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Comparative Study of 3rd Generation V/S 4th Generation ELISA in Blood Donors for Early Diagnosis of HIV in Rural Population

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A B S T R A C T

The present study has been conducted in the rural population attended to MNR medical college for blood donation. Among the 100 voluntary donors screened for HIV the mean age group being taken 19 to 50 yrs. Among the gender the male donors were more in number. The first Screening test for detecting HIV infection is the third-generation ELISA 3rd (GENERATION ELISA TEST) IVD used to screen blood donors for HIV infection proved to be 100% sensitive, and 100% specific for the detection of Abs to HIV-1 and HIV-2. The fourth-generation ELISA (Erbasure 4th generation) also showed 100% Sensitivity and Specificity for Antibody detection. However, it was able to detect p24 Ag.

Introduction

The sero-prevalence of HIV among blood donors in India is 0.28 % (NACO 2013) although it varies from 0.16 % to 0.8 % in various Indian studies. The HIV prevalence in Andhra Pradesh is 1.07 per cent among males, and 0.73 among females, which again is higher than in other states. This grim scenario, and the need for early detection, formed an impetus for our study. Early diagnosis of HIV infection is the cornerstone of prevention and care strategies for HIV-infected individual (Diagnostic window with a new combined p24 antigen and human immunodeficiency virus antibody screening assay). Early detection of HIV is important for reasons of Infection security, prevention, and individual prognosis. Antiretroviral combination therapy started early, during Primary HIV infection, reduces the likelihood of a rapid progression to the AIDS stage. Moreover, the frequency of opportunistic infections later, is reduced, enhancing the quality of life (Van Binsbergen *et al.*, 1998). Expensive assays that can be applied both in developed and developing countries for early diagnosis are needed to determine prognosis, guide therapy. Hence, this study was carried out to evaluate the sensitivity and specificity of various techniques for Antibody detection and Antigen detection, and to determine the technique most suitable for early diagnosis of HIV infection (Schreiber *et al.*, 1996).

The main aim and objectives of this study includes, Qualitative determination of Antibodies to HIV1/HIV2 and ii) p24 Antigen, Bya) Third-Generation ELISA test, (indirect technique using Microtitre wells coated with immune dominant epitopes of HIV env protein gp41, C terminus of gp120 for HIV-1, and gp36 for HIV-2.b) Fourth-Generation (double antigen/antibody Sandwich with solid micro wells pre-coated with Recombinant HIV-1 gp41, HIV-1 Group O gp41, HIV-2 gp36 and Monoclonal Anti p24Antibodies.

Materials and Methods

A total of 100 serum samples of voluntaryblood donors from the blood bank, M.N.R Hospital, Sangareddy were collected (Table 1). This study was conducted after approval by the Institutional Research and Ethics Committee. Informed consent was obtained from the patients for the sample collection and for enrolment in the study.

Inclusion criteria: one hundred Voluntary blood donors attending the blood Bank, MNR Hospital, were included in the study,

Exclusion criteria: Persons who were not willing to participate in the present study were excluded. Professional blood donors were excluded from the study.

Blood sample of 2ml was collected using a sterile 24G disposable needle and syringe in plain tubes.TheSerawereseparated after centrifugation.All one hundred blood donors enrolled in the study were counselled and consent was obtained prior to testing. As per Drugs and Cosmetics Act (3rd amendment 2001) (Sheetal *et al.,*) Govt. of India, the hundred blood donors enrolled in the present study were tested for HIV antibodies using 3rd generation ELISA (Microlisa – HIV microwell ELISA kits manufactured by J. Mitra and Co. Pvt. Ltd.If a sample was found positive, the test was repeated with another 3rd generation ELISA kit. Those which were found negative by both these methods were subjected to Fourth- Generation ELISA for detection of p24 Ag, if any, by the ErbaSure HIV Gen-4. Kit.

Principle and procedure of diagnostic techniques employed

3rd generation ELISA test (J. Mitra & Co. Pvt. Ltd) IVD

Principle

Microlisa HIV test is an enzyme immunoassay based on Indirect ELISA.

HIV envelope proteins gp41, C terminus of gp 120 for HIV-1, and gp 36 for HIV-2 representing immunodominant epitopes are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated. Antibodies to HIV-1 and HIV-2 if present in the specimen, will bind to the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen-antibody complex present.

Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of HIV-1 and/or HIV-2 antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

Procedure

The serum samples were diluted and 100 ul of control (RTU) was added.The plate was covered and incubated at 37 0 c for 30 mins. After 5 cycles of washing,100ul of conjugate was added, and the plate was covered and again incubated at 37 0 c for 30 mins. After 5 more cycles of washing, 100ul of chromofenic substrate was added and the plate was covered and incubated in the dark at room temp for 30 mins.100ul of stop solution was then added, and the results were read with an ELISA reader, using a 450 nm/630nm filter

Combaids - RS Advantage-STHIV 1+2 IMMUNODOT TEST KIT (IVD) Span Diagnostics Ltd.)

Dot immunoassay intended for the qualitative detection of IgG/IgM antibodies to the HIV type 1 and / or 2 in human Whole Blood, Serum or Plasma.

Procedure

All kit components and samples to be tested were brought to room temperature. Samples to be tested were clearly marked, and their identity was recorded before starting the test. Each sample number was marked on the corresponding microtest well and two drops of Sample Diluent (Reagent 3) were added to each microtest well to be used for Samples as well as Controls.

Two drops (0.1 mL) of each Sample/control were then added with the help of disposable plastic dropper to each of the above wells containing Sample Diluent.

All Samples and controls were mixed with diluent by repeated aspirating and expelling or stirring with disposable plastic dropper tip. The position and identity of all Samples or Controls was recorded as they were added.

The required number of blister packs was taken, and the combs were kept ready. The remaining blister packs were stored in a tightly sealed zip lock pouch provided with silica gel.

The comb was marked with the sample numbers and placed into rows of corresponding microtest wells.

The comb was placed in the first row of diluted samples by holding it vertically with the teeth pointing down.

The timer was set for 10 minutes, and the wells were incubated for 10 minutes. The comb was gently rocked back and forth 2-3 times at the beginning, for 5 minutes, in the middle and the end of incubation. In the meantime, 4 drops (0.2 mL) of Colloidal Gold Signal Reagent (Reagent 2) was dispensed into each of another set of unused microtest wells. The comb was removed from the sample containing wells, the tips of the teeth, blotted on absorbent material. The comb was held vertically with tips pointing down and rocked forward and backward in the Wash Solution fora total oftentimes. The tips of the arms were botted again.

The comb was placed into wells containing colloidal gold signal reagent, and gently rocked back and forth 2-3 times at the beginning, at middle 5 minute, and at the end of exactly 10 minutes, on incubation at room temperature. After incubation, the washing repeated. The comb was then placed on a clean surface, reactive labelled side up, and allowed to air-dry completely before reading the results.

Pareekshak ® HIV 1/2 Triline card test (IVD)

This is an immunochromatographic based assay for the detection of antibodies to HIV-1 and HIV-2 in human serum or plasma. Since

HIV antigens are used for both binding and capturing, this test can detect all classes of HIV antibodies, hence detects early sero conversion.

AIDSCANe HIV-1/2 TRISPOT test [idbarathbiotechindia (p)]

This is the 2nd confirmatory test for Antibody detection, as per NACO Guidelines AIDSCANe HIV-1/2 TRISPOT Test is an immunoassay which employs r-proteins for the detection of antibodies to HIV in human serum or plasma. These proteins, which are corresponding to highly antigenic segments of both the structural and non-structural proteins of the HIV constitute the solid phase antigenic absorbent. The use of r-proteins offers the advantage of high degree of specificity and sensitivity due to multiple epitopes. AIDSCANe HIV-1/2 TRISPOT test utilizes a unique combination of HIV-1 and 2 antigens of the virus to selectively detect all subtypes of HIV-1 and 2 virus in human serum/plasma with a high degree of sensitivity and specificity.

Procedure

All reagents, devices and specimens were brought to room temperature. 2 drops of buffer solution were first added to the test devices, 2 drops of either serum or plasma were then added, followed by 2 drops of gold conjugate, and later 4 drops of buffer solution the results were read immediately.

