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Evaluation of 4th Generation Elisa HIV Testing Kit and its Comparison with the 3rd Generation Kit

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A B S T R A C T

The study was organized in the population of Dehradun, Uttarakhand at IMA Blood Bank to ascertain the sensitivity and specificity of 4th generation antibody and antigen p24 combo Elisa kit atop of 3rd generation HIV antibody Elisa kit. Methods: A total of 2045 voluntary donors were screened for HIV over a span of 6 months. The mean age group included 18 to 50 yrs with male preponderance. Each sample was assessed twain with the 3rd and the 4th generation HIV ELISA testing kit of Bio-Rad concomitantly and HIV positive samples were further authenticated with PCR. Results: Out of these 2045 samples, two samples were detected reactive with the 4th generation ELISA kit but nonreactive with the 3rd generation kit. Both these were found to be negative on PCR. One sample was found to be reactive with both 3rd and 4th generation kits and was confirmed positive by PCR. Conclusion: The study does not culminate the precedence of the 4th generation kit atop of the 3rd generation kit. However, we propound that in order to draw a logical conclusion furthermore samples should be evaluated. The study assures that the 3rd generation kit is likewise considerable as the 4th generation ELISA kit, none of the reactive samples (by 4th generation ELISA) was mislaid (by 3rd generation ELISA) which were further authenticated by PCR.

Introduction

Due to scarcity of cognizance about AIDS and presupposition, people have consternation from the victims of AIDS.AIDS patients and their relatives do not divulge their HIV status because of social stigma. Many clinicians (due to fear of getting infection) also discriminate these patients. (Kumar *et al.*, 2017) Everybody has been thrown into peril of contracting the AIDS and without proper cognizance it is hard to avert contracting the disease. Causative of agent of AIDS is human immunodeficiency virus (retrovirus) of two types, HIV-1 and HIV-2, which is spread by the exposure of HIV contaminated body fluids like blood, genital secretions and by mother to fetus. Accurate diagnosis of HIV is required to ensure the safer blood supply and HIV prevention.

HIV-1 infection has been depicted globally, HIV-2 infection has been delineated occurring predominantly in West Africa and some parts of European countries. (Clavel, 1987) Both HIV virus

exhibit significant antigenic cross reactivity in their core proteins, but envelope glycoproteins are slightly cross reactive, it is needed for screening purposes to use epitopes from the envelope proteins of the two viruses in addition to the major cross reacting core proteins to ensure detection of antibodies against both types of viruses at every stage following infection. (Simon *et al.,* 1998; Denis *et al.,* 1988)

Many commercial assays are acquirable for the detection of HIV. Among them Elisa is the most routinely used screening assay. There is a constant demand for high quality Elisa kits. Studies for quality evaluation of Indian kits are very less. (Nandi *et al.,* 2014; Maity *et al.,* 2012)

These assays include four categories i.e. testing for detecting HIV antibody, detecting p24 antigen, detecting viral nucleic acids and assessing T-lymphocyte numbers. (Saville *et al.,* 2001)

According to the modifications in the Elisa kits manufacturers use the term first generation, second generation, 3rd generation and fourth generation ELISA. The 3rd generation HIV antibody ELISA tests includes the detection of anti-HIV IgM antibodies along with IgG in plasma samples. The 4th generation HIV antibody and p24 ELISA (Combo) tests is supposed to enhance the sensitivity of the assay for the detection of acute HIV infection by including detection of p24 antigen with IgM and IgG.

A Study by Nandi *et al.,* compared the performance of different Elisa kits on 100 samples suggested the need for a routine mechanism for evaluation of kit for obtaining standards for its use. (Nandi *et al.,* 2014)

A study done by of voluntary blood donors Sujatha *et al.,* tested 100 samples with rapid, HIV 3rd generation test and 4th generation test and found 4th generation assay a better diagnostic tool as compare to the 3rd generation assay. (Sujatha and Vaisakhi, 2017)

Sarma *et al.,* in a study on 140 blood donors, compared 3rd and 4th generation kit and recommended h4th generation Elisa kit for better clinical diagnosis. (Sarma *et al.,* 2019)

Materials and Methods

This study, conducted in the population of Dehradun, Uttarakhand at IMA Blood Bank (2008-2009) to ascertain the sensitivity and specificity of 4th generation antibody and antigen p24 combo Elisa kit atop of 3rd generation HIV antibody Elisa kit. Highest no. of 2045 voluntary donor was screened for HIV over a span of 6 months. Each sample was assessed twain with 3rd and the 4th generation HIV ELISA testing kit of Bio-Rad concomitantly and HIV positive samples were further authenticated with PCR.

The study was carried out with two main objectives.

