Table 3
Reproducibility of quantitation of HIV-1 proviral DNA in clinical samples.

Sample[a]	Subtype	Proviral DNA (copies/μg PBMC DNA)	Intra-assay (n=3) SD (CV)	Inter-assay (n=3) SD (CV)	Plasma viral load (copies/ml)	CD4 count (cells/μl)	Antiretroviral treatment (duration in month)
P20	B	<8	ND[b]	ND	<50	728	d4T,ddI,EFV (66)
P21	B	13	2.9 (50%)	1.5 (26%)	<50	587	d4T,3TC,EFV (12)
P22	B	<8	ND	ND	<50	516	d4T,ddI,EFV (31)
P23	CRF01_AE	<8	ND	ND	<50	775	d4T,3TC,EFV (37)
P24	CRF01_AE	15	2.9 (50%)	1.5 (26%)	<50	116	ddI,3TC,EFV (31)
P25	CRF01_AE	<8	ND	ND	<50	450	d4T,3TC,EFV (33)
P26	CRF01_AE	<8	ND	ND	<50	509	AZT,3TC,LPV/RTV (37)
P18	F	<8	ND	ND	<50	120	AZT,3TC,EFV (7)
P27-1	B	55	1.2 (6%)	6.1 (31%)	61,000	200	Untreated
P27-2	B	<8	ND	ND	<50	414	AZT, ddI, EFV (20)
P2	B	34	4.7 (36%)	4.1 (31%)	3,700	389	Untreated
P28	B	18	2.8 (40%)	2.0 (28%)	13,000	715	Untreated
P29	B	60	6.0 (26%)	4.7 (21%)	100,000	417	Untreated
P30	B	<8	ND	ND	3,600	571	Untreated
P31-1	CRF01_AE	77	5.8 (18%)	12.3 (39%)	500,000	184	Untreated
P31-2	CRF01_AE	20	3.7 (50%)	2.7 (37%)	<50	587	ddI,3TC,NFV (21)
P32-1	CRF01_AE	19	1.9 (26%)	2.8 (38%)	200,000	72	Untreated
P32-2	CRF01_AE	12	1.8 (37%)	0.3 (7%)	<50	759	d4T,ddI,EFV (41)
P9-1	CRF01_AE	64	6.8 (28%)	9.0 (37%)	54,000	209	Untreated
P9-2	CRF01_AE	38	4.3 (30%)	3.9 (27%)	<50	300	AZT,3TC,EFV (22)
P33	CRF01_AE	11	1.6 (42%)	1.1 (29%)	21,000	712	Untreated
P17-1[c]	F	<8	ND	ND	2,800	294	Untreated
P17-2[c]	F	67	8.2 (36%)	6.8 (30%)	150,000	167	Untreated
P17-3[c]	F	494	20.0 (13%)	30.0 (20%)	690,000	233	Untreated

[a] The number before the hyphen denotes a patient code, and the number after the hyphen denotes a sample code.
[b] Standard deviation could not be calculated because measurements were below the lower limit of quantitation.
[c] The samples P17-1, P17-2, and P17-3 were collected 15 days, 8 months, and 22 months, respectively, after termination of HAART for prevention of mother-to-child transmission.

and 7–39% for inter-assay. These results indicate that the present assay is performed well in testing of clinical samples.

4. Discussion

Several real-time PCR protocols for proviral HIV-1 DNA quantitation have been described (Désiré et al., 2001; Kabamba-Mukadi et al., 2005; Saha et al., 2001; Yun et al., 2002; Zhao et al., 2002), but the ability of these assays to quantify non-B subtypes has not been studied extensively. Désiré et al. (2001) tested amplification for non-B subtype strains and Kabamba-Mukadi et al. (2005) showed similar amplification efficiencies for non-B subtype reference isolates, but the accuracy of the obtained quantitation values was not evaluated. In this study, a TaqMan real-time PCR assay was developed by using primers and a probe with some degenerate nucleotides for the quantitation of proviral DNA of HIV-1 group M. The introduction of the degenerate primers enabled a considerable improvement in the quantitation of group M subtypes: (i) it enabled DNA amplification for four non-B subtype isolates that had not been amplified with the non-degenerate primers; (ii) the quantitation values agreed with those determined using a Poisson distribution-based method; and (iii) the quantitation values for subtype B isolates were similar when the degenerate primers and the subtype B-based primers were used. Although the number of non-B samples in this study was limited, the present assay may be generally applicable for group M subtypes because the degenerate nucleotides were incorporated in a manner such that the primers and probe matched more than 90% of all the reported variants belonging to group M. To our knowledge, this is the first case where degenerate primers are used for HIV-1 DNA quantitation and the obtained values were evaluated by comparison with a Poisson distribution-based assay.

The reproducibility of the present method (<36% for isolates and <50% for clinical samples) was similar to that of commercially available viral load kits (Coste et al., 1996; Murphy et al., 1999). Good linearity was achieved in the range of 4–5000 copies in 0.5 μg DNA for both subtypes B and CRF01_AE. All of the 40 HIV-1 negative samples were below the lower quantitation limit. The assay could monitor changes in proviral loads using clinical samples before or after antiretroviral therapy. Therefore, it can be concluded that the present TaqMan real-time PCR method has excellent sensitivity, specificity, reproducibility, and accuracy. The assay will be useful for studying the relationship between HIV-1 proviral loads and the long-term efficacy of antiretroviral therapy for subtype B as well as non-B subtype strains.

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Inhibition of human immunodeficiency virus type 1 (HIV-1) nuclear import via Vpr–Importin α interactions as a novel HIV-1 therapy

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ABSTRACT

The development of multidrug-resistant viruses compromises the efficacy of anti-human immunodeficiency virus (HIV) therapy and limits treatment options. Therefore, new targets that can be used to develop novel antiviral agents need to be identified. One such target is the interaction between Vpr, one of the accessory gene products of HIV-1 and Importin α, which is crucial, not only for the nuclear import of Vpr, but also for HIV-1 replication in macrophages. We have identified a potential parent compound, hematoxylin, which suppresses Vpr–Importin α interaction, thereby inhibiting HIV-1 replication in a Vpr-dependent manner. Analysis by real-time PCR demonstrated that hematoxylin specifically inhibited nuclear import step of pre-integration complex. Thus, hematoxylin is a new anti-HIV-1 inhibitor that targets the nuclear import of HIV-1 via the Vpr–Importin α interaction, suggesting that a specific inhibitor of the interaction between viral protein and the cellular factor may provide a new strategy for HIV-1 therapy.

Macrophages are a major target of human immunodeficiency virus type 1 (HIV-1) and serve as a viral reservoir for the release of small amounts of viral particles in symptomatic carriers [1]. HIV-1 in latently infected macrophages in some lymphoreticular tissues cannot be eradicated by highly active anti-retroviral therapy (HAART), and these cells may produce viral particles that can spread throughout the body [2]. A striking feature of HIV-1 is its ability to replicate in non-dividing cells, in particular, macrophages. Replication in non-dividing cells depends on the active nuclear import of the viral pre-integration complex (PIC) [3]. The HIV-1 PIC exhibits biophysical properties typical of a large nucleo–protein complex and contains the viral proteins reverse transcriptase, integrase (IN), nucleocapsid, Vpr, and small amounts of matrix (MA), in addition to the viral nucleic acids [4–7]. Three PIC-associated proteins, MA, IN, and Vpr, have been proposed as karyophilic agents that act probably via their interactions with Importin (Imp) α, Imp β, and/or Imp 7 [8]. In addition, a recent study indicates that transportin-SR2, which shuttles the essential splicing factor, mediates PIC nuclear import, thereby facilitating HIV infection [9]. Moreover, a novel partner, tRNA, has been shown to facilitate PIC nuclear import [10]. However, the molecular mechanisms of PIC nuclear import and its role in viral replication in macrophages are still not completely understood.

Several studies have shown that Vpr is essential for the nuclear import of PIC in macrophages [11,12], while others do not support such observations [13]. However, our studies have clearly shown that Vpr is targeted to the nuclear envelope and then transported into the nucleus by Imp α alone, in an Imp β-independent manner [12,14]. Furthermore, the interaction between Imp α and the N-terminal α-helical domain (αH1) of Vpr, amino acid residues 17–34, is indispensable, not only for nuclear import of Vpr, but also for HIV-1 replication in macrophages [12]. Thus, it appears that Vpr–Imp α binding precedes a novel nuclear import process, which is a potential target for therapeutic intervention in macrophages, which is crucial for subsequent viral spread to lymphoid organs and T-helper lymphocytes [15]. In addition to nuclear transport, Vpr also has other important functions, including the induction of cell cycle arrest at the G_2 phase [16], the regulation of apoptosis [16,17] and splicing [18,19], carried out through interactions with a variety of cellular partners. These observations suggest that drug targeting of Vpr may lead to pleiotropic effects on the HIV life cycle.

As a promising target for blocking HIV-1 replication in macrophages, by screening a large collection of chemical compounds,

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we here discovered several compounds that selectively inhibit the nuclear import of Vpr in an Imp α-dependent manner. Importantly, hematoxylin blocks HIV-1 replication in a Vpr-dependent manner in macrophages by blocking the nuclear import of PIC, but does not block Vpr-induced G_2 cell cycle arrest, the virion incorporation function of Vpr and nuclear import of karyophilins, which possess the classical nuclear localization signal (cNLS).

Materials and methods

Enzyme linked immuno-sorbent assay (ELISA)-based binding assay. The wells of 96-well microplates (NUNC) were coated with an anti-glutathione *S*-transferase (GST)-specific monoclonal antibodies (MAb) (Sigma) in 50 mM $NaHCO_3$ (pH 9.8) for 6 h at 4 °C. After washing the wells, GST or GST-N17C74 (0.5 μg/well) in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) were added to the wells and incubated for 2 h at 4 °C. Imp α-histidine tag_6 (His_6) was added to the wells together with Imp β or test compounds and incubated for 2 h at 4 °C. The horseradish peroxidase (HRP)-conjugated anti-His tag MAb (Sigma) was added. Following incubation at 22 °C for 1 h, the microplates were washed three times and tetramethylbenzidine (TMB) (Pierce) was added. After incubation at 37 °C for 30 min, the amount of surface-bound Imp α was estimated by monitoring the optical density of the wells at 450 nm using an ELISA plate reader (Wallac ARVO™ SX 1420; Perkin-Elmer).

