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Design of cocktail peptide vaccine against *Cytomegalovirus* infection

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ABSTRACT

Objective(s): *Human Cytomegalovirus* (HCMV) remains a major morbidity and mortality cause in immuno suppressed patients. Therefore, significant effort has been made towards the development of a vaccine. In this study, the expression of the pp65 and gB fusion peptides and Fc domain of mouse IgG2a as a novel delivery system for selective uptake of antigens by antigen-presenting cells (APCs) in *Pichia pastoris* yeast system were studied.

Materials and Method: In this study, four immune dominant sequences in pp65 protein and 3 immuno dominant sequences in gB protein were selected according to literature review. Peptide linker -GGGGS- was used for construction of fusion peptide. This fusion peptide was cloned in the pPICZαA expression vector and transfected into *P. pastoris* host cells.

Results: Dot blot and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) techniques showed that a high level of pp65-gB-Fc fusion peptide was expressed.

Conclusion: This CMV pp65-gB-Fc fusion peptide could be a promising candidate for the development of a novel peptide vaccine.

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Introduction

Human Cytomegalovirus (HCMV) is one of the prevalent opportunistic pathogen that causes a wide spectrum of clinical manifestations (1). This virus is regarded as the main morbidity and mortality cause in immune suppressed patients (ie; organ transplant recipients, human immunodeficiency virus (HIV) patient) (2). Newborns with congenital infection have a mortality rate of about 5 percent, and neurologic morbidity occurs in 50 to 60 percent of survivors (3).

In spite of the fact that CMV is a widespread pathogenin the human population, prevention of its disease is rather difficult. Hence, vaccination is the ideal way to prevent the spread of CMV infections. Although several vaccine approaches have been developed, none have yet been put into routine clinical practice. The cell-mediated immune response is necessary in the recovery of infections (4). The best way to protect females before the onset of puberty is a CMV vaccine that stimulates cellular and humoral immune response. This would reduce the risk of congenital CMV infection in the fetus and newborn (5).

In previous studies, many peptides had been reported that had been used as vaccine against CMV. The most abundant tegument protein (pp65 protein) is a major target of the cytotoxic T-cell (6). Besides, the most abundant envelope protein (the glycoprotein B, gB) is a major target of the humoral immunity (1, 3). It seems that the development of a suitable vaccine for CMV, synchronously using epitopes specific for the immune dominant protein derived from these proteins, are the best choice.

A major limitation for using vaccines to induce protective immune responses against CMV infection is related to achieving efficient uptake and processing antigen-presenting cells (APCs). One way to overcome this problem is the use of the Fc-fusion peptides in which one or more antigenic peptides are fused into the Fc domain of immunoglobulin (Ig). Fusion of peptides to Fc domain can increase harvesting and delivering them to the T lymphocyte, which is critical for protection against CMV. The most effective method of antigen uptake in APCs such as macrophages and dendritic cells (DCs) is receptor-mediated uptake. For this reason, DCs have several types of Fc

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receptors (FcγR) that bind the Fc domain of IgG molecules (7). Therefore, to facilitate the binding of peptide to APC, Fcγ receptor was added to desired fusion peptide. In recent decade, *Pichia pastoris* was used as a useful expression system in vaccine development (8).

In this study, a cocktail peptide vaccine using pp65, and gBwas designed against Cytomegalovirus infection. Cloning and expression of this cocktail peptide vaccine fused with Fc domain of mouse IgG2a in *P. pastoris* was reported.

Materials and Methods

Epitopes sequences

T-cell epitopes for pp65 and gB proteins were selected by literature review. These epitopes were $gB_{431-450}$, $gB_{471-490}$ and $gB_{571-590}$ (9) for gB protein (Gen Bank access number GI: 138192) and $pp65_{361-376}$ (10), $pp_{340-355}$ (11), and $pp65_{417-427}$ (12) for pp65 protein (Gen Bank access number GI: 130714). A universal-GGGGS-linker was used for conjunction of cocktail epitopes. Finally, the Fc fragment of mouse IgG2a (Gen Bank access number GI: 51835) (7) was fused to C-terminal of cocktail epitopes.

