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HIV p24 Antigen Assay in the Diagnosis of Immunological Failure Among Patients with HIV Infection in Mwanza City

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ABSTRACT

Background: Despite the great efforts to expand anti-retroviral therapy (ART) services in Tanzania since 2004, data shows that the proportion of patients on second line is still very low. This may be due to the fact that patients who fail the first line are not recognized in time.In a resource limited country like Tanzania quantification of the human immunodeficiency virus type 1 (HIV-1) RNA levels (viral load) in plasma is limited. This is due to lack of enough health facilities to performreal-time based PCR assays.This study aimed at evaluating an Ultrasensitivep24 antigen assay as a potential alternative to HIV-1 RNA viral load assay.

Methodology: HIV infected patients attending care and treatment clinics in selected Mwanza city hospitals (Buzuruga, Nyamagana and Sekou-Toure) were recruited. Aseptic venipuncture wasdone to obtain 5mls of blood. This was used for p24 antigen assay, viral load assay and CD4 determination. Laboratory results were then compared to detect the utility of p24 assay compared to real time based PCR assay in establishment of immunological failure.

Results: A total of 270 HIV- infected participants were recruited (192 ART naïve and 78 (29%) on ART). HIV experienced ART had lower median (IQR) p24 antigen level of 3.4 (1.53; 4.43) pg/mL compared to 10.6 (3.34; 39.32) pg/mL median p24 antigen level of HIV ARV naïve. In the period of 6 months and above of being on ARV, 14.5 % of the participants were categorized as immunological failure while virological suppressions was 69.2 %. CD4+ lymphocyte count at recruitment and heat denatured p24 antigen level were significantly correlated with each other (r = -0.38; 95%CI, -0.48; -0.27, p<0.001). The gradual increase of p24 antigen level with time correlated with an increase of proportion of immunological failure. Only CD4+ lymphocyte was highly predicative of subsequent immunological failure. In cox multivariate models the age and CD4+ lymphocyte were factors that were highly predictive of subsequent immunological failure.

Conclusions: p24 antigen level in our study population was well correlated with CD4 lymphocyte count. The rise in p24 antigen levels was correlated with the proportion of immunological failure. Although p24 was not found to be statistically significant predictor of immunological failure but its correlation with CD4 count still makes it a useful indicator of immunological failure. p24 antigen assay can easily be measured in our resource limited environment with minimum challenges.

KEYWORDS

p24 antigen assay; Immunological failure; Virological failure; Mwanza; Tanzania.

BACKGROUND

In Tanzania, the HIV/AIDS prevalence by 2012 was about 5.1% and was higher amongst women 6.2% than that of men 3.8% [1]. The prevalence was higher in urban (8.9%) than in rural (5.1%) areas as well [1]. It was reported that there is about 90,000 – 130,000 new HIV infections annually and the new

infections exceeds deaths. The national HIV/AIDS Care and Treatment Plan for Tanzania was launched in 2004, charged with the responsibility of providing quality HIV care and treatment to as many patients as possible. At the start, 71,439 (13.6%) of the estimated 527,357 AIDS cases commenced ART, by the end of the 2008, the operational plan stipulated that, 400,000 AIDS patients be on antiretroviral drugs.

Despite the great efforts to expand ART services in Tanzania, data has shown that the proportion of patients on second line is still very low in most health facilities away from Dar es Salaam and consultant hospitals. This is because patients who fail are not recognized in time due to the fact that most patients are not monitored on their immunological status after initiation of ART, which is the main way to detect failure in Tanzania. On the other hand clinicians do not regularly assess patients for clinical failure and the viral load is not done in many centres because it is expensive in terms of resources and human capacity [2]. ELISA assay of HIV-1 p24 antigen can be used in the place where viral load assay is not available.

