Diagnosis of acute HIV is done by patient history and examination and testing of RNA, proviral DNA, and serology using fourth-generation antigen/antibody detection assays. We describe an HIV-1 primary infection with a second diagnostic window of 18 to 34 days on a fourth-generation immunoassay, which would have been missed using some current algorithms. Caution must be exercised when fourth-generation HIV-1 immunoassays are interpreted in isolation, and additional testing should be considered depending on patient risk assessment.

CASE REPORT

A 43-year-old circumcised Caucasian man who regularly attended a sexually transmitted infection (STI) clinic presented for testing, reporting a recent mild flu-like illness. His sexual risk factors included regular local bathhouse contact, mostly unprotected oral sex, unprotected insertive anal sex over several years, and recent unprotected receptive anal sex. The patient’s medical history included treated syphilis 2 years earlier, treated Neisseria gonorrhoeae infection, treated Chlamydia trachomatis infection, and recurrent anal infection with herpes simplex virus (type unknown, with no recent symptoms). Previous regular screening for HIV infection was negative, most recently 3 months earlier. The screening was performed at South Eastern Area Laboratory Services (SEALS), where a fourth-generation screening immunoassay (Abbott Architect HIV Ag/Ab Combo [Abbott Laboratories, Abbott Park, IL, USA]) was incorporated into the routine diagnostic algorithm (Fig. 1), consistent with national and international standards of testing. The patient denied use of intravenous drugs or prophylactic antiretrovirals, although confirmatory testing of blood for antiretroviral use was not performed.

Screening for HIV-1 and HIV-2 was performed using the routine testing algorithm. Initial screening (day 0 of testing, following recent high-risk activities) was consistent with HIV viremia without seroconversion (Table 1). Specifically, the Abbott Architect HIV Ag/Ab Combo assay and Genscreen HIV p24 Ag assay (Bio-Rad, Marnes-la-Coquette, France) were reactive, with the latter result confirmed by neutralization. However, the Serodia HIV-1 Ab assay (Fujiirebio, Tokyo, Japan) was nonreactive, and no bands were present on Western blots (MP Biomedicals, Illkirch, France). HIV Western blots were interpreted using accepted criteria for diagnosis in Australia, which differ from criteria proposed by the Centers for Disease Control and Prevention in the United States (1, 2). On day 7, the Abbott Architect HIV Ag/Ab Combo assay, Bio-Rad Genscreen HIV p24 antigen assay, and Serodia HIV-1 antibody assay showed no reaction, and no bands were present on the Western blot. Given the day 0 result, an HIV viral load test (COBAS AmpliPrep/COBAS TaqMan HIV-1 test, v.2.0 [Roche Molecular Systems, Inc., Branchburg, NJ, USA]) was performed on plasma, revealing 11,352 copies/ml. The patient’s serum transaminases were mildly elevated (AST 61 [upper limit of normal, 45 IU/liter] and ALT 97 [upper limit of normal, 45 IU/liter]), with later testing showing variable results. The viral load was initially very high and then progressively fell to 175 copies/ml by day 35 (Table 1). The Serodia HIV-1 antibody assay became positive on day 10. The Western blot was indeterminate on day 10, with only p24 antibody detected, followed by the gp160 band (day 21), p18 band (day 49), p65 and p51 bands (day 62), and gp41 to gp43, gp120, and p55 bands (day 175). The Abbott Architect HIV Ag/Ab Combo assay (tested using two different i2000SR machines) became reactive on day 35. Throughout the testing period of 175 days, the OraSure HIV-1/2 Ab (OraSure Technologies, Bethlehem, PA, USA) point-of-care test (POCT) remained unreactive when oral fluid was used (blood-derived specimens were not tested).

The second diagnostic window was investigated by performing nonroutine retrospective testing. Stored samples demonstrated viral loads of 2,888,068 copies/ml on day 0 and 0 copies/ml 98 days prior to this. Two additional fourth-generation assays (Elecsys HIV Combi PT [Roche Diagnostics, Penzberg, Germany] and Genscreen Ultra HIV Ag-Ab [Bio-Rad, Marnes-la-Coquette, France]) were performed on sera from all available dates and demonstrated consistently reactive results, without a second diagnostic window. Retroviral testing with a Determine HIV 1/2 Ag/Ab Combo (Alere, Waltham, MA, USA) POCT did not detect antigen and demonstrated antibody only after day 49.

