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A simple and high-throughput luciferase immunosorbent assay for both qualitative and semi-quantitative detection of anti-HIV-1 antibodies

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ABSTRACT

In this study, we described an ultrasensitive and high-throughput luciferase immunosorbent assay (LISA) for qualitative and quantitative detection of anti-HIV-1 antibody. Anti-HIV antibody in serum or plasma samples was captured by protein A/G-coated microtiter plate and detected with crude cell lysates expressing Nanoluc luciferase (Nluc) enzyme fused with HIV-1 p24 or gp41 antigen without the need of protein purification. After the addition of furimazine substrate, anti-HIV antibodies were quantitatively measured as luciferase light units. LISA showed a wide linear range of detection and was about 10^4-fold more sensitive than ELISA. For the detection of both anti-p24 and anti-gp41, LISA showed extraordinary sensitivity (99.5% and 100%, respectively) and equivalent specificity (100%). LISA could also monitor the change in the anti-HIV-1 antibody response over time in antiretroviral therapy (ART) treated individuals, and can sufficiently distinguish between recent and long-term HIV-1 infections. Our preliminary results indicate that LISA may provide a novel universal immunoassay platform for simultaneous HIV-1 detection, quantitative measurement of anti-HIV antibodies as well as the differentiation of HIV-1 infection stages.

1. Introduction

So far, enzyme-linked immunosorbent assay (ELISA) is still the most convenient and routine antibody detection method (Aydin, 2015; Engvall, 2010). However, there are several factors limiting its application. Antigens applied in the ELISA are usually recombinant proteins expressed in prokaryotic (*Escherichia coli*) or eukaryotic expression system (yeast, mammalian cells or insect cells) (Mohajeri et al., 2017). Purification of recombinant antigens is always time-consuming and laborious, which in turn affect the rapid development of antibody detection methods for emerging infectious pathogens. In addition, *E. coli* expression systems usually produce inactive or insoluble proteins and lack post-translational modifications (Baeshen et al., 2015). The amount of antigens expressed in eukaryotic systems is usually low and thus more laborious post-expression modification and enrichment are needed (Wurm and Bernard, 1999). Furthermore, ELISA detection relies on optical density produced by horseradish peroxidase (HRP) enzyme and the corresponding colorigenic substrate, detection is mainly qualitative and detection range is narrow (Burbelo et al., 2011b). In addition, species-specific secondary antibodies are needed but difficult to obtain in particular for some animals, which affect its application in detection of animal-related emerging and reemerging infectious diseases.

Burbelo et al. have reported luciferase immunoprecipitation system (LIPS) for the quantitative measurement of antibody responses (Burbelo et al., 2011a, 2015). In this assay, crude Renilla luciferase (Ruc)-fused antigen extract generated from the mammalian cells, diluted serum samples and protein A/G beads were incubated together, and specific antibody responses were then quantitatively determined by the addition of the substrate of Ruc after multiple washing steps to remove the unbound Ruc-fusion antigen. By using the light-producing enzyme, a wide range of antibody detection can be achieved, thereby eliminating the time-consuming purification of antigens. However, there are a number of shortcomings of the high-throughput antibody detection assay. Firstly, pre-incubated Ruc-antigen and serum mixture need to be transferred to a special 96-well filter plate containing the protein A/G beads for antibody capture. Secondly, a plate washer with vacuum is also required for capturing the antigen-antibody-beads complex (Burbelo et al., 2009).

In the current study, we developed a luciferase immunosorbent assay (LISA) by generating fusion proteins of antigens with a more sensitive luminescent reporter Nanoluc luciferase (Nluc). This assay was developed by coating protein A/G on microtiter 96-well plate for capturing total IgG antibody in serum or plasma samples. The crude cell extracts expressing Nluc-antigen fusion protein were added and the specific antibody was then measured quantitatively by the addition of

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the corresponding substrate. For a proof-of-concept study, we chose detection of antibody against p24 and gp41 proteins of human immunodeficiency virus type one (HIV-1) using LISA system. Our preliminary results indicated that LISA could be a universal platform for detecting HIV-1 infection, discriminating recent and long-term HIV-1 infection and monitoring antibody response related to antiretroviral therapy (ART).

