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Characterization of HIV-1 CRF01_AE *env* genes derived from recently infected Indonesian individuals

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Running head: CRF01_AE *env* genes derived from recent infection

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Abstract

To eradicate human immunodeficiency virus type 1 (HIV-1) infection, a comprehensive strategy including preventive vaccine development is needed. Envelope glycoproteins (Env) play a central role in viral infection and are the major targets of humoral immune responses. Therefore, Env is a candidate vaccine antigen, and its characterization is necessary for vaccine development. The characterization of the transmitted/founder (i.e. recently infected) virus that is responsible for the establishment of infection and induction of primary anti-HIV-1 immune responses is important. We herein established HIV-1 *env* clones derived from recently infected Indonesian individuals. All *env* genes were classified into CRF01_AE. The immunological characterization of *env* clones was performed by neutralization tests using a series of broadly neutralizing antibodies. The present study is the first to immunologically characterize the CRF01_AE transmitted/founder virus circulating in Indonesia.

Key words: Recent HIV-1 infection, Transmitted/founder virus, HIV-1 envelope glycoproteins, CRF01_AE, Indonesia

Human immunodeficiency virus (HIV) is a major causative agent of acquired immune deficiency syndrome (AIDS). In 2017, 1.8 million individuals were newly infected with HIV and the total number of HIV-infected individuals reached 36.9 million worldwide[1]. As a strategy to combat HIV, several antiretroviral drugs have been developed. Combination antiretroviral therapy (cART), which combines multiple types of antiretroviral drugs, has been implemented worldwide. HIV-infected individuals who receive cART need life-long therapy to suppress their viral load; however, several issues are associated with long-term cART, such as the adverse effects of drugs and emergence of resistant viruses. Thus, to eradicate HIV infection, comprehensive strategies including not only ART, but also the development of preventive HIV vaccines are needed.

HIV is divided into two types: HIV type 1 (HIV-1) and type 2 (HIV-2)[2]. HIV-1 infection is responsible for the majority of AIDS cases globally. HIV-1 is classified into four groups: M (major), O (outlying), N (new or non-M, non-O), and P (pending). The viruses in group M are further classified into many subtypes and circulating recombinant forms (CRFs)[2]. HIV-1 subtype B is the predominant strain in the Americas, Europe, and Australia, while CRF01_AE is a major CRF circulating throughout Southeast Asian countries, including Indonesia[2]. In Indonesia, 48,000 individuals were newly infected with HIV in 2016, and the total number of HIV-infected individuals reached 620,000[3].

The envelope glycoproteins (Env), gp120 and gp41, mediate specific interactions with the CD4 receptor and chemokine co-receptors CCR5 and/or CXCR4 on the surface of target cells, and play a central role in viral transmission. In addition, Env is a major target of humoral immune responses against HIV-1. Therefore, Env is a candidate vaccine antigen[4]. Env gp120 and gp41 are the most variable HIV-1 proteins; therefore, humoral immune responses against Env vary among different subtypes and CRFs.

Several potent anti-HIV-1 broadly neutralizing antibodies (nNAbs) that inhibit the infection of various subtypes and CRFs of HIV-1 have been isolated[5]. These bNAbs need to be examined in more detail for the development of effective vaccines. In the early phase of HIV-1 infection, the virus within an infected individual is genetically homogeneous due to the selection pressure of host immune responses[6]. The transmitted/founder virus (T/F virus) in the early phase of HIV-1 infection is responsible for the establishment of HIV-1 infection and induction of primary ant-HIV-1 immune responses. Therefore, the T/F virus is regarded as a target of anti-HIV-1 vaccines. In the present study, we amplified *env* genes (early *env* clones) that may represent the characteristics of *env* derived from the T/F virus from recently infected Indonesian individuals and established expression vectors. The immunological characterization of early Env clones was then performed using lentiviral vectors expressing Env clones and

a series of anti-HIV-1 Env bNAbs.

