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## Rapid and Simultaneous Detection of *bla*<sub>KPC</sub> and *bla*<sub>NDM</sub> by Use of Multiplex Real-Time PCR

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# Molecular Evolution of the HIV-1 Thai Epidemic between the Time of RV144 Immunogen Selection to the Execution of the Vaccine Efficacy Trial

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**The RV144 HIV-1 vaccine trial (Thailand, 2003 to 2009), using immunogens genetically matched to the regional epidemic, demonstrated the first evidence of efficacy for an HIV-1 vaccine. Here we studied the molecular evolution of the HIV-1 epidemic from the time of immunogen selection to the execution of the efficacy trial. We studied HIV-1 genetic diversity among 390 volunteers who were deferred from enrollment in RV144 due to preexisting HIV-1 infection using a multiregion hybridization assay, full-genome sequencing, and phylogenetic analyses. The subtype distribution was 91.7% CRF01\_AE, 3.5% subtype B, 4.3% B/CRF01\_AE recombinants, and 0.5% dual infections. CRF01\_AE strains were 31% more diverse than the ones from the 1990s Thai epidemic. Sixty-nine percent of subtype B strains clustered with the cosmopolitan Western B strains. Ninety-three percent of B/CRF01\_AE recombinants were unique; recombination breakpoint analysis showed that these strains were highly embedded within the larger network that integrates recombinants from East/Southeast Asia. Compared to Thai sequences from the early 1990s, the distance to the RV144 immunogens increased 52% to 68% for CRF01\_AE Env immunogens and 12% to 29% for subtype B immunogens. Forty-three percent to 48% of CRF01\_AE sequences differed from the sequence of the vaccine insert in Env variable region 2 positions 169 and 181, which were implicated in vaccine sieve effects in RV144. In conclusion, compared to the molecular picture at the early stages of vaccine development, our results show an overall increase in the genetic complexity of viruses in the Thai epidemic and in the distance to vaccine immunogens, which should be considered at the time of the analysis of the trial results.**

A major obstacle for the development of a safe and globally effective HIV vaccine is the extensive genetic diversity of the virus (1, 2). Most infections in the pandemic are due to HIV type 1 (HIV-1) group M, which is classified into nine subtypes (A to D, F to H, J, and K) differing by roughly 15% in their full-genome nucleotide sequence and up to 35% in the sequences of the gp120 envelope protein. Numerous recombinants have been characterized in the pandemic (3). Some of them have achieved wide dissemination (i.e., circulating recombinant forms [CRFs]), while others have been retrieved from only a single individual (i.e., unique recombinant forms [URFs]). HIV-1 subtypes and recombinants have distinct geographic distributions (4); for example, subtype B is the predominant strain in North America and Western Europe, while infections in South, East, and Southeast Asia are mostly due to subtypes B, C, and CRF01\_AE and their recombinants.

Due to the early and continuous participation in the planning and execution of HIV-1 vaccine trials, the Thai epidemic is one of the best studied (5). The early 1990s saw a great expansion of the HIV-1 epidemic, with a nationwide seroprevalence among young military conscripts of 3.7%, reaching 12% among those from northern Thailand (6, 7). At that time, HIV-1 strains segregated on the basis of risk factors, with subtype B predominating among injecting drug users (IDUs) and CRF01\_AE being the main clade among those with heterosexual exposure risk (8–12), though through ensuing years, CRF01\_AE has come to predominate the epidemic among IDUs as well (13–21).

From the mid-1990s to the mid-2000s, a series of molecular processes (e.g., the expansion of the genetic diversity within circulating subtypes, shifts in the proportions of different molecular forms, the emergence of intersubtype recombinants, and the influx of new strains from other epidemics) played major roles in the evolution of this epidemic. First, it was noted that the within-subtype interhost genetic divergence had increased significantly compared to that noted in earlier surveys (9, 10, 17, 22–25). Second, the barriers that maintained the segregation of subtypes on the basis of risk factors started to efface; the representation of CRF01\_AE among IDUs increased, especially among new infections (26), to the point where CRF01\_AE accounted for 80 to 90%

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of the cases in Bangkok and the northern province of Chiang Mai (27, 28). Coinfections (29) and superinfections (30) with subtype B and CRF01\_AE were documented, and intersubtype recombinants were detected among individuals with either IDU (28, 31) or heterosexual (32, 33) exposure. While most of the recombinants were URFs, a new CRF, CRF15\_01B, was found throughout the country among individuals with either risk factor, reinforcing the linkage between the different epidemics in Thailand (31, 32). Finally, the connection of the Thai HIV-1 epidemic with other epidemics in the region, through travel and trafficking of drugs and persons, has been reflected in the genetic relatedness of Thai strains to those from other countries in the Golden Triangle (i.e., a major illicit opium-producing area spanning Myanmar [Burma], Vietnam, Laos, and Thailand) (34, 35) and China (36). Subtypes A, C, D, and CRF02\_AG—which are more common in African epidemics—have been detected sporadically (37–39); however, to date there have been no indications that these strains have spread locally.

In preparation for the RV144 trial, community cohort surveys were conducted from 1998 to 2001 in Rayong and Chon Buri Provinces (i.e., the areas where the trial was later to be executed) and found that 90.5% of prevalent infections were due to CRF01\_AE, 2.4% were due to subtype B, and 7.2% were due to recombinants (including CRF15\_01B) or dual infections (40). Prospective volunteers for RV144 were enrolled in screening protocol RV148 to assess eligibility (41); those with preexisting HIV infection were deferred from enrollment in the RV144 trial and are the subject of the current study. This study attempts (i) to provide a molecular snapshot of the HIV-1 epidemic in eastern Thailand between 2003 and 2005, (ii) to correlate the viral genetic information in this cohort with the epidemiological characteristics of the volunteers for RV144, and (iii) to determine the genetic relationship between the immunogens incorporated in the vaccine and the viral strains that challenged the trial participants. The HIV-1 strains characterized in RV148 were drawn at the same time, from the same location, and from the same social networks as those in the RV144 trial, and they represent one of the closest descriptions we may have to the HIV-1 strains to which the RV144 participants might have been exposed during the trial. This study is complementary to the recent work of Rolland et al. (42) that describes a variable region 2 (V2)-delimited sieve analysis of HIV-1 strains infecting RV144 trial participants, as it describes viral strains that are the nearest possible approximations to the viral strains to which the placebo and vaccine recipients in RV144 might have been exposed. The thorough analysis of near-full-length RV144 breakthrough viruses is the focus of a separate report (M. Rolland, unpublished data).

## MATERIALS AND METHODS

**Population under study.** From September 2003 to December 2005, 26,676 volunteers in Rayong and Chon Buri Provinces in East Thailand who consented for the RV144 trial were assessed for eligibility in the setting of the screening protocol RV148. One hundred twenty-eight individuals were not referred for protocol-defined HIV testing because they met at least one exclusion criterion and/or failed to meet at least one inclusion criterion. Out of the 26,548 volunteers who were tested for HIV, 418 had HIV infection (see reference 41 for details on the testing reagents and algorithms), were counseled and referred to designated Ministry of Public Health (MOPH) hospitals for evaluation of the status of their infection, and were considered for standard treatment and care on the basis of the published Thai MOPH guidelines (43; see reference 41 for details on

counseling). Samples from 390/418 individuals were available for HIV-1 genotyping. CD4<sup>+</sup> T cell enumeration was performed using standard flow cytometry (FACSCalibur; Becton, Dickinson, Palo Alto, CA), and HIV-1 RNA quantification was performed using reverse transcription-PCR technology (Roche Amplicor version 1.5 test; Roche Diagnostics, Indianapolis, IN).

**Ethical review.** The screening protocol RV148 was reviewed and approved by the ethical committees of the Thai MOPH, the Royal Thai Army, Mahidol University, and the Human Subjects Research Review Board of the U.S. Army Medical Research and Materiel Command. The study was conducted in accordance with U.S. and Thai FDA regulations as well as the International Conference on Harmonisation (ICH) technical requirements for registration of pharmaceuticals for human use guideline on good clinical practice (GCP) for clinical trials, guideline E6 (<http://www.ich.org/products/guidelines/efficacy/article/efficacy-guidelines.html>).

**HIV-1 genotyping.** Viral RNA was extracted from serum samples by robotic extraction (MagnaPure; Roche Diagnostics), and the determination of HIV-1 subtype and recombinants was achieved through a multi-region hybridization assay (MHAbce) of the whole available sample set and near-full-genome amplification and sequencing of a subset of samples.

(i) **MHAbce version 2.** The presence of infections with subtypes B, C, and CRF01\_AE, and recombinants and dual infections combining these strains was evaluated using MHAbce version 2, as previously published (32). Briefly, 8 regions throughout the HIV-1 genome (p17 in *gag*; protease, reverse transcriptase, and integrase in *pol*; the first exon of *tat*; gp120 and gp41 in *env*; and *nef*) were reverse transcribed and PCR amplified with universal primers. Subsequently, each region was subject to real-time PCRs with respective fluorescent TaqMan probes specific for subtypes B, C, and CRF01\_AE. Samples with concordant patterns of probe reactivity throughout the genome were assigned to the corresponding subtype or CRF, while those samples showing discordant patterns of probe reactivity were considered intersubtype recombinants. Patients with samples exhibiting dual-probe reactivity in the same genome region were considered dually infected after verification by cloning and real-time PCR subtyping of at least 20 clones per sample. Using typeability criteria determined at assay validation, only samples showing probe reactivity in 4 or more genome regions were considered typed (32).

(ii) **Near-full-genome amplification and sequencing.** Near-full-genome HIV-1 sequences were obtained as either a complete HIV-1 genome or two half genomes overlapping by 1.5 kb, as previously described (44).

**Phylogenetic analyses.** Alignments of RV148 near-full-genome HIV-1 sequences and subtype and CRF reference sequences (3) were obtained using the HIVALign program (<http://www.hiv.lanl.gov/content/sequence/VIRALIGN/viralalign.html>) and were manually edited with BioEdit version 7.0.9.0 software (45). RV148 sequences were assigned to the corresponding subtype or CRF through phylogenetic analyses of nucleotide sequences conducted in the MEGA5 program (46) using maximum likelihood trees; bootstrap values supporting relevant clusters were computed through the Kimura 2-parameter model (47) and neighbor-joining trees (48) with 1,000 iterations. The structure of recombinant forms was determined using the jumping profile hidden Markov model (jpHMM; <http://jpHMM.gobics.de/>) (49) and was confirmed through subgenomic phylogenetic analysis. The location of all the recombination breakpoints is reported in the HXB2 coordinate system.

Nucleotide sequence diversity was determined through a maximum likelihood estimation using the general reversible model, as implemented in *Hypothesis Testing Using Phylogenies* (HyPhy) (50). The distance of the deduced protein sequences of RV148 immunogens and published sequences from East and Southeast Asia to RV144 immunogens was computed using the p-distance, Poisson (51), Jones-Taylor-Thornton (JTT) (52), Dayhoff (53), and HIV-Between (54) models implemented in MEGA5 and HyPhy. None of these models was specifically tailored to weigh the impact of amino acid substitution on immune recognition. For the current analysis, we selected the p-distance model, which is the one

that makes the fewest *a priori* assumptions, as we found that it had a very strong linear correlation with the more complex models (range of  $r^2$  values, 0.9557 to 0.9999) (data not shown).

**Network analysis.** The sharing of common recombination breakpoints between RV148 intersubtype recombinants and published HIV-1 strains from East and Southeast Asia was studied using the RecDraw program (55), allowing 27 bp of mapping imprecision (i.e., the average distance between the jpHMM-predicted and previously published breakpoints, as determined by Schultz et al. [49]). Network diagrams were built using the UCINET version 6.275/NetDraw version 2.091 (Analytic Technologies, Lexington, KY) and NodeXL (Social Media Research Foundation; <http://www.smrfoundation.org>) programs.