2. Materials and methods

2.1. Patient samples

A total of 191 de-linked cross-sectional serum or plasma samples were collected from HIV-1 infected individuals including 65 people who inject drugs (PWIDs) and 126 men who have sex with men (MSMs) in Guangzhou, China. Serum samples from 76 healthy blood donors who are negative for HIV-1 were also included in this study as negative controls. A total of 156 serum or plasma specimens collected from 31 recent HIV-1-infected individuals and 125 chronic HIV-1-infected individuals in Guangzhou, China were included. Another 40 longitudinal serum samples from 10 HIV-1 infected treatment naïve MSMs and 10 serial serum samples received ART from 2 HIV-1-infected MSMs were used in this study. Serum or plasma samples from 100 hepatitis C virus (HCV) - and 100 hepatitis B virus (HBV)-infected patients were included to test cross-reactivity. All the samples were stored at −80 °C immediately after collection. The study has been approved by Ethics Committees of Guangzhou Eighth People's Hospital and Southern Medical University and was performed according to the approved research protocols.

2.2. Generation of NanoLuc-antigen fusion constructs

A mammalian NanoLuc (Nluc) luciferase expression vector, pNLF1-N (Promega, USA) was used to generate fusion plasmid (Fig. 1A). HIV-1 p24, gp41 and gp41-p1 fragments were amplified from the plasmid pNL4-3 by PCR using the following primers: 5′-CGGAATTCCCTATAG TGCAGAACATCCAG-3′ and 5′-GCTCTAGATTACAAAACTCTTGCCTTA TGGC-3′ for HIV-1 p24; 5′-CGGAATTCATGGGCGCAGCGTCAATGAC-3′ and 5′- GCT CTAGATTATCCCCTCGGGATTGGGA-3′ for gp41; 5′-CGG AATTCGAGGCGCA GCAGCATCTGTT-3′ and 5′-GCTCTAGATTAATTAC TCCAAGAGGAGT-3′ for gp41-p1, respectively. The gene sequence of gp41-MP4 which was consisted of four gp41-p1 fragments derived from predominant HIV-1 genotypes (CRF01_AE, CRF07_BC/CRF08_BC and subtype B) in China and subtype D was synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The PCR products or the synthesized gene sequence were both double digested with EcoRI and XbaI restriction enzymes (Takara Biotechnology Co., Ltd, China) and then subcloned into the EcoRI-XbaI-cut pNLF1-N vector downstream of Nluc luciferase gene. DNA Sequencing was used to confirm the integrity of the Nluc-antigen fusion constructs. The resulting plasmid DNA was prepared by using DNA purification kit (TIANGEN Biotech, China) and used for cell transfection.

2.3. Preparation of cell lysates with nanoluc-antigen fusion proteins

Hela cells seeded in 100-mm^2 dishes were transfected with 5 μg plasmid DNA by using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturers' protocols. Forty-eight hours after transfection, cells were washed twice with cold 0.01 M phosphate-buffered saline (PBS) and treated with trypsin. The cells were then lysed on ice for 30 min with lysis buffer composed of 50 mM Tris, pH7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50% glycerol and protease inhibitors (Roche, Germany). Supernatants were collected after centrifugation at 12,000 rpm for 4 min and stored at -80℃. Luciferase activity of crude extracts was measured by adding an equal volume of Nano-Glo Luciferase assay reagent (Promega, USA) in a Tecan infinite M200 PRO microplate luminometer.

