV/AIDS and more than 3 million new cases being reported each year (UNAIDS, 2013). In Kenya, the overall HIV prevalence is 6.0% with the prevalence of 2.2% among young people (15-24yrs) and 1.6 million people living with AIDS (NASCOP, 2014).

HIV genome, with about 9749 nucleotides, encodes for three structural genes (*pol, gag,* and *env*) and six regulator genes (*nef, vpu, vif, tat, rev* and *vpr*). The *env* gene encodes for gp160 that is enzymatically cleaved into two peptides, *env* gp120 and *env* gp41. The *env* gp41 peptide contains the Immunodominant Region (IDR) in the amino-terminal portion from which more than 99% of HIV-1infected individuals produce antibodies directed to the region (Kartekayan et al, 1998; Los Alamos, 2015; Masciotra et al*,* 2000). HIV is known to exhibit high rates of genetic variability *in vivo* due to the poor proof-reading mechanisms during reverse transcription (that result in mutations and recombinations) and rapid viral turnover in patients with an active disease. These mutations and recombinations have resulted in evolution of HIV-1 into various groups ("major" group M that constitutes more than 90% of all HIV-1, the "Outlier" group "O", N and P). Group M is further divided into nine genetically distinct subtypes / clades: A, B, C, D, F, G, H, J and K. The virus has also evolved into various Circulating Recombinant Forms (CRFs) and Unique Recombinant Forms (URFs). Majority of the recombinants, though, do not survive for long. The nucleotide variations within the *env* gene are 5 to 15% intra-clade and 15 to 30% inter-clades (Leitner, 1996; Korber et al*,* 1997; Quinones-Mateu et al*,* 1999; Bobkov et al*,* 2004; Plantier, 2009).

Arising from the diversity within the *env* gene of HIV-1, some studies have demonstrated that the performance of various HIV immunoassays have been affected by the prevailing subtypes of HIV infections in a specific region (Thorstensson et al*,* 1998*;* Wei et al*,* 2010;Kilembeet al*,* 2012). A study by Thorstensson et al (1998) compared the performance of 14 commercially available HIV-1/2 immunoassaysfrom patient sera from Tanzania, Sweden and Guinea-Bissau and found that 85.7% (n = 12) of these kits identified correctly all HIV-1 and HIV-2 antibody positive sera and that one Tanzanian HIV-1 antibody positive sample was not detected by two of the ELISAs employing synthetic peptides. Wei et al (2010) also found that HIV kits developed using individual *env* gp41 IDR peptides derived from respective subtypes failed to detect some HIV infections by other subtypes. In Zambia and Rwanda a study by Kilembe et al (2012) found that a new rapid antigen and antibody test could only detect less than 2% of p24 antigen positive samples. It is also known that the main challenge in the current global efforts to develop HIV vaccine is the extensive diversity of HIV *env* glycoprotein and one of the possible solutions that has been considered is to design region-specific vaccines to protect populations infected by a specific viral strains circulating in discrete geographic areas (Salemi, 2011).

However, other studies have demonstrated that the performance of various HIV testing kits were not affected by the prevailing subtypes of HIV infections in a specific region (Masciotra et al*,* 2000; Vallari et al*,* 2010). A study by Masciotra et al (2000) established that Consensus *env*gp41 from group M peptides was able to detect all 130 group M sera subtype A, 21 subtype B, 13 subtype B9, 20 subtype C, 21 subtype D, 14 subtype E, 25 subtype F, and 6 subtype G) resulting in a test sensitivity of 100%. Vallari et al (2010) noted that in spite of the high genetic divergence between HIV-1 groups M and N, all group N infections were detected using five commercial HIV immunoassays.

Most of the studies on the effects of diversity of HIV on their detection have been based on diagnostic/screening kits. However, when Wei et al (2010) were developing their HIV Incidence kit, they found that those kits that were developed using individual *env* gp41 peptides derived from respective subtypes were unable to work well with samples containing other subtypes, and so they had to design multisubtype gp41 recombinant antigen (rIDR-M) in order to improve their results

Amidst the above controversies, this study was designed to develop immunoassays for HIV based on Consensus *env* gp41 IDR peptide from HIV infections in Kenya and to assess the effect this could have on detection of the HIV infections and determination of its Incidence.

2. Materials and Methods

2.1 Study design and Sampling

The study design was the Laboratory-based Experimental Design.

A total of 400 samples (200 HIV positive and 200 HIV negative) were picked from the four Regional Blood Transfusion Centers (uniformly spread among the four centers over a period of three months). This was the minimum sample size that is recommended by World Health Organization (WHO) and Centers for Diseases Control of United States (CDC) for Evaluation of HIV LFA and ELISA (WHO and CDC, 2002). The sample size selected for sequencing was 91 based on the calculations done using Fisher's formula and taking into consideration the HIV prevalence of 6% in Kenya then.

2.2 PCR amplification, sequencing, sequence analysis and peptide synthesis

HIV viral RNA was extracted from 91 HIV positive samples using the QIAamp viral RNA kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol. Primers were picked from the study by Dachraoui et al (2008) and synthesized by Bioneer Corporation (Seoul, South Korea). For reverse transcription (RT) and primary PCR, the primers used were GP40F1 (forward; 5′TCTTAGGAGCAGCAGGAAGCACTATGGG) and GP41R1 (reverse; 5′AACG ACAAAGGTGA GTATCCCTGCCTAA). For the nested PCR, the primers used were GP46F2 (forward; 5′ACAATTATTGTCTGGTATAGTGCAACAGCA) and GP47R2 (reverse; 5′TTAAACCTATCAA GCCTCC TACTATCATTA). Three to 10 µl of the RNA extract were used to synthesize cDNA with primer GP41R1 (20 µM) and the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) following the manufacturer's protocol. The 20-µl cDNA reaction mixture was then added to a PCR mixture containing 50 µM GP40F1 and 30 µM

