

## HIV-1 Gag specific IgG response in mice immunized with Vp22-Gag vaccine candidate

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### Abstrak

**Latar Belakang:** Stimulasi respon sel T CD8<sup>+</sup> spesifik Gag, terkait dengan penurunan viremia, kontrol replikasi virus, dan perkembangan penyakit yang lambat. Respon T CD8<sup>+</sup> yang efektif juga dipengaruhi oleh sel T CD4<sup>+</sup>. Protein rekombinan Gag dapat diklonasi dan diekspresikan pada sistem prokariota, dan pada saat diimunisasi pada hewan coba atau manusia akan bersifat sebagai antigen eksogen. Antigen eksogen dapat menjadi antigen endogen dengan menambahkan protein yang mempunyai kemampuan bertranslokasi kedalam membran sel, salah satunya protein Vp22.

**Metode:** Transformasi Plasmid rekombinan didapatkan dari Pusat Penelitian dan Layanan Virologi Kanker Patobiologi Fakultas Kedokteran Universitas Indonesia-Rumah Sakit Pusat Nasional dr. Cipto Mangunkusumo (PPLVKP FK UI-RSCM), yang dilakukan transformasi pada sistem ekspresi prokariota dengan metode heat shock, yang dilanjutkan dengan ekspresi protein rekombinan. Purifikasi protein rekombinan dilakukan dengan kromatografi afinitas. Analisa berat molekul protein rekombinan dilakukan dengan SDS-PAGE. Western blotting dilakukan untuk mengetahui reaktivitas protein rekombinan dengan antibodi poliklonal terhadap antigen p24. Transfeksi sel CHO dan imunisasi mencit DDY dengan protein rekombinan, untuk mengetahui kemampuan migrasi intraseluler serta stimulasi respon imun spesifik.

**Hasil:** Uji western blotting, menunjukkan protein rekombinan dapat berinteraksi dengan antibodi poliklonal terhadap antigen p24. Pengamatan mikroskop konfokal menunjukkan protein rekombinan berlokalisasi dengan endosom. Uji ELISA, menunjukkan respon IgG spesifik Gag setelah imunisasi pada mencit DDY.

**Kesimpulan:** Protein rekombinan dapat diekspresikan pada sistem ekspresi prokariota. Kemampuan migrasi intraseluler protein rekombinan pada sel CHO belum dapat dibuktikan. Protein rekombinan dapat menstimulasi respon IgG spesifik Gag. (*Health Science Journal of Indonesia 2017;8(2):59-67*)

**Kata Kunci:** Protein rekombinan Gag dan Vp22-Gag; Migrasi intraseluler, Respon IgG spesifik Gag.

### Abstract

**Background:** Stimulation of Gag-specific CD8<sup>+</sup> T-cell response, associated with reduction in viremia, viral replication control, and slow disease progression. Effective CD8<sup>+</sup> T cell response is also influenced by CD4<sup>+</sup> T cells. Gag recombinant protein may be cloned and expressed in the prokaryotic system and when they are immunized in experimental animals or human will have property as exogenous antigens. Exogenous antigens may become endogenous antigens by adding proteins that have the ability to translocate into the cell membrane, one of which is the Vp22 protein.

**Methods:** Recombinant plasmids were obtained from Research and Services Centers of Virology and Cancer Pathobiology Medical Faculty Universitas Indonesia-dr. Cipto Mangunkusumo National Central General Hospital (PPLVKP FK UI-RSCM) which transformation to prokaryotic expression system with heat shock method was followed by expression of recombinant proteins. Purification of recombinant proteins was performed with affinity chromatography. The molecular weight analysis of recombinant proteins was performed with SDS-PAGE. Western blotting was performed to determine the reactivity of recombinant proteins with polyclonal antibodies against p24 antigens. Transfection of Chinese Hamster Ovary (CHO) cell and immunization of Deutschland, Denken, and Yoken (DDY) mice with recombinant proteins was conducted to determine intracellular migration ability and stimulation of specific immune response.

**Results:** Western blotting test, indicating recombinant protein may interact with polyclonal antibody against p24 antigens. The observation of a confocal microscope showed recombinant proteins localized with endosomes. The Enzyme-linked Immunosorbent Assay (ELISA) test indicates Gag-specific IgG response after immunization in DDY mice.

