Detecting Seronegative-Early HIV Infections Among Adult Versus Student Kenyan Blood Donors, by Using Stimmunology

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***Background*: Undetectable HIV infection in blood banks poses a serious threat to public health. Thus, donations from high school students are preferred over adult samples in Kenyan blood banks, due to lower HIV infection prevalence within this population, as detected by conventional serology testing. However, the number of recently infected individuals remains difficult to identify, as HIV-induced immunological window periods can span months. This study focuses on the potential contribution of a novel mode of diagnostic testing in revealing early, seronegative HIV carriers. *Methods and Findings:* Stimmunology, an *in vitro* lymphocyte stimulation technique, was used to detect early HIV infection among random samples of adult and adolescent blood donors. The Stimmunology protocol unveiled a significant number of early, pre-seroconversion HIV carriers both among adult and teenage Kenyan populations, undetected by typical serological diagnostic kits. Both populations demonstrated a significant increase in HIV-specific antibody formation following activation using the Stimmunology assay. The younger population exhibited a higher proportion of early HIV infection (0.45) than the adult (0.27) population. *Conclusions*: While blood samples of young donors are preferred over adult donations, these data demonstrate a worrisome ratio of early, seronegative HIV carriers within this population. This simple, cost-effective, and reliable HIV-boosting antibody assay can be used in a resource-poor setting to increase blood supply safety and quality. Incorporation of Stimmunology into basic blood bank testing and into diagnostic protocols can also decrease undesirable disease transmission.**

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Introduction

Due to the threat of human immunodeficiency virus (HIV) transmission, the past two decades have witnessed significant modifications in transfusion medicine. New policies demand more stringent donor risk factor screening and vigorous donation testing (1). In areas of high HIV incidence, donor selection based on risk factors is quite limited due to both the shortage of blood donors and immeasurable risk factors.

To date, the internationally accepted method for screening and diagnosis of HIV carriers relies on the detection of HIV-specific antibodies. Enzyme-linked immunoadsorbent assay (ELISA) and other antibody detection methods have been improved to detect significantly low levels of early IgM antibodies and display excellent specificity and sensitivity. However, during the early stages of the "window period," when viruses lay latent without prompting immune responses, serum antibody screening has proven ineffective in identifying the majority of low-level HIV carriers (2, 3). Therefore, any method that facilitates the detection of HIV carriers during this window period is critical both as a measure of the incidence and risk level of a given population, and as a tool for reducing that risk. In the developed world, many countries have supplemented their blood unit screening with viral particle-detecting tests. Initially, a p24 antigen test was developed (4–7) to identify HIV carriers, and proved to reduce the window period by 5–7 days. The existing tests, while clearly very effective, need improvement and require the investment of time, energy,

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and resources by the companies in efforts to improve their standardized tests. These developments include molecular amplification and detection tests (8–10), which will further shorten the window period by successfully detecting carriers 10–12 days prior to seroconversion (11, 12). However, these assays are quite complex, requiring highly trained individuals, and very expensive, which are not viable options for resource-poor countries. Unfortunately, it is precisely those countries that are at the highest risk for transfusion-based HIV transmission and infections. Clearly, a dire need exists for a more substantial means of testing seronegative HIV carriers within the window period, which can span months (13–15). Such a test should best fit within the current testing paradigm and tools of antibody/antigen testing available in the countries that need it most. However, 3rd- and 4th-generation ELISA-based assays, which have narrowed the window period (WP), continue to rely on the presence of sufficient amounts of HIV-1–specific antibodies in the blood and/or presence of sufficient amounts of HIV-1 proteins such as gag (requiring sufficient amount of virus replication to occur).

It has been previously reported that virally induced immune suppression plays a significant role in defining the serological window period during which HIV-specific antibody production is inhibited (3, 16). Our major focus has been to identify a method which shortens the WP and is more relevant to populations where HIV-1 incidence and prevalence continue to be a problem. In these populations, a major issue involves individuals who are in a state of "relative immunosuppression" secondary to either poor nutrition and/or due to chronic parasitemias endemic in these select populations. Thus, following exposure, a small but significant number of individuals become infected while being "immunosuppressed." Since their CD4+ T cells do not respond with the same degree of vigor as otherwise "healthy" individuals, the virus does not replicate to the same levels. Their B cells, which get primed to HIV-1 antigens, do not proliferate as well due to lack of optimal CD4+ T cells help and thus require artificial stimulants to induce them to synthesize and secrete HIV-1–specific antibodies.