**Statistics.** Differences in the distribution of gender, HIV-1 subtypes, recombinants, and dual infections across sociodemographic characteristics were assessed using the chi-square test and Fisher's exact test (if the expected cell count was less than 5) for categorical variables and through the Wilcoxon rank sum test for continuous variables; crude odds ratios and 95% confidence intervals (CIs) were calculated using JMP version 10.0 (SAS Institute, Cary, NC). JMP was also employed to assess differences in genetic diversity among time intervals and country of sampling using the Wilcoxon rank sum test and to assess the linear fit between genetic distances, computed by least-squares regression using different models. All of the presented *P* values are unadjusted for multiple comparisons.

**Nucleotide sequence accession numbers.** The HIV-1 sequences obtained in this study have been deposited in GenBank under accession numbers JN248316 to JN248357.

## RESULTS

**Sociodemographic characteristics of the population under study.** In order to determine eligibility for enrollment in RV144, volunteers were first enrolled in the screening protocol RV148. Out of 26,548 volunteers who were tested for HIV, 418 individuals (1.57%) had a confirmed HIV infection and were deferred from enrollment in RV144 (see Materials and Methods) (56). The sociodemographic risk factors for HIV seropositivity have been previously reported (41) and included female sex, older age, lower education, widowed/divorced/separated marital status, and fisherman/sex worker/entertainment worker self-reported occupation.

Samples from 390/418 volunteers who were ineligible for RV144 because HIV infection was detected during screening were available for HIV-1 genotyping. Table 1 shows their sociodemographic and clinical characteristics. Among the seropositive volunteers, comparable numbers of female ( $n = 192$ ) and male ( $n = 198$ ) individuals were represented. Statistically significant differences in sociodemographic characteristics between male and female volunteers were observed. Male seropositive volunteers were enriched for transgender individuals ( $P < 0.001$ ), volunteers who were single ( $P < 0.001$ ), volunteers who had completed at least senior high school ( $P = 0.02$ ), or volunteers who were office/factory workers ( $P = 0.02$ ). Among females there was enrichment in volunteers who had not completed primary school ( $P = 0.03$ ) or who were married ( $P < 0.001$ ), divorced ( $P < 0.001$ ), entertainment workers ( $P = 0.04$ ), or housewives ( $P < 0.001$ ) (data not shown). The distribution of plasma viral load (pVL) and CD4 cell counts did not differ significantly between males and females (data not shown).

**HIV-1 subtype distribution among RV148 seropositive individuals.** Out of the 390 specimens tested with MHAbce, 375 samples (96.2%) showed probe reactivity in 4 or more regions, which was the preestablished typeability requirement (32) (see Materials and Methods). The main reason for untypeability was undetect-

**TABLE 1** Sociodemographic and clinical characteristics of screened volunteers who were deferred from enrollment in the RV144 vaccine trial due to preexisting HIV infection

Characteristic	Female ( $n = 192$ )	Male ( $n = 198$ )	Total ( $n = 390$ )
No. (%) of volunteers by gender			
Female	192 (100)	10 (5.1)	202 (51.8)
Male	0 (0)	187 (94.4)	187 (48.0)
Missing	0 (0)	1 (0.5)	1 (0.2)
Age (yr)			
Median	25	26	26
Interquartile interval	22–28	23–27	22–28
No. (%) of volunteers by the following characteristic:			
Birthplace			
Chon Buri	44 (22.9)	52 (26.3)	96 (24.6)
Rayong	45 (23.4)	44 (22.2)	89 (22.8)
Other	103 (53.7)	102 (51.5)	205 (52.6)
Screening site			
Chon Buri			
Panthong	8 (4.2)	16 (8.1)	24 (6.2)
Sri Racha	11 (5.7)	17 (8.6)	28 (7.2)
Bang Lamung	48 (25.0)	54 (27.3)	102 (26.1)
Sattahip	30 (15.6)	23 (11.6)	53 (13.6)
Rayong			
Ban Chang	12 (6.3)	20 (10.1)	32 (8.2)
Mueang	23 (12.0)	29 (14.6)	52 (13.3)
Ban Khai	23 (12.0)	19 (9.6)	42 (10.8)
Klaeng	37 (19.2)	20 (10.1)	57 (14.6)
Marital status			
Single	21 (10.9)	97 (49.0)	118 (30.2)
Married	123 (64.2)	89 (44.9)	212 (54.4)
Separated	2 (1.0)	0 (0.0)	2 (0.5)
Divorced	40 (20.8)	11 (5.6)	51 (13.1)
Widowed	6 (3.1)	1 (0.5)	7 (1.8)
Education			
Did not complete primary school	13 (6.8)	4 (2.0)	17 (4.3)
Primary school	107 (55.8)	99 (50.0)	206 (52.9)
Junior high school	54 (28.1)	60 (30.3)	114 (29.2)
Senior high school/vocational school	15 (7.8)	28 (14.1)	43 (11.0)
Vocation school	2 (1.0)	5 (2.5)	7 (1.8)
Bachelor's degree or higher	1 (0.5)	2 (1.0)	3 (0.8)
Occupation			
Laborer	56 (29.1)	62 (31.3)	118 (30.2)
Office/factory	35 (18.2)	57 (28.8)	92 (23.6)
Merchant/business	15 (7.8)	17 (8.6)	32 (8.2)
Housewife	26 (13.5)	0 (0.0)	26 (6.7)
Entertainment	15 (7.8)	6 (3.0)	21 (5.4)
Agriculture	8 (4.2)	10 (5.1)	18 (4.6)
CSW	8 (4.1)	9 (4.5)	17 (4.4)
Fishery	3 (1.6)	5 (2.5)	8 (2.0)
Government officer	1 (0.5)	4 (2.0)	5 (1.3)
Military/police	0 (0)	4 (2.0)	4 (1.0)
Student	0 (0)	3 (1.5)	3 (0.8)
Other	0 (0)	2 (1.0)	2 (0.5)
Unemployed	25 (13.0)	19 (9.6)	44 (11.3)
Plasma viral load (no. of RNA copies/ml)			
Median	30,371	33,841.5	32,919
Interquartile interval	7,137–121,999	14,518.8–127,640	9,629–124,361
No. (%) of patients with VLs of <400 copies/ml	8 (4.1)	6 (3.0)	14 (3.6)
CD4 <sup>+</sup> cell count (no. of cells/ $\mu$ l)			
Median	401	397.5	399
Interquartile interval	260–568.75	276.75–583.25	272–577.5
No. (%) of patients with CD4 counts of:			
<200 cells/ $\mu$ l	33 (17.1)	27 (13.6)	60 (15.3)
<350 cells/ $\mu$ l	73 (37.8)	77 (38.9)	150 (38.4)

able pVL (data not shown). Results from MHAbce showed a predominance of infections due to CRF01\_AE (90.2%), followed by B/CRF01\_AE recombinants (6.1%) and subtype B (3.2%) (Table 2). Two cases showed dual-probe reactivity for subtype B and CRF01\_AE. Additionally, among the untyped samples, there was one specimen showing probe reactivity with subtype C probes in two regions and with CRF01\_AE probes in one region. A near-

TABLE 2 HIV clade distribution among 375 volunteers deferred from enrollment in the RV144 vaccine trial due to preexisting HIV infection, genotyped by MHAbce and full-genome sequencing

Clade	No. (%) of volunteers	
	MHAbce only	MHAbce with sequencing confirmation
CRF01_AE	338 (90.2)	344 (91.7)
Subtype B	12 (3.2)	13 (3.5)
B/CRF01_AE recombinants	23 (6.1)	16 (4.3)
B/CRF01_AE dual infection	2 (0.5)	2 (0.5)

full-genome sequence was retrieved, revealing that the sample corresponded to subtype C, clustering within the African radiation (data not shown). All typeable samples showing reactivity with subtype B probes in at least one region were subject to near-full-genome sequencing

(Fig. 1). All 12 samples identified as pure subtype B by MHAbce showed a concordant subtype designation by near-full-genome sequencing. Out of the 23 samples identified as B/CRF01\_AE recombinants by MHAbce, 16 were confirmed to be recombinants by sequencing, while 1 and 6 strains were determined by near-full-genome sequencing to be pure subtype B and CRF01\_AE strains, respectively. Detailed inspection of near-full-genome sequences revealed that the main reason for heterotypic probe reactivity in MHAbce was the presence of infrequent polymorphisms in the probe binding sites, which had not been anticipated in the design of the assay (32). The subtype distribution combining MHAbce- and near-full-genome sequencing-based genotyping is shown in Table 2. Overall, CRF01\_AE strains accounted for 91.7% of the infections, and pure subtype B strains were only slightly outnumbered by

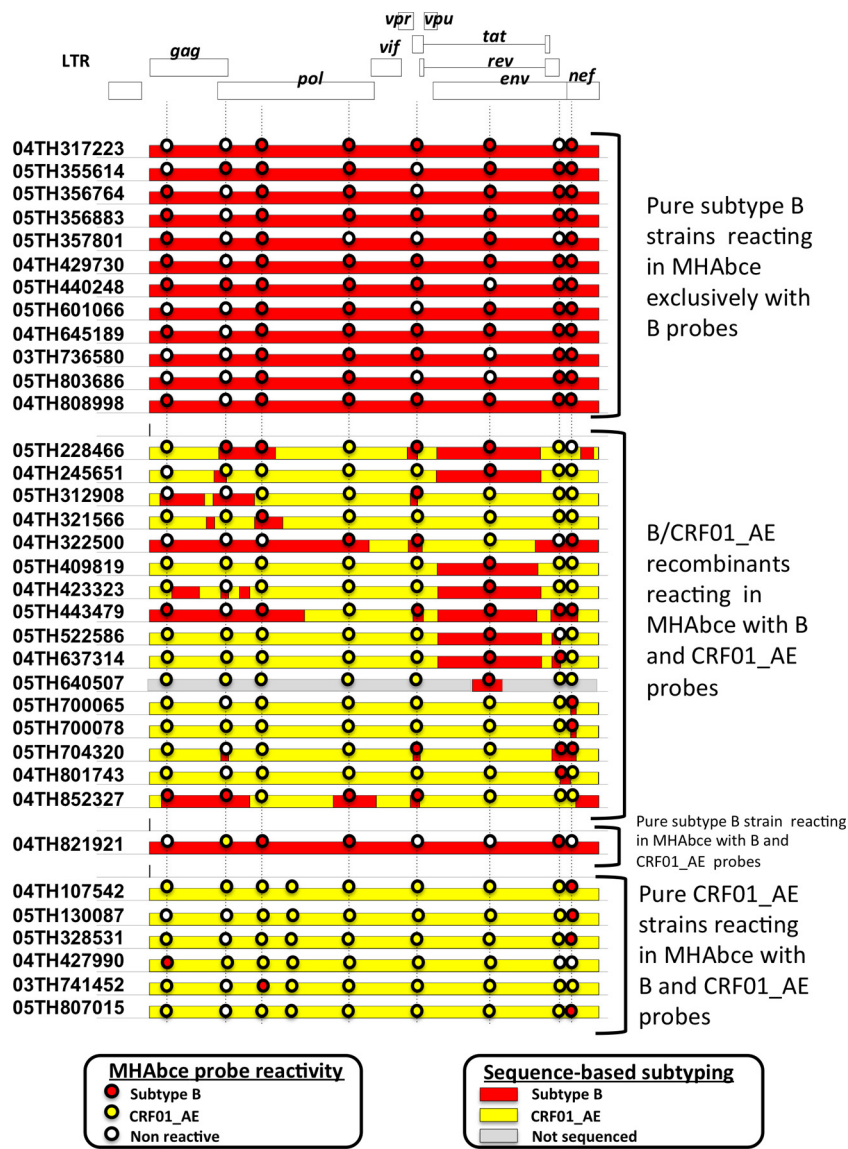
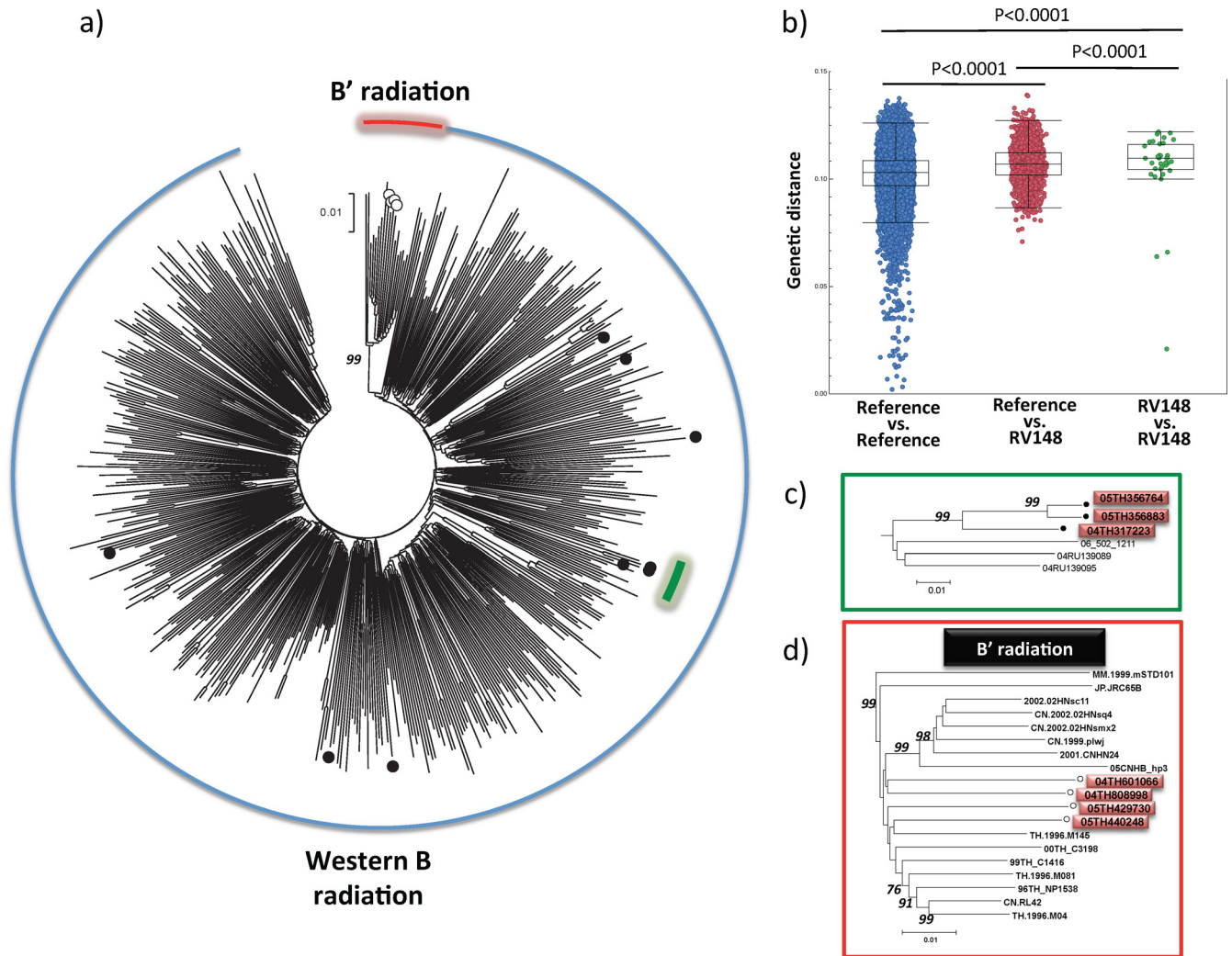