GP41R1, 1× GeneAmp PCR buffer II, 1.25 mM $MgCl_2$, 1.25 mMdNTPs mix and 2.5U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Foster City, Calif.) and brought to a final volume of 100 µl with sterile distilled water. After initial denaturation at 94 °C for 2 min, 35 cycles of PCR were performed in the GeneAmp 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Each cycle consisted of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s, with a final extension at 72°C for 5 min. For nested PCR, 5 µl of the primary PCR product were added to a 100-µl PCR mixture containing reagents similar to those in the primary PCR, except that the primers were replaced by 25 µM each GP46F2 and GP47R2. The PCR mixture was subjected to 35 cycles under similar conditions as the primary PCR. After PCR, the nested PCR products were electrophoresed in 1.5% agarose gels along with a 100-bp ladder (Gibco, Grand Island, N.Y.) and visualized under UV light by ethidium bromide staining. The Complementary DNA was sent to Macrogen Europe (Amsterdam, Netherlands) for sequencing using a big dye terminator v. 3.1 (PE) and the same primers that were used for initial nested PCR (GP46F2 and GP47R2). The resultant sequences were aligned using CLUSTALW program within MEGA 4 Program (Tamura et al, 2007). The prevailing HIV Consensus sequence was generated by use of the software "Advanced Consensus Maker" in the Los Alamos Database (Los Alamos, 2009). The Global Consensus was obtained from the literature (Plantier, 2009). The Consensus sequence of gp41 IDR (Kenya) established in this study and the Global Consensus gp41 IDR were then sent to LifeTein LLC (Hillsborough, NJ) for synthesis of *env* gp41 IDR peptides.

2.3 Development of ELISA using the synthetic peptide derived from the *env* gp41 region representing the Consensus sequence for HIV in Kenya

The two synthetic HIV *env* gp41 IDR peptides (Kenya: WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTNVP WNSSW and Global: WGIKQLQAR VLAVERYLKDQQLLGIWGCSGK LICTTAVPWNASW) were used to develop ELISA kits. Briefly, polyvinyl plates (ImmulonII; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 5µg of synthetic peptide per ml (100 ml/well) in 0.01 M carbonate buffer (pH 9.6) and incubated overnight at 4°C.The plates were washed 6 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST); excess reactive sites were blocked by the addition of 5% Bovine Serum Albumin in PBST. This blocking buffer was arrived at through a series of optimization steps that involved various types of blocking buffers and their different concentrations. This step was followed by the addition of a 1:100 dilution of each test plasma samples. The plates were incubated overnight at 4°C. After six more washes, Fc-specific, HRP-conjugated goat antibody to human immunoglobulin G (Sigma, St. Louis, Mo.) was added and the plates left at room temperature for 1 hr. They were washed 6 times with PBST after which 100µl of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) / hydrogen peroxide substrate (MP Biomedical, Santa Ana, Calif.) was added and incubated for 30 minutes in the dark. To stop the reaction, 100µl of 1M sulfuric acid (Sigma, St. Louis, Mo.) was added and the Optical Density (OD) measured at 450nm with Multiscan ELISA Reader (Thermo Fisher Scientific, Waltham, Mass.). The cutoff values were calculated by adding 0.1 to the mean optical densities plus 3 standard deviations of normal control sera in the assay. The p24-antibody and *env* gp36 IDR peptides were incorporated and also optimized.

2.4 Development of Lateral Flow Assay (LFA) using synthetic peptide derived from the gp41 region representing the Consensus sequence for HIV in Kenya

The key step in the process of preparation LFA was the preparation viable colloidal gold conjugates (p24-antibody, *env* gp41 IDR peptides (Kenya and Global) and *env* gp36 IDR peptide). These colloidal gold solutions were prepared by controlled reduction of a boiling solution of 0.02% chloroauric acid with 1% Sodium citrate according to the method described by Frens (1973). The LFA was optimized through a series of experiments as described by Crowther et al (1995).

2.5 Development of Incidence/Recency testing system

The Two-well avidity index assays were conducted using ELISA wells coated with 2.5 µg HIV *env* gp41 peptides rIDR-M, Consensus *env* gp41 (Kenya) and Consensus *env* gp41 (Global) respectively as previously described by Wei et al (2010). Briefly, HIV *env* gp41 peptides were reconstituted in 0.1M PBS, applied on ELISA wells and incubated overnight. Plates were washed with PBS containing 0.1% Tween-20 and blocked with 5% nonfat dry milk powder in PBST (milk buffer). A 100 ml aliquot of diluted sample in ratio of 1 to 400 with milk buffer was added to the two wells and incubated for 1hr at 37 °C. The plate was then washed 4 times with 300 µl well of PBST. One well was incubated with dissociation buffer pH 3.0 while the second well (control) was incubated with wash buffer at 37 °C for 15 min. The plate was then washed 4 times with 300ml of PBST. One hundred microliters of goat-antihuman IgG peroxidase, diluted 1:5000 in milk buffer was added and incubated at 37 °C for 30 min, followed by 4 washes with 300 µl of PBST. One hundred microliters of TMB was added and incubated at 25 °C for 15 min. The color development was stopped by the addition of 100 µl of 1N Sulphuric acid to each well and the OD was read at 450 nm. The Avidity Index (AI) was calculated as a ratio of OD of the treated well divided by the OD of the control well, expressed as a percent, with a cut-off score of above 80% being graded as recent (an overall mean duration of recency of 141 days according to the study by Wei et al, 2010)

2.6 Data analysis

The bioinformatics data in this study was analyzed using Los Alamos, NCBI and MEGA bioinformatics suites. Correlation of coefficient, p-values, levels significance, One-way ANOVA and F-Test values of various data were also calculated as applicable.

2.7 Ethical Considerations

The study was approved by KEMRI Ethical Review Committee (**Protocol no. 2170** dated April 2012).

Table 1: The frequency of various types of amino acid substitutions in HIV gp41-IDR peptide (Kenya)