Fusion peptides were flanked by *XhoI/ XbaI* restriction enzymes. Sequences optimization and colon simulating in *P. pastoris* were checked by DNA 2.0, GenScript and Genius softwares. pPICZαA expression vector carries *α*-factor signal sequence *(α-MF)* to drive fusion peptide secretion. Besides, this vector contains the zeocin resistance gene, *Shble,* which is used for selection of *Escherichia coli* and *P. pastoris* recombinants. Also, a C-terminal polyhistidine (6xHis) tag was added to C-myc in order for fusion peptide to be detected by dot blot assay. This designed peptide sequence was ordered in pGH vector by Gene Raye company.

Strains and media

Escherichia coli JM109 strain was used as a host for cloning and plasmid manipulation. This strain was cultured at 37 ˚C in LB (Luria Broth) medium supplied with ampicillin. pPICZαA was used as shuttle vector for expression of fusion peptide, and the *P. pastorisGS115* strain (Invitrogen, USA) was used as an expression host. The yeast cells were grown at 30 ˚C on YPD (Yeast extract Peptone Dextrose) medium (1% yeast extract, 2%Bac to-peptone 2% glucose) and YPDS (Yeast Extract Peptone Dextrose medium with Sorbitol) (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol) supplemented with 100 µg/ml zeocin when necessary. Inclusion of sorbitol in YPD plates stabilizes electroporated cells as they appear to be somewhat osmotically sensitive.

Restriction enzymes used for cloning were purchased from Promega and used in accordance with manufacturer's recommendations.

Preparation of fusion peptide sequence

In order to amplify this synthetic fusion sequence, pGH vector was transformed into *JM109 E. coli* strain using calcium chloride method. Plasmid extraction was carried out by commercial kit (Bioneer, Korea). The presence of desired segment was confirmed by double digestion with related restriction enzymes and sequencing.

Construction of pPICZαA-CMV

After minipreparation of pGH containing fusion sequence and double digestion, gel extraction and purification of desired segment were carried out by commercial kit according to manufacturer's recommendations (5 prime, Germany). This segment was cloned into pPICZαA shuttle vector under control of AOX1 promoter, and the resulting vectors were called pPICZαA-CMV. The construction of vector was confirmed by restriction digestion, PCR with AOX1 and α-Factor primers, which amplified entire recombinant fusion peptide sequence. PCR was performed with the primers 3′AOX1 5′-GCAAATGGCATTCTGACATCC-3′, and α-Factor, 5′-TACTATTGCCAGCATTGCTGC-3′. PCR was carried out by 10 pmol of each primers and PCR mix. Temperature profile was one cycle in 94 ˚C for 3 min followed by 30 cycles in 94 ˚C for 1 min, 55 ˚C for 1 min, and 72 ˚C for 2.30 min and finally 72˚C for 1 min.

Electroporation of yeasts

Stable integration into the AOXI (Aldehyde Oxidase 1) of *P. pastoris* was achieved after linearization of the 5 µg pPICZαA- CMV peptide expression vectors with SacI. This recombinant plasmid was purified by phenol-chloroform-propanol alcohol. *P. pastoris* competent cells were provided and transformed by sorbitol method. After colony selection on YPDs media supplemented with 100 µg/ml zeocin, the recombinant *P. pastoris* cells were subjected to BMGY (Buffered Glycerol-complex Medium) medium for fusion peptide expression. In this step, a total of 10 colonies were obtained and subsequently screened for methanol utilization phenotype.

