# NEUTRALIZATION ACTIVITY PATTERN OF RECENT HIV-1 INFECTION IN YOUNG THAI MEN, 1999-2013

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**Abstract.** Strength and breadth of neutralizing antibodies of 133 HIV-1 infected young Thai men, randomly selected from the annual serosurveillance, 1999-2013, were classified using a multiassay algorithm (ELISA, IgG-Capture HIV-EIA and western blotting) into 38 sera in Fiebig stage V (late acute infection phase), 71 in Fiebig stage VI (early chronic infection phase) and 24 in chronic infection sample groups. TZM-bl neutralizing antibody (Nab) assay against five HIV pseudoviruses, namely, MN subtype B/1990, CM235/CRF01_AE/1990, 0503M02138/CRF01_AE/1996, 620354CRF01_AE/2005 and 427299.c12/CRF01_AE/2006, was employed to study neutralizing activity, revealing neutralization strength of chronic infection group is significantly higher than those of Fiebig stage V and VI groups. Of the five pseudoviruses, MN/B pseudovirus, a tier 1A virus, was the most neutralizing sensitive, both in neutralization strength and breadth by all three serum groups. Among CRF01_AE pseudoviruses, CM235/E was the most sensitive to the neutralizing ability of Fiebig stage VI and chronic serum groups. Neutralization frequency against each pseudovirus increased from Fiebig stage V to Fiebig stage VI and then to chronic infection serum groups, with the latter being highest against MN/B pseudovirus (92%). Neutralization ability of Fiebig stage VI serum group (33.33%) from 1999-2005 is significantly broader than that (12.00%) from 2006-2013 ($p$=0.008), but chronic infection serum group had the most neutralization breadth. Neutralization frequency (29%) of Fiebig stage VI sera from 1999-2005 against 620354/E pseudovirus is significantly higher than that (8%) from 2006-2013 ($p$=0.018). Neutralization pattern of recently HIV- infected Thai sera showed potency and breadth to neutralize tier 1 clade-mismatched viruses and, thus, may be used for further vaccines against non-clade specific HIV.

**Keywords:** Fiebig staging, HIV-1 infection, neutralizing antibody, TZM-bl neutralization antibody assay

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## INTRODUCTION

Seroprevalence of HIV-1 in young Thai men has steadily increased from 0.5% in 1989 to a peak 3.7% in 1993 (Nelson *et al*, 1996; Phoolcharoen, 1998). Following

an effective HIV-prevention campaigns lauched by the Ministry of Public Health, Thailand prevalence of HIV-infected young Thai men has slightly decreased and is stable at 0.6% in 2013 (BOE, 2015). However, 280,000 new infections were reported in Asia and the Pacific in 2017 (UNAIDS, 2017). HIV CRF01_AE is prevalent throughout Southeast Asia and is responsible for >80% of infection cases in Thailand (Arroyo *et al*, 2010; Hemelaar *et al*, 2011).

Antibodies are produced against the majority of HIV proteins but neutralizing antibodies (Nab) are only detected against Env glycoprotein (Mascola and Montefiori, 2010). The first antibodies are detected 12-15 days after HIV-1 infection (Euler *et al*, 2010; Hemelaar *et al*, 2011) and antibodies that can mediate antibody-dependent cellular cytotoxicity (ADCC) appear during the first three months of acute infection (Euler and Schuitemaker, 2012). At the same time, autologous neutralizing antibodies arise that are mostly strain-specific and cannot neutralize heterologous viruses (Hemelaar *et al*, 2011). Only 10-30% of HIV-1 infected individuals develop broadly neutralizing anti-HIV antibodies (bNAbs), which are vital component of protective immunity required for developing an effective HIV-1 vaccine (Girard *et al*, 2011; Koff, 2012).

Since HIV-1 was first recognized, there has been only one phase III vaccine trial (RV-144) (Haynes *et al*, 2012; Koff, 2012). Only Tier 1 viruses are neutralized in the RV144 trial with Nabs against V3 loop of gp120 (Haynes *et al*, 2012; Montefiori *et al*, 2012). Moreover, binding antibodies to clade B and E gp120 are present in 99% of vaccinated subjects but titers wane 15 folds over a period of 20 weeks (Haynes *et al*, 2011).

This study investigated HIV-1 neutralizing activity against subtype B and CRF01_AE HIV Env pseudoviruses of sera from 133 HIV-1-infected Thai males collected during 1999-2013 using a TZM-bl Nab assay. Pseudovirus *env* were examined from CRF01_AE viruses at various time points of an HIV-1 epidemic in Thailand (1990-2006) including CM235/CRF01_AE/1990, 0503M02138/CRF01_AE/1996, 6203 54CRF01_AE /2005 and 427299c12/CRF01_AE/2006. These data could be used to gain a better understanding over time of the neutralizing antibodies against HIV-1 and contribute to the development of a more successful HIV-1 vaccine.

## MATERIALS AND METHODS

### Serum samples collection, classification and HIV subtyping

Since 1991 the Royal Thai Army (RTA) has evaluated countrywide the prevalence of HIV-1 infection in young Thai men enrolled as conscripts (Nelson and Rangsin, 2017). Approximately 60,000 sera were collected and serologically tested for anti-HIV antibodies by ELISA each year. Western blot (WB) assay or ELISA were used to confirm HIV seropositivity according to CDC (2012) guidelines. According to the nQuery Advisor program (Statistical Solutions, Cork, Ireland), 9-10 HIV-1 seropositive serum samples from each year were selected (except when unavailable in 2000, 2003, 2006, and 2008).