Amino acid and position	Frequency	Unique or common substitution(s)	Amino acid and position	Frequency	Unique or common substitution(s)
A101→S	68	Not unique	Q^{79}→R	1	Not unique
K77→R	31	Not unique	S^{88}→A	1	Not unique
A96→ N	50	Not unique	K^{90}→V	1	Unique
I84→L	17	Not unique	A^{101}→Y	1	Unique
A96→ T	16	Not unique	G^{83}→A	1	Unique
V72→L	15	Not unique	I^{84}→F	1	Unique
T94→P	12	Not unique	R^{99}→W	1	Not unique
A96→T	9	Not unique	V^{77}→I	1	Not unique
R68→S	8	Unique	I^{84}→V	1	Unique
L91→H	7	Not unique	C^{87}→S	1	Unique
V69→I	7	Not unique	Q^{66}→K	1	Unique
A71→G	5	Unique	Q^{66}→H	1	Unique
L91→I	10	Not unique	Q^{66}→R	1	Not unique
K90→R	5	Not unique	R^{74}→S	1	Not unique
A101→T	5	Not unique	L^{91}→R	1	Unique
K77→Q	4	Not unique	P^{98}→R	1	Unique
T94→S	4	Unique	A^{101}→V	1	Unique
K77→V	3	Unique	A^{67}→ T	1	Not unique
V72→I	3	Not unique	R^{68}→E	1	Unique
V69→I	7	Not unique	Q^{66}→ P	1	Not unique
A71→G	5	Unique	A^{96}→ S	1	Not unique
K90→R	5	Not unique	R^{74}→A	1	Not unique
A101→T	5	Not unique	K^{77}→T	1	Not unique
K77→Q	4	Not unique	Q^{80}→R	1	Not unique
T94→S	4	Unique	L^{91}→P	1	Unique
K77→V	3	Unique	L^{70}→Q	1	Unique
V72→I	3	Not unique	G^{83}→R	1	Not unique
A96→F	3	Not unique	R^{68}→K	1	Not unique
A67→T	3	Not unique	Q^{79}→H	1	Unique
I62→V	2	Unique	C^{93}→S	1	Unique
L70→ Q	2	Unique	L^{81}→P	1	Unique
C87→L	2	Unique	L^{82}→P	1	Not unique
R74→K	2	Not unique	K^{77}→G	1	Not unique
R74→G	2	Not unique	V^{69}→L	1	Not unique
L81→I	2	Not unique	Y^{75}→F	1	Not unique
K77→E	1	Not unique	T^{94}→A	1	Not unique
D78→H	1	Unique	V^{69}→M	1	Unique

ISSN 2303-9841

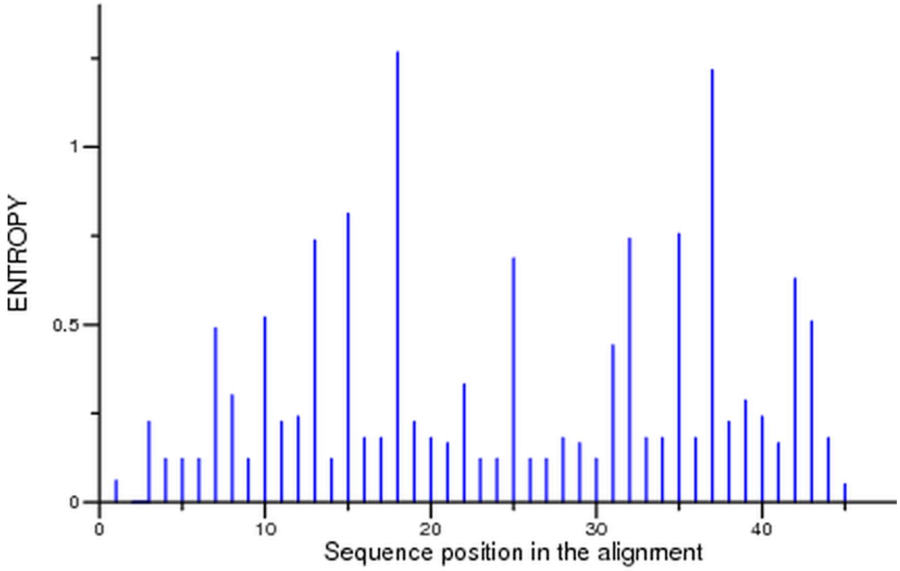

Figure 1: Shannon-Two Entropy of alignments of 91 HIV gp41 IDR peptide sequences

3. Results

3.1 Consensus peptide sequence for HIV gp41 IDR (Kenya)

This study established that the Consensus peptide sequence for HIV *env* gp41 IDR (Kenya) was WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTNVP WNSSW. The study found only two amino acids differences between HIV Consensus *env* gp41-IDR peptide (Kenya) and HIV Consensus *env* gp41-IDR peptide (Global) where A96→N and A101→S giving a similarity of 95.3% between the two sequences. There was 100% sequence similarity in the key sub-regions of IDR of *env* gp41: the CTL epitope (aa 71 to 82; AVERYLKDQQLL) and the Cysteine Loop (aa 87 to 93; CSGKLIC).When BLAST analysis of the HIV Consensus *env* gp41-IDR peptide (Kenya) was carried out at the NCBI database, more than 200 sequences with 100% sequence similarity were found implying that this Consensus sequence was not unique. However, when individual sequences were analyzed, 74 different substitutions (in comparison with HXB2 sequence) were noted among the *env* gp41 IDR (Kenya), out of which 29 (39.2%) were unique (**Table 1**).

When Shannon-Two Entropy of alignment of 91 HIV *env* gp41 IDR peptide sequences obtained in this study was determined using Los Alamos "Entropy" software (Foley et al, 2013) with HXB2 gp41 IDR peptide as a background sequence, position 77R was the most variable position with the Shannon Entropy value of 1.266 followed by N96 (1.217), 74R (0.813), 94T (0.755), 91L (0.742), 84 (0.686) and 101S (0.629). Sixty seven point four percent (67.4%) of the amino acid positions were conserved with the Entropy value of below 0.25 (baseline Entropy) with positions 60W (entropy value of 0.06) and 61G (entropy value of 0.0) being the most conserved (**Figure 1**).

3.2 HIV incidence in Kenya

The Standard ELISA procedure carried out using wells coated with rIDR-M peptide gave the HIV Incidence of 31.9% while the tests that were carried out using wells coated with HIV Consensus *env* gp41 IDR (Global) and Consensus *env* gp41 IDR (Kenya) peptides gave the HIV Incidences of 76.9% and 41.8% respectively using the Avidity Index (AI) cut off point of 80% (**Figure 2**). The results of the tests that were carried out using wells coated with Consensus *env* gp41 IDR (Kenya) peptide were closer to the Standard than those from Consensus *env* gp41 IDR (Global) peptide.

