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Original Research Article

Occurrence of false positivity in a fourth generation (Ag/Ab) HIV screening assay: horns of a dilemma

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ABSTRACT

Background: Universal screening for HIV with Ag/Ab combo assays has reduced the window period significantly due to its high sensitivity. However, occurrence of false positives is common and in a country like India, it may lead to social distress and loss of livelihood. We wanted to ascertain the occurrence of false positives in our laboratory.
Methods: There were 21817 samples analysed retrospectively from Jan 2015 to Jul 2017 (31 months). Architect HIV Ag/Ab Combo (Abbott Laboratories, Abbott Park, IL) was used as the first test. Repeatedly reactive samples with a signal to cutoff (S/CO) ratio greater than or equal to 1.00 was considered reactive. Strategy III (3 test algorithm) of NACO guidelines was followed uniformly.
Results: In this study, 147 samples tested reactive (0.67%) and 40 samples tested false positive (repeatedly reactive; 0.18%). 6 samples were indeterminate (0.027%). Overall, the sensitivity of the Architect HIV Ag/Ab Combo was 100%, the specificity varied between 99.74% and 99.91%. The PPV from June 2016 to July 2017 was 68.63% (32 false positives). The S/CO values of 72.8% (117/147) reactive samples ranged between 201- 800, whereas 72% (29/40) of false positive samples, the S/CO values ranged between 1.0-2.0. The specificity of the test improved to 99.98% when S/CO value was adjusted at 2 and 100% when adjusted at 5. Similarly, the PPV too improved to 93.04% and 98.66% at S/CO values of 2 and 5 respectively.
Conclusions: Further studies are needed to ascertain the optimal or 'grey-zone' S/CO values for India to minimise the false positive results and avoid further supplemental tests routinely.

Keywords: Ag/Ab combo assay, False positive, HIV, Test sensitivity, S/CO

INTRODUCTION

Detection of HIV infection, at the earliest, has been the primary focus of the HIV screening tests since its inception. The first screening test for HIV was introduced in 1985 that was based on the detection of anti-HIV antibodies. Subsequently, many improved versions of the antibody tests and p24 antigen based EIA tests were introduced that improved the detection limits. In 1997, antigen/antibody combination (Ag/Ab) assays were launched that resulted in a significant decrease in the window period by 4-5 days as compared to the antibody based tests alone.[1] Consequently, Ag/Ab based combination assays have been implemented in many countries as the primary screening test for HIV.[2]

The sensitivity and specificity of Ag/Ab combo HIV testing worldwide is around 100% and 99.9% respectively.[3] To identify the maximum number of HIV infected persons effectively, universal screening for HIV

with Ag/Ab combo assays among adults aged between 13-64 years was recommended by The Centers for Disease Control and Prevention (CDC).[4] This recommendation was specially targeted for countries with low prevalence of HIV infection.

However, it was soon realised that a significant number of tests reactive for HIV infection were false-positives. Detection of false-positive in an asymptomatic population causes major distress and social trauma. The stigmata associated with HIV infection in a developing country such as ours can be catastrophic. In India, many overseas job employment agencies conduct HIV tests on their potential candidates as part of a comprehensive medical examination. False positive results by Ag/Ag combo tests often lead to rejection of the candidate leading to loss of livelihood combined with social stigma.

In India, HIV tests on asymptomatic persons are conducted strictly as per the guidelines, issued by National AIDS Control Organization (NACO). In brief, asymptomatic persons are screened under 'Strategy-III' wherein, the first test is conducted using a highly sensitive test assay followed by two confirmatory tests using different test principles and/or antigens, if the first test is reported as reactive. The reports are released in accordance with the NACO approved format.[5]

Among populations where the expected prevalence of HIV infection could be extremely low e.g. ante-natal cases, the rate of false positivity could be markedly higher. In a study conducted by Shima-Sano et al, the authors reported a positive predictive value (PPV) of only 3.7% in pregnant females.[6]

A number of Ag/Ab combo test on automated platform employing different assay technologies are now available in India. The data on false positives on such automated platforms are limited. We conducted a retrospective study to analyse the rate of false positivity in Architect HIV Ag/Ab Combo screening assay on an automated platform in a standalone laboratory in New Delhi, India.

