

Cloning and Expression of HA1 Gene of H1N1 Influenza Virus 2009 Pandemic (H1N1pdm09) Indonesia Strain in the *Pichia pastoris* Expression System for the Development of Influenza Vaccine

ASRI SULFIANTI^{1*}, ANDI YASMON², BUDIMAN BELA^{2,3}, AND FERA IBRAHIM^{2,3}

¹*Biomedical Science Program, Faculty of Medicine, Universitas Indonesia, Jalan Salemba Raya No. 6 Jakarta, Indonesia;*

²*Departement of Microbiology, Faculty of Medicine, Universitas Indonesia, Jalan Salemba Raya No. 6 Jakarta, Indonesia;*

³*Research Center of Virology and Cancer Health Service Pathobiology, Universitas Indonesia, Jalan Salemba Raya No. 6 Jakarta, Indonesia*

Among influenza viral proteins, hemagglutinin 1 (HA1) is the target for neutralizing antibodies which inhibit virus binding to receptor of target cells. This protein is widely developed as subunit recombinant vaccine. In this research, we expressed HA1 protein recombinant from DK1271/2011 Indonesian strain in *Pichia pastoris*. The identity of this protein was confirmed by western blotting using anti-His Tag and mouse specific antibody HA H1N1pdm09. The use of yeast *P. pastoris* as an alternative strategy to solve the problems which commonly found in influenza vaccine productions. Expression protein in *E. coli* has been known to have many problems, while mammalian and insect cells requires special skills and relatively high cost. The analysis of HA1 gene sequences showed no mutation in epitope region which recognized by T dan B cells. Further, this recombinant protein can be used as vaccine candidate in influenza vaccine development.

Key words: H1N1pdm09, hemagglutinin 1, influenza virus, *Pichia pastoris*, vaccine

Dari sejumlah protein virus influenza, hemagglutinin 1 (HA1) adalah target antibodi penetralisasi yang menghambat pelekatan virus pada reseptor sel target. Protein ini banyak dikembangkan sebagai vaksin subunit rekombinan. Pada penelitian ini, telah diekspresikan protein rekombinan HA1 dari strain Indonesia DK1271/2011 pada *Pichia pastoris*. Identitas protein telah dikonfirmasi dengan *western blotting* menggunakan *anti-His Tag* dan antibodi spesifik HA H1N1pdm09 tikus. Penggunaan *P. pastoris* berperan sebagai strategi alternatif yang dapat mengatasi masalah umum pada produksi vaksin influenza. Ekspresi protein di *E. coli* diketahui memiliki banyak masalah, sementara sel mamalia dan insekta membutuhkan teknik khusus dan relatif mahal. Analisis sekuens gen HA1 menunjukkan tidak adanya mutasi di daerah epitope yang dikenali oleh sel T dan B. Selanjutnya, protein rekombinan ini dapat digunakan sebagai vaksin kandidat dalam pengembangan vaksin influenza.

Kata kunci: H1N1pdm09, hemagglutinin 1, *Pichia pastoris*, vaksin, virus influenza

H1N1 Influenza A viruses in pandemic 2009 are from Mexico, South of America (Xu *et al.* 2010). This virus firstly emerged in early April 2009 and spread rapidly around the world in June (Belser *et al.* 2011; Strengell *et al.* 2011). World Health Nation (WHO) assed the impact of this virus was moderate because the majority of infected patients experienced mild influenza like illness, but sporadic death found in individual with poor pre-existing immunity to H1N1 influenza virus. According to WHO, 18.000 cases of death caused by H1N1 in the world. (Xu *et al.* 2010).

H1N1 influenza A viruses belong to the Orthomyxoviruses family (Setiawan 2008; Athmaram *et al.* 2011). This virus is an enveloped virus with eight

segmented negative sense RNA genome surrounded by a helical symmetry shell. Influenza A virus genome encode eleven viral proteins which has a specific functions. Hemagglutinin (HA), Neuramidase (NA), Matrix M1 and M2 are known to have effects on the morphology of influenza virus particles. Internal to the M1 matrix are found the nuclear export protein (NEP; also called nonstructural protein 2, NS2) and the ribonucleoprotein (RNP) complex, which consists of the viral RNA segments coated with nucleoprotein (NP) and the heterotrimeric RNA-dependent RNA polymerase, composed of two "polymerase basic" and one "polymerase acidic" subunits (PB1, PB2, and PA) (Bouvier and Palese 2008).