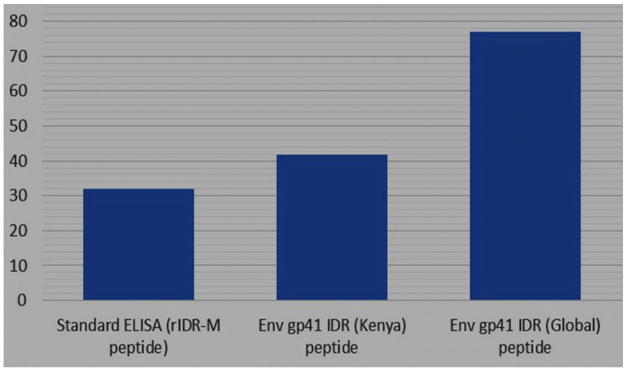

Figure 2: HIV Incidence using wells coated with Consensus *env* gp41 IDR Kenya), Consensus *env* gp41 IDR (Global) and the Standard (r-IDR-M peptide) respectively

3.3 The comparison of the performance of Control wells (without the dissociation of immune-complex)during the determination of HIV Incidence

The optical densities of wells which were treated with PBS (control wells) in ELISA system coated with three peptides {rIDR-M, Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global) respectively} showed close correlation of the performance of the three ELISA systems (**Figure 3**). The correlation between the Control ELISA system of Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global) was the

closest (R = 0.9823, P-Value < 0.00001 and significant at p < 0.05). The correlation between the Control ELISA system of Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (rIDR-M) was the (R = 0.7206, P-Value < 0.00001 and significant at p < 0.05) one which was closer than that of Consensus *env* gp41 IDR (Global) and Consensus *env* gp41 IDR (rIDR-M) (R = 0. 0.7086, P-Value < 0.00001 and significant at p < 0.05). When One-way ANOVA was computed with results from rIDR as dependent variables the F-Test value for Control ELISA system of Consensus *env* gp41 IDR (Kenya) was 2.299 (significant value of 0.103) while that of Control ELISA system of Consensus *env* gp41 IDR (Global) was 2.015 (significant value of 0.144) implying that the difference among the three ELISA systems was not significant at p-value of 0.05).

3.4 Comparison of performance of wells treated with a dissociation buffer (Citrate buffer at pH 3) during determination of HIV Incidence

The optical densities of wells which were treated with dissociation buffer (Citrate buffer pH 3) in ELISA system coated with three peptides respectively rIDR-M, Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global) showed an existence correlation of the performance of the three ELISA systems (**Figure 4**). The correlation between the ELISA system of Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global) after using Dissociation buffer was the closest (R = 0.9739, P-Value < 0.00001 and significant at p < 0.05). The correlation between the Control ELISA system of Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (rIDR-M) was the (R = 0.8044, P-Value < 0.00001 and significant at p < 0.05) which was closer than that of Consensus *env* gp41 IDR (Global) and Consensus *env* gp41 IDR (rIDR-M) was the (0.7588, P-Value < 0.00001 and significant at p < 0.05). When One-way ANOVA was computed with results from rIDR as dependent variables the F-Test value for Consensus *env* gp41 IDR (Kenya) was 4.550 (significant value of 0.006) while that of Control ELISA system of Consensus *env* gp41 IDR (Global) was 6.677 (significant value of 0..001) implying that the significant difference among the three ELISA systems at p-value of 0.05).

This computation also show that Consensus *env* gp41 IDR (Global) deviated more from the Standard than Consensus *env* gp41 IDR (Kenya) a fact that is also demonstrated in **Figure 2**.

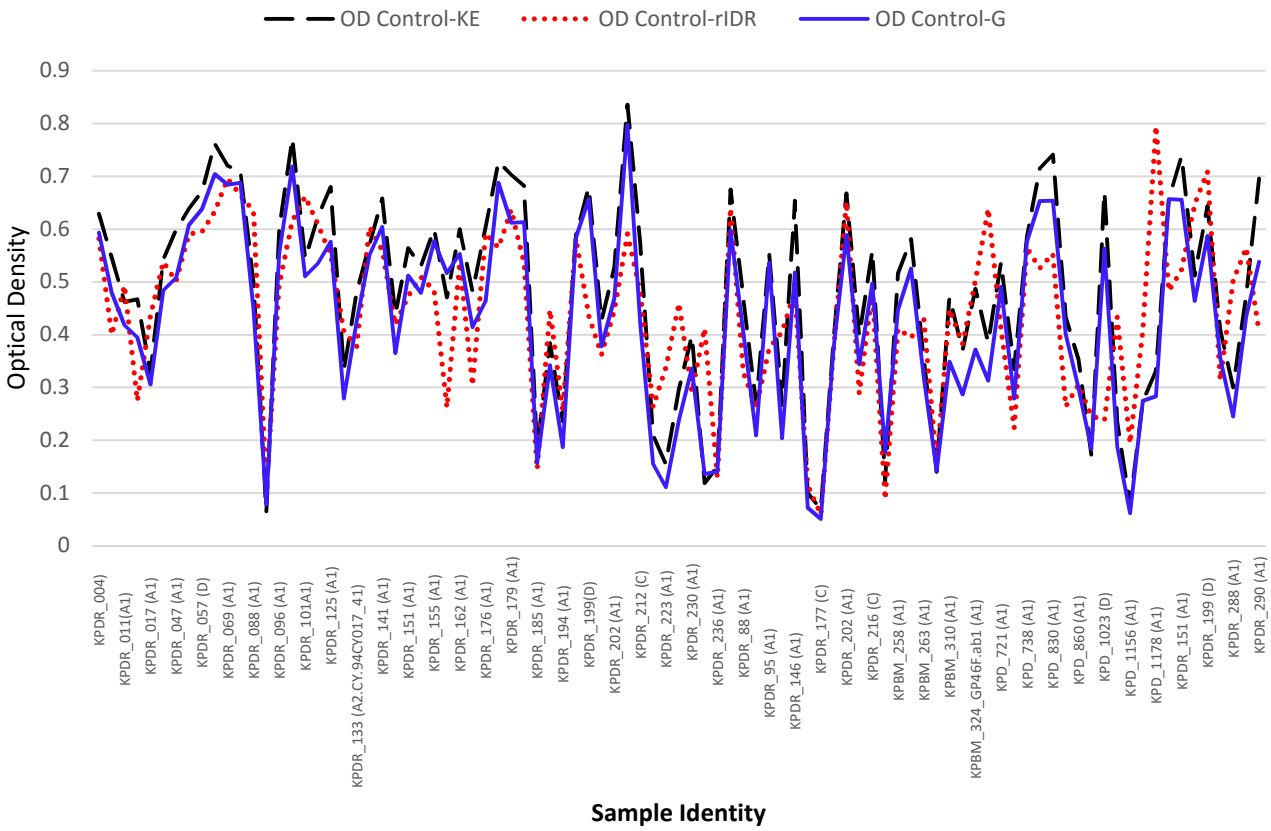

Figure 3:. Comparison of the HIV incidence test results of ELISA system coated with three peptides: rIDR-M, Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global) respectively.