Hence, to overcome this immune suppression, an *in vitro* lymphocyte stimulation technique has been developed to stimulate a small sample of unprocessed blood from potential donors (16–20). During the incubation period, immunosuppression is overcome by a process termed "Stimmunology" (ST), where *in vivo*–primed, HIV-specific lymphocytes are stimulated *in vitro* to produce HIV-recognizing antibodies. The antibody-containing supernatant fluid can then be tested with commercially available HIV-detecting ELISA-based assay kits.

The HIV pandemic has ruthlessly struck Kenya, grading it one of the nine African countries with the most widespread occurrence of AIDS (21), where the number of AIDS-related deaths has tripled over the past decade (21). Effective blood donor screening is a key factor in the worldwide attempt at reducing further transmission of the disease within Kenya and beyond. Young blood donors in rural areas have been considered the donors of choice as they exhibit lower HIV seroprevalence. Thus, in this study, two blood donor populations were examined in Nairobi, Kenya: adult walk-in donors (WD) and high school students from the outskirts of the city (HD). The Stimmunology system was employed to attempt to detect potentially additional HIV-infected, seronegative individuals in both populations and to test the efficacy of standard blood screening protocols in blood banks. The ST methodology detected alarmingly high ratios of adolescent and adult seronegative HIV carriers. By detecting such previously undetected carriers, this method promises to curb viral transmission and enhance prospects of early intervention and medical care for infected individuals.

Methods

Studied Populations. Two populations in Nairobi, Kenya, were studied: 513 adult WD, estimated ages 20–40, and 332 teenage HD. Random blood samples of blood donors entering the blood bank over the months of the study were collected in heparin-containing vacuum tubes and sent for HIV antibody testing at the Immunology laboratory of the Kenyata Hospital (Nairobi, Kenya), in conformance with the guidelines and approval of the Kenyata Hospital Institutional Review Board as well as by the ethics committee of the Health Ministry of Kenya. Tubes were identified by a letter (K for WD and N for HD) and a serial number only, with no other identification details.

Routine HIV Antibody Testing. Plasma (pre-ST) collected from 1 ml of centrifuged blood was tested for HIV-specific antibodies using an ELISA-based diagnostic kit (Sanofi Pasteur, Freiburg, Germany, and/or Genetic Systems Corp., Redmond, Washington, USA). The remaining plasma was frozen in two aliquots. The procedure utilized followed the requirements of the kit manufacturer's stringent criteria for identifying positive values by the ELISA-based assays, with the addition of four known negative local samples for possible shift in cut-off values due to the local viral or immune variance. In all runs, the cutoff value remained as per the kit's criteria.

Stimmunology. Based on the fact that the long window period between HIV infection and detectable seroconversion is due, at least in part, to specific immune suppression (16), an assay termed "Stimmunology" was developed. The assay is directed primarily at overcoming the *in vivo* specific immune suppression by providing the antigen-primed lymphocytes *in vitro* with a cocktail of highly activating stimuli that promote the proliferation and differentiation of B cells, leading to antibody production *in vitro*. This whole process is carried out by a short incubation (3–5 days) of 1 ml of whole blood in the stimulating media in a tissue culture tube. The formulation of the active ingredients in the Stimmunology solution, as it is used in the

SMARTube™ HIV&HCV for example, is patented by its manufacturer and subject to legal restrictions in terms of disclosure. In order to evaluate both the technical and diagnostic feasibility of using Stimmunology as a blood pre-treatment step prior to testing for antibodies by the currently used diagnostic kits, testing for HIV antibodies was done in parallel on both regular donor plasma and plasma after the Stimmunology process.

The blood sample to be tested was thoroughly mixed and 1 ml of blood was transferred into a Stimmunology culture tube (16) and placed in a 5–7% CO_2-humidified incubator at 37°C, for five days. At the end of the incubation period, the plasma of the supernatant fluid (herein termed post-ST) was collected from the top of the tube and divided into two aliquots. One aliquot was frozen and the other was tested for the presence of HIV-specific antibodies, using the same diagnostic kits as for the routine plasma testing. Sample dilution during ST treatment was taken into account when further tested in the ELISA plates and appropriate adjustments were made accordingly. A preliminary study with 8 known positives and 12 known negatives was run on several commercial diagnostic kits, using the post-ST supernatant fluid to guarantee that the Stimmunology step does not affect the cutoff (>98% CI) values. ELISA-positive samples were re-tested to eliminate the potential false-positive results and the remaining supernatant fluid was frozen for future study.

