THE SENSITIVITY OF AN HIV P24 ANTIGEN AND ANTIBODY ASSAY USED IN PARALEL (3RD GENERATION) COMPARED TO HIV ANTIGEN/ANTIBODY COMBINATION ASSAYS (4TH GENERATION) TO DETECT RECENT HIV INFECTION IN SOUTH AFRICAN BLOOD DONORS AS IDENTIFIED THROUGH NUCLEIC ACID TESTING

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ABSTRACT

Introduction

Although Nucleic Acid Testing has become the gold standard for the detection of HIV it remains an expensive technology. In Africa the use of sensitive serological assays might be a more viable option. HIV Combo assays have been on the market for several years and have been evaluated. Four cases of a 2nd diagnostic window period have been reported by the use of these assays. This raised questions regarding the sensitivity of these Combo assays compared to stand-alone serological assays for HIV detecting antigen and antibodies separately. In this study the sensitivity of three HIV Combo assays were compared to stand-alone assays by the use of 2 unique sample groups of early HIV infections as identified by NAT since 2005 by the South African National Blood Service (SANBS).

Methods

A retrospective study using two sample groups of archived plasma from HIV positive blood donors;

A) 153 HIV NAT yield samples B) 87 low antibody ratio (Abbott Prism) recent HIV samples. These groups were tested by three HIV Combo assays (Abbott, Biorad and Roche) and one p24 antigen assay (Innogenetics). The Abbott Prism HIV O Plus assay results were already available as reference method for antibody detection.

Sensitivity to detect HIV antigen and/or antibodies were evaluated. The significance of donor demographic indicators of donor group (new, re-joined or active) race, age, gender and region were analysed in relation to the presence/absence of p24 antigen between the two groups.

Results

The HIV Combo assays showed equal sensitivity with regards to p24 antigen detection compared to the p24 antigen assay with only the Roche missing 3 additional samples. All low ratio HIV antibody samples were detected by the HIV Combo assays indicating equal sensitivity to detect antibodies to HIV compared to the antibody only assay.

There were significantly more p24 negative NAT yields amongst new donors, donors presenting for a 2nd donation, donors aged between 20 to 24 and female donors.

Conclusion

HIV Combo assays can be used as a testing strategy in Africa and the possibility of a 2nd diagnostic window period could probably be ignored in decision making. Compared to a NAT strategy these assays would still be able to detect about 4 out of 10 early pre-seroconversion infections by detection of p24 antigen. For countries already using NAT, the Combo assays could probably be used for its ability to detect HIV antibodies. From the analysis of demographic indicators it seems being a new donor, making the 2nd donation, being female or being between the ages 20 to 24 makes it more likely that one would present for a blood donation in the window period prior to p24 antigen being detectable (approximately 16 days) raising the possibility of test seaking amongst these groups.

Introduction

Testing and treatment strategies for Human Immunodeficiency Virus (HIV) are under constant development since the discovery of the virus in 1981 [1] and the release of the first diagnostic test approved by the Centre of Disease control (CDC) in 1985 [2].HIV testing strategies changed drastically over the last 10 years due to the availability of direct viral genome detection using nucleic acid testing (NAT) and Individual Donation (ID)-NAT has become the gold standard for the detection of early HIV infections [3, 4]. The importance of anti-HIV testing in combination with NAT testing remains critical due to potential HIV elite controllers with low or undetectable virus in circulation, but usually detectable antibodies [5]. This is emphasised in the data reported by SANBS which indicated 21 potential HIV elite controllers identified by anti-HIV testing only in an ID NAT setting [6].

A testing strategy which includes NAT remains an expensive option which requires extensive resources. In an African setting a testing strategy with NAT is not always a viable option. Serological assays like HIV p24 and 4th generation HIV antigen/antibody combination assays (HIV combo) are a more affordable option [7]. The sensitivity of various serological assays and systems to detect HIV infection at an early stage are well described [3, 4] and a study by Ly *et al* concluded that HIV 4th generation assays could be able to replace p24-only assays [8].

When considering a "window period" for HIV infection where infection will not be detected, this term is primarily used to refer to the first few days after infection when the amount of virus is still too low to detect and antibodies have not been produced. HIV Combo assays have been reported to have another possible limitation where false negative results may be obtained due to a second diagnostic window period and four cases of this second diagnostic window have been described [9-11].