Antiviral treatment was not initiated after HIV diagnosis, given that the patient’s HIV load rapidly dropped to 175 copies/ml by day 35, his CD4 count remained around 1.0 × 10⁹ throughout the testing period.

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period, the patient was clinically well, and he adhered to strict uptake of condom use. The patient’s known sexual contact was alerted, was reportedly HIV negative on initial screening, and advised to repeat screening after 3 months.

Viral genotyping (Trugene HIV-1 and vircoTYPE HIV-1) indicated an HIV-1 type M subtype CRF01-AE infection based on a 96.8% protease and 96.3% reverse transcriptase match to the closest reference isolate in the Stanford University HIV Drug Resistance Database.

Fourth-generation HIV immunoassays, which include both antibody and p24 antigen detection components, were first used for HIV screening in Australia in 2003. Several studies have reported sensitivities of 100% (3–6), with calculations based on various testing algorithms and reference standards, in different population subsets, and consistent with manufacturer’s guidelines (7–9). In contrast, studies of recent infections have reported transient sensitivities of 62% to 89% (10, 11) when assessed against HIV-RNA tests. Although it is generally regarded as rare, there are many published cases where at least one fourth-generation assay has failed to diagnose an HIV-positive patient on at least one test date (4, 10–20). In some cases, this may be due to the presence of a second diagnostic window, defined as a period where a fourth-generation assay becomes nonreactive after the antigen component drops below the limit of detection and before the antibody component exceeds the limit of detection. One case report describes a permanent failure to detect antibody with two fourth-generation assays in an HIV group O infection (20). Other cases are possibly due to a primary diagnostic window caused by relative insensitivity of the antigen detection component compared with assays such as those for RNA detection (4) or for p24 antigen (14). The literature describes numerous cases where fourth-generation-assay failures could conceivably be due to a second diagnostic window (10–18, 21). Many different assays may be implicated, including the Abbott Architect HIV Ag/Ab Combo (10–12), Abbott AxSYM HIV Ag/Ab Combo assay (14, 17, 18), Abbott Murex HIV Ag/Ab Combo (14), bioMérieux Vidas HIV Duo (14, 15), Bio-Rad Genscreen plus HIV Ag/Ab (13, 14), Dade Behring Enzygnost HIV Integral (14, 15), Organon Teknika Vironostika Uniform HIV Ag/Ab (16), Organon Teknika Vironostika Uniform II HIV Ag/Ab (14), Roche Cobas Core HIV Combi (21), and Roche Elecsys Combi assay (17) (a summary is provided in Table S1 in the supplemental material). Given limitations in published sample data, many cases cannot be definitively classified. However, irrespective of cause and designation, they confirm that fourth-generation assays do occasionally fail to detect recent infection.

This case, which involved an individual who remained seronegative over many years of high-risk activity, has several unique features. To our knowledge, this represents the longest reported second diagnostic window for a fourth-generation assay. Further, a supplemental assay using a first-generation whole-virus lysate was assessed throughout the testing period and was positive from...