The Abbott Architect HIV Ag/Ab Combo assay (Abbott Combo; Abbott Laboratories, Abbott park, IL, USA) is an automated modular platform based on chemiluminescent microparticle immunoassay (CMIA) that simultaneously detects HIV-1 p24 antigen (Ag), HIV-1 gp41 antibody (Ab), and HIV-2 gp36 Ab. The signal generated by this assay is reported as a signal-to-cutoff (S/CO) ratio. S/CO ratio greater than or equal to 1.00 is considered reactive.[7-9]

METHODS

A total of 21817 samples were analysed retrospectively for a period of 31 months from 1 January 2015 to 31 July 2017. Approximately 3 ml of whole blood was received in vacutainers with clot activator transported and stored under recommended conditions and processed without delay at a large super specialty laboratory in New Delhi, India. The samples were processed after ensuring the correctness of the test requisition form (TRF) and the HIV consent form duly signed by the individual undergoing the test and the counsellor.

The following testing protocol was adopted uniformly for each sample. Each sample was initially tested by Architect HIV Ag/Ab Combo (HIV Combo; Abbott Laboratories, Abbott Park, IL). The test was run on an automated modular random-access immunoassay platform (Architect i2000; Abbott Laboratories) strictly as per the manufacturer's instructions. S/CO ratio greater than or equal to 1.00 is considered reactive.

For each initially reactive sample, fresh sample was requisitioned and retested after centrifugation at 10000 rpm. Reactive samples were again retested on the same platform. Samples found repeatedly reactive were further tested by HIV TRI-DOT (J. Mitra and Co Pvt. Ltd) and Alere Determine™ HIV-1/2 (Alere, USA) with principles based on immunofiltration and immunochromatography respectively for secondary screening; confirmatory test and for differentiation between HIV 1 and HIV 2. Based on the reporting algorithm provided by NACO, the results were reported as non-reactive, reactive or indeterminate.

Reports were released in a standard format. Median S/CO value was released. Necessary advisories regarding repeat and confirmatory tests were incorporated. Each report also included the necessity of a mandatory post-test counselling. Strict confidentiality was maintained at all stages. Unfortunately, patients could not be followed up and we could not confirm the sero-status of the patient at a later date.

The results were analysed in two distinct groups. The first group comprised of 9363 samples received between 01 Jan 2015 and 31 June 2016 (18 months). The second group included rest of the 12459 samples processed between 01 Jul 2016 and 31 July 2017 (12 months). These two groups were made as there were differences in the rate of false positivity on initial scrutiny of the results.

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), disease prevalence, outlier detection and the 'p' value were calculated using two online statistical calculators simultaneously to ensure accuracy and reproducibility (vassarstats.net/clin1.html and https://www .medcalc.org/calc/diagnostic_test.php).

RESULTS

Of a total of 21817 samples processed for HIV infection, 147 (0.67%) samples tested reactive by all three test modalities. 40 (0.18%) samples tested positive by Architect HIV Ag/Ab combo alone (repeatedly reactive). However, the second as well as the third test were non-reactive (negative). These tests were reported as non-

reactor for HIV. Six (6; 0.027%) samples tested positive by Architect HIV Ag/Ab Combo of which five (5) tested reactive by Alere Determine™ HIV-1/2 but non-reactor by HIV TRI-DOT. The sixth sample tested positive by HIV TRI-DOT but non-reactor by Alere Determine™ HIV-1/2. These six samples were reported as 'indeterminate' as per NACO guidelines and were advised further follow up.

