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Screening and confirmation of human immunodeficiency virus type 1 infection solely by detection of RNA

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Diagnosis of human immunodeficiency virus (HIV) infection by antibody-based testing allows for some recently infected individuals to be falsely assessed as non-infected. Since such individuals often have high viral loads and are capable of transmitting HIV, it is an imperative public health need to identify these individuals. We investigated the feasibility and capability of a diagnostic algorithm which included screening and confirmation of HIV infection using only nucleic-acid-based tests. This investigation involved screening 1361 prospectively collected specimens using antibody-based methods in parallel to simultaneously testing the same specimens by a qualitative HIV RNA detection method (APTIMA HIV-1). Specimens that were positive by antibody screening were confirmed by either immunofluorescent assay or Western blotting, while specimens positive by RNA screening were confirmed by real-time RT-PCR. In the course of the study, 27 specimens were found to contain either HIV antibody or HIV RNA. Twenty-six of the 27 specimens were HIV RNA positive, while 23 of the 27 specimens were antibody positive. One specimen was found which possessed HIV antibody but was assessed as negative by the HIV RNA screening test. Four specimens were found to contain detectable HIV RNA but were negative by the antibody screening test. Three of these four patients were negative at point-of-care by rapid test, while one was negative by enzyme immunoassay. These data indicate that screening and confirmation of HIV infection by RNA methods alone, if affordable, may constitute an effective alternative HIV diagnostic algorithm in certain settings.

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INTRODUCTION

Infection with human immunodeficiency virus type 1 (HIV-1) has routinely been determined through the use of antibody screening tests, which include enzyme immuno-assay (EIA) or rapid, point-of-care (POC) methods. Confirmation of reactive (positive) antibody screening tests is accomplished either by Western blotting or by immunofluorescent assay (IFA). Positive screening and confirmation tests require that the patient has produced an antibody-based, immunological response to HIV proteins. However, it is well established that infection with HIV is followed immediately by a window period, in which HIV-specific antibody cannot be detected by any currently utilized antibody test (Constantine *et al.*, 1994; Fiebig *et al.*, 2003). During this period, HIV is present in the blood and other bodily fluids in considerably higher concentrations than at any other time during the infectious course (Daar, 1998; Little *et al.*, 1999). Simultaneous with such periods of high viraemia is a commensurate increase in capability to transmit HIV infection (Pilcher *et al.*, 2004; 2007; Pinkerton, 2007; Wawer *et al.*, 2005). The use of direct virus testing methods for detection of recent HIV infection has been explored. Primarily due to cost and labour constraints, such investigations have involved antibody screening followed by analysis of pooled, antibody-negative specimens which are then analysed by direct virus detection (HIV RNA-based) methods. Such studies have routinely, albeit rarely, identified specimens that contain HIV particles but not detectable anti-HIV antibody (Fiscus *et al.*, 2007; Louie *et al.*, 2006; Patel *et al.*, 2006; Pilcher *et al.*, 2005; Priddy *et al.*, 2007; Stekler *et al.*, 2005). Such specimens have been assumed to be from recently infected individuals, and the levels of HIV RNA in such specimens are often high.

The diagnostic use of HIV RNA detection methods has thus far been restricted to the analysis of pooled samples of specimens which have previously been tested by antibody-based methods (EIA or rapid test). When tested this way, the HIV RNA results do not become available until several

Abbreviations: EIA, enzyme immuno-assay; IFA, immunofluorescent assay; POC, point-of-care; qRT-PCR, real-time reverse transcriptase-PCR; TMA, transcription-mediated amplification.

days after the initial blood draw. As such, the benefit of detecting early HIV infection by RNA-based methods is reduced. The time that elapses while a recently HIV-infected individual is ignorant of their HIV status is critical time that allows for subsequent HIV transmission events. This concern is heightened in consideration of the increased infectiousness of an infected individual during this early stage, perhaps due to high viraemia and the absence of neutralizing antibodies.