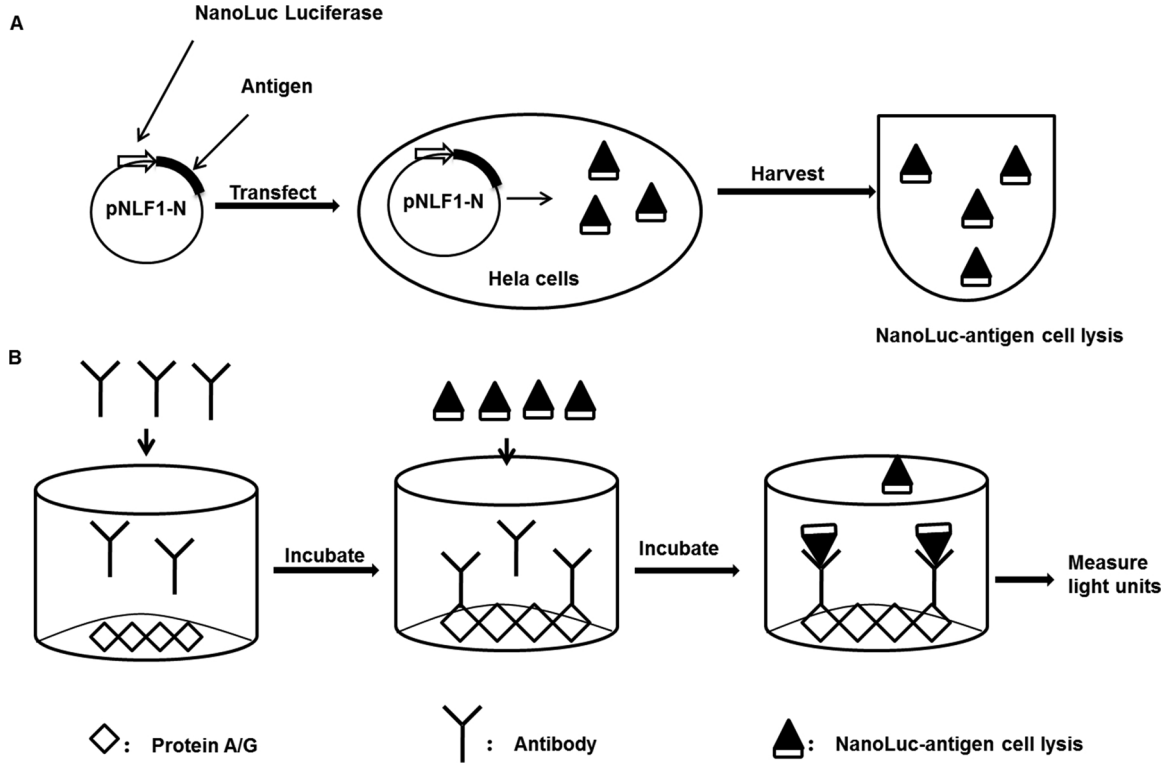

Fig. 1. Schematic of the steps involved in LISA. (A)Nano-luc-antigen recombinant plasmids were constructed and transfected to the Hela cells for the generation of luciferase-antigen crude cell extracts. (B) Protein A/G coated on the plate was incubated with serum or plasma samples and diluted luciferase-antigens were then added for specific antibody capture. Antibody titers were then determined by the addition of the Nano-luc luciferase substrate.

2.4. LISA analysis

As shown in Fig. 1B, white microtitre plates (Corning, USA) were coated with 5 μg/ml of protein G (Genscript Co., China) in 0.01 M PBS and incubated overnight at 4 °C. The plates were then washed five times with 0.01 M PBS containing 0.05% Tween 20 (PBS-T) and blocked with 5% non-fat dry milk (NFDM) for 1 h at 37 °C followed by incubation with serum samples (1:100 dilution in 2% NFDM) for 1 h at 37 °C. Nanoluc-antigens crude cell extract with 10^5 luciferase light units (LU) were added in 50 μL of 2% NFDM and incubated at 37 °C for 30 min. After washing with PBS-T, 50 μL of the Nano-Glo Luciferase assay reagent was added to each well to determine the LU according to the manufacturer's protocol. The cut-off value was derived from the average value plus 3 standard deviations (SD) of the negative controls. The cut-off value of MP4-based LISA for discrimination of recent and long-term HIV-1 infection was determined by receiver operating characteristic curve (ROC curve).

2.5. ELISA analysis

The in-house ELISA for the detection of anti-HIV p24, gp41 and gp41-p1 has been described previously (Li et al., 2016).

2.6. Data analysis

The data were analyzed with SPSS 20.0 statistical software package (SPSS Inc., Chicago, USA) and GraphPad Prism 5.0 (GraphPad Software, California, USA). All the LU data presented in this study were obtained from the average of triplicate tests. Antibody levels were reported as the geometric mean titer (GMT) ± 95% confidence intervals (CI). Group comparison was done by using the Mann-Whitney U test. The kappa coefficient was used to represent the correlation between LISA and ELISA.

3. Results

3.1. LISA is more sensitive than ELISA

To illustrate the ultrasensitivity of LISA, we made a serial dilution of a commercially available monoclonal anti-p24 antibody (C65690) to assess the detection ability of both LISA and ELISA. A low limit of detection (LOD) of 10 pg/mL and 100 ng/mL was reached for LISA and ELISA, respectively (Fig. 2). Thus, LISA was about 10^4-fold more sensitive than ELISA.

