Sensitivity of a rapid point of care assay for early HIV antibody detection is enhanced by its ability to detect HIV gp41 IgM antibodies

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ABSTRACT

Background: Anti-HIV-1 IgM antibody is an important immunoassay target for early HIV antibody detection.

Objectives: The objective of this study is to determine if the early HIV antibody sensitivity of the 60 s INSTI test is due to detection of anti-HIV-1 IgM in addition to IgG.

Study Design: To demonstrate HIV gp41 IgM antibody capture by the INSTI HIV-1 gp41 recombinant antigen, an HIV-IgM ELISA was conducted with commercial HIV-1 seroconversion samples. To demonstrate that the INSTI dye-labelled Protein A-based colour developer (CD) has affinity to human IgM, commercial preparations of purified human immunoglobulins (IgM, IgD, IgA, IgE, and IgG) were blotted onto nitro-cellulose (NC) and probed with the CD to observe spot development. To determine that INSTI is able to detect anti-HIV-1 IgM antibody, early seroconversion samples, were tested for reduced INSTI test spot intensity following IgM removal.

Results: The gp41-based HIV-IgM ELISA results for 6 early seroconversion samples that were INSTI positive determined that the assay signal was due to anti-HIV-1 IgM antibody capture by the immobilised gp41 antigen. The dye-labelled Protein-A used in the INSTI CD produced distinct spots for purified IgM, IgA, and IgG blotted on the NC membrane. Following IgM removal from 21HIV-1 positive seroconversion samples with known or undetermined anti-HIV-1 IgM levels that were western blot negative or indeterminate, all samples had significantly reduced INSTI test spot intensity.

Conclusions: The INSTI HIV-1/HIV-2 Antibody Test is shown to detect anti-HIV-1 IgM antibodies in early HIV infection which enhances its utility in early HIV diagnosis.

1. Background

Early detection of HIV is an important component of HIV pre-vention and control programs since identification of HIV infected individuals in the early stages can support effective transmission reduction, intervention and care strategies [1,2]. Different diag-nostic testing algorithms exist for HIV detection; however, those that utilize rapid point-of-care tests (RPOCTs) for HIV have advan-tages in terms of time-to-results, accessibility to testing programs, and ease of use in comparison to the standard laboratory-based tests [3,4]. Among these, the FDA-approved, US Health and Human Services Clinical Laboratories Improvement Amendment (CLIA) waived INSTI™ HIV-1/HIV-2 Antibody Test ("INSTI", bioLytical Lab-oratories, Richmond BC) is capable of providing results in as little

as 60 s [5]. The INSTI test employs a flow-through design format, which facilitates the more rapid time to results while maintaining a high degree of sensitivity and specificity [6,7]. Moreover, INSTI has shown an ability to detect early HIV-1 seroconversion in a com-parative study using characterized, sequentially collected, archived plasma samples from individuals who seroconverted for HIV-1 in British Columbia, Canada [2]. Additionally, in a report posted by the US Centers for Disease Control, Atlanta, INSTI was more sen-sitive during early infection in comparison to other FDA approved second and third generation rapid HIV antibody tests, detecting HIV antibody a median 13.5 days after RNA detection (CDC unpub-lished data) [5]. Under the Fiebig classification system based on test results for markers of the virus and the immune response, only the most sensitive HIV antibody tests are able to detect HIV antibody in samples that meet Fiebig class III staging, while HIV Western Blot is still negative, and anti-HIV-I-IgM is positive [8] (Table 1). We hypothesized that INSTI capacity for early HIV antibody detection, equivalent to a Fiebig class III staging, is due to its ability to detect

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Table 1
Stages of primary HIV infection based on laboratory tests [8].