To remove inhibitory effect of other carbon sources, the recombinant *P. pastoris* strain *GS115* was cultured in BMGY medium (containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base, 4 x 10-5% biotin and 1% glycerol). After acquiring BMGY turbidity to OD600 = 1.5, the cells were harvested through centrifuge. In order to induct AOX1, the product in question was transferred into the BMMY medium (the same as BMGY medium but with 0.5% methanol instead of 1% glycerol). In this system, the inducer was 0.5% methanol, which was added into the medium from day 2 up to day 7. To confirm the

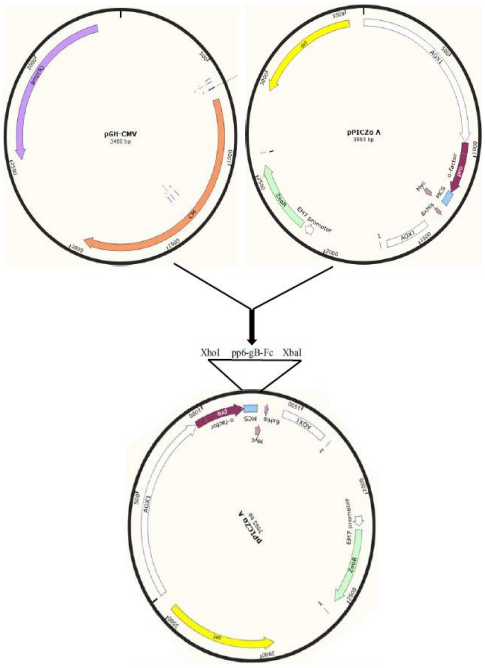

Figure 1. Schematic diagram for cloning of modified pp65-gB-Fc segments in pPICZαA (pPICZαA-pp65-gB-Fc)

expression of pp65-gB-Fc, dot blot and SDS-PAGE were used. SDS-PAGE was performed in a 10% (W/V) polyacrylamide gel. As a control, *P. pastoris* cells were transformed with the vector pPICZαA, without any insert.

Results

Bioinformatics analysis

In attempting to obtain secretion of pp65-gB-Fc fusion peptide at high levels in the culture medium, a cod on-optimized *α*-MF along with the pPICZαA based vector was employed. In this paper, the AOXI promoter sequence was used to allow integration of the pPICZαA-CMV expression vector via homologous recombination, as well as a cod on-optimized *α*-factor from *Saccharomyces cerevisiae* to drive the secretion of fusion peptide (13). In this study, *P. pastoris* strain *GS115* was used as the host and the pPICZαA was used as an expression vector. Pp65-gB-Fc segments were cloned in XhoI/XbaI site of MCS (multiple cloning site) from pPICZαA (Figure 1). Amino acid sequences of final recombinant protein were shown in Figure 2.

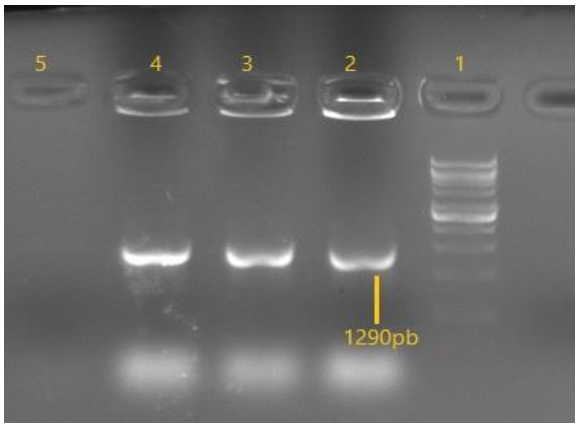

Figure 3.PCR analysis of integrated *Cytomegalovirus* fusion peptide into the pPICZαA expression vector. Line1: 1 kb DNA ladder, Line 2-4: 1290 bp PCR product, Line 5: Negative control