Furthermore, LISA showed a wide linear range of detection from 10^2 to 10^7 LU/reaction or from 10 pg/ml to 100 ng/ml when detecting anti-p24 antibody (Fig. 2). A wide dynamic range of detection from 5×10^2 to 7×10^6 LU was also observed when detecting HIV-1-infected clinical samples (Fig. 3A and B). Among 191 HIV-1 positive and 76 HIV-1 negative sera, the GMT of anti-p24 antibodies was 224,578 LU (95% CI, 158765–317673) and 112 LU (95% CI, 96–130), respectively (Fig. 3A), indicating 2000 times difference in the GMT between HIV-1 positive and negative samples. In contrast, the corresponding value for anti-p24 ELISA was only 10-fold difference (Fig. 3C). The sensitivity and specificity of LISA were further determined as 99.5% and 100%, respectively. A strong correlation between LISA and ELISA was obtained (kappa = 0.963) when testing both HIV-1 positive and negative clinical samples. Similar results were observed for the detection of anti-gp41 (Fig. 3B). The calculated sensitivity and specificity for gp41 LISA were both 100% and the kappa coefficient between LISA and ELISA was 0.991. No cross-reactivity was observed when testing serum samples from 100 HCV- and 100 HBV-infected patients (Supplemental Fig. 1).

3.2. HIV-1 seroconversion determined by LISA

A total of 40 longitudinal samples from 10 untreated HIV-1-infected patients were used to determine the time of HIV-1 seroconversion by LISA and ELISA (Fig. 4). When using the full length of HIV-1 p24 or gp41 protein as coating antigen, all the samples tested showed anti-HIV positive 50 days post-infection and the titers of anti-HIV gradually increased over time by both LISA and ELISA (Fig. 4A, B, C and D). However, when a 57-mer peptide gp41-p1 was used to measure the anti-HIV antibody, different seroconversion time was observed: 67(50, 77) days post-infection for LISA and 116(84, 206) days for ELISA. Of the 10 individuals tested, 4 subjects were seronegative for anti-gp41-p1 until 120 days post-infection by ELISA (Fig. 4F) whereas all the 10 patients were anti-gp41-p1 positive by LISA at day 50 post-infection (Fig. 4E). These results indicated earlier detection of anti-HIV and shorter window period obtained by LISA than ELISA.

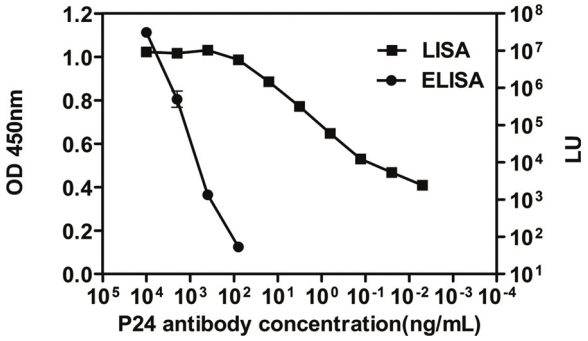

Fig. 2. Validation of LISA and ELISA with HIV-1 p24 commercial antibody. In the LISA assay, Protein G was coated onto the plate and then incubated with different concentration of p24 commercial antibody and diluted luciferase-p24 lysates were added for antibody quantitation. In the ELISA assay, recombinant p24 protein was coated on plates and then incubated with different concentration of p24 antibody. HRP conjugated goat anti-human IgG secondary antibody was added for measurement of the optical density. All the data presented in the figures were obtained from the average of triplicate tests and error bars indicated the s.d. of the triplicate results. LU, Light Units; OD450 nm, optical density at 450 nm.

3.3. LISA can distinguish HIV-1 recent and long-term infection

We previously reported that gp41-p1 located in the loop region of HIV-1 CRF01_AE gp41 could distinguish recent and long-term HIV-1 infection (Li et al., 2016). Here, the LISA method was adapted using gp41-MP4 fusion protein to detect 156 samples previously classified as recent or long-term HIV-1 infection by a commercially available LAg-Avidity EIA (Maxim Biomedical, Inc., USA). Significant statistical difference of anti-HIV titers was found between the samples from HIV-1 recent infection (GMT, 12,878 LU; 95%CI, 8568 LU-19354 LU) and chronic infection (GMT, 77,181 LU; 95%CI, 70,219 LU-84834 LU) ($P < 0.001$) (Fig. 5). Compared to LAg-Avidity EIA, the sensitivity and specificity of LISA were 96.8% and 87.1%, respectively with a high correlation (kappa = 0.839), indicating that gp41-MP4-based LISA can distinguish between recent and chronic HIV-1 infection with a wide detection range.