In developed countries, the diagnosis of ART failure is based on HIV RNA concentration and the detectable level in two consecutive tests is used to define virological failure. In a study on ART follow up in 14 African countries it was found that 27% of patients who had been on ART for 6 months to 4 years had plasma RNA of greater than 500 copies/ml. Single country study in Botswana, Malawi, Uganda and Cameroon showed that 15-25% of patients had plasma HIV RNA greater than 400 copies/ml [3-6].

Systemic review and meta-analysis showed that there might be high levels of genotypic resistance to NRTIs in resource poor settings particularly if viral load monitoring is not available. Few studies have been done to detect virological failure in Tanzania, the study in Mbulu reported the virological failure of 12% of which 23% were associated with thymidine analogue mutations (TAMs) that are associated with cross resistance to all NRTIs [7]. The prevalence of immunological treatment failure in Tanzania is approximately 17% in the study that was done at Bugando Medical Centre and Muhimbili National Referral Hospital. In Bugando study, there was a significant association between baseline CD4 count less than 100cells/µl and immunological treatment failure. Also the average time to treatment failure was found to be 20 months and 59% of failed patients had a lag time of 5 weeks before changing the ART regimen [8, 9, 10]. This formed the basis for evaluation of simple assay thatcan be used in lower health facilities and rural areas to monitor treatment so that early intervention can be done in order to reduce accumulation of mutations conferring resistance to various ARVs.

In the 1990s, new technologies, including the polymerase chain reaction (PCR) assay [11], the branched DNA (bDNA) assay [12] and the nucleic acid sequence-based amplification (NASBA) assay [13] were developed. These have made it possible to quantify accurately, human immunodeficiency virus (HIV) RNA in plasma. HIV RNA plasma levels predict powerfully the risk for disease progression [1, 3, 4]. They are also used to determine the effectiveness of antiretroviral drugs in clinical trials [11, 14, 15]. Quantification of viral load in plasma is necessary to assess patients' prognosis and manage HIV antiretroviral therapy [16, 17]. The importance is reflected in the revised WHO guidelines for HIV treatment in resource-limited settings [12]. For the first time, viral load data were included in the criteria to define treatment failure, and viral load thresholds for resource-limited settings were suggested. Apart from the obvious benefits of viral load measurements in resource-limited settings such as: (a) Treatment failure, (b) Adherence monitoring, resistance surveillance and vaccine efficacy studies, (c) Diagnosis of HIV infection in infants, viral load testing is an important assay in any laboratory running expensive sensitive assays such as HIV Genotyping and HIV Drug Resistance Testing. It serves as a quality control measure prior to testing and will save a lot of time and resources. In the developed countries, there are four systems to measure viral load: the Abbott real-time HIV-1 PCR assay, the Bayer Versant HIV-1 RNA 3.0 (bDNA) assay, the bioMe´rieuxNucliSens HIV-1 QT (NASBA) assay, and the Roche Amplicor HIV-1 Monitor 1.5 (RT-PCR) assay (which can also be run in a real-time format) [18, 19]. Versions of the real-time assays have been successfully transferred to the south using generic, low-cost reagents, with performance comparable to existing assays [20], and at a reduced cost [21], however expansion of viral load testing capacity has been highly variable in resource limited countries [22]. Any of the real-time based assays require expensive machines, refrigerated reagents, isolation rooms to prevent cross-contamination and highly skilled laboratory technicians.

The Ultrasensitive p24 Antigen Assay [23] detects HIV-1 p24 antigen (qualitative and quantitative) in HIV-1 infected patients using plasma specimen in EDTA tubes. This assay was developed by PerkinElmer, Inc. (Waltham, Massachusetts, USA) and termed the ultrasensitive p24 antigen assay (Up24) [24]. Up24 has 100% sensitivity of detection at a viral load of > 30,000, with sensitivity of 46.4% at a viral load of < 30,000. The assay was highly reproducible, with excellent correlation between duplicates and among three laboratories [23- 25].

This study evaluateda simple technique to assess virological failure that can be expanded in most centres in Tanzania. This study intended to determine correlation between p24 antigen and viral load and determine applicability of p24 assay in the

diagnosis of immunological and clinical failure. The information gathered can be used to guide intervention regarding monitoring of patients on ART in Tanzania.