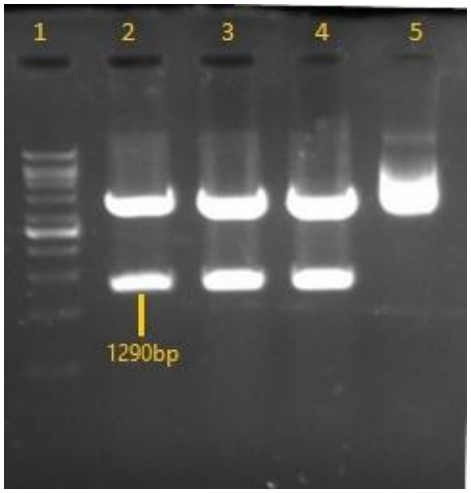

Figure 4. Double restriction digestion analysis of pPICZαA-*CMV* with XhoI/XbaI restriction enzymes. Line1: 1Kb DNA Ladder, Line 2-4 digested recombinant plasmid. Line 5: undigested plasmid

Construction and confirmation of pp65-gB-Fc

PCR (Figure 3) and digestion (Figure 4) showed that pp65-gB-Fc segments were ligated with pPICZαA expression vector. The PCR and restriction digestion of the recombinant plasmid pPICZαA-pp65-gB-Fc produced a fragment with the expected length of ~1300 bp (Figure 3). DNA fragments with the expected length of the target gene (1290 bp) were produced in the transformed yeast clones.

Figure 2. Amino acid sequences of final recombinant protein

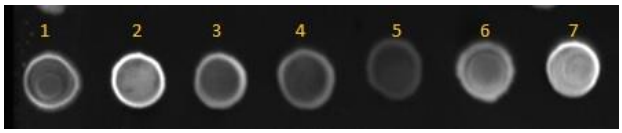

Figure 5. Dot blot results. Fusion peptide was present in the supernatant during total course of cultivation (7 days)

Expression of pPICZαA-CMV in *Pichia pastoris*

Digestion with SacI generates a fragment, which can integrate by homologous recombination into the AOXI region. Cells of *P. pastoris* strain *GS115* were transformed with linear *(*SacI-digested) form of the expression vector, pPICZαA-*CMV*. After expression of pPICZαA-*CMV* in induction media, supernatant of the media was studied by dot blot and 10% SDS-PAGE. In dot blot, we used antibody against c-myc epitopes in terminal segment of cassette gene (Figure 5). By using the anti-cmy antibody, it was demonstrated that a fusion peptide was present in the supernatant during the cultivation course, while no detection was observed in the control extracts. In SDS-PAGE, coomassie blue staining showed that six of the ten induced clones produced a fusion peptide, which was not present in the control strain and had the expected molecular weight (~50 kDa) for pp65-gB-Fc. (Figure 6).

Discussion

Nowadays, development of effective, cheap and safe vaccines is one of the major challenges in medical science. In order to design effective peptide vaccines, identification of proper viral antigens is important for inducing protective immune response (14). There is little data about using *P. pastoris* for HCMV peptide vaccine. In this research, it was shown that *P. pastoris* yeast can be successfully used as an expression system for generating CMV fusion peptide. *P. pastoris* has several advantages over *E. coli* as a recombinant gene expression system. *P. pastoris* performs some posttranslational modifications such as glycosylation and refolding on heterologous eukaryotic proteins (13). One of the advantages of *P. pastoris* is glycosylation of expressed peptide. This glycosylation can affect the stability and activation of recombinant protein that is used for studies of some aspects of recombinant protein functions (15).