Viral infection assay. Primary macrophages in 24-well plates were inoculated with vesicular stomatitis virus G (VSV-G) pseudotyped reporter virus [NL-Luc-E^-R^+ (VSV-G) or NL-Luc-E^-R^- (VSV-G); 4 ng of p24 antigen], cultured in the absence or presence of hematoxylin for 2 days, harvested, and lysed in luciferase assay substrate (Promega). Luciferase activity was measured using a Wallac ARVO™ SX 1420 luminometer (Perkin-Elmer). Moreover, primary macrophages were exposed to diluted virus stocks (containing 2 or 20 ng of p24 antigen), the JR-CSF strain of the HIV-1 macrophage-tropic virus for 3 h at 37 °C. The cells were then washed three times and seeded in a 24-well tissue-culture plate (NUNC) for primary macrophages. Cells were maintained in RPMI-1640 that contained 10% fetal calf serum. Culture supernatants were harvested at 4-, 8-, 12-, 16-day intervals for primary macrophages and viral production was monitored by sequential quantitation of p24 antigen in cell-free supernatants using an HIV-1 $p24^{gag}$ ELISA kit (LUMIPULSE; Fuji REBIO).

Quantitative real-time PCR. Real-time PCR for quantification of total viral DNA, 2-long terminal repeat (LTR) circular DNA, was performed as follows. Differentiated primary macrophages were infected with VSV-G pseudotyped reporter viruses [NL-Luc-E^-R^+ (VSV-G) or NL-Luc-E^-R^- (VSV-G)] containing 4 ng of p24 antigen and genomic DNA was isolated at 24 h. Total DNA, 2-LTR DNA, and β-globin were quantified with specific primers (2-LTR-forward, ccctcagaccctttagtcagtg; 2-LTR-reverse, tggtgtgtagttctgccaatca; U5gag forward, gtagtgtgtgcccgtctgttg; U5gag reverse, caagccgagtcctgcgt; β-globin primers were those used by Yamamoto et al. [20]).

Other assays. Other assays were described in Supplementary materials.

Results

To identify compounds inhibiting HIV-1 replication, we focused on the nuclear entry of HIV-1 via the Vpr–Imp α interaction as a target for therapeutic strategies and performed ELISA-based binding assays using natural product libraries derived from microbial and fungal metabolites. For these experiments, we used the Vpr N17C74 fragment (containing residues 17–74), because this is a functionally transportable region [12,14]. Among 49 compounds that specifically inhibited the interaction between N17C74 and Imp α, as measured by the ELISA-based binding assay (Fig. 1B), one compound, hematoxylin, inhibited specific binding of Vpr to Imp α in a dose-dependent manner, as assessed by *in vitro* pull-down assays (Fig. 1A and C). Next, we tested the effect of this compound in an *in vitro* nuclear import assay using digitonin-permeabilized HeLa cells. Interestingly, Imp α-mediated nuclear import of Vpr was dose-dependently inhibited by the addition of hematoxylin with a mean 50% inhibitory concentration (IC_{50}) of 5 μM, suggesting that hematoxylin is a potent small-molecule inhibitor of Vpr nuclear entry. Furthermore, we demonstrated the effect of hematoxylin on Vpr-mediated G_2 phase cell cycle arrest, which is one of the major roles of Vpr in HIV-1 replication (Fig. 2B). Flow-cytometry analysis showed that hematoxylin failed to inhibit the Vpr-induced G_2 phase cell cycle arrest. The αH1 which is essential for the nuclear import of Vpr appeared to be critical for the expression, stability and incorporation of Vpr into the viral particle [14,21]. Therefore, we analyzed whether hematoxylin has an effect on the incorporation of Vpr into viral particle using the virion incorporation assay. Hematoxylin had no effect on the virion incor-

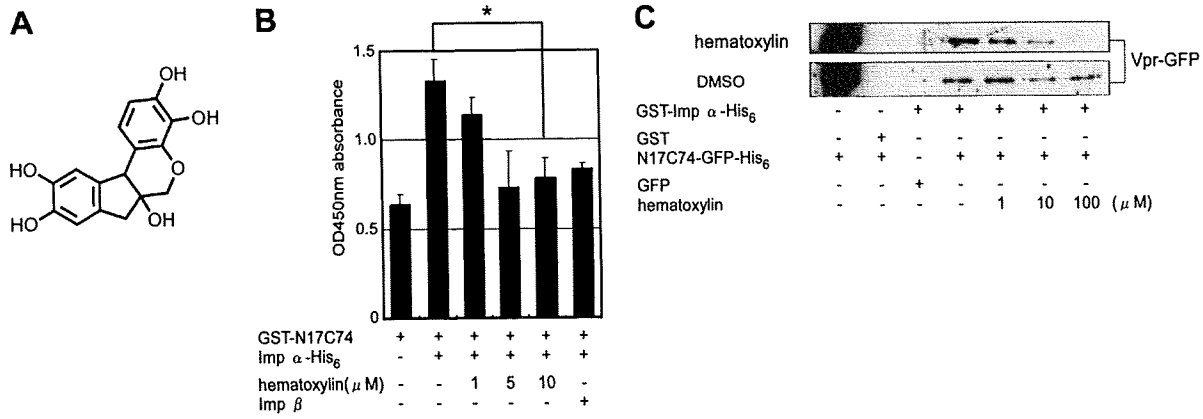

Fig. 1. Identification of a small-molecule that inhibits the interaction between Vpr and Imp α. (A) Chemical structure of hematoxylin. (B) Binding of GST-N17C74 to Imp α-His_6 was quantified using an ELISA-based binding assay. The bound GST-N17C74 was incubated with Imp α-His_6 in the absence (−) or presence (+) of Imp β or hematoxylin. Bound Imp α-His_6 was detected using a HRP-conjugated anti-His MAb. The data are presented as means ± standard deviation of three independent experiments. The asterisk (*) indicates a statistically significant difference ($p < 0.05$). (C) Glutathione-Sepharose beads coupled with GST–Imp α-His_6 or GST were incubated with N17C74-green fluorescence protein (GFP)-His_6 or GFP in the absence (−) or presence of 1, 10 or 100 μM hematoxylin, or 1, 10 or 100 μl dimethyl sulfoxyde (DMSO). The bound fractions were analyzed by Western blotting with anti-GFP MAb.

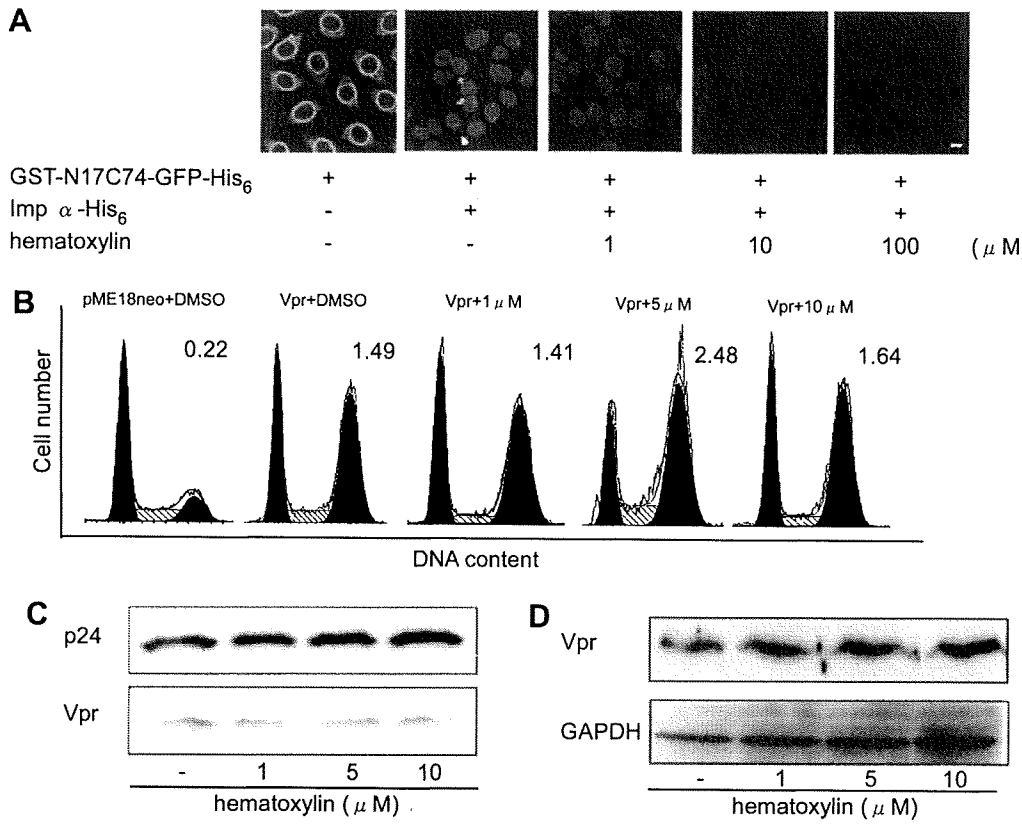

Fig. 2. Hematoxylin inhibits the nuclear import of Vpr by Imp α. (A) Nuclear import of Vpr by Imp α. Digitonin-permeabilized HeLa cells were incubated with 1 μM of GST-N17C74-GFP-His_6 in the absence (−) or presence (+) of 2 μM Imp α, or the indicated concentration of hematoxylin. After fixation, cells were analyzed by confocal laser-scanning microscopy. Bar = 10 μm. (B) Vpr-mediated G_2 arrest. HeLa cells were transfected with pME18Neo encoding Flag-tagged Vpr, or the pME18Neo-Flag, together with pEGFP-N1. At 42 h after addition of 1, 5 or 10 μm hematoxylin, cells were fixed and stained with propidium iodide (PI) for analysis of DNA content and GFP-positive cells were analyzed by flow-cytometry. The proportion of cells in the G_2 phase is indicated at the upper right in each panel. (C) Incorporation of Vpr into viral particles. At 6 h post transfection with pNL4-3, 293T cells were cultured in the absence or presence of hematoxylin, and viral particles were analyzed by Western blotting with anti-Vpr antibody or anti-HIV-1 p24 MAb. (D) Stability of Vpr in HeLa cells. At 6 h post transfection with pME18Neo encoding Flag-tagged Vpr, HeLa cells were cultured in the absence or presence of hematoxylin cells were harvested, lysed and analyzed by Western blotting with anti-Flag M2 MAb and anti-GAPDH MAb.

poration function of Vpr (Fig. 2C). As regards the stability of Vpr, Western blotting analysis showed that the expression level of Vpr in HeLa cells was not affected by hematoxylin 48 h post transfection (Fig. 2D). These results clearly indicate that although hematoxylin has a specific effect on the nuclear import of Vpr, it does not affect the ability of the Vpr to induce G_2 cell cycle arrest or the virion incorporation function of Vpr.