This second diagnostic window period is possible at a specific point in time after infection when the amount of HIV antigen declines and the HIV antibodies are still at an early stage of development with perhaps very little Immunoglobulin G (IgG)(Figure 1) [12]. A short "window" is possible where the amount of p24 antigen (HIV viral antigen) and/or antibodies to HIV is below the detectable limit of a specific assay. High amount of antibody/antigen complexes is also a possibility at this specific time and might affect the ability of the HIV Combo assay to detect either the p24 antigen and/or antibody [11]. Most HIV Combo assays do however also detect Immunoglobulin M (IgM) now and has a p24 sensitivity of less than 30 pg/ml which increases the sensitivity [12].

In this study we took to a step back from NAT testing by evaluating the sensitivity of a stand-alone p24 assay and 4th generation HIV Ag/Ab combo assays by their ability to detect primarily clade C in recently infected donors. A group of early ID-NAT positive, antibody negative (NAT Yield) samples as well as a group of low level anti-HIV samples (LLaHIV) was used to challenge the assays' ability to detect p24 antigen and HIV antibodies and investigate the possibility of a 2nd diagnostic window period.

Methods

Sample groups

Frozen plasma samples stored at the SANBS Plasma Unit Repository (PUR) were used. Over a 5 year period (Oct 2005 – Sept 2010) 165 confirmed window period donors (NAT repeat positive, anti-HIV negative) were identified [13]. These donors were all confirmed NAT positive by repeat testing (Procleix ULTRIO Assay, Gen-Probe Incorporated 10210 Genetic Centre Drive San Diego, CA 92121 USA)[14], which included 3 times ULTRIO and a discriminatory HIV screen on the index donation. Confirmation was performed by repeating the same tests on a separate sample obtained from the stored plasma bag from the donor and/or recalling the donor for testing. Discrepant results on the plasma bag and/or donor retest with the index would exclude the donor from this data. From this group 153 samples were used in this study which had sufficient plasma stored.

The second group was of samples were selected based on the following criteria:

- NAT and anti-HIV positive with Prism anti-HIV signal/cut off ratio (S/CO) of less than 10 (Abbott laboratories, Abbott Park, IL)
- Negative on a detuned HIV assay (Alere Determine™ HIV 1/2, Alere Medical Co., Ltd.357 Matsuhidai, Matsudo-shi, Chiba, 270-2214 Japan)

HIV positive data from Oct 2005 until Dec 2012 was reviewed to identify low ratio (Abbott Prism) samples. Only 87 samples could be found with plasma available. The method described by Kshatriya *et al* [15] which involves the use of a 1:1000 dilution was adopted in this study to screen Group 2 samples.

Testing methodology

The following assays were evaluated in this study:

1. Innogenetics INNOTEST HIV Antigen mAb (INNOGENETICS N.V. Technologiepark 6, 9052 Gent, Belgium)
2. Abbott Architect HIV Ag/Ab Combo (Abbott, Max-Planck-Ring 2, 65205 Wiesbaden, Germany)
3. Biorad Genscreen ULTRA HIV Ag-Ab (BIORAD, 3 bd Raymond Poincaré, 92430 Marnes-la-Coquette-France)
4. Roche HIV Combi PT (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim)

ELISA plates from INNOTEST and Genscreen were sampled manually and processed on the Siemens BEP III analyser (Siemens Healthcare Diagnostics GmbH Ludwig-Erhard-Straße12 65760 Eschborn) or alternatively processed manually by use of a Biorad PW40 washer and Absorbances determined on the Tecan Sunrise reader. Roche Combi tests were performed on the Roche Cobas e411 analyser. The Abbott HIV Ag/Ab tests were performed on the Abbott Architect i2000 instrument.

Results from the samples that were not in agreement across the 4 assays were repeated in duplicate to confirm the initial result.

The difference in sensitivity across the 4 different assays was be analysed by Chi-square analysis. The INNOTEST in combination with the Abbott HIV O Plus assay (Anti-HIV) was compared to the 3 HIV Combo assays. The significance of age, gender and donation type (new, re-joined or active) on the presence of p24 antigen within the two study sample populations were analysed by use of the Chi-square test.