IgG-Capture HIV-EIA or Sedia™ BED HIV-1 Incidence enzyme immunoassay (EIA) method (Sedia biosciences, Portland, OR) was employed for distinguishing recent HIV-1 infections from those which are long-term. HIV-1 acute infection was classified into Fiebig stages I-VI (Fiebig *et al*, 2003; Fiebig *et al*, 2005; McMichael *et al*, 2010) based on stepwise gain in HIV-1

antigens positivity and HIV-1-specific antibodies. Fiebig stage V, last phase of acute infection phase, occurs approximately 100 days after infection and is defined as recent seroconversion of WB positivity without presence of p31, while Fiebig stage VI, the last stage of chronic infection phase, is characterized by recent seroconversion with WB assay positivity together with p31. Peptide ELISA method was used to determine HIV subtypes: subtype B, CRF01_AE, non-B, non-E, and undetermined (McMichael *et al*, 2010; Gray *et al*, 2011; Karasavvas *et al*, 2015).

**HIV pseudoviruses**

Five HIV-1 Env-pseudoviruses used in the study were kindly provided by Dr Nicos Karasavvas, AFRIMS, Bangkok. HIV-1 Env-pseudoviruses are classified by their sensitivity to antibody-mediated neutralization into four subgroups, namely, very high (tier 1A), above-average (tier 1B), moderate (tier 2) and low (tier 3) (Davis *et al*, 2009). They were tier 1A subtype B (MN/subtype B/1990), tier 2 subtypes CRF01_AE (620354/CRF01_AE/2005 and 427299.c12/CRF01_AE/2006) and undetermined tier subtypes CRF01_AE (CM235/CRF01_AE/1990 and 0503M02138/CRF01_AE/1996) (Table 1). HIV-MN was isolated from the mother to child transmission in USA in 1990 and the four CRF01_AE pseudoviruses were isolated from HIV-1 infected Thais including CM-235, R5 virus from 1990, HIV-1-0503M02138, X4 virus from 1996 and HIV-620354 and HIV-427299.c12 from 2005 and 2006, respectively (Cao *et al*, 1995; Montefiori *et al*, 1996; Doria-Rose *et al*, 2009). HIV *env* nucleotide sequences of all five HIV-1 Env-pseudoviruses were aligned, translated and compared with the consensus sequence (HIV-CM-240/CRF01_AE/1990) by Clustal W multiple alignment (Bio edit program).

**Titration of Env-pseudoviruses**

Env-pseudovirus stocks were titrated by performing serial 5-fold dilutions in quadruplicate in growth medium [Dulbecco's Modified Eagle's Medium with L-glutamine, sodium pyruvate, glucose, pyridoxine and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Gibco BRL Life Technologies, Grand Island, NY)], in 96-well culture plates (Naldini *et al*, 1996; Wei *et al*, 2002; Li *et al*, 2005; Polonis *et al*, 2008; Ozaki *et al*, 2012). In brief, freshly trypsinized epithelial HeLa-derived TZM-bl cells (NIH AIDS Reagent Program, Germantown, MD, USA) ($10^3$ cells/100 µl) were added to each well in growth medium containing an optimized concentration of DEAE-dextran (Polonis *et al*, 2008) and incubated at 37°C for 48 hours. Virus-induced syncytium formation and cell killing were monitored by microscopic examination. Culture fluid (100 µl) was removed from each well and replaced with a Luc reporter gene assay system reagent (Britelite; Perkin-Elmer Life Sciences, Shelton, CT, or Brite-Glo; Promega, Madison, WI). After a 2-minute incubation at ambient temperature to allow cell lysis, 150 µl aliquot of cell lysate was transferred to a 96-well black solid plate (Corning-Costar, Corning, NY) for measurements of luminescence using a Victor 2 luminometer (Perkin-Elmer Life Sciences, Shelton, CT).

**TZM-bl HIV neutralization antibody (Nab) assay**

TZM-bl assay was performed as previously described (Naldini *et al*, 1996; Wei *et al*, 2002; Li *et al*, 2005). In short, TZM-bl target cells carrying luciferase reporter gene sensitive to the presence of HIV Tat protein and reporter gene expression is induced in *trans* by viral Tat protein after

Table 1
Genetic and biological characteristics of HIV-1 Env-pseudoviruses used in the study.

| Name[a] | Subtype | GenBank accession number | | Isolation year | Biological phenotype | | Tier (TZM-bl) |
|---|---|---|---|---|---|---|---|
| | | Full-length | *Env* | | Syncytium induction | Co-receptor | |
| MN | B | M17449 | AY736819 | 1990 | SI | X4 | 1A |
| CM235 | CRF01_AE | AF259954, AF259955 | AY736837 | 1990 | NSI | R5 | NA |
| 0503M02138 | CRF01_AE | AY713424 | NA | 1996 | SI | X4 | NA |
| 620354 | CRF01_AE | JN944656 | NA | 2005 | NA | R5 | 2 |
| 427229.c12 | CRF01_AE | JN944655 | NA | 2006 | NA | R5 | 2 |

[a]Cao *et al* (1995), Montefiori *et al* (1996), Doria-Rose *et al* (2009). NA, not available; NSI, non-syncytium induction; R5, CCR5 co-receptor usage; SI, syncytium induction; X4, $CXCR_4$ co-receptor usage.

a single cycle infection. TZM-bl Nab assay was used to avoid the diversity of target cells and viruses and only a single round of HIV Env-pseudoviral replication was used (Naldini *et al*, 1996; Wei *et al*, 2002; Li *et al*, 2005; Polonis *et al*, 2008; Ozaki *et al*, 2012). The results are quantified as relative luminescence units (RLU). The 80% inhibitory dose ($ID_{80}$) is defined as the reciprocal of sample concentration that causes 80% reduction in RLU compared to that from control virus well after subtraction of background RLU (control well). Neutralization titer >20 is used as criterion to classify a positive neutralization activity. Conversely, neutralization titer <20 indicates a negative neutralization activity. All 133 HIV-1 positive sera were tested for neutralizing activity against the five HIV pseudoviruses.