**Conclusion:** Recombinant proteins may be expressed on a prokaryotic expression system. The ability of recombinant protein intracellular migration in CHO cell has not been proven. Recombinant proteins may stimulate Gag-specific IgG response. (*Health Science Journal of Indonesia 2017;8(2):59-67*)

**Keywords:** Gag and Vp22-Gag recombinant proteins; intracellular migration, Gag-specific IgG response.

HIV is a virus of the Retroviridae family, Orthoretrovirinae subfamily, the lentiviruses genus (which comes from the Latin, lentus, meaning slow),<sup>1</sup> which is the main cause of AIDS, ie: a state of decreasing immune function, which thus increases the vulnerability to a number of infections with fatal consequences, called opportunistic infections.<sup>2,3</sup> In 2014, there were 35 million people with HIV and 25,000,000 people with HIV-related deaths worldwide, 90% cases of which occur in developing countries, which treatments for the infection generally were not available.<sup>4</sup> Indonesia reported 150,296 HIV cases and 9,796 HIV-related deaths in 2014.<sup>5</sup>

HIV infection control was done with ARV treatment. ARV administration may reduce the number of virus in circulation to a level that may not be detected by the existing examination system,<sup>6</sup> but ARV may not eliminate the virus. Prolonged ARV administration may cause side effects, including: toxicity and resistance.<sup>7-9</sup> Thus, the infection development should be controlled, by developing vaccine candidates.<sup>10</sup> One way to produce vaccine candidates that have a good effectiveness is by developing vaccine candidate based on viral subtypes which are predominant in the population.<sup>3,4,11,12</sup> The dominant HIV subtype in Indonesia is CRF01\_AE.<sup>13</sup>

Gag protein, is a virion-building structural protein, which is relatively more sustainable than other viral proteins, and may stimulate the immune response, so it can be developed as a vaccine candidate.<sup>14,15</sup> Gag recombinant protein subunit vaccine candidates may be cloned and expressed in prokaryotes system. The vaccine when injected to the experimental animal or human will have property as an exogenous antigens.<sup>16,17</sup>

In developing a vaccine, an adjuvant which is molecule that may increase the vaccine potential, is generally added.<sup>18</sup> Vp22 protein has been used as DNA vaccine adjuvant, due to its ability to induce the proteins spread intercellularly. Some studies indicated that DNA vaccine whose antigens is expressed in a fusion manner with Vp22 will improve the antibodies response.<sup>19</sup> Vp22 protein is a HSV-1 tegumen protein encoded as gene ul49,<sup>20,21</sup> which have translocation capability into cell.<sup>22,23</sup> The intracellular migration ability of Vp22 protein has been demonstrated in several researches, including among others: transfection in COS-1 cell,<sup>24</sup> transfection in BT549 cell,<sup>25</sup> and transfection in MCF-7 cell.<sup>23</sup>

The stimulation of Gag-specific CD8<sup>+</sup> T cell response is associated with reduction in viremia, viral replication control and slow disease progression.<sup>14</sup>

The effective CD8<sup>+</sup> T response was also influenced by the CD4<sup>+</sup> T cells.<sup>26,27</sup> This study is aimed at obtaining Vp22-Gag recombinant protein HIV-1 subtype CRF01\_AE, and knowing the intracellular migration ability of recombinant protein as well as specific immune response stimulation of recombinant protein.

## METHOD

### Recombinant plasmids

pQE-801 plasmid (Invitrogen, Carlsband, CA) which have been inserted with *gag* and *vp22-gag* genes were obtained from PPLVKP FK UI-RSCM. Recombinant plasmid is confirmed by sequencing.

### *E.colli* BL-21CP

*E. coli* BL-21CP (DE3)-RIPL, with genotype BF<sup>-</sup> *optM* *hsdS* (rB<sup>-</sup> mB<sup>-</sup>) dcm<sup>+</sup> Tet<sup>r</sup> gal  $\square$  (DE3) ENDA The [*argU proL CamT*] [*argU ileY leuW Strep/Spec<sup>r</sup>*] (Aglient, Santa Clara, CA). The bacteria were cultured in liquid LB medium, with the addition of 0.1 M MgCl<sub>2</sub> as much as 1/5 culture volume, incubated in ice for 15 minutes and then centrifuged 3.500 g, 4°C, for 10 minutes. The supernatant was removed and added with 100 mM CaCl<sub>2</sub> as much as 1/100 culture volume. Competent bacteria may be used as a recombinant protein expression system.<sup>28</sup>

### Chinese Hamster Ovary (CHO) cell

CHO cell were obtained from the American Type Culture Collection (Manassas, VA). CHO cell were cultured in DMEM medium, which was added with 15% fetal bovine sera, 2 mmol/l L-glutamine, 2 mmol/l HEPES, 100 pmol/l non-essential amino acids, 10 mg/l phenol red, and 1,000 mmol/l peniciline-streptomycine.<sup>28</sup>

### Deutschland, Denken, and Yoken (DDY) mice

Male DDY mice at 5 weeks of age which were bred inbreeding manner were obtained from PT. Biofarma-Bandung. They were kept for 2 weeks in the laboratory before the treatment was administered.