Results

The BIORAD, Abbott and Roche antigen/antibody combination assays were able to detect all 87 LLaHIV samples (100%). Only 1 sample (Abbott Prism S/CO ratio 1.24) gave negative results on the initial screen on the BIORAD, Abbott and Roche assays but was positive on all 3 assays after repeat testing from a new sample. The INNO assay (antigen only) detected 46 of the 87 (52.9%) of the samples, indicating that 47.1% of the samples probably had undetectable p24 antigen and was

found positive by the antigen/antibody assays by detection of the low level HIV antibodies present (Table 1).

Compared to nucleic acid testing the INNO, BIORAD and Abbott assays failed to detect 91 (59.5%) stage 1 HIV infections whereas the Roche assay failed to detect 97 stage 1 infections (Table 1).

The HIV Yield group had significantly more p24 negative results with 62% (31 of 50) amongst New donors compared to 36.4% (8 of22) of the LLaHIV group (p=0.04) (Table 2). Proportions amongst re-joined and repeat donor were not significantly different (p=0.48 and 0.21).

Females donors in the NAT yield group had more p24 negative results with 67% (61 of 91) compared to the LLaHIV with 50% (20 of 41) but this was shown not to be significant (p=0.06). Proportions amongst male donors were not significantly different.

Race or geographic region had no influence on the proportion of p24 positive vs negative results when comparing the two groups.

The HIV Yield group had significantly more p24 negative results in the 20 to 24 year old age group with 78.3% (18 of 23) compared to 25% (3 of 9) in the LLaHIV group. The proportions amongst the other groups were not significantly different (Table 2).

It was already mentioned that new donor had significantly more p24 negative results in the HIV Yield group. When grouping donation numbers together (see table 2 for grouping) donors with 1 (new) or 2 donations had significantly more p24 negative results in the NAT Yield group compared to the LLaHIV group (p=0.005). Proportions in the other grouped donation numbers were not significantly different amongst the two groups.

Not surprisingly a donation interval of zero was shown as significant just as new donors mentioned. No other donation interval significantly influenced proportions of p24 positives vs negatives within the NAT yield group compared to the LLaHIV group.

Discussion

The sensitivity of the combo assays were similar to that of the INNO assay in the NAT yield sample group indicating that the assays were able to detect p24 antigen equally. The sensitivity of the combo assays were also similar in the LLaHIV group indicating that the combo assays were able to detect antibodies to HIV with equal sensitivity to the Abbott Prism HIV antibody only assay .The use of a stand-alone p24 antigen assay (INNO assay) together with a stand-alone anti-HIV assay (Abbott Prism) therefore did not prove to be more effective than using an HIV antigen/antibody assay.

Although one LLaHIV sample was found negative initially on the combo assays, this result was positive when the testing was repeated on new plasma samples indicating possible sample deterioration in the initial plasma sample used. The presence of a 2nd diagnostic window period with the use of HIV antigen/antibody assays therefore seems unlikely as this could not be demonstrated with the LLaHIV samples used in this study.

The negative results obtained by the combo assays on the LLaHIV sample initially does however demonstrated that sample age and quality plays an extremely important role in detection of an early HIV seroconversion and extreme care should be taken to ensure sample integrity

An even distribution of HIV positive donors throughout the Fiebig stages of HIV infection would be expected if donor behaviour was independent of any factors. Distribution in relation to p24 results would comprise of HIV RNA positive/p24 negative donors in the NAT Yield group, followed by HIV RNA positive/p24 positive donors. Seroconversion and development of HIV antibodies would lead to p24 antigen positive/anti-HIV positive donors followed eventually by p24 negative/anti-HIV positive donors in the LLaHIV group.

From the analysis of the p24 antigen reactivity amongst the two groups it seems like new donors, donors making the 2nd donation, female donors or donors between the ages 20 to 24 were more likely to present for a blood donation in the window period prior to p24 antigen being detectable (approximately 16 to 17 days) raising the possibility of test seeking amongst these groups [16].