Stages	HIV-RNA	P24 Ag	EIA-enzyme immunoassay	Western blot (IgG)
I	+	−	−	−
II	+	+	−	−
III	+	+	IgM positive	−
IV	+	+/−	IgM positive	Indeterminate
V	+	+/−	+	+
VI	+	+/−	+	+

the presence of anti-HIV-1 IgM antibody while HIV-specific IgG is still negative or indeterminate. The stages of primary HIV infection, according to the Fiebig model, are presented in Table 1.

As with every viral infection, IgM is the first immunoglobulin product of the humoral system in HIV-1 infection [1,9]. The ability of a gp41 antigen-based HIV antibody assay to specifically detect anti-HIV-1 IgM antibody would be an important characteristic of a RPOCT for the early diagnosis of HIV infection [1]. Therefore, accurate detection of anti-HIV-1 IgM antibody can narrow the serologic window for early detection of HIV infection and reduce the gap between p24Ag detection and HIV antibody detection even further [1,9].

2. Objectives

The objective of this study was to determine if the sensitivity of INSTI for early HIV-1 antibody detection is based on the assay's ability to capture and visualize reactions from the IgM class of antibodies to HIV-1 gp41 during early seroconversion. To demonstrate this, it was necessary to: (1) provide evidence that the recombinant gp41 protein used in INSTI has affinity for the IgM class of specific HIV antibodies; (2) demonstrate that the protein-A/indigo blue dye conjugate used to visualize HIV antibody reactions in the INSTI method also has affinity to human IgM; (3) demonstrate that early seroconversion samples that were reactive with INSTI became nonreactive or had significantly reduced signal intensity upon removal of anti-HIV-1 IgM antibody.

3. Method & materials

3.1. INSTI

INSTI is a manual, visually read, flow-through RPOCT for the qualitative detection of HIV-1and/or HIV-2 antibodies in 50 μl of human blood, whole blood, serum and plasma in as little as 60 s [10].

3.2. Anti-HIV-1 IgM enzyme-linked immunosorbent assay (ELISA) (Fig. 1)

In this ELISA method, 0.5 μl of gp41 recombinant protein at a concentration of 1 mg/ml (GenWay Biotech Inc., San Diego, CA) was spotted onto unbacked Hi-Flow (HF-180) nitrocellulose membranes (EMD Millipore Billerica, MD, USA). Bovine Serum Albumin (BSA, 1.0%) in Tris-NaCl buffer (TBS, Tris 50 mM, NaCl 150 mM, PH 2.7) was used as a blocking agent. Primary antibodies, diluted 1:100 in 1% (BSA-TBS) were applied to the antigen-spotted membranes. Commercial seroconversion panel specimens (SeraCare Life Sciences HQ, MA) that indicated high levels of HIV-IgM antibody on the panel datasheets as shown in Fig. 2, HIV-IgG antibody positive serum (ProMedDx, MA), and HIV negative serum (ProMedDx, MA) were used as primary antibodies. Mouse monoclonal anti-human IgM (Novus Biologicals), diluted 1:10,000 in 1% BSA-TBS, was used as a secondary antibody, and finally horseradish peroxidase-labelled (HRPO) rabbit polyclonal anti mouse IgG (Sigma–Aldrich),
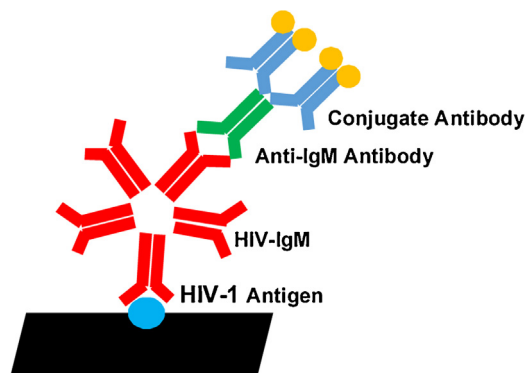

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) method for detecting anti-HIV-1 IgM antibody captured by membrane-bound recombinant gp41.
In this method the primary HIV-IgM antibody bound to the gp41 is detected by mouse anti-human anti-IgM antibody and visualized by the HRPO conjugated antibody/substrate.

was used as a conjugate antibody and developed with a chemiluminescence reagent for 30 s. The assay design is provided in Fig. 1.