Given the key role played by Vpr in the nuclear import of PIC in non-dividing cells, treatment of infected macrophages with hematoxylin might block HIV-1 replication. Initial experiments were designed to determine a concentration of hematoxylin that could be used with minimal effects on cell viability and cell cycle progression. We investigated the survival of hematoxylin treated human primary macrophages. Macrophages were incubated with hematoxylin at various concentrations in complete culture medium for 15 days and cell viability was evaluated using a 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Fig. 3A). For primary macrophages, hematoxylin was toxic with a 50% cytotoxity concentration (CC_{50}) of >40 μM, respectively. Hematoxylin had no effect on the host cell cycle progression, as indicated by flow-cytometry analysis (Fig. 3B). Given the results here, hematoxylin had no adverse effect on the cell growth and viability of the cells at the IC_{50} concentrations for the binding and nuclear import assays, determined in Figs. 1 and 2. Furthermore, we tested whether nuclear import of the cNLS, which requires the formation of a ternary complex of Imp β and Imp α, was inhibited by hematoxylin. The cNLS-Imp α interaction and classical transport were not inhibited by hematoxylin (Fig. 3C and D), implying that hematoxylin has a specific action on the Imp α-mediated nuclear import of Vpr.

Vpr is essential for the nuclear import of PIC in macrophages. Therefore, specific inhibition of nuclear import by hematoxylin via the Vpr–Imp α interaction led us to investigate whether hematoxylin blocks HIV-1 replication in macrophages. Primary macrophages were infected with the macrophage-tropic JR-CSF HIV-1 strain at low viral input (2 ng of p24 antigen) and cultured for 8 days in the presence or absence of hematoxylin at indicated concentrations (Fig. 4A, left panel). Virus replication was monitored at 4 or 8 days post infection by p24 ELISA. Hematoxylin blocked virus replication efficiently and in a dose-dependent manner, reaching inhibition levels up to about 50% and 70% in 4 and 8 days, respectively, at 20 μM (IC_{50} = 1.64 μM). Similarly, results of infection at high viral input (20 ng of p24 antigen) showed same tendency (Fig. 4A, right panel). Furthermore, to confirm the effect of hematoxylin on HIV-1 replication in macrophages, macrophages were infected with a VSV-G-pseudotype HIV-1 strain that encoded either the wild-type Vpr or a truncated Vpr which can only support a single round of HIV-1 replication, and cultured for 2 days in the absence or presence of 2.5, 5, 10 or 20 μM hematoxylin (Fig. 4B). When hematoxylin was added at the time of infection, the replication of Vpr^+ virus was suppressed by hematoxylin in a dose-dependent manner with a mean of IC_{50} of 5 μM (Fig. 4B). Importantly, levels of luciferase activity of the Vpr^- virus were not effective in each concentration of hematoxylin, indicating that inhibition of viral replication by hematoxylin is a Vpr-dependent manner.

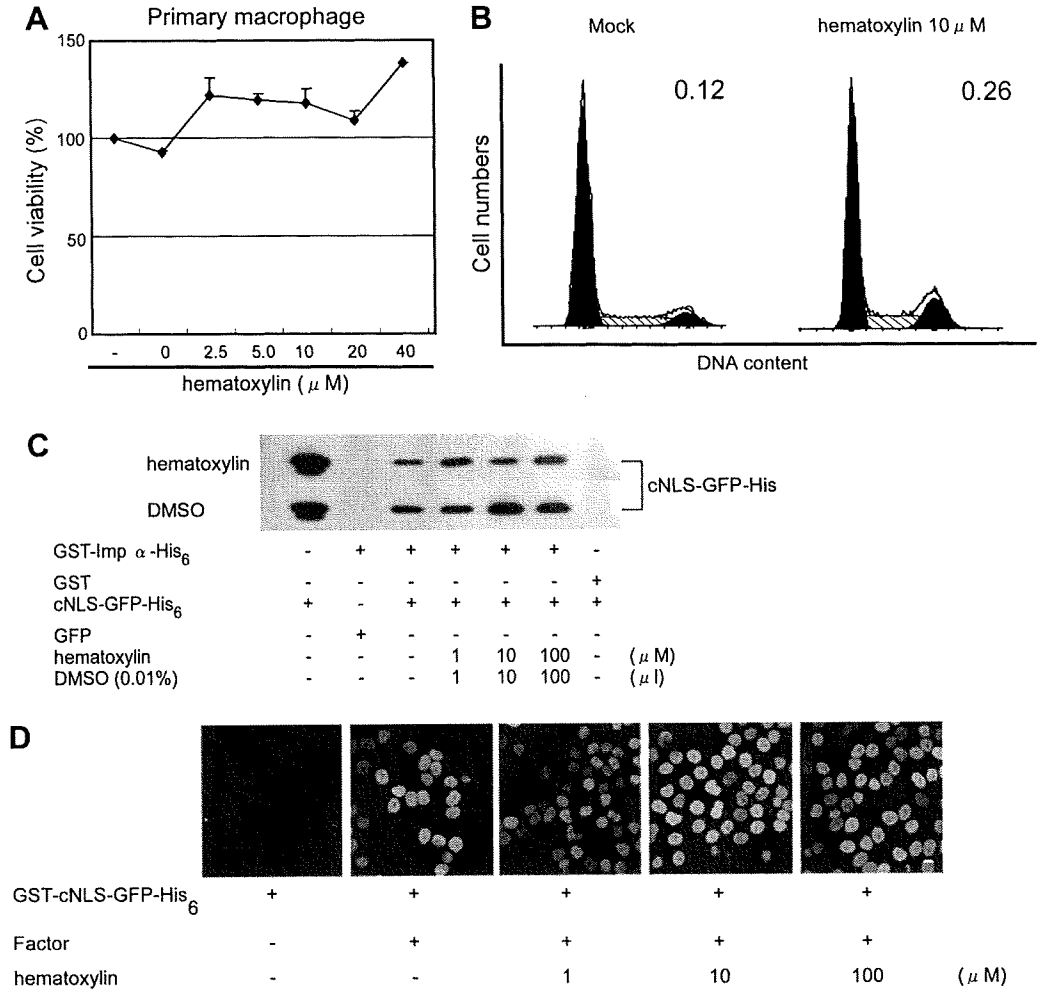

Fig. 3. Analysis of the effect of hematoxylin on normal cell functions. (A) Differentiated primary macrophages were treated with the indicated concentrations of hematoxylin for 15 days. Cell viability was determined using an MTT assay. (B) HeLa cells were treated with 10 μM hematoxylin for 48 h and cell cycle profiles were analyzed by flow cytometry. (C) Glutathione-Sepharose beads coupled with GST-Imp α-His_6 or GST were incubated with cNLS-GFP-His_6 or GFP as a control, in the absence (−) or presence of hematoxylin or DMSO at indicated concentrations. The bound fractions were analyzed by Western blotting with a GFP-specific MAb. (D) Digitonin-permeabilized HeLa cells were incubated with GST-cNLS-GFP-His_6 in the absence (−) or presence (+) of hematoxylin and soluble factors as described previously [12], and then analyzed by confocal laser-scanning microscopy. Bar = 10 μm.

To determine the target of hematoxylin in HIV-1 life cycle, we quantified the viral nucleic acid species present in macrophages after infection. We analyzed the effect of hematoxylin on the formation of late reverse transcripts, as an indicator of total viral DNA (U5 gag as shown in the text), 2-LTR circular DNA, as a marker for successful nuclear import of viral genomic DNA [22]. Treatment with hematoxylin dramatically reduced the amount of 2-LTR circular forms without affecting the copy numbers of U5 gag total DNA in a Vpr-dependent manner (Fig. 4C). These results strongly indicate that the anti-retroviral activity of hematoxylin is likely to be mediated by inhibition of HIV-1 nuclear transport, in macrophages, rather than other steps, such as virus entry and virus maturation.

Discussion

In this study, we explored the anti-HIV activity of hematoxylin, a compound screened by ELISA-binding assays to target the interaction between Vpr and Imp α. By targeting the Vpr–Imp α interaction, we successfully identified a compound inhibiting HIV-1 replication. This result indicates that a specific inhibitor of an interaction between a viral protein and a host cellular factor may provide a new therapeutic strategy for blocking HIV-1 replication. Our results further demonstrate that hematoxylin can efficiently block the nuclear import of Vpr and PIC, and potently blocked HIV-1 replication with Vpr-dependent manner in macrophages. By contrast, hematoxylin, did not inhibit HIV-1 replication in peripheral blood mononuclear cells (PBMCs), which limits the effectiveness of nuclear import inhibitors (unpublished data). This result strongly suggests that Vpr–Imp α interaction is a valuable new drug target which can be exploited for the development of HIV-1 therapies targeting macrophages which cannot be eradicated by HAART.