Sub-saharan Africa is still the most heavily burdened region in terms of HIV and deaths related to AIDS [7]. Testing strategies in Africa range from NAT testing (Only 4 countries including South Africa), Enzyme Linked Immunosorbant Assays (ELISAs), rapid HIV assays and in some instances no testing at all [7]. Many challenges also exist in terms of Good Laboratory Practice (GLP) and adherence to quality standards. Organisations like WHO, TREC, AfSBTS, BSRI and many other have been committed to education and funding in Africa in order to address these critical issues. Many African countries have stated the desire to implement NAT testing, but will require international financial assistance to achieve this which might not be sustainable. The use of HIV Ag/Ab Combo assays would have a significant impact on the reduction of transfusion transmitted HIV in countries where Nucleic Acid Testing is not a viable option as this option could be as much as 20 times cheaper than NAT testing. With limited resources available for medical care, decision makers should weigh primary health care needs against testing costs and strategies. Based on the results in this study, these serological assays would still be able to detect about 4 out of every 10 early pre-antibody

seroconversion infections in donors by p24 antigen detection. This early stage of infection is also the most infectious stage of the virus as viral loads are highest during this stage (10,000 copies/ml or higher). The minimum infectious dose for HIV infection is estimated in the region of 20 copies [3]. Early detection of HIV antibodies (IgM and IgG) is also important as p24 antigen becomes undetectable once the immune response reacts and viral load declines. The serological assays used in this study instilled confidence in its ability to detect antibodies to HIV as it detected all low level anti-HIV samples in this study. This study indicated that the likelihood of missing an HIV infection due to a 2nd diagnostic window period by the use of HIV Combo assays are unlikely and that this possibility could probably be excluded from risk modelling and decision making.

Antibody detection remains an important aspect even when using NAT testing due to the possibility of "Elite controllers" who are able to suppress the HIV viral load to levels undetectable even by NAT. Currently not all NAT tests are designed to detect the presence of HIV-2, therefore this should be considered in countries where HIV-2 is prevalent. Based on the results in this study, these HIV Combo assays can also be used in parallel with NAT testing purely for its ability to detect antibodies to HIV. A HIV Combo assay might be more practical as part of a testing strategy for all donations due to instrumentation, availability or cost in comparison to an antibody only assay. A suggested algorithm could be to test all donations with NAT and HIV Combo assay. If both assays are positive, no further testing is not required for HIV confirmation. Stand-alone anti-HIV and p24 assays could be however be performed for research purposes to establish the presence of p24 antigen or antibodies to HIV. If the NAT test is negative and the HIV Combo assays positive, this is probably indicating the presence of antibodies to HIV. This should be confirmed with a Line Immuno assay (LIA) or Western blot specific to HIV antibodies. A reactive NAT test with a negative HIV Combo will indicate an early HIV infection pre-seroconversion.

The SANBS currently use NAT Testing in combination with an anti-HIV only assay. If a HIV Combo assay was to be used it would probably not influence detection of HIV antibodies. It will however mean that all early HIV infections where p24 antigen is detectable would be reported positive by the HIV Combo assay, which would change the classification of such a donor from *Possible HIV window period* to *HIV confirmed positive.* The relevance of this change in classification relates to donor counselling and the decision to repeat testing on a donor as well as risk calculations. Current risk calculations were based on the Busch model as well as the Weusten model [3]. The Busch model uses HIV NAT yields in its calculation whereas the Weusten model uses the total number of seroconverting repeat donor in its calculation. The relevance of this relates to the fact that both these factors will be influenced by the use of a HIV combo assay. This might complicate comparative

year to year risk comparisons. A new strategy would have to be used to marginalise early HIV infection especially in new donors. Assays indicating recent HIV infections are already in use for research purposes and would play an important role if a HIV Combo assay were to replace the current anti-HIV assay [17].

Table 1: Detection of HIV antigen and/or antibody amongst 1) known low level anti-HIV (LLaHIV) and 2) NAT positive, anti-HIV negative (HIV Yield) sample groups

Sample group and result	Inno p24 antigen	Roche Ag/Ab	Biorad Ag/Ab	Abbott Ag/Ab
LLaHIV: % negative	41 of 87 (47.1%)	0.0%	0.0%	0.0%
LLaHIV: % positive	46 of 87 (52.9%)	(87 of 87) 100.0%	(87 of 87) 100.0%	(87 of 87) 100.0%
HIV Yield: % negative	(91 of `153) 59.5%	(97 of 153) 63.4%	(91 of `153) 59.5%	(91 of `153) 59.5%
HIV Yield: % positive	(62 of 153) 40.5%	(56 of 153) 36.6%	(62 of 153) 40.5%	(62 of 153) 40.5%