3.3. Detection of different human Igs by INSTI Colour Developer

To provide indication that the standard colour developer solution from INSTI has binding affinity to human IgM, 0.5 μl of five different classes of commercial purified single human immunoglobulin preparations, IgM (Sigma–Aldrich), IgD (Athens Research & Technology Inc.), IgA (Bethyl laboratories Inc.), IgE (AbD Serotec Bio-Rad), and IgG (Bethyl laboratories Inc.) at a concentration of 1 mg/ml were blotted onto nitrocellulose membranes and allowed to dry. The standard volume of 1.5 ml of INSTI colour developer was added and allowed to flow through the membrane followed by 1.5 ml of INSTI clarifying solution.

3.4. Removal of IgM antibody

The MACS Human anti-IgM MicroBeads and magnetic separation (MS) with MS column system (Miltenyi Biotec Inc., San Diego CA), was applied for total IgM removal. Aliquots of 50 μl from each seroconversion or control sample, or 50 μl of purified human IgM were diluted in 1.5 ml of INSTI solution 1, and 20 μl of anti-human IgM MicroBeads was added to the diluted seroconversion sample. After 30 min incubation at 4 °C, IgM from the diluted samples that was bound to human the anti-IgM MicroBeads was extracted using an MS column equipped with a magnetic separator. The IgM extraction step was repeated three times to ensure maximum yield. Finally, the IgM-extracted seroconversion and control samples were tested with INSTI. The same untreated samples at equivalent dilution were also tested with INSTI to compare to the IgM-extracted counterparts for visible signal intensities.

4. Study design

4.1. Anti-HIV-1 IgM ELISA to detect the affinity of gp41 to HIV-1 IgM

HIV-1 positive seroconversion samples containing high levels of HIV-IgM (SeraCare Life Sciences HQ, MA, Fig. 2) were used as primary antibody. As a negative control, seroconversion panel samples which were HIV antibody negative, but HIV-RNA or P24 antigen positive, and three commercial HIV negative plasma samples (ProMedDx, MA), were used as the primary antibody. Any anti-HIV-1 IgM antibody present in the primary specimen would produce a visible colour reaction at the antigen spot location and indicate that

the solid phase-bound gp41 antigen effectively captures anti-HIV-1 IgM antibody.

4.2. Detection of human IgM by INSTI colour developer

The presence of any visible blue dots at the Ig blotted spot locations indicate a direct affinity of the INSTI colour developer to the immunoglobulin class [11,12].

4.3. Detection of anti-HIV-1 IgM antibody by the INSTI

To demonstrate that the INSTI detects anti-HIV-1 IgM antibody due to the combined affinities of its HIV-1 gp41 antigen and protein-A indicator solution to human IgM, total IgM was removed by anti-IgM microbeads from 21 known INSTI positive seroconversion samples that were WB (IgG) negative or indeterminate to observe for significant reduction or disappearance of spot intensity following INSTI testing. As a control, 5 later-bleed samples from the seroconversion panels, containing little or no anti-HIV-1 IgM antibody but high levels of anti-HIV-1 IgG antibodies [11,12] were used in the same method.

5. Results

5.1. ELISA method to demonstrate INSTI gp41 antigen affinity to HIV-IgM

Seroconversion samples 944-06 and 943-07, each containing high levels of IgM were reacted with duplicate spots of the membrane-bound INSTI gp41 antigen and observed for the presence of visible colour reaction upon completion of the ELISA method. As shown in Fig. 2, both samples produced visible purple-coloured spots when reacted with the secondary mouse anti-human IgM followed by conjugated rabbit polyclonal anti-mouse antibody and substrate. As a control for anti-HIV-1 IgM antibody specificity, HIV negative samples (B6390, B6392, B6888) and HIV antibody negative/RNA, P24Ag positive plasma samples (PRB945-02), and HIV antibody negative and RNA positive plasma sample (PRB944-02) showed no visible test spots (non-reactive) in this ELISA procedure.

