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Protocols

Prokaryotic expression and purification of HA1 and HA2 polypeptides for serological analysis of the 2009 pandemic H1N1 influenza virus

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Hemagglutinin (HA) is an important influenza virus surface antigen that is highly topical in influenza research. In the present study, the genes encoding the HA1 and HA2 proteins from the 2009 pandemic influenza virus H1N1 (A/California/04/2009(H1N1)) were cloned into a prokaryotic expression plasmid pCold-TF, and soluble fusion proteins containing H1N1 HA1 and HA2 were produced by transformed *Escherichia coli*. Western blot assays were used to examine the immunoreactivity of the recombinant proteins using polyclonal and monoclonal antibodies derived against the whole virus A/California/04/2009(H1N1). Recombinant protein immunoreactivity was also analyzed qualitatively by ELISA and hemagglutination inhibition using human serum samples. These results will aid future immunological and serological studies of the 2009 pandemic H1N1 virus HA.

1. Introduction

Influenza is considered to be one of the most severe threats to human health and animal welfare. Influenza viruses are divided into three different types (A–C) serologically according to the antigenicity of conserved inner virus structures. Influenza A viruses (Family Orthomyxoviridae, genus Influenza virus A) are single-stranded, negative-sense RNA viruses, comprising eight genome segments that are enveloped by a lipid bilayer containing hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Both surface proteins are key antigens targeted by human humoral immunity and are used for virus subtyping: 16 H (H1–H16) and 9 N (N1–N9) subtypes (Fouchier et al., 2005; Rohm et al., 1996).

Influenza A outbreaks occur almost annually although the magnitude and severity are highly variable. In the 20th century, influenza A virus caused three pandemics: the 1918 Spanish influenza, the Asian influenza in 1957 and the Hong Kong influenza in 1968 (Reid et al., 2003). During April 2009, a new influenza A virus outbreak occurred in North America that originated from Mexico and the southwestern United States (Dawood et al., 2009; Peiris et al., 2009). The virus was identified as an H1N1 virus and genetic analysis revealed a unique reassortment of genes that were of swine origin (Garten et al., 2009; Lu et al., 2009; Trifonov et al., 2009). This new pathogen spread faster than any previous virus and was distributed worldwide in only few months (Naffakh and van der Werf, 2009; Smith et al., 2009). In the case of swine-origin influenza A virus, considerable changes in both HA and NA surface antigens may result from "antigenic shift". Such antigenic changes enable the virus to escape pre-existing antibodies and the "herd immunity" (Gallaher, 2009). Subsequent research found that persons under the age of 30 years had little evidence of cross-reactive antibodies to the pandemic virus (Hancock et al., 2009) and may explain why the new virus spread quickly worldwide. The World Health Organization (WHO) raised its pandemic alert to the highest level, phase 6, to announce that the world was facing the challenge of an influenza pandemic (WHO, 2009). Until 4 June 2010, more than 214 countries have reported laboratory confirmed cases of pandemic influenza H1N1 2009, including over 18138 deaths (WHO, 2010). Evidence suggests that "antigenic drift" mutations of the 2009 pandemic virus could produce more virulent virus strains (Glinsky, 2010).

Neutralizing antibodies directed against the HA glycoprotein are the primary mediators of protection against influenza virus infection (Mozdzanowska et al., 2003; Wiley and Skehel, 1987). Three HA monomers, each consisting of an HA1 and an HA2 subunit, form the trimeric HA spike protruding from the viral membrane. The HA1 subunit contains the receptor-binding site that mediates viral attachment to the cell membrane, whereas the HA2 subunit contributes to membrane fusion (Eisen et al., 1997; Skehel and Wiley, 2000). The HA1 domain contains most of the antigenic sites and is more prone to mutations and antigenic drifts than other parts of the protein (Shih et al., 2007). As membrane proteins, HA1 and HA2 are usually expressed in the form of inclusion bodies in a prokaryotic expression system, thereby impeding the study of

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their antigenicity and immunoreactivity. In this study, a fusion protein expression vector pCold-TF (Takara, Otsu, Japan) was used, which expressed the trigger factor (TF) as a soluble tag. TF is a prokaryotic ribosome-associated chaperone protein that facilitates co-translational folding of newly expressed polypeptides. TF is also highly expressed in *Escherichia coli* expression systems due to its *E. coli* origin. With this vector, the soluble HA1 and HA2 proteins of the 2009 influenza H1N1 virus were expressed successfully in *E. coli*. ELISA and Western blot analysis demonstrated that both recombinant proteins exhibited high immunoreactivity.

2. Materials and methods

2.1. Construction of expression vector

The HA gene of influenza A virus (A/California/04/2009(H1N1)) was assembled by PCR using synthetic oligonucleotides and was cloned into a cloning vector pMD18 (Takara, Dalian, China). After sequence confirmation, the recombinant plasmid was used as a PCR template to produce the HA1 and HA2 gene fragments. Two HA1 primers (HA1-F-XhoI, 5′-GGC TCG AGG ACA CAT TAT GTA AGG-3′; HA1-R-EcoRI, 5′-CGG AAT TCT AGC CTC TAG ATT GA-3′) were used to amplify the HA1 gene fragment (1002 bp). PCR products were digested with *Xho*I and *Kpn*I and inserted into the prokaryotic expression vector pCold-TF (Takara, Dalian, China) to create the HA1 expression plasmid pCold-TF-HA1 (Fig. 1). HA2-F-KpnI (5′-GGG GTA CCT CTA TTC AAT CTA GAG-3′) and HA2-R-BamHI (5′-CCG GAT CCT AAA TAC ATA TTC TAC-3′) primers were used to amplify the HA2 gene (699 bp). PCR products were digested with *Eco*RI and *BamH*I and then inserted into pCold-TF to create the HA2 expression plasmid pCold-TF-HA2.