Repeat Testing as Confirmation and Additional Testing in Israel. One aliquot from each pre-ST and post-ST sample was sent to Israel for confirmatory testing, using the same diagnostic kits along with a third one (Recombigen HIV 1/2, Cambridge Diagnostics, Galway, Ireland). Those samples that were positive only after the ST step were also analyzed for HIV-positive antibodies by Western blot analysis (New Lav Blot I, Bio-Rad, France, and Inno-LIA, Innogenetics, Ghent, Belgium). All ST-positive samples were also sent to the National Reference Laboratory in Israel to test for the presence of viral particles using RT-PCR using pre-ST samples. Pre-ST samples from those identified as HIV infected who are still in the window period [defined so by the pre-ST antibody negative result coupled with a post-ST antibody positive result] and both all-negative samples and all-positive samples were added to the sets of samples sent to be run blindly at the reference laboratory. The RT-PCR analysis was performed with highly specific sets of HIV-1 "gag" primer pairs and obtained a single band on each of the PCR-positive samples (22). Both negative and positive controls of the reference laboratory were always included with each analysis, further verifying the specificity of the results obtained (17, 23).

Data Collection and Compilation. All the data were stored as Excel sheets, which were locked upon reading and kept in the HIV testing laboratory in Kenyata Hospital with a hard and electronic copy sent to the laboratory in Israel. Initial HIV antibody screening of the samples in Israel was executed independent of the results obtained in Kenya and the two were later compared.

Table 1. HIV Antibody ELISA of Walk-In, Adult Donor Samples in Regular Plasma (pre-ST) and Plasma After Pre-Incubation and Stimulation In-Vitro (post-ST)[a]

Patient ID	Pre-ST Kenya	Post-ST Kenya	Pre-ST Israel	Post-ST Israel
K006	0.66	2.87	0.91	7.64
K016	0.34	2.73	0.34	2.56
K042	0.75	1.49	0.44	1.42
K052	0	1.79	0.43	3.00
K054	0.53	1.08	0.63	0.73
K059	0.83	1.09	0.81	1.77
K160	0.37	1.02	0.54	1.52
K174	0.86	1.65	0.95	2.52
K196	0.91	1.17	0.83	1.97
K201	0.55	2.27	0.85	4.82
K212	0.60	1.82	0.65	5.14
K217	0.57	1.08	1.15	1.35
K240	0.76	1.30	not run	1.02
K286	0.51	1.74	0.55	2.63
K294	0.57	1.48	0.97	4.06
K296	0.51	1.39	0.71	5.44
K302	0.97	1.49	0.87	2.20
K365	0.65	2.46	0.67	1.89
K467	8.57	2.52	0.67	1.89

[a] Fresh blood, 1 ml, was incubated in a SMARTube for the Stimmunology process and the resulting "plasma" (post-ST) was tested for HIV-specific antibodies in parallel to the regular plasma, on the routinely used screening and diagnostic kits of the blood-bank's laboratory (Sanofi Pasteur). Repeat testing was done, on a small aliquot, in Israel, on a different HIV antibody kit (Genetic Systems). Since cutoff value is a relative one, since the samples were run on different days, the comparison between them is done by presenting all of the OD readings as ratio of the cutoff. Values >1.00 are positive for HIV antibodies according to the kit's algorithm.

Results

To determine individuals during the early stages of HIV infection, two Kenyan blood donor populations were studied. The first group included walk-in donors (WD), mostly replacement donors of family members hospitalized in the Kenyata Hospital. The estimated age range was 20–45 years old. The second group consisted of blood donations from high school students (HD), collected in a mobile van in rural areas, where HIV seroprevalence was known to be lower. All samples were processed and tested.

Adult Walk-in Donors. Of the 513 WD plasma samples tested for HIV-specific antibodies, 8.8% (45 samples) proved to be seropositive for HIV using standard serological testing. However, upon ST activation of the blood samples, an additional 17 samples displayed positive anti-HIV antibody activity, raising the percentage of carriers within the donor group by 3.3%. Both pre- and post-ST ELISA tests were performed in the Kenyata Hospital, and a frozen aliquot of each sample was later shipped to and retested in Israel to confirm the findings. Table 1 summarizes the ELISA readings of pre- vs. post-ST samples

Table 2. Western Blot and PCR Analysis of HIV Seronegative yet Post-ST-Positive Samples from WD Blood Donor Samples[a]