3.4. LISA can monitor the change of anti-HIV titers over time after ART

It has been well known that ART can inhibit anti-HIV response in particular during acute HIV-1 infection (Stefic et al., 2018). In our study, anti-HIV-1 p24 and gp41 antibody were measured by both LISA and ELISA in serum samples collected from 10 HIV-1-infected patients at pretreatment, or 3, 6, 12 and 24 months post-ART. The results from two representative subjects were presented (Fig. 6). As expected, the levels of anti-p24 and gp41 antibody declined over time after ART (Fig. 6), and paralleled with the decrease of HIV-1 RNA (data not shown). However, due to the differences in the detection sensitivity and range between LISA and ELISA (Fig. 2), both anti-p24 and gp41

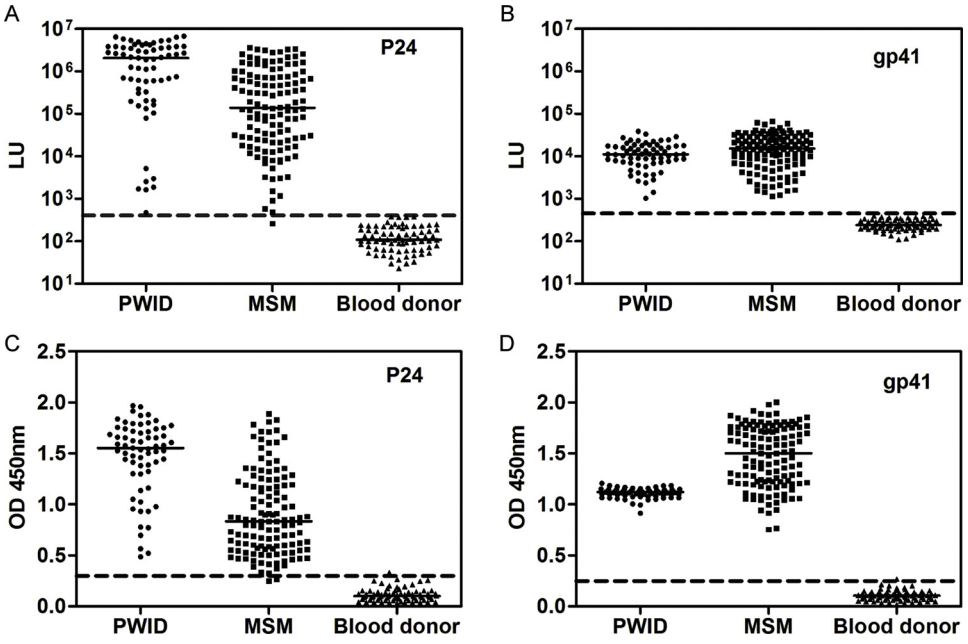

Fig. 3. Distribution of antibody levels to p24 and gp41 as determined by LISA and ELISA. Serum or plasma samples from 65 PWIDs, 126 MSMs and 76 blood donors were included. (A, B) shows the antibody levels against p24 and gp41 detected by LISA respectively. A significant difference was found between the HIV-1 infected individuals and negative control subjects ($P < 0.0001$, Mann-Whitney U test). (C, D) shows the antibody responses against p24 and gp41 as determined by ELISA. All the data presented in the figures were obtained from the average of triplicate tests. The coefficient of the correlation for LISA-p24 and LISA-gp41 detection from two independent experiments was 0.991 and 1.000, respectively. PWID, persons who inject drugs; MSM, men who have sex with men; LU, Light Units; OD450 nm, optical density at 450 nm. The horizontal dashed lines show cut-off values.

antibody remained positive by LISA at a serum dilution of 10^5, while ELISA could only detect anti-HIV antibody at a dilution of 10^3 after two years' treatment. These results further indicated that LISA was not only more sensitive than ELISA, but also more suitable for monitoring ART-related anti-HIV response.

4. Discussion

In the current study, we evaluated and provided data to highlight the potential use of our newly established LISA as a high-throughput, ultrasensitive immunoassay for both qualitative and semi-quantitative detection of anti-HIV antibodies. This assay can reliably distinguish HIV-1 infected patients from uninfected healthy subjects and reflect antibody changes over time in serial samples post-ART and distinguish recent and long-term HIV-1 infection.

Recently, Burbelo et al. (Burbelo et al., 2015) reported a luciferase immunoprecipitation system (LIPS) assay and demonstrated improved sensitivity and specificity for measuring antibodies in autoimmune and infectious diseases. However, LIPS needs pre-incubation of Ruc-antigen and samples followed by the transfer of the mixture to a special 96-well filter plate containing the protein A/G beads. A plate washer with vacuum is also needed for capturing the antigen-antibody-beads complex (Burbelo et al., 2009). In the current study, we dramatically simplified the assay system by coating the 96-well microtiter plate with protein A/G to capture the total IgG antibody from serum or plasma samples, followed by the addition of luciferase-antigen fusion proteins and luciferase specific reagent. Thus, LISA and ELISA are operated in a similar manner. Furthermore, in the LISA system, we adopted nanoluc luciferase (Nluc) to replace the renilla luciferase used in LIPS. Nluc has been reported to be brighter than either firefly or renilla reniformis luciferase (England et al., 2016; Loh and Proft, 2014). In addition, relative small Nluc enzyme (171 amino acids) makes it an excellent reporter protein for constructing fusion proteins (Hall et al., 2012).

