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Clearance of False-positive Antigen-Antibody Reactions of a Diagnostic Antigen Produced in *Escherichia coli* with Human Sera

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Although many pharmaceutically useful proteins are produced in *E. coli* expression system, it is very rare for the system to be used in the production of diagnostic antigen due to a major problem, *i.e.*, false-positive reaction of *E. coli* host-derived proteins contaminating purified diagnostic antigen with human sera. The N (nucleocapsid) protein of Seoul virus causing haemorrhagic fever with renal syndrome (HFRS) was produced in *E. coli* BL21 (DE3), and used for the detection of N protein-specific antibodies in human sera. Using the N protein as a diagnostic antigen of HFRS, the false-positive reaction was cleared by merely mixing the test sera with the extract of *E. coli* host strain not harboring expression plasmid.

Key words: diagnostic antigen, *E. coli*, false-positive reaction

INTRODUCTION

Although nucleic acid-based sensitive PCR (polymerase chain reaction) is available as a diagnostic method nowadays, it has some restrictions if the systems are to be used routinely. First, a pathogen has to be isolated from the serum of each individual and its genomic DNA or RNA should be prepared. Second, the diagnostic procedure is multi-step, time-consuming and inappropriate for the simultaneous testing of many samples. Thus many of infectious diseases are still diagnosed by the detection of pathogen-specific antibodies in the serum of an infected individual.

For the detection of pathogen-specific antibodies, diagnostic antigens of the pathogens should be prepared. Diagnostic antigens can be prepared either by the expression of some structural proteins using recombinant DNA technique or by cultivating the pathogens in some culture media or host cells. Among these two methods, recombinant expression systems provide a lot of advantages, such as avoiding of handling infectious material, ease of securing antigens in enough quantities at a lower preparation cost, and so on. Among many recombinant expression systems, *E. coli* system is most convenient for mass-production of foreign proteins. However, the antigen produced by recombinant *E. coli* system renders us a high probability of false-positive signals unless it is highly purified, since many individuals have high titers of antibodies against proteins of *E. coli*-origin. Here, we introduce a method to lessen a false-positive signal significantly when *E. coli*-produced protein is used as a diagnostic antigen with an example with N (nucleocapsid) protein of Seoul virus and human sera of haemorrhagic fever with renal syndrome (HFRS). Seoul virus is one of the major serotypes causing HFRS.

Hantaviruses are rodent host-borne viruses belonging to the family *Bunyaviridae*. They are etiologic agents for two acute diseases, *i.e.*, haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) [1,2]. N protein of hantavirus is a major viral antigen, and both anti-N IgM and IgG antibodies are detected in the sera at the onset of the symptoms of HFRS as well as HPS. Therefore anti-N antibodies are potentially useful targets for early diagnosis of HFRS and HPS.

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MATERIALS AND METHODS

Cloning of Hantaviral Nucleocapsid Gene

Seoul viral cDNA pool was prepared from Seoul viral genomic RNA using random hexamers. The gene encoding Seoul viral N protein was amplified by two different PCR procedures. Primer 1 and 2 were used for the first PCR from the cDNA pool, and primer 2 and 3 were used for the second PCR from the first PCR product.

Primer 1(5′-TAGTAGTAGACTCCCTA-3′)
Primer 2(5′-CCAGATCTATGGCAACTATGGAG-3′; *Bgl* II site underline)
Primer 3(5′-GGAATTCTTAGAGTTTCAAAGG-3′; *Eco*R I site underline)

The second PCR product was digested with *Bgl* II/*Eco*R I and inserted into *Bam*H I/*Eco*R I sites of plasmid pET-3a. The resultant recombined plasmid was designated as a pET-sNP (Fig. 1).