Patient ID	WB Innogenetics (Kenya)	WB Bio-Rad (Israel)	RT-PCR
K006	not run	p55/gp41/p18	pos
K016	not run	p55/gp40/p24	neg
K052	gp40	none	neg
K054	gp40	none	neg
K059	p68/p55/p52/p25/p18	p55/gp41/p24	neg
K160	not run	p24	neg
K174	gp160/p68/gp40/p18	p65	pos
K196	not run	gp160/gp120/p65/p55/p43/p24/p18	pos
K201	None	p55/gp41/p24	neg
K212	gp40	gp40	pos
K217	not run	p18	pos
K286	None	none	weak pos
K294	gp40	p24	pos
K296	p25	p24	pos
K302	not run	p65/p55/p51/p24/p18	neg

[a] Fresh blood, 1 ml, was incubated in a SMARTube for the Stimmunology process and the resulting "plasma" (post-ST) was tested for HIV-specific antibodies in parallel to the regular plasma, on the routinely used screening and diagnostic kits of the blood-bank's laboratory. Confirmation was done on a second (different) ELISA. Western blot was done both in Kenya and in Israel. RT-PCR was done (blindly) in the National Reference Laboratory in Israel using a very sensitive (1–5 copies) assay. True negative and seropositive samples were included for additional controls (data not included).

obtained from 19 WD, comparing the results received in two different laboratories, using two different commercial kits. Two samples yielded post-ST positive results in only one of the two kits and are displayed in the table, but not included in the ensuing calculations. Furthermore, any samples producing OD readings within 10% of the manufacturer's cut-off value were rendered HIV-positive when the competing ELISA kit gave OD readings that were clearly above this grey zone.

Although all post-ST–positive samples were confirmed by a second ELISA run, further testing was carried out in quest of the epitope specificity of the antibodies synthesized by these carriers. Of the 17 newly discovered HIV carriers, 15 were tested twice by Western blot (WB) analysis, using two commercially available kits (Table 2). Of the WB carried out in Israel, 12/15 of these samples contained HIV-peptide–specific antibodies, with 6/12 conforming to the criteria for a positive WB. The WB analysis performed in Kenya, with a different commercial kit, yielded 7/9 HIV-specific antibody–containing samples, where 2/7 were considered HIV-positive according to the manufacturer's guidelines. RT-PCR (and/or PCR) (23)-positive results were demonstrated for 8/15 of these samples analyzed by the National Reference Laboratory in Israel (Table 2).

All 45 WD samples displaying sero-positivity prior to ST treatment remained positive following ST culture. However, a measurable increase in antibody levels was observed in 40% of these samples upon ST treatment. A continued escalation in antibody levels was noted over the 3–5 days of incubation, indicating the stimulatory effect of this immune response–activating process (Table 3).

To exclude any possibility that the mare mixture of the blood/plasma and the Stimmunology media could affect the readings on the antibody testing, leading to increase in "noise" and false-positive readings, 100 seronegative blood samples were just added to the Stimmunology media and their OD readings were compared to those of regular plasma (=pre-ST). All readings were low negative and no differ-

Table 3. Increase in HIV-Specific Antibody Levels Post-ST in 18/45 WD Seropositive Samples (signal/cutoff)[a]

Patient ID	Pre-ST, day0	Post-ST, day3	Post-ST, day4	Post-ST, day5
K032	1.49	1.93		
K033	3.07	8.09		
K049	17.81	19.47	23.84	
K086	2.08	2.47	2.53	
K100	3.89	5.77	not run	6.45
K120	1.46	2.21	2.27	3.02
K138	1.64	2.34	NR	3.05
K190	1.88	2.07	1.98	2.14
K209	16.88	17.77	21.84	26.04
K213	15.56	22.69	23.97	
K290	19.76	22	not run	30.62
K408	17.1	20.8		
K419	15.81	20.08		
K468	16.85	19.8		
K492H	16.53	24.08		
K496H	15.14	24.58		
K498H	17.6	22.6		
K509	17.88	20.69		

[a] Fresh blood, 1 ml, was incubated in a SMARTube for the Stimmunology process and the resulting "plasma" (post-ST) was tested for HIV-specific antibodies in parallel to the regular plasma, on the routinely used screening and diagnostic kits of the blood-bank's laboratory. Results are expressed as signal/cutoff values (pos = >1.00).