LISA has several advantages over classical ELISA technology. 1) LISA is a more convenient and straightforward technology that uses crude cell extracts expressing luciferase-antigen fusion proteins directly. Unlike ELISA, the need for use of purified protein samples is eliminated. Therefore, LISA is quite suitable for the rapid development of immunoassays for detection and diagnosis of emerging infectious diseases. 2) LISA measures luciferase light units rather than optical density, therefore, it displays an extremely wide dynamic range of detection and is capable of semi-quantitation without the need for serial dilution of test samples. Our results indicated higher sensitivity and shorter window period of LISA than ELISA for detection of anti-HIV antibody. 3) The specificity of LISA relies on the specific binding of antibody and antigen without the need of species-specific secondary antibody. Therefore, LISA can detect antibody reaction from a wide range of species. Except for the detection of anti-HIV antibody by LISA, we have tried to detect antibody against zika virus and MERS virus in the samples collected from human, mice, monkey and camel with the same system (Wang J, et al., manuscript in preparation). Our results indicate that LISA is a very convenient immunoassay and can be used for the detection of and monitoring of the epidemic of emerging infectious diseases in all kinds of animals.

As we know, HIV-1 gp41 is the most important antigen for developing HIV-1 diagnosing assays. The immunodominant epitopes of HIV-1 gp41 are also capable of differentiating recent and long-term HIV-1 infections (Li et al., 2016). Our study further indicates that by using a more sensitive platform such as LISA and the HIV-1 gp41 peptides, it is possible to develop an assay for the diagnosis of HIV-1 infection and determination of recent HIV-1 infection. It has been well known that anti-HIV antibody response increases overtime during seroconversion and decreases post-ART (Fournier et al., 2002; Keating et al., 2017; Killian et al., 2006; Voltersvik et al., 2003). Consistent with previous reports obtained by ELISA, changes in anti-HIV response were readily detected by LISA. Our preliminary results indicate that LISA could provide a suitable platform for quantitative measurement and monitoring of antibody response with respect to antiretroviral therapy. Further evaluation is ongoing to determine whether ART-related anti-HIV antibody inhibition could be used to monitor the response to ART since the parallel inhibition of both anti-HIV antibody and HIV-1 RNA has been observed in our study.

Regarding the improved detection sensitivity of HIV-1 diagnosis, LISA is quite different from the current 4th generation of HIV-1 detect assays, which can detect both HIV-1 p24 antigen and anti-HIV antibodies (De Paschale et al., 2018). The addition of p24 antigen detection significantly shortens window period of acute HIV-1 diagnosis and has been proven to be more effective in identifying new cases than the 3rd generation antibody tests (Stafylis and Klausner, 2017). However, LISA decreases the low limit of detection of anti-HIV antibody, which in turn increases the detection sensitivity of anti-HIV antibody.