H1N1 influenza A was a novel influenza virus which derived it gene from human, *classical swine*, and *avian influenza* (Nelli *et al.* 2010). Currently, available

*Corresponding author; Phone: +62-85885266350; Email: asrisulfianti88@gmail.com

control measures for this virus are by antiviral and vaccine. However, because of drugs resistance and difficulties in threatening patient with severe, make antiviral is become not effective in controlling H1N1 virus in population (Zu *et al.* 2012). So vaccine is another alternative tools to solve this problem (Maikel *et al.* 2012). It is because not only can prevent the spread of viruses, vaccine also can mitigate the severity of illness and the impact of the disease. Unfortunately, current available vaccine was produced in chicken embryo and cell culture which has a risk of allergic reaction, time consuming, and relative high cost. It has been a challenging task for pandemic influenza because of the insufficient time in preventing wide scale morbidity or mortality (Athmaram *et al.* 2011). An approach to overcome the problem is development of vaccine based on genetic engineering.

Among viral proteins, HA1 is the target for neutralizing antibodies which inhibit virus binding to receptor of target cells. Previously, we reported no substitution on 7 amino acid of HA1 gene of viruses isolated in 2009 (Yasmon *et al.* 2012). Recently, in 2013 we also reported the phylogenetic tree of virus isolated from 2009-2011 (Sulfiанти, 2013). Based on this studies, all of viral strain showed conserved region of HA 1 gene particularly on 7 amino acid 2009 that are playing an important role in viral attachment to the host receptor.

Previous studies on bacterially-expressed HA protein of H5N1 avian influenza virus (AIV) reported an absence in it post translational modification such as glycosylation. This newly synthesized HA protein also showed are not likely to fold properly or trimerize like native HA molecule, which change the conformational epitope used to be recognized by immune responses. Commonly, *E. coli* expressed recombinant protein is also characterized as inclusion bodies and requires careful optimization of the refolding condition. This may have an impact on the loss of recombinant protein and an increased costs of manufacture (Sahdev *et al.* 2007; Khow *et al.* 2012).

Other protein expression system that used in HA1 production is by mammalian and insect cells. But because the systems are still under development and clinical trial, these two systems are rarely used (Athmaram *et al.* 2011). Recently, *Pichia pastoris* yeast become a new ideal organism to express viral antigens. This yeast has many advantages as eukaryotic expression (eg: protein processing, folding and post-translational modification) and are technically faster, easier, and cheaper than another eukaryotic expression

systems, such as mammalian and baculovirus (Krainer *et al.* 2007). *Pichia pastoris* can also express protein as secreted protein. This protein comprises the vast majority of the total protein in the medium, and serves as the first step in purification of the protein (Athmaram *et al.* 2011).

As one of the strategy to control pandemic influenza virus in Indonesia, in this research we have demonstrated the cloning and expression of HA1 from HA1 DKI271/2011 Indonesian strain in *Pichia pastoris*. The purpose of this research is to produce HA1 subunit recombinant protein which used as a subunit vaccine candidate in the development of influenza vaccine in Indonesia.

MATERIALS AND METHODS

Viral HA1 Gene. The HA1 gene of H1N1pdm09 strain A/Jakarta/271/2011(H1N1pandemic2009) was amplified from pPICZ α -A-HA1-02 recombinant vector. It was obtained from the Department of Microbiology, Faculty of Medicine, Universitas Indonesia.

Cloning of HA1 Gene into pPICZ α -A. The HA1 gene was re-amplified from pPICZ α -A-HA1-02 following standard PCR conditions using a forward and reverse primer consisting of *EcoRI* and *SfiI* restriction sites at their 5' end, respectively. pPICZ α -A-HA1-02 was a recombinant vector contains tree fused genes, namely HA1, MA2, and NS1 of H1N1pdm09. The probability of mutation during amplification of HA1 gene was minimalized by used *Pfu High fidelity DNA polymerase* which has good accurate as DNA polymerase. The amplicon was then digested by *EcoRI* and *SfiI*, and cloned into pPICZ α -A in *E.coli*. The bacterial clones containing new recombinant vector were verified by restriction digestion (*EcoRI* dan *NotI*) and DNA sequencing. This new recombinant vector was named by pPICZ α -A-HA1 (H1N1pdm09).