METHODOLOGY

Study area

This study was conducted in Mwanza city Tanzania. The city consists of Nyamagana and Ilemela districts. The study involved HIV patients attending Care and Treatment Clinics (CTC) in the two districts. CTC activities in Mwanza started in 2004 and have been extended to all hospitals in Mwanza city.

Study population and inclusion criteria

This study was conducted among HIV infected patients attending CTC services in Mwanza and participants were included in the study if : (1) aged above 18 years (2) is HIV positive (3) is a resident of Mwanza city (4) written informed consent was given and were excluded if:

1) Pregnant women (2) severely ill (3) had psychiatric disorders (4). on second line ART

Study design

This wasa cross-sectional study among HIV infected patients (naïve and ART experienced) attending CTC clinics in Mwanza city.

Sample size and sampling

The sample size calculation was based on the WHO HIV drug resistance (HIVDR) threshold survey methods that recommend a sample size of at least 47 patients in a surveillance of transmitted HIV-DR [26].To increase the power of the study, we collected 270 samples and patients were recruited serially until the sample size was attained.

Study outcomes

The primary outcome was proportion of HIV-infected patients (naïve and ART experienced) with virological failure as extrapolated by ultrasensitive p24 Antigen assay (Alliance HIV-p24 ELISA kit and ELAST ELISA amplification system, PerkinElmer, Inc.) as potential alternative to human immunodeficiency virus Type 1 RNA Viral Load Assay.

Data collection

Prior to study implementation, training was provided to data collectors and laboratory technicians to ensure quality data. Data were collected using questionnaires, checklists and laboratory forms. At time of enrolment to the study, information on baseline characteristics such as age, sex, ART regime were noted. CD4 count and ARV adherence of the patients were retrieved from clinic case report forms and electronic databases where appropriate.

Patients, samples and laboratory procedures

Blood samples were collected in a 5ml EDTA stabilizing tube from consenting participants and stored at -800C. The p24 assay was performed according to the manufacture's instruction as provided with the p24-specific viral load ELAST amplification system kit (catalog no. NEP116VL; Perkin Elmer Life Sciences), which is used in combination with an HIV-1 p24 enzyme-linked immunosorbent assay kit (catalog no.NEK050) for Up24. After the addition of orthopenylenediamine- HCl substrate the plate is read kinetically for 30 min by using Quanti-Kin detection system software (Rilab, Genoa, Italy) as described previously [27]. The colorimetric reaction is stopped after 30 min, and the endpoint reading is determined for final calculations with the Quanti- Kin software. Log10 transformations are then used for extrapolating viral load. All analyses were done at the NIMR, Mwanza Centre Microbiology Laboratory which complies with good clinical laboratory practices.

Statistical analysis

The stata version 12.0 (Stata Corp LLC, College Station, Texas, USA) was used for data analysis. The collected data was firstly double entered using Epidata software version 3.1 before transferring to stata software. Descriptive statistics were computed and comparisons of associations were done using appropriate statistic tests. In this analysis we defined immunologic failure if the participant met one of the following criteria:

1) Persistent CD4 below 100 cells/mm3. 2) A drop of CD4 cell count below baseline pre-treatment level or 3) a drop of CD4 cell count of 50% from peak on treatment value all in the absence of an ongoing co-infection and after a minimum of 6 months of ART [28].Whilevirological suppression was defined as HIV viral load <400 copies/mL, since this was the detection limit of the assay used in this study [29]. Association among p24 antigen level, CD4+ lymphocyte count, and viral load were compared by using Spearman's rank correlation coefficients with confidence intervals that were calculated by using Fisher's Z-transformation in stata version 6. Log transformation was used to improve skewness, and other measure of normality for CD4+ lymphocyte count, viral load, and p24 antigen level. Figures were done using two-way scatter plotting. Cox proportion hazards models was used to determine univariate and multivariates predictors of immunological failure and Kaplan-Meier survival analysis of time to immunological failure was performed and probability value (p-value), where applicable, of less than 0.05 was considered significant.