### Transformation of recombinant plasmids

The transformation was conducted with heat shock method, with composition of 50  $\mu$ l and 5  $\mu$ l plasmid (concentration of 100 ng) were added. 200  $\mu$ l SOC medium were added and incubated at 37°C, for 60

minutes, at 150 rpm. A total of 50  $\mu$ l suspension was cultured at LB agar (added with antibiotic ampicillin, at a concentration of 1.000  $\mu$ g, as much as  $1/_{1,000}$  volumes of suspension) and incubated at 37° C for 18 hours.<sup>28</sup>

### Expression of recombinant proteins

Expression of recombinant proteins was made by culturing the bacteria which had been transformed with the recombinant plasmid in 100 ml TB broth (added with antibiotic ampicillin at concentration of 1,000  $\mu$ g, as much as  $1/_{1,000}$  suspension volume) and incubated for 6 hours, at 37° C, 150 rpm. In the 2<sup>nd</sup> hour, 100 mM IPTG was added (as much as  $1/_{1,000}$  volume of bacterial suspension).<sup>28</sup>

One ml bacterial culture was taken in the 2<sup>nd</sup> hour before IPTG was added and every hour after was IPTG added. It was centrifuged for 1 minute, at 4° C, 12,000 rpm and the supernatant was discarded. The pellets obtained were stored at 4° C for SDS-PAGE analysis.<sup>28</sup>

### Purification of recombinant proteins

The recombinant protein was purified with native methods, in accordance with the procedures of The QIA Expressionist: A handbook for high-level expression and purification of 6xHis-tagged proteins 5th ed.<sup>29</sup>

### Western blotting of recombinant proteins

Blotting was done with semidry method, which was constant 25 volts, for 30 minutes. Ponceau was added to nitrocellulose membrane to look at the blotting efficiency.<sup>28</sup> The first antibodies used were: (1) HIV-infected patient sera; (2) HIV-un infected patient sera; (3) rabbit antibodies against p24 antigens; (4) and rabbit antibodies against *E.colli*. The second antibodies were: (1) antibody against biotin-labelled human antibodies; (2) and antibody against biotin-labelled rabbit antibodies.<sup>28</sup> A 70  $\mu$ l subrat (imun-star reagent [Biorad] with reagent ratio A: B = 1: 1) was added. It was incubated in the dark for 1 minute and observed at Quant LS4000.<sup>28</sup>

### Transfection of CHO cell

300  $\mu$ l CHO cell at concentration of  $5 \times 10^5$  cell/ml were added with 1 ml complete DMEM medium that had been added with dextran tetramethylrhodamine. It was incubated at 37° C, 5% CO<sub>2</sub>, for 48 hours. CHO cell were added with recombinant proteins

and immunostaining was then performed. The CHO cell that were added with *eGFP* and *gag* plasmids were incubated at 37° C, 5% CO<sub>2</sub>, for 48 hours and immunostaining was then performed.<sup>28</sup>

### Immunostaining of CHO cell

CHO cell were washed with PBS 1x, and fixated with 3,7% formaldehyde in PBS 1x. It was permeabilized with 0,2% triton x-100 in PBS 1x. The 1<sup>st</sup> antibodies, namely: rabbit antibodies against p24 antigens, at concentration of  $1/_{10,000}$  in 2% BSA, and washed with PBS 1x. The 2<sup>nd</sup> antibodies, namely: antibody against FITC-labeled rabbit antibodies, at concentration of  $1/_{10,000}$  in 2% BSA, washed with PBS 1x. The cover slide was moved to a glass object with mouwil. The observation was performed with a confocal microscope.<sup>28</sup>

### Mice immunized

The total DDY mice were calculated with Federer colculation, namely:  $(t-1) (n-1) \geq 15$ .<sup>30</sup> Mice blood was collected from the submandibular vein one week before the first immunization. Immunization was done three times with interval of each immunization of two weeks. Immunization was done intramuscularly on quadriceps muscle.<sup>28</sup> Each mice was given with 100  $\mu$ g antigens at 5  $\mu$ g concentration in each immunization and 10  $\mu$ l DMRIE-C was added.<sup>31-33</sup>

### Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed on plate 96 well which was added with 70  $\mu$ l Gag recombinant protein with concentration 12,5  $\mu$ g/ml in carbonate/bicarbonate pH 9,6 and incubated for 60 minutes, at 37° C. It was washed with 100  $\mu$ l 0,05% tween-20 in PBS 1x. 80  $\mu$ l 5% skim milk was added in PBS 1x and incubated for 60 minutes, at 37° C. It was washed with 100  $\mu$ l 0,05% tween-20 in PBS 1x. 50  $\mu$ l mice sera was added (each sera was diluted with  $1/_{27}$ ,  $1/_{81}$ ,  $1/_{243}$  and  $1/_{729}$  in 0,1% skim milk). It was incubated for 60 minutes, at 37° C and was washed with 100  $\mu$ l 0,05% tween-20 in PBS 1x. 50  $\mu$ l antibody against biotin-labeled mice sera were added at concentration of  $1/_{5,000}$  in 0,1% skim milk and incubated for 60 minutes, at 37° C. It was washed with 100  $\mu$ l 0,05% tween-20 in PBS 1x and added with 50  $\mu$ l streptavidin-HRP at concentration of  $1/_{10,000}$  in 0,1% skim milk and incubated for 60 minutes, at 37° C and washed with 100  $\mu$ l tween-20 0,5% in 1x PBS. 48  $\mu$ l OPD substrates were added and incubated for 10 minutes at room temperature and added with 24  $\mu$ l 2,5M H<sub>2</sub>SO<sub>4</sub> in PBS 1x. Then read with spectrophotometer  $\lambda = 450\text{nm}$ .<sup>28</sup>

## Statistical analysis

Statistical analysis was performed using SPSS software (v 20.0). Mice antibody titers data before and after immunization were tested with paired t-test if the data were normally distributed, if not normally distributed then the data were tested with Wilcoxon test. Mice antibody titers data after immunization in each treatment group were tested with one-way anova test followed with LSD test, if the data were not normally distributed and not homogeneous, then Kruskal-Wallis test was performed, followed with Mann-Whitney test. The value of  $p < \alpha$ , showed data was significantly different. The value of  $\alpha$  used is 0,05 with 95% confidence level.<sup>34, 35</sup>

## RESULTS

### Transformation of recombinant plasmids

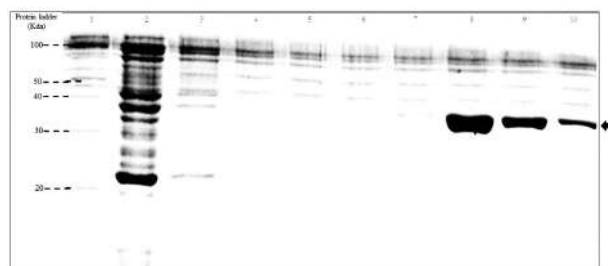
*E.colli* BL-21CP which had been transformed with the recombinant plasmids might grow in LB agar that contained the antibiotic ampicillin. Meanwhile, the bacteria control, namely: *E.colli* BL-21CP which was not transformed with the recombinant plasmids could not grow on the LB agar that contained the antibiotic ampicillin (Data not showed).

### Expression of recombinant proteins

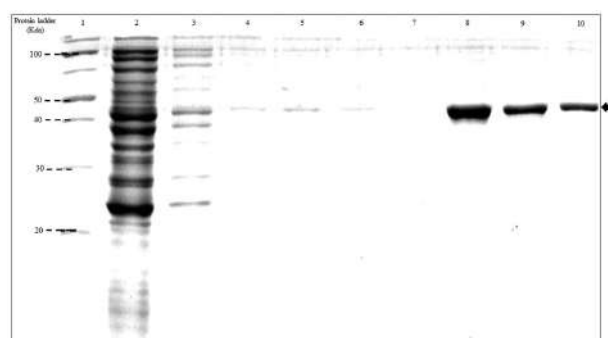
SDS-PAGE of Gag protein-expression bacteria there were dominant bands located between the protein ladder with the molecular weight of 30 KDa and 40 KDa. On the other hands, the Vp22-Gag protein-expression bacteria had dominant bands located between the protein ladder with the molecular weight of 40 KDa and 50 KDa. At the 1<sup>st</sup> hour after induction, the protein band continued to thicken when the incubation period increased (Data not showed).

### Purification of recombinant proteins

SDS-PAGE analysis of recombinant proteins after purification showed that in the flowthrough there were some bands, in the wash buffer there was band, and in the elution there was one band. In the 1<sup>st</sup> elution, the band was the thickest and in the further elution the band was getting thinner. The Gag recombinant protein bands migrated between protein ladder with molecular weight of 30 KDa and 40 KDa (**Figure 1**). Meanwhile, the Vp22-Gag recombinant protein band migrated between the protein ladder with molecular weight of 40 KDa and 50 KDa (**Figure 2**).