Serum samples were collected in Surabaya, Indonesia between 2012 and 2014[7, 8]. Detailed information on study participants was described previously[7, 8]. Serum samples were subjected to the captured BED-enzyme-linked immunosorbent assay (ELISA) to estimate the incidence of HIV infection[9]. This assay measures the proportion of HIV-1-specific IgG in blood samples with respective total IgG. Normalized optical density (ODn) was calculated by the OD of samples divided by the median OD of the calibrator on captured BED-ELISA. Based on previous findings, samples with an ODn of <0.8 were estimated to be from recently (<127 days) seroconverted individuals. Full-length, early *env* genes were then amplified from serum samples derived from recently infected individuals, as described previously[10]. Viral RNA was reverse transcribed to cDNA using the SuperScript III First-Stand Synthesis kit (Invitrogen, Carlsbad, CA, USA) with the reverse primer, K-env-R1[10]. Full-length *env* genes were then amplified by a polymerase chain reaction (PCR) from cDNA using PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies, Santa Clara, CA). Amplified full-length, early *env* genes were cloned into the HIV-1 *env* shuttle/expression vector, pCI-envCT to generate early Env-expression vectors, as described previously[11]. Based on the results obtained, expression vectors for the full-length, early *env* clones SM11-8, SM11-13, SM15-1, SM18-K5, SM18-N11,

SM26-3, SM26-5, SM56-1, UA4,-1 UA18-6, UA18-9, PJ39-9, and PJ90-1 were established. Early *env* genes were named by two alphabetical characters and two digits followed by one digit. The first two alphabetical characters and two digits in the ID of *env* genes denote patient IDs. For example, *env* clones SM11-8 and SM11-13 were derived from the same patient, SM11. In addition, K and N in the IDs, SM18-K5 and SM18-N11, denote the primer sets used to amplify *env* genes[10]. In order to evaluate the Env function of amplified *env* genes, luciferase reporter lentiviral vectors expressing the Env clones were generated by transfecting Lenti-X 293T cells (Takara, Shiga, Japan) with an Env-expression vector, the lentiviral packaging plasmid, psPAX2 (Addgene plasmid #12259), and luciferase-expressing lentiviral vector plasmid, pLenti CMV Puro LUC (w168-1) (Addgene plasmid #17477) using polyethylenimine (Polysciences, Warrington, PA) or the FuGENE HD transfection reagent (Promega, Madison, WI, USA). Viral titers were measured using the HIVp24 antigen ELISA kit (Rimco, Okinawa, Japan). The infectivity and second receptor usage of Env-expressing lentiviral vectors were evaluated using U87.CD4.CXCR4 (U87.X4) and U87.CD4.CCR5 (U87.R5) cells, as described previously[11]. U87 cell lines were provided by Dr. HongKui Deng and Dan R. Littman through the NIH AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH. Luciferase activity in infected cells was measured using a Steady-

Glo Luciferase Assay Kit (Promega) and LB 962 Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany). The relative infectivity of the Env-expressing lentiviral vector was estimated by comparing it with the luciferase activity of U87.X4 cells infected with a lentiviral vector expressing the Env of pNL4-3 (Genbank accession number; AF324493.2) (pNL4-3 Env) or the luciferase activity of U87.R5 cells infected with a lentiviral vector expressing the Env of pBa-L (GenBank accession no. AB253432) (pBa-L Env)[11]. The results obtained showed that 10 lentiviral vectors expressing the early Env clones SM11-8, SM11-13, SM18-K5, SM18-N11, SM26-3, SM26-5, SM26-7, UA18-6, UA18-9, and PJ39-9 showed high or moderate levels of infectivity, while the 4 remaining lentiviral vectors expressing the early Env clones, SM15-1, SM56-1, UA4-1, and PJ90-1, showed no infectivity (data not shown). Therefore, we considered the 10 Env clones that conferred infectivity to lentiviral vectors to be functional, and subjected them to further genotypic and phenotypic characterization. The co-receptor usages of 10 early Env-expressing lentiviral vectors were assessed based on their infectivity to U87.X4 and U87.R5 cells. SM11-8, SM11-13, and PJ39-9 were X4-tropic, while SM18-K5, SM18-N11, SM26-3, SM26-5, SM26-7, UA18-6, and UA18-9 were R5-tropic.