**TABLE 1 Comparative performance of assays**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Roche COBAS AmpliPrep/TaqMan</th>
<th>Bio-Rad Genscreen p24 Ag (pg/ml)</th>
<th>Abbott Architect HIV Ag/Ab Combo</th>
<th>Roche Elecsys HIV Combi PT</th>
<th>Bio-Rad Genscreen Ultra HIV Ag/Ab</th>
<th>Determine HIV 1/2 Ag/Ab Combo</th>
<th>Orasure OraQuick HIV-1/2 Ab (titer)</th>
<th>Fujirebio Serodia HIV-1 Ab (band[s] present)</th>
<th>MP Biomedicals Western blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicon</td>
<td>(copies/ml)</td>
<td></td>
<td></td>
<td>Band(s) present</td>
<td>(s/co)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-98</td>
<td>0.07</td>
<td>0.07</td>
<td>3.9</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>0</td>
<td>268,806</td>
<td>R (44)</td>
<td>R (17.78)</td>
<td>R (15.08)</td>
<td>R (4.87)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>7</td>
<td>11,352</td>
<td>NR</td>
<td>NR (0.82)</td>
<td>R (1.31)</td>
<td>R (2.23)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>10</td>
<td>7,591</td>
<td>NR</td>
<td>NR (0.89)</td>
<td>R (2.20)</td>
<td>R (3.41)</td>
<td>NR</td>
<td>NR</td>
<td>NR (1:128)</td>
<td>IND (p24)</td>
</tr>
<tr>
<td>21</td>
<td>2,648</td>
<td>NR</td>
<td>NR (0.90)</td>
<td>R (11.08)</td>
<td>R (8.57)</td>
<td>NR</td>
<td>NR</td>
<td>IND (p24, gp160)</td>
<td>IND (p24, gp160)</td>
</tr>
<tr>
<td>24</td>
<td>1,809</td>
<td>NR</td>
<td>NR (0.93)</td>
<td>R (12.86)</td>
<td>R (7.30)</td>
<td>NR</td>
<td>NR</td>
<td>IND (p24, gp160)</td>
<td>IND (p24, gp160)</td>
</tr>
<tr>
<td>35</td>
<td>175</td>
<td>NR</td>
<td>NR (1.13)</td>
<td>R (27.32)</td>
<td>R (4.26)</td>
<td>NR</td>
<td>NR</td>
<td>IND (p24, gp160)</td>
<td>IND (p24, gp160)</td>
</tr>
<tr>
<td>49</td>
<td>254</td>
<td>NR</td>
<td>R (1.23)</td>
<td>R (51.40)</td>
<td>R (5.66)</td>
<td>R (Ab only)</td>
<td>NR</td>
<td>IND (p25, p40)</td>
<td>IND (p24, gp160)</td>
</tr>
<tr>
<td>62</td>
<td>99</td>
<td>NR</td>
<td>R (2.04)</td>
<td>R (117.30)</td>
<td>R (9.10)</td>
<td>R (Ab only)</td>
<td>NR</td>
<td>IND (p25, p40)</td>
<td>IND (p24, gp160)</td>
</tr>
<tr>
<td>98</td>
<td>609</td>
<td>NR</td>
<td>R (3.01)</td>
<td>R (130.15)</td>
<td>R (10.27)</td>
<td>R (Ab only)</td>
<td>NR</td>
<td>IND (p24, gp160)</td>
<td>IND (p24, gp160)</td>
</tr>
<tr>
<td>175</td>
<td>294</td>
<td>NR</td>
<td>R (6.81)</td>
<td>R (272.7)</td>
<td>R (6.94)</td>
<td>R (Ab only)</td>
<td>NR</td>
<td>IND (p24, gp160)</td>
<td>IND (p24, gp160)</td>
</tr>
</tbody>
</table>

* The Abbott Architect HIV Ab/Ag Combo remained nonreactive from day 7 through 24, becoming reactive at day 35. The Roche Elecsys HIV Combi PT and Bio-Rad Genscreen Ultra HIV Ag/Ab were reactive throughout the testing period. R, reactive; NR, nonreactive; NB, no bands; IND, indeterminate; P, positive; s/co, signal-to-cutoff ratio.
* Retrospectively tested from serum.
* Retrospectively tested from plasma.
day 10. Finally, comparison of routine serology with two point-of-care assays revealed that they remained nonreactive until day 49 in the case of the Determine HIV 1/2 Ag/Ab Combo and on all test dates in the case of the Oraquick HIV-1 Ab.

The second diagnostic window was described previously (15, 17, 18), although the lack of data in many cases limits critical appraisal. However, review of those reports shows that the failure to detect infection cannot be consistently predicted by factors such as timing postinfection, HIV-1 subtype, patient demographics, or assay manufacturer. An analysis of the sensitivity of the Abbott Architect HIV Ag/Ab Combo in acute HIV infection described a second diagnostic window associated with a lower limit of detection correlating with a viral load of 14,000 to 30,000 RNA copies/ml in antibody-negative patients (11). Consistent with this, the likely explanation for the failure of the Architect HIV Ag/Ab Combo in this case was that the assay detects HIV p24 antigen and antibody to gp41 but not HIV p24 antibody. HIV-1 gp41 antibody was first detected by Western blotting at day 175 in this case, whereas p24 antibody was detected at day 10 (Table 1). The antigens and antibodies detected by assays vary, including HIV-1 p24 antigen, HIV-1 gp41, gp120, and gp160 antibodies, HIV-1 and HIV-2 reverse transcriptase, and HIV-2 gp36 and gp105 antibodies (3, 5, 21), with no published data indicating an assay that detects p24 antibody.