In present study, by targeting the interaction between Imp α and αH1, we identified a compound inhibiting HIV-1 replication. However, whether hematoxylin directly interacts with Imp α or Vpr remains to be clarified. Recently, using a new hematoxylin derivative and photo-cross-linked small-molecule affinity matrix assay, we obtained evidence to suggest that hematoxylin derivative directly interacts with the αH1 domain of Vpr, but does not interact with Imp α (unpublished data). We postulate that hematoxylin directly bind αH1 and that specifically inhibit Vpr–Imp α interaction without disrupting the global structure of Vpr, because hematoxylin did not inhibit the incorporation of Vpr into viral particle, which is mediated by the αH1 domain. Moreover, the modified Wu-Kabat variability index clearly showed that the αH1 of Vpr is highly conserved among various HIV-1 strains, compared to the

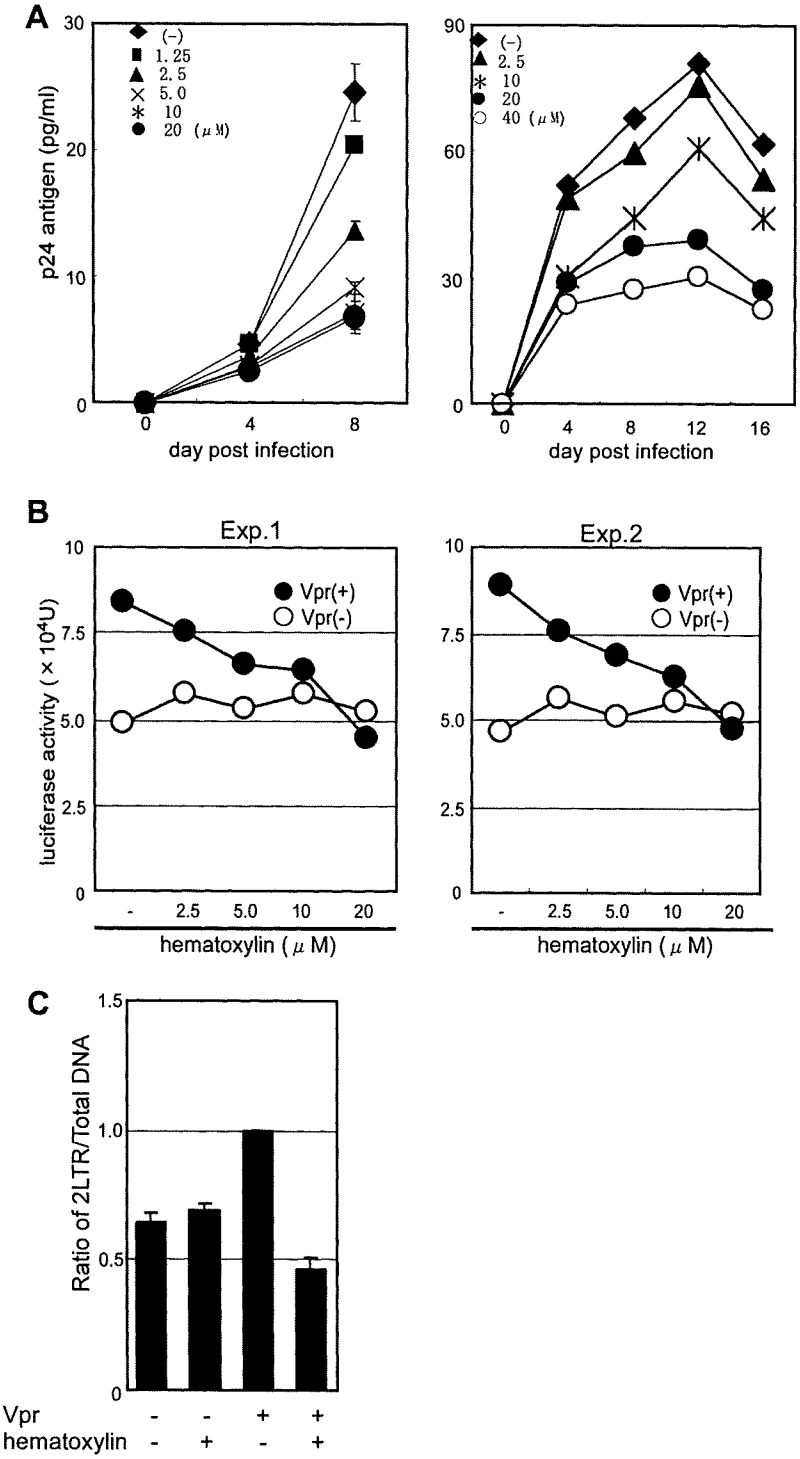

Fig. 4. Hematoxylin inhibition of HIV-1 replication depends on the Vpr in macrophages. (A) Macrophages were infected with HIV-1 (JR-CSF strain) at 2 ng (left panel) or 20 ng (right panel) of p24 antigen in the absence or presence of hematoxylin. Cells were maintained for 4, 8, 12 and 16 days, and the levels of virus production in the culture supernatants were measured by p24 antigen ELISA. (B) Macrophages from two healthy donors were infected with VSV-G-pseudotype virus encoded wild-type Vpr (closed circle) or truncated Vpr (open circle) at 4.0 ng of p24 antigen, and cultured in the absence or presence of hematoxylin. Proviral gene expression was analyzed by luciferase assays 2 days after the infection. (C) Macrophages were infected with the VSV-G-pseudotype virus encoding wild-type Vpr or truncated Vpr at 4 ng of p24 antigen and cultured in the absence or presence of 10 μM hematoxylin. Total HIV-1 DNA and 2-LTR DNA were determined using real-time PCR at 24 h post infection. All samples were tested in duplicate or triplicate, and the data are presented the mean levels of p24 antigen or luciferase activity.

α-helix 2 and α-helix 3 domains, suggesting that the αH1 domain may play crucial roles in HIV-1 survival (unpublished data). Although these data are preliminary, the fact that hematoxylin directly binds to αH1 predicts that HIV-1 resistance to hematoxylin would occur far less frequently than resistance to other conventional drugs. More importantly, understanding of the detailed mechanism of the interaction between Vpr and hematoxylin is essential.

Three HIV-1 proteins, Vpr, MA, and IN, have been proposed as karyophilic agents that recruit the cellular nuclear import machin-

ery to the PIC [8]. Other machineries in addition to the Vpr likely contribute to HIV-1 nuclear import; nevertheless, potent inhibition of HIV-1 replication was achieved by hematoxylin inhibition of Vpr–Imp α interaction. As shown in Fig. 4A, although hematoxylin blocked viral replication efficiently, resulting in 70% on 8 or 16 day, it did not block viral replication completely. This may indicate that there are PIC nuclear import pathways that mediate PIC nuclear import independently of the Vpr; for example, Imp β, Imp 7, Imp β/Imp 7 heterodimer and transportin-SR2 are involved in the nuclear import of PIC into macrophages. In addition, IN, MA, and Vpr either work sequentially or synergistically to regulate PIC nuclear import.

Our results demonstrate that the Vpr–Imp α interaction, a virus protein–cellular protein interaction, is a potential target for an antiviral agent that inhibits nuclear entry. To clarify the mechanism of action of hematoxylin, we are conducting ongoing studies to analyze the crystal structures of the hematoxylin–αH1 domain complex and the αH1 domain–Imp α complex. Detailed descriptions of these interactions will provide new therapeutic strategies for rational drug design. Nuclear import-blocking drugs may be useful not only against HIV, but also against other viruses that require nuclear import for replication, such as the influenza virus, hepatitis B virus, or herpes viruses. The development of such compounds is likely to provide a valuable enrichment of our arsenal of antiviral drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.180.

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A Human Immunodeficiency Virus Screening Algorithm to Address the High Rate of False-Positive Results in Pregnant Women in Japan

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Abstract

Background: Prenatal human immunodeficiency virus (HIV) testing is essential for the prevention of mother-to-child transmission. However, false-positive results of screening testing are a concern as they may cause unnecessary emotional stress to pregnant women waiting for confirmatory test results. In regions with an extremely low prevalence, the positive predictive values of screening are unacceptably low rate. Here, we propose a HIV screening algorithm consisting of serial two fourth-generation enzyme immunoassays to reduce the number of false-positive screening results.

Methodology/Principal Findings: When 6461 pregnant women presenting to two maternity hospitals located in the Tokyo metropolitan area of Japan from September, 2004 to January, 2006 were tested using Enzygnost HIV Integral as a first screening test, 27 showed positive reactions. When these positive reaction samples were tested using VIDAS HIV DUO Quick as a second screening test, only one of them had a positive reaction, and the remaining 26 were nonreactive. Confirmatory Western blots and nucleic acid amplification test also showed that one was positive and the remaining 26 were negative; the subject who was positive with the confirmatory tests was identical to the subject who was positive with the second screening test. Thus, by adding the second screening test, the false-positive rate was improved from 0.4% to 0%, and the positive predictive value from 3.7% to 100%, compared with the single screening test.

Conclusion: By applying our serial screening algorithm to HIV testing in maternity hospitals, many uninfected pregnant women would not need to receive confirmatory tests and be subjected to emotional turmoil while waiting for their confirmatory test results. This algorithm would be suitable for HIV testing of pregnant women living in low prevalence regions such as Japan.

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Competing Interests: Two authors are employed by Health Science Research Institute, Incorporated. However, the company has not supported this study in terms of grants, consultancy, patents, products in development or marketed products. There is no competing interest with the company. The authors declare that the cooperation with the company in this study does not alter their adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

The human immunodeficiency virus (HIV) epidemic in Japan is still at a low level compared with other developed countries, but the number of newly identified infections is increasing every year. For earlier detection and clinical and preventive services, much effort is made to implement voluntary HIV counseling and testing in a variety of health-care settings including public health centers, STD clinics, and outreach medical services. According to the report of the National AIDS Surveillance Committee, 13,894 persons with HIV/AIDS were reported between 1985 and 2007, and 1,500 new cases were reported in 2007 alone [1]. The HIV prevalence in Japan was estimated at 0.008% in 2007 [2]. Of all the HIV-infected persons reported in Japan, 71.0% were Japanese men; 11.4% were non-Japanese men; 6.2% were Japanese women; and 11.4% were non-Japanese women. Currently, about 70% of the Japanese men with HIV infection are men who have sex with men.

Although the HIV prevalence in women is very low in Japan (about 0.004%), universal HIV testing has been performed for pregnant women to prevent mother-to-child transmission since 1999

[3]. Nationwide questionnaire surveys on HIV testing in pregnant women are conducted every year. The HIV testing rate has gradually increased from 73.2% in 1999 to 97.2% in 2007. Over the 21 years between 1987 and 2007, mother-to-child transmission has occurred in only one in 219 (0.5%) HIV-infected pregnant women who received both antiretroviral therapy (ART) and a cesarean section, one in 17 (6%) women who had a cesarean section without ART, and 14 in 36 (39%) women who delivered vaginally [3].

Although prenatal HIV testing is essential for the prevention of mother-to-child transmission, there are concerns about false-positive results of screening tests [4,5]. Positive test results may cause anxiety of HIV infection and emotional stress in pregnant women waiting for confirmatory test results. Some severe cases were covered by the mass media in 2007, leading to an official notification on the frequent observation of HIV false-positive screening results from the Ministry of Health, Labour and Welfare of Japan [6].

There has been little study on the rate of false-positive results in HIV screening testing of pregnant women in Japan. Thus, we conducted a prospective study at two maternity hospitals in the Tokyo metropolitan area to evaluate the performance of screening test, including the prevalence, false-positive rate, and positive predictive value, and proposed a new HIV screening algorithm composed of two serial tests to enable a substantial reduction in the number of false-positive results at this stage.