All samples found to be negative for Antibodies to HIV-1 and HIV-2 by COMBAIDS- RS Advantage-ST HIV1+2 Immunodot test were subjected to Fourth-Generation ELISA Erba Sure HIV-Gen4 for detection of p24 Ag if any.

Table.1 Samples studied

Samples studied from :	No. Tested	Percentage
Blood donor	100	39%

Table.2 Age-group – incidence

Age group	No. tested	Percentage
19-20	4	4%
21-25	20	20%
26-30	28	28%
31-35	18	18%
36-40	19	19%
41-45	8	8%
46-50	3	3%
Total	100	100%

Table.3 Gender incidence

Sex	No. tested	Percentage
Male	87	87%
Female	13	13%
Total	100	100%

Table.4 PHBD/HOI/STI/NO Risk factors

	Number tested	percentage
PHBD	20	20%
HOI	3	3%
STI	1	1%
No Risk factors	76	76%
Total	100	100%

Table.5 Test results in blood donors

Name of the test	Number Tested	Number tested Positive	Number tested Negative	Percentage Positive
Third Generation ELISA	100	Nil	100	0%
Fourth Generation ELISA	100	1	99	1%

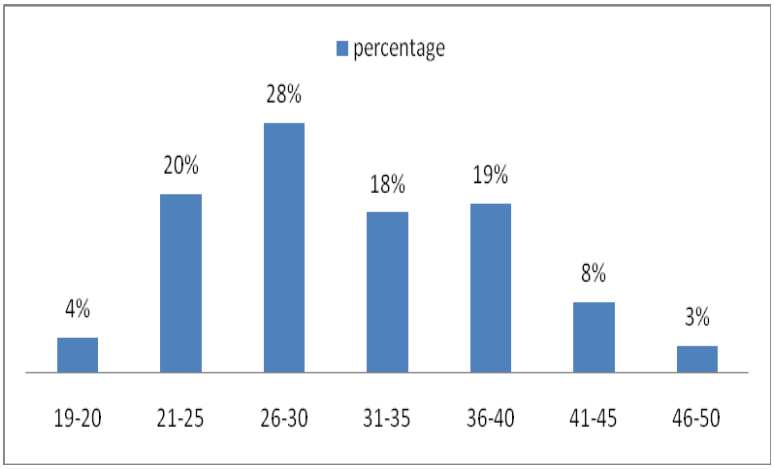
Table.6 Results of the present study

Type of samples	No. tested Positive	Percentage Positive	No. tested Negative	Percentage Negative
Voluntary Blood Donors	1	1%	99	99%

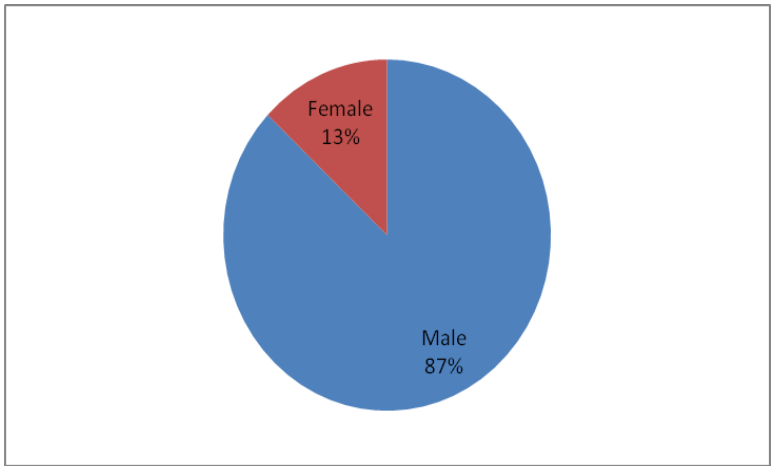
Table.7 HIV seroprevalence among voluntary blood donors in other studies

Authors	Seroprevalence
Srikrishna *et al.*, (1999)	0.44%
Garg *et al.*, (2001)	0.44%
Singha *et al.*, (2004)	0.8%
Singh *et al.*, (2005)	0.54%
Farnandes *et al.*, (2010)	0.06%
Arora *et al.*, (2010)	0.3%
Kaur *et al.*,(2010)	0.6%
Pallavi *et al.*,(2011)	0.44%
Our study(2014)	1%