A strategy that includes the use of HIV RNA detection as the primary screening test for HIV infection merits consideration. The primary benefit of this strategy is the ability to detect infected individuals during the window period, when they are potentially most able to transmit HIV infection. A secondary benefit of this strategy is the reduction in the time that patients must wait prior to getting the results of an HIV RNA-based test, as specimens would not be pooled and tested only after the results of an antibody test. A potential drawback of an HIV RNA-only method of screening and diagnosing HIV infection is the possibility of defining people who do not possess a measurable viraemia in the plasma at the time of HIV testing as falsely HIV-negative. Such individuals have been identified at relatively low frequency (Hubert *et al.*, 2000; Lefrere *et al.*, 1999; Madec et al., 2005; Sajadi *et al.*, 2007). An additional drawback is the potentially high cost of using RNA-based methods as the screening test. We sought to explore the feasibility of an HIV-RNA-only based strategy for HIV diagnosis. This involved the prospective screening of patient specimens using an HIV RNA detection method as the primary screening test. The testing method used as a screening test is a qualitative, highly sensitive, transcription-mediated amplification (TMA)-based method, the APTIMA HIV-1 RNA Qualitative Assay Aptima (Gen-Probe, San Diego, CA). Confirmation of HIV RNA detection in specimens was done by using a real-time reverse transcriptase-PCR (qRT-PCR)-based method (*m*2000, Abbott Molecular) with nearly equal sensitivity to the APTIMA HIV-1, thus ensuring that the confirmation test would have little chance of failing in the confirmation of initial screening results. In parallel, the same specimens were tested by the established algorithms of serological testing, which include either EIA or rapid POC test followed by confirmation by Western blot or IFA. Based upon the results of previous work with the detection of HIV RNA in pooled, antibody-negative specimens, we hypothesized that an RNA-only based method of screening and confirmation of HIV infection would detect more HIV-infected individuals in the community than antibody-based testing alone (Louie *et al.*, 2006; Patel *et al.*, 2006). We further hypothesized that an RNA-only method of screening may result in the incorrect assessment of a small number of individuals that are truly infected but lack a measurable viral load. This study presents the results from the HIV RNA-only screening strategy on 1361 prospectively collected specimens for 118 days in parallel to a standard, serological screening algorithm. We have found that each of the above hypotheses is true: RNA-only testing did result in the detection of more cases of HIV infection than antibody-based screening and had the potential for faster turnaround time for results. However, during the course of the study, one specimen out of 1361 tested was identified which contained HIV antibodies but was below the detectable threshold for the HIV RNA screening test.

METHODS

Specimen collection and storage. Specimens were collected by venipuncture into plasma separator tubes (Grenier-Bio One). After preparation of plasma, specimens were transported to the laboratory within 24 h of collection and stored at −70 °C until analysis.

Antibody testing. Specimens were screened either at the clinic site for HIV antibody by OraQuick Advance (OraSure Technologies) using either the oral swab or finger-stick protocol, or in the laboratory by EIA. For EIA analysis, the Genetic Systems HIV-1/HIV-2 PLUS O EIA (Bio-Rad) was used. Positive antibody screening tests were confirmed using IFA (Fluorognost HIV-1 IFA; Sanochemia Pharmazeutika). Conflicting specimens were also tested by Western blot using the Genetic Systems HIV-1 Western Blot (Bio-Rad).

HIV-1 RNA screening. Prepared, frozen plasma (100 µl) was combined into pooled samples consisting of five individual specimens. Pooled samples were analysed by TMA using the APTIMA HIV-1 RNA Qualitative Assay (Gen-Probe). The test is a qualitative method of HIV-1 RNA detection with a lower limit of detection of approximately 30 copies per ml plasma. Positive pools were identified and the pool constituents were analysed by qRT-PCR (HIV-1 *m*2000, Abbott Molecular). This quantitative test has a lower limit of quantification of 40 copies per ml plasma. The qRT-PCR method provided identification of RNA-positive pool constituent(s). Specimens that were positive by both TMA and qRT-PCR were considered to have been taken from HIV-infected individuals.

RESULTS AND DISCUSSION

For 118 days, 1361 specimens (plasma) that were collected for HIV testing from a municipal STD clinic were analysed both by an RNA-only detection-based algorithm (Fig. 1a), and by either of two antibody-based detection algorithms.

EIA-based algorithm 1 (Fig. 1b). A portion of the specimens (581/1361; 43 %) were subjected to third-generation EIA, with all reactive specimens being tested two additional times. Repeatedly reactive specimens were then subjected to either IFA or Western blotting for confirmation. Non-reactive specimens were subjected to RNA pooling and screening as below.

POC test-based algorithm 2 (Fig. 1c). A portion of specimens (780/1361; 57 %) were initially screened by rapid, POC antibody test (OraQuick) at the clinic site. All patients were also subjected to venipuncture; blood specimens were delivered to the laboratory for RNA testing as described below (if they were negative at POC) or for confirmation by EIA and IFA or Western blotting (if they were positive by rapid test at POC).