Materials and Methods

Study Setting

The study was conducted from September, 2004 to January, 2006 in two maternity hospitals located in the Tokyo metropolitan area. Each of the hospitals conducts more than 1,000 deliveries each year.

HIV Testing

Blood samples were initially tested using Enzygnost HIV Integral (Siemens Healthcare Diagnostics, Deerfield, Illinois, USA), a fourth-generation enzyme-linked immunosorbent assay with the ability to detect HIV-1 gp41 antibody, HIV-2 gp36 antibody, and HIV-1 p24 antigen at a reference laboratory of the Health Science Research Institute Inc. (Yokohama, Japan). The Enzygnost HIV Integral can test 880 samples during each run lasting 240 min. The samples that tested positive in the initial screening were subjected to a secondary screening test and confirmatory tests, which were conducted at the Kanagawa Prefectural Institute of Public Health. The second screening test was performed using VIDAS HIV DUO Quick (bioMérieux, Marcy l'Etoile, France), a fourth-generation enzyme-linked fluorescent assay with the ability to detect HIV-1 gp160 antibody, HIV-2 gp36 antibody, and HIV-1 p24 antigen. The VIDAS HIV DUO Quick can test 60 samples during each run lasting 80 min. Confirmatory tests were performed using Western blot tests (Lab blot 1 and Lab blot 2; Bio-Rad Laboratories, Hercules, California, USA), and a nucleic acid amplification test (NAT), Amplicor HIV-1 Monitor test version 1.5 (Roche Molecular Systems, Branchburg, New Jersey, USA). HIV typing was performed using SERODIA•HIV-1/2 PA (Fujirebio, Tokyo, Japan). All the tests were conducted and interpreted as recommended by the manufacturers.

Samples for Evaluating the Sensitivity of the Screening Tests

Ten HIV-1 seroconversion panels (PRB 936, 937, 938, 939(E), 945, 951, 952, 953, 954, and 955) and two samples from HIV p24 Antigen Mixed Titer Performance panels (PRA 201-05 and 201-17) were obtained from SeraCare Life Sciences (formerly Boston Biomedica, West Bridgewater, Massachusetts, USA). Seroconversion panels were used to evaluate the sensitivity in the early phase of infection. Three samples (PRB 936-04, PRA 201-05, and PRA 201-17) were diluted twofold serially with HIV negative human pooled plasma and were used to evaluate the antigen detection sensitivity of the enzyme immunoassay (EIA).

Screening Algorithm

We proposed an algorithm (Fig. 1) to reduce the number of false-positive screening results in prenatal HIV testing. In our algorithm, each blood sample is tested serially with two EIA tests that should be highly sensitive and have different detection formats. The first screening test was performed on the day or next day of blood sampling during the first trimester, and the second screening test was done as soon as possible after the sample in the first test was found to be positive. If the result of the first screening test is negative, the test is reported as negative; if a result of the first screening test is positive, the same sample is tested using the second screening test. If the result of the second test is negative, the test is reported as negative; if the result of the second test is positive, confirmatory tests using Western blots and NAT are conducted.

Statistical Analysis

Specificity was calculated using a combination of Western blots and NAT as the gold standard. Confidence intervals (CIs) were estimated using approximation to the normal distribution.

Research Ethics

This study was jointly approved by the ethics committees at the two maternity hospitals and the Kanagawa Prefectural Institute of Public Health. The verbal informed consent for study participation including screening and confirmatory tests was obtained from study participants and recorded by the physician on a separate study-participation sheet. As blood samples used in this study had been collected as routine tests and thus no additional invasive action was required for participants, the committees approved this procedure according to the Ethical Guideline of the Ministry of Health, Labour and Welfare of Japan. All links between the test results and personal identifiers were removed and were known only to the physicians in charge of the subjects.

Results

Comparative Assays

The sensitivities of the Enzygnost HIV Integral and VIDAS HIV DUO Quick in the early phase of infection were compared using 10 HIV-1 seroconversion panels. HIV infection was detected with the VIDAS HIV DUO Quick earlier than with the Enzygnost HIV Integral in eight out of ten panels; the interval was an average of 4.5 days (Table 1). Next, the antigen detection sensitivities of the two tests were compared using serial twofold dilutions of three HIV-1 antigen samples (PRB936-04, PRA 201-05, and PRA201-17). The VIDAS HIV DUO Quick was 16–32 times more sensitive than the Enzygnost HIV Integral (Table 2).

Results of HIV Testing

Of the 6,461 study participants, 27 (0.42%) showed positive results for the first screening test performed using the Enzygnost HIV Integral. When the positive samples were tested with the second screening test performed using the VIDAS HIV DUO Quick, only one sample exhibited a positive reaction and the remaining 26 samples were nonreactive. When the samples that tested positive in the first screening were tested using confirmatory Western blots and NAT, only one sample was positive and the

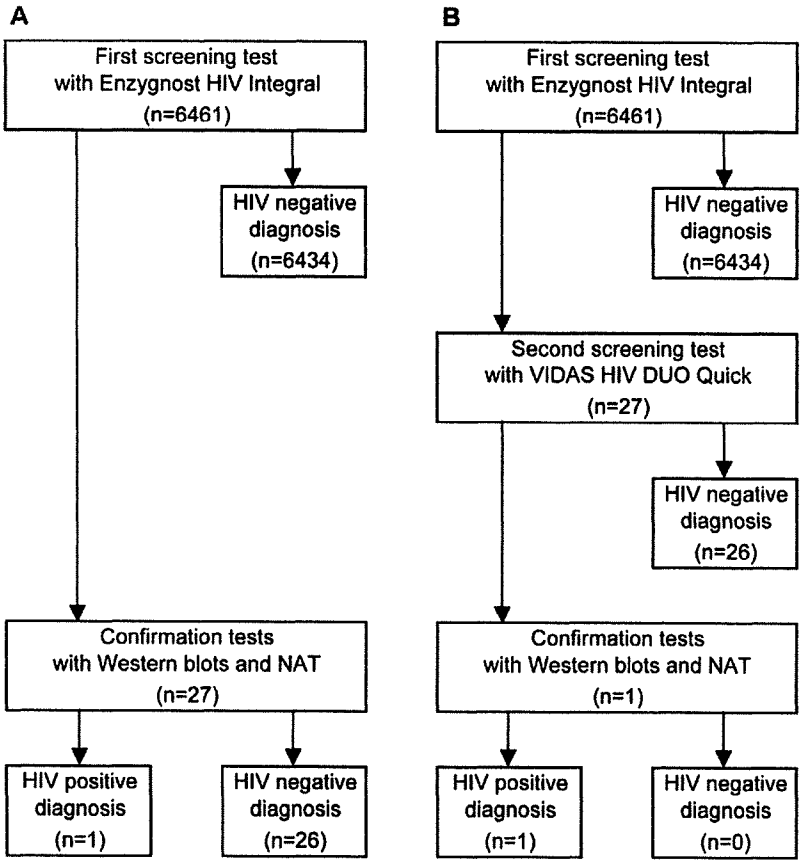

Figure 1. Comparison of the results obtained by two HIV testing algorithms. A, algorithm containing single test screening. **B**, algorithm containing serial two-test screening.
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Table 1. Test performance to detect bleed day with first positive result using seroconversion panel members.

Panel	Bleed day with first positive result		Difference in bleed days between the two tests
	Enzygnost HIV integral	VIDAS HIV DUO Quick	
PRB936	12	12	0
PRB937	21	14	7
PRB938	3	0	3
PRB939(E)	21	16	5
PRB945	13	13	0
PRB951	11	8	3
PRB952	17	10	7
PRB953	10	3	7
PRB954	21	17	4
PRB955	12	3	9
Average			4.5

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other 26 samples were negative (Fig. 1). The subject whose confirmatory test results were positive was the same as the one whose second screening test result was positive. The sample from this subject was positive with an HIV-1 Western blot, indeterminate with an HIV-2 Western blot, and HIV-1 positive on HIV typing. As for the signal-to-cutoff (S/CO) ratio on the Enzygnost HIV Integral used as the first screening test, the one positive sample was 6.47; of 26 false-positive samples, two were ≥6.0, four were 2.0–6.0, and 20 were <2.0. Western blots of the 26 negative samples showed that one was indeterminate with both HIV-1 and HIV-2 Western blots (S/CO ratio, 1.24), two were indeterminate with only HIV-1 Western blots (0.92 and 5.87), and one was indeterminate with only HIV-2 Western blots (6.34): all were negative on HIV typing.

In the standard protocol using a single screening test, the false-positive rate was 0.40% (95% CI, 0.25–0.56%), and the positive predictive value was 3.7%. However, when an additional screening test was introduced, the overall specificity of the screening was improved dramatically, and the above values were changed to 0% and 100%, respectively.

Discussion

According to the guideline for prevention of mother-to-child transmission of HIV in Japan [7], women who are found to be pregnant at hospital are generally tested for HIV during the first

Table 2. Antigen detection limits by antigen-antibody combined detection tests using 3 antigen positive specimens in the panels.

Panel No.	Antigen-antibody combined detection test		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
PRB936-04	VIDAS HIV DUO Quick	TV[1]	9.84	5.55	3.01	1.53	0.89	0.47	0.32	0.21
		Result	POS	POS	POS	POS	POS	POS	POS	NEG
	Enzygnost HIV Integral	S/CO[2]	4.52	2.32	0.90	0.64	0.38	0.21	NT[3]	NT
		Result	POS	POS	IND	NEG	NEG	NEG	NT	NT
PRA201-05	VIDAS HIV DUO Quick	TV	3.27	1.69	0.98	0.56	0.31	0.23	0.15	0.13
		Result	POS	POS	POS	POS	POS	NEG	NEG	NEG
	Enzygnost HIV Integral	S/CO	1.58	0.63	0.34	0.19	NT	NT	NT	NT
		Result	POS	NEG	NEG	NEG	NT	NT	NT	NT
PRA201-17	VIDAS HIV DUO Quick	TV	2.98	1.58	0.84	0.45	0.28	0.22	0.17	NT
		Result	POS	POS	POS	POS	POS	NEG	NEG	NT
	Enzygnost HIV Integral	S/CO	1.77	0.78	0.36	0.19	NT	NT	NT	NT
		Result	POS	NEG	NEG	NEG	NT	NT	NT	NT

POS, positive; NEG, negative; IND, indeterminate.
[1]TV, test value. TV<0.25 was judged as negative, and TV≥0.25 was judged as positive.
[2]S/CO, signal-to-cutoff ratio.
[3]NT, not tested.
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trimester, and HIV-positive women are treated with antiretroviral therapy from the second trimester and intravenous administration of zidovudine during labor. Babies are treated with zidovudine syrup for 6 weeks after birth. Testing of women in labor is performed with a rapid antibody test, and positive women are regarded as infected with HIV, and zidovudine administration was initiated to the woman and a newborn baby.