A sequencing analysis of the 10 full-length, early *env* genes was performed by Macrogen Japan (Kyoto, Japan), and the data obtained were assembled using Genetyx

ver. 10 software (Genetyx, Tokyo, Japan). The nucleotide sequences of the early *env* genes were deposited in the GenBank database under accession numbers MG839510 - MG839519. The subtype classification of early *env* genes was then performed using the Recombinant Identification Program (RIP) available on the website of the HIV sequence database (www.hiv.lanl.gov). Furthermore, a phylogenetic analysis of early *env* genes was performed using MEGA7 software. The nucleotide distance matrices generated using the Kimura two parameter model were used to construct a phylogenetic tree by the neighbor-joining method. The *env* genes of HIV-1 subtypes A1, A2, B, C, D, and G as well as those of the CRF01_AE, CRF02_AG, CRF15_01B, and CRF33_01B reference strains were included in the analysis. Furthermore, phylogenetically close *env* genes, including 3 CRF01_AE or CRF33_01B *env* genes isolated in Indonesia, pPRD320-11AE61 (GenBank accession no. AB485652), 07IDJKT194-C (GenBank accession no. AB547464), and 07IDJKT189-C (GenBank accession no. AB547463), were selected using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and included in the analysis. Viral subtyping by RIP was consistent with that by the phylogenetic analysis (data not shown). Regarding the results obtained, 10 early *env* genes derived from recently infected Indonesian individuals were classified into CRF01_AE. The phylogenetic analysis also revealed that 10 early *env* genes were divided into two groups: SM11-8, SM11-13, SM26-

3, SM26-5, SM26-7, and PJ39-9, and SM18-K5, SM18-N11, UA18-6, and UA18-9; however, none of the 10 early *env* genes were closely related to the 3 previously reported Indonesian CRF01_AE or CRF33_01B *env* genes (Fig. 1). Moreover, 2 SM18 *env* clones were closely related to HM089 (GenBank accession no. KY213724) isolated in Singapore in 2008 (Fig. 1). These results suggested that HIV-1 strains in Indonesia had not only spread domestically, but had also come from other countries in Southeast Asia.

The deduced amino acid sequences of 10 early Env clones were aligned by the Clustal Omega program available on the website of EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/). The Showalign program at the EMBOSS explorer (http://www.bioinformatics.nl/cgi-bin/emboss/showalign) was used to display the alignments of amino acid sequences. Potential N-linked glycosylation (PNLG) sites on the deduced amino acid sequences were identified using N-Glycosite (www.hiv.lanl.gov). The deduced amino acid sequences of 10 early *env* genes were shown in Fig. 2. The number of amino acid residues in the V1, V2, V3, V4, and V5 regions and the number of PNLG sites were manually counted (data not shown). The numbers of amino acid residues in each V1, V2, V3, V4, and V5 region and PNLG sites in gp160 (gp120 and gp41) were then compared with those from 14 Thai early CRF01_AE Env clones derived from recently infected Thai individuals[11]. Although the lengths of the V1,

V3, V4, and V5 regions were similar between the early CRF01_AE Env clones derived from recently infected Indonesian and Thai individuals (data not shown), the lengths of the gp120 V2 regions of 10 Indonesian early CRF01_AE Env clones were significantly longer (p= 0.021) than those of 14 Thai early CRF01_AE Env clones[11] (Fig. 3). Previous studies reported that the lengths of the V2 regions of 14 Thai early CRF01_AE Env clones were significantly shorter than those of 35 Thai chronic CRF01_AE Env clones derived from chronically infected Thai individuals[10, 11]. In contrast, the lengths of the gp120 V2 regions of 10 Indonesian early CRF01_AE Env clones were similar to those of 35 Thai chronic CRF01_AE Env clones (data not shown). In addition, the average number of PNLG sites in gp160 among 10 Indonesian early CRF01_AE Env clones was 29, which was similar to that in 14 Thai early CRF01_AE Env clones[11] (data not shown). These results suggested that the phenotypic characteristics of 10 Indonesia early CRF01_AE Env and 14 Thai early CRF01_AE Env were different.