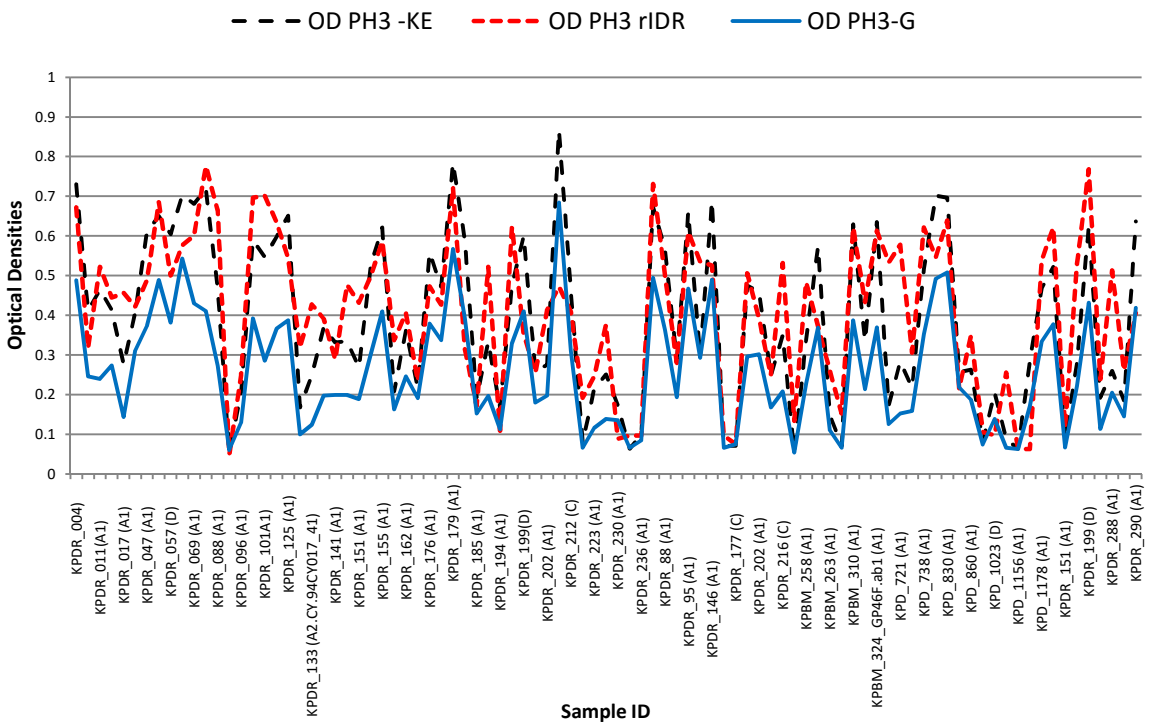

Figure 4: Relationship between the performances of samples treated with dissociation buffer (Citrate buffer pH 3) in ELISA system coated with three peptides respectively: rIDR-M, Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global)

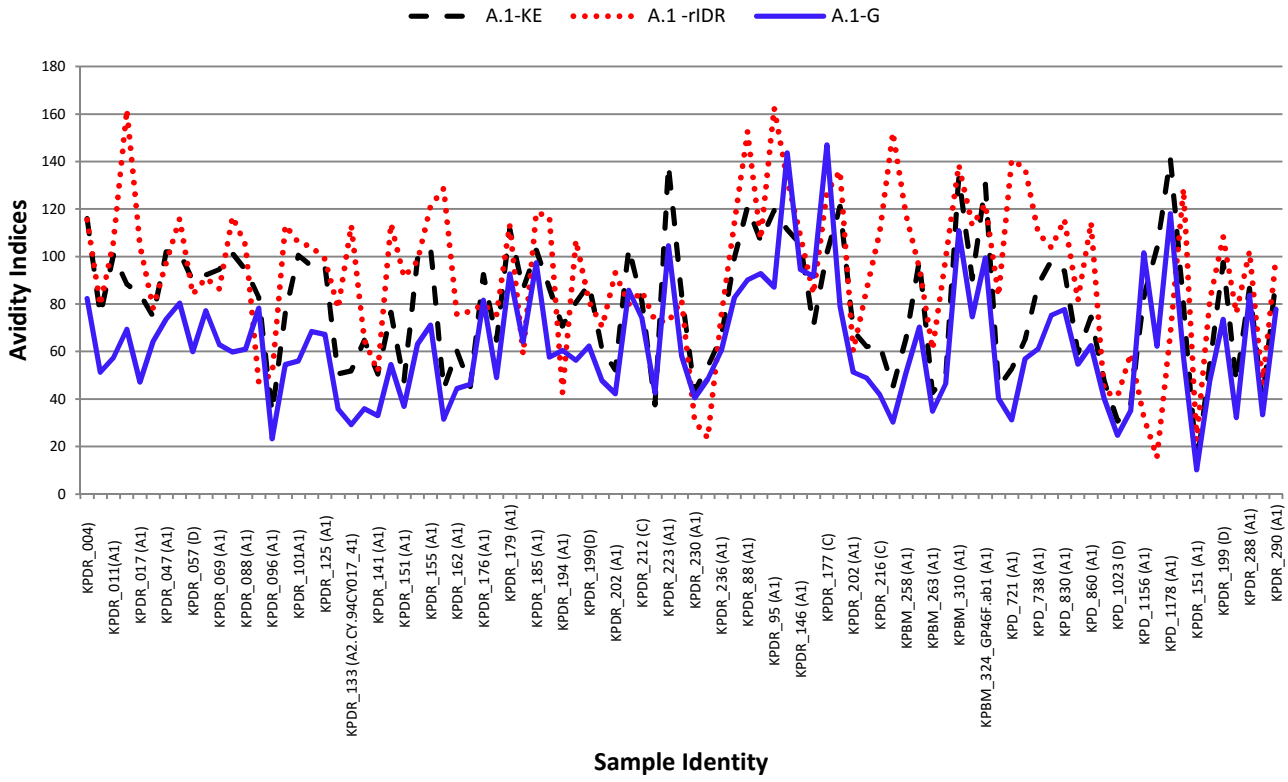

Figure 5: Relationship between the Avidity indices in ELISA system coated with three peptides: rIDR-M, Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global)respectively

Table 2: Performance of various Rapid test kits in respect to Consensus status with LFAs