FIG 1 MHAbce reactivity pattern and genomic structure of typed samples showing reactivity with subtype B probes in one or more regions. Color-coded horizontal bars represent the genomic structure based on near-full-genome sequencing, and the circles depict the corresponding MHAbce probe reactivity. Vertical dotted lines indicate the genomic locations of the MHAbce probes. For sample 05TH640507, only a subgenomic sequence was retrieved (HXB2 residues 6991 to 7573). LTR, long terminal repeat.



**FIG 2** Location of RV148 subtype B strains in the global subtype B phylogeny. (a) Black circles depict RV148 B strains from the Western B radiation, and hollow circles depict RV148 strains from the B' radiation. (b) Distribution of pairwise genetic distance within the cosmopolitan Western B epidemic (blue circles), among RV148 sequences from the Western B radiation (green circles), and between these two groups (red circles). This analysis was based on near-full-genome sequences from 344 cosmopolitan Western B strains that were sampled between 2000 and 2007. (c) Detail of a trio of RV148 strains with low genetic diversity (red boxes). (d) Subtree of the B' radiation, where the RV148 strains are highlighted (red boxes). The phylogenetic trees were built using near-full-genome sequences. Numbers on the branches of the phylogenetic trees represent the percentage of bootstrap replicates supporting the relevant clusters.

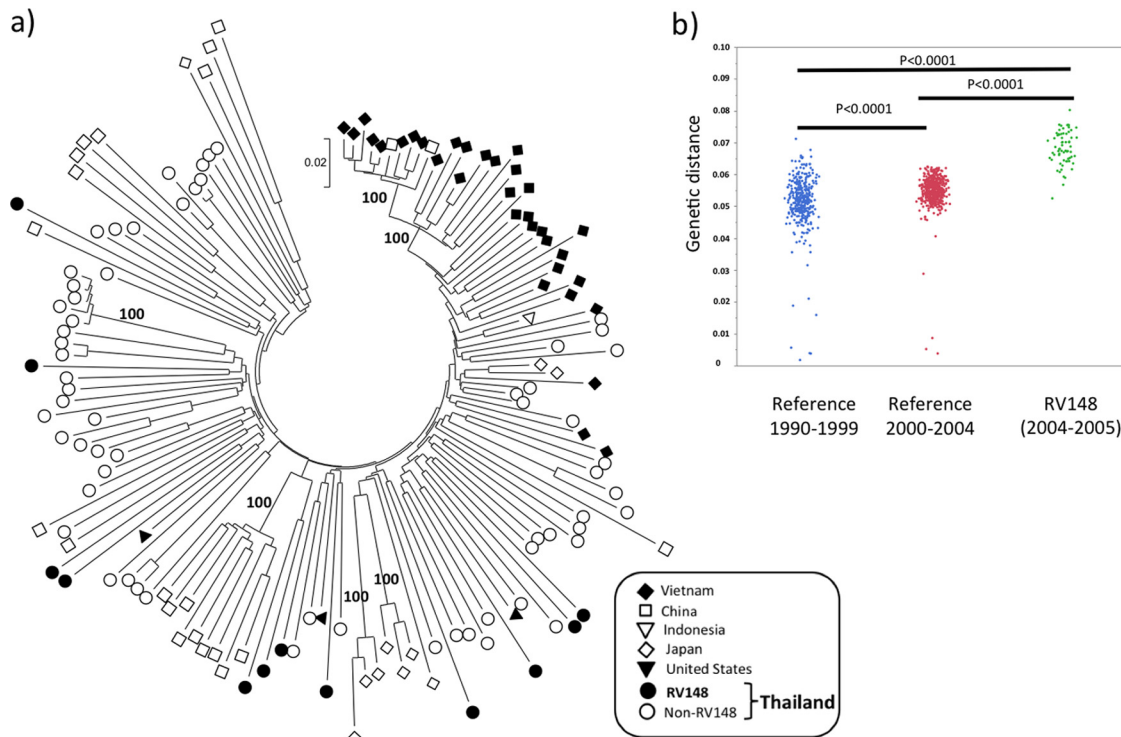
B/CRF01\_AE intersubtype recombinants (3.5% and 4.3%, respectively). While B/CRF01\_AE dual infections were detected, they were very infrequent (0.5%).

**Analysis of near-full-genome sequences. (i) Subtype B.** Sequences from the 13 subtype B samples were analyzed (Fig. 2). The subtype B strains circulating in Southeast Asia can be subclassified into two different clades on the basis of their genetic sequences and especially their motif at the crown of the V3 loop in gp120: sequences clustering with strains from the Americas and Western Europe carrying the GPGR motif in V3 (which are designated Western B throughout the text) and a separate cluster of sequences identified primarily in Southeast Asia and China and carrying the GPGQ motif in V3, referred to in the literature as B' or Thai B (57, 58). In RV148, 9 sequences clustered with strains from the Western B radiation, while the remaining 4 sequences clustered within the B' radiation (Fig. 2a). There were no significant differences in the distribution of sociodemographic characteristics between car-

riers of Western B and B'; however, there was a trend toward a higher prevalence of Western B strains in the Bang Lamung area of Chon Buri Province that borders on Pattaya (5/9 for Western B versus 0/4 for B',  $P = 0.10$ ), an international tourist destination.

The genetic diversity among RV148 Western B strains (median, 0.11; interquartile range [IQR], 0.105 to 0.116) was only slightly higher than the genetic diversity between RV148 and contemporaneous reference Western B strains (median, 0.107; IQR, 0.102 to 0.112;  $P < 0.0001$ ) or among contemporaneous reference Western B strains (median, 0.103; IQR, 0.097 to 0.109;  $P < 0.0001$ ) (Fig. 2b). Overall, these results support the hypothesis of multiple introductions of Western B strains in the area. The genetic diversity observed among RV148 B' strains (median, 0.069; IQR, 0.068 to 0.070; data not shown) was significantly lower than the one observed among Western B RV148 strains ( $P = 0.001$ ).

Among the Western B strains, there was a pair of sequences (05TH356764 and 05TH356883) that showed a high level of se-



**FIG 3** Genetic diversity of near-full-genome CRF01\_AE sequences in RV148. (a) Location of CRF01\_AE RV148 strains in the context of the Asian CRF01\_AE radiation. The different symbols depict the country of sampling of each sequence, as indicated in the box. Sequences sampled in the United States, but likely acquired in Southeast Asia (85), were also included. Numbers on the branches of the phylogenetic tree represent the percentage of bootstrap replicates supporting the relevant clusters. (b) Distribution of pairwise nucleotide genetic distances among CRF01\_AE strains in different time periods in the history of the Thai HIV-1 epidemic: 1990 to 1999 ( $n = 30$ ) and 2000 to 2004 ( $n = 32$ ).

sequence identity (distance, 0.021) (Fig. 2c). They were both sampled in the same site 2 weeks apart. Neither the remaining Western B RV148 strains nor the four strains from the B' radiation showed a high level of sequence identity to other strains from the same cohort or to reference strains (Fig. 2d).

(ii) **CRF01\_AE.** CRF01\_AE sequences from 12 individuals were analyzed, including the 6 pure CRF01\_AE strains depicted in Fig. 1 and 6 randomly selected specimens (Fig. 3a). The median genetic distance among these sequences was 0.068 (IQR, 0.065 to 0.073). The genetic diversity among CRF01\_AE RV148 strains was significantly higher than the genetic diversity in Thailand from 1990 to 1999 (median, 0.052; IQR, 0.049 to 0.056;  $P < 0.0001$ ) and 2000 to 2004 (median, 0.055; IQR, 0.053 to 0.058;  $P < 0.0001$ ), indicating that the Thai CRF01\_AE epidemic has undergone a significant expansion in the past 2 decades (Fig. 3b); no contemporaneous reference full-genome CRF01\_AE sequences from Thailand were available for comparison. None of the sequenced RV148 strains showed high similarity with either reference or other RV148 sequences. Overall, the RV148 sequences were distributed throughout the East and Southeast Asian CRF01\_AE radiation, intermingling with the sequences sampled throughout the region.

(iii) **B/CRF01\_AE recombinants.** In RV148, by combining MHAbce and sequencing, we were able to identify 16 recombinants, all of them between subtype B and CRF01\_AE; near-full-genome sequences were generated for 15 of them (Fig. 1). CRF01\_AE comprised the majority of the genomic content of the RV148 intersubtype recombinants (Fig. 4); 13/15 sequences had a

CRF01\_AE genomic content greater than 50% (range, 59.0% to 98.8%). Some genomic areas were CRF01\_AE in all of the recombinants (e.g., *vif* and *vpu*), while in other genomic areas (e.g., *gp120* in *env*) there were equivalent numbers of strains carrying either clade. In 14/15 recombinants, the subtype B components belonged to the B' radiation. Strain 04TH322500 was the only intersubtype recombinant whose subtype B content was contributed by the Western B radiation (data not shown). The number of breakpoints observed among RV148 B/CRF01\_AE recombinants did not differ significantly from the number observed among previously described B/CRF01\_AE recombinants (data not shown).