***Pichia pastoris* Transformation and Selection of Transformants.** The recombinant vector (pPICZ α -A-HA1 (H1N1pdm09)) was linearised by *BstXI*, and integrated at 5'AOX1 locus on the *P. pastoris* GS115 genome (Invitrogen) by electroporation (25 μ F, 200 Ω , 1500 V) (Biorad, CA). This yeast was then plated onto yeast extract peptone dextrose (YPD) agar plates containing 100 μ g/ml of zeocin (Invitrogen, USA). The yeast transformants were subcultured on YPD broth. Gene integration was verified by yeast genomic DNA PCR with specific primers (5'SWFLSEQF2 5'A GCTCAGTGTGCATCATTTGAA-3' and SWFLSEQ-R2

5'-CCGGCTCTACTAGTGTCCAG-3'). Selection of putative transformants and differentiation a copy number of integrated were did by streaked this yeast transformants on the replica YPD agar plates containing of 500 $\mu\text{g ml}^{-1}$ zeocin. The incubation was performed at 30 °C for two days.

Expression of rHA1 in *P. pastoris*. The positive yeast transformants were inoculated into 2 ml of YPD broth + ampicillin 50 $\mu\text{g ml}^{-1}$ and grown at 30 °C for 48 h at 250 rpm. Addition of ampicillin to inhibit bacterial contamination during the protein expression. After 48 h, the cells were harvested by centrifugation at 5000 rpm for 5 m. The cells were washed three times with distilled water and moved to 10 ml buffered with 100 mM potassium phosphate medium pH 8,2 (BMMY). The cultures were induced with 1% methanol (v/v) for every 24 h until 120 h. After 120 h, the cells were harvested by centrifugation at 5000 rpm for 5 m in room temperature. The pellet contained cells were stored in -80 °C, and the supernatant contained soluble protein in medium were analyzed on 12% SDS-PAGE.

Western Blot. The expressed of HA1 protein was confirmed by western blotting using Anti-*His detector*TM *nickel-HRP* (KPL) and mouse anti HA1 polyclonal antibodies. 20x concentration of supernatant protein by PEG (Polyethylene glycol) was run on SDS gel and transferred onto a nitrocellulose membrane. The membrane was blocked with blocking buffer containing BSA 1% and TBST 1X (KPL) at 4 °C for 16-18 h. The membrane was then incubated with a 1:8000 anti His (*His detector*TM *nickel-HRP*) or 1:8000 mouse anti HA1 polyclonal antibodies at room temperature for 1 h. The membrane was washed twice with washing buffer containing TBST 1X for 5 min. Membrane was then incubated in TMB membrane substrate at room temperature for 5-15 min. For membrane that detected by mouse anti HA1 polyclonal antibodies, it was incubated with *Hrp anti mouse IgG* as secondary antibody at room temperature for 30 min after washing twice. The reaction was terminated by adding sterile distilled water after protein band was detected in the membrane.

RESULTS

HA1 amplicon was showed as an expected single DNA band corresponding to 1.076 bp (Fig 1). The DNA fragment consisted of HA1 gene (1.071 bp) and extension sequences (25 bp) at 5 and 3' end for *EcoRI* dan *SfiI* sites. The amplicon was digested with *EcoRI* dan *SfiI* and cloned into similiary digested pPICZ α -A

vector (Invitrogen). The clones which brings recombinant plasmid (pPICZ α -A-HA1 (H1N1pdm09)) were confirmed by restriction digestion with *EcoRI* dan *NotI*. It released a 3.593 bp pPICZ α -A for vector and 1.129 bp for rHA1 (Fig 2). Based on DNA sequencing, this recombinant plasmid showed an inserted HA1 gene which had a correct in-frame with vector (data not shown).