The intensity of the anti-HIV-1 IgM antibody positive test spot was proportional to the relative amount of IgM present in the sample; Fig. 3 illustrating the relative sensitivity of INSTI to HIV-1-IgM.

Sequential samples from two commercial seroconversion panels with progressively reduced levels of anti-HIV-1 IgM antibody

Panel Members PRB944	1	2	3	4	5	6
Day since first bleed	0	2	7	9	14	16
INSTI HIV1/HIV2	N	N	N	N	P	P
In-House IgM (S/CO)	N-0.3	N-0.3	N-0.4	N-0.9	P-12.9	P-15.2

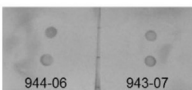| Panel Members PRB943	1	2	3	4	5	6	7
Day since first bleed	0	5	7	12	14	19	21
INSTI HIV1/HIV2	N	N	N	N	N	P	P
In-House IgM (S/CO)	N-0.2	N-0.3	N-0.4	N-0.3	P-1.0	P-11.7	P-12.4

Fig. 2. The ability of INSTI HIV-1 gp41 antigens to bind anti-HIV-1 IgM antibody. Anti-HIV-1 IgM antibody ELISA results for HIV positive seroconversion samples with a high amount of HIV-IgM (944-06, 943-07) showed clearly visible spots on the membrane. HIV antibody negative plasma samples from seroconversion panels and HIV negative serum showed no visible spot on membrane. In-House IgM data was provided with the panel datasheet from SeraCare Life Sciences. N: negative; P: positive; S/CO: signal/cutoff.

Panel Members PRB940	1	2	3	4	5	6	7	8
Day since first bleed	0	7	11	15	18	22	25	29
INSTI HIV1/HIV2	N	N	P	P	P	P	P	P
In-House IgM (S/CO)	N-0.2	N-0.3	P-3.1	P-10.4	P-6.8	P-4.5	P-2.3	P-2.0

Panel Members PRB934	1	2	3
Day since first bleed	0	7	11
INSTI HIV1/HIV2	N	P	P
In-House IgM (S/CO)	N-0.5	P-10.9	P-5.0

Fig. 3. Relative sensitivity of INSTI gp41 antigen to HIV-IgM antibody levels. The intensity of the anti-HIV-1 IgM antibody ELISA positive test spot is proportional to the relative IgM levels found in the seroconversion panel samples 940-04, 940-08, 934-02, 934-03. N: negative; P: positive; S/CO: signal/cutoff.

across patient bleeds as shown in Fig. 3 Table were tested (PRB934 and PRB940, Fig. 3). Anti HIV-1 ELISA testing showed significant reduction or disappearance of test spot intensity across sequential samples, indicating that anti-HIV-1 IgM antibody ELISA spot intensity was proportional to the level of anti-HIV-1 IgM antibody present in the primary antibody sample.

5.2. The ability of INSTI colour developer to detect human purified IgM antibody

In INSTI, the test spot captures HIV-1/2 antibodies which are present in the test sample and then visualized by the protein-A-indigo blue colour developer producing a blue colour signal on the membrane. After reacting this protein A-indigo colour developer directly against the different human immunoglobulin classes blotted onto nitrocellulose, purified IgM, IgA and IgG showed a blue spot while IgD and IgE did not show any spot (Fig. 4). Thus, protein-A-indigo in the INSTI colour developer binds to human IgM, IgG, and IgA antibodies but not to IgD or IgE antibodies.

5.3. The ability of INSTI to detect anti-HIV-1 IgM

The results for the IgM-extracted samples before and after processing with the anti-IgM MicroBeads followed by magnetic separation are presented in Table 2.