**Statistical analysis**

Statistical analysis was carried out using standard functions of Statistics Package for the Social Sciences (SPSS) version 25. Statistical significance between two groups was determined using Mann-Whitney *U* test and among three groups using independent-samples Kruskal-Wallis test. Differences in neutralizing activity against the five HIV pseudoviruses were determined using related-samples Friedman's two-way analysis of variance by ranks. A $p<0.05$ is considered significant.

## RESULTS

**Serum samples**

Of 947,992 serum samples collected from RTA conscripts enrolled during 1999-2013, HIV serosurveillance using ELISA and WB assay detected 6,025 (0.64%) positive samples. Seroprevalence of HIV infection in Thai conscripts gradually decreased from 2.9% in 1991 to 0.6% in 2013 (Table 2). Among 133 randomly selected HIV-1 positive sera, 109 (82%) and 24 (18%) were identified by Sedia™ BED HIV-1 Incidence EIA as recent seroconversion and chronic infection samples, respectively.

Table 2
Annual HIV seroprevalence survey of the Royal Thai Army conscripts, 1991-2013.

| Year | Number of surveillance samples | Number of HIV seropositive samples | Number of studied samples | Serum group | | | Prevalence (%) |
|---|---|---|---|---|---|---|---|
| | | | | Fiebig V (*n*=38) | Fiebig VI (*n*=71) | Chronic (*n*=24) | |
| 1991 | 61,686 | 1,816 | NA | NA | N A | NA | 2.9 |
| 1992 | 59,574 | 2,103 | NA | NA | NA | NA | 3.5 |
| 1993 | 52,228 | 1,916 | NA | NA | NA | NA | 3.7 |
| 1994 | 55,455 | 1,652 | NA | NA | NA | NA | 3.0 |
| 1995 | 55,193 | 1,354 | NA | NA | NA | NA | 2.4 |
| 1996 | 55,054 | 1,131 | NA | NA | NA | NA | 2.0 |
| 1997 | 56,585 | 1,072 | NA | NA | NA | NA | 1.9 |
| 1998 | 51,514 | 821 | NA | NA | NA | NA | 1.6 |
| 1999 | 60,625 | 801 | 10 | 1 | 0 | 9 | 1.3 |
| 2000 | 64,884 | 756 | 3 | 0 | 1 | 2 | 1.2 |
| 2001 | 66,238 | 456 | 10 | 3 | 3 | 4 | 0.7 |
| 2002 | 58,755 | 389 | 10 | 2 | 4 | 4 | 0.7 |
| 2003 | 59,492 | 341 | 5 | 2 | 1 | 2 | 0.6 |
| 2004 | 57,730 | 299 | 10 | 4 | 3 | 3 | 0.5 |
| 2005 | 62,031 | 300 | 10 | 1 | 9 | 0 | 0.5 |
| 2006 | 57,564 | 268 | 6 | 5 | 1 | 0 | 0.5 |
| 2007 | 58,016 | 267 | 10 | 5 | 5 | 0 | 0.5 |
| 2008 | 61,475 | 299 | 9 | 4 | 5 | 0 | 0.5 |
| 2009 | 61,835 | 331 | 10 | 0 | 10 | 0 | 0.5 |
| 2010 | 62,138 | 324 | 10 | 3 | 7 | 0 | 0.5 |
| 2011 | 71,757 | 365 | 10 | 5 | 5 | 0 | 0.5 |
| 2012 | 77,611 | 436 | 10 | 1 | 9 | 0 | 0.6 |
| 2013 | 67,841 | 393 | 10 | 2 | 8 | 0 | 0.6 |

NA, not available.

Fiebig stage V (last phase of acute infection phase) and stage VI (early chronic infection) is characterized by positive WB assay without and with p31 protein respectively (McMichael *et al*, 2010). Of 109 recent seroconversion samples, 38 (29%) and 71 (53%) were in Fiebig stage V and VI group respectively. Fiebig stage V, Fiebig stage VI and chronic infection sera were subsequently used for neutralizing activity study. As the chronic infection sera were collected during 1999-2005, Fiebig

Table 3
Comparison of env similarity among HIV-1 Env pseudoviruses used in the study.

| HIV-1 pseudovirus[a] | CM235/E/ 1990 | 0503M02138/E/ 1996 | 620354/E/ 2005 | 427299.C12/E/ 2006 | MN/B/ 1990 |
|---|---|---|---|---|---|
| CM235/E/1990 | 100% | 94.5% | 93.2% | 91.4% | 83.2% |
| 0503M02138/E/1996 | | 100% | 92.5% | 90.9% | 83.3% |
| 620354/E/2005 | | | 100% | 90.8% | 83.1% |
| 427299.C12/E/2006 | | | | 100% | 82.7% |
| MN/B/1990 | | | | | 100% |

[a]From Table 1.

stages V and VI samples were divided into those collected during 1999-2005 [*n*=13 (10%) and 21(16%), respectively] and during 2006-2013 [*n*=25 (19%) and 50 (38%), respectively].

Peptide ELISA showed 6 (4%), 105 (79%), 16 (12%) and 6 (4%) were subtype B, CRF01_AE, non-B, non-E and undetermined subtype, respectively.