The genomic structures of intersubtype recombinants isolated in RV148 were compared to those of recombinants containing subtypes B and CRF01\_AE from the published literature. All of the RV148 recombinants, with the exception of strain 04TH801743, shared at least one breakpoint with previously reported CRFs and URFs. Strain 04TH409819 was the only B/CRF01\_AE recombinant that shared a full pattern of recombination breakpoints with a previously described CRF, CRF15\_01B, which has been found in Thailand in individuals attending antenatal/family planning clinics in Lampang and Rayong Provinces (32, 40), an IDU attending an opiate treatment clinic in Chiang Mai (59), and a female commercial sex worker (CSW) in Bangkok (59) (Fig. 5). There was a group of six additional strains (i.e., 04TH228266, 05TH245651, 04TH423323, 04TH443479, 05TH522586, and 05TH637314) that shared both of the CRF15\_01B recombination breakpoints in the envelope region; all of these recombinants incorporated additional breakpoints in other genomic areas.

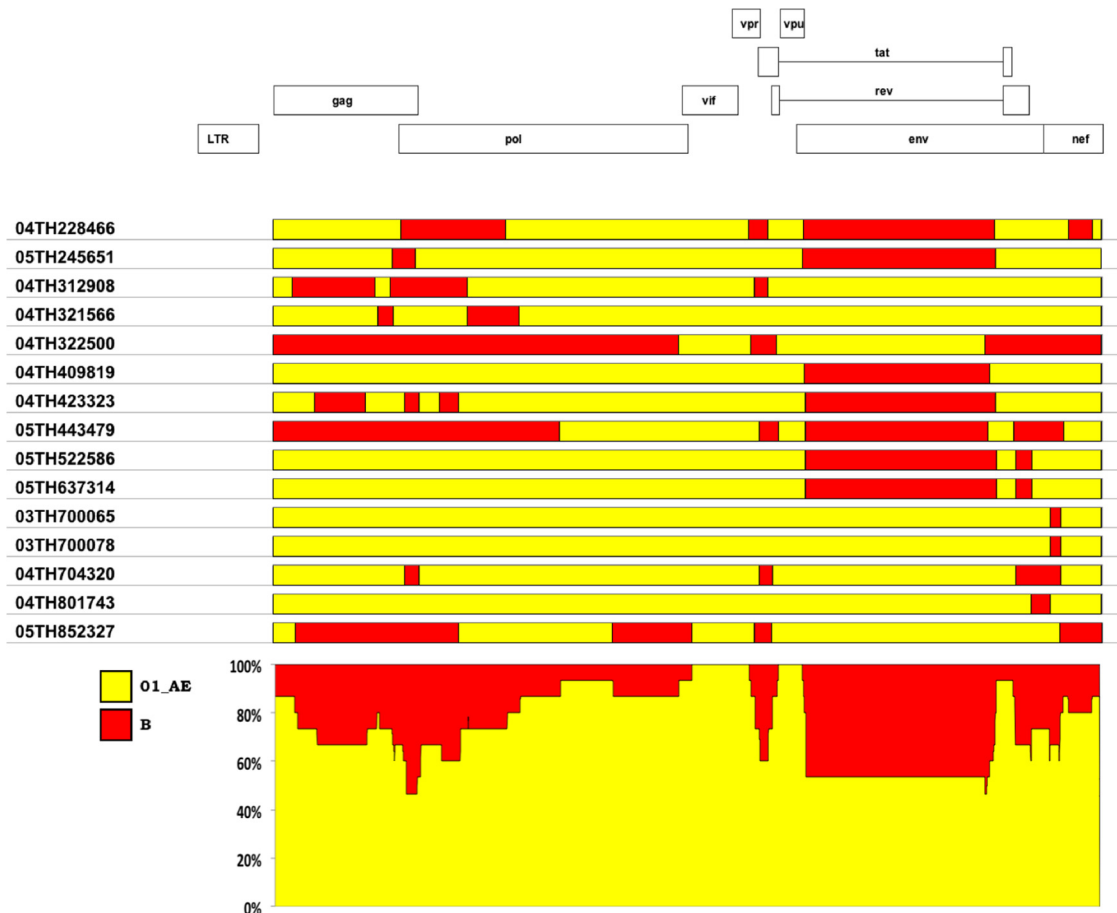


FIG 4 CRF01\_AE comprises the majority of the genomic content of B/CRF01\_AE recombinants in RV148. On the basis of recombination breakpoint mapping, the subtype composition of all the recombinants was added at each genomic position. Some areas showed equivalent contributions from CRF01\_AE and subtype B (e.g., gp120 in *env*), while other areas were predominantly CRF01\_AE (e.g., *vif*).

There were two pairs of B/CRF01\_AE recombinants that shared their complete recombination patterns. Strains 05TH522586 and 05TH637314 were mostly CRF01\_AE and contained two subtype B segments in *env* (Fig. 1). These two segments corresponded, respectively, to the gp120 segment present in CRF15\_01B and the gp41/second exon of the *rev* segment present in CRF34\_01B. The genetic distance between these two strains was 0.039. The volunteers from whom these strains were recovered were screened at different sites located 12 km apart in Rayong Province, and their visits occurred 6 months apart. The other pair of strains sharing their complete recombination pattern was 03TH700065 and 03TH700078, which were composed of a single 109-bp subtype B fragment in *nef* embedded in a CRF01\_AE background. The genetic distance between these two strains was 0.026. This recombinant structure was fully shared with strain 02TH.OUR840I, which had been retrieved from a 2002 incident infection within an IDU cohort in Chiang Mai, Thailand (28); the genetic distances from 02TH.OUR840I to 03TH700065 and 03TH700078 were 0.055 and 0.058, respectively. Individuals 03TH700065 and 03TH700078 were screened on the same day at the same site in Rayong Province, and the possibility of a direct epidemiological linkage between them cannot be excluded. For this reason, and despite the shared recombination pattern with 02TH.OUR840I, this recombinant should not be designated a CRF at this time.

#### Location of RV148 intersubtype recombinants in the context of the East and Southeast Asian HIV-1 recombinant network.

The irreversible nature of HIV-1 recombination makes recombination breakpoints extremely useful markers in molecular epidemiology studies; recombinants are generated within multiply infected individuals, and when transmitted to new individuals, their genomic structure is preserved as a stable form. Additionally, transmitted recombinants may further recombine with other strains in subsequent multiply infected individuals, and some of the breakpoints from the parental strains may be preserved. We have previously shown that if shared recombination breakpoints are used to compare different sequences and strains that share common recombination breakpoints are grouped together, the vast majority of the recombinants sampled in East and Southeast Asia are part of a single, structured, and intricate network (55). Here we analyzed the RV148 recombinants in the backdrop of the network composed of all the reported recombinants containing subtypes B and CRF01\_AE (Fig. 6). RV148 strains were an integral part of the larger East/Southeast Asian network. With the exception of strain 04TH801743, all of the RV148 strains were linked to at least one reported strain. RV148 recombinants were directly linked to reported recombinants sampled in diverse countries (i.e., Malaysia, Myanmar, Singapore, and Thailand) and from individuals with diverse reported risk factors for HIV-1 infection (i.e., IDU and sexual contact). While there were clear connections

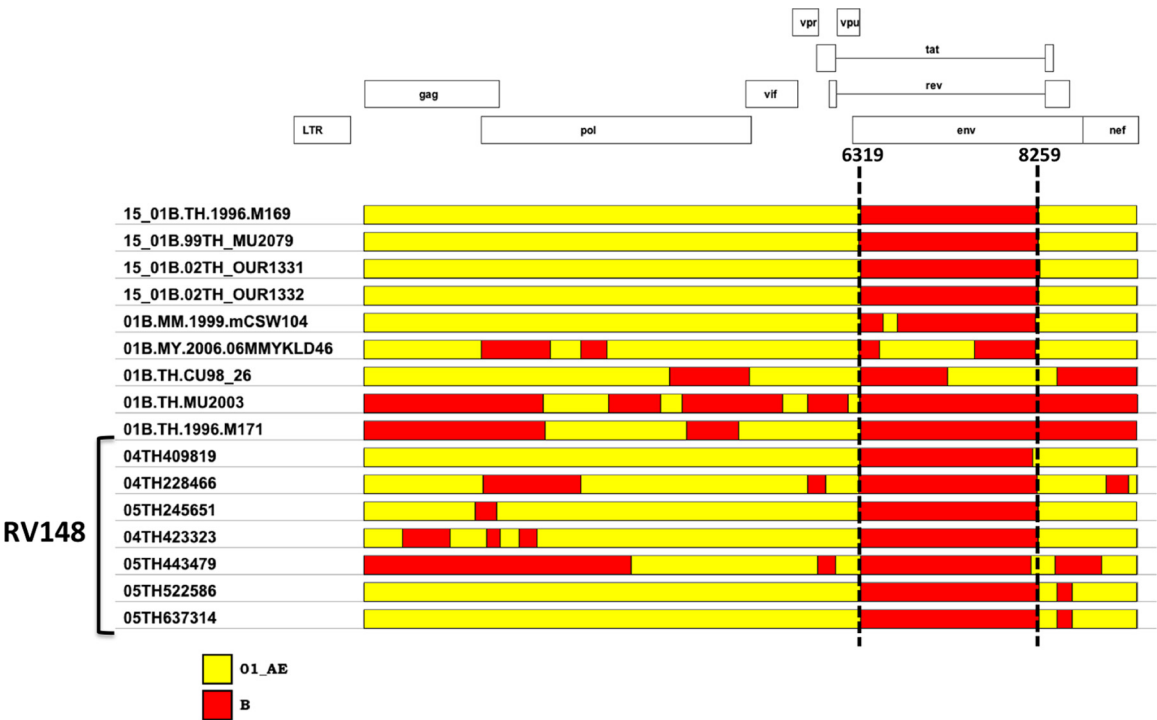


FIG 5 Seven RV148 B/CRF01\_AE intersubtype recombinants share common recombination breakpoints with CRF15\_01B and with other published Southeast Asian recombinants. The vertical dashed lines indicate the location of CRF15\_01B breakpoints in the HXB2 coordinate system. See the text for details.

linking RV148 strains to other strains from the same cohort, RV148 strains showed a higher level of connection to reference strains ( $P = 0.001$ ; data not shown).

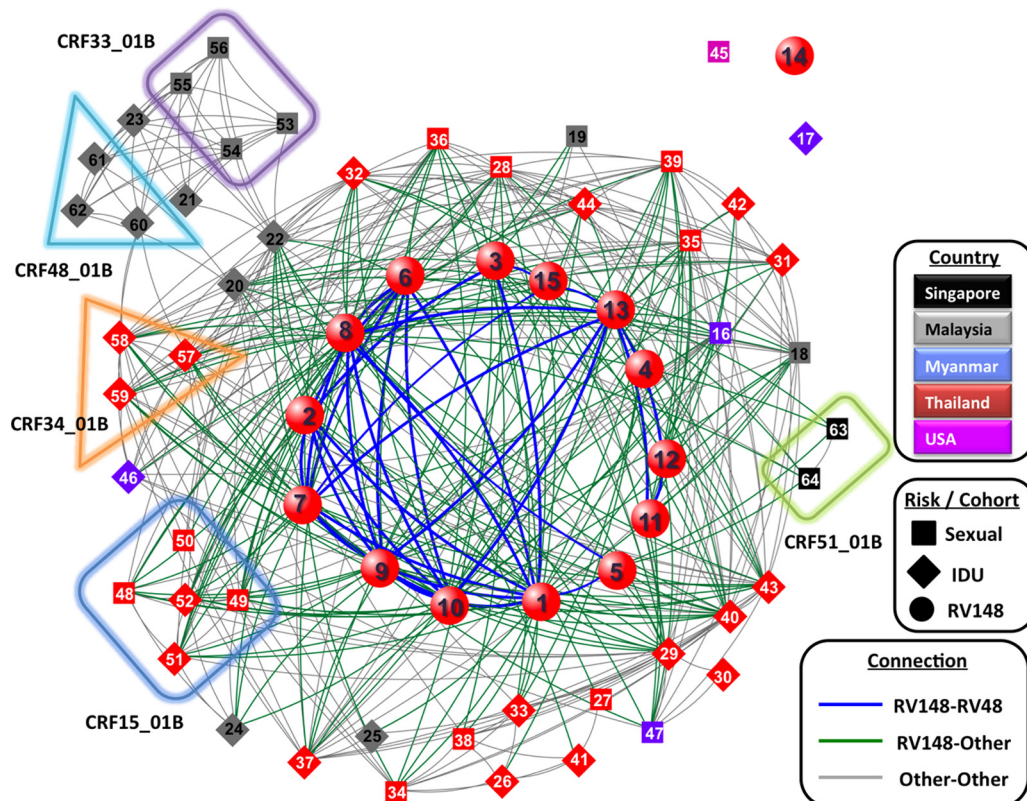
**HIV-1 subtype distribution and its association with sociodemographic variables.** In East Africa and Thailand, it has previously been reported that sociodemographic characteristics can be associated with more genetically complex HIV-1 epidemics (60–66). In the current study, we explored the association between recorded sociodemographic variables and the infecting HIV-1 strain. There were no statistically significant differences in the distribution of subtypes and recombinants on the basis of sex, gender, age, birthplace, marital status, education level, or occupation (Table 3).