Of the 21 HIV-1 positive, WB negative or indeterminate commercial seroconversion samples from which IgM was extracted, all showed a significant reduction of INSTI spot intensity when tested following IgM extraction compared to test spot intensity before extraction. Of note, 10/21 samples became INSTI negative after IgM extraction, indicating that the antibody response to HIV in these early seroconversion samples was likely entirely due to anti-HIV-1 IgM antibody. Interestingly four of the seroconversion samples tested in this study, 924-06, 943-06, and 927-03 and 928-02 provided datasheet information consistent with the Fiebig class III staging description. Three out of four samples (ie. 924-06, 943-06, and 928-02) showed dot disappearance, and the fourth sample (i.e., 927-03) showed more the 50% spot intensity reduction following IgM removal, indicating that INSTI is capable of being reactive during class III staging of early HIV seroconversion where Western Blot is negative. Two additional Western Blot negative Seroconversion samples (922-02, and 922-03) that did not undergo IgM removal also showed INSTI positive results.

The 5 HIV antibody positive samples from late seroconversion, which contain only HIV IgG antibodies, showed no difference in INSTI test spot intensity before and after IgM extraction. Additionally, to demonstrate the ability of the MACS kit to remove only IgM, the IgM extraction process was applied to a purified human IgM (Sigma) preparation as well as an HIV-IgG antibody positive serum
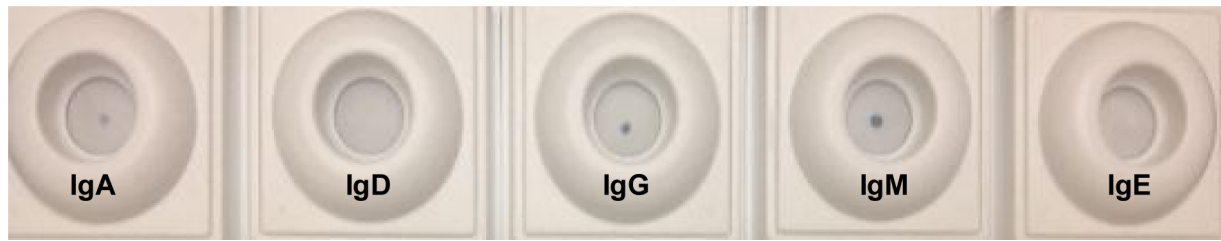
Fig. 4. The affinity of INSTI solution 2 (solution 2-ProA) to bind to human immunoglobulin classes.
INSTI protein-A/indigo solution produces visible reactions to human IgA, IgG, IgM, but not to IgD, IgE.

Table 2
Summary of INSTI test results on Commercial HIV-1 Seroconversion Samples before and after IgM removal. A 100% reduction in INSTI intensity is interpreted as non-reactive, ie no visible test spot. 21 INSTI positive samples that were WB (IgG) indeterminate or negative showed reduction or disappearance of dot intensity; 5 samples that were WB (IgG) positive did not show any reduction in spot intensity after IgM removal. P: positive; N: negative; IND: indeterminant; ND: in-house IgM assay not determined; *: meets Fiebig class III staging definition.

Sample #	Western Blot (IgG) for INSTI positive samples	In house-IgM	Test dot disappeared after IgM removal in%
914-01	IND	ND	100%
914-02	IND	ND	>%50
914-03	IND	ND	100%
924-06*	N	P	100%
924-07	IND	P	>%50
924-08	IND	P	>%50
925-05	IND	P	100%
925-06	IND	P	>%50
927-03*	N	P	>%50
927-04	IND	P	100%
928-02*	N	P	100%
934-02	IND	ND	>%50
934-03	IND	P	>%50
938-03	IND	P	100%
940-03	IND	P	100%
940-04	IND	P	>%50
940-05	IND	ND	<%50
941-04	IND	P	>50%
943-06*	N	P	100%
944-05	IND	P	100%
950-04	N	ND	>%50
914-04	P	ND	0%
914-05	P	ND	0%
904-04	P	ND	0%
904-05	P	ND	0%
919-03	P	ND	0%

sample with no known HIV IgM as a control. The results are shown in Fig. 5.