Regarding the immunological characterization of early Env clones, neutralization tests against bNAbs were performed for 7 early Env-expressing lentiviral vectors that showed high infectivity to U87.R5 cells. The neutralization susceptibilities of Env-expressing lentiviral vectors were examined for bNAbs, IgG1 b12, VRC01, N6, 2F5, 4E10, and 10E8, as described previously[11]. IgG1 b12, VRC01, and N6 are bNAbs

against the CD4-binding domain of Env gp120, while 2F5, 4E10, and 10E8 are bNAbs against Env gp41[5]. Neutralization levels were evaluated as a reduction in luciferase activity in infected cells. The 50% inhibitory concentrations (IC50) of bNAbs were calculated using a standard function of GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The antibodies IgG1 b12, 2F5, and 4E10 were purchased from Polymun Scientific (Vienna, Austria). VRC01, N6, and 10E8 were prepared using the expression vectors of the heavy and light chains. Briefly, FreeStyle 293-F cells were co-transfected with the expression vectors using 293fectin Transfection Reagent (Life Technologies, Oslo, Norway). Antibodies were then purified from culture supernatants using the MAbTrap Kit (GE Healthcare, Buckinghamshire, United Kingdom). The N6 mAb Heavy Chain Expression Vector and N6 mAb Light Chain Expression Vector were provided by Drs. Jinghe Huang and Mark Connors through ARRRP. The expression vectors of the heavy and light chains, CMVR 10E8 H and CMVR 10E8 L, were provided by Dr. Mark Connors through ARRRP. Neuralization tests revealed that all early Env-expressing lentiviral vectors were resistant to neutralization by IgG1 b12, while the lentiviral vector expressing subtype B, pBa-L Env was neutralized well by IgG1 b12 (Table 1). IgG1 b12 is derived from a HIV-1 subtype B-infected individual. A previous study reported that CRF01_AE showed low susceptibility to IgG1 b12[12], presumably due

to some intersubtype differences in protein structure-related properties. The present results were consistent with previous findings[12]. The neutralization susceptibilities of early Env-expressing lentiviral vectors to VRC01 markedly differed between 2 SM18 Env clones; SM18-K5 and SM18-N11, and 5 other early Env clones; SM26-3, SM26-5, SM26-7, UA18-6, and UA18-9 (Table 1). SM18-K5 and SM18-N11 were susceptible to VRC01, while SM26-3, SM26-5, SM26-7, UA18-6, and UA18-9 were resistant to VRC01-mediated neutralization (Table 1). A previous study showed that the lack of 2–3 amino acid residues in the V5 region played a role in conferring VRC01 resistance to CRF01_AE Env-expressing lentiviral vectors[13]. Consistent with these findings, the number of amino acid residues in the V5 region differed between 2 SM18 Env clones and 5 other early Env clones (Table 1). SM18-K5 and SM18-N11 have 2 more glycine residues in the V5 region relative to the remaining 5 early Env clones (Fig. 2). N6 showed lower IC50 values for inhibiting infection by all lentiviral vectors than those for VRC01 or IgG1 b12 (Table 1). N6 was established in 2016 and recognizes the CD4-binding site of gp120[5]. N6 susceptibility was not affected by the V5 mutations that altered VRC01 susceptibility[14]. Consistent with these findings, N6 neutralized all early Env-expressing lentiviral vectors, including VRC01-resistant vectors, at lower concentrations (Table 1). In addition, most early Env-expressing lentiviral vectors were neutralized by bNAbs

against Env gp41, except for SM26-3, which was not neutralized by 4E10 (Table 1). 10E8 showed a lower IC50 value for inhibiting infection by all lentiviral vectors than those for 2F5 and 4E10 (Table 1). A single amino acid mutation, alanine (A) to threonine (T), was detected in the 2F5 core epitope, ELDKWA, in the MPER of the gp41 of SM26-5 and SM26-7 (Fig. 2). In addition, a single amino acid mutation, aspartic acid (D) to serine (S) [or asparagine (N)], was found inside the 4E10 core epitope NWFDI in the MPER of the gp41 of SM18-K5, SM18-N11, and UA18-9 (Fig. 2). However, these mutations did not affect the neutralization susceptibilities of these lentiviral vectors to 2F5 and 4E10.