Ethical consideration

Consent for participation was sought from all study participants. The researchers undertook to protect the confidentiality of all informants utilizing the information only for the

purpose stated in the consent form. No real names of informants or specific information onplace of residence was used in reports or publications. Ethical approval was granted by the National Institute for Medical Research in Tanzania. Participation to the study was voluntary and subjects were free to withdraw from the study at any point during study implementation and this was meant not to affect their right to receive routine health care.

RESULTS

Of the HIV- infected participants recruited, only 78 (29%) were on Anti- retro viral therapy (ARV). Viral load results were only available for HIV experienced ARV participants (78), while CD4+ lymphocyte counts and p24 antigen level results were available in 266 and 270 HIV infected participants respectively. Of those HIV ARV naïve patients recruited 74.7% were female and for HIV experienced ARV, female were 64.6 %. Over all median (IQR) p24 antigen level was 5.81 pg/mL. However, HIV experienced ARV had low median (IQR) p24 antigen level of 3.4 (1.53; 4.43) pg/mL compared to 10.6 (3.34; 39.32) pg/mL median p24 antigen level of HIV ARV naïve. Median CD4+ lymphocyte counts across the group and other background characteristics of study population are summarized in Table 1.

Table 1: Background characteristics of study sample as representative of naïve and experienced HIV patients1.

variable	HIV naïve patients (n = 192)	HIV experienced ARV patients(n =78)
Mean age (range), y	37.3 (14.7; 68.5)	40.3 (18.1; 63.0)
Female sex, No. (%)		
	145 (74.7)	51 (64.6)
Occupation, No. (%)		
Salaries employee	8 (4.1)	11 (13.9)
Self employee	118 (60.8)	51 (64.6)
Housewife	51 (26.3)	12 (15.2)
Student/unemployed	17 (8.7)	5 (6.3)
Education level		
No formal education	44 (22.8)	10 (12.7)
Primary school	128 (66.3)	54 (68.4)
Secondary school	19 (9.8)	14 (17.7)
College/university	2 (1.0)	1 (1.3)
Mean weight (range), Kg	55.3 (20; 105)	60.5 (44;95)
Median CD4count at recruitment (IQR), cell/µl	335 (104; 511)	352 (214; 471)
BMI at recruitment (kg/m2)		
< 18.5	46 (25.7)	5 (6.4)
>18.5	133 (74.3)	73 (93.6)

[1]Experience represents HIV patients on ARV for 6 months and above

In the period of 6 month and above of being on ARV in this study population, 14.5 % of the participants were categorized as immunological failure while virological suppressions (defined as HIV viral load <400 copies/mL) was 69.2 %. As time of being on ARV increased the risk of ending up with immunological failure was decreasing (Figure 1). Compared to male, female immunity got weaker especially in first 40 months of being on ARV (Figure 2).

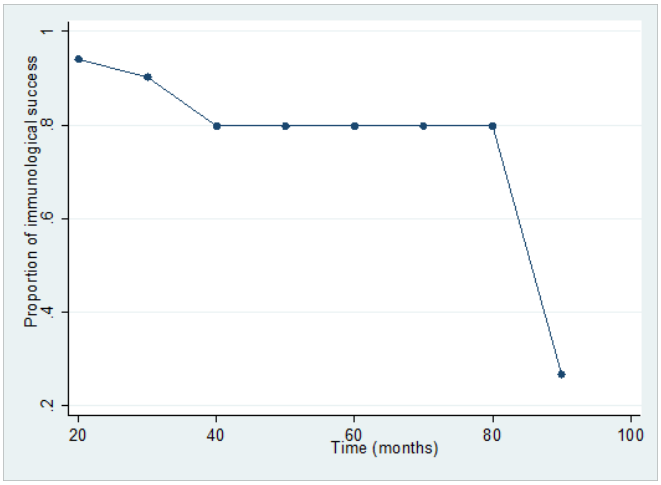

Figure 1: Curve showing progression to immunological failure starting six months after being on ARV.