HIV Rapid Test Kit	D-SN (95% CI)	D-SP (95% CI)	PPV (95% CI)	NPV (95% CI)
Determine™ HIV-1/2	100 (97.4-100.0)	96.8 (83.2 - 99.5)	99.3 (96.1 - 99.9)	100 (88.3-100.0)
KHB Colloidal Gold	100 (97.4-100.0)	100 (88.7-100.0)	100 (97.4-100.0)	100 (88.7 - 100.0)
Uni-Gold™ HIV test	96.4 (91.8 - 98.8)	100.0 (88.7-100.0)	100.0 (97.3-100.0)	86.1 (70.5-95.3)
First Response™	100 (97.4-100.0)	96.8 (83.2 - 99.5)	99.3 (96.1- 99.9)	100 (88.3 - 100.0)
Aware™ HIV-1/2 BSP	99.3 (96.0 - 99.9)	100.0 (88.7-100.0)	100.0 (97.3 - 00.0)	96.9 (83.7-99.5)
In-House HIV 1/2 LFA	99.3 (96.0 - 99.9)	100.0 (88.7-100.0)	100.0 (97.3 - 00.0)	96.9 (83.7-99.5)

**D-SN Diagnostic Sensitivity; D-SP Di*agnostic specificity; PPV Positive Predictive value; NPV Negative Predictive value

Table 3: Performance of ELISA kits developed using Consensus *env* gp41 IDR (Kenya & Global) and Vironostika™ Uni-Form II Ag/Ab ELISA

ELISA	D-SN (95% CI)	D-SP (95% CI)	PPV (95% CI)	NPV (95% CI)
Consensus *env* gp41 IDR (Kenya)	97.24 (93.1 - 99.2)	100 (86.3-100.0)	100 (97.4-100.0)	86.2 (68.4 – 96.1)
Consensus *env* gp41 IDR (Global)	97.24 (93.1 - 99.2)	100 (86.3-100.0)	100 (97.4-100.0)	86.2 (68.4 – 96.1)

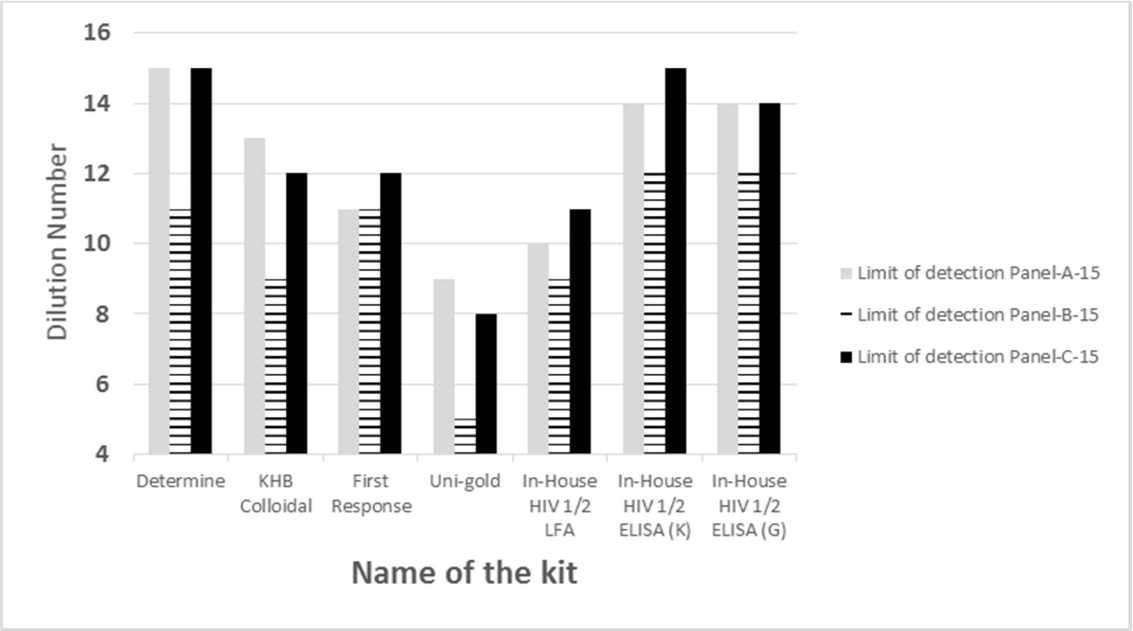

Figure 6: The relative Analytical Sensitivity of HIV LFAs in Kenya using panels that was prepared in-house

3.5 Comparison of Avidity indices during determination of HIV Incidence

The Avidity Index (AI) of wells in ELISA system coated with the three peptides rIDR-M, Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global)respectively showed an existence of correlation of the performance of the three ELISA systems (**Figure 5**). The correlation between the ELISA system of Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (Global) was strong (R = 0.8349, P-Value < 0.00001 and significant at $p < 0.05$). The correlation between the ELISA system of Consensus *env* gp41 IDR (Kenya) and Consensus *env* gp41 IDR (rIDR-M) was weak (R = 0.4535, P-Value < 0.00001 and significant at $p < 0.05$) which was closer than that of Consensus *env*

 ISSN 2303-9841

gp41 IDR (Global) and *env* gp41 IDR (rIDR-M) was weak (R = 0.3546, P-Value < 0.000563 and significant at p < 0.05). When One-Sample T-Test was computed it was found that the T-Statistic values were 23.980, 27.885 and 28.041 for Consensus *env* gp41 IDR (Global), *env* gp41 IDR (rIDR-M) and Consensus *env* gp41 IDR (Kenya) respectively indicating a greater deviation of the Avidity index of the Consensus *env* gp41 IDR (Global) from the Standard *env* gp41 IDR (rIDR-M) than with Consensus *env* gp41 IDR (Kenya).

3.6 Evaluation of the developed immunoassays

The LFA that was developed using Consensus *env* gp41 IDR (Kenya) showed a Diagnostic Sensitivity (D-SN) of 99.3 (95% CI: 96.0 - 99.9) and Diagnostic Specificity (D-SP) of 100% (95% CI: 88.7-100.0) which was similar to Aware™ HIV-1/2 BSP and better performance than Uni-Gold™ HIV test (**Table 2**).

3.7 Analytical Sensitivity of the developed immunoassays

Determine™ HIV-1/2 showed the highest sensitivity in comparison with other HIV LFAs (**Figure 6**). The performance of KHB Colloidal Gold and First Response™ 1-2.0 were comparable (13 and 11 for panel P-A-15; 9 and 11 for panel P-B-15 and 12 and 12 for P-C-15). Uni-Gold™ HIV showed the lowest Analytical Sensitivity (with detection limit at 9, 5 and 8 dilutions of the three HIV panels respectively).