All peptides in our research were immunogenic and have been used in previous studies, separately. In this respect, *P. pastoris* cells are attractive tools to use as antigen carrier, which subsequently induces specific CTL (Cytotoxic T Cell) immune responses against viral antigens. There are many ways to enhance vaccines immunogenicity. A recent study looks into improving the immunogenicity of the peptides based on the fast development of multi-epitopes vaccines, which showed immunological activity against bacteria and

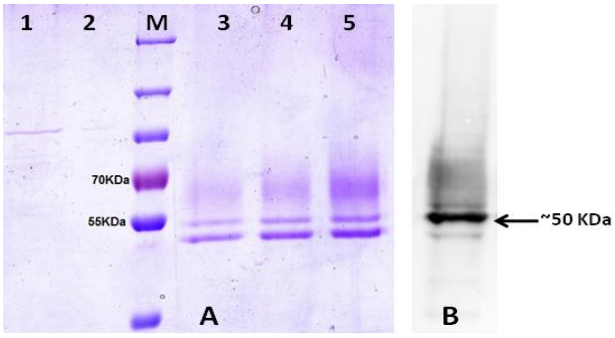

Figure 6. Expression of the *Cytomegalovirus* recombinant fusion protein. (A) The analysis of the *P. pastorisGS115/* pPICZαA-*CMV* cells by 10% SDS-PAGE stained with coomassieblue revealed a protein band profile with a molecular weight of ~50 kDa. No protein was found in the *P. pastoris GS115*/pPICZαA cells. Line 1: pPICZαA without fusion peptide, Line 2: *P. pastoris* without vector. Line M: Protein ladder. Line 3-5: pPICZαA with *CMV* fusion peptides. (B)The expression of the recombinant pp65-gB: Fcγ2a protein was analyzed by immunoblot using anti mouse IgG-HRP

virus (16). In this study, we choose eight epitopes on two immunologic proteins of HCMV.

In this study, emphasize was put on *P. pastoris* as one of the best characterized yeasts, which is also

becoming increasingly important in the field of eukaryotic cell biology and gene technology.

Based on the metabolization of methanol, *P. pastoris* strains were divided into three distinct phenotypes: The first phenotype is wild-type, methanol utilization plus (Mut+) that is characterized by the presence of the alcohol oxidase 1 gene (AOX1), which is responsible for 85% of the methanol utilization ; The second is methanol utilization slow phenotype (Muts), characterized by the absence of AOX1 gene and presence of the AOX2 gene, which is about 97% homologous to AOX1 but much less expressed; and the third is methanol utilization minus phenotype (Mut–), where both AOX genes are absent. Among these strains, the Mut+ strains have a greater growth rate on methanol as the sole carbon source. Expression of the CMV cocktail peptide was efficiently achieved for the first time through an easier production schedule than when an AOX1inducible promoter is employed (17).

In this research, we utilized an expression vector, pPICZαA, which has the AOX1 gene promoter to drive peptide expression, which can be induced by adding methanol to the growth medium. In this vector, the native signal peptide was replaced with that of the *S. cerevisiae*α-factor, allowing secretion of the peptide into the extracellular medium (18-20). We have shown that fusion CMV peptide can be expressed in a yeast system by dot blot and SDS-page assays.

Currently, there is a growing need for CMV vaccines that are capable of eliciting cell-mediated and humoral immunity. Previously, the expression of DNA encoding pp52, as a non-fusion protein, in *P. pastoris* had shown good level of intracellular pp52, which has good reactivity with human sera (21).

Cellular and humoral immune responses are also important in protection against CMV. According to recent studies, pp65 protein recognized by CD^{+}_{8} T cells from the majority of CMV-infected individuals, and evidence from preclinical and clinical studies indicates that this protein is principal target of protective cellular and humoral immune response (17, 22, 23). In this context, it has been shown that $pp65_{495\text{-}503}$ cytotoxic CD_{8}^{+} T-cell epitope can stimulate pp65 T-cells in healthy volunteers (24).

In this research, we selected four immunodominant epitopes in pp65 protein and fused by -GGGGS- linkers. Moreover, gB protein can stimulate cellular and humoral immune response. Recently, a subunit vaccine based on CMV glycoprotein B (gB) formulated with MF59 adjuvant successfully terminated phase II clinical trials (25).