**Genetic distance of RV148 strains to immunogens from RV144 regimen.** The cocirculation in Thailand of subtype B, CRF01\_AE, and their recombinants poses an added level of difficulty to the process of HIV-1 vaccine development, and the incorporation of subtype B and CRF01\_AE immunogens into vaccine candidates was implemented early on (reviewed in reference 67). However, genetic changes in the local epidemic occurring in the time span elapsing from immunogen selection to vaccine testing might have had profound implications for the success of vaccine trials and need to be considered in the analysis of vaccine efficacy. Here we assessed the genetic distance of the RV148 protein sequences to the different immunogens, and we analyze these results in the context of the evolving HIV-1 epidemics in East and Southeast Asia (Fig. 7).

**(i) Distance to CRF01\_AE Env immunogens: 92TH023 gp120 linked to LAI gp41 (ALVAC-HIV) and CM244 gp120 (AIDSVAX B/E).** Figures 7a and b depict the genetic distance of 21 RV148 strains—13 CRF01\_AE B and 8 B/CRF01\_AE recombinants

whose corresponding genomic region is CRF01\_AE—along with those of 284 CRF01\_AE reference sequences sampled in Thailand, China, and Vietnam between 1990 and 2009 to the CRF01\_AE Env immunogens. The median distances of RV148 strains to the CRF01\_AE ALVAC-HIV and AIDSVAX B/E Env immunogens were 0.136 (IQR, 0.127 to 0.145) and 0.155 (IQR, 0.142 to 0.163), respectively. Interestingly, when analyzing sequences sampled in Thailand from 1990 to date, there is a clear, progressive, and significant increase in the distance to both CRF01\_AE Env immunogens: the median distances of sequences from 1990 to 1994 to ALVAC-HIV and AIDSVAX B/E were 0.081 (IQR, 0.071 to 0.108) and 0.102 (IQR, 0.081 to 0.120), respectively (see Fig. S1a and b in the supplemental material). These represent significant increases in the distance to immunogens of 68% and 52% ( $P < 0.0001$ ), respectively. Sequences sampled in Thailand and Vietnam between 1995 and 1999 fit in this progression, and those sampled in Thailand between 2005 and 2009 were farther away from the immunogens than the RV148 samples (median distance to ALVAC-HIV, 0.146 [IQR, 0.138 to 0.158;  $P = 0.01$ ]; median distance to AIDSVAX B/E Env, 0.166 [IQR, 0.152 to 0.179;  $P = 0.03$ ]).

**(ii) Distance to gp120 MN (AIDSVAX B/E).** Figure 7c depicts the genetic distance of 20 RV148 strains—13 subtype B and 7 B/CRF01\_AE recombinants whose corresponding genomic region is subtype B—along with those of 128 subtype B reference sequences sampled in Thailand, China, Japan, and South Korea between 1986 and 2009 to the subtype B Env immunogen. The median distance of RV148 strains to the MN immunogen in AIDSVAX B/E was 0.220 (IQR, 0.201 to 0.239). This reflected only a 12% increase from the measurement evidenced on sequences sampled in Thailand from 1990 to 2002 (median, 0.196; IQR, 0.186 to 0.203;  $P = 0.0002$ ) (see Fig. S1c in the supplemental



**FIG 6** RV148 recombinants are embedded in the larger network of Southeast Asian HIV-1 recombinants. In the network diagram, each node represents a recombinant color coded by country of sampling and shape coded by risk factor for HIV-1 acquisition (risk factors in RV148 were not recorded and thus are depicted as a separate category). Lines connecting nodes link strains that share common recombination breakpoints, and they are color coded to help distinguish relationships within RV148, within published strains, and between these two groups. Boxes group together strains that belong to the same CRF. Numbers within the nodes identify the strains, as follows: 1, 04TH228466; 2, 05TH245651; 3, 04TH312908; 4, 04TH321566; 5, 04TH322500; 6, 04TH409819; 7, 04TH423323; 8, 05TH522586; 9, 05TH522586; 10, 05TH637314; 11, 03TH700065; 12, 03TH700078; 13, 04TH704320; 14, 04TH801743; 15, 05TH852327; 16, 01B.MM.1999.mCSW104; 17, 01B.MM.2000.mIDU502; 18, 01B.MY.2003.03MYKL018\_1; 19, 01B.MY.2004.04MYKL016\_1; 20, 01B.MY.2004.04MYKL019\_1; 21, 01B.MY.2005.05MYKL043\_1; 22, 01B.MY.2006.06MMYKLD46; 23, 01B.MY.2007.07MYKLD47; 24, 01B.MY.2007.07MYKLD48; 25, 01B.MY.2007.07MYKLD49; 26, 01B.TH.-0.1269\_12\_69; 27, 01B.TH.-CM237; 28, 01B.TH.-CU98\_26; 29, 01B.TH.-MU2003; 30, 01B.TH.-NP1623; 31, 01B.TH.-TH1326; 32, 01B.TH.-TH283; 33, 01B.TH.-TH9\_95; 34, 01B.TH.1996.M005; 35, 01B.TH.1996.M043; 36, 01B.TH.1996.M171; 37, 01B.TH.1999.OUR2574; 38, 01B.TH.2000.00TH\_C2254\_BE; 39, 01B.TH.2000.00TH\_R1741; 40, 01B.TH.2001.OUR0331; 41, 01B.TH.2002.OUR7401; 42, 01B.TH.2002.OUR8401; 43, 01B.TH.2002.OUR8461; 44, 01B.TH.2002.OUR8471; 45, 01B.US.1998.99US\_MSC5043; 46, 01B.MM.1999.mIDU107; 47, 01B.MM.2000.mCSW503; 48, 15\_01B.TH.1996.M169; 49, 15\_01B.TH.1999.99TH\_MU2079; 50, 15\_01B.TH.1999.99TH\_R2399; 51, 15\_01B.TH.2002.02TH\_OUR1331; 52, 15\_01B.TH.2002.02TH\_OUR1332; 53, 33\_01B.MY.05.05MYKL045\_1; 54, 33\_01B.MY.2005.05MYKL007\_1; 55, 33\_01B.MY.2005.05MYKL015\_2; 56, 33\_01B.MY.2005.05MYKL031\_1; 57, 34\_01B.TH.1999.OUR1969P; 58, 34\_01B.TH.1999.OUR2275P; 59, 34\_01B.TH.1999.OUR2478P; 60, 48\_01B.MY.2007.07MYKT014; 61, 48\_01B.MY.2007.07MYKT016; 62, 48\_01B.MY.2007.07MYKT021; 63, 51\_01B.SG.2011.11SG\_HM021; 64, 51\_01B.SG.2011.11SG\_HM091. See the text for details.

material); too few reference sequences were available to conduct a comparison with shorter temporal intervals. The median distance from sequences from China, Japan, and South Korea to the immunogen fell within the same range as the RV148 measurement (0.204, 0.218, and 0.221, respectively).

**(iii) Distance to HIV-1 Gag and Pro of LAI (ALVAC-HIV).** Figure 7d depicts the genetic distance of 15 RV148 strains—13 subtype B and 2 B/CRF01\_AE recombinants whose corresponding genomic region is subtype B—along with those of 140 subtype B reference sequences sampled in Thailand, China, Japan, and South Korea between 1986 and 2009 to the subtype B Gag/Pro immunogen. The median distance of RV148 strains to LAI was 0.084 (IQR, 0.066 to 0.088), which is 29% greater than the distance from sequences retrieved in Thailand from 1990 to 2002 (median, 0.065; IQR, 0.060 to 0.074;  $P = 0.0005$ ) (see Fig. S1d in the supplemental material). The distance of subtype B strains sampled in East Asia—most of them contemporaries of RV148 strains—to the LAI immunogen was equivalent to that of RV148.

**Variation in V1 and V2 of HIV-1 Env.** Previous studies have shown that sequence variation in variable regions 1 and 2 (V1V2) can dramatically impact antibody binding and neutralization (68–72). Most recently, analyses in RV144 revealed that the level of binding of IgG antibodies to V1V2 correlated inversely with the rate of HIV-1 infection (73) and that there was an increased vaccine efficacy against viruses with genetic signatures in V2 (42). Given the importance of this variable region, we compared the V1V2 sequences from RV148 and reference isolates to those of the vaccine inserts. We focused our analysis on CRF01\_AE sequences from Thailand (1990 to 2009) and the corresponding CRF01\_AE immunogens (i.e., 92TH023 presented in ALVAC-HIV and CM244 presented in AIDSVAX B/E), as this constituted the largest data set. After excluding areas with extensive length variation, there were 18 positions where more than 20% of the RV148 sequences presented amino acid residues different from those of RV144 immunogens (i.e., residues 130, 133, 148, 152, 154, 155, 158, 161, 165, 166, 169, 170, 171, 173, 178, 181, 183, and 192 in the

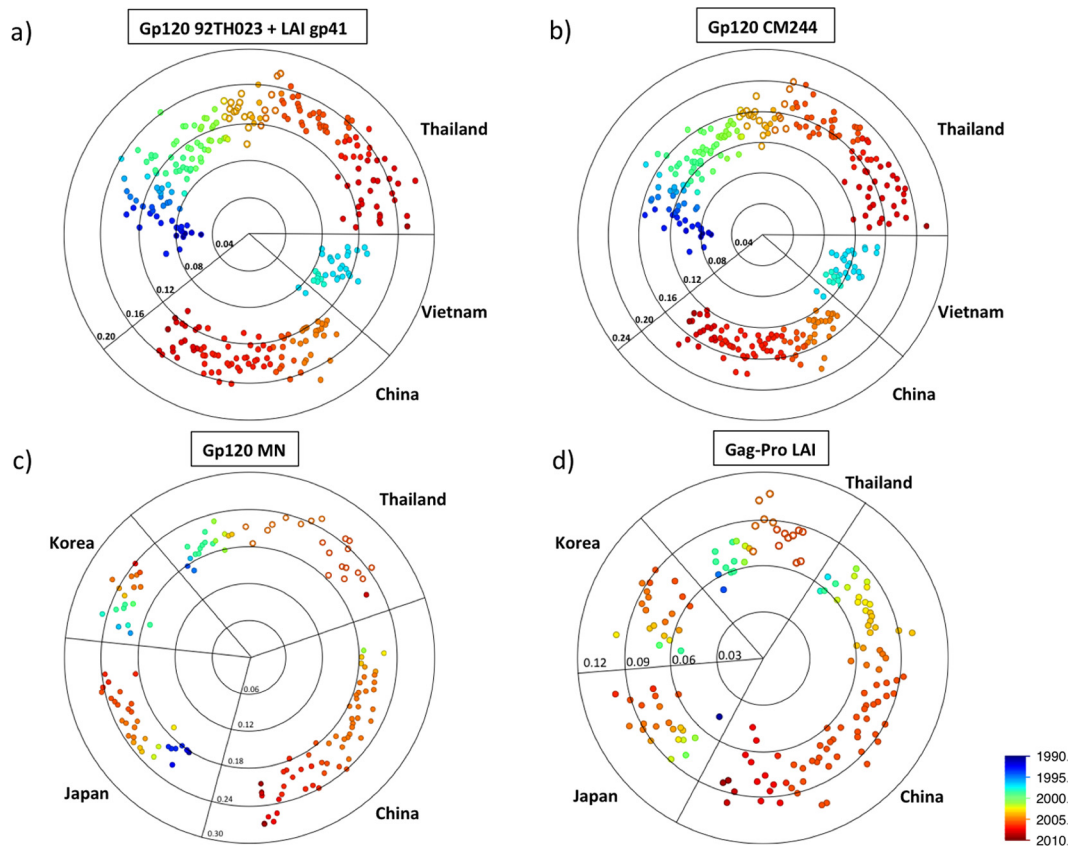
**TABLE 3** Comparison of sociodemographic variables across the different genotypes detected among 375 HIV-infected volunteers deferred from enrollment in RV144