**Figure 1. SDS-Page of recombinant protein Gag after purification.** Line 1: protein ladder (Page ruler BR unstained), line 2: flowthrough, line 3: wash 1, line 4: wash 2, line 5: wash 3, line 6: wash 4, line 7: wash 5, line 8: elution 1, line 9: elution 2, and line 10: elution 3. Polyacrylamide gel 12%, electrophoresis 60 minute and 150 V. The arrows showed band of recombinant protein that migrated between the protein ladder with a molecular weight of 30 KDa and 40 KDa.<sup>Primary data</sup>



**Figure 2. SDS-Page of recombinant protein Vp22-Gag after purification.** Line 1: protein ladder (Page ruler BR unstained), line 2: flowthrough, line 3: wash 1, line 4: wash 2, line 5: wash 3, line 6: wash 4, line 7: wash 5, line 8: elution 1, line 9: elution 2, and line 10: elution 3. Polyacrylamide gel 12%, electrophoresis 60 minute and 150 V. The arrows showed band of recombinant protein that migrated between the protein ladder with a molecular weight of 40 KDa and 50 KDa.<sup>Primary data</sup>

### Concentration of recombinant proteins

The concentration of recombinant proteins that had been was measured with Bradford method. The results of the measurement were as follows: Gag recombinant protein was 0,080 mg/ml and Vp22-Gag recombinant protein was 0,1318 mg/ml. The recombinant protein was added with 10% glycerol (of the total volume) and stored in a freezer at -20°C, to be used in transfection of CHO cells and DDY mice immunization.

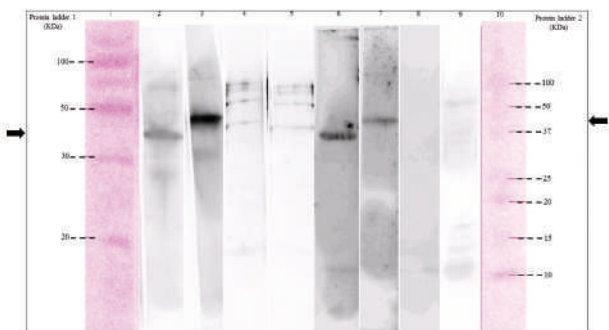
### Western blotting of recombinant proteins

Western blotting showed that the rabbit antibodies against p24 antigens and the HIV-infected patients

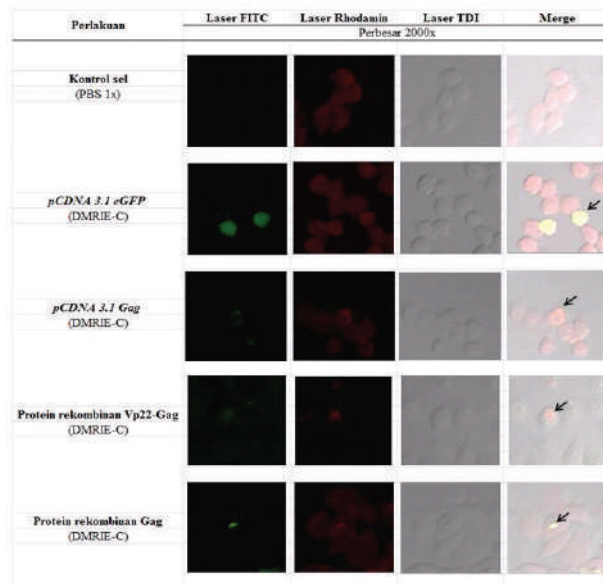
sera interact with Gag and Vp22-Gag recombinant protein, which was indicated by the presence of bands between protein ladder with molecular weight of 30 KDa and 50 KDa. In the recombinant proteins added with antibodies againsts *E.colli* antigens and sera of HIV-uninfected patients sera, there were several protein band patterns, but the protein band patterns formed were different from the band pattern of the recombinant proteins that were added with antibodies against p24 antigens (Figure 3).