4. Discussion

This study established the Consensus peptide sequence for HIV env gp41 IDR (Kenya) to be: WGIKQLQARVLAVERYLKDQQLLGIWGCSGKLICTTNVP WNSSW. When BLAST analyses of this peptide sequence were carried out at the NCBI data base more than 200 sequences with 100% sequence identity were found implying that this consensus sequence is not a unique Consensus sequence. However, the study found that this Consensus peptide differed from the HIV Consensus *env* gp41-IDR peptide (Global) (Masciotra et al, 2000) by two amino acids where A96→N and A101→S giving a similarity of 95.3%. Further examination of each sequence yielded 74 substitutions of which 29 (39.2%) were unique ([a]Kimotho et al, 2015). Sixty seven point four percent (67.4%) of the amino acid positions were conserved with the Entropy value of below 0.25 with the most conserved positions being positions 60W and 61G. The study also found that the HIV sub-type distribution among the blood donors in Kenya was A1 (76.9%), C (6.6%), D (14.3%) and CRF A2.CY.94CY017_41 (2.2%), which was not significantly different from the results that are recorded in Los Alamos data base for HIV (P-Value = 1; p < 0.05) (Los Alamos, 2015).

The HIV Incidence (or Recency) testing conducted in this study using the Two-Well Avidity-Based assays (Wei et al, 2010) found that 31.9% of 91 samples tested using ELISA wells coated with Consensus *env* gp41 rIDR-M peptide (as the standard) were recent. When the same samples were tested for recency using wells coated with Consensus *env* gp41 IDR (Global) and Consensus *env* gp41 IDR (Kenya) peptides the HIV Incidence was found to be 76.9% and 41.8% respectively. Previous studies in Kenya reported the rate of recent HIV infection of 11% among the HIV seropositive individuals (WHO, 2009), using the BED CEIA testing protocol, with males aged 25-35 years old showing the highest percentage of recent HIV infection at 47%. The study also found that the age group with the largest discrepancy in recent infection between males and females was the 15-24 year old age group (36% in females vs. 13% in males) (WHO/UNAIDS, 2013). According to NASCOP (2015) the current HIV Incidence in Kenya is 0.44% of the general population (or 7.3% of the HIV positive cases). In Kenya, about 70% of blood donors are aged between 15-19 years old while 30% are about 20-64 years old. This may partly explain the HIV Incidence of 31.9% obtained in this study. The False Recency Rate (FRR) is reported to be about 5% due to presence of Elite controllers (individuals who naturally maintain low or undetectable HIV RNA levels and have low antibody responses) (UNAIDS, 2013)hence after adjustment the HIV Incidence is 26.9%. The study established that although the three peptides (Consensus *env* gp41 IDR (Kenya), Consensus *env* gp41 IDR (Global), *env* gp41 IDR (rIDR-M), showed insignificant difference when they were used to develop HIV detection ELISA they displayed significant difference when they were used to develop HIV incidence kits. These results imply that the three peptides that were used to run the three ELISA are likely to have different Immune-Complex Dissociation kinetics in the presence of the dissociation buffer.

When the developed kits were evaluated it was found that there was no significant difference between the performance of the HIV ELISA Testing kit that was developed with Consensus *env* gp41-IDR peptide (Kenya) and that which was developed using Consensus gp41-IDR peptide (Global) with both having a Diagnostic sensitivity of 97.2%. This finding seems to be in favour of findings by Masciotra et al. (2000) who established that Consensus gp41-IDR from group M peptides (WGIKQLQARVLAVERYLKDQQLLGIWGCSGKL ICTTAVPWNASW) was able to detect all 130 group M sera (10 subtype A, 21 subtype B, 13 subtype B9, 20 subtype C, 21 subtype D, 14 subtype E, 25 subtype F, and 6 subtype G). The study also agrees with the findings by Vallari et al (2010) who noted that despite the high genetic divergence between HIV-1 groups M and N, all group N infections were detected using five commercial HIV immunoassays (Vallari et al, 2010). Dorn et al (2000) noted that substitutions observed within the IDR of gp41 of HIV-1group M subtypes do not affect antibody recognition and that all HIV-1-seropositive specimens containing the observed substitutions reacted with the FDA-licensed ELISA kits regardless of viral genotype and geographic origin. This may be explained from the finding in this study that the three Consensus peptides had 100% sequence similarity in the key sub-regions of IDR of gp41: the CTL epitope (aa 71 to 82; AVERYLKDQQLL) and the Cysteine Loop epitope (aa 87 to 93; CSGKLIC). During the evaluation process it was established that the Analytical Sensitivity of Determine™ HIV-1/2 Combo Ag/Ab LFA (Abbott Diagnostic Division, Hoofddorp, The Netherlands) in respect to testing for HIV p24 antigen was the same as that of Vironostika™ Uni-Form II Ag/Ab ELISA (bioMérieux, Marcy-l'Etoile, France). Also during the process of evaluation it was established that

the overall D-SN of using LFAs in HIV testing in Kenya was found to 96.0% (95% CI: 92.3-98.3%). Mine et al (2015) found a D-SN and D-SP of 98.2% and 100% respectively for *KHB Colloidal Gold* against Vironostika™ Uni-Form II Ag/Ab ELISA as Gold Standard and D-SN and D-SP of 98.2% and 98.1% respectively for Uni-Gold™ HIV test against the same ELISA kit. However, Fabiani et al (2005) found a low sensitivity of the HIV Testing Algorithm using Rapid Tests of 90.6% (95% CI: 78.6 -96.5) when compared with an ELISA system.

The evaluation of the developed LFA kits was done using plasma samples thus restricting the future use of these kits to testing of HIV in the plasma matrix only. This was due to the limitations of funds. It is recommended that the LFA developed in this study be evaluated with whole blood samples in future.

In conclusion this study developed diagnostic immunoassays whose performance was not affected by use of Consensus *env* gp41-IDR peptides (Kenya) and*env* gp41-IDR peptides (Global). However, the study demonstrated that Consensus *env* gp41-IDR peptide (Kenya) could be more suitable for development of HIV Incidence assays in Kenya than *env* gp41-IDR peptide (Global).

Conflict of Interest declaration

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to KEMRI-CDC for facilitating procurement of the HIV diagnostic kits used in this study, Director Kenya National Blood Transfusion Services for allowing us to use rejected donors' blood for the study and staff of KEMRI Production Department for their moral support of this study. Director KEMRI for permission to publish the findings.