Table 2: Distribution of LLaHIV and HIV yield group by donor type and p24 reactivity

	LLaHIV group		**HIV yield group**		P-Value
	p24 antigen negative	p24 antigen positive	p24 antigen negative	p24 antigen positive	
Donor group					
New	8 (36.4%)	14 (63.6%)	31 (62.0%)	19 (38.0%)	***0.04***
Re-Join	4 (66.7%)	2 (33.3%)	8 (50.0%)	8 (50.0%)	0.48
Repeat	29 (49.2%)	30 (50.8%)	52 (59.8%)	35 (40.2%)	0.21
Gender					
Female	20 (50.0%)	20 (50.0%)	61 (67.0%)	30 (33.0%)	0.06
Male	21 (44.7%)	26 (55.3%)	30 (48.4%)	32 (51.6%)	0.7
Race					
Asian	0 (0%)	0 (0%)	2 (40.0%)	3 (60.0%)	NA
Black	37 (48.7%)	39 (51.3%)	73 (60.8%)	47 (39.2%)	0.09
Coloured	1 (33.3%)	2 (66.7%)	4 (66.7%)	2 (33.3%)	0.34
White	3 (37.5%)	5 (62.5%)	12 (54.5%)	10 (45.5%)	0.41
Zone					
Eastern Cape	2 (40.0%)	3 (60.0%)	3(30.0%)	7 (70.0%)	0.7
Egoli	7 (43.8%)	9 (56.3%)	19 (67.9%)	9 (32.1%)	0.12
FreeState/North Cape	3 (33.3%)	6 (66.7%)	5 (62.5%)	3 (37.5%)	0.23
KwaZulu Natal	3 (42.9%)	4 (57.1%)	28 (59.6%)	19 (40.4%)	0.4
Mpumalanga	11 (64.7%)	6 (35.3%)	9 (50.0%)	9 (50.0%)	0.37
Northern	8 (47.1%)	9 (52.3%)	13 (59.1%)	9 (40.9%)	0.45
Vaal	7 (43.8%)	9 (56.3%)	14 (70.0 %)	6 (30.0%)	0.11
Age					
16-19	0 (0%)	0 (0%)	4 (33.3%)	8 (66.7%)	NA
20-24	3 (25.0%)	9 (75.0%)	18 (78.3%)	5 (21.7%)	***0.002***
25-29	14 (50.0%)	14 (50.0%)	21 (63.6%)	12 (36.4%)	0.28
30-34	11 (61.1%)	7 (38.9%)	21 (70.0%)	9 (30.0%)	0.52
35-39	3 (37.3%)	8 (72.7%)	11 (61.1%)	7 (38.9%)	0.07
40-44	2 (66.7%)	1 (33.3%)	4 (33.3%)	8 (66.7%)	0.29
45-49	5 (62.5%)	3 (37.5%)	3 (50.0%)	3 (50.0%)	0.64
50+	3 (42.9%)	4 (57.1%)	9 (47.4%)	10 (52.6%)	0.84
Donation numbers					
1 to 2	12 (33.3%)	24 (66.7%)	45 (61.6%)	28 (38.4%)	***0.005***

3 to 10	18 (58.1%)	13 (41.9%)	31 (68.9%)	14 (31.1%)	0.93
11 to 20	6 (54.5%)	5 (45.5%)	9 (45.0%)	11 (55.0%)	0.61
21 or more	5 (55.6%)	4 (44.4%)	6 (40.0%)	9 (60.0%)	0.46
Donation interval					
0 (No previous)	7 (66.7%)	14 (66.7%)	29 (64.4%)	16 (35.6%)	***0.018***
<60 days	5 (35.7%)	9 (64.3%)	12 (70.6%)	5 (29.4%)	0.05
61 to 100 days	15 (62.5%)	9 (37.5%)	15 (45.5%)	18 (54.5%)	0.2
101 to 365 days	11 (45.8%)	13 (54.2%)	28 (62.2%)	17 (37.8%)	0.19
>365 days	3 (75.0%)	1 (25.0%)	7 (53.8%)	6 (46.2%)	0.45

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