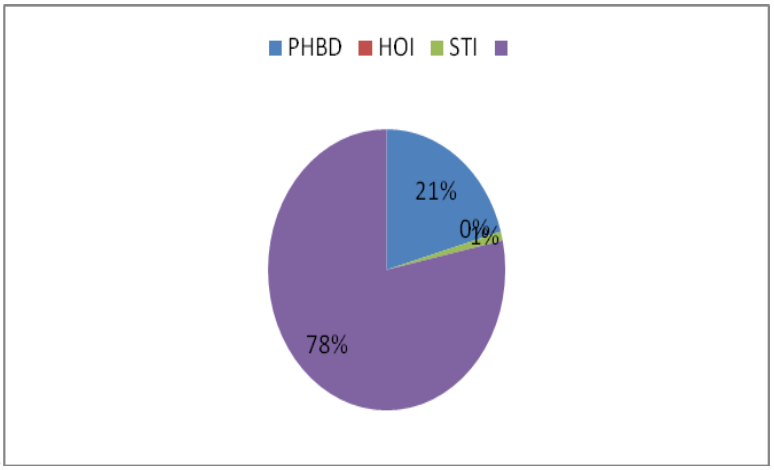
Graph.1 Age-group – incidence



Graph.2 Gender incidence

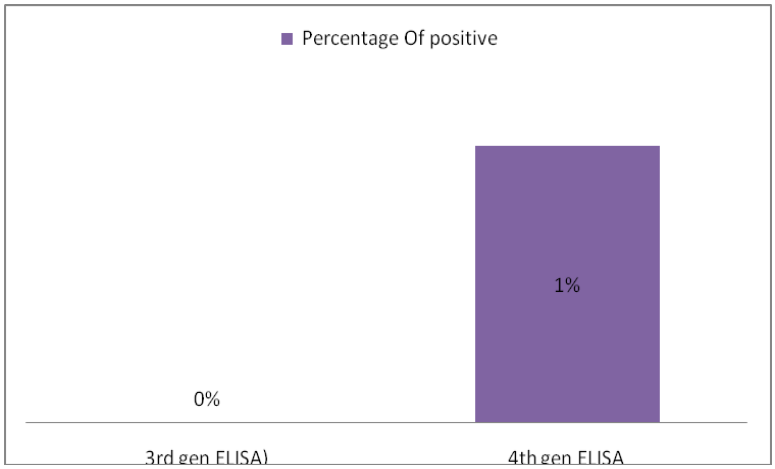


Graph.3 PHBD/HOI/STI/NO Risk factors



Note: PHBD: Previous history of blood donation, HOI: history of injections, STD: Sexually transmitting diseases

Graph.4 Percentage of positive results of the present study



Evaluation of fourth generation ELISA (Erbasure HIV Gen-4)

In comparison with the diagnostic kits mentioned above

Intended use

The fourth - generation MICROLISA HIV Ag and Ab is an *in- vitro* qualitative enzyme immunoassay for the detection of Abs to HIV-1, and/or Abs to HIV-2, and HIV-1 p24 Ag, detectable in human serum or plasma. The blood samples of all blood donors who tested negative with third generation ELISA, and all antenatal mothers who tested

Procedure

25 ul of sample diluent was added to each well. 100ul of the samples and the controls were then added to the respective wells. The plate was covered and incubated at room temp for 60 mins After 6 cycles of washing, 100ul of working conjugate was added to each well. The plate was covered, and again incubated at room temp for 30 mins. After 6 more cycles of washings, 100ul of working substrate is added to each well. The plate wash then incubated in the dark for 30 mins at room temp.100ul of stop solution was then added to each well, and the results were read at 450nm/630nm. All serum samples that tested negative for Abs to HIV-1 and HIV-2 by the methods prescribed for their respective groups were tested for p24 Ag by the 4th generation ELISA. The importance of testing for p24Ag, using 4th generation kits was then evaluated.

Results and Discussion

The present study was carried out in M.N.R Hospital, Sanga Reddy, in the Department of Microbiology during the period of August 2012 to July 2014. In this Study the Rapid HIV test and 3rd Generation ELISA test for detection of antibody was evaluated and compared with a fourth-generation ELISA detecting both p24 antigen and antibody.