In conclusion, we established 10 functional early *env* clones from recently HIV-1-infected Indonesian individuals. To the best of our knowledge, this is the first study to establish Indonesian early CRF01_AE *env* clones. CRF01_AE Env is being used as a vaccine antigen in recent vaccine development, including a clinical trial conducted in Thailand, RV144. Therefore, we consider the established Indonesian early AE Env clones to be useful for evaluating the efficacy of immune responses elicited by these vaccine candidates. Furthermore, 10 Indonesian early CRF01_AE Env-expressing lentiviral vectors may be useful for elucidating the phenotypic properties of the T/F CRF01_AE virus circulating in Indonesia. Subtype B viruses prevalent in Western countries have been extensively examined[15], whereas CRF01_AE viruses in Southeast Asian countries have

not. We hope to conduct this research in the future.

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Author disclosure statement

The authors declare that no competing interests exist.

Sequence Data

Nucleoside sequences are available under GenBank accession numbers

MG839510 - MG839519.

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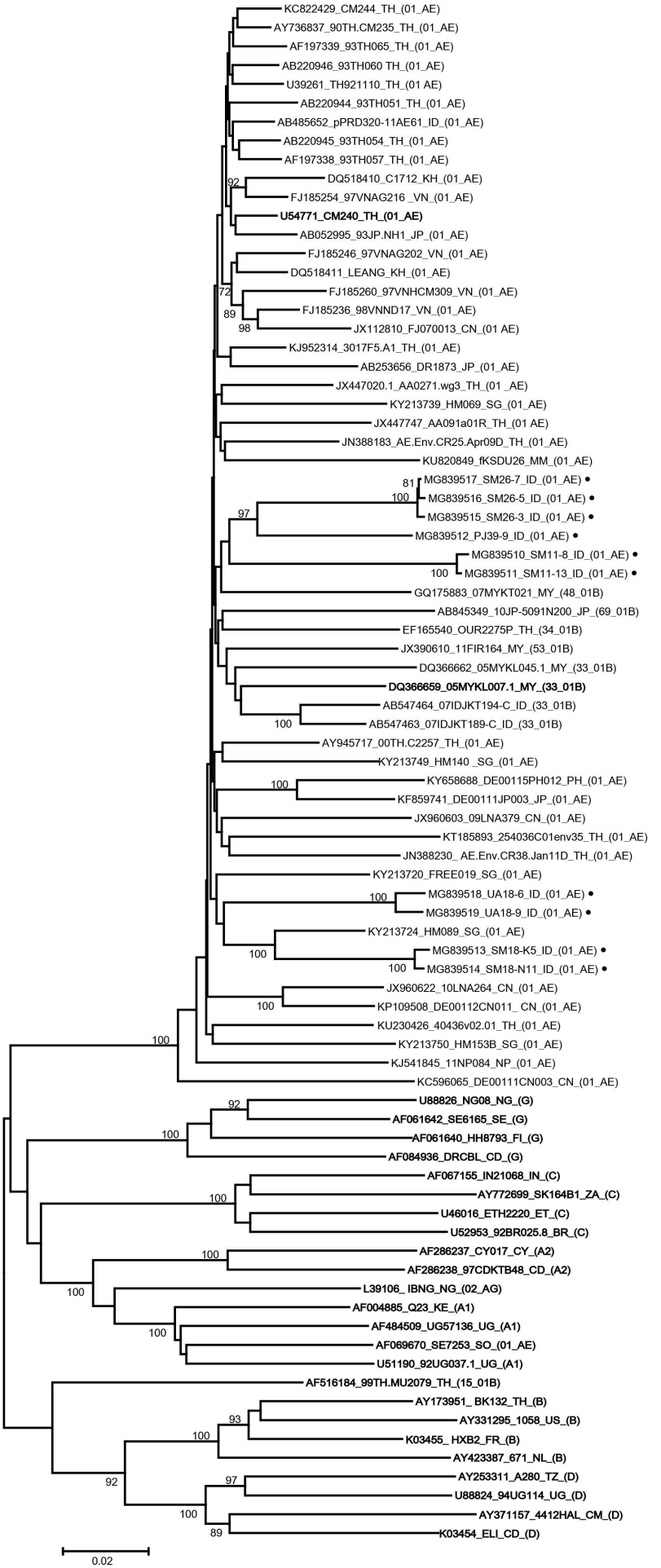
Figure legends