2.2. Expression of the His-tagged HA1 and the HA2 fusion proteins

E. coli BL21 (DE3) (Novagen, Shanghai, China) transformed with pCold-TF-HA1 or pCold-TF-HA2 was cultured in LB medium supplemented with 100 μg/ml ampicillin, grown at 37 °C until the logarithmic phase (at OD600 of 0.5–0.6) and induced by isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1.0 mM for 24 h at 15 °C. Bacterial lysates were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Bandscan 5.0 software was used to assess fusion protein expression.

2.3. Characterization of the solubility of the His-tagged HA1 and the HA2 fusion proteins

To assess His-tagged protein solubility, induced bacterial cultures were pelleted, resuspended in 20 mM Tris–HCl lysis buffer (pH 8.0) supplemented with 100 mM NaCl, 1.0 mM EDTA, 50 mg/ml lysozyme and subjected to sonication on ice until clear. Total bacterial proteins were then partitioned into soluble and insoluble fractions by centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatant (soluble fraction) was collected and the pellets (insoluble fraction), which contained the inclusion bodies, were resuspended in deionized water. Both fractions were analyzed in parallel by 10% SDS-PAGE to characterize the solubility of the His-tagged HA1 and HA2 fusion proteins.

2.4. Purification and thrombin cleavage of the His-tagged HA1 and HA2 fusion proteins

The supernatant was filtered through a 0.45 μm membrane and then loaded onto a gravity-flow column packed with 3 ml $Ni2^{+}$-NTA resin slurry (Novagen, Shanghai, China). His-tagged HA1 and HA2 fusion proteins were purified following the manufacturer's instructions and the yield was quantified using a Coomassie Protein Assay Kit (Biomed, Beijing, China) (Bradford, 1976). 10% SDS-PAGE was performed to validate the identity and evaluate the purity of the target fusion protein. The elution buffer was replaced with phosphate-buffered saline (PBS) and the solution was treated with thrombin (Novagen, Shanghai, China 10 U thrombin/mg fusion protein) for 16 h at 20 °C to remove the TF domain from the fusion protein. Finally, the cleavage product was analyzed using 12% SDS-PAGE.

2.5. Immunological analysis with polyclonal and monoclonal antibodies produced against whole virus lysates

Polyclonal and monoclonal antibodies were prepared from mice that were injected with whole influenza A virus strain (A/California/04/2009(H1N1) lysates. After immunization three times, antisera were harvested and stored at −80 °C for further use and spleen cells were collected for hybridoma generation. Protein samples treated with thrombin were subjected to 12% SDS-PAGE and transferred electrophoretically to a PVDF membrane. Membranes were then blocked at 4 °C overnight with 10% non-fat milk in Tris-buffered saline-Tween (TBST) (50 mM Tris–HCl, 200 mM NaCl, 0.1% (v/v) Tween-20, pH 7.5) and probed with the polyclonal antibody (1:3000 dilution) or monoclonal antibodies (1:500 dilutin) at 37 °C for 1 h. After washing with TBST, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:3000 dilution) (ZSGB-BIO, Beijing, China) at 37 °C for 1 h. Immunoreactive proteins were then visualized using the ECL Western blotting analysis system (Pierce, Rockford, USA).

2.6. ELISA and Western blot analysis of the fusion proteins with human sera

Human serum samples ($n = 92$) were obtained from a hospital, including three pandemic H1N1 positive samples with a hemagglutination inhibition (HI) titer greater than 160 and four pandemic H1N1 negative HI samples which are positive for seasonal H1N1 (HI). The fusion proteins TF-HA1 (300 ng per well) and TF-HA2 (400 ng per well) were coated on ELISA plates (Nunc, Roskilde, Denmark) at 4 °C overnight. Plates were then blocked at 37 °C for 2 h with 10% non-fat milk and washed four times with PBST. Human sera were added as the primary antibody (1:1000 dilution) and incubated overnight at 4 °C. Plates were then washed four times with PBST and incubated with HRP-conjugated goat anti-human IgG (ZSGB-BIO, Beijing, china) at 37 °C for 1 h. Color was developed using TAB solution (Sigma, Shanghai, China) and absorbance was calculated using an ELISA reader at 450 nm.

For Western blot analysis, protein samples treated with thrombin were first subjected to SDS-PAGE and then transferred electrically to a PVDF membrane. A pandemic H1N1 positive serum sample (1:1000 dilution) was used as the primary antibody and the HRP-conjugated goat anti-human IgG (1:3000 dilution) as the secondary antibody. Immunoreactive proteins were visualized using the ECL Western blotting analysis system. (Pierce, USA).

2.7. Hemagglutination inhibition assay

HI assays were performed according to established procedures using influenza virus A/California/07/2009(H1N1) vaccine strain. Briefly, patient sera were treated with receptor destroying enzyme (RDE) and absorbed with the erythrocytes to remove non-specific hemagglutination. Two-fold serial dilutions of each serum were prepared in a V-bottom microtiter plate. Virus suspension containing 4 HA units/25 μl was added to each well and mix thoroughly. The plate was incubated for 15 min at room temperature. Fifty microliters of 0.75% red blood cell (RBC) suspension was then added to each well. Results were observed after an incubation of 60 min