Characteristic	No. (%) of volunteers with the following genotype:					Odds ratio for non-CRF01_AE (95% CI)
	Total	CRF01_AE	Subtype B	B/CRF01_AE recombinants	B/CRF01_AE dual infection	
Sex						
Female	183	172 (94.0)	5 (2.7)	6 (3.3)	0 (0.0)	Reference
Male	192	172 (89.6)	8 (4.2)	10 (5.2)	2 (1.0)	1.82 (0.85–3.91)
Gender						
Female	192	181 (94.3)	5 (2.6)	6 (3.1)	0 (0.0)	Reference
Male	182	162 (89.0)	8 (4.4)	10 (5.5)	2 (1.1)	2.03 (0.94–4.4)
Missing	1	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA <sup>a</sup>
Age (yr)						
18–25	185	170 (91.9)	8 (4.3)	6 (3.2)	1 (0.5)	Reference
26–30	190	175 (91.6)	5 (2.6)	10 (5.3)	1 (0.5)	1.04 (0.50–2.17)
Birthplace						
Rayong	85	80 (94.1)	1 (1.2)	4 (4.7)	0 (0.0)	Reference
Chon Buri	92	82 (89.1)	6 (6.5)	3 (3.3)	1 (1.1)	1.95 (0.66–6.50)
Other	198	182 (91.9)	6 (3.0)	9 (4.6)	1 (0.5)	1.40 (0.53–4.42)
Marital status						
Single	112	103 (92.0)	4 (3.6)	4 (3.6)	1 (0.9)	Reference
Married	206	187 (90.8)	7 (3.4)	11 (5.3)	1 (0.5)	1.16 (0.52–2.78)
Separated	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Divorced	49	46 (93.9)	2 (4.1)	1 (2.0)	0 (0.0)	0.75 (0.16–2.63)
Widowed	6	6 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Education						
Primary	196	177 (90.3)	7 (3.6)	11 (5.6)	1 (0.5)	Reference
Did not complete primary school	17	15 (88.2)	1 (5.9)	1 (5.9)	0 (0.0)	1.24 (0.19–4.87)
Junior high school	111	103 (92.8)	5 (4.5)	2 (1.8)	1 (0.9)	0.72 (0.29–1.66)
Senior high school/vocational school	41	39 (95.1)	0 (0.0)	2 (4.9)	0 (0.0)	0.48 (0.07–1.74)
Vocational school	7	7 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Bachelor's degree or higher	3	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Occupation						
Office/factory	87	80 (92.0)	1 (1.1)	5 (5.8)	1 (1.1)	Reference
Laborer	117	108 (92.3)	2 (1.7)	6 (5.1)	1 (0.9)	0.95 (0.34–2.77)
Merchant/business	31	30 (96.8)	1 (3.2)	0 (0.0)	0 (0.0)	0.38 (0.02–2.27)
Housewife	24	24 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Entertainment	20	19 (95.0)	1 (5.0)	0 (0.0)	0 (0.0)	0.60 (0.03–3.68)
Agriculture	16	14 (87.5)	1 (6.3)	1 (6.3)	0 (0.0)	1.63 (0.23–7.63)
Male/female CSW	15	13 (86.7)	1 (6.7)	1 (6.7)	0 (0.0)	1.76 (0.24–8.28)
Fishery	8	7 (87.5)	1 (12.5)	0 (0.0)	0 (0.0)	1.63 (0.08–11.3)
Government officer	5	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Military/police	4	3 (75.0)	1 (25.0)	0 (0.0)	0 (0.0)	3.81 (0.18–34.7)
Student	3	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Other	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Unemployed	43	36 (83.7)	4 (9.3)	3 (7.0)	0 (0.0)	2.22 (0.71–6.95)

<sup>a</sup> NA, not applicable.

HXB2 numbering system). In all of these sites, sequence variation was not restricted to RV148 but could be evidenced throughout the 2 decades that spanned molecular epidemiology studies in Thailand, with no consistent temporal trend (data not shown). Also, the amino acid residues involved in toggling at each position coincided among the compared periods, probably reflecting convergent elicitation of immune responses and pathways of viral

escape during natural HIV-1 infection. The sieve analysis conducted on RV144 breakthrough cases revealed significant differences between vaccine and placebo recipients in the amino acid residues represented at positions 169 and 181; the vaccine efficacy was higher against viruses carrying a lysine at position 169 (169K) and against viruses not carrying an isoleucine at position 181 (181X) (42). Figure 8 shows that, in RV148, 11/21 individuals



**FIG 7** Temporal and geographical differences in the distance of southeast Asian strains to RV144 immunogens. (a) Protein distance of RV148 CRF01\_AE and B/CRF01\_AE recombinants whose gp120 region is CRF01\_AE and published HIV-1 CRF01\_AE strains sampled in East and Southeast Asia to the 92TH023 gp120 linked to the LAI gp41 immunogen presented in the ALVAC-HIV. (b) Protein distance of RV148 CRF01\_AE and B/CRF01\_AE recombinants whose gp120 region is CRF01\_AE and published HIV-1 CRF01\_AE strains sampled in East and Southeast Asia to the CM244 gp120 immunogen presented in the AIDSVAX B/E. (c) Protein distance of RV148 subtype B and B/CRF01\_AE recombinants whose gp120 region is subtype B and published HIV-1 subtype B strains sampled in East and Southeast Asia to the MN gp120 immunogen presented in the AIDSVAX B/E. (d) Protein distance of RV148 strains to subtype B and B/CRF01\_AE recombinants whose *gag-pro* region is subtype B and published HIV-1 subtype B strains sampled in East and Southeast Asia to the LAI Gag immunogen presented in the ALVAC-HIV. Each point in the radar chart depicts an RV148 (hollow circles) or published strain (full circle), and its radial distance to the center represents the protein p-distance to the immunogen. Concentric circles show the distance scale. The country and year of sampling are indicated.

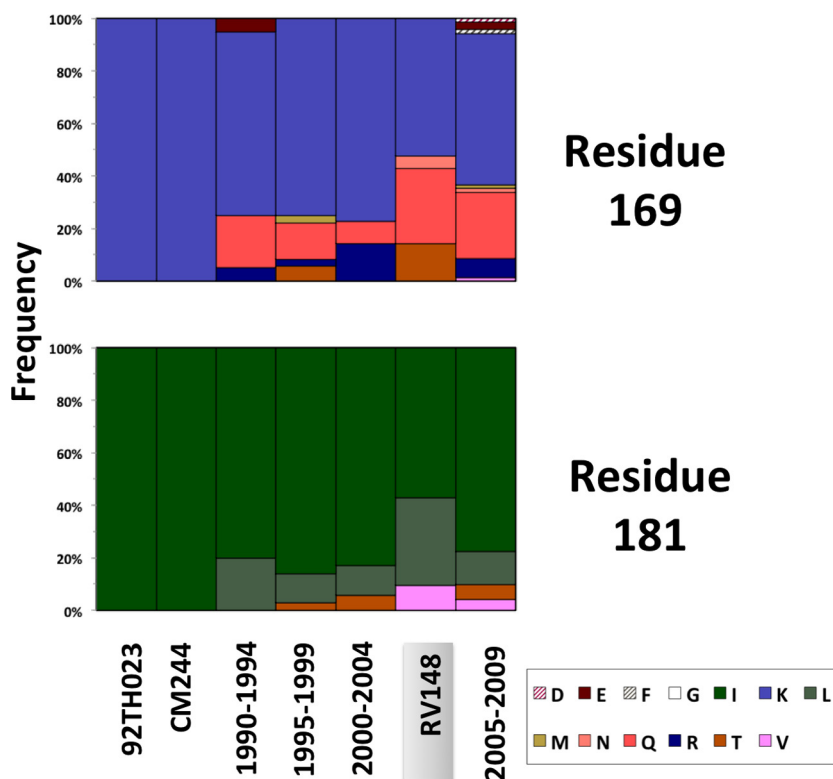
carried 169K, which was less than the proportion evidenced among viruses sampled in Thailand from 1995 to 1999 and 2000 to 2004 (unadjusted  $P = 0.07$  and  $0.053$ , respectively). Similarly, 9/21 individuals carried the 181X signature in RV148, which represented a proportion greater than the proportions evidenced from 1990 to 1995, 2000 to 2004, and 2005 to 2009 (unadjusted  $P = 0.02$ ,  $0.04$ , and  $0.07$ , respectively). Due to the large numbers of comparisons carried out, these unadjusted  $P$  values should be interpreted as trends.

V1V2 is notable for its high level of N-glycosylation; RV148 sequences presented a median of 8 potential N-glycosylated sites (IQR, 7 to 10), which did not differ significantly from that for reference sequences (data not shown). Despite V1V2 sequence variation, the N-glycosylation site 160 and the cationic nature of the C strand, which have been shown to be critical for neutralization by PG9 and related antibodies, were conserved among RV148 and reference sequences (data not shown). Overall, this analysis shows that there have been no notable changes in the patterns of V1V2 sequence variation among CRF01\_AE strains in Thailand in the past 2 decades.

## DISCUSSION

RV144 was the first vaccine trial to demonstrate a significant, though modest, protection from HIV-1 acquisition (modified intent-to-treat vaccine efficacy, 31.2% [95% CI, 1.1 to 52.1%]) (56). Subsequent studies have defined correlates of risk, importantly, vaccine-induced IgG to the V1V2 region (73), and analysis of breakthrough infections (42) has further identified genetic signatures in Env, particularly at HXB2 amino acid positions 169 and 181, which are associated with vaccine efficacy. In the broader context, understanding the molecular epidemiology of volunteers deferred from enrollment in RV144 due to preexisting HIV-1 infection (i) provides a cross-sectional representation of the HIV-1 epidemic in Thailand at the time of recruitment for RV144, (ii) helps better define who volunteered for the trial, and (iii) is an important approximation to the viral strains that challenged the vaccine and placebo recipients in RV144 and their relationship to the immunogens.

**Cross-sectional representation of the HIV epidemic in Thailand at the time of recruitment for RV144.** In RV148, we observed that the predominant strain, with a prevalence of over 90%,



**FIG 8** Amino acid variation in V1V2 positions 169 and 181 among CRF01\_AE sequences sampled in Thailand between 1990 and 2009 and their relation to the RV144 vaccine immunogens. The frequency of each amino acid residue at the corresponding position is depicted for published Thai CRF01\_AE gp120 sequences sampled between 1990 and 1994 ( $n = 20$ ), 1995 and 1999 ( $n = 36$ ), 2000 and 2004 ( $n = 35$ ), and 2005 and 2009 ( $n = 71$ ), as well as CRF01\_AE and B/CRF01\_AE recombinants whose gp120 region is CRF01\_AE from RV148 ( $n = 21$ ). The amino acid residues presented in 92TH023 and CM244 RV144 immunogens are depicted for comparison.

was CRF01\_AE, followed by equivalent proportions of subtype B and B/CRF01\_AE recombinants and only minor occurrences of dual infections. These results are in high concordance with the findings of the community cohort studies performed in the same area between 1998 and 2001 in preparation for RV144 (40). Regarding the level of genetic diversity among full-genome CRF01\_AE sequences in RV148, we noticed increases of 31% and 24% compared to the measurements from Thailand from 1990 to 1999 and 2000 to 2004, respectively. This observation supports previous observations indicating that in the past 2 decades the CRF01\_AE Thai epidemic has experienced a continued genetic expansion (25).