Also we selected three immunodominant and MHC class I restricted epitopes in gB protein and fused by -GGGGS- linkers. In order to activate antigen-specific T-cells, antigens must be taken up by antigen presenting cells (APCs), and must be consequently presented by MHC class I and II proteins. Immediately, antigen-specific T cells were activated. The development of system to deliver peptide antigen to APC (ie. dendritic cells, DCs) including Fc fusion protein will improve availability of protein antigen (10, 14).

Previous studies demonstrated that targeting of immunogenic peptide to Fc on APCs can increaseits selectively uptake and induce effective protective immune response both *in vitro* and *in vivo*. These studies indicated that Fc domain of immunoglobulin (specially IgG) to form fusion with peptide (such as pp65 and gB from CMV in this research) can increase the immunogenicity of Fc-fused antigens and could be considered as a delivery system (26, 27).

Recently, it was shown that FcRn-targeted mucosal immunization produced durable memory immune responses. Immunological memory is exemplified by increased levels of effector T and B cells and, functionally, by the ability to respond faster and more vigorously to a second encounter with the vaccine antigens (7).

To the best of our knowledge, this is the first time that the expression of HCMV-pp65-gB-Fc protein was achieved in *P. pastoris.* There is no data for expression of pp56 and gB in *P. pastoris* system.

Conclusion

In this study, we have shown that the pp65-gB-Fc can be produced at appropriate levels by *P. pastoris*. Current study provides a starting point for future attempt to immunological studies on this vaccine. We believe that secretion of *HCMV* pp65-gB-Fc in the culture media could help downstream processing. Although further experiments are needed to determine immunogenicity of this cocktail peptide, the data outlined here underline that this vaccine could be a promising candidate for the development of a novel peptide vaccine.

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Conflict of interests

All authors declare that they have no conflicts of interest.

References

1. McVoy MA. Cytomegalovirus Vaccines. Clin Infect Dis 2013; 57:196–199.
2. Kotton CN, Kumar D, Caliendo AM, Asberg A, Chou S, Danziger-Isakov L, *et al*. Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. Transplantation 2013; 96: 333-360.
3. Tomtishen JP 3rd. Human cytomegalovirus tegument proteins (pp65, pp71, pp150, pp28). Virol J 2012; 9:22.
4. Li W, Joshi MD, Singhania S, Ramsey KH, Murthy AK.Peptide Vaccine: Progress and Challenges. Vaccine 2014; 2:515-536.