FIG. 1. Phylogenetic tree analyses of 10 HIV-1 *env* genes derived from recently infected Indonesian individuals. Phylogenetic trees were constructed for the HIV-1 *env* gene newly sequenced in the present study (denoted by black circles), as described in the text. The *env* genes of reference HIV-1 strains representing subtypes A1, A2, B, C, D, and G, as well as CRF01_AE (01_AE), CRF02_AG (02_AG), and CRF33_01B (33_01B) were included in the analysis (denoted in bold). In addition, phylogenetically close *env* genes were selected using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and also included in the analysis. Sequence IDs are presented as a GenBank accession number, an ID of the HIV-1 strain, the country of origin, and the subtype or CRF of the strain (shown in parentheses) in that order. Bootstrap values were shown if they were >70. The country of origin of each sequence is indicated by the ISO 3166 two-letter code (https://www.iso.org/obp/ui/#iso:pub:PUB500001:en) as follows: BR, Brazil; CD, Democratic Republic of the Congo; CM, Cameroon; CN, China; CY, Cyprus; ET, Ethiopia; FI, Finland; FR, France; ID, Indonesia; IN, India; JP, Japan; KH, Cambodia; MM, Myanmar; MY, Malaysia; NG, Nigeria; NL, the Netherlands; NP, Nepal; VN, Viet Nam; PH, Philippines; SE, Sweden; SG, Singapore; SO, Somalia; TH, Thailand; TZ, Tanzania; UG, Uganda; US, the United States of America; ZA, South Africa.

FIG. 2. Deduced gp160 amino acid sequences of 10 early *env* genes derived from recently infected Indonesian individuals. The nucleotide sequences of *env* genes were translated, aligned, and compared with the consensus sequence, as described in the text. The positions of the Env signal peptide, gp120 and gp41, as well as the variable (V1, V2, V3, V4 and V5) and conserved (C1, C2, C3, C4 and C5) regions of gp120 are denoted above the aligned sequences. In addition, the membrane-proximal external region (MPER) of gp41 is indicated. The numbering of amino acid residues begins with the first residue of the Env signal peptide. Dots denote amino acid identities, whereas dashes represent gaps introduced to optimize alignment. PNLG sites are shown by underlining.