Table 4. HIV Antibody ELISA of High School Adolescent Donor Samples in Regular Plasma (pre-ST) and Plasma After Pre-Incubation and Stimulation *In Vitro* (post-ST)[a]

Patient ID	Pre-ST Kenya	Post-ST Kenya	Pre-ST Israel	Post-ST Israel
N329–4	0.40	1.85	0.65	1.33
N335–9	0.90	1.38	0.94	1.19
N340–7	0.65	1.95	0.64	1.56
N363–8	0.47	1.03	0.59	1.39
N372–4	0.58	1.79	0.74	2.41
N419–8	0.78	1.40	0.99	1.09
N432–6	0.95	18.11	0.60	20.70
N460–8	0.65	1.86	0.77	1.04
N464–7	0.94	1.65	0.78	1.12
N322–9	0.43	2.82	1.04	3.26

[a] Fresh blood, 1 ml, was incubated in a SMARTube for the Stimmunology process and the resulting "plasma" (post-ST) was tested for HIV-specific antibodies in parallel to the regular plasma, on the routinely used screening and diagnostic kits of the blood-bank's laboratory. Repeat testing was done, on a small aliquot, in Israel, on a different HIV antibody kit. Results are expressed as signal/cutoff values; thus, values >1.00 are positive for HIV antibodies according to the kit's algorithm.

ences were found between the two types of samples (data not shown).

High School Donors. Among the 332 high school donors (HD), only 12 (3.6%) plasma samples were seropositive using standard commercially available ELISA-based diagnostic kits. However, the ST pre-treatment allowed for the identification of an additional 10 (3%) as being HIV-specific antibody–positive (Table 4), increasing the actual percentage of HD HIV-infected blood to 22 teenagers (6.6%). Apart from detecting seronegative carriers, dismissed as virus-free by typical serological assays, the ST technique also suggests a significantly higher ratio of recent infection (antibodies detectable only following the ST pre-treatment) among the total seropositive donors in this high school population (0.45) versus the adult population (0.27).

Follow-Up Study. A follow-up study of those detected during the "window period", i.e., Stimmunology-positive/regular ELISA-negative cases, was performed in a separate population of pregnant women at high risk for HIV. Seven of the 20 women tested at the antenatal clinic were seropositive (both before and after Stimmunology). Among the 13 seronegative women, five were antibody positive after Stimmunology (Table 5). Serology testing in follow-up visits showed that 4/5 of the post-ST positive (pre-ST negative) seroconverted (as defined by standard plasma-based ELISA) during the following 4–6 months. The fifth one was lost to follow-up after 2 months, at which time it was still seronegative. All eight post-ST negative women remained seronegative through out the follow-up (9–11 months).

Discussion

Kenyan blood banks prefer blood donations from younger populations, where the seroprevalence of HIV infection is believed to be considerably lower. This study confirms that while HIV seroprevalence among the adult Kenyan population is double that of high school students (8.8% vs. 3.6%), the ratio of newly infected individuals is significantly higher among the adolescent population studied (0.45 vs. 0.27). These results set the actual HIV prevalence of the adult vs. adolescent groups at 12.1% vs. 6.6%, respectively. These data support the finding of a study of nearly 10,000 Kenyan schoolgirls, which concluded that a female typically experiences her first sexual encounter, a central mode of viral transfer, at the age of 14–15. Thus, upon reaching this age range, HIV incidence in this population mirrors that of the adult population (3.3% vs. 3%) and can no longer be viewed as a safer source for blood

Table 5. Serological Follow-Up of Post-ST Positive Seronegative Pregnant Women in Kenya [a]

Patient ID	1st testing post-ST	1st testing pre-ST	2nd sample	3rd sample
KP32	2.46	0.46	0.35 (3 months)	**18.75 (5 months)**
KP26	2.87	0.45	**6.12 (4 months)**	10.18 (10 months)
KP56	1.58	0.13	0.04 (4 months)	**6.18 (6 months)**
KP64	5.65	0.93	**1.37 (5 months)**	11.80 (10 months)
KP24	1.61	0.65	0.42 (2 months)	None

[a] Women were tested for HIV antibodies in their visits to the antenatal clinic. At the initiation of the study, fresh blood, 1 ml, was incubated in a SMARTube for the Stimmunology process and the resulting "plasma" (post-ST) was tested for HIV-specific antibodies in parallel to the regular plasma, on the routinely used screening and diagnostic kits of the blood-bank's laboratory. Repeat testing was done, confirming the positive antibody results. Upon repeat visits, antibodies were tested using regular (pre-ST) plasma only. Eight HIV-negative (both pre- and post-ST) women also completed the follow up (>9 mo) and all remained negative (data not shown). Results are expressed as signal/cutoff values; thus, values >1.00 are positive for HIV antibodies according to the kit's algorithm. Seroconversion time was calculated as the middle time point between the last seronegative and the first seropositive samples.

bank donors. Although the incidence of the window period individuals was similar in these two populations, the incidence/prevalence ratios differed markedly. Thus, when considering cost effectiveness, taking advantage of the lower prevalence rate among younger Kenyans may preserve their status as the preferred donor source for blood banks.