When the INSTI solutions were applied to 50 μl of purified human IgM added to a protein

A-dot blotted nitrocellulose membrane before and after IgM extraction, a significant reduction in protein-A spot intensity was observed. Conversely, no reduction of protein-A spot or HIV-1 gp41 antigen spot intensities were observed following INSTI testing of the IgM-extracted HIV-IgG positive sample (Fig. 5B).

An additional example where INSTI reactive results are determined by the presence of anti-HIV-1 IgM antibodies is presented in Fig. 6. In this example, INSTI was reactive for a commercial seroconversion sample (PRB 928-2) that contained measureable levels of anti-HIV-1 IgM but both HIV-IgA and Western Blot (IgG) negative were negative according to sample datasheet provided by SeraCare Life Sciences. Upon MACS extraction of IgM, this sample became INSTI non-reactive.
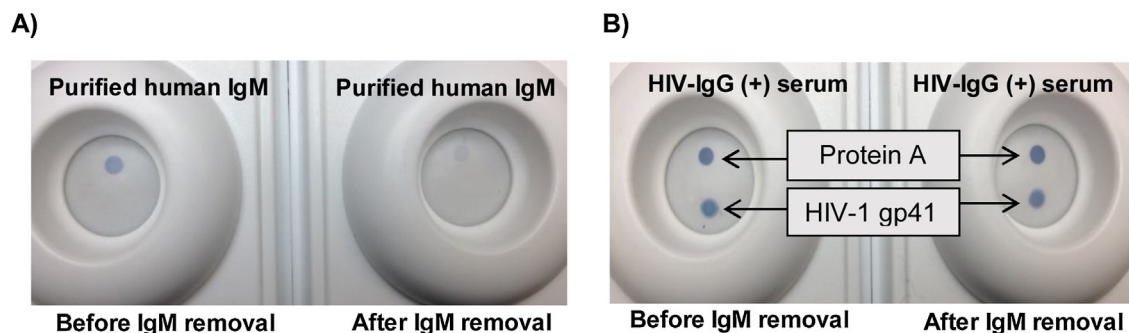

Fig. 5. Images of INSTI results before and after the IgM removal process for purified human IgM (5A) and HIV-IgG positive serum (5B).

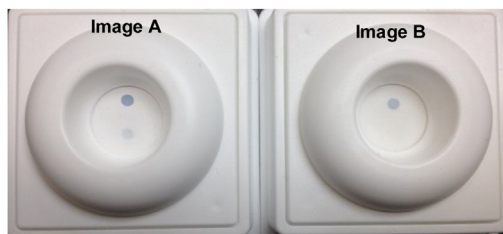| Panel Members PRB928	1	2	3	4	5
Day since first bleed	0	111	120	125	130
INSTI HIV1/HIV2	N	P	P	P	P
In-House IgM (S/CO)	N-0.5	P-5.9	P-9.1	P-9.1	P-9.1
In-House IgA (S/CO)	N-0.6	N-0.9	P-3.5	P-3.4	P-3.9
Western blot (IgG, S/CO)	N	N	P	P	P

Fig. 6. The ability of INSTI to detect anti-HIV IgM antibody.
INSTI results for a commercial seroconversion sample PRB928-2 with measureable anti-HIV IgM, and negative western blot before (Image A) and after (Image B) MACS extraction of IgM. N: Negative, P: Positive, S/CO: signal/cutoff.