**HIV pseudoviruses**

In order to overcome the heterogeneity of primary isolated and laboratory-adapted viruses, which may influence the results of neutralizing antibody assay, HIV pseudoviruses were used as substitutes of HIV isolates. Even though 24 circulating CRF01_AE HIV-1 Env-pseudoviruses are used in neutralization assays, few of these HIV pseudoviruses are constructed from HIV CRF01_AE isolates from Thailand as were those used in this study (Table 1) (Karasavvas *et al*, 2015). The average number of amino acid residues in V1, V2, V3, V4 and V5 regions of gp120 in Env-pseudovirus MN/subtype B/1990, 620354/CRF01_AE/2005, 427299.c12/CRF01_AE/2006, CM235/CRF01_AE/1990 and 0503M02138/CRF01_AE/1996) were 17, 32, 27, 19 and 7 amino acids, respectively (Table 3). Percent similarity of HIV pseudoviruses CRF01_AE env nucleotide sequences among all five isolates was higher in older compare to more recent isolates. For instance, similarity of 1990 (CM235/E) compared to 2005 (620354/E) and 2006 (427299.c12/E) isolates was 93.2% and 91.4%, respectively and that of 1996 (0503M02138/E) compared to with recent isolates from 2005 (620354/E) and 2006 (427299.C12/E) isolates was 92.5 and 90.9%, respectively. These results were higher than the similarity (90.8%) between the 2005 (620354/E) and 2006 (427299.C12/E) isolates. Genetic identity of these five HIV *env* showed the least similarity when compared to subtype B pseudovirus, MN/B.

**Neutralizing strength of Fiebig stage V, Fiebig stage VI and chronic serum samples**

Neutralization activities of sera were determined by measuring serum inhibition on a single round of replication of the HIV Env-pseudovirus MN/B, CM235/E, 0503M02138/E, 620354/E and 427299.c12/E. The neutralization strength of chronic sera against four pseudoviruses, namely, MN/B, CM235/E, 0503M02138/E and 620354/E is significantly higher than those of Fiebig stage V group ($p<0.001$, <0.001, 0.015 and 0.014, respectively (Fig

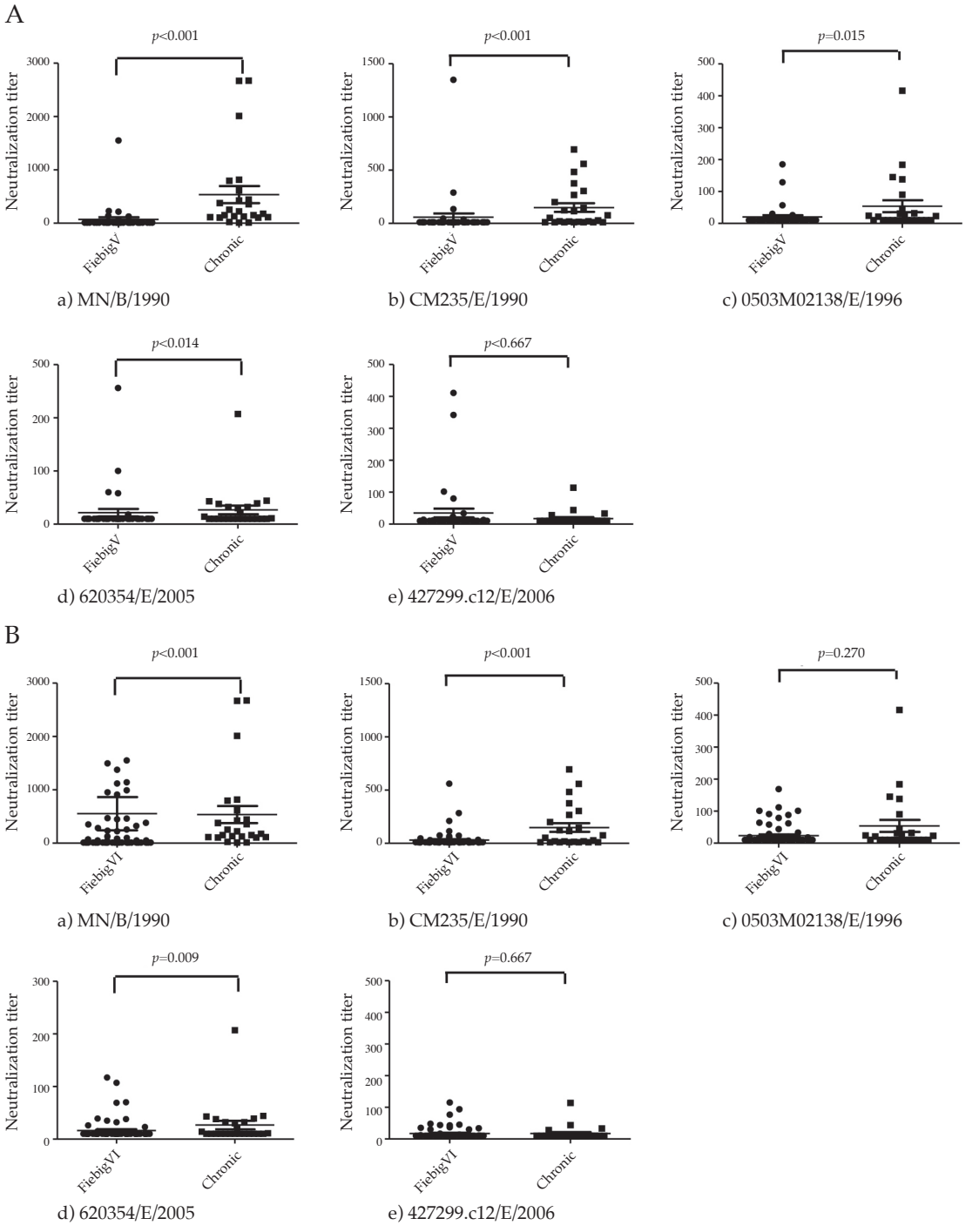


Fig 1 - Comparison of neutralizing activity of (A) Fiebig stage V and (B) Fiebig stage VI with that of chronic HIV infection serum groups against five HIV-1 Env-pseudoviruses. Properties of the HIV-1 Env-pseudoviruses are listed in Table 1. Neutralization antibody assay was performed according to a TZM-bl Nab assay as described by Naldini *et al* (1996), Wei *et al* (2002) and Li *et al* (2005).