5. Polz-Dacewicz M, Fołtyn S, Macieląg P, Polz D. Cytomegalovirus (CMV) – a new prospect for prevention. Pre-Clinical and Clinical Research 2013; 7:118-123.
6. Rieder F, Steininger C. Cytomegalovirus vaccine: phase II clinical trial results. Clin Microbiol Infect 2013; 10.1111:1469-0691.
7. Lu L, Palaniyandi S, Zeng R, Bai Y, Liu X, Wang Y. A neonatal Fc receptor-targeted mucosal vaccine strategy effectively induces HIV-1 antigen-specific immunity to genital infection. Virology 2012; 85:10542–10553.
8. Fickers P. *Pichia pastoris*: a workhorse for recombinant protein production. Curr Res Microbiol Biotechnol 2014; 2:354-363.
9. Dasari V, Smith C, Zhong J, Scott G, Rawlinson W, Khanna R. Recombinant glycoprotein B vaccine formulation with Toll-like receptor 9 agonist and immunestimulating complex induces specific immunity against multiple strains of cytomegalovirus. Gen Virol 2011; 92:1021–1031.
10. Kern F, Bunde T, Faulhaber N, Kiecker F, Khatamzas E, Rudawski I , *et al.* Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T Cell repertoire in CMV-exposed individuals. Infect Dis 2002; 185:1709–1716.
11. Provenzano M , Sais G, Bracci L, Egli A, Anselmi M, Viehl C, Schaub S, *et al.* A HCMV pp65 polypeptide promotes the expansion of $CD_4^+$and CD_8^+ T cells across a wide range of HLA specificities. Cell Mol Med 2009; 13:2131-2147.
12. Slezak S, Bettinotti M, Selleri S, Adams S, Marincola F, Stroncek D. CMV pp65 and IE-1 T cell epitopes recognized by healthy subjects. J Translat Med 2007; 5:17.
13. Mariz F, Coimbra E, Jesus A, Nascimento L, Torres F, Freitas C. Development of an IP-Free biotechnology platform for constitutive production of HPV16 L1 capsid protein using the pichia pastoris PGK1 promoter. Hindawi 2015; 594120.
14. Li X, Guo L, Kong M, Su X, Yang D, Zou M, Liu Y, Lu L. Design and Evaluation of a Multi-Epitope Peptide of Human Metapneumovirus. Intervirology. 2015; 58: 403-412
15. Ahmad M, Hirz M, Pichler H, Schwab H. Protein expression in Pichia pastoris: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 2014; 98:5301–5317.
16. Cai M, Zhu F, Wu H, Shen P. Expression, purification and glycosylation analysis of chicken infectious bursal disease virus VP2 in yeast. Iran J Vet Res 2013; 3:211-219.
17. Brein F, Heintel T, Schumacher A, Meyerhans A, Schmitt M. Specific activation of CMV-primed human T lymphocytes by cytomegalovirus pp65 expressed in fussion yeast. FEMS Immunol Med Microbiol 2003; 38:231-239.
18. Do BC, Dang TT, Berrin JG, Haltrich D, To KA, Sigoillot JC, *et al.* Cloning, expression in *Pichia pastoris*, and characterization of a thermostable GH5 mannan endo-1,4-β-mannosidase from *Aspergillusniger*BK01. Microb Cell Fact 2009; 8:59.
19. Krainer FW, Dietzsch C, Hajek T, Herwig C, Spadiut O, Glieder A. .Recombinant protein expression in Pichia pastoris strains with an engineered methanol utilization pathway. Microb Cell Fact 2012; 11:22.
20. Fazlalipour M, Fazlalipour H, Monavari H, Mollaie H. Recombinant Coree1e2 protein expressed in pichia pastoris yeast a candidate vaccine for hepatitis C virus. Antiretrovir 2014; 6:3.
21. Battista M, Bergamini G, Campanini F, Landini M, Ripalti A. Intracellular production of a major cytomegalovirus antigenic protein in the methylotrophic yeast Pichia pastoris. Gene 1996; 176:197-201.
22. Reap EA, Dryga SA, Morris J, Rivers B, Norberg PK, Olmsted RA, *et al*. Cellular and humoral immune responses to alphavirus replicon vaccines expressing cytomegalovirus pp65, IE1, and gB proteins. Clin Vaccine Immunol 2007; 14:748–755.
23. Pass RF. Development and evidence for efficacy of CMV glycoprotein B vaccine with MF59 adjuvant. J Clin Virol 2009; 46:73–76.
24. La Rosa C[1], Longmate J, Lacey SF, Kaltcheva T, Sharan R, Marsano D, *et al.* Clinical evaluation of safety and immunogenicity of PADRE-cytomegalovirus (CMV) and tetanus-CMV fusion peptide vaccines with or without PF03512676 adjuvant. J Infect Dis 2012; 205:1294–12304.
25. Yamada A, Yamada T, Noguchi M, Itoh K. Next-generation peptide vaccines for advanced cancer. Cancer Sci 2013; 104:15-21.
26. Baker K, Qiao SW, Kuo T, Kobayashi K, Yoshida M, Blumberg RS.. Immune and non-immune functions of the (not so) neonatal Fc receptor, FcRn. Semin Immunopathol 2009; 31:223–236.
27. Lu L, Palaniyandi S, Zeng R, Bai Y, Liu X, Wang Y, *et al*. A neonatal Fc receptor-targeted mucosal vaccine strategy effectively induces HIV-1 antigen-specific immunity to genital infection. J Virol 2011; 85:10542-10553.