It was found that out of 9363 samples processed between 01 Jan 2015 and 31 June 2016 there were 77 true positives, 8 false positives and 2 indeterminate, whereas, of the 12459 samples processed between 01 Jul 2016 and 31 July 2017, there were 70 true positives, 32 false positives and 4 indeterminate. The difference between the rate of false positivity between these two groups was found to be statistically significant (p = 0.0033).

Of a total of 21817 samples, 10254 were males (47%) and 11563 were females (57%). Overall disease prevalence was found to be 0.67% (Males: 1.12%; Females: 0.28%) (Table 1). Half yearly distribution of samples (depicted as HY-1, HY-2 etc) (Figure 1) revealed significant increase in samples from Jan 2016 onwards. The HIV infection values too were higher during this period (Figure 2).

Table 1: Gender wise distribution of sample and results.

	Total samples	True negative	True positive	False positive	Indeterminate
Male	10254	10133	115	37	6
Female	11563	11531	32	3	0
Total	21817	21664	147	40	6

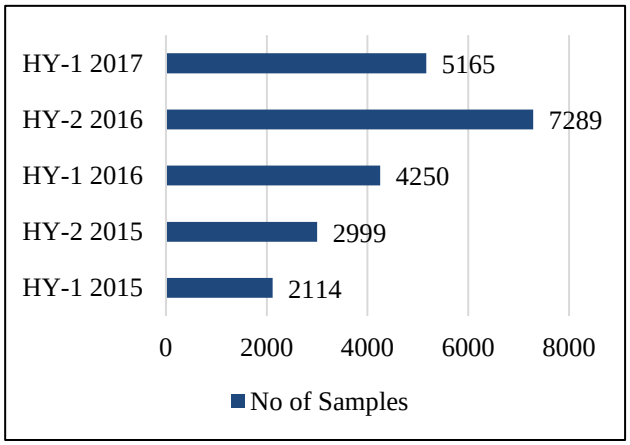

Figure 1: Half yearly distribution of samples.

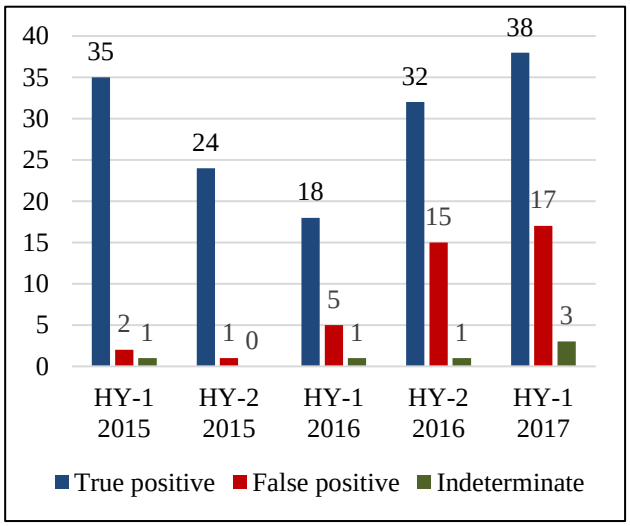

Figure 2: Half yearly distribution of results.

Architect signal to cutoff values (S/CO)

S/CO values more than 1 (one) by Architect HIV Ag/Ab Combo is considered positive. Accordingly, the S/CO values were analysed. For samples reported reactor by all three tests, it was found that the median value was 405.28 and ranged between 22.67 and 1639.12. 136 samples had a S/CO value >300; 07 were between 100-200 and only 4 below 100. The sample found reactive with the lowest S/CO value was 4.91. The median S/CO value for the 40 false positive samples was 1.24 and ranged between 1.01 and 16.47. It was found that 35 samples had a value between 1-2 and 3 samples had values between 2-5. Only two samples had higher S/CO value at 14.12 and 16.47. Calculated at significance value of 0.05, there were no outliers in both the series. The radar figure representation of the combined S/CO values revealed a wider variation of S/CO values in the reactive samples as compared to the small concise dot in false positive cases as represented by a bluish-green ovoid area and a red dot respectively (Figure-3).