It is not immediately clear why a second diagnostic window was observed only with the Architect HIV Ag/Ab Combo. However, several possibilities require consideration. It is unclear if p24 antigen-antibody complexes could result in p24 antigen levels falling below the limit of detection in some assays while remaining detectable in others. It is noteworthy that the Genscreen Ultra HIV Ag-Ab assay includes gp160 recombinant protein. We first detected gp160 antibody by Western blotting on day 21. Given the relative insensitivity of Western blotting, we cannot exclude the possibility that antibody to gp160 was detected by the Genscreen Ultra HIV Ag-Ab assay earlier than day 21. Detection of HIV reverse transcriptase antibody (seen as p51 and p66 in Western blots) on the Elecsys HIV-1 Combi does not immediately explain the lack of window in that assay, given we did not see relevant bands until relatively late (day 62).

Other testing modalities may be susceptible to a second diagnostic window. For example, failure to detect has been reported with nucleic acid methods (21), although notably, the sensitivity of the assays used was inferior to that of currently available assays. For our case, we note that the diagnostic window of the Serodia HIV-1 Ab assay partly overlapped the second diagnostic window of the Abbott Architect HIV Ag/Ab Combo.

Current Australian guidelines for STI and HIV testing in men who have sex with men recommend HIV testing every 3 months for men reporting unprotected anal sex (22), during which time secondary contacts can become infected. Neither the Oraquick HIV-1 Ab nor Determine HIV 1/2 Ag/Ab Combo (also a fourth-generation assay) would have assisted in diagnosis during the diagnostic window of the Architect HIV Ag/Ab Combo, and their limited sensitivity for the detection of acute HIV infections has been noted elsewhere (23–25). Given these factors, we believe that fourth-generation assays are insufficient for identifying some acute infections in high-risk individuals. Alternative screening algorithms require reconsideration of the role of nucleic acid and other testing, especially in the context of recent high-risk exposure. The issue has been previously raised (26), with benefits demonstrated in pooled samples (27) or in individual testing in high-risk populations (25). Ideally, the testing structure adopted by a revised algorithm would account for the possibility of delayed seroconversion and rapid viral-load decreases in patients, especially given recent cases of unreported use of antiretroviral therapy in HIV study participants (28).

The use of signal-to-cutoff ratios between 0.5 and 1.0 in the Abbott Architect HIV Ag/Ab Combo assay has been proposed as a potential method of improving sensitivity (29). While this could potentially allow detection of otherwise missed cases, this would constitute an off-license practice and would therefore be inappropriate. Additionally, the effect of such reporting on specificity would require detailed analysis. Our laboratory employs an informal gray zone of 0.9 to 1.0 when flagging results for review, based on the measurement of uncertainty of the Abbott Architect HIV Ag/Ab Combo assay. However, all results are strictly interpreted and reported according to manufacturer’s specifications in accordance with licensing requirements.

Many second diagnostic windows experienced with fourth-generation assays have been detected only because concurrent HIV RNA nucleic acid testing was undertaken. Our observations support this finding, and we are not aware of any case where current-generation nucleic acid testing has given a false-negative result during a second window in a fourth-generation assay. In Australia, proviral DNA in the context of a group IV indeterminate Western blot provides sufficient definitive laboratory evidence to confirm a case of newly acquired HIV (30). In addition to excluding patients based on history, the Australian Red Cross Blood Service screens blood using both the Abbott PRISM (Abbott Diagnostics, Wiesbaden-Delkenheim, Germany) chemiluminescent assay and the Novartis HIV-1/HCV/HBV Procleix Ulitro assay using the fully automated Procleix TIGRIS system (Gen-Probe/Novartis Diagnostics, San Diego, CA, USA). As these are screening tests, subsequent confirmatory assays are required. Regarding diagnosis of newly acquired infection, HIV RNA testing is not included in the Australian case definition, HIV proviral DNA testing is not currently a licensed test even though it is included in the case definition, and as such neither is available for routine diagnosis in Australia.

The costs associated with including an additional test in the initial screening panel must be weighed against the public health costs of potentially undiagnosed HIV-positive patients. Consideration must also be given to urgent testing for individuals such as organ transplant donors, where accurate, timely, and sensitive testing is critical. More research into the cause and prevalence of second diagnostic windows, ongoing mutation and its impact on detection, use of illicit and prescribed prophylactic antivirals potentially delaying seroconversion, the impact of self-testing, the effect of cell-mediated immunity on detection, and the effect of inhibitors of immunoassays is required. Consequently, it is imperative that laboratories and clinicians be aware that false-negative interpretations are possible when current-generation immunoassays are relied on solely and that they consider alternative strategies where appropriate.

**REFERENCES**