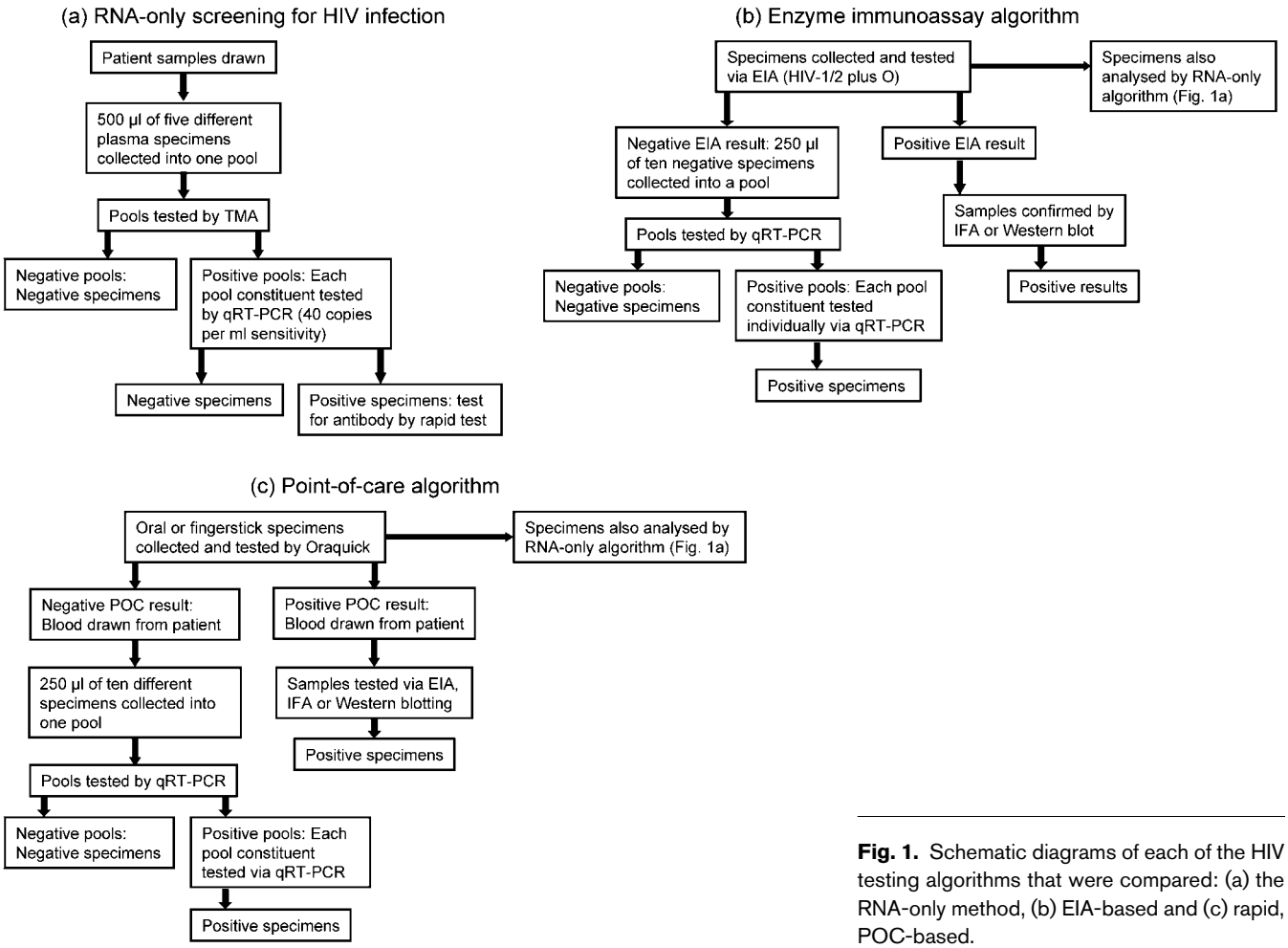

Fig. 1. Schematic diagrams of each of the HIV testing algorithms that were compared: (a) the RNA-only method, (b) EIA-based and (c) rapid, POC-based.

All specimens that were non-reactive (negative) for HIV antibody by either of the two antibody-based algorithms, above, were subsequently assessed for the presence of HIV RNA by a previously described pooling method (Patel *et al.*, 2006). In this method, samples from 10 specimens were combined and tested by qRT-PCR; positive-pool constituents were subjected to an additional round of qRT-PCR for identification of the RNA-positive individual specimen.