Electroporation *P.pastoris* with recombinant plasmid yielded ≥ 300 transformant per 2 μg of DNA used. Selection of this transformants resulted in 44 colonies showing resistance to YPD plate with 500 $\mu\text{g ml}^{-1}$ zeocin. Genomic DNA PCR was performed by using specific primers resulted in amplification of 384 bp of HA1 gene (detection of HA1 gene). Of 44 selected transformants, 23 clonal cells showed positive results (Fig 3). The DNA from non-recombinants did not show any amplification. Unfortunately, variations in the amplicon band intensities of different clones cannot be attributed to difference in the transgene copy number. It happened because there was no standardization in DNA templates which were used in PCR reaction.

Of 23 clones positive integration, only one clone (number 41) showed better expression of the HA1 recombinant protein. The HA1 recombinant was detected as a single band on SDS-PAGE with approximately 40 kDa (Fig 4). Confirmation by western blotting using HA1 H1N1 polyclonal antibody from mice serum specific H1N1 and anti-his tag also showed a protein band with size of approximately 40 kDa. The weight of the protein was smaller than the amino acid sequence prediction which yielded 48 kDa.

DISCUSSION

Pichia pastoris expression system in this research has been successfully expressed HA1 protein as soluble protein in supernatant culture. This protein comprises the vast majority of the total protein in the medium, and serves as the first step in purification of the protein (Athmaram *et al.* 2011). The identity of this protein was confirmed by western blotting using anti-His Tag and mouse specific antibody HA H1N1pdm09. Based on these detections, HA1 recombinant showed as a protein band with lower than 40 kDa. This anti-tag recognized the marker of poly His tag on the downstream of recombinant HA1 protein, and the mouse specific antibody HA H1N1pdm09 detect the recombinant HA1 protein in supernatant culture.

The size of these recombinant proteins was smaller

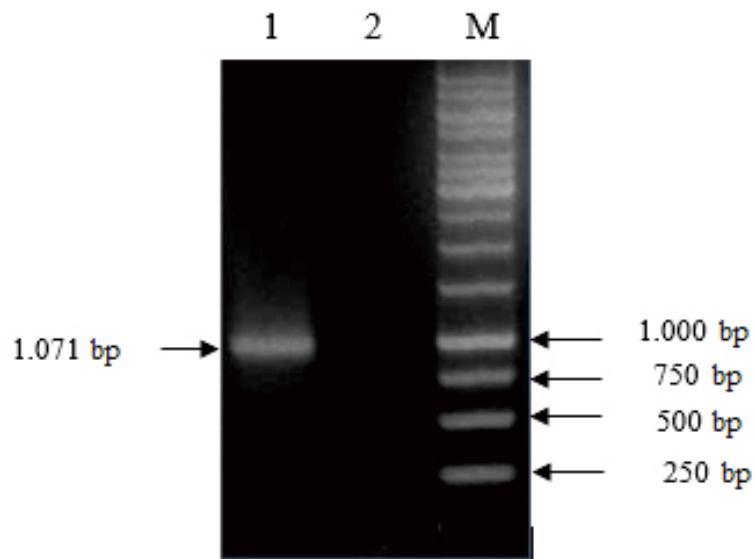


Fig 1 Amplification of HA1 gene from pPICZ α -A-HA1-02 by PCR. Lane 1: PCR with pPICZ α -A-HA1-02 as DNA template, Lane 2: PCR negative control, Lane M: DNA ladder. bp: base pair.