Routine HIV testing for pregnant women has been underway since 1999 in Japan. A questionnaire survey conducted in 2003 reported that only 7 out of 82,290 pregnant women were diagnosed as being HIV-1 positive [8]; thus, the prevalence was 0.009%, which is extremely low compared with 0.15%–5% in the United States [9]. This survey also reported that the false-positive rate of screening tests was 0.094%, and that its positive predictive value was 8.3%. These values agree with those obtained in the present study. Since about one million pregnant women are tested each year in Japan, about 1,000 women are probably notified of false-positive results after screening tests.

Identifying such false-positive results using confirmatory testing is not easy. With the introduction of fourth-generation EIA tests for screening, the confirmatory test sequence has become very complicated. Because these EIA tests can detect antibodies against HIV-1 and HIV-2 as well as HIV-1 antigen, the confirmation of positive results requires an HIV-1 Western blot, HIV-2 Western blot, and NAT for HIV-1 RNA. Western blots result in a high percentage of indeterminate results [10]. Furthermore, even if the result of HIV-2 Western blot is negative, HIV-2 infection cannot be denied because the sensitivity of HIV-2 Western blots is lower than those of EIA tests for the detection of HIV-2 antibody. Therefore, even if samples are shown to be negative with any one of the three tests, the subjects should be retested one month later. Consequently, once a pregnant woman is assigned a positive screening test result by a false-positive reaction, she must undergo two rounds of confirmatory tests in one month.

HIV-tested pregnant women have been reported to encounter various problems associated with false-positive screening results [3,9–13]. In Japan, pregnant women who were notified of positive screening results felt strong anxiety and depression while waiting for the results of the confirmatory tests; some of the women became suspicious of their partners, and some considered abortion or divorce [3]. One woman was notified of an HIV positive result by her clinician without receiving sufficient explanation about the screening testing; later, when confirmatory testing showed that she was HIV negative, she became upset and untrusting of medical services [3]. Such emotional disturbances have been reported in other countries [9,10]. However, the situation in Japan may be somewhat different from those in most of developed countries because the HIV prevalence (0.009%) is extremely low. Most obstetricians have never treated an HIV-infected individual and thus have little chance of learning HIV infection and its diagnosis. Therefore, it is the most important to help obstetricians to understand the nature of HIV testing and to provide clients with counseling and information, including the high frequency of false-positive results in screening testing and the necessity of confirmatory testing to obtain a decisive result.

As an alternative approach to resolving these problems from a technical point of view, we proposed a screening algorithm consisting of serial two fourth-generation enzyme immunoassays to reduce the number of false-positive test results. When this algorithm was applied to the 6,461 pregnant women who participated in this study, the specificity of screening was improved from 99.6% to 100% and the positive predictive value was improved from 3.7% to 100%, compared with the standard protocol. Although the two screening tests were conducted at separate reference laboratories in this clinical trial, these tests can be sequentially done at the same place. By applying this algorithm to clinical settings, many uninfected clients would not need to receive confirmatory tests and thus would not be subjected to emotional turmoil while waiting for their confirmatory test results. In addition, because extensive confirmatory tests and repeat visits are not required, it results in cost savings. Although the number of participants in this study is limited, the increase in specificity and positive predictive value can likely be extrapolated to a larger population of pregnant women.

It has been suggested that false positives may be caused by alloantibodies resulting from pregnancy, transfusions, or trans-

plantation [10]. We did not collect other medical conditions of the study participants, which may influence testing results. The false-positive rate of Enzygnost HIV Integral in this study was 0.40%, which is within a range (0.3%–0.8%) of previously reported false-positive rate of this kit [14–16]. Therefore, it is unlikely that the false-positive rate observed in the first screening was influenced by medical conditions including pregnancy and testing factors such as quality control and performance in the reference laboratory.

The proposed protocol is characterized by a specificity-optimized serial two-test algorithm. When the specificity is optimized, a serial testing algorithm and a parallel testing algorithm are equally sensitive and specific. A parallel testing algorithm is time-saving for diagnosis of HIV infection; a serial testing algorithm is cost-effective and less laborious. Because the HIV prevalence was very low in Japan, most of positive results are due to false positives of the tests. Furthermore, simultaneous two screening tests are not covered the public medical insurance. We think that a serial testing is more acceptable in our country.

The order of the two tests could be determined based on several factors including throughput, cost, labor intensity, sensitivity, and specificity. In this study, the VIDAS HIV DUO Quick was used as the second screening test because it is less suitable for large-scale testing and has been shown to be more sensitive in the early phase of infection than the Enzygnost HIV Integral. The latter characteristic may help to reduce the number of false-negative results in the second screening. However, it should be noted that the overall sensitivity and specificity of a serial screening algorithm are determined only by the combination of the two tests and their order is irrelevant.

Inevitably, the sensitivity of the serial screening algorithm is lower than those of the individual tests employed therein, and the specificity is higher. However, if the two tests employed are highly sensitive, the decrease in sensitivity is expected to be marginal and less than the difference among currently available screening tests [17]. On the other hand, the adoption of the algorithm improves the specificity dramatically. Theoretically, the sensitivity of the first test should be as high as possible to ensure the detection of the largest possible number of HIV-positive samples [17,18]. However, recent EIA tests have undergone dramatic improvements, and most fourth-generation tests have achieved nearly 100% sensitivity on HIV-1/2 reference positive samples and are capable of detecting early infection two to seven days after NAT [15,19,20]. The ranking of these tests on seroconversion panels varies among individual panels [15,19,20], and is probably indecisive for field samples. Therefore, as long as two highly sensitive fourth-generation tests are used, which test is more sensitive may not be the primary determinant of the test order.

We should be cautious in applying the proposed screening algorithm to other health-care settings. In clinical settings specializing in HIV infection, clinicians are likely to diagnose their patients with a high sensitivity and specificity. The HIV-1 prevalence in this setting would likely be relatively high, and the clinicians would be well trained with regard to providing counseling and information regarding HIV testing. In such a setting, the present algorithm may not be required. Meanwhile, voluntary HIV counseling and testing have been implemented chiefly in public health centers on a free-of-charge basis, and rapid antibody tests are widely used for screening testing. The sensitivity and specificity of third-generation rapid test were shown to be lower than those of fourth-generation EIA tests [16,17]. In these cases, the introduction of a fourth-generation EIA test as the second screening would not miss true positive samples and would enable a great reduction in false-positive screening samples.

As the performance of HIV diagnostic tools evolve, the diagnostic algorithms should also be changed to be accurate as well as beneficial to the clients, and they need to be developed specifically for individual health-care settings. The screening algorithm presented in this study provides improved specificity and positive predictive value, and cost savings, which is suitable and beneficial for HIV testing in low prevalence settings such as for maternity hospitals in Japan.

Author Contributions

Conceived and designed the experiments: TSS KK SK MI. Performed the experiments: TSS KS KS MK. Analyzed the data: TSS RY YT NI SK MI. Contributed reagents/materials/analysis tools: TSS HH HS YT NI. Wrote the paper: TSS SK MI. Coordinated with the patients, physicians, laboratory stuffs and data analyzer: RWH.

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【健康危険情報の概要】

従来日本国内においてはほとんど報告のなかったHIV-2感染症例が近年愛知県内において複数見つかっている。確認されたHIV-2感染症例数は2007年に2例，2008年に2例の合計4例であるが，遡って2004年も1例感染者がいたことが判明している。確認された5症例のうち3名は来日中のアフリカ系の外国人男性であるが，残りの2例は日本人女性であり，来日中のアフリカ系外国人※との性交渉により日本国内において感染したと思われる。

ヒト免疫不全ウイルス1型（HIV-1）感染が同性愛者間で主に拡大しているのに対して，今回のHIV-2感染症例，特に日本人女性2症例は異性間の性交渉で感染しており，今後HIV-2感染が性的嗜好にかかわらず拡大する危険を孕んでいると思われる。

国内におけるHIV-2感染拡大の恐れについてはすでに過去に危険情報が出されており，保健所等におけるHIV検査体制，日赤におけるスクリーニングのいずれもHIV-2感染の存在を念頭においた対策が取られている。しかし，日本国内において実際にHIV-2感染伝播が確認されたのは今回の2症例が初めてであり，検査実施時にHIV-2への注意を改めて喚起する必要がある。

※前記HIV-2感染が確認されたアフリカ系外国人とは別人

＜特集関連情報＞

HIV検査法の現状と課題

近年，HIV感染症の検査技術は飛躍的な進歩を遂げている。それにともなって，従来の診断プロトコールの改変が余儀なくされたり，薬剤耐性検査のような新しい検査が導入されたりしている。一方では，HIV-2の診断のような，診療における新たなニーズに検査技術の実用化が追いついていないという問題もある。本稿では，HIVの免疫学的検査法，RNA定量法，薬剤耐性検査法について，それぞれの現状と課題を考察する。

1．免疫学的検査法

従来のHIV感染症の診断では，EIA（酵素免疫測定法）あるいはPA（粒子凝集法）によるスクリーニング検査の陽性者に対してWB（ウエスタンブロット法）で確認検査を行うことが一般的であった。当初のEIAは，WBと比較して感度は同等であったが，特異度の点で劣っていたため，低コストのEIAをまず行い，それで陽性と判定された検体をWBで再検査するというプロトコールは理論的にも経済的にも合理性があったと思われる。しかし，EIAの性能はその後格段の進歩を遂げ，第4世代と呼ばれる最新の検査試薬では，HIV-1の抗体と抗原，それにHIV-2の抗体を同時に検査することができ，また検出感度自体も向上したため，WBに比べて約20日間も早期にHIVを診断できるようになった。そのため，最新のEIAで陽性となった検体をWBで確認検査をすると，急性期の感染を見落としてしまう可能性が高くなってきた。このような問題に対処するため，日本エイズ学会と日本臨床検査医学会は「診療におけるHIV-1/2の診断ガイドライン　2008」を今年公表した。このガイドラインでは，抗原抗体同時スクリーニング検査を行ったあと，陽性例に対しては確認検査としてHIV-1のWBとRNA定量法を同時に行うことを推奨している。また，HIV-2の診断もWBを用いてできる限り正確に行うことを求めている。ただし，HIV-2の診断に関しては，抗原検出が可能なEIAやRNAを検出できる核酸増幅法が市販されていない現状では，HIV-1と同じレベルで正確に診断するのは実際上非常に難しい。この問題に対処するための一つの有効な方法として，献血液の検査で用いられているような，HIV-1 RNAとHIV-2 RNAの同時検出法の導入が考えられる。