Expression of Seoul Viral Nucleocapsid Protein in *E. coli*

The *E. coli* BL21 (DE3) carrying the T7 RNA polymerase gene under the control of *lac*UV5 promoter [3] was transformed with pET-sNP (Fig. 1). The recombinant *E. coli* was cultured at 37 ℃ in LB medium supplemented with 0.2% dextrose and 100 μg/mL ampicillin. The expression of N protein was induced by the addition of 1 mM IPTG when absorbance (600 nm) of the culture reached 0.5~0.7. The cells were cultured

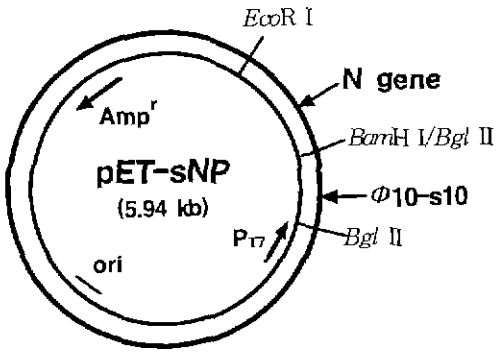

Fig. 1. Structure of plasmid pET-sNP for the expression of Seoul virus nucleocapsid gene in *E. coli* BL21(DE3).

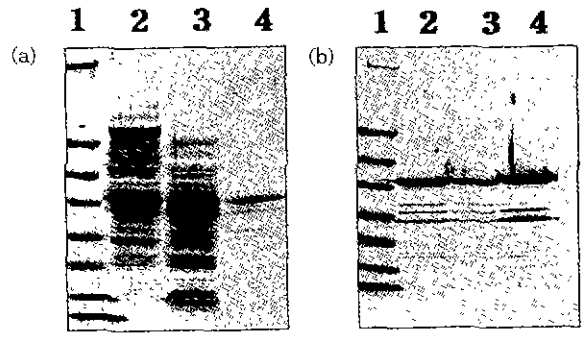

Fig. 2. SDS-PAGE (a) and western blot (b) analyses of N protein samples with anti-N protein monoclonal antibody following purification steps. Lane 1 is a molecular weight standard. Lane 2 is an ammonium sulfate precipitate of supernatant of whole cell lysate. Lane 3 is supernatant of whole cell lysate. Lane 4 is a flow through of phenyl Sepharose column chromatography.

further for four hours.

Purification of Nucleocapsid Protein

The cultured *E. coli* cells were disintegrated by sonication in 50 mM Tris/HCl (pH 8.0). The supernatant taken after centrifugation of the cell lysate was precipitated with 30% ammonium sulfate. The precipitate was re-dissolved in 40 mM KH_2PO_4/Na_2HPO_4 (pH 8.0) and chromatographed through a column of phenyl Sepharose. The flow-through fraction was taken and concentrated by ultrafiltration with a membrane filter having cut-off value of 50,000. The purified N protein was analyzed with SDS-PAGE and western blotting. *E. coli* extract for the pretreatment of human sera was prepared according to the same procedure for the purification of N protein from the culture of *E. coli* host strain not harboring the plasmid.

Western Blot Analysis for Reaction of N Protein with Human Sera

Western blot was performed according to the standard protocol [4]. Purified N protein was boiled in a loading buffer (5 mM Tris/HCl pH 6.8, 2% w/v SDS, 2.5% v/v glycerol, 0.005% w/v Bromophenol Blue, 2.5% v/v beta-mercaptoethanol) for 5 min. The samples were loaded on SDS-12% w/w polyacrylamide gels. Immunostaining was carried out with human sera and peroxidase conjugate of anti-human IgG, and by using coloring material 4-chloro-1-naphtol. For the pretreatment of human serum, 300 μg of *E. coli* extract was added in 10 mL of PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 per liter of distilled water, pH 7.4) together with 10 mL of human serum. The mixture was used as a primary antibody for reaction with N protein transferred to nitrocellulose membrane. HFRS-patient sera were obtained from Korean NIH where they confirmed the sera with indirect immunofluorescence antibody assay.