FIG. 3. Comparison of lengths of gp120 V2 regions between early CRF01_AE Env clones derived from recently infected Indonesian and Thai individuals. The numbers of amino acid residues in the V2 region of Env gp120 were manually counted and plotted. Horizontal solid lines show median values, and the number of samples studied (n) is shown below the panels. Differences among groups were analyzed with the unpaired *t*-test, and p value (p) is shown in the panel.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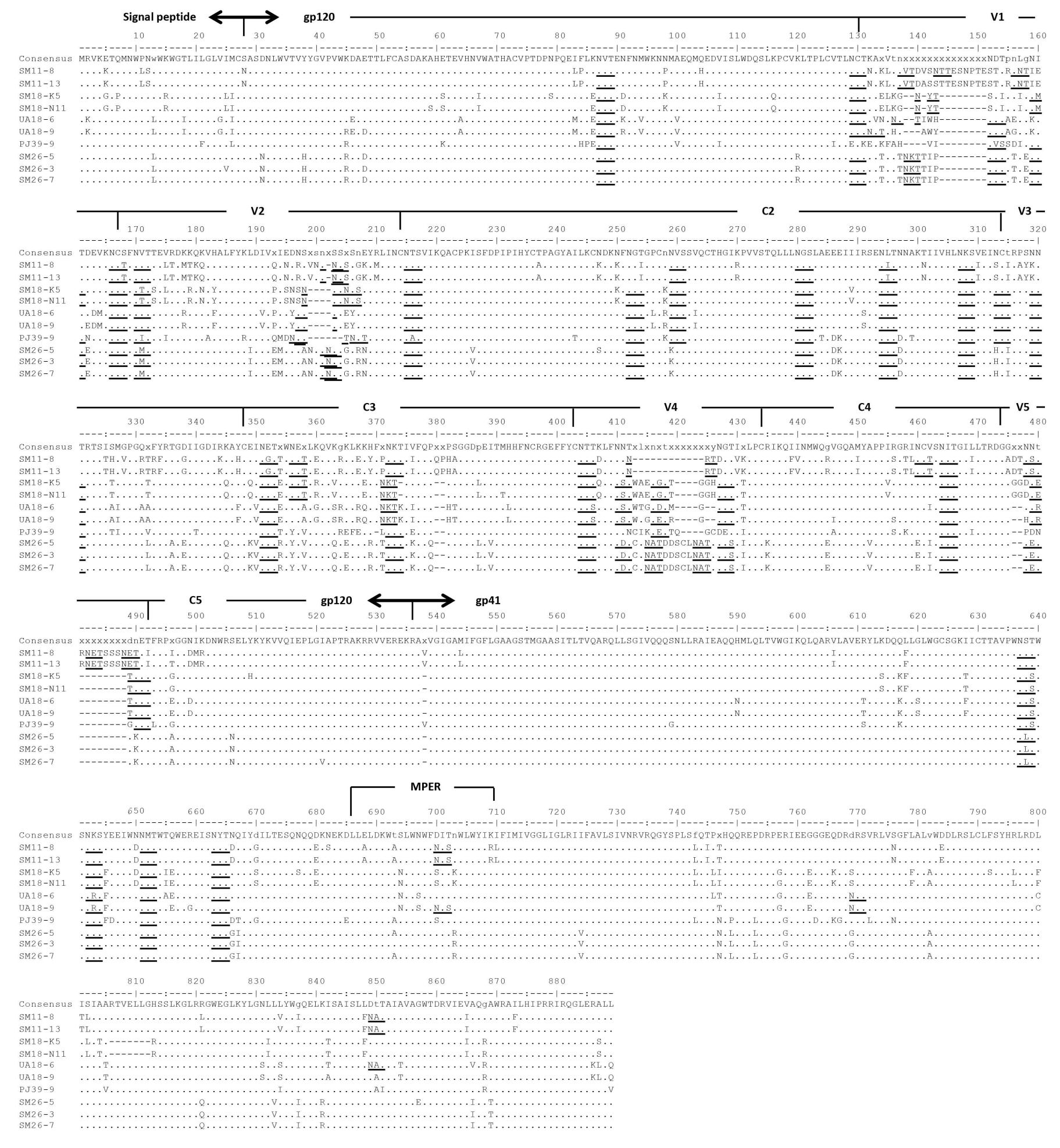
AF484509_UG57136_UG_(A1)
AF069670_SE7253_SO_(01_AE)
U51190_92UG037.1_UG_(A1)
AF516184_99TH.MU2079_TH_(15_01B)
AY173951_ BK132_TH_(B)
93
AY331295_1058_US_(B)
100
K03455_ HXB2_FR_(B)
AY423387_671_NL_(B)
92
AY253311_A280_TZ_(D)
97
U88824_94UG114_UG_(D)
100
AY371157_4412HAL_CM_(D)
89
K03454_ELI_CD_(D)
0.02


Signal peptide
gp120
V1
V2
C2
V3
C3
V4
C4
V5
C5
gp120
gp41
MPER
Consensus
SM11-8
SM11-13
SM18-K5
SM18-N11
UA18-6
UA18-9
PJ39-9
SM26-5
SM26-3
SM26-7