イムノクロマトグラフィーを原理とするHIV-1/2の迅速検査法が保健所等の検査施設や民間クリニックで使用されている。現在使われている迅速検査法は，第4世代スクリーニング法に比べて感度が若干低いが，15分で検査結果が出るため，陰性の場合は即日で結果を受検者に返すことができる。最近，HIV-1の抗原も同時に検査ができる迅速検査法（富士レビオ）が認可されたが，販売直後にHIV-2抗体に対するプロゾーン反応の問題が見つかったため，今のところ販売が中止されている。今後，抗原抗体同時迅速診断法が，他のメーカーからのものも含め，販売されるようになるであろうが，その使用にあたっては，抗原の検出感度がどの程度であり，それがHIV-1の早期診断にどれぐらい役立つかを十分検討する必要がある。

2．RNA定量法

HIV-1 RNA定量法は，HIV-1感染症のフォローアップ，特に抗HIV薬による治療効果を判定するために重要な検査手段である。HIV-1診断においても利用されていることは先に述べた。わが国においては，RT-PCRとハイブリダイゼーションを原理とするロシュ・ダイアグノスティクスのアンプリコアHIV-1モニター（以下，アンプリコア）が長らく使用されてきたが，2007年12月，リアルタイムPCRを原理とするコバスタックマンHIV-1（以下，コバスタックマン）が同じ会社から発売された。また，アボットからも2009年1月，同じくリアルタイムPCRを原理とする製品が発売されている。リアルタイムPCR法は，従来の方法に比べて自動化が進み，迅速で定量範囲が広いという特徴がある。

さて，2008年3月頃から検査センターにおけるHIV-1 RNA定量は，アンプリコアからコバスタックマン

に次第に置き換わって行ったが，その過程でコバスタックマンによる患者の血中 HIV-1 RNA 測定値がアンプリコアによる測定値よりも 2～3 倍高くなることが多くの医療施設から報告された。このことは，エイズ臨床研究センターや東京医科大学の大規模な研究においても確かめられた。特に，アンプリコアでは検出限界以下（<50コピー/m*l*）であった症例で，コバスタックマンで測定されるようになると，50コピー/m*l* 以上になることが多くなったため，患者や臨床医の間に困惑が広がった。ロシュからの最新の情報によると，血漿検体を用いた場合の測定値の乖離は，血漿分離管の不具合による，感染リンパ球の血漿への混入が原因である可能性が高いらしい。しかし，より一般的に用いられている血清検体における測定値の乖離の原因については今なお不明のままである。

一方，ヨーロッパでは逆の現象が起こっていた。すなわち，コバスタックマンの測定値がアンプリコアのそれより有意に低いというのである。最近，その原因の一つが，コバスアンプリコアで使われている下流プライマーと一部の HIV-1 との特定の 1 塩基ミスマッチにあり，それが定量値を1/100以下に低下させるという論文が発表された（Korn ら，2009)。このミスマッチはデータベースに集められた HIV-1 塩基配列の 2 %で見られるらしい。国内においても，このミスマッチを原因とする HIV RNA 定量値の低下が観察された（近藤ら，エイズ学会発表予定)。ロシュでは，以上のような問題に対処した，新しいバージョンのコバスタックマンを申請することを予定しているようだ。

3．薬剤耐性検査法

薬剤耐性検査法は，抗 HIV 薬による初回治療やサルベージ治療における薬剤選択に広く利用されている。一般には，血漿中 HIV-1 RNA の逆転写酵素やプロテアーゼなどのコーディング領域を RT-PCR で増幅させたあと，この増幅産物を用いて直接シーケンシングを行い，薬剤耐性に特異的な変異を見つけることによりウイルスの薬剤耐性を評価している。一方，HIV は遺伝子的に非常に多様な個体からなる集団であり，薬剤耐性変異に関しても野生型と耐性型が混在している場合が多い。薬剤耐性検査に直接シーケンシングを用いた場合，存在割合が20～30%以下の野生型あるいは耐性型の塩基を検出することは非常に難しい。直接シーケンシングでは見つけられないような微少薬剤耐性ウイルスの存在が抗 HIV 治療にどの程度影響を及ぼすかを，アリール特異的リアルタイム PCR などの最新技術を用いて現在盛んに調べられている。今のところまだ明確な結論は出ていないが，3TC/FTC や EFV/NVP のような，それらに対する耐性変異の遺伝的障壁が低い薬剤に関しては，微少薬剤耐性ウイルスの存在と治療効果の低下の間に有意な相関があると報告されている。今後，わが国においても，治療失敗例における微少薬剤耐性ウイルスの役割について詳細に検討する必要があると考えられる。

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＜特集関連情報＞

ウイルス性肝炎と HIV 感染症

HIV 感染者における死因の変化

HIV 感染症は，強力な多剤併用療法（highly active anti-retroviral therapy: HAART）が可能となった1990年代後半より，長期生存が可能となり，慢性疾患へと変化してきている。海外からの報告では，HIV 感染症の免疫低下を背景に出現する日和見感染症による死亡率の低下が報告される一方で，B 型肝炎ウイルス（HBV)，C 型肝炎ウイルス（HCV）による肝硬変，肝細胞癌や，非 AIDS 関連悪性腫瘍を原因とした死亡者の増加が指摘されている。

図 1 は，1985～2007年に当院で死亡した HIV 感染者200例の死因の推移を示したものである。AIDS 指標疾患，細菌感染症，肝疾患（肝細胞癌を含む)，非 AIDS 関連悪性腫瘍に着目し，HAART 出現前の1985～1996年（Pre HAART 期100例)，HAART 出現早期の1997～2002年（early HAART 期51例)，最近の2003～2007年（late HAART 期49例）の 3 期に分けて検討してみると，AIDS 指標疾患は，依然として多数を占めているが，HAART が可能となった1996年以降は，肝疾患と非 AIDS 関連悪性腫瘍の増加が明らかである。この傾向は，海外の報告と軌を一にするものと考えられる。

HIV 感染症と慢性肝炎

以下に，HIV 感染症と慢性 B 型肝炎および慢性 C 型肝炎の関連について触れてみたい。

B 型肝炎は，HIV感染症と同様に，主に性行為により感染が伝播し，HIV/HBV 重複感染例は少なくない。このため，HIV 感染者には，定期的な HBV マーカーの確認が推奨されている。米国・西ヨーロッパでは，HIV 感染者の 6～14%に B 型肝炎の合併が報告され

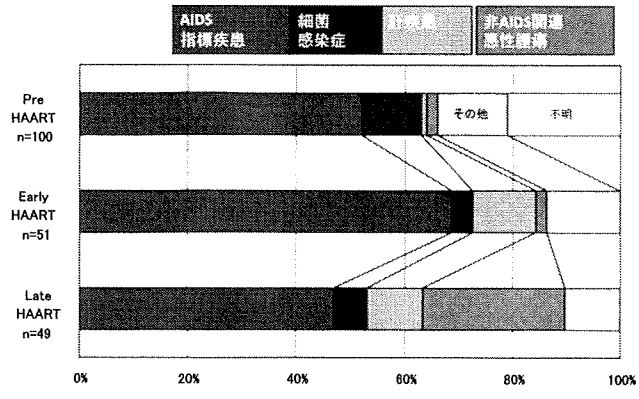

図1. 当院で死亡したHIV感染者の死因の推移

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特集：HIV/AIDS

HIV検査
—最近のスクリーニング検査と遺伝子検査の進歩—

今井光信　　加藤真吾

HIV検査
—最近のスクリーニング検査と遺伝子検査の進歩—

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Recent progress in HIV screening tests and nucleic acid tests

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Abstract

HIV testing plays a crucial role in detecting and monitoring HIV infection. Diagnosis of HIV infection is basically made by sequential two tests : a screening test with an enzyme immunoassay(EIA) and a confirmatory test with Western blot. The most recent EIAs, used in commercial laboratories, identify HIV infection earlier because they detect both HIV-1 antibody and antigen. Rapid tests represent another advance for HIV screening. They are widely used in voluntary counselling and testing at public health centers and private clinics. An assay for detection of HIV-1 RNA was approved as a confirmatory test of reactive screening tests to diagnose early infection. These new testing technologies offer more accurate, reliable, and convenient diagnosis of HIV infection.