Blood donors

A total of 100 blood donors from the Blood bank, M.N.R Hospital, belonging to the following age groups were randomly chosen for the study.

The highest number of blood donors included in our study were in the 26-30yrs age group (28%), followed by the 21-25 yrs age group (20%), the 36-40 yrs age group (19%), the 31-35yrs age group (18%), the 41-45 yrs age

group (8%), the 19-20yrs age group(4%),and then, lastly the 46-50yrs age group (3%) (Table 2).

Eighty seven (87%) of the one hundred blood donors included in the present study were males, and 13 (13%) of them were females (Table 3).

Out of the 100 blood donors in the present study categorised on the basis of possible risk factors, 20 (20%) gave a history of blood donation previously, 3 (3%) gave history of having taken injections previously, and 1 (1%) gave history of STI (Table 4). The rest (76%) gave no history of any risk factors. 65 (65%) of them were married, and 35% were not married.

All one hundred sera were found to be negative for antibodies to HIV-1 and HIV-2 by the Third-Generation Microlisa-HIV test, but one of the 100 sera tested positive by the Fourth-Generation ELISA (Erba Sure HIV Gen 4). Both the tests were repeated twice immediately and were found to yield the same results (Tables 5 and 6).

First screening test for detecting HIV infection in the third-generation ELISA used to screen blood donors for HIV infection proved to be 100% Sensitive, and 100% Specific for the detection of Abs to HIV-1 and 2. The fourth-generation ELISA also showed 100% Sensitivity and Specificity for Antibody detection. However, it was able to detect p24 Ag.

The inclusion of anti-p24 Antibodies in the solid phase ELISA have enabled the diagnosis of p24 Antigen to reduce the window period in fourth generation assays by 8-9 days.5 It is reported that by implementing Antigen screening of blood, an estimated four to six cases of transfusion- associated HIV infections may be prevented per year, lowering the estimated risk per unit transfused to a range of one in 562,000 to one in 825,000 Therefore, it appears that Fourth-Generation Assays have greater utility in screening for HIV infection, due to their ability to detect p24 antigen. In the present study, we report the performance characteristics of three different screening tests – COMBAIDS, third-generation ELISA, and fourth-generation ELISA, for the diagnosis of HIV infection, the results with characterized specimens from voluntary blood donors.

The present study was conducted in the Department of Microbiology, MNR Medical College, Sangareddy, A total of 100 Voluntary blood donors from the Blood Bank, M.N.R Hospital, Sangareddy.

As per Drugs and Cosmetics Act (3rd amendment 2001, Govt. of India, One hundred blood units in the present study were tested for HIV antibodies using 3rd generation ELISA (Microlisa – HIV microwell ELISA kits manufactured by J. Mitra and Co. Pvt. Ltd. If a sample was found positive, the test was repeated with another 3rd generation ELISA, Positive sera were subjected to one more Third-Generation ELISA. All samples in our study tested Negative for Antibodies to HIV-1 and 2. In addition, these donor units were also screened with 4th generation ELISA, ErbaSure HIV-Gen4 in order to detect p24 Ag if any. Manufacturer's instructions were strictly followed while performing each assay.

The highest number of voluntary blood donors in the present study (28%) was in the 26-30 yrs. age group. Patel Piyus *et al.*, (2010), Sangeeta, Shah Jigesh reported their highest age-group incidence to be 21-40 yrs, slightly similar to this study (Patel Piyus *et al.*, 2010). Many of the older people suffer from hypertension, diabetes mellitus, low hemoglobin and chemic heart diseases and

hence may abstain from donating or considered unfit during pre- donation counseling, 92%. Of the voluntary blood donors tested in the present study were males, and 8% were females. Our findings are similar to those of Patel Piyush *et al.,* (2010) Van Binsbergen *et al.,* (1998) reported that 95.48% of accepted donors in their study were male and 4.52% donors were female and also compared with Srikrishna *et al.,* who reported that males were 95.4% and females were 4% which is also similar to our study (Gisselquist *et al.,* 2004) (Table 7). The trend is similar in most blood banks, probably because Indian women have a very high incidence of anaemia, especially in the child bearing age and hence, are likely to face disqualification while being screened for blood donation (Microbiology and Immunology, 2nd edition). Other reasons for less female participation may be the lack of awareness, motivation and education regarding blood donation.