Table 4
Neutralization strength, neutralization titer and percent reactivity of HIV-1 infected serum samples against five HIV-1 Env-pseudoviruses used in the study, 1999-2013.

| Serum group | | Neutralization activity (titer) against HIV-1 Env-pseudovirus[a] | | | | | % |
|---|---|---|---|---|---|---|---|
| | | MN/B/ 1990 | CM235/E/ 1990 | 0503M02138/E/ 1996 | 620354/E/ 2005 | 427299.C12/E/ 2006 | reactivity[b] |
| Fiebig | Mean | 71 | 57 | 20 | 22 | 35 | |
| stage V | SD | 252 | 221 | 34 | 43 | 84 | |
| 1999-2013 | Median | 10 | 10 | 10 | 10 | 10 | |
| (*n* = 38) | % reactivity | 21 | 13 | 13 | 10 | 16 | 13 |
| Fiebig | Mean | 29 | 22 | 21 | 17 | 16 | |
| stage V | SD | 46 | 35 | 33 | 18 | 19 | |
| 1999-2005 | Median | 10 | 10 | 10 | 10 | 10 | |
| (*n*=13) | % reactivity | 15 | 15 | 15 | 15 | 15 | 15 |
| Fiebig | Mean | 93 | 76 | 20 | 23 | 44 | |
| stage V | SD | 309 | 271 | 36 | 52 | 102 | |
| 2006-2013 | Median | 10 | 10 | 10 | 10 | 10 | |
| (*n*=25) | % reactivity | 24 | 12 | 12 | 8 | 16 | 12 |
| Fiebig | Mean | 553 | 31 | 24 | 16 | 17 | |
| stage VI | SD | 2,619 | 77 | 31 | 20 | 19 | |
| 1999-2013 | Median | 16 | 10 | 10 | 10 | 10 | |
| (*n*=71) | % reactive | 49 | 15 | 22 | 14 | 17 | 18 |
| Fiebig | Mean | 283 | 39 | 29 | 17 | 20 | |
| Stage VI | SD | 708 | 64 | 33 | 11 | 18 | |
| 1999-2005 | Median | 50 | 10 | 10 | 10 | 10 | |
| (*n*=21) | % reactivity | 62 | 29 | 29 | 29 | 29 | 33 |
| Fiebig | Mean | 666 | 27 | 21 | 16 | 16 | |
| Stage VI | SD | 3,090 | 82 | 30 | 23 | 20 | |
| 2006-2013 | Median | 10 | 10 | 10 | 10 | 10 | |
| (*n*=50) | % reactivity | 44 | 12 | 20 | 8 | 12 | 12 |
| Chronic | Mean | 537 | 148 | 54 | 27 | 17 | |
| 1999-2005 | SD | 780 | 198 | 91 | 40 | 22 | |
| (*n*=24) | Median | 199 | 40 | 15 | 10 | 10 | |
| | % reactivity | 92 | 54 | 50 | 37 | 17 | 50 |

[a]From Table 1. [b]Percent sera containing neutralizing antibodies against >2 pseudoviruses.

1A). Chronic serum group also has significantly higher neutralizing titer than Fiebig stage VI group against three pseudoviruses, namely, MN/B, CM235/E and 620354/E ($p$<0.001, <0.001 and 0.009, respectively) (Fig 1B). However, neutralization ability of Fiebig stage V serum group against 427299.c12/E is not significantly different than that of Fiebig stage VI or chronic serum group (Fig 1A). Pseudovirus MN/B was the most sensitive virus to neutralization with significantly higher sensitivity to chronic serum group than to Fiebig V or VI stage serum group ($p$<0.001 and <0.001, respectively) (Fig 1Aa and Ba). Among CRF01_AE pseudoviruses, CM235/E was the most sensitive virus with mean neutralization titer of 148 ±194, 57±220 and 30±77 from chronic, Fiebig stage V and Fiebig stage VI serum groups, respectively (Table 4).

The neutralizing activity against the five HIV pseudoviruses were also compared in each group. Chronic serum group had higher neutralization ability against MN/B than 0503M02138/E, 620354/E and 427229.c12/E (all $p$<0.001) (Fig 2Aa, b and c). Also, the neutralization titer of chronic serum group against CM235/E is significantly higher than that against 427229.c12/E ($p$=0.003) (Fig 2Ad). There are no significant differences in neutralization ability from Fiebig stage V serum group against all five pseudoviruses. Fiebig stage VI serum group has neutralization strength significantly different against MN/B compared to CM235/E, 620354/E, 0503M02138/E and 427299.c12/E ($p$=0.003, <0.001, 0.027 and <0.001, respectively) (Fig 2B).

**Neutralization breadth of Fiebig stage V, Fiebig stage VI and chronic serum samples**

Among the 1999-2005 HIV seropositive serum groups, the number of neutralizable sera against each pseudovirus gradually increased from Fiebig stage V to Fiebig stage VI and then chronic infection sample groups with the highest titer being among the chronic serum group (91.67%) (Table 4). In addition, 50% of sera from chronic infection group showed a broad range of neutralization activity. The neutralization range (33%) of Fiebig VI serum group from 1999-2005 is significantly broader than that (12%) from 2006-2013 ($p$=0.008) but there is no significant difference in neutralization breadth between the Fiebig VI serum groups.