Key words: HIV, screening test, antibody, nucleic acid test, real-time PCR

はじめに

HIV感染の有無を調べるための検査は，基本的にスクリーニング検査と確認検査の二段階により行われる．スクリーニング検査には抗HIV抗体を調べる方法と，抗HIV抗体とHIV抗原(p24抗原)を同時に調べる方法がある．現在，保健所などで即日検査に使用されている迅速検査キット(ダイナスクリーン)は抗HIV抗体を検出し，民間検査センターや病院などで行っている通常の酵素免疫測定法(EIA)などの検査試薬のほとんどは，抗HIV抗体と同時にHIV抗原を検出できる．また，最近は，抗HIV抗体とHIV抗原の双方を同時に検出できる迅速検査キットも開発され，近々発売が予定されている．確認検査としては，抗体の陽性を確認する方法としてウエスタンブロット(WB)が最も確実で現在も広く用いられている．ただし，感染初期の抗体の弱陽性の時期や抗体検出前の抗原陽性期については，WBによる確認は困難であり，血中のウイルス量を測定するために開発されたHIV遺伝子検査法が，非常に重要な役割を果たしている．

本稿の前半では，免疫学的検査法を中心に，後半では遺伝子検査を中心に，HIV検査法の最近の進歩と問題点や活用法について紹介する．

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1. 免疫学的検査

a. HIVスクリーニング検査法の進歩

HIVスクリーニング検査法は，1984にAIDSの病原体がHIVと確認されて以来，年々改良が重ねられ目覚ましい進歩を遂げてきた．最初のキットは培養ウイルス(HIV-1)由来の抗原を用いたEIA法でIgG抗体のみの検出であった．その後，抗原としてリコンビナントタンパクや合成ペプチド(HIV-1，HIV-2)を用い，また，抗原-抗体-抗原のサンドイッチ法などによりIgM抗体の検出も可能になった．このため，感染初期のウインドウ期間も大幅に短縮され，また，HIV-1に加えてHIV-2に対する抗体の検出も可能となった．

また，抗体検査に加え，抗原検査(HIV-1)も同時にできる抗原抗体同時検出キットも開発され，抗体の陽転前の抗原陽性期の検出も可能となり，ウィンドウ期は更に短縮された．現在，我が国で市販されているほとんどのHIVスクリーニング検査キット(**表1**)は抗原抗体同時検出キット(HIV-2については抗体のみ検出)である．

一方では，血液または血清を1滴加え，15分間静置するだけで，結果の得られる，簡易迅速キット(イムノクロマト法など)が開発されるなど，検査法の簡易化と迅速化に関しても急速な進歩がみられた．現在，我が国で市販されている迅速検査キットは抗HIV抗体を検出する1種類(ダイナスクリーン)だけであるが，抗原と抗体の両方を一つのキットで個別のラインとして検出可能な簡易迅速キットが開発され，認可もされており，近々，利用が可能になると思われる．

b. 確認検査法の進歩

HIVスクリーニング検査では，感染初期のウィンドウ期間内にある場合を除き，HIV感染者はすべて陽性となる．ただし，検査キットで使用している抗原や抗体との交差反応により，およそ0.3％程度の人が，感染していなくとも陽性(偽陽性)となる．

このため，スクリーニング検査で陽性となった場合には，感染による真の陽性か交差反応などによる偽陽性かを鑑別するため確認検査が必要となる．

抗体の確認検査法としては，HIVの構成タンパクそれぞれに対する抗体の有無を調べるWB法が広く使われており，WB法で典型的な陽性パターンを示す場合はHIV感染が確実となる．ただし，WB法で1-2本のバンドが出て判定保留となるケースや，陰性であっても感染初期が疑われるケースでは，遺伝子検査により感染初期か否かを確認する必要がある．

感染初期で抗体が弱陽性のケースや抗体の陽転前で抗原が陽性のケースでは，血液中のウイルスを鋭敏に検出できる遺伝子検査で陽性となる．このため，スクリーニング検査陽性で，WBの結果が保留あるいは陰性のため結論の得られないケースについては，遺伝子検査を行うことで，感染初期かスクリーニング検査の偽陽性例かを確定することが可能である．

c. 偽陽性を除外するための追加検査法

保健所などで行っている即日HIV検査(迅速検査)では，現在迅速検査キット(ダイナスクリーン)を使用しているが，このキットではほぼ1％の偽陽性がある．これら比較的頻度の高い偽陽性を除外するため，迅速検査キットで陽性となった場合には，より検出感度の優れている他の検査キット(抗原抗体検査キットやPA法)で追加のスクリーニング検査を行い，追加検査陰性であれば，スクリーニング検査陰性と判断する．追加検査で陽性であればスクリーニング検査陽性として，確認検査を行う．

この追加検査法で迅速検査の偽陽性例のほとんどを陰性と判定できる．

通常のHIVスクリーニング検査法の偽陽性率0.3％に比べて，感染率の著しく低い集団(妊婦など)を対象にHIV検査を行う場合にも，この追加検査により偽陽性例をスクリーニング検査段階で除外することにより，偽陽性によりもたらされる多くの無用な負担が避けられる．

d. HIV-2の検査

現在，我が国で使用されているHIVスクリーニング検査キットのすべてが，抗HIV-2抗体

表1 HIV検査試薬(2009年9月現在)

スクリーニング検査試薬

検査法	キット名	メーカー名	測定方法
抗体検査	ダイナスクリーン・HIV-1/2	インバネス・メディカル・ジャパン	イムノクロマト
	ジェンスクリーンHIV1/2	バイオ・ラッド ラボラトリーズ	ELISA
	ジェネディアHIV-1/2 ミックスPA	富士レビオ	PA
	セロディア・HIV-1/2(HIV型別用)	富士レビオ	PA
	ルミパルスオーソ HIV-1/2	オーソ・クリニカル・ダイアグノスティックス	CLEIA
	ビトロス HIV-1/2抗体	オーソ・クリニカル・ダイアグノスティックス	CLEIA
	ランリーム HIV-1/2	シスメックス	ラテックス定量
抗原抗体同時検査	アキシム HIV Ag/Ab コンボアッセイ・ダイナパック	アボットジャパン	MEIA
	アーキテクト・HIV Ag/Ab コンボアッセイ	アボットジャパン	CLIA
	ジェンスクリーンHIV Ag-Ab	バイオ・ラッド ラボラトリーズ	ELISA
	エンザイクノストHIVインテグラルII	シーメンス	ELISA
	バイダスアッセイキットHIVデュオII	シスメックス・ビオメリュー	ELFA
	エスプラインHIV-Ag/Ab	富士レビオ	イムノクロマト
抗原検査	ルミパルスI HIV-1p24(感染初期検出)	富士レビオ	CLEIA

確認検査試薬

検査法	キット名	メーカー名	測定方法
抗体検査	ラブブロット1	バイオ・ラッド ラボラトリーズ	WB
	ラブブロット2	バイオ・ラッド ラボラトリーズ	WB
	ペプチラブ1, 2(HIV型別用)	バイオ・ラッド ラボラトリーズ	イムノブロット
遺伝子検査	アンプリコアHIV-1モニターVer.1.5	ロシュ・ダイアグノスティックス	RT-PCR
	コバスアンプリコアHIV-1モニターVer.1.5	ロシュ・ダイアグノスティックス	RT-PCR
	コバスTaqMan HIV-1「オート」	ロシュ・ダイアグノスティックス	RT-PCR
	コバスTaqMan HIV-1「マニュアル」	ロシュ・ダイアグノスティックス	RT-PCR

を検出できるが，日本国内で見いだされたHIV-2感染例はいまのところ10例に満たない．しかしながら，HIVスクリーニング検査で陽性となりHIV-1感染の可能性が否定されたケースについてはHIV-2感染の可能性を考慮し，抗HIV-2抗体の確認が必要となる．通常HIV-2のWBを行い陽性であればHIV-2感染，陰性であればHIV-2の感染は否定される．ただし，WB-2の検査で1-2本のバンドが検出され判定保留となった場合，他の方法による確認が必要となる．一つはHIV-2のPA検査であり，もう一つは，最初のスクリーニング検査に用いたのとは異なるスクリーニング検査キットを用いた再検査である．これらの検査で陰性であれば，抗HIV-2抗体は陰性と判定できる．もし，これらの方法でも陽性の場合や，HIV-2の初期感染

表2 コバスTaqMan HIV-1とアキュジーンm-HIV-1の性能比較表

	コバスTaqMan HIV-1	アキュジーンm-HIV-1
方 法	リアルタイムPCR	リアルタイムPCR
プローブ	TaqManプローブ	部分的2本鎖プローブ
検体量	0.85mL	0.6 mL, 0.2mL
検体前処理	自動処理(AmpliPrep)	自動処理(m2000sp)
検体種	血清・血漿(EDTA)	血漿(ACD-A, EDTA)
測定下限	<40コピー/mL	40コピー/mL(0.6mL使用時) 150コピー/mL(0.2mL使用時)
測定上限	10^7コピー/mL	10^7コピー/mL
サブタイプ検出能	グループM	グループM, O, N
ターゲット領域	*gag*遺伝子	*pol* integrase遺伝子
データ管理	QS(内部標準)により抽出・増幅効率を補正	ICにより増幅阻害を確認
コンタミネーションのモニタリング	不要(AmpEraseあり)	定期的なモニタリング(AmpEraseなし)
試薬調製の要否	自動処理	要用手調製
試薬保存	冷蔵(2-8℃)	冷凍(<-10℃)

の可能性が高いと思われる場合には，HIV-2の遺伝子検査などでHIV-2感染の確認を行う必要がある．

2. 遺伝子検査

a. PCRによるHIV遺伝子の検出・定量

レトロウイルスの一種であるHIVは，遊離ウイルス粒子の状態ではRNAゲノムをもつが，ヒトの細胞に感染するとDNA(プロウイルス)となり染色体DNAの中に組み込まれる．したがって，血漿中のHIV RNAを測定することによりウイルスの存在やその量を，末梢血単核球DNA中のHIV DNAを測定することにより感染細胞の存在やその量を調べることができる．

HIV-1が最初に分離されたのは1983年のことであるが，この年はPCRが考案された年でもある．当初，HIV-1のDNAとRNAの検出にはそれぞれサザンブロットとノーザンブロットが使われていたが，臨床検体からの検出には感度が不足していることがすぐに判明した．そこで，はるかに検出感度が高く，操作性にも優れたPCRが，HIV-1のDNAとRNAの検出・定量に応用され，感染者の診断と病態把握のため大きな貢献を果たした．HIV-1の発見とPCRはその後どちらもノーベル賞を受賞している．

先にも述べたように，HIV-1のDNAとRNAはHIV感染の異なった側面を反映するものであり，どちらも病態把握のためには重要な指標である．しかし，血中HIV-1 RNA量(遊離ウイルス量)が病期の進行や抗HIV治療により大きく変動するのに対し，末梢血単核球中DNA量(感染細胞数)はその変動が小さく，測定誤差や日内変動以上の有意な変化を得ることが困難であることがわかった．そのため，発症予測や抗ウイルス効果判定の指標として血中HIV-1 RNA量が主に使われることになった．

1996年，商業的HIV-1 RNA定量検査試薬として，RT-PCRとハイブリダイゼーションを原理とするロシュ・ダイアグノスティックス社(ロシュ社)のアンプリコアHIV-1モニター(以下，アンプリコア)が，米国で初めて認可・販売された．その後，この検査法はサブタイプB以外のHIV-1の定量に問題のあることがわかり，2002年に改良が加えられた．また，PCR以外にも核酸を高感度検出する方法が開発され，転写介在増幅(TMA)あるいは枝分かれDNA(bDNA)を原理とするHIV-1 RNA定量検査法が実用化されている．

b. 我が国におけるHIV-1 RNA定量の現状

我が国においては，先述のアンプリコアが唯