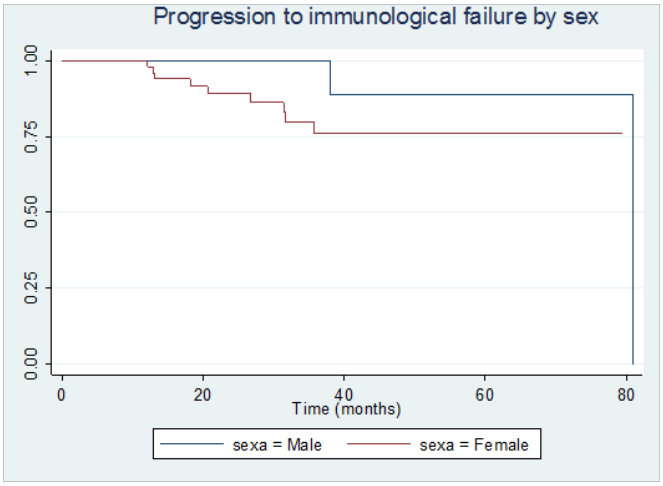

Figure2: Kaplan-Meier analysis stratified by sex with respect to progression of immunological failure among HIV ART experienced patients.

CD4+ lymphocyte count at recruitment and heat denatured p24 antigen level were correlated with each other. CD4+ lymphocyte counts correlated with p24 antigen level by r = -0.38 (95%CI, -0.48; -0.27, p < 0.001). CD4+ lymphocyte show weakest trend correlation with viral load by r = 0.07 although this was not statistically significant (p = 0.53). Other examined correlation between p24 antigen level and viral load at recruitment did not appear statistically significant. (Table 2, Figure 3-5). The gradual increase of p24 antigen level with time correlated with an increase of proportion of immunological failure (Figure 6).

Table 2: Association of CD4 lymphocyte count, p24 antigen level, and viral load level using spearman's rank correlation with, 95% confidence interval (95% CI) and p-values.

	n	Variable	r (95% CI)	p
Model A	266	CD4 lymphocyte count vs p24 antigen level	-0.38 (-0.48; -0.27	<0.001
Model B[1]	75	CD4 lymphocyte count vs Viral load	0.07 (-0.16; 0.29)	0.54
Model C[1]	78	p24 antigen level vs viral load	-0.03 (-0.25; 0.19)	0.77

[1]Viral load data available for only HIV ARV experienced participants

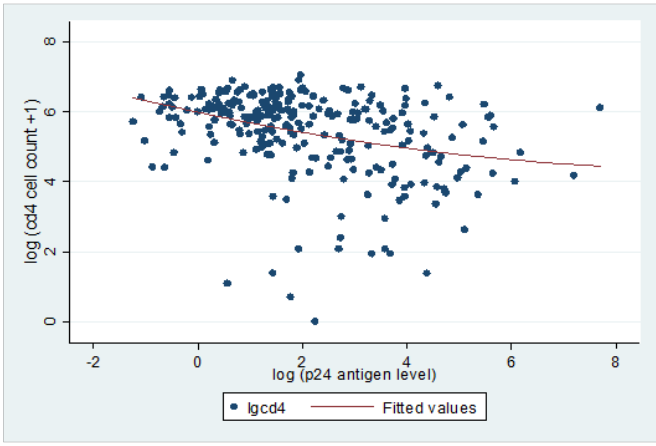

Figure 3: Model A, showing relationship between CD4+ lymphocyte count and p24 antigen level.