Of the five pseudoviruses, MN/B pseudovirus, a tier 1A virus, was the most sensitive to neutralization both in strength and breadth by all serum groups (Table 4). Among the HIV-1 CRF01_AE pseudoviruses, CM235/E was the most neutralization sensitive but had less strength and breadth than MN/B pseudovirus. Of the 24 HIV-1 chronic infection sera from 1999-2005, 13 (54%), 12 (50%), 9 (37%) and 4 (17%) were able to neutralize CM235/E, 0503M02138/E, 620354/E and 427299.c12/E, respectively but with lower strength than against tier 1 MN/B virus (Table 4). However, chronic infection sera from 1999-2005 give significantly higher neutralizing titer against the older CRF01_AE virus, E/CM235/1990 (148±198) than the more recent E/427299.c12/2006 (17±22) ($p$=0.003) but not significantly different against the other two CRF01_AE viruses, E/0503M02138/1996 (54 ± 90) and E/620354/2005 (27±40). The neutralization strength of Fiebig stage V 2006-2013 serum group against the older CRF01_AE virus (E/CM235/1990) is not significantly different than Fiebig stage VI group. The neutralization titers of Fiebig stage VI sera of 1999-2005 sera against the four HIV-1 CRF01_AE pseudoviruses are not significantly different to those from 2006-2013

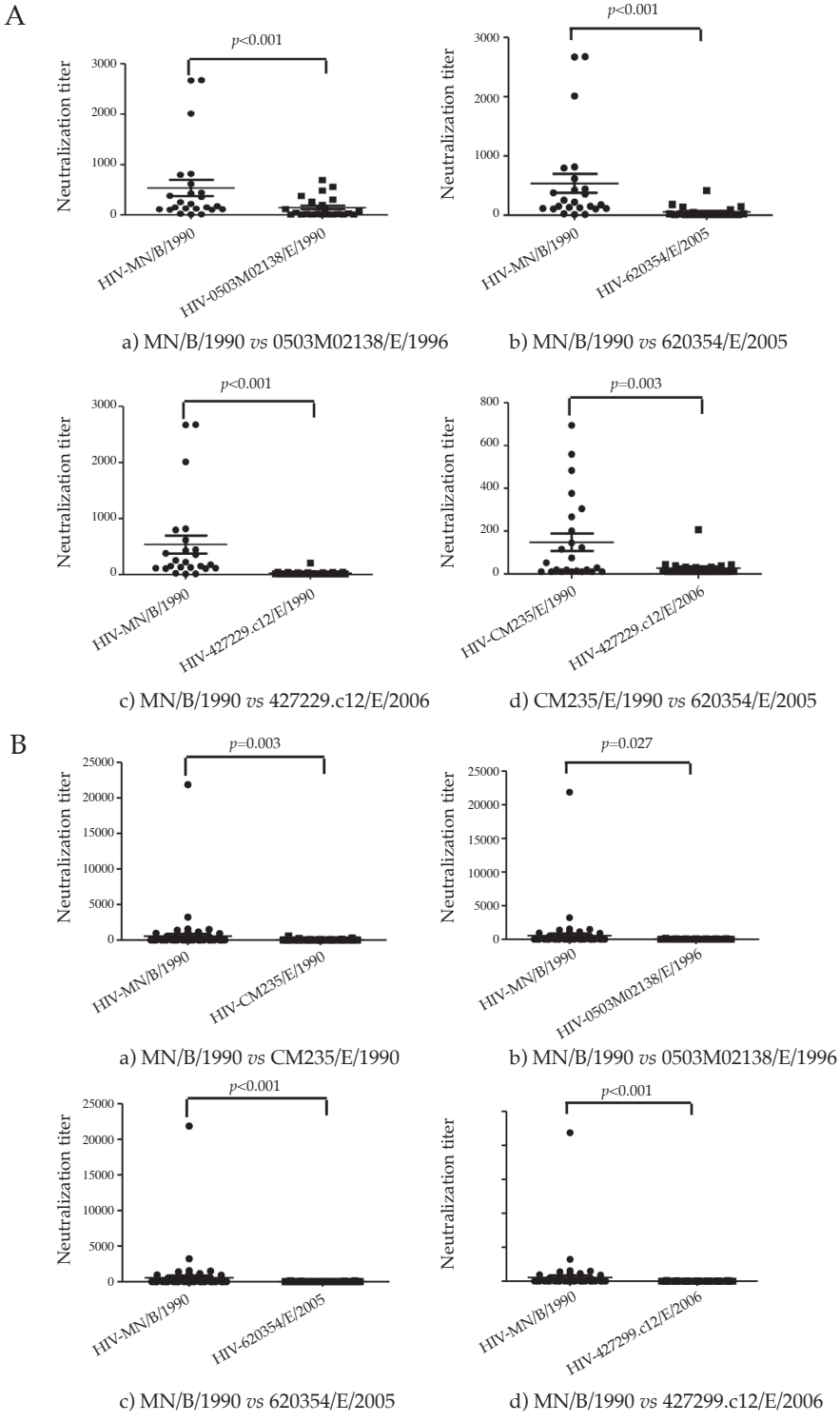


Fig 2 - Pair-wise comparison of HIV-1 Env-pseudoviruses in neutralizing activity assay using (A) chronic HIV infection and (B) Fiebig stage VI sera. Properties of the HIV-1 Env-pseudoviruses are listed in Table 1. Neutralization antibody assay is described in legend to Fig 1.