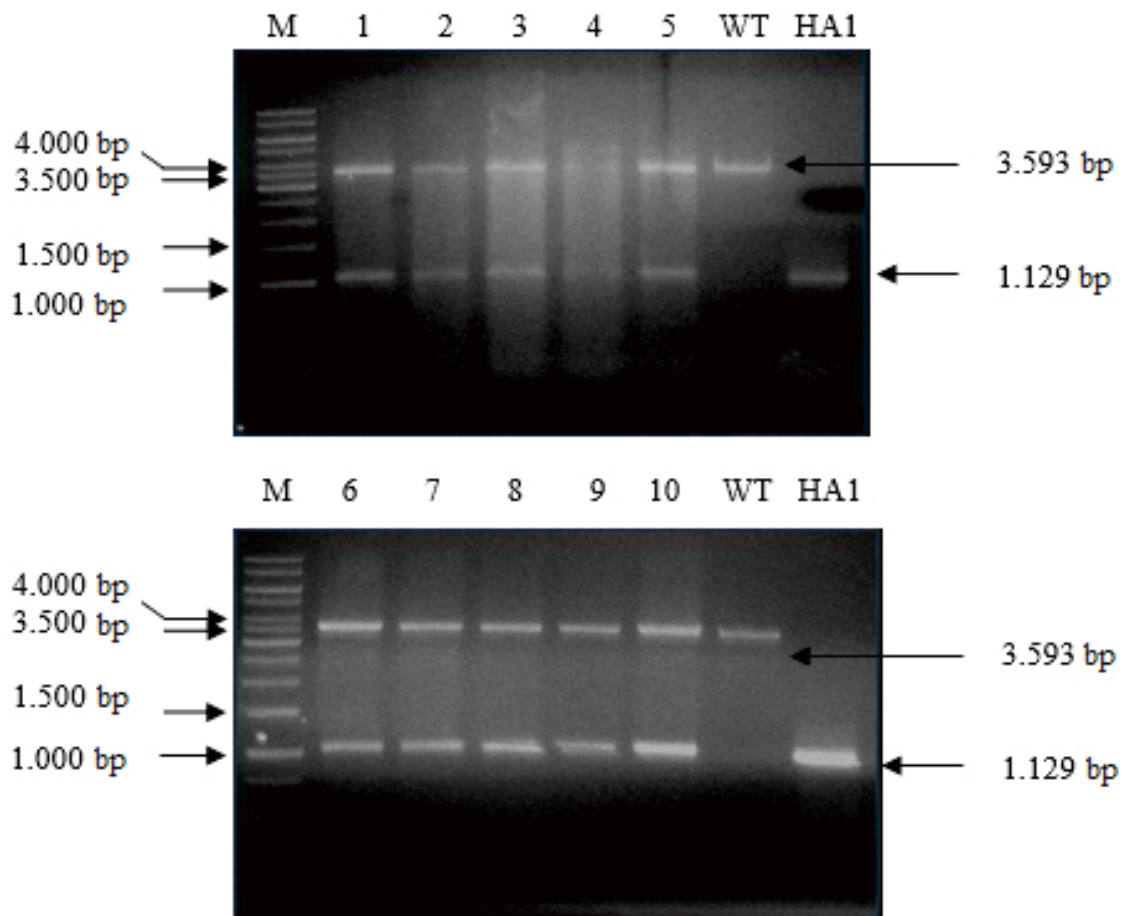


Fig 2 Restriction analysis of recombinant pPICZ α -A-HA1 (H1N1pdm09) using *EcoRI* and *NotI*. Lane 1-10: digestion product of DNA recombinant plasmid. Lane WT: digestion product of wild type DNA pPICZ α -A vector. Lane HA1: amplification product of HA1 gene. Lane M: DNA ladder. bp: base pair.