The proportion of RV148 strains that were subtype B was 3.5%. An interesting aspect of the East and Southeast Asian HIV-1 epidemics is the cocirculation of two different clusters within subtype B: Western B and B' (57, 58). The latter is widespread throughout the region and has been incorporated by the vast majority of subtype B-containing recombinants (e.g., CRF07\_BC, CRF08\_BC, and CRF15\_01B). In RV148, the Western B strains outnumbered the B' strains in a 2:1 ratio. Our phylogenetic analyses indicate that the Western B strains did not proceed from a single source but seemed to be part of the larger, cosmopolitan, and contemporary Western B epidemic. The fact that more than half of the Western B strains in RV148 were sampled near Pattaya, a hub for international tourism, suggests that this area may be an important port of entry for these strains. While B' strains continue to be detected in Thailand, they do not seem to constitute the

majority of the subtype B strains in Rayong and Chon Buri Provinces. It is possible that the populations that used to bear B' strains were high-risk groups (e.g., IDUs) and became superinfected with CRF01\_AE strains, which led to the emergence of numerous B/CRF01\_AE URFs (28). It can be expected that if the process of absorption of B' strains into B/CRF01\_AE recombinants persists and the influx of Western B strains continues, pure B' strains may eventually be replaced by Western B strains in the Thai HIV-1 epidemic. In contrast to the evolution of B', the incorporation of Western B strains in B/CRF01\_AE recombinants has been restricted to a few examples (e.g., CRF51\_01B [74], CM237 [75], and strain 04TH322500 in the current study), which suggests that Western B circulates in social networks where dual infection and dissemination of recombinants is not frequent.

The cocirculation of subtype B and CRF01\_AE in Southeast Asia has led to the emergence of intersubtype recombinants. While some of them expanded in the population (e.g., CRF15\_01B and CRF34\_01B) (59, 76), the majority of recombinants have been sampled only on single occasions (i.e., URFs). An interesting feature of the URFs in Thailand is that most of them share common recombination breakpoints. Molecular epidemiological studies support the hypothesis that these similarities reflect common ancestry (28, 77–79), though some common breakpoints may represent independent and convergent events (80). In RV148, 1 out of 15 sequenced B/CRF01\_AE recombinants belonged to a CRF, CRF15\_01B, which is the CRF that has spread among different provinces and social networks throughout Thai-

land (32, 59). Additionally, 6 URFs shared with CRF15\_01B both of the signature recombination breakpoints in *env*, suggesting that this CRF plays a central role in the Thai HIV-1 epidemic, not only by spreading to some extent as a stable form but also as a major contributor of genetic material to new recombinants. The genomic structure of CRF15\_01B can be compared to a pseudotype virion (81), as it is composed of a single B' segment extending from the beginning of gp120 (excluding the leader peptide, which is CRF01\_AE) to the end of the ectodomain of gp41 in a CRF01\_AE background. The retention of these motifs in the CRF15\_01B-derived URFs reiterates a phenomenon observed in other subtype B-containing recombinants sampled worldwide (e.g., CRF14\_BG [82]) and may indicate that these motifs interact better in this configuration, which warrants further structural and functional analyses (A.-L. Chenine et al., unpublished data).

It has previously been shown that the sharing of recombination breakpoints among CRFs and URFs in East and Southeast Asia links virtually all of these recombinants in a single network, which connects strains from different geographic locations and strains sampled from individuals with different routes for HIV-1 acquisition (55). Here we have shown that all but one of the RV148 recombinants are fully integrated within this larger network. The fact that the direct connectedness of RV148 to other reference recombinants was higher than the direct connectedness to other members of RV148 indicates that recombinants in RV148 did not proceed from a single pocket or subpopulation but, rather, that they represent a more diverse pool of sequences. This may shed light on the social networks from which the RV148 volunteers were drawn (see below).

**Who volunteered for the trial?** The information retrieved from RV148 volunteers included basic sociodemographic characteristics, but the volunteers were not asked questions regarding behaviors that may increase the risk for HIV acquisition (41). In cohorts in Thailand and worldwide, it has been shown that there were associations between the complexity of HIV-1 strains and sociodemographic characteristics and behaviors that may increase the risk of HIV infection (e.g., more frequent needle sharing among IDUs [61], the presence of sexually transmitted infections [63], exchanging sex for money [65, 66], and having high numbers of sex partners [66]). We compared the frequency of different molecular forms—pure subtypes and recombinants—between RV148 and other low-risk cohorts (i.e., RV109, a mother-to-child transmission [MTCT] study conducted in Lampang between 1996 and 1997 [32]) and high-risk cohorts (i.e., a study conducted among IDUs seeking treatment in Chiang Mai between 1999 and 2000 [61] and the Thai Red Cross [TRC] study conducted among high-risk men who have sex with men [MSM] and heterosexuals in Bangkok between 2006 and 2007 [83]) from Thailand that have also been genotyped using MHABce. In RV148, the frequency of non-CRF01\_AE strains (9.8%) was higher than that in the low-risk MTCT study (5.1%) but lower than the ones observed in higher-risk cohorts: IDUs (18.7%) and high-risk MSM and heterosexuals (20%). In RV148, the combined frequency of complex molecular forms (i.e., recombinants and dual infections) was 6.6%; likewise, these complex strains were more frequent in RV148 than in the low-risk MTCT cohort (2.3%) but less frequent in RV148 than in the high-risk Opiate-Users Research (14.7%) and TRC (13%) cohorts. Overall, these results are consistent with the idea that the level of risk for HIV-1 infection among RV148 HIV-infected individuals was at the level of what is expected from

a community-based cohort, which was the aim of RV144 (56). While individuals belonging to groups with high-risk behaviors may also have volunteered for the trial, their representation in RV148 seems to be only minor. This is an important point, as the level of risk of vaccine trial populations can impact the observed vaccine efficacy (84).

The fact that in RV148 we observed a large number of URFs along with a limited number of multiply infected individuals can be explained through the model through which, in the region, there are small groups with high levels of exposure to HIV-1, where recombinants are being generated in multiply infected individuals. The linkage of these groups with the larger, lower-risk community then leads to the dissemination of URFs. Following this model, in order to limit the expansion in HIV-1 genetic diversity, public health efforts should be directed at identifying these high-risk groups and strengthening effective intervention campaigns.

**An approximation to the viral strains that challenged the vaccine and placebo recipients in RV144 and their relationship to the immunogens.** The complex activities involving vaccine development can take more than a decade from the time of immunogen selection until testing in efficacy trials. Even if the immunogens were chosen to minimize the mismatch to the circulating strains at the time of vaccine development, the molecular characteristics of the local epidemic can dramatically change by the time of vaccine testing in efficacy trials. Here we showed that the distance to the immunogens increased 52% to 68% for CRF01\_AE Env, 12% for subtype B Env, and 29% for subtype B Gag-Pro from the early 1990s to the mid-2000s. The CRF01\_AE Thai epidemic in the early 1990s was very compact and continued expanding throughout the decade. Considering the data for the period from 2005 to 2009 from Thailand, the increase in genetic diversity had not plateaued at the time of RV148. On the other hand, it seems that the subtype B epidemic is not expanding at such a fast pace. It is important to continue the molecular characterization of communities where vaccine trials are planned, as this phenomenon can impact the analysis of vaccine efficacy in future trials. At the time of RV148, the distance of CRF01\_AE and subtype B strains from other countries in East and Southeast Asia to the immunogens was equivalent to that measured in Thailand. This means that the distance of circulating HIV-1 strains to the immunogen should not be a major concern at the time of extending the testing/usage of the RV144 vaccine regimen to other countries in the region.

Of note, the above-mentioned protein distance calculations were based on the entirety of the immunogen sequence. The analysis of immunological correlates of protection from acquisition in RV144 has revealed a significant inverse association between binding of IgG antibodies to V1V2 and infection risk (73). This region of gp120 is recognized as one of the most variable areas of the HIV-1 proteome. Our analysis of CRF01\_AE strains indicates that the nature and level of sequence variation in highly polymorphic sites in V1V2 have been maintained since the early 1990s to date, in contrast to the results from the analyses based on the whole immunogen. Some V1V2 sites showed an extremely high level of mismatch between RV148 sequences and immunogens; 48% and 43% of the sequences differed from the sequence of the vaccine insert in positions 169 and 181, respectively, with some trends suggesting differences with previous time points. Interestingly, these are two sites where recent analyses by Rolland et al.

have detected significant sieve effects in RV144 (42), further supporting the need for continuing molecular epidemiological surveys in communities where vaccine efficacy trials are planned or under way.

**Limitations of the study.** The current study, involving nearly 400 individuals, constitutes one of the largest HIV-1 molecular surveys conducted in Southeast Asia to date; however, it has several limitations. The use of high-throughput MHAbce allowed the cost-effective genotyping of virtually the entire cohort and allowed the detection of recombinants that would have been missed by partial-genome sequencing; nevertheless, it is possible that some of the samples typed as pure CRF01\_AE may in fact be recombinants incorporating other subtypes in genomic areas not interrogated by MHAbce. Additionally, MHAbce is not capable of detecting multiple infections due to the same clade (e.g., coinfection with two distinct CRF01\_AE strains). Thus, the current tallies of recombinants and multiple infections should be considered lower-limit estimates. Another limitation of the current study arises from the fact that near-full-genome sequences were retrieved from only 10% of the individuals, with special emphasis on subtype B and intersubtype recombinants. For this reason, the study was not powered to systematically address the presence of direct epidemiological linkage among HIV-1 infected individuals, especially among those infected with CRF01\_AE.

Finally, the current work focused exclusively on the RV144 volunteers deferred from enrollment in the vaccine trial due to preexisting HIV-1 infection. The thorough analysis of RV144 breakthrough cases is the focus of a separate report (Rolland, personal communication) and will provide wider context to the current observations.

**Conclusion.** In summary, our results show that at the time of enrollment for RV144, the HIV-1 epidemic in Rayong and Chon Buri Provinces was dominated by CRF01\_AE strains. Some representation of subtype B was noted, with an increase in the Western B frequency due to multiple entries in the region and waning of B' being detected. Most of the intersubtype recombinants were URFs embedded within the larger regional network of recombinants. The level of genetic complexity observed in this study strongly indicates that the RV148 volunteers presented risk levels consistent with those for a community-based cohort. Compared to the molecular picture at the early stages of the process of vaccine development, we evidenced substantial changes in the epidemic (i.e., the expansion of the genetic diversity within CRF01\_AE, the increase in the frequency and complexity of B/CRF01\_AE recombinants, and shifts in the Western B/B' balance) which had the potential to impact the efficacy of the vaccine and should be considered at the time of the analysis of the trial results.