In conclusion, we developed a LISA assay with well-characterized

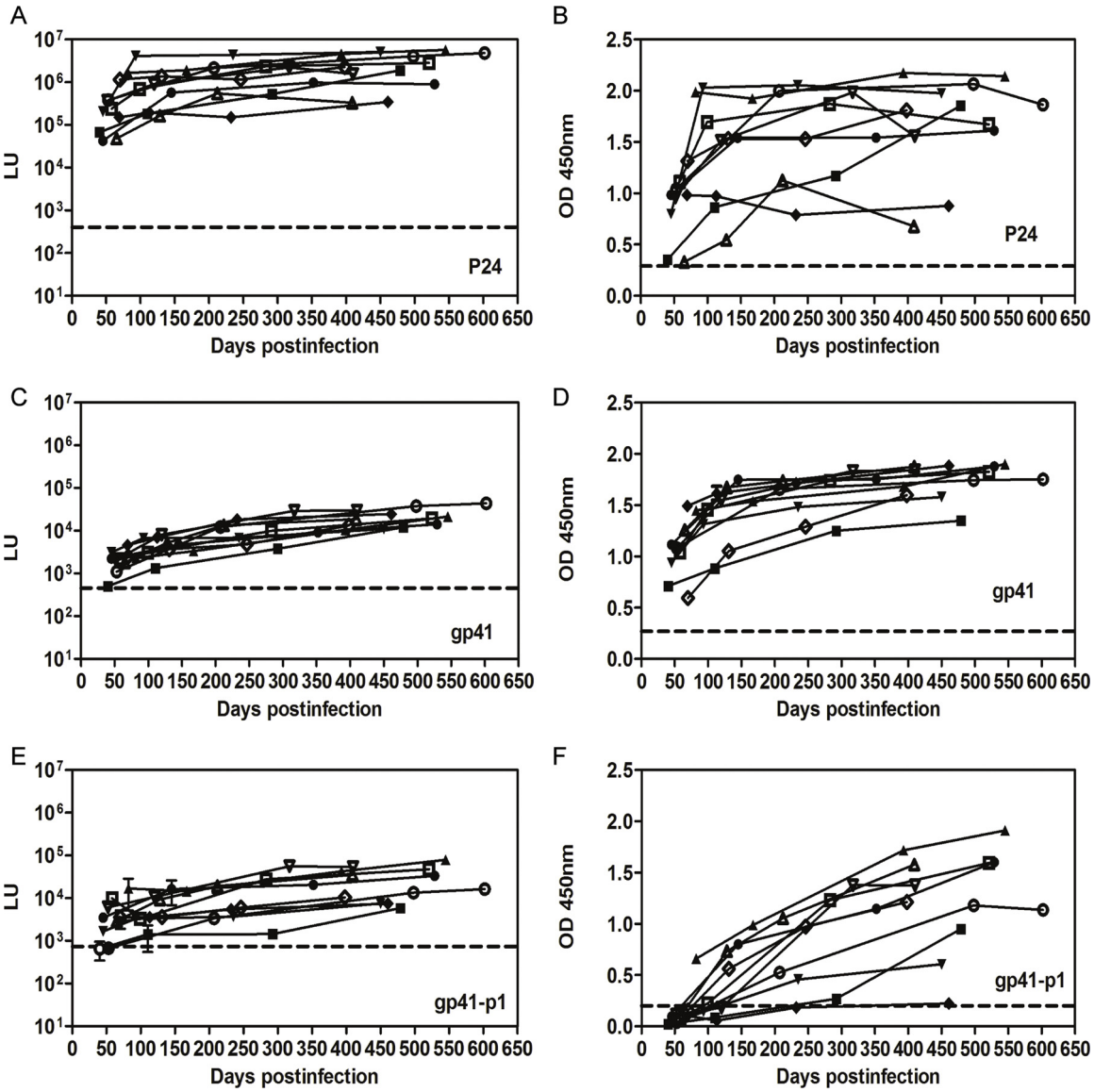

Fig. 4. Antibody titers against p24 (A, B), gp41(C, D) and gp41-p1 (E, F) in 40 longitudinal serum or plasma samples from 10 HIV-1 infected patients at different time points, as determined by LISA(A,C,E) and ELISA(B,D,F). LU, Light Units; OD450 nm, optical density at 450 nm. The horizontal dashed lines show cut-off values. All the data presented in the figures were obtained from the average of triplicate tests and error bars indicated the s.d. of the triplicate results.

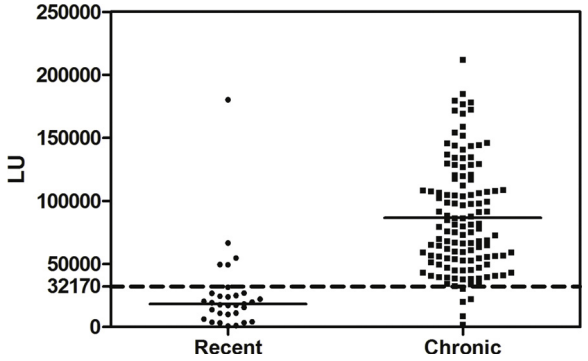

Fig. 5. LISA detection of antibodies against gp41-MP4 in 156 recent and chronic HIV-1 infected samples. The horizontal dashed line shows the cut-off values of "recent/chronic" infection. A short line in each plot indicates the median of LU value. All the data presented in the figures were obtained from the average of triplicate tests. The coefficient of the correlation for recent and chronic infection detection from two independent experiments was 0.888. LU, Light Units.