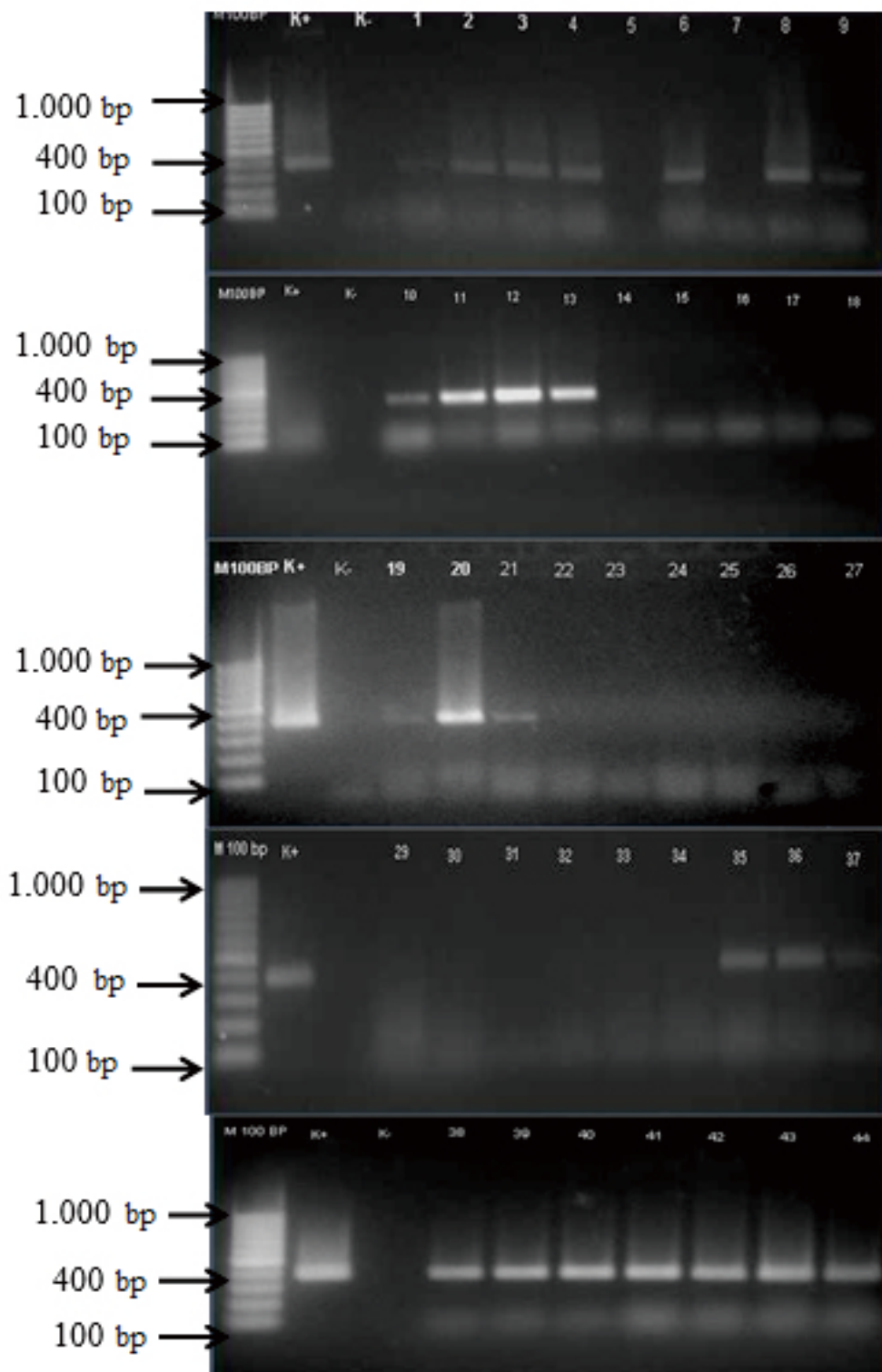


Fig 3 Amplification HA1 gene from genomic DNA of 44 yeast transformants by PCR. Lane 1-44: amplification product of genomic DNA PCR from *Pichia pastoris* that transformed by pPICZ α -A-HA1 (H1N1pdm09), clones no. 1 to 44. Lane K+: amplification product of DNA recombinant plasmid (pPICZ α -A-HA1 (H1N1pdm09)). Lane K-: amplification product of DNA genomic yeast which transformed by pPICZ α wild type (negative control). Lane M 100BP: DNA ladder. bp: base pair.