**Transfection of CHO cell**

Confocal microscopic showed that Gag and Vp22-Gag recombinant proteins which were formulated with DMRIE-C, were localized with endosomes as indicated by yellow fluorescence, which were a combination of green (antibodies against FITC-labeled p24 antigens) and red (endosome organelles dye, namely: dextran tetramethylrhodamine) (Figure 4).<sup>31</sup>



**Figure 3. Western blotting of recombinant protein.** Line 1: protein ladder (Page ruler BR unstained), line 2: recombinant protein Gag added with antibody that reacted to p24 antigens, line 3: recombinant protein Vp22-Gag added with antibody that reacted to p24 antigens, line 4: recombinant protein Gag added with antibody that reacted to *E.colli* antigens, line 5: recombinant protein Vp22-Gag added with antibody that reacted to *E.colli* antigens, line 6: recombinant protein Gag added with HIV-infacted patiens sera, line 7: recombinant protein Vp22-Gag added with HIV-infacted patiens sera, line 8: recombinant protein Gag added with HIV-uninfacted patiens sera, line 9: recombinant protein Vp22-Gag added with HIV-uninfacted patiens sera, and line 10: protein ladder (Precesion plus protein TM all blue standart). The arrows showed band of recombinant protein that laid between the protein ladder with a molecular weight of 30 KDa and 50 KDa.<sup>Primary data</sup>



**Figure 4. Observation of CHO cell transfected recombinant proteins with confocal microscope.** Immunostained of CHO cell performed with dextran tetramethylrhodamine that can be colored endosome (red). Recombinant proteins that laid within cytosol will have green fluorescence (fitc fluorescence). On the other hand, recombinant proteins that laid within endosome will have yellow fluorescence (combination of red and yellow).<sup>Primary data</sup>

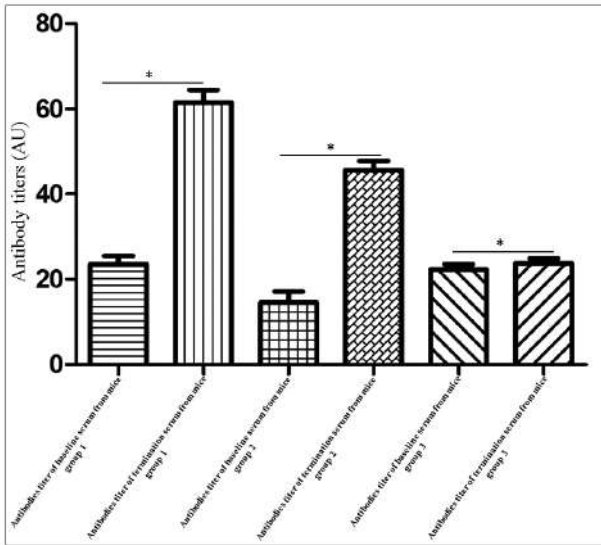
**Mice immunized**

The results of antibodies titers were significantly different between baseline and termination, as expressed with  $p < 0,05$ , when it is done with paired t test (Figure 5). While Mann-Whitney test showed a comparison of termination sera between treatment groups, showing that the group two had the highest and most significant antibodies titers than other treatment groups, as expressed with  $p < 0,05$ (Figure 6).

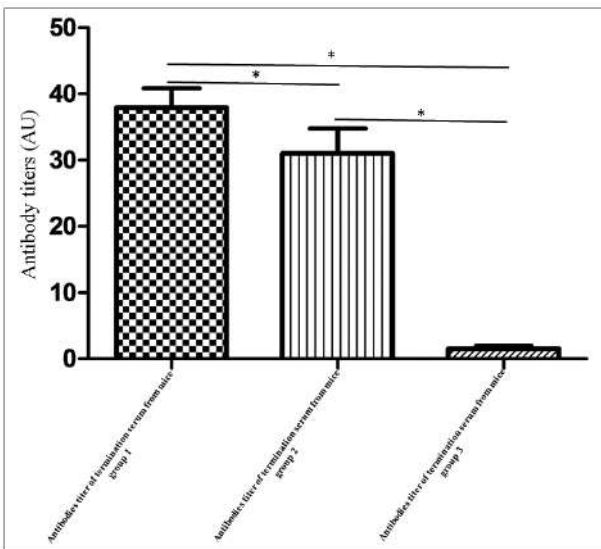
**DISCUSSION**

**Expression of recombinant proteins in prokaryotic expression system**

Plasmids encoded Gag and Vp22-Gag recombinant proteins that used in this study was the DNA resulted from RNA reverse transcription of virus that did not undergo the codon optimization for the mammalian expression system. The differences in viral organisms as DNA to be expressed and prokaryotes that acted as vectors of protein expression may become obstacles in the process of protein translation. This is due to differences in codons between the two organisms. In this study, *E.colli* BL-21CP was used to express the Gag dan Vp22-Gag recombinant proteins with the aim of overcoming the problems of codon differences between the organisms.