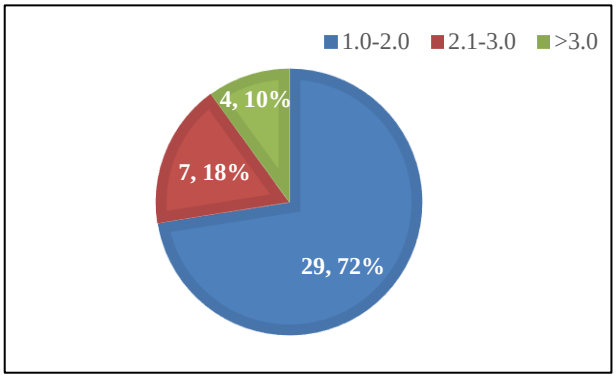

Figure 3: Distribution of S/CO values in false positive samples.

Of the 147 true reactor cases, S/CO values of 107 samples ranged between 201- 800 (72.8%) whereas 29 (72%) out of a total of 40 false positive samples, the S/CO values ranged between 1.0-2.0 (Figure 4).

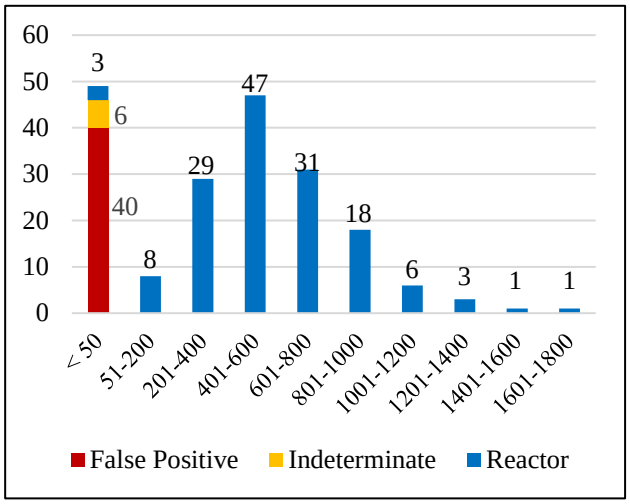

Figure 4: Distribution of S/CO values in reactor, indeterminate and false positive samples.

Sensitivity, specificity, PPV and NPV

Overall, we found that the sensitivity of Architect HIV Ag/Ab Combo was uniformly 100% as there were no false negatives. However, the specificity varied between 99.74% and 99.91%. A lower specificity was seen in the second group where 32 samples tested false positive over a period of one year. Interestingly the PPV was only 68.63% in the second group as compared to a PPV of 90.59% overall (Table 2).

DISCUSSION

The primary goal of HIV screening tests has been to detect HIV infection as early as possible to enable appropriate therapeutic intervention coupled with prevention of its spread. Subsequent improvements, refinements in technology and addition of antigen (p24) detection has led to the reduction of the window period. Accordingly, the focus has been on high sensitivity of the test, as near to 100% to fully obviate any false negative results. In most developed countries, fourth generation Ag/Ab combo assay is preferred because of its high sensitivity. This approach has paid rich dividends in conditions such as transfusion and organ donation services, prevention of mother to child transmission (PMTCT) and so forth. This has also been successful in countries where prevalence of HIV infection in the general population is low and individuals being tested are usually healthy and asymptomatic. Occurrence of false positive results are known and these are further investigated and confirmed by various supplemental tests namely Western blot and nucleic acid amplification tests (NAAT).

Table 2: Test parameters for architect HIV Ag/Ab Combo.