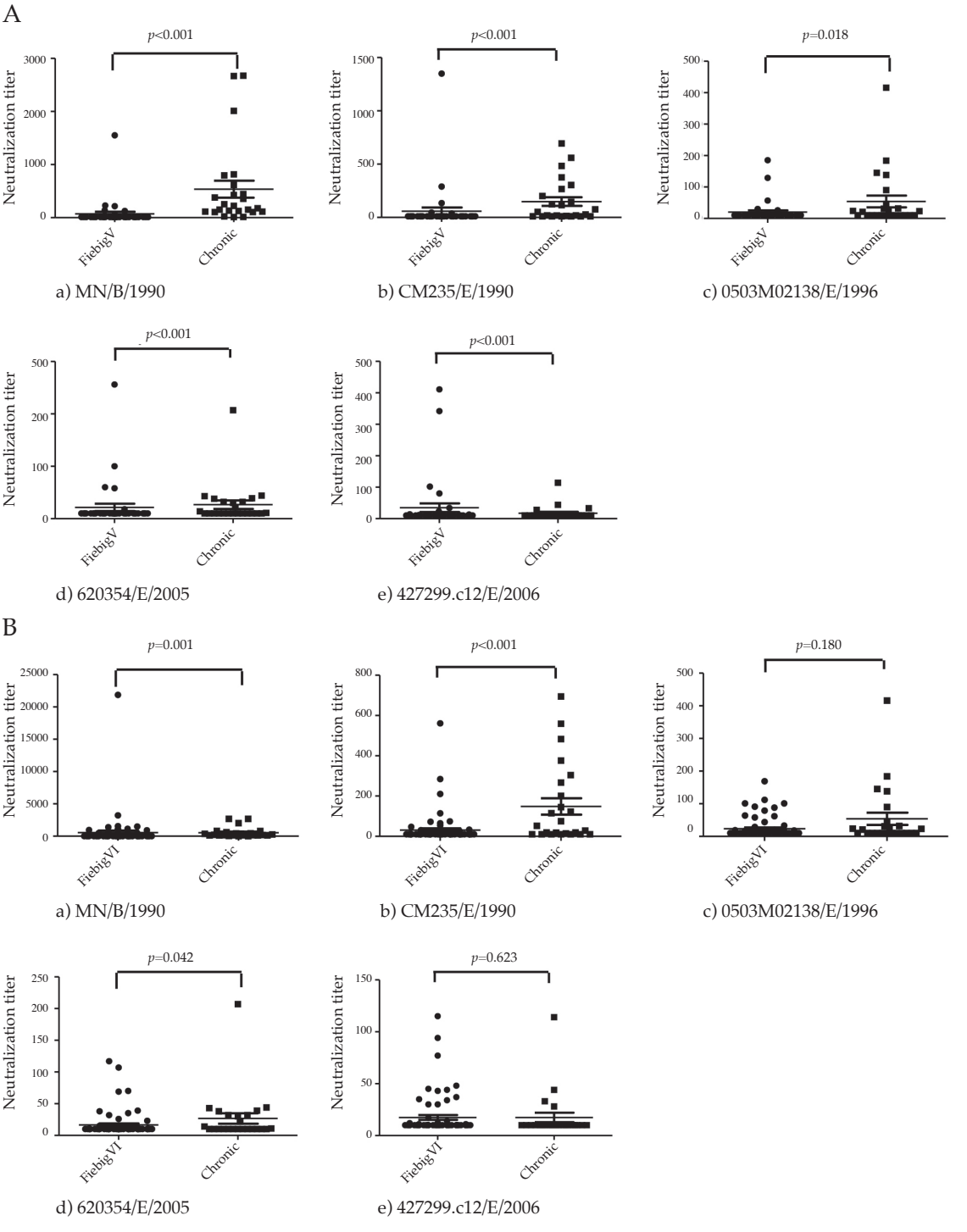


Fig 3 - Comparison of neutralizing activity of HIV-1 subtype E infected sera from chronic HIV infection with (A) Fiebig stage V and with (B) Fiebig stage V serum groups against five HIV-1 Env-pseudoviruses. Properties of the HIV-1 Env-pseudoviruses are listed in Table 1. Neutralization antibody assay is described in legend to Fig 1.

but the fraction of neutralizable sera from 1999-2005 against 620354/E pseudovirus (29%) is significantly higher than that (8%) from 2006-2013 ($p$=0.018).

**Neutralizing activity of subtype E serum from HIV-1-infected young Thai men**

Of the neutralizing activity of HIV-1 subtype E serum samples [Fiebig stage V ($n$=27), Fiebig stage VI ($n$=57) and chronic stage ($n$=21)] against five HIV-1 pseudoviruses, chronic serum samples showed the highest neutralizing activity against all HIV-1 pseudoviruses except 427299. c12/E.The neutralizing antibody titers of chronic subtype E sera against MN/B, CM235/E, 0503M02138/E and 620354/E are significantly higher than those from Fiebig stage V subtype E sera ($p$<0.001, <0.001, 0.018 and 0.008, respectively (Fig 3A). In addition, the neutralizing activity of chronic HIV-1 subtype E sera against MN/B, CM235/E and 620354/E is significantly higher than those of Fiebig stage VI subtype E sera ($p$=0.001, <0.001 and 0.042, respectively) (Fig 3B). However, the neutralization strengths of chronic, Fiebig stage V and Fiebig stage VI subtype E serum groups are not significantly different from those of all serum groups.

## DISCUSSION

It is well known WB assay is not sensitive enough in most cases to identify recent HIV-1 infection (Hecht *et al*, 2011) and a multiassay algorithm (MMA) is required to detect recent HIV-1 infections which false misdiagnosis is easily found due to low avidity and specificity of HIV-specific antibodies (Laeyendecker *et al*, 2013).