References

Bobkov AF, Kazennova EV, Selimova LM, Khanina TA, Ryabov GS and Bobkova, M.R. (2004). Temporal trends in the HIV-1 epidemic in Russia: predominance of subtype A. *J. Med. Virol.,* **74**: 230-237.

Crowther JR (1995). Methods in Molecular Biology. In: ELISA, Theory and Practice Vol. 42, Humana Press, New Jersey, pp 66

Dachraoui R, Brand D, Brunet S, Barin F and Plantier JC. (2008). RNA amplification of the HIV-1 Pol and env regions on dried serum and plasma spots. *HIV Med.* **9**:557-561

Dorn J, Masciotra S, Yang C, Downing R, Biryahwaho B, Mastro TD, Nkengasong J, Pieniazek D, Rayfield M, Hu DJ, Lal and RB.(2000). Analysis of genetic variability within the Immunodominant epitopes of envelope gp41 from Human Immunodeficiency Virus Type 1 (HIV-1) Group M and its impact on HIV-1 antibody detection. *J. Clin. Microbiol.* **38**: 773–780.

Fabiani MN, Biryahwaho B, Ouma J, Ninci A and DeclichS. (2005).Evaluating HIV testing algorithms for research, diagnosis and surveillance. *Health Pol. &Dev:* **3:** 28-31.

Foley B, Leitner T, Apetrei C, Hahn B, Mizrachi I, Mullins J, Rambaut A, Wolinsky S and Korber (2013). HIV Sequence Compendium Published by Theoretical Biology and Biophysics Group Los Alamos National Laboratory NM LA-UR 13-26007.

Frens G (1973). Collodial nucleation for the regulation of particle size in monodisperse gold suspension *Nat. Phy. Sci:* **241:** 20-25

Kartekayan S, Bharmal RN, Tiwari RP and Bisen PS (1998). Human Immunodeficiency Virus In: *HIV and Aids: Basic elements and priorities* 7th ed. Dordrecht, Springer:pp39-51

Kilembe W, Keeling M, Karita E, Lakhil S, Chetty P and Price M A (2012). Failure of a Novel Rapid Antigen and Antibody Combination Test to Detect Antigen-Positive HIV Infection in African Adults with Early HIV Infection PLoS ONE **7**: e37154

Korber B, Hahn B and Foley B (1997). Human Retroviruses and AIDS: A compilation of analysis of nucleic acid and amino acid sequences. *Theoretical Biology and Biophysics group* Los Alamos National Library Los Alamos NM **3**: 28-40

[a]Kimotho JH, Ng'ang'a Z, Nyairo E, Ochwoto M, Nzioka N,Ogolla F and Kiptoo M (2015).Diversity within the Immunodominant Epitopes of *env*elope gp41 HIV-1 in Kenya and Its Effects on Performance of the HIV-1 Antibody-Based Detection Kits. *Am. J. Int. Med.* **3**: 15-22

[b]Kimotho JH, Ng'ang'a Z, Nyairo E, Ochwoto M, Nzioka N,Ogolla F, Kiptoo M (2015). Laboratory Evaluation of the Validity of the Current HIV Testing Algorithm in Kenya. *Am. J. Int. Med.* **3**: 23-28.

Leitner T (1996). Genetic subtypes of HIV-1 In: *Human Retroviruses and AIDS*: A compilation of analysis of nucleic acid and amino acid sequences Theoretical Biology and Biophysics group Los Alamos National Library Los Alamos NM **3**: 28-40

Los Alamos Website (2015). http://www.hivla.nl.gov Accessed on: 30th September 2014

Los Alamos (2009). Advanced Consensus Maker HIV Sequence Database http://www.hivla.nl.gov/content/sequence/CONSENSUS/AdvConhtml. Accessed on: 30th September 2014

Masciotra S, Rudolph D L, Der Groen G V, Yang C and LalRB (2000). Serological Detection of Infection with Diverse Human and Simian Immunodeficiency Viruses Using Consensus *env* Peptides *Clin. Diag. Lab. Imm.* **7**: 706–709

Mine M, Chishala S, Makhaola K, Tafuma TA, Bolebantswe J, Merrigan MB (2015).Performance of rapid HIV testing by lay counselors in the field during the behavioral and biological surveillance survey among female sex workers and men who have sex with men in Botswana. *J. Acquir. Immune. Defic. Syndr.* **68**:365-8.

NASCOP Ministry of Health KENYA (2014) Kenya HIV Estimates. http://www.nacc.or.ke Access date: 12th February 2015

 ISSN 2303-9841

National AIDS and STI Control Programme Ministry of Health Kenya September 2014 Kenya AIDS

Plantier JC (2009). A new human immunodeficiency virus derived from gorillas. *Nat. Med.* **15**: 871-872

Quinones-Mateu M E and Arts E J (1999). Recombinations in HIV-1: Update and Implications. *AIDS Rev.* **1:** 89-100

Salemi M (2011).Toward a robust monitoring of HIV subtypes distribution worldwide. *AIDS*. **25**: 713-714

Tamura K, Dudley J, Nei M and Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**: 1596-1599

Thorstensson R, Andersson S, Lindbaeck S, Dias F,Mhalu F, Gaines H and Biberfeld G (1998). Evaluation of 14 commercial HIV-1/HIV-2 antibody assays using serum panels of different geographical origin and clinical stage including a unique sero-conversion panel. *J. Virol. Methods.* **70**: 139-151

UNAIDS (2013). AIDS epidemic update: December 2013

Vallari A, Bodelle P, Ngansop C, Makamche F, Ndembi N, Mbanya D, Kaptue L, Gurtler LG, McArthur CP, Devare SG, and Brennan CA (2010). Four new HIV-1 group N isolates from Cameroon: Prevalence continues to be low. *AIDS Res. Hum. Retrovir.* **26**: 109-15

Wei X, Liu X, Dobbs T, Kuehl D, Nkengasong J, Hu D, and Bharat PB (2010). Development of Two Avidity-Based Assays to Detect Recent HIV Type 1 Seroconversion Using a Multisubtype gp41 Recombinant Protein. *AIDS Res. Hum. Retrovir*. **26**(1): 61-71

WHO (2009). WHO Technical Working Group on HIV Incidence Assays. Meeting Report. http://www.who.int/ Accessed on 15th April 2015

WHO/UNAIDS (2013). Technical Update on HIV incidence assays for surveillance and epidemic monitoring. http://www.unaids.org Accessed on 15th April 2015

 ISSN 2303-9841