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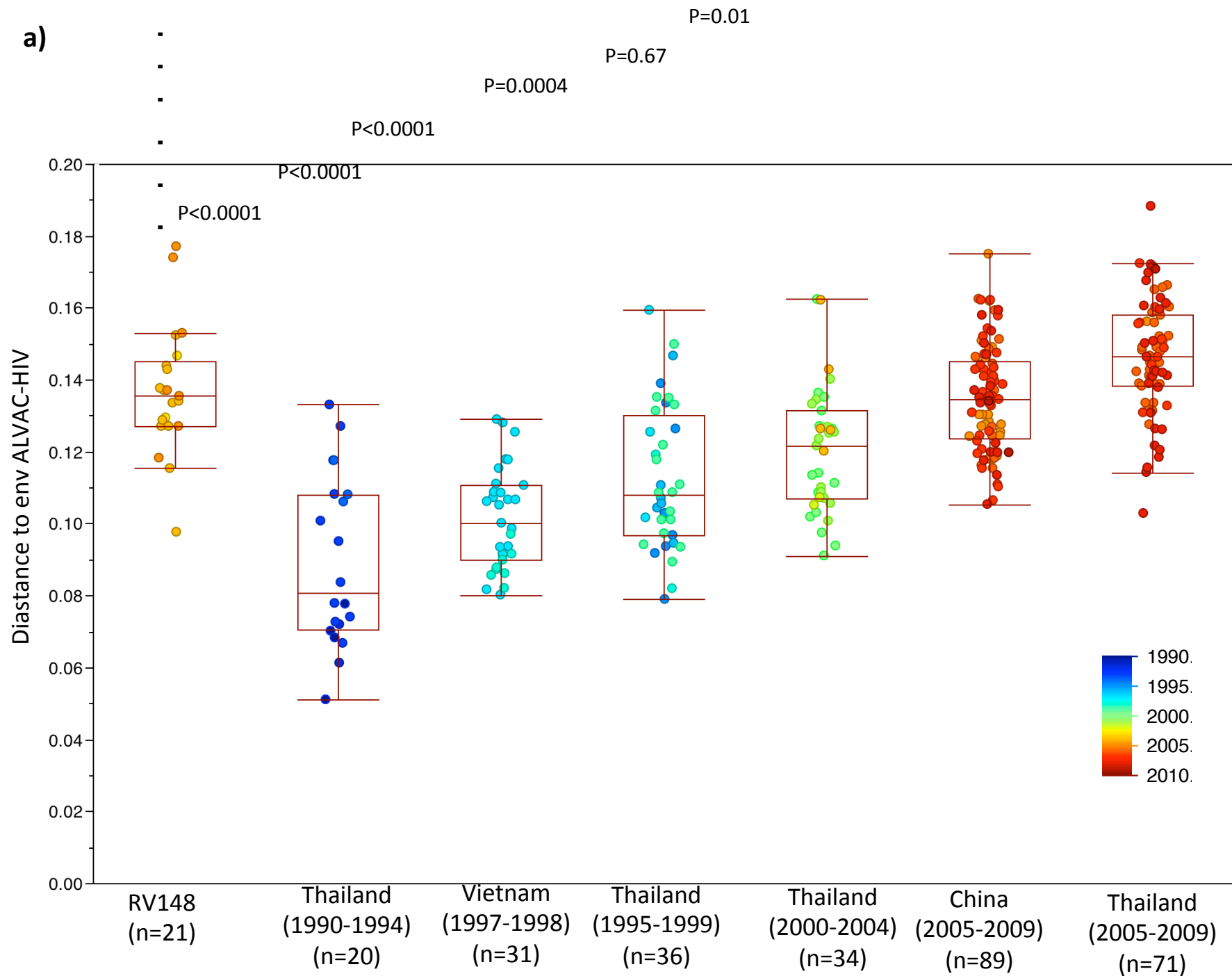
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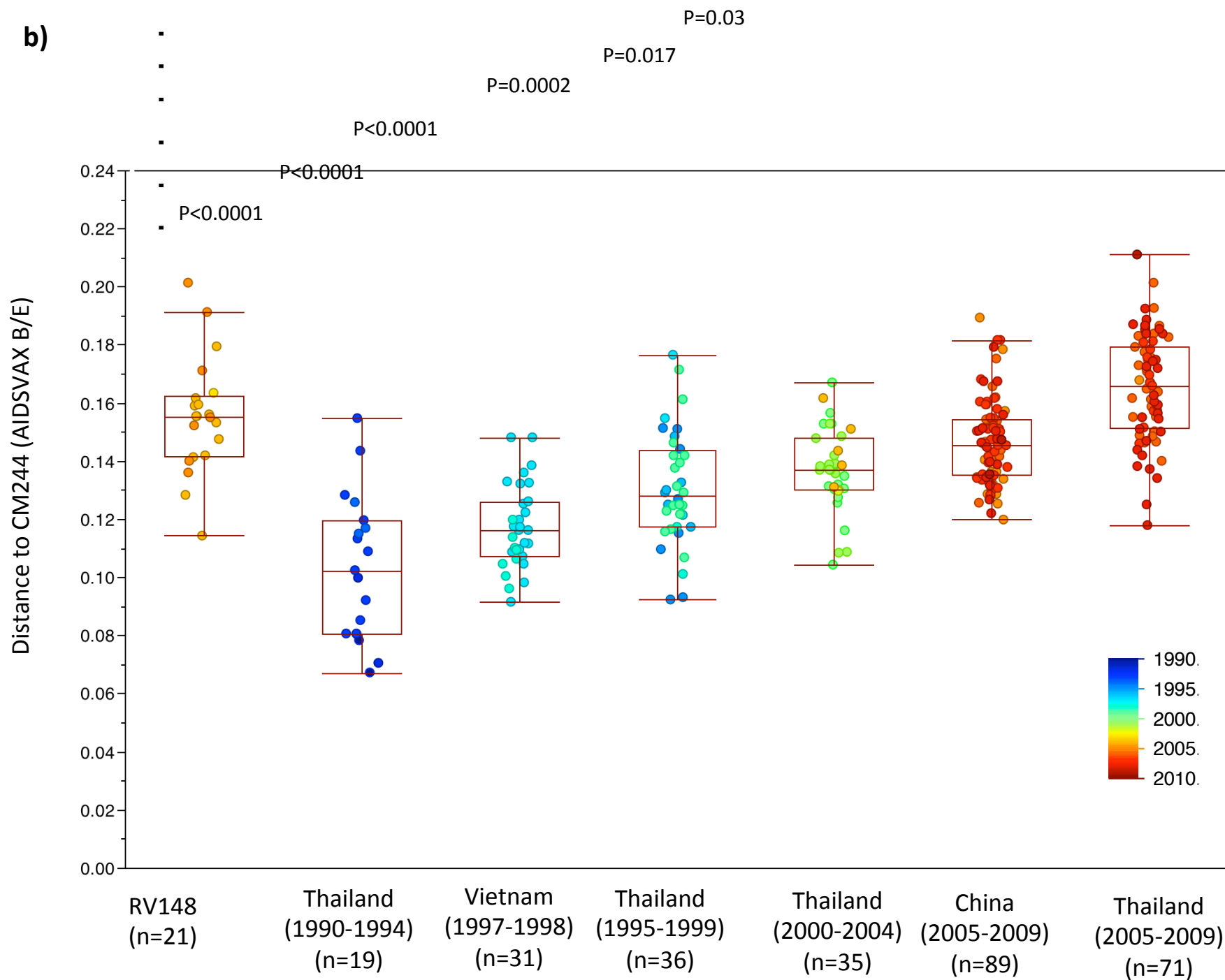
## Supplemental figure legends

**Figure S1.** Temporal and geographical differences in the distance of southeast Asian strains to RV144 immunogens. a) Protein distance of RV148 CRF01\_AE and B/CRF01\_AE recombinants whose *gp120* region is CRF01\_AE, and published HIV-1 CRF01\_AE strains sampled in East and Southeast Asia, to the 92TH023 Gp120 linked to LAI gp41 immunogen presented in the ALVAC-HIV. b) Protein distance of RV148 CRF01\_AE and B/CRF01\_AE recombinants whose *gp120* region is CRF01\_AE, and published HIV-1 CRF01\_AE strains sampled in East and Southeast Asia, to the CM244 Gp120 immunogen presented in the AIDSVAX B/E. c) Protein distance of RV148 subtype B and B/CRF01\_AE recombinants whose *gp120* region is subtype B, and published HIV-1 subtype B strains sampled in East and Southeast Asia, to the MN Gp120 immunogen presented in the AIDSVAX B/E. d) Protein distance of RV148 strains to subtype B and B/CRF01\_AE recombinants whose *gag-pro* region is subtype B, and published HIV-1 subtype B strains sampled in East and Southeast Asia, to the LAI Gag immunogen presented in the ALVAC-HIV. Each point in the charts depicts an RV148 (hollow circles) or published strain (full circle). The country and year of sampling are indicated.

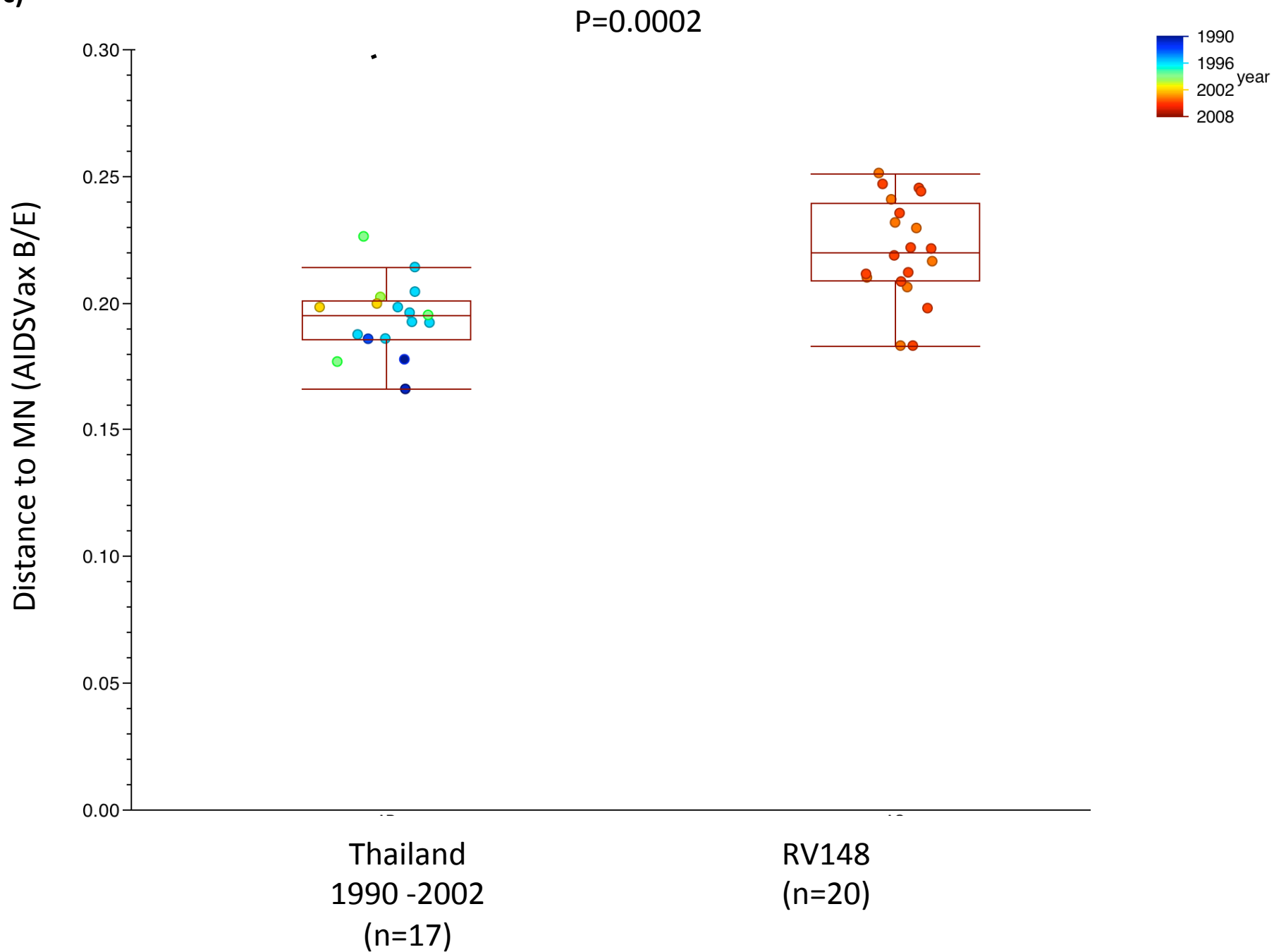
a)



b)



c)



d)

P=0.08

P=0.38

P=0.07

P=0.0005