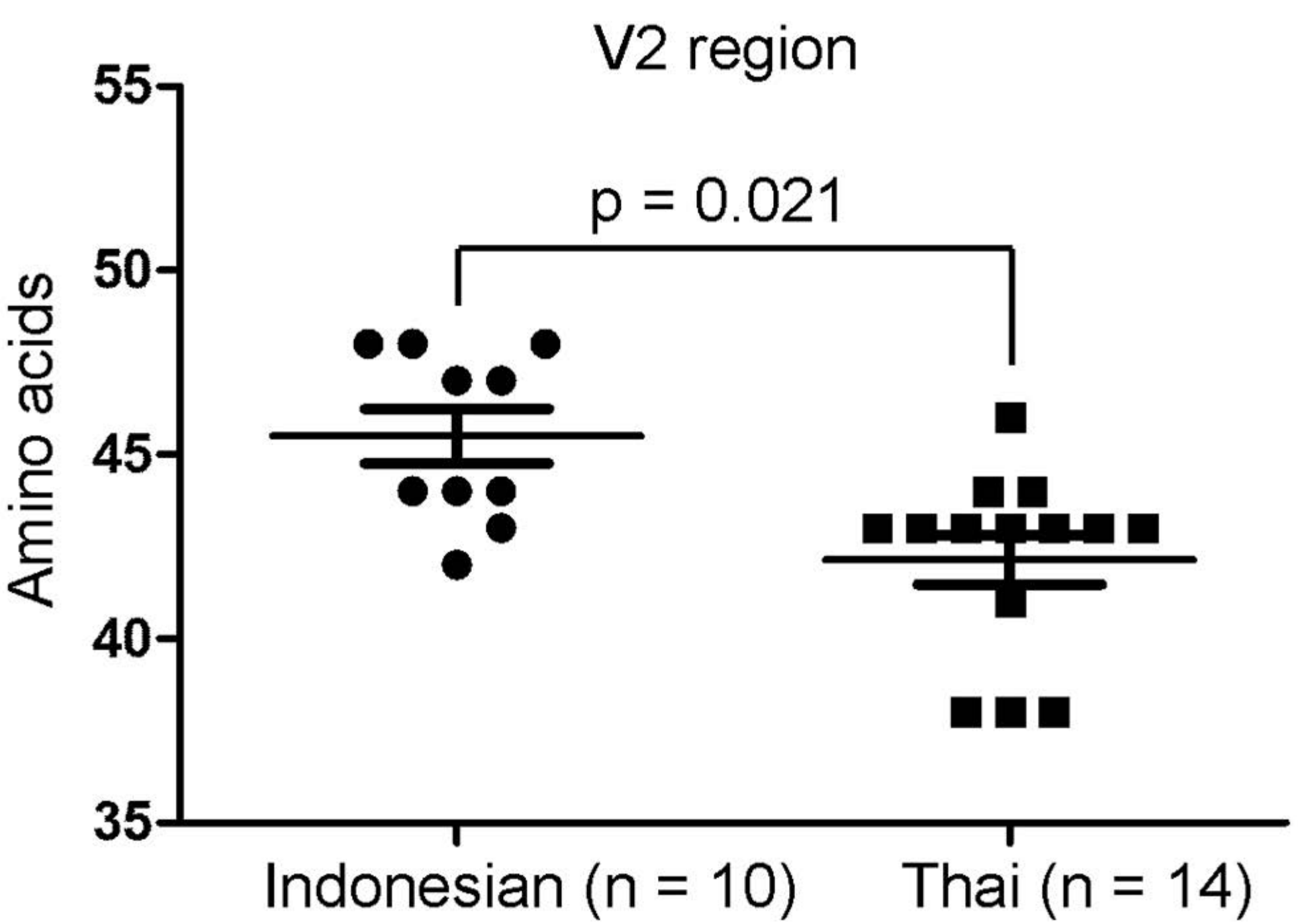

V2 region
p = 0.021
Amino acids
55
50
45
40
35
Indonesian (n = 10)
Thai (n = 14)

TABLE 1. Neutralization susceptibilities of 10 early Env-expressing lentiviral vectors to bNAbs.[a]

env gene	IC50 of bNAbs (μg/ml)[b]					
	IgG1 b12	VRC01	N6	2F5	4E10	10E8
pBa-L	0.07	0.28	0.06	4.55	7.32	0.60
SM18-K5	>10	0.23	0.10	7.84	1.69	0.25
SM18-N11	>10	0.43	0.12	2.23	2.57	1.07
SM26-3	>10	>2.5	1.42	6.46	>10	2.29
SM26-5	>10	>2.5	1.63	2.00	4.54	0.31
SM26-7	>10	>2.5	1.05	1.01	2.24	2.01
UA18-6	>10	>2.5	0.46	2.26	0.64	0.03
UA18-9	>10	>2.5	0.47	1.11	1.35	0.10

[a]The neutralization susceptibilities of Env-expressing lentiviral vectors were examined using U87.CD4.CCR5 cells.

[b]The IC50 values of bNAbs for suppressing lentiviral infection were calculated using GraphPad Prism 5 software.