Evaluation of 4th generation HIV testing kit

Comparison of efficacy of 4th generation HIV testing kit with the 3rd generation HIV testing kit.

3rd generation Elisa kit (Bio-Rad)

This is an Enzyme immunoassay kit for the detection of antibodies to human immunodeficiency virus type 1 (HIV-1, HIV-1 group O) and 2 (HIV-2) in human serum or plasma.

Principle

This enzyme immunoassay is based on microwells solid phase coated with a synthetic peptide depecting an immunodominant region of HIV-1 (O), recombinant protein acquired from the envelope proteins of HIV-1 and HIV-2 and an HIV core protein. The Conjugate is a combination of the same epitopes all labeled with horseradish peroxidase. Test samples and control sera are incubated in the well and antibodies to HIV in the samples or control sera bind to the antigens on the microwell, samples and any excess antibodies are then washed away. In

the next step, Conjugate is added which in turn binds to any specific antibody already bound to the antigen on the well. Samples not containing specific antibody will not cause in the Conjugate to bind to the well. Unbound Conjugate is washed away and at this step a solution containing 3,3,'5,5'-Tetramethylbenzidine (TMB) and hydrogen peroxide is added to the wells. Wells with bound Conjugate develop a purple color, which is converted to an orange color when the reaction is stopped with Sulphuric acid. After incubation the enzymic reactions are halted with sulphuric acid and the color is read spectophotometrically at 450 nm. The amount of Conjugate and hence color, in the wells is directly proportional to the concentration of antibody to HIV in the sample.

Procedure

Serum samples were used. 50µl of Sample diluent was added to each well. For each plate first column of wells was assigned for the assay controls. Controls were added to the assigned wells after dispensing the 50µl samples to the designated sample wells. 50µl of the Negative Control into each three wells A1 to C1 and 50µl of the anti-HIV-1 and HIV-2 Positive Controls into wells D1 and E1 were added respectively.

After covering with lid the wells were incubated for 30 minutes at 37ºC. After incubation plate was washed with wash buffer. Immediately, after washing 50µl of Conjugate was added to each well. Again the wells were covered and incubated for 30 minutes at 37ºC. After incubation plate was washed with wash buffer.

After washing, 100µl of Substrate Solution was added to each well. The wells were covered with the lid and incubated for 30 minutes at 37ºC kept unexposed to direct sunlight. A purple color developed in wells containing reactive samples.50µl Stop Solution was added (0.5 M to 2M Sulphuric acid) to each well. Absorbance was taken at 450 nm using 620 nm to 690 nm within 15 minutes as the reference wavelength.

Calculation and interpretation of the results

Mean absorbance of the negative control was calculated. Cut-off Value was calculated by adding 0.2 to the mean of the Negative Control replicates. The absorbance of each of the Positive Controls was more than 0.8 above the mean absorbance of the Negative Control.

Non- reactive Results

Samples showing an absorbance less than the Cut-off value were considered negative in the assay.

Reactive Results

Samples showing an absorbance equal to or greater than the Cut-off value are considered initially reactive in the assay.

4th generation Elisa kit (Bio Red)

This is an Enzyme immunoassay kit for the detection of HIV p24 antigen and antibodies to HIV-1 and HIV-2 in human serum or plasma by enzyme immunoassay.

Principle

This is an enzyme immunoassay based on the principle of sandwich technique for the detection of HIV antigen and of the various antibodies associated with HIV-1 and/or HIV-2 virus in human serum or plasma. The solid phase is coated with Monoclonal antibodies against p24 HIV-1antigen, Purified antigens: gp 160 recombinant protein, a synthetic peptide imitating a totally artificial (i.e. encoded by no existing virus) HLV-1 group O-specific epitope, and a peptide mimicking the immunodominant epitope of the HIV-2 envelope protein. The conjugates are based upon the use of Biotinylated polyclonal antibodies to HIV Ag (conjugate 1), Streptavidin and HIV antigens–peroxidase conjugate (gp41 and gp36peptides imitating the immunodominant epitopes of the HIV-1 and HIV-2 envelope glycoproteins, and the same synthetic

peptide mimicking a totally artificial HIV-1 group O-specific epitope used for the solid phase) (conjugate 2)