HIV-1 positive samples and provided data to demonstrate its application in detecting HIV-1 infection, discriminating recent and long-term HIV-1 infection and monitoring antibody responses. This assay is highly sensitive, easy to use and does not need special equipment. Thus, it has the potential in monitoring immune responses to different infectious agents.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

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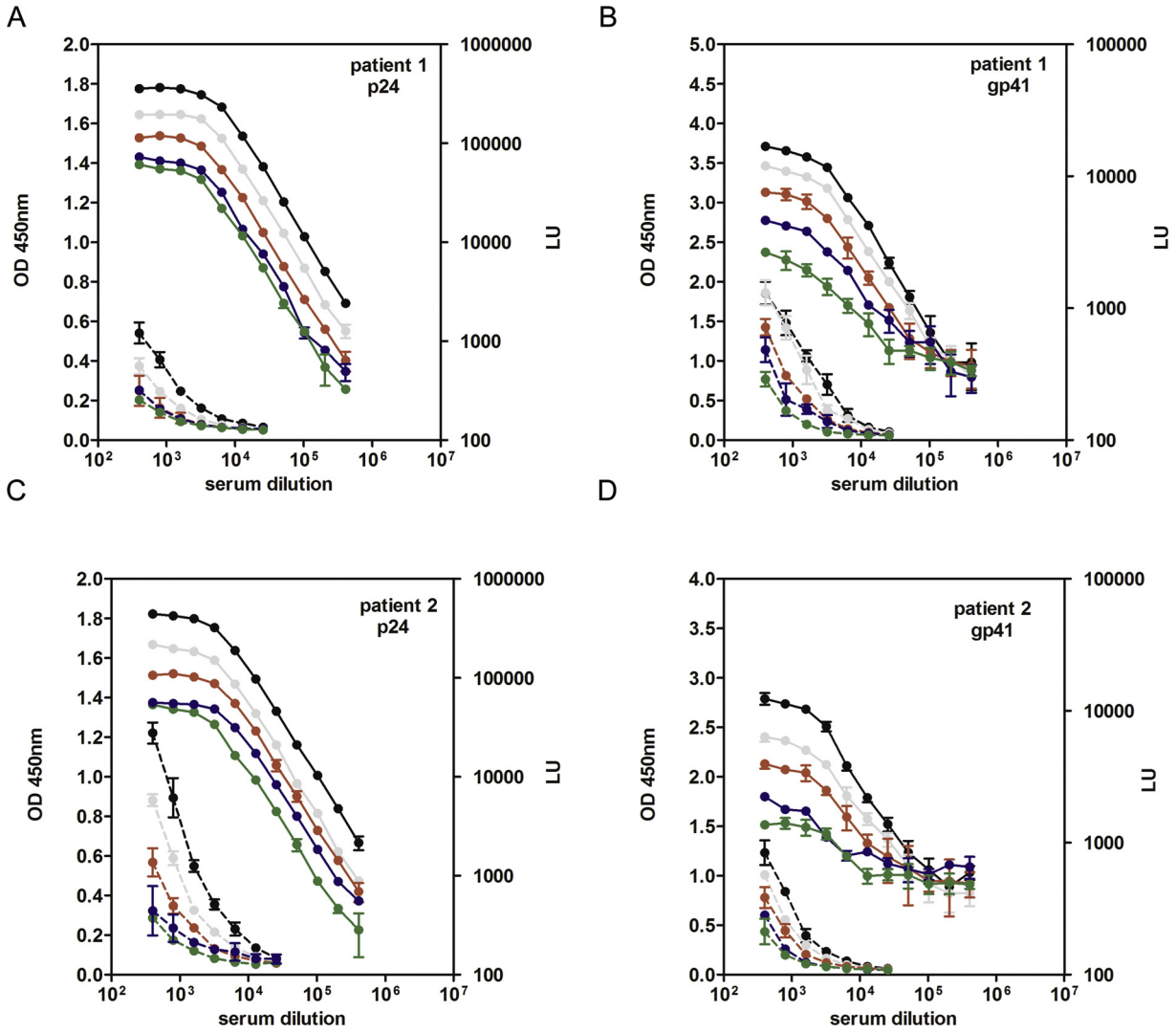

Fig. 6. Antibody level changes against p24(A,C) and gp41(B,D) over time post antiretroviral therapy(ART) in two representative patients measured by ELISA (dash lines) and LISA(solid lines).black, gray, red, blue, green lines represent pretreatment and 3, 6,12,24 months post ART, respectively. LU, Light Units; OD450 nm, optical density at 450 nm. All the data presented in the figures were obtained from the average of triplicate tests and error bars indicated the s.d. of the triplicate results (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.12.017.

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