This study stresses the importance of detecting those infected individuals who are still in the window period and the value of the Stimmunology technique in detecting seronegative HIV-infected individuals, typically missed by standard serological testing methods (16–18, 24) due to potential immune-suppression within the window period.

The results of this study are further supported by those determined on samples from another African blood transfusion service, where an additional 2.2% of tested samples were ST-detected from conventional seronegative HIV carriers (unpublished results). A later study, carried out on samples from the same blood bank, led to similar conclusions, where 1.8% of the seronegative samples were determined to be positive, following ST pretreatment, and later confirmed to be infected with HIV using standard serology (unpublished results). Additionally, routine testing of Ethiopian immigrants arriving in Israel in 1992 and 1998 demonstrated an ST-enhanced identification of 0.3% and 2.7%, respectively, as seronegative HIV carriers (manuscript in preparation). Thus, the ST culture step offers a means of increasing blood safety, vital in countries where HIV prevalence and incidence are high.

As set by World Health Organization guidelines, any blood sample yielding positive recordings on an HIV-Ab ELISA must be regarded as contaminated and should be discarded. These internationally accepted regulations do not require further confirmatory testing of any sort. Nevertheless, the surprisingly high rate of missed infections and the wish to confirm the HIV-positive diagnosis of the blood donors prompted us to perform additional confirmatory testing. Aside from the repeat testing performed for each post-ST–positive sample in Kenya, these samples were also tested on an additional ELISA kit by the technical staff of a different laboratory in Israel. At that time, kits detecting only anti-HIV IgG antibodies were also used, showing that a few samples had only HIV-specific IgM, while most had both IgM and IgG. WB analysis, run both in Kenya and in Israel, offered additional information regarding the antibody repertoire of ST-treated samples. While the WB analyses of the pre-ST samples of all seronegative carriers were negative, 28.5–50% of post-ST ELISA-positive samples gave rise to bands on WB consistent with the presence of core and envelope protein-specific antibodies of the IgG isotype, as that is the only Ig isotype that the WB detects. As demonstrated in these results, WB analysis can hardly be viewed as a confirmatory assay, as such wide variations exist between the kits used. These variations often stem from differences in the choice of epitopes used to represent a specific protein of interest. Such variances can be of great significance when testing the narrow, epitope-limited spectrum of antibodies expressed by the early-stage HIV carrier. The IgG-specific WB kits will remain insensitive to the many IgM antibodies expressed throughout the early window period, especially after ST pretreatment, which is assumed to propagate antibody expression by cells silenced during the initial stages of infection. Furthermore, while the IgG-specific WB kits may provide additional information regarding the immunological status of the HIV carriers at early stages of infection, the IgM+IgG sensitive ELISA kits available today display much more sensitivity in early detection of HIV. Due to the lack of information concerning the precise point of viral contraction, any attempt at correlating appearance of HIV-specific IgG antibodies with more advanced stages within the window period would be completely speculative. Furthermore, the presence of silenced, primed B cells that have already undergone class switching (16) may reflect the course of initial infection and its entry to latency without attesting to the point along the window period at which the carrier stands. In addition, ELISA basically measures the presence of both conformational and non-conformational antibodies against the antigen in question. On the other hand, the Western blot primarily detects antibodies directed against denatured antigen and thus does not normally involve detection of conformational antibodies. Thus, differences do exist between Western Blot and ELISA, especially in antibody responses during early primary immune responses which appear to be directed more at conformational epitopes than antibodies against linear epitopes of processed antigens. It has previously been shown that early primary immune responses are directed at env and gag and later on, multiple bands against the other viral proteins are detected, which is likely due to levels of the appropriate antigen. Thus, there is a gradual maturation of the humoral response.

Viral particle presence was assessed via RT-PCR analysis using highly sensitive assays (1–5 copies/ml), and HIV was detectable in 53.3% of the seronegative but HIV-1–infected carriers. It has been reported that RT-PCR or nucleotide amplification tests, with a sensitivity threshold of 50–200 copies/ml, can detect HIV in the blood 10–12 days prior to seroconversion, and sometimes even earlier (25). However, following exposure, the virus primarily appears to target gastro-intestinal lymphoid tissues (GALT) where it replicates and causes pathology. During this initial acute infection period, the frequency of HIV-1–infected cells in the peripheral blood has been estimated to be less than 1 in a million CD4+ T cells, according to Dr. Robert Siliciano's data and others who have studied the SIV-infected rhesus macaques. Thus, performing RT-PCR assays for HIV-1 can provide reliable information regarding the presence of infection only when the levels reach 100–500 viral copies.