6. Discussion

Studies have shown that newer-generation assays, including third-generation assays, can detect HIV antibody in as little as 3–4 weeks after infection [13]. Combination HIV antibody/p24 Antigen tests, commonly referred to as fourth-generation tests, have become the gold standard for laboratory-based testing algorithms due to their ability to capture p24Ag in the pre-antibody window period of infection [14]. However, performance of fourth generation assays for HIV antigen detection in RPOCT formats has been inconsistent [15–18]. Most approved rapid HIV antibody tests currently on the market are highly sensitive for detecting HIV infection, but they vary in their ability to detect low levels of antibody, such as those occurring before complete seroconversion [19]. During this early seroconversion period, IgM antibodies can reach detectable levels within the first three weeks following infection [11]; however, these HIV-specific IgM antibodies are not produced consistently during early infection [11,20]. Nevertheless, the ability of some HIV assays to detect IgM antibody simultaneously or in advance of immunoglobulin G (IgG) may be responsible for their higher analytical sensitivity. One such assay, INSTI, has repeatedly shown sensitivity to HIV-1 antibody detection in samples collected during early seroconversion [2,7].

Our results have shown that INSTI's design format enables it to capture anti-HIV-1 IgM antibodies consistently when present in early seroconversion samples. The results from this study as shown in Fig. 2 and Fig. 3 clearly demonstrated the ability of INSTI's recombinant HIV-1 gp41 antigen to bind anti-HIV-1 IgM in characterized commercial early seroconversion samples. These findings are consistent with a determination by Tomaras et al. that the first free plasma anti-HIV-1 antibody was to gp41, which appeared 13 days after the appearance of plasma virus [21]. Studies have shown that Protein A has strong affinity for IgG and variable affinity for IgM and IgA with no affinity for IgD and IgE [23]. Coupled with the IgM-affinity of the protein-A based INSTI Colour Developer, as shown in Fig. 4, INSTI is highly optimized for anti-HIV-1 IgM detection. In addition, the relatively large sample size of 50 μl may compensate for the lower affinity for IgM compared to IgG.

This ability of INSTI to detect anti-HIV-1 IgM was conclusively established when INSTI was conducted on early seroconversion samples before and after IgM extraction. As shown in Table 2, all INSTI initially reactive results for these seroconversion samples became non-reactive or showed significant loss of test spot intensity when re-tested after IgM extraction. In contrast, late seroconversion and long term anti-HIV positive samples showed no loss of INSTI test spot intensity following IgM extraction. Therefore, we

conclude that the HIV-1 antibody detectable by INSTI in very early seroconversion is primarily IgM. In total, 39 of the 41 INSTI reactive commercial early seroconversion samples tested in this study contained detectable anti-HIV-1 IgM antibodies according to the sample panel datasheets. Of these, six samples (922-02, 922-03, 924-06, 943-06, 927-03, and 928-02) were consistent with Feibig Class-III HIV-1 staging of primary infection.

Although a specific anti-HIV-1 IgM response is variable across individuals with early HIV infection [11,20], and generally does not persist beyond 3 months following its development in vivo [22], the clinical application of a RPOCT assay such as the INSTI that detects anti-HIV-1 IgM in addition to anti-HIV-1 IgG allows for reliable early diagnosis of HIV infection.

Competing interests

None declared.

Ethical approval

Not required.

Authors contribution

Noushin Moshgabadi: The conception and design of the study, and acquisition of data and analysis and interpretation of data. Drafting the article and revising it critically for important intellectual content. Final approve of the version to be submitted.

Rick A. Galli: The conception and design of the study, and analysis and interpretation of data. Drafting the article and revising it critically for important intellectual content. Final approve of the version to be submitted.

Amelia C. Daly: Drafting the article and revising it critically for important intellectual content. Final approve of the version to be submitted.

Sze Mun Shirley Ko: The study, and analysis and interpretation of data.

Tayla E. Westgard: The study, and analysis and interpretation of data.

Ashley F. Bulpitt: The study, and analysis and interpretation of data.

Christopher R. Shackleton: The study, and analysis and interpretation of data.

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