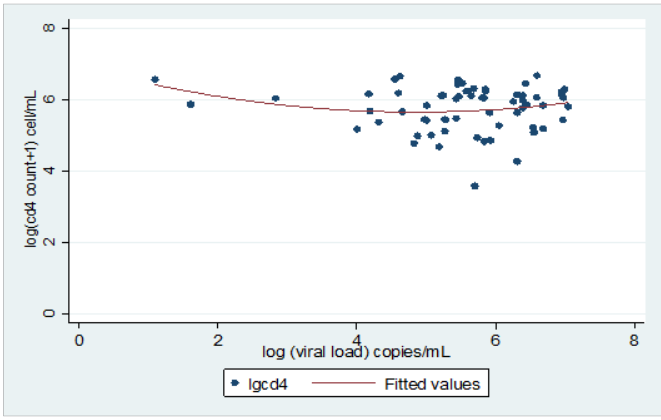

Figure 4: Model B showing relationship between CD4+ lymphocyte count and viral load level.

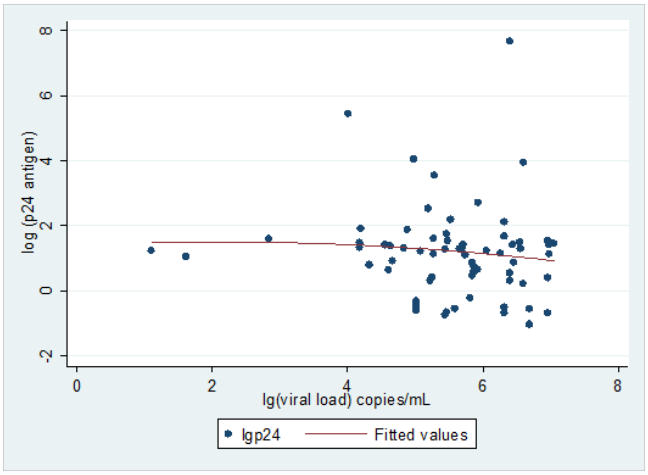

Figure 5: Model C showing relationship between CD4+ lymphocyte count and viral load level.

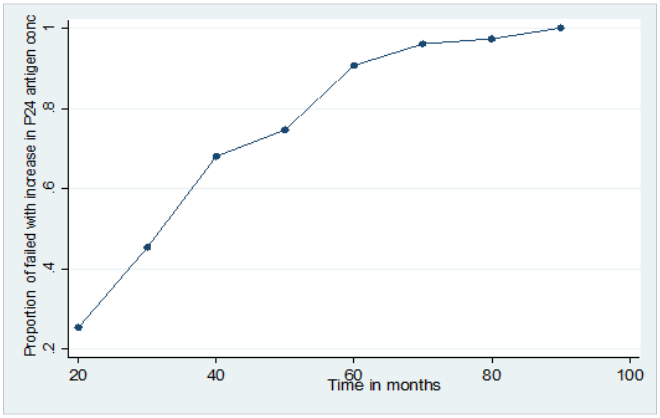

Figure 6: Progression to immunological failures with increase of p24 antigen level.

The predictive value of immunological failures was assesed among 78 HIV ARV experienced participants. Cox proportional hazard models were constructed to assess predictors of progression to immunological failures (Table 3). In univariate analysis variables tested were age, gender, CD4+ lymphocyte, p24 antigen level, BMI and viral load at recruitment, Of these factors, only CD4+ lymphocyte was highly predictive of subsequent immunological failure. For each increase change in unit CD4+ lymphocyte resulted in decrease of incidence of immunological failure by hazard ratio of 0.2 (95%CI; 0.09; 0.41, p<0.001). In cox multivariate models the age and again CD4+ lymphocyte, persisted as factors that were highly predictive of subsequent immunological failure. Increase of unit age was associated with immunological failure by hazard ratio of 0.86(95%CI; 0.75; 0.99, p < 0.04) while unit CD4+ lymphocyte by hazard ratio of 0.04(95%CI; 0.03; 0.43, p < 0.01) (Table3). Other factors in multivariate model did not appear statistically significant as predictors of the immunological failure progression.

Table 3: Predictors of immunological failure among experienced HIV patients on ART for more than 6 months with odd ratio, 95% confidence interval (95% CI) and p-values.