In the studies reported herein, there were 7 (+1 weak) RT-PCR positive among the seronegative HIV-1–infected donors [using very sensitive, not commercial, RT-PCR, and no pooling] and those 7 plus 10 additional donors were

detected as HIV antibody positive using the Stimmunology pre-treatment step for the blood samples prior to testing on approved diagnostic kits. The difference stems from the fact that Stimmunology has been proven to enable detection of HIV carriers at the initial lymphocyte priming stages (16, 20), which is believed to occur within days of immunogen exposure. It must be noted that while ST enhances antibody generation *in vitro*, it bears no effect on the sample's viral count, allowing the diagnostician to analyze early-stage antibodies despite viral latency. As the results clearly demonstrate, some post-ST–positive samples proved to be RT-PCR negative. This phenomenon further widens the gap between viral content–dependent methods of HIV detection compared to antibody-dependent methods.

In addition to the fact that Stimmunology enables earlier detection, independent of viral latency or its levels, there are also issues of cost effectiveness. It is readily acknowledged by the WHO and other international organizations that RT-PCR–based assays are too expensive to set up and maintain in resource-poor countries and especially in countries where the HIV incidence and prevalence are high. There is a continued effort to enable detection of infected yet seronegative donors within the realm of the antibody detection assays (e.g., 4th generation ELISA-based assays that combine antigen + antibody detection). We are extremely sensitive and aware of this issue and have thus chosen to focus on attempts to identify a method that in fact can be relatively easy to perform and is cost effective.

When comparing the results of two different assays used to detect the infection in a given population, the issue of "sensitivity" should be considered. Since Stimmunology is not an assay, one would be comparing the positive antibody testing using post-ST samples to those of PCR or NAT results (DNA or RNA) in the pre-ST sample. Since the comparison is between two different assays that detect two different things, analytical sensitivity and/or diagnostic sensitivity are not comparable. However, statistical/ diagnostic sensitivity (population wise) can be addressed by looking at two parameters: 1. How many of those detected by antibodies in post-ST samples were also detected by PCR (pre-ST). 2. On the time line, which signal appears first in a newly infected person.

Table 2 addresses the first parameter. Using very sensitive PCR (1–5 copies/ml), only 7/15 of the window period samples (i.e., antibody-positive post-ST only), had detectable virus. Thus, the Stimmunology process enables the antibody testing to become more sensitive than super sensitive RT-PCR. We humbly submit that less analytically sensitive PCR/NAT (or other systems for the detection of viral genome) would be even less statistically sensitive. As to the second parameter, Table 5 addresses this issue in a follow-up study in a Kenyan population, where antibodies in post-ST samples were detectable several months prior to seroconversion. The PCR/NAT tests have been shown to detect virus in the blood 10–12 days prior to seroconversion. Following an infectious needle stick injury, the health professional remained seronegative for the 5 months' duration of the follow-up, while virus was detected intermittently after 3 months (with borderline positivity at 2 months), with the signal lost at 5 months. However, antibodies were detectable form the second week onwards when using Stimmunology as a pre-treatment of the blood prior to the routine antibody testing (data not shown). Thus, to conclude, our findings, as described in this manuscript, demonstrate a higher sensitivity of Stimmunology + antibody testing than ultrasensitive PCR.

Incidence values are currently calculated based on confirmed seropositive cases. In this study we were able to detect a 3.3% increase in the number of samples that were HIV antibody positive post-ST over those detected by the regular pre-ST ELISA. Thus, using the ST step, two complementary pieces of information can be derived: 1. the incidence rate based on the % of seronegative Stimmunology positive (post-ST sample) in the population tested—i.e., the % of HIV carriers in the window period. 2. the % of newly seroconverted among the total seropositive. The stimulation index is utilized herein to suggest that this can potentially be used as a tool for incidence calculations based on cross-sectional studies and assays set for differentiating between recent and old seropositives. This tool will be further evaluated in the future and compared with detuned and BED assays, etc.