HIV-1 Env glycoproteins (gp120 and gp41) play crucial rules in viral transmission and infection of target cells through specific interactions with CD4 receptor and chemokine co-receptors (Engelman and Cherepanov, 2012). Env is the most variable HIV-1 protein with a typical intersubtype and intrasubtype difference of 35% and 20%, respectively (Gaschen *et al*, 2002; Utachee *et al*, 2009). Moreover, in Thailand recent CRF01_AE strains are 31% more diverse than those from past HIV-1 epidemics (Kijak *et al*, 2013). Mutations of amino acid residues involved in N-linked glycosylation affect protein structure and affect neutralization susceptibility of HIV-1 Env (Mascola and Montefiori, 2003; Hwang *et al*, 2010). Here we characterized the neutralization activity of sera from HIV-1 infected Thai men at different stages of HIV-1 infection and show HIV pseudovirus CRF01_AE isolates from 1990 to 1996 were less similar in *env* nucleotide sequences than recent isolates from 2005 to 2006.

High-throughput assay for immune responses will be required to evaluate and compare vaccine candidate immunogens. Thus, standardized panels of Env-pseudoviruses were established to assess the potency and breadth of NAb elicited by vaccine immunogens. A three-tier algorithm for the evaluation of novel immunogen (s) was approached to evaluate Nab responses (Mascola *et al*, 2005). HIV-1 Env-pseudoviruses were classified based on their neutralization sensitivity into tiers, with tier 1 viruses being the most sensitive to neutralization, tier 2 moderately sensitive and tier 3 the least sensitive (Seaman *et al*, 2010). Fiebig stage V, Fiebig stage VI and chronic sera had high mean titer of neutralizing activity against tier 1 Env-pseudovirus, with the neutralization strength of chronic HIV-1 infected sera showing higher neutralization titer and breadth against tier 1 than tier 2 Env-pseudovirus (as expected despite the heterologous subtypes and stages of HIV infection). Although 80% of tested sera

were subtype E, their neutralizing activities against tier 1 heterologous virus were higher than homologous CRF01_AE pseudoviruses. Previously, NAb responses in two HIV-1 vaccine efficacy trials, RV144 and Vax003, showed only tier 1 heterologous pseudoviruses are neutralized in RV144 trial and weak neutralization of tier 2 pseudoviruses is occasionally seen in Vax003 trial (Montefiori *et al*, 2012). Hence, exposed neutralizing epitopes of highly sensitive tier 1 Env protein might be more important for neutralization than consensus sequence of the same subtypes. A single amino acid change in gp41 membrane proximal external region is capable of transforming a tier 2 into tier 1 virus (Bradley *et al*, 2016).

When NAb responses in acute and chronic HIV-1-infected plasma are compared, Nab responses against both autologous and heterologous virus are lower among individuals with acute infection than among those with chronic infection (Deeks *et al*, 2006). In our study, the neutralization potency of chronic infection sera against tier 1 pseudoviruses were higher than sera of early infection, in line with previous studies (Sapsutthipas *et al*, 2013; Chaitaveep *et al*, 2016). Recent CRF01_AE viruses might be more diverse in gp120 V1V2 loop length, net charge and number of N-linked glycans, all associated with neutralization escape, than older viruses (Hraber *et al*, 2014).

Interestingly, neutralization by Fiebig stage VI serum group from 1999-2005 is significantly broader than those from 2006-2013, but there is no difference in neutralization breadth of Fiebig stage V serum groups from both periods. However, the breadths of Fiebig stage V and VI serum groups were less than that of chronic infection serum group. The broad NAbs in sera of recent (29%) and chronic (42%) HIV-1-infected Thai individuals against recombinant viruses with both homologous and heterologous subtypes were reported (Chaitaveep *et al*, 2016). The neutralization potency and breadth of chronic HIV-1 infection sera against 219 Env-pseudoviruses (A, B, C, D, G and CRFs) display some level of cross-neutralization and approximately half of the number of sera neutralize >50% of the tested viruses (Doria-Rose, 2010; Hraber *et al*, 2014), but the NAb breadth can vary widely among chronic infected patients (Doria-Rose *et al*, 2010, Euler *et al*, 2010). One third of patients with chronic HIV infection have broad NAbs (Sather *et al*, 2009; Simek *et al*, 2009). In addition, >50% of chronic HIV-1 clade B-infected plasma donors neutralize at least half of the viruses tested and 2% neutralize all while 5% neutralized none of the viruses tested (Hu *et al*, 2012). All of these reports support the neutralization activity responses observed in our study.

A significantly higher number of NSI subtype E viruses are able to neutralize sensitive more than SI subtype E (Polonis *et al*, 2003). In our study, the neutralizing activities against NSI (CM235/E/1990) and SI (0503M02138/E/1996) pseudoviruses were not different. In heterosexual HIV-1 infections (80%) established by one transmitted/founder virus, the first NAb against this virus arises 3 months after transmission and is strain-specific (Keele *et al*, 2008). However, this Nab drives viral escape and virus mutants become resistant to neutralization by autologous plasma, and then 80% of patients have poor or restricted specificity of Nabs (Richman *et al*, 2003; Goo *et al*, 2014). On the other hand, 20% of the patients have broad Nabs (Bonsignori *et al*, 2011; Walker *et al*, 2011; Goo *et al*, 2014).

In summary, this study characterized

the strength and breadth of neutralizing antibodies of early HIV-1-infected sera against tier 1 virus subtype B and two tier 2 CRF01_AE viruses. Although the strength and breadth of recent HIV-1 infected sera have been reported, the sample sizes are limited (Chaitaveep *et al*, 2016). This study also tested the neutralizing activity of 133 HIV-1 infected sera from 1999-2013 and finds early infection sera contained sufficient potency and breadth to neutralize tier 1 clade-mismatched virus. Thus, further studies should be conducted on immunogens for development of non-clade specific HIV vaccines.

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