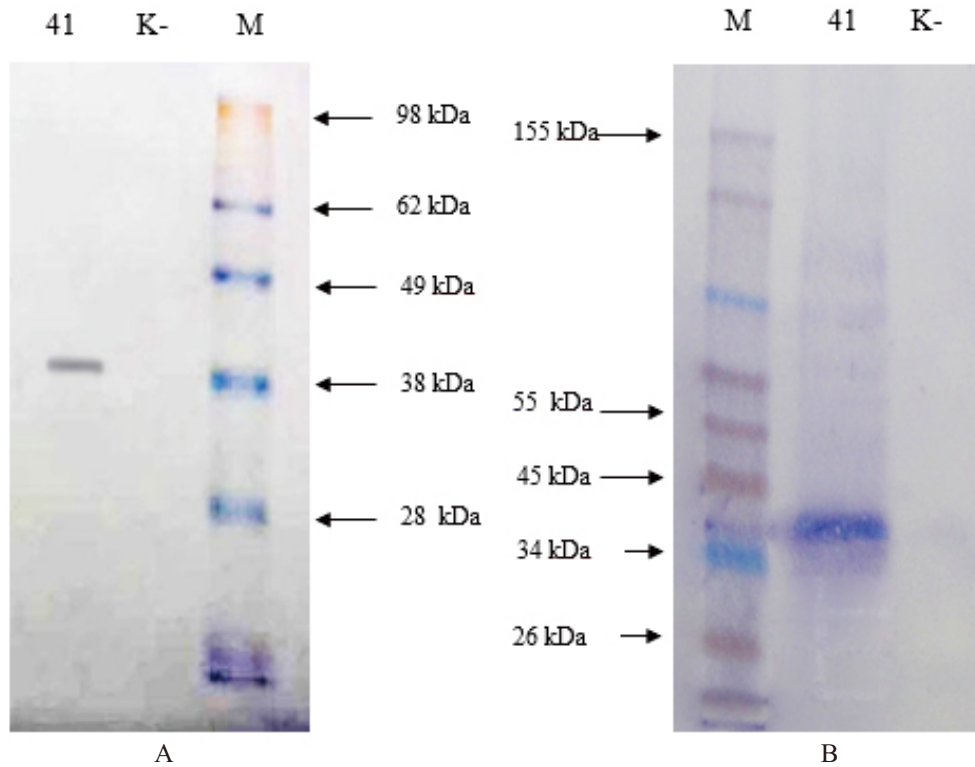


Fig 4 Detection of HA1 expression secreted from recombinant *P. pastoris* by western blot using anti-His Tag (A) and mouse specific antibody HA H1N1 pdm09 (B). Lane 41: yeast clone no. 41, showed HA1 recombinant protein band HA1 approximately 40 kDa, Lane K- : yeast was transformed by pPICZ α wild type (negative control), Lane M (Fig 4A): protein ladder (See blue Plus2TM), Lane M (Fig 4B): protein ladder (Chromatein prestained protein ladder). kDa: kilo dalton.

than amino acid sequence prediction, 48 kDa. Flint *et al.* (2000) described that it might happened because during the secretion of the recombinant HA1 protein, there was a process of cutting in the protein's alpha signal factor sequence. The cutting process resulted in a smaller protein size. The alpha signal factor cutting involves a signal peptidase enzyme and it happens during translocation in the reticulum endoplasm.

The HA1 protein was only found in one clone (number 41). It was suggested that there had been a multicopy integration between HA1 gene and *Pichia* genome in that particular clone. The multicopy integration caused the protein to be expressed and secreted more to the medium culture (Athmaram *et al.* 2012).

The recombinant protein was not expressed in the other clones. The first possible reason is spontaneous mutation in the AOX1 locus or in the alpha signal factor sequence. Spontaneous mutation in the AOX1 locus of the recombinant plasmids prevented the process of homolog recombination between the plasmid and the yeast genome. Therefore, the HA1 gene cannot integrate to the yeast genome and resulted in no protein expression (Athmaram *et al.* 2012). The second reason

is mutation in the alpha signal factor by means of PCR process. This mutation causes a failure to cut and bring the recombinant protein into the reticulum endoplasm. It prevents the protein in entering the reticulum endoplasm and therefore cannot be secreted through normal secretory pathway (Murugan *et al.* 2013). The third reason is HA1 protein was retained in the reticulum endoplasm as inclusion bodies. This accumulation of this recombinant HA1 protein in the ER limits the yield of HA1 secreted into the extracellular environment (Athmaram *et al.* 2012).

Analysis of HA1 gene sequence showed variation that cause 4 amino acid differences to HA1 from influenza strain isolated in Mexico 2009. Two of these amino acids were suggested in changing the structural protein because it changed the hydrophobicity of the protein's amino acids (data not shown). Comparison of amino acid sequences between HA1 recombinant, HA1 from Mexico 2009, and several other HA1 sequences which were isolated in 2010 and 2011 (from Jakarta, Surabaya, China, India, Thailand, and Vietnam) showed no mutation on epitopes for B and T cells (data not shown); therefore, the recombinant HA1 protein will induce immunity responses that are cross-

reacted with other viral strains.

With this ability, the subunit vaccine derived from this recombinant HA1 has a potential to be in charge in neutralization of influenza virus H1N1 pandemic 2009 that circulated in future. The recombinant HA1, which was secreted as an extra-cellular protein, also gives several advantages, such as easier and more economical method of isolation. It can increase effectivity of vaccine production, which in turn can prevent mortality and morbidity of the H1N1 influenza patients (Murugan *et al.* 2013).

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