Statistic	Jan 2015-June 2016	Jul 2016-Jul 2017	Jan 2015- Jul 2017 (Cumulative)
Total Samples	9363	12459	21817
True Positive	77	70	147
False Positive	8	32	40
Indeterminate	2	4	6
Sensitivity	100% (95.32% to 100.00%)	100% (94.87% to 100.00%)	100% (97.52% to 100.00%)
Specificity	99.91% (99.83% to 99.96%)	99.74% (99.64% to 99.82%)	99.82% (99.75% to 99.87%)
PPV	90.59% (82.80% to 95.06%)	68.63% (60.75% to 75.56%)	78.61% (72.95% to 83.36%)
NPV	100%	100%	100%
Disease prevalence	0.82% (0.64% to 1.02%)	0.56% (0.44% to 0.71%)	0.67% (0.57% to 0.79%)

In India, we test asymptomatic individuals under strategy I and III as laid down by National AIDS Control Organization (NACO). Due to economic constraints and lack of infrastructure, it is not possible to routinely confirm a single positive test by more expensive and technically demanding tests like Western blot and/or NAAT. NACO, in its guidelines for asymptomatic individuals have laid down the testing protocol that mandates if, the first test, conducted by a highly sensitive assay is positive then either the unit of donated blood is discarded (strategy I) or is further confirmed by two more tests employing different technology platforms (Strategy III). Reportage of the results are done as per the NACO format wherein the details of all the test kits and the test results are mentioned. Detailed advice is incorporated for future follow up and post-test counselling in the report.

While perusing the kit insert enclosed in the Architect HIV Ag/Ab Combo, it was found that specificity in the low risk population was 99.77% with an exact 95% confidence interval of 99.62% to 99.88%.[10] Different specificity rates in various studies conducted worldwide is represented in Table 3.

Table 3: Specificity rates reported in various studies for architect HIV Ag/Ab Combo.

Sample profile	Total sample	Specificity	Reference
Healthy individuals	6,165	99.77%	ARCHITECT HIV Ag/Ab Combo Insert (FDA, US)[10]
Pregnant females	448	100.00%	ARCHITECT HIV Ag/Ab Combo Insert (FDA, US)[10]
Blood donors	6,365	99.89%	ARCHITECT HIV Ag/Ab Combo Insert (B4J2S0, India)[11]
Paediatric population	558	99.83%	ARCHITECT HIV Ag/Ab Combo PMA Summary of Safety and Effectiveness Data (SSED)[12]
Multicentre, sample-based study, USA	10,995	99.50%	P. Chavez et al, Journal of Clinical Virology[7]
Hospital based study, South Korea	155,339	99.78%	Kim et al, Clinical and Vaccine Immunology[13]
Our study, New Delhi	21,817	99.82%	-

Table 4: Alterations in specificity and PPV at different S/CO values.

	S/CO: 1	S/CO: 2	S/CO: 5
Total samples	21817	21817	21817
True positive	147	147	147
False positive	40	11	2
Sensitivity	100% (97.52% to 100.00%)	100% (97.52% to 100.00%)	100% (97.52% to 100.00%)
Specificity	99.82% (99.75% to 99.87%)	99.98% (99.91% to 99.97%)	100% (99.97% to 100.00%)
PPV	78.61% (72.95% to 83.36%)	93.04% (88.10% to 96.02%)	98.66% (94.84% to 99.66%)
Disease prevalence	0.67% (0.57% to 0.79%)	0.67% (0.57% to 0.79%)	0.67% (0.57% to 0.79%)

On further analysis of the results, we tried to ascertain the changes in sensitivity, specificity, PPV, NPV and disease prevalence rates, if the values of the S/CO values were modified. We adjusted the S/CO values at 2 and 5 and recalculated the values. We found that the specificity of the test from the initial value of 99.82% was improved to 99.98% for S/CO value adjusted at 2 and 100% when adjusted at 5. Similarly, the PPV from its initial value of 78.61% improved to 93.04% and 98.66% at S/CO values of 2 and 5 respectively (Table 4).