Procedure

Serum samples were used. Negative, HIV-1 Ab positive and HIV Ag positive controls were used for each series of determinations to validate the test results. 25µl of conjugate 1 in each well, 75µl of HIV Ag positive control in well A1, 75µl of HIV Ab positive control in well B1, 75µl of negative control in well C1, D1 and E1, 75µl of specimen 1 in well F1, 75µl of specimen 2 in well G1, etc. The mixture was Homogenized the by a minimum of 3 aspirations with 75µl pipette or by shaking the microplate after the pipetting step. The wells were covered with the lid and incubated for 30 minutes at 37ºC. After incubation plate was washed with wash buffer. Immediately after incubation 100µl of conjugate 2 solution was dispensed into all wells. When possible, cover the plate with new adhesive film and incubate for 30 minutes at Room temperature (18-30ºC). The wells were covered with the lid and incubated for 30 minutes at 37ºC. After incubation plate was washed with wash buffer. Immediately after incubation 80µl of prepared substrate solution was dispensed into each well. The wells were covered with a lid and incubated for 30 minutes at 37ºC kept away from direct sunlight. 100µl stopping solution was added into each well. After 2 minutes of stopping solution addition, optical density was taken at 450/620-700 nm using a plate reader within 30 minutes of stopping the reaction.

Calculation and interpretation of the results

The presence or absence of detectable HIV antigen or antibodies to HIV-1 and/or HIV-2 is determined by comparing the absorbance measured for each sample to the calculated cut- off value. Mean absorbance of the negative control was calculated. Cut off value was calculated by adding 0.2 to the mean of the Negative Control replicates. The mean of the absorbance of the negative controls should be less than 0.150 (NC < 0.150).The absorbance of HIV Ab positive control should be greater than 0.9 (PC > 0.9)

Non-reactive Results

Samples showing an absorbance less than the Cut-off value are considered negative in the assay.

Reactive Results

Samples showing an absorbance equal to or greater than the Cut-off value are considered initially reactive in the assay.

PCR

HIV positive samples were further confirmed by PCR. The primers utilized were specific to the targeted sequences in the DNA of HIV virus. (PCR, 2022) The high sensitivity of PCR made possible HIV virus detection immediately after infection and even prior to the outbreak of disease. PCR was taken as a confirmatory test in this study. (Cai *et al.*, 2014)

Such early detection helps physicians significantly in treatment. The viral load in a patient can also be measured by PCR-based DNA profiling methods. (Berger *et al.*, 1998)

Results and Discussion

A total of 2045 samples were evaluated over a period of 6 months. All these samples were tested with both the 3rd and the 4th generation HIV ELISA testing kit of Bio-Rad simultaneously. Further all samples, which tested positive with either 3rd or 4th generation, were confirmed by nucleic acid based test, using PCR as the Gold Standard.

Out of these2045 samples

Two samples were found to be reactive with 4^{th} generation ELISA kit but nonreactive with 3^{rd} generation kit. Both these were found to be negative on PCR.

Table.1 Age group distribution of blood donors

Age group	No. of Donors	Percentage
18-25	612	29.9%
26-30	470	22.9%
31-35	332	16.2%
36-40	325	15.8%
41-45	204	9.9%
46-50	102	4.9%
Total	2045	100%

Table.2 Gender incidence

Gender	No. of Donors	Percentage
Male	1575	**77.1%**
Female	470	**22.9%**
Total	**2045**	**100%**

Table.3 Positive Results

S.No.	Sample/Tested by	3rd generation ELISA	4th generation ELISA	PCR
1	Sample 1	NEG	POS	NEG
2	Sample 2	NEG	POS	NEG
3	Sample 3	POS	POS	POS

Table.4 Results %

Testing method	No. of samples tested	No. of samples tested Positive	No. of samples tested Negative	Positive%
3rd generation ELISA	2045	01	2044	**0.048%**
4th Generation ELISA	2045	03	2042	**0.146%**
PCR (Confirmatory test for positive samples only out of 2045)	**03**	**01**	**02**	**33.33%**