		Univariate Adjusted OR1		Multivariate OR	
	n	HR (95% CI)	p	HR (95% CI)	p
Age (y)	77	0.96 (0.89;1.04)	0.38	0.86 (0.75; 0.99)	0.04
Gender, %		1	0.15	1	0.72
Male	78	4.53 (0.57; 35.32)		1.54 (0.0.14; 16.97)	
Female					
CD4 count	75	0.20 (0.09; 0.41)	<0.001	0.04 (0.03; 0.43)	0.01
P24 antigen	78	1.23 (0.91; 1.67)	0.17	1.46 (0.91; 2.36	0.12
BMI at baseline (kg/m2)	78				
< 18.5		1		1	0.05
>18.5		1.57 (0.33;7.44)	0.56	43.01 (1.02; 1850.08)	
Viral load at recruitment1	66	1.02 (0.57; 1.81)	0.95	0.96 (0.37; 2.46	0.93

[1]Viral load data are not summing to 78 due to some missing data

DISCUSSION

The overall p24 antigen level at baseline among our study population was 5.81pg/ml, it was 10.6pg/ml among HIV naïve and 3.4pg/ml among ARV experienced patients. The decline in the p24 antigen levels is expected and is similar with other findings as this drop with an increase in CD4 lymphocyte counts [30].

For patients who were on ARV for six months and above, immunological failure was found to be 14.5% and virological suppression (viral concentration less 400copies/ml) was found among 69.2%. The prevalence of immunological failure in our study was lower than that detected in Bugando by Jakaet al [10]. Virological suppression was found in 69.2% of the study population on ART for 6 months and above. This level of suppression was lower compared to other studies in Africa (studies in Botswana, Malawi, Uganda and Cameroon) where the patients who had copies above 400copies/ml were between 75-85% [3-6]. This can be attributed to an increase in primary drug resistance among the HIV patients in our group and poor adherence.

In this study there was a correlation of CD4 lymphocyte count and p24 antigen in plasma at recruitment (r = -0.38 and p<0.001). The increase in p24 antigen level in plasma correlated with an increase in the proportion of immunological failure. This finding in our population is similar to what has been reported in developed countries [30] and other developing countries [31]. In most studies done, there are always good correlations between CD4 lymphocyte count and p24 antigen [30, 31]. This shows that if the baseline p24 antigen level is known then the increase in p24 antigens correlates well with immunological failures. This finding shows that the heat denatured p24 antigen assay can be useful in determination of immunological failure and decline in CD4 lymphocytes in our population contrary to the said theory that p24 antibodies impairs detection in African populations.

The rise in CD4 count and p24 antigen levels did not correlate with viral load in our study patients. This can be explained by the fact that p24 can still be found in plasma from viral particles destroyed in the lymphatics. It has been clearly stated that in stably suppressed viraemiap24 antigen may still be detected in significant amount from viruses destroyed in the lymphatic system. The destroyed viruses release both viral RNA and p24 proteins but the RNA in blood is quickly destroyed by RNAse while the proteins remain for longer time [30]. The shorter half life of the viral particles is also another explanation for this.

The predictors of immunological failures in our study were found to be only CD4 lymphocyte count and age. There was 46% chance of immunological failure with a unit increase in p24 antigen levels though was not statistically significant (p = 0.12). We think if a study with larger sample size is done this may be significant. This is similar to the study done previously in Bugando [10].

Due to limited funding our study could not do sequencing of the viral RNA to detect both primary and secondary drug resistance. The samples have been kept in our refrigerators and this can be analysed pending availability of funds.

CONCLUSION

p24 antigen level in our study population was correlated with CD4 lymphocyte count. The rise in p24 antigen levels was correlated with the proportion of immunological failure. Al-

though p24 was not found to be statistically significant predictor of immunological failure but its correction with CD4 count still makes it a useful indicator of immunological failure. P24 antigen assay can easily be measured in our resource limited environment with minimum challenges. We recommend further studies especially with larger sample sizes in our environment

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