All 1361 plasma specimens that were screened by either of the antibody-based algorithms above (Fig. 1b, c) were also analysed by the RNA-only algorithm (Fig. 1a). Pooled samples were immediately subject to TMA using APTIMA HIV-1 for the detection of HIV-1 RNA. The pool constituents of samples found to contain HIV RNA were tested individually by qRT-PCR. Specimens found to contain HIV RNA by qRT-PCR were identified. The results of RNA-based testing were compared with the results of the antibody testing algorithms.

Of the 1361 prospective specimens analysed in parallel by both antibody and RNA-only methods, 23 specimens were confirmed positive for HIV antibody using the antibody detection algorithms (EIA or POC test followed by IFA or Western blot). All 23 specimens were confirmed positive by either IFA or Western blotting, with no indeterminate results. Simultaneously, 26 specimens were found to be positive by the RNA-only detection algorithm. Some discordance existed between the results generated by the algorithms (Table 1). The RNA-only detection algorithm identified four specimens which contained HIV RNA that were negative by antibody-based screening test (numbers 3, 12, 25, 27 in Table 1). Of these four specimens, three had been screened by an oral, POC rapid test (12, 25 and 27) and one had been screened by third generation EIA (3). Two of the three RNA-positive specimens that were non-reactive by POC test (12 and 27 in Table 1) were found to be positive by third-generation EIA. Specimens 12 and 27 were also positive by IFA and Western blotting. Specimen 25 was found to be negative by third-generation EIA and was also negative both by IFA and Western blotting. Specimen 3, which was negative by initial screen by third-generation EIA, was found to be negative by both IFA and Western blotting. Plasma of specimen 3 was negative when analysed by OraQuick (data not shown).

Table 1. Positive specimens, detected by either RNA or antibody-based screening.

Specimens were screened by third-generation EIA or POC test (oral rapid test), followed by IFA or Western blot (WB). ND, Assay not done.

Specimen	TMA screen	Antibody screen	Viral load (copies ml^{-1})	WB	IFA
1	−	+	<40	+	+
2	+	+	46 505	ND	+
3	+	−*	363 061	−	−
4	+	+	507 723	ND	+
5	+	+	68 366	ND	+
6	+	+	14 534	ND	+
7	+	+	4478	ND	+
8	+	+	81 136	ND	+
9	+	+	20 762	ND	+
10	+	+	3455	ND	+
11	+	+	3898	ND	+
12	+	−*	303	+	+
13	+	+	219	ND	+
14	+	+	6635	ND	+
15	+	+	68 735	ND	+
16	+	+	190	ND	+
17	+	+	64 992	ND	+
18	+	+	146 789	ND	+
19	+	+	52 657	ND	+
20	+	+	20 354	ND	+
21	+	+	42 087	ND	+
22	+	+	22 082	ND	+
23	+	+	3065	ND	+
24	+	+	870	ND	+
25	+	−*	58 164	−	−
26	+	+	116 638	ND	+
27	+	−*	4 347 211	+	+

*Specimen 3 was screened by third-generation EIA, specimens 12, 25 and 27 by POC (Oraquick).

One specimen (specimen 1) was identified which was reactive by antibody screening test, but was negative for the presence of RNA by HIV RNA-only screening (Table 1). Because all specimens tested by the RNA-only methods were anonymous, information could not be acquired in order to rule out that the subject who submitted this specimen had previously received a positive HIV test result or whether the subject was using antiretroviral agents. Moreover, when this antibody-positive, TMA-negative specimen was analysed in undiluted form by qRT-PCR, it was found to contain detectable RNA at <40 copies per ml.

Using the RNA-only-based method of screening and confirming HIV infection, results could be made readily available within 5 days of specimen submission. All four specimens that were detected by the RNA-only method (Fig. 1a) that had failed to be detected by antibody screening algorithms were also found to be RNA-positive by the routine qRT-PCR pooling and screening method that is normally performed in association with our negative antibody tests. The time to results ranged from 7 to 12 days.

These data indicate that screening and diagnosing HIV infection solely by HIV RNA detection methods is a feasible alternative to screening by antibody-based methods. As indicated by previous studies, the detection of RNA, when used as part of an HIV screening and diagnosis strategy, does augment the number of infected individuals that are identified. Hence, it is not surprising that the RNA-only screening and confirmation algorithm investigated here identied more infected individuals over a fixed time period compared with antibody-based screening alone.