Fig.1

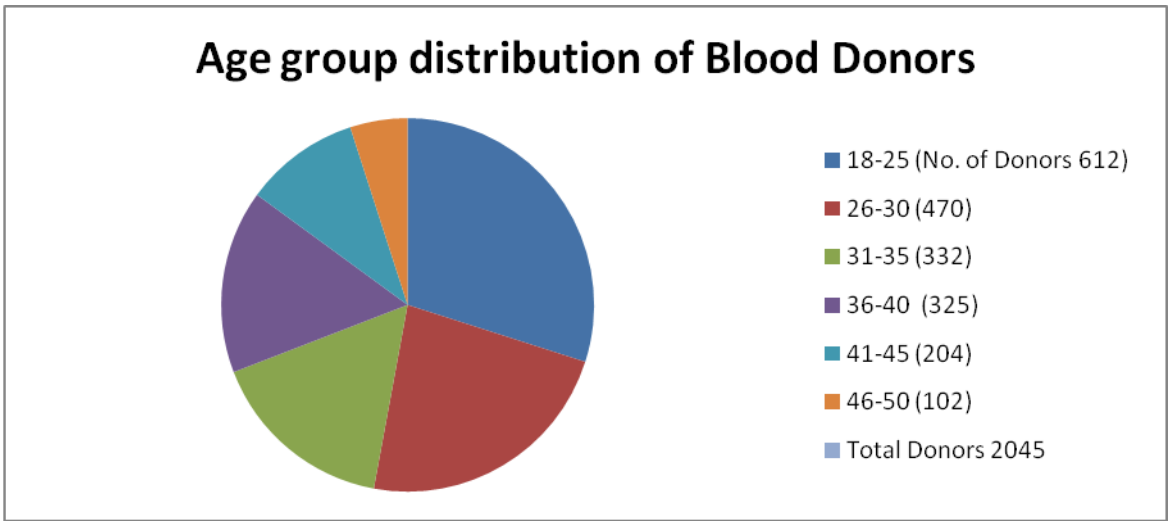
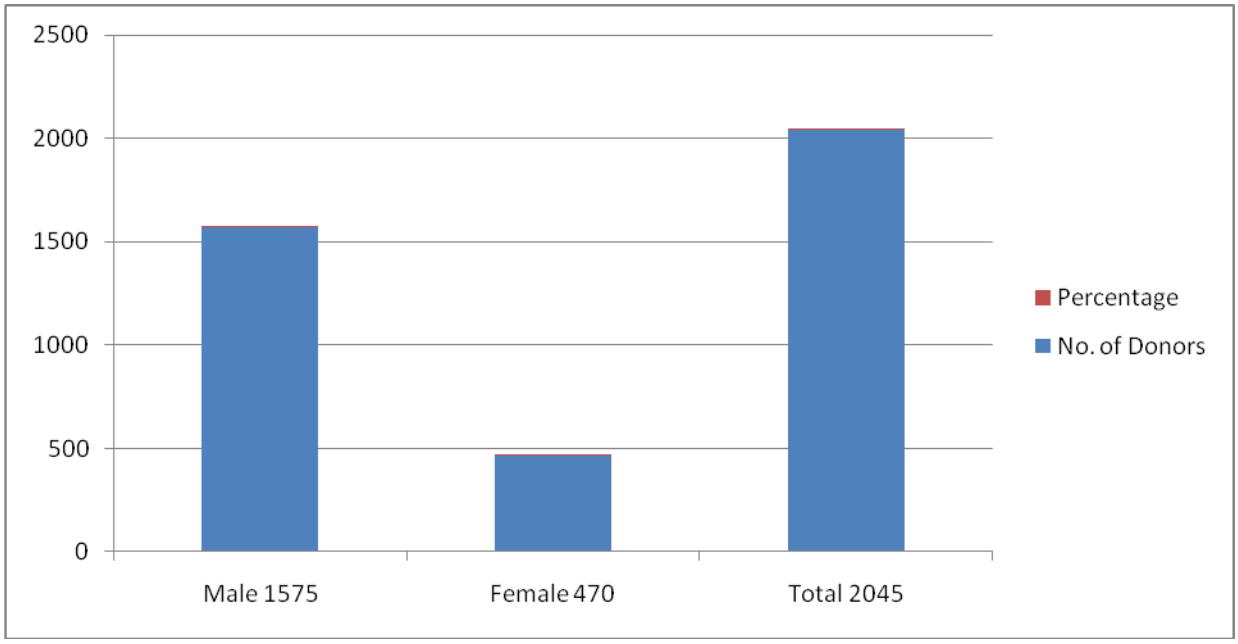


Fig.2



One sample was found to be reactive with both 3rd and 4th generation kits and was confirmed positive on PCR.

Over a time of 6 months a total of 2045 samples were evaluated with the 3rd and the 4th generation ELISA kit simultaneously. And the reactive samples were confirmed by the PCR. During our study we found 2 samples reactive with 4th generation kit but not confirmed positive by PCR. 1 sample that was confirmed positive with PCR was found to be reactive with both 3rd and 4th generation kit. Therefore the study does not conclude the superiority of 4th generation kit over 3rd generation kit however we suggests that in order to reach a logical conclusion more samples should be evaluated. However it gives assurance that the 3rd generation kit is fairly good as no reactive sample was disparaged which was attested positive by PCR. Study does not culminate the precedence of 4th

generation kit atop of 3rd generation kit however we propound that in order to draw a logical conclusion furthermore samples should be evaluated. Study assures that the 3rd generation kit is likewise considerable as 4th generation ELISA kit, none of reactive samples (by 4th generation ELISA) was mislaid (by 3rd generation ELISA) which were further authenticated by PCR.

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