**Figure 5. Comparison of antibody titers between termination and baseline mice sera.** Group 1: immunized with recombinant protein Gag formulated DMRIE-C, group 2: immunized with recombinant protein Vp22-Gag formulated DMRIE-C, and group 3: immunized with PBS 1x. Symbol (\*) showed that p value < 0,05. Analysis data with paired t-test. Antibodies titers based on ratio to highest poll sera.<sup>Primary data</sup>



**Figure 6. Comparison of antibody titers of termination sera between mice group.** Antibodies titers of termination corrected with each antibodies titers of baseline. Group 1: immunized with recombinant protein Gag formulated DMRIE-C, group 2: immunized with recombinant protein Vp22-Gag formulated DMRIE-C, and group 3: immunized with PBS 1x. Symbol (\*) showed that p value < 0,05. Analysis data with Mann-Whitnet test. Antibodies titers based on highest poll sera.<sup>Primary data</sup>

The recombinant protein expression utilized the IPTG inducer, which is thio-galactosidase. IPTG will bind with *LacI* and activates the *operon lac*. The *operon lac* will increase the expression of T7 RNA polymerase, and may bind with the T5 promoter contained in the recombinant plasmids.<sup>36-38</sup> The low of IPTG concentration, ie: 1,5 to 2,5  $\mu\text{M}/\text{cell}$  may alter expression of operon lac activities. At high of IPTG concentrations, transacetylase will be performed by LacA in the cell, which converts IPTG into isopropyl 6-o acetyl-pD thio-galaktopironiside and exerted from the cell and has no properties as an inducer.<sup>37</sup> IPTG concentrations used in this study was 0,1 mM/ml.

The expression level of recombinant proteins was also influenced by the incubation temperature, time after induction, and volume of media culture. The volume of culture medium used is determined by the level and location of protein expression. The advantages of IPTG as an inducer, among others are as follows: it may produce high bacteria density, relatively simple use, and does not require a bacterial growth monitoring, as well as a relatively short induction time (where the incubation time of 36 hours means the highest protein expression, while > 36 hours means protein declining). The decreasing concentration of IPTG may reduce cell density (decrease of 0,01 mM IPTG may decrease expression level of 10 to 15 times).<sup>39, 40</sup>

The recombinant proteins were denaturated, when analyzed with SDS-PAGE. SDS is a anionic detergent contained in the SDS-PAGE sample buffer, and instrumental in cutting the hydrophobic protein bonds. Denaturation process is also done by heating (> 60° C), and addition of other reducing agents (eg: in-thiothreitol or DTT and 2-  $\beta$  mercapethanol), which reduces the disulfide bonds. Denaturation of recombinant proteins will eliminate the protein's tertiary (even quaternary) structure, so the proteins are in the primary or linear structure.<sup>41,42</sup> The weaknesses in determination of proteins molecular weight, using SDS-PAGE include error rate of 20%.<sup>43,44</sup>

The Gag and Vp22-Gag recombinant proteins are expressed in fusion state with *6xHistaq*. The *6xHistaq* fused with a recombinant protein has a small molecular weight, and not charged at physiological pH, so that it did not affect the protein structure expressed in fusion manner both on the end of amino and carboxyl. Additionally, the *6xHistaq* is not immunogenic,<sup>40,45</sup> and so will not induce an immune response that may affect the vaccine effectiveness

The Gag and Vp22-Gag recombinant proteins were successfully purified using Ni-NTA in the native

state. The protein was purified from supernatant of bacterial sonication results. The successful protein purification in the native state indicated a big chance that the protein when expressed in bacteria was not in the inclusion bodies.<sup>40</sup> In addition, that the protein may be purified in a native state showed that the 6xHistaq could be accessed by Ni-NTA and not covered by the protein's tertiary structure.

The concentration of purified protein with Ni-NTA was measured with Bradford method. The protein concentrations were calculated based on the linear equations of BSA standard curve that were made based on the ratio of OD with known BSA concentrations. The results of measurement of concentration of Gag and Vp22-Gag recombinant proteins are as follows: 0,080 mg/ml and 0,1318 mg/ml. Ni-NTA resins were capable of binding 5 to 10 mg 6xHis Tag proteins/ml (eg 1 ml agarose Ni-NTA was capable of binding 300 to 400 nmol proteins).<sup>29,40,45</sup>

The recombinant proteins that had been purified still contained proteins contamination from *E. coli*. This is because *E. coli* proteins may bind with Ni-NTA resin through hydrophobic interactions. The contamination of *E. coli* proteins may be overcome with addition of detergent (eg Triton X-100 or Tween-20 with a concentration of less than 1%), with addition of adding NaCl salt solution with a concentration of less than 500 mM NaCl, and addition of glycerol or ethanol with a concentration of <2 0%.<sup>40,45</sup>

Western blotting indicated that Gag and Vp22-Gag recombinant proteins may interact with antibodies against p24 antigens from HIV-1 subtype CRF01\_AE and HIV-infected patients sera. This implied that Gag and Vp22-Gag recombinant proteins were confirmed as HIV proteins and most likely they may stimulate the immune response.