Twenty (20%) of the voluntary blood donors gave history of previous H/O blood donation compared with Guoing *et al.,* (Schreiber *et al.,* 1996) 10% given, which is lower than our study. This is an alarming situation requiring immediate action in appropriate counselling of the donor before and after testing.

It further shows the need to communicate the test result to the donor (Centers for Disease Control, 1989). These precautions not only inform donors of their health status, but also prevent them from donating infected blood (Kaur *et al.,* 2010). Furthermore, unnecessary expenditure from the superfluous testing and proper disposal of the infected blood product are also eliminated, thereby lowering the costs involved.

All the one hundred sera tested were found to be negative for Antibodies to HIV-1 and HIV-2 by 3rd Generation ELISA Microlisa, but one of the 100 sera tested positive (1%) by 4th gen ELISA (Erba Sure HIV Gen 4). Both the tests were repeated immediately, and were found to yield the same results. Although the micro titre wells in the 4th generation ELISA (Erba Sure HIV Gen 4) are pre-coated with both Monoclonal p24 Antibody, and Recombinant HIV-1 and HIV-2 Antigens, it is inferred that this particular voluntary blood donor who tested positive with 4th-Generation ELISA, was actually positive for p24 Antigen, since he was already found to be negative for Antibodies to HIV- 1 and HIV2, with the 3rd Generation Microlisa-HIV.

This person was a 22year-old married man, with Intermediate qualification, holding a job in a private firm. He weighed 52kgs, and had Haemoglobin of 13.5gms%. His Blood group was B+ve. He was also HBS Ag positive. Since he tested positive for p24 Ag by Fourth Generation ELISA, indicating that he was in the Acute HIV infection stage, or in the window period, it enabled early diagnosis, and early initiation of treatment. He was given posttest counselling and was advised to refrain from high risk behavior and to self-exclude from future donations. He was referred to VCTC for counseling and further confirmatory testing. A similar study done by Courouce *et al.,* (1992) indicated that the safety of donated blood could be improved by fourth-generation assays, since they permit an earlier diagnosis of HIV infection than the third-generation double-Ag sandwich assays, by detecting p24 Ag which may be present in samples from individuals with recent HIV infection prior to seroconversion.

The seroprevalence of HIV among voluntary blood donors in the present study was 1%, more when compared to National data (0.28%).

The seroprevalance of HIV in blood donors in various Indian studies ranges from 0.06 to 3.8%. In Medak, the sero prevalence of HIV in the general population is reported to be 2.5

%, and in Sangareddy, the prevalence is 5.1% in the general population, and 0.2% among blood donors. Kaur *et al.,* (2010) reported 0.8%, which is lower than what we reported (1%). Garg *et al.,* (2001) reported 0.44% and Pallavi *et al.,* (2011) also reported a seroprevalence of 0.44%, which is lower than that in our study. All one hundred blood donors tested Negative for Antibodies to HIV-1 and HIV-2 by Third-Generation ELISA, but, ONE of them tested Positive for p24 Antigen by Fourth-Generation ELISA.

The results of the present study show that p24 Antigen detection by Fourth-Generation ELISA is more sensitive for the detection of HIV infection, compared to Antibody detection by third-Generation ELISA and COOMBAIDS Dot Immuno Assay. The present study emphasizes the importance of using Fourth-Generation assays, which permit the simultaneous detection of p24 antigen and antibodies to HIV-1 and 2, in the screening of all groups of persons for HIV infection, in order to ensure early detection of HIV infection by detecting p24 Ag, present in recent HIV infection prior to seroconversion. The detection of p24 Antigen by Fourth-Generation ELISA used in the present study has thus been found to be a better diagnostic tool in facilitating Early Diagnosis, Early initiation of treatment, and early change in the lifestyle of high risk persons, thereby reducing the prevalence of HIV infection in the communities worldwide. Simultaneous Antigen and Antibody detection help in reducing the diagnostic window, the time, personnel, and costs involved, (comparable to tests for HIV-1 RNA), and will therefore, in many ways, benefit clinical laboratories in hospitals or private organizations, in the early diagnosis of HIV infection.

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