A similar study conducted by Kim et al, in 2010 arbitrarily adjusted the cut off value to 6.6 and demonstrated a significant improvement in specificity (99.99%) and PPV (89.70%).[13] Similarly, Jenson et al, examined 138,911 tests with Abbott Architect HIV Ag/Ab Combo assay (3,705 reactive). As per their report a S/CO ratio of >151.17 had a PPV of 100% but a sensitivity of only 67.4%.[14] During analysis, we also noticed that in our study there was a significant difference between the sample load as well as the false positivity rate between the two groups of study period we made i.e. between 01 Jan 2015 and 31 June 2016 (18 months) and the second group from 01 Jul 2016 and 31 July 2017 (12 months). The increase in the samples was attributed to higher number of samples forwarded by an overseas recruiting agency based in New Delhi.

At this stage, it appears that false positives are higher due to a lower S/CO cut off value at 1. This value has specifically been determined for countries with low prevalence rates for HIV. However, the same value may not be suitable for countries with moderate to high prevalence rates that include India. Although most of the cases are finally declared non-reactor using the 3-test algorithm established by NACO, it does create a lot of dilemma and distress among the individuals. Many recruitment agencies screening for jobs for overseas appointments are unwilling to recruit such individuals even if a single test in the algorithm is reactive. This causes loss of job opportunity and creates social complications. This is over and above the laboratory aspects of repeat tests, delay in reporting and requirement of fresh samples.

Despite extensive search, we found no significant study from India on this subject. Munshi et al, in their report from Dacca, Bangladesh have cautioned the users of 4th generation assays against the possibilities of false positives.[15] Liu et al, reported two cases that highlight the spectrum of false positivity in the Abbott Architect p24 antigen/antibody assay.[16]

Klarkowski et al, propose that there is an increased CD5+ response along with polyclonal cross reactivity due to early B cell response in patients with concurrent parasitic infestations in African populations. This is a major cause of false positivity with rapid diagnostic tests (RDT).[17] A study sample of 1517 patients in south-western Uganda revealed 43.9% false positive rates that used a 'tie-

breaker' algorithm (129/295 positive results) similar to NACO guidelines prevalent in India.[18]

During interaction with sister laboratories in the city, we have been apprised that a major confusion exists due the false positive reports in this platform though the precise data is unavailable. Incidentally, our laboratory receives a large number of samples from other laboratories for inter-laboratory confirmation (ILC). Many of these assays turn out to be false positives with significant frequency. Since this platform is being used pan India, we anticipate that the cumulative number of false positives on Architect HIV Ag/Ab Combo may be staggering.

In view of the above, it appears prima facie that a revision in the S/CO values may be required to minimise the occurrence of false positives without compromising on the sensitivity. However, the matter is complex and the principal manufacturer of the system may explore the possibility of conducting a large scale multicentre test in India to ascertain the optimal S/CO value for this country. India being a multi-racial nation with different ethnic groups, a detailed analysis of the S/CO values need to be established or revised to minimise the false positive results. In addition, it may also be worthwhile to establish a 'grey-zone' similar to the S/CO cut-off for anti HCV antibodies by the same manufacturer.

Our study is primarily a retrospective analysis retrieved from the database. The samples were received from various peripheral centres and laboratories to our centre and therefore, the individual cases could not be followed up. We were also unable to carry out confirmatory supplemental tests. However, our study has pointed towards the magnitude of the problem with regards to false positives. More detailed study is required to establish the facts beyond doubt and remedial actions may be initiated. Considering the specificity of 99.75% to 99.87%, we expect 130-250 false positives for every 100,000 samples tested and that is a matter of concern.

HIV infection continues to be non-curable and early laboratory detection is of paramount importance. The ideal test must be able to detect all true positives (i.e. no false negatives) in a population. Therefore, lower cut-offs leave the possibility of false positives a reality. In developed countries with lower HIV prevalence rate, the priority remains on high sensitivity and false positives are followed up by various advanced supplemental tests. In India, it may not be possible to follow up all false positives in a resource scarce setting such as ours by molecular methods due to lack of infrastructure and the cost involved. A false positive result may lead to severe mental agony in an individual with accompanying social and economic consequences. The choice remains between eliminating all false negatives or accepting false positives. It's truly "Horns of a Dilemma".

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