RESULTS AND DISCUSSION

N protein of Seoul virus was expressed in *E. coli* harboring plasmid pET-sNP shown in Fig. 1 in a soluble form, and was purified by salting-out with ammonium sulfate followed by the phenyl Sepharose hydrophobic interaction chromatography. As shown in Fig. 2, the purity of the purified N protein was

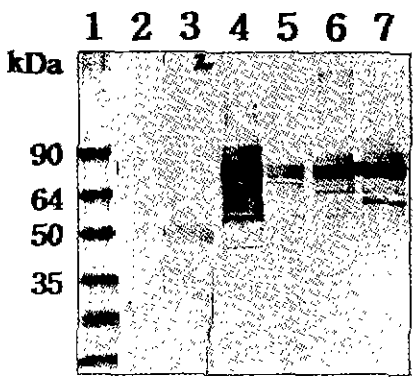

Fig. 3. Western blot analyses of the purified N protein with human sera. Lane 1 is a molecular weight standard. Normal human serum does not show any signals in lane 2. HFRS-patient serum shows positive signal at 50 kDa in lane 3. Normal human sera show false-positive signals at 90 kDa in lane 4 to 6.

estimated to be about 90%. For the confirmation of purified N protein, western blot analyses were carried out using mouse anti-N monoclonal antibody.

When the specificity of the purified N protein was studied by immunodot blot using 20 healthy human sera, four of them showed positive signals. However, when the result was reconfirmed with western blot, all the positive signals were found to be false. There were no color bands at 50 kDa, the expected location of Seoul viral N protein. Meanwhile, color bands were observed at 90 kDa (Fig. 3). The protein of 90 kDa seems to be derived from cytoplasmic proteins of *E. coli* and to remain in the purified N protein. We tried to remove the contaminating protein by several methods such as ion-exchange, gel-filtration, and ultrafiltration, *etc.*, but failed to clear the false-positive reaction completely by improving the purity of N protein.

We introduced another technique, *i.e.*, pretreatment of human sera with *E. coli* extract, to remove antibodies showing antigen-antibody reaction with contaminating proteins of *E. coli*-origin in the purified N protein. This trick was very successful and false-positive reaction was cleared close to completion without affecting the sensitivity. The results of western blot analyses were shown in Fig. 4.

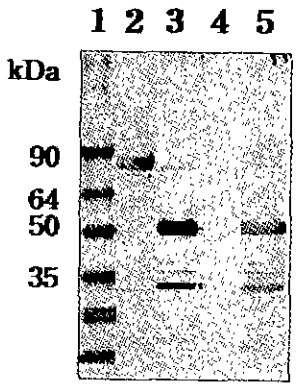

Fig. 4. The effect of pretreatment of human sera with *E. coli* extract to clear false-positive reaction of purified N protein. Lane 1 is a molecular weight marker. Lane 2 is reacted with normal human serum without pretreatment. Lane 3 is reacted with HFRS-patient serum without pretreatment. Lane 4 is reacted with pretreated normal human serum with *E. coli* extract. Lane 5 is reacted with HFRS-patient serum pretreated with *E. coli* extract.

Purified N protein was applied to SDS-PAGE and transferred to nitrocellulose membrane. The membranes were reacted with HFRS-patient human serum or normal human serum showing false-positive signal, both of which were pretreated or not pretreated with *E. coli* extracts to observe an effect of pretreatment of *E. coli* extract. As shown in Fig. 4, a band at about 90 kDa was observed (lane 2) when normal human serum without pretreatment with *E. coli* extract was used. Meanwhile the band disappeared (lane 4) when normal human serum pretreated with *E. coli* extract was used, implying that the pretreatment of human sera with *E. coli* extract is very effective to clear false-positive reaction of purified N protein with human serum. Also as it can be seen in lane 3 and lane 5 of Fig. 4, there is no big difference in the strength of band color at 50 kDa between pretreated and non-pretreated patient sera. It implies that the pretreatment does not interfere with a reaction of purified N protein with anti-N antibodies in HFRS-patient serum.

In conclusion, a pretreatment of sera samples with *E. coli* extract is very effective to clear false-positive signal without decreasing sensitivity when *E. coli*-produced protein was used as a diagnostic antigen. The method was still effective (data not shown), even when the purity of diagnostic protein was as low as about 50%.

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