Calculation of the incidence, solely based on the number of ST-detected carriers, is impossible, as the serological window period length of the population remains unknown. Several models have been proposed for estimating the length of the window period, but the majority is based on large-scale studies in low-risk blood donor populations. Alternatively, some models are based on calculations of seroconversion in individuals transfused with infected blood, where the time of exposure and seroconversion are known (26). However, these models do not reflect African or other populations at high risk for HIV infections, neither in their mode and dose of infection nor in their genetic and environmental background. The increase in seroprevalence reported by Kenya's National AIDS and STDs Control Programme (27) and other international agencies is lower than that which is implied by the percentage of window period individuals detected in this study. However, the incidence cannot be calculated based on the rise in seroprevalence of a given population, as the number of deaths over the period of interest must also be considered. Furthermore, a longer window period than that which is typically calculated for non-African populations can explain the significantly higher "incidence" reported herein. Such a model, describing a longer window period, is supported by results seen in an additional African population (28). This model also infers that the HIV replication rate seen in the active viremia (29) stage prior to seroconversion is not constant, where long periods of low-level viremia and even longer periods of latent infection

(13–15) can exist. Those in the window period are those recently infected, but to actually calculate the incidence one would have to have a good estimate of the length of the window period and the length of the asymptomatic period in this population. Another key factor would be whether the donors represent the general population (e.g., general risk–associated life style and recent change in life style) or rather a skewed population representing a higher risk of recent exposures. Using Stimmunology-based results and comparing pre-ST and post-ST antibody results, both qualitative and quantitative, has enabled the calculation of the window period length in the Ethiopian cohort, indicating it to be much longer (mean of 5 months) than in the Western population (29). Since this population had a recent marked change in the level of risk associated with their "life style" and exposure to sexual workers—this population would resemble more the high school donors. The difference could be accounted to loss of virginity and frequency of sexual contacts in the younger population.

Studies are currently being done to assess the incidence rates in given populations based not only on the rate of window period samples, but also on the "Stimmunology index," i.e., the increase in antibody levels in the sample following the Stimmunology step. This ratio of OD reading post-ST/pre-ST is higher in recent seroconvertors versus those that have been seropositive for a longer time and antibody production *in vivo* is fully expressed.

A follow-up study of such Stimmunology-positive/regular plasma ELISA-negative women has shown that 4/5 of them became positive, using regular plasma on the ELISA, within 4–6 months. These data provide further support both for the validity of the Stimmunology-based assay for early detection of HIV infection and an additional indication regarding the length of the seronegative window period in the study population. Studies in other high-incidence, high-risk populations in Sub-Sahara countries also indicate the existence of a window period longer than the 3 months (for 95% seroconversions) calculated based on other populations.

The Stimmunology blood pre-treatment step is a novel and new approach to earlier and more complete detection of HIV-infected individuals. Much work has been done to try and understand the changes in the cell populations' profile when suppression is overcome that leads to production of suppressed antibody. Due to the limitations of clinical trials in resource-poor settings, especially with unlinked samples, this was further studied using the monkey model of AIDS—the SIV infection. The frequency of various major subsets pre- and post-Stimmunology using FACS analysis was compared. While the frequency of total CD4+ cells did not change (34.5–39.4, the frequency of CD4+ T cells that expressed CD25+ rose from 3.5 to 19.4. The CD8+ cells were reduced from 37.5 to 13.4, with most of them being CD25+. CD20+ cells increased three-fold (from 11.9 to 37.4), with CD14+ reduced from 6.3 to 3.2. Most B cells were large blasts.

The avidity of IgG antibody is well known to increase progressively with time after exposure to an immunogen (1). The avidity of the antibodies produced in ST culture of cells from SIV-infected monkeys was measured using an indirect ELISA and running each sample in two wells, one of which was treated with 8M urea. The results were expressed as an Avidity Index (AI), which was determined as the ratio of the OD value obtained following urea treatment divided by the OD obtained without urea treatment, expressed as a percentage. The post-ST samples from monkeys ($n = 12$) 7 days after infection were clearly positive, but the avidity was much lower ($44.9 \pm 9.4\%$) than that of chronically infected monkeys ($n = 21$, $AI = 99.8 \pm 6.5\%$).

In summary, results of this study reveal a higher-than-expected percentage of window period HIV carriers in young Kenyans living on the outskirts of Nairobi. These results suggest that even among young donors, more caution must be taken when screening potential blood donors. Addition of the Stimmunology technique to standard antibody testing could bridge the detection gap which is due to the window period of seronegative HIV carriers. The addition of the Stimmunology-based blood pre-treatment step and the analysis of the results does not require sophisticated automated machines nor ultragrade pure reagents to perform. However, since the five-day culture process is too long in a blood bank routine setting, it has been reduced to 3 days following a large-scale comparative study showing high (albeit not optimal) detection of the window period donors after 3 days.

As Stimmunology enables a more comprehensive detection of HIV carriers, it can serve as a means of significantly limiting the risk of HIV transmission via blood banks in areas where HIV is epidemic. Furthermore, early detection can allow for immediate enrollment of HIV carriers into prevention and education programs, hopefully stemming the incidence and prevalence of HIV infection.

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