### Interacellular of recombinant proteins on CHO cell

Localization of recombinant proteins with endosomes suggested that recombinant proteins translated into the cell in endocytosis manner, and likely would be destroyed in the lysosome. This is consistent with the results of analysis of the Gag-specific IgG response in mice immunized with Gag and Vp22-Gag recombinant proteins, indicating an increase in antibodies titers. The formation of antibodies in the body is affected by the type of antigens presented by the antigens presenting cell (APC). The exogenous antigens originating from outside the cell will be presented to CD4<sup>+</sup> T cells through the class I MHC molecules after passing through the endocytosis and

proteolysis process in lysosomes. Meanwhile, the endogenous antigens produced by the cell itself will be presented to CD8<sup>+</sup> cells through the class I MHC molecule, without prior processing in the lysosome.

In this study the ability of Gag and Vp22-Gag recombinant proteins to translocate into the intracellular environment were not proven yet, because in this study, the process of delivery of both proteins was done with the help of a delivery system such as DMRIE-C.

### Reactivity of DDY mice sera after immunized

Reactivity of sera against Gag recombinant protein indicated an increase antibody titers before and after immunization with some antigens formulations. It showed that the antigens formulation of recombinant proteins used for immunization of DDY mice might activate CD4<sup>+</sup> T cell that contribute to the process of antibody class switching on B cells. CD4<sup>+</sup> T cell activation occurred as a result of activation by exogenous antigens. This indicated that the addition of Vp22 to the construction of the recombinant protein and formulation with DMRIE-C did not affect the ability of exogenous antigens to remain being processed via endocytosis and proteolysis in the lysosome. The vaccine formulation with DMRIE-C was expected to help the antigens incoming through endocytosis pathway to perform endosomal escape mechanism so it may be 'transformed' into endogenous antigens that activated the CD8<sup>+</sup> T cell.

Formulation of Gag recombinant protein and formulation with DMRIE-C, it was indicated that the antibodies titers was the highest compared with the Vp22-Gag recombinant protein and formulation with DMRIE-C. This lower antibodies titers when compared with the group of mice likely was due to a number of Gag recombinant protein that slip out of the endosome and existed in the cytosol.

The Vp22 addition to the construction of Vp22-Gag recombinant protein was shown to improve the ability of the recombinant protein to translocate across the cells, through the cell junction.<sup>22</sup> The Vp22 ability to translocate to the cell around it will increase the cell which expresses the Vp22-Gag recombinant protein as an endogenous antigens. The Vp22-Gag recombinant protein both formulated with DMRIE-C may induce the formation of Gag-specific IgG response and activate CD4<sup>+</sup> T cell, although the resulting antibodies titers is not as high as the antigens formulation of Gag recombinant protein with DMRIE-C. The utilization of Vp22 as adjuvant of sub unit protein vaccine with the aim of improving the response of antibodies formation has not been

reported. The results of the study also showed that the addition of Vp22, although it did not improve the antibody formation response, proved that the Vp22 did not inhibit the activation of CD4<sup>+</sup> T cells and the formation of antibody responses.

The study by Saha (2006), proved that the complete sequence of Vp22 protein had the ability to stimulate the antibodies response when fused with the nucleoprotein. However, Vp22 protein might not stimulate antibodies response when expressed incompletely, ie: Vp22<sub>1-267</sub><sup>19</sup>. The study by Nishikawa (2010) proved that Vp22 protein with incomplete sequences, ie: Vp22<sub>268-301</sub>, had transmembrane migration capabilities, and could increase the cytosol delivery from of fused molecules. The cytosol delivery was important process in stimulating the peptide-mediated CTL response.<sup>22</sup>

The highest antibodies response was generated by a group of mice immunized with Gag recombinant protein formulation with DMRIE-C. This suggested that the addition of Vp22 were not able to improve the antibodies response, but were able still to induce the activation of CD4<sup>+</sup> T cell. This is in line with the purpose of utilization of ALMR delivery systems and the addition of Vp22 adjuvants successively to increase protein insertion from the endosome and increase the intracellular migration.

In conclusion, in this study, the Gag and Vp22-Gag recombinant proteins were able to be expressed in prokaryotic expression system. The ability of the Vp22-Gag recombinant protein translocation in mammalian cell had not been proven. The Vp22-Gag recombinant protein was able to stimulate the Gag-specific IgG response.

### Competing interest

The authors declare that they have no competing interest

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