In this study, the relatively poor performance of oral, rapid antibody testing as a screening method, compared with third-generation EIA, was notable. This raises questions regarding the value of oral POC testing in high-prevalence areas. While such screening is convenient and easy, it is a public health concern that the oral, rapid POC tests are failing to detect recently infected individuals. Other, more sensitive rapid testing options are available, and their sensitivities for detecting infection in acutely infected individuals have been assessed (Louie *et al.*, 2008). Note that the municipal STD clinic where this study was performed services a very high-risk community within San Francisco, which is itself a city with relatively high HIV prevalence. For this reason, the community studied herein may have a greater proportion of recently infected individuals who are within the window period of the OraQuick Advance rapid test.

It is not known how well a fourth-generation EIA would have performed in comparison with the algorithms used here. Since fourth-generation EIA possesses the ability to detect antigen in addition to antibody, it has the ability to detect acute HIV infection and as such, has window periods that are smaller than antibody tests alone. Such antigen–antibody tests are approved for patient use in much of the world, but remain unavailable in the United States. We seek to determine, in future studies, the performance of such tests relative to antibody screening and RNA-pooling strategies. We hypothesize that the majority of acute infections detected by RNA screening methods would be detectable by a fourth-generation EIA. One specimen found in the course of our study (number 12, Table 1) possessed a viral load of 303 copies ml^{-1} and as such, might have a relatively small amount of antigen present. However, that specimen was positive when tested using a third-generation EIA, and hence, would most likely have been detected by a fourth-generation EIA. Certainly, use of a fourth-generation EIA would be much cheaper than antibody screening combined with RNA pooling, and would require far less labour.

The benefits of screening and confirming HIV infection solely by RNA methods include the ability to detect HIV

infections during the antibody window period and to acquire these data without having to execute the lengthy process of pooling after antibody testing. Since the confirmation test of the RNA-only diagnostic algorithm includes a quantitative viral load assessment, it may obviate the need for the newly diagnosed individual to have their viral load assessed upon entry into medical care. A key drawback of an RNA-only-based detection algorithm is that there will be occasional individuals who will be tested who possess no measurable HIV viral load upon seroconversion. It is not clear how often this phenomenon occurs within the population. Studies which have investigated the viral loads of newly diagnosed, treatment-naïve patients have shown that 6–16 % of such individuals will present with consecutive undetectable viral load measurements (Hubert *et al.*, 2000; Lefrere *et al.*, 1999; Madec *et al.*, 2005; Sajadi *et al.*, 2007). Such studies used viral load tests with a sensitivity of 400 or 500 HIV RNA copies per ml plasma. One study has investigated the viral loads of non-treated individuals with known dates of seroconversion, using a test with a sensitivity of 40 copies per ml plasma and found that 5.4 % of treatment-naïve, HIV-infected individuals possessed below-detectable viral loads (Lefrere *et al.*, 1999). In the data shown here, 4 of the 27 specimens identified as HIV-positive had viral loads less than 400 RNA copies per ml plasma, while one specimen had a viral load less than 40 copies. Hence, these data agree with those observed in earlier studies. These data demonstrate that even the use of current, highly sensitive viral load tests as screening tools would fail to identify certain infected individuals. An additional drawback of the RNA-only method is that it would fail to detect HIV-2 infection. In the setting where this work was performed, infection with HIV-2 is not prevalent; however, this would most certainly not be the case worldwide.

Previous studies have shown that viral load correlates well with the ability to transmit HIV infection (Pilcher *et al.*, 2004, 2007; Pinkerton, 2007; Wawer *et al.*, 2005). In light of such findings, individuals who would be incorrectly assessed by an RNA-only screening and testing algorithm (because they did not have a measurable viral load) would happen to be those who possess the least ability to transmit HIV infection. This is opposed to antibody-based testing, which would fail to identify as HIV-infected those who typically have the greatest ability to transmit infection (high-viraemic, antibody-negative individuals). An obvious drawback of an RNA-only screening algorithm is the cost of RNA methods compared to antibody detection. Executed as described here (to analyse approximately 60 specimens per week), it is estimated that the screening of one HIV-negative individual by TMA using the 5-pooled format would cost US$10.20 for reagents, controls and supplies, with approximately 35 h of labour per week. To screen the same number of specimens using antibody-based testing costs approximately US$5.20 per negative specimen and requires approximately 15 h per week. Clearly, the monetary cost of screening is much higher. However, consideration must also be given to the cost–benefit that would arise from detecting and notifying the individuals who are most capable of generating new HIV infections in the community.

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