Journal of Science and Technology ***54*** *(4A) (2016) 331-339*

PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST RECOMBINANT VP6 ANTIGEN OF ROTAVIRUS

Do Thi Thu Ha[1], Ngo Thu Huong[2], Nguyen Dang Hien[2], Truong Quoc Phong[1, *]

[1]*Hanoi University of Science and Technology, 1 Dai Co Viet road, Hanoi*
[2]*Center for Research and Production of Vaccines and Biological, 418 Vinh Hung, Thanh Tri, Hanoi*

[*]Email: *phong.truongquoc@hust.edu.vn*

Received: 15 August 2016; Accepted for publication: 7 October 2016

ABSTRACT

Rotavirus is the most common cause of diarrhea in infants and young children under five years old. The VP6 protein of rotavirus was considered as an important antigen in the development of novel vaccine generation and diagnostic tools. Polyclonal antibodies of VP6 antigen could be used to generate a rapid test based on the lateral flow immunoassay. In the present study, we showed the results of synthesis of polyclonal antibodies against recombinant VP6 protein of rotavirus. Recombinant VP6 protein was strongly produced by *E. coli* in suitable conditions. The properties of recombinant VP6 protein were characterized by Western blot and ELISA methods. Pure recombinant VP6 protein was used as an antigen to inject into the rabbits. The recombinant VP6 protein was highly immunogenic in rabbits. Anti-VP6 antibodies were purified from the rabbit serum using the Protein A affinity chromatography. The specificity of the antibody towards its antigen was characterized by Western blot and ELISA immunoassay. The purity of antibody was reached as 97 %. The efficiency of antibody production in rabbit was 3.94 mg/ml serum. The amount and quality of the obtained anti-rotavirus VP6 antibody was suitable for application in the development of the diagnostic kits.

*Keywords:*diarrhea, polyclonal antibody, recombinant, rotavirus,VP6 protein.

1. INTRODUCTION

Rotavirus is one of the major causes of pediatric gastroenteritis and diarrhea. Untreated, rotavirus infection may result in severe illness with dehydration and disturbance of the body's normal electrolyte balance, and a major cause of infant mortality in the developing countries. The report of surveillance showed that rotavirus infection explains for up to 55 – 60 % of the hospitalized cases of diarrheal illness in infants and young children [1 – 3]. Estimated by WHO, 20 % of deaths in children under 5 years old was due to rotavirus infection [3, 4]. These factors make it necessary to develop a diagnostic system that permits the specific detection of rotavirus [5]. Group A rotavirus is formed by three concentric layers of protein in which the intermediate layer is formed by VP6 [6, 7] Protein VP6 is the most abundant protein (51 %) and highly

conserved with approximately 90 % homology at the amino acid level among group A rotavirus [8]. It is also highly immunogenic rotavirus protein [9]. The VP6 protein of rotavirus was considered as an important antigen in the development of novel vaccine generation and diagnostic tools [10, 11]. Several diagnostic tools were successfully developed to detect rotavirus on basis of immunosensor techniques. Immunoassay methods are highly dependent on the quality of its detection agent, i.e antibodies, which are often conjugated with a tracer, such as enzymes (ELISA), nanoparticles (LFA). Therefore, the ability to produce sensitive and specific antibodies to rotavirus has become very important. Although monoclonal antibody can be produced and they provide more specificity, the production of polyclonal antibody is simple and can be performed with adequate specificity when coupled with lateral immunosensors. In this paper we describe the preparation of recombinant VP6 protein and its specific polyclonal antibody.

2. MATERIALS AND METHODS

2.1. Materials

The recombinant *Escherichia coli* BL21(DE3) strain harboring recombinant plasmid pET22*::vp6* for expression of VP6 was from [12]. Native VP6 protein was extracted from pure rotavirus. Standard rotavirus sample from ProSpecT Rotavirus Microplate Assay (Oxoid Ltd, UK). White rabbits 3 months old were supported by POLYVAC.mAb anti-VP6 antibody, goat anti-rabbit IgG antibody conjugated alkaline phosphatase or horse radish peroxidase (HRP) were purchased from Abcam (USA), Genscript (USA), Sigma-Aldrich (USA), respectively. Chemicals for analyses were purchased from Sigma-Aldrich (USA), Merck (Germany), Thermo Scientific (USA).

2.2. Methods

2.2.1. Preparation of antigen

After 4 hours induction with 0.1 mM IPTG at 30 ^{0}C, the cell biomass of *E. coli* BL21(DE3)/pET22*::vp6* was harvested. The cells were lysed with A buffer (20mM phosphate buffer,0.5 M NaCl, lyzozyme 100µg/ml, pH 7.4) at 37 °C for 1 hours. The pellet containing VP6 inclusion body protein collected after centrifugation was further washed with B buffer (20 mM phosphate buffer, 0.5 M NaCl, 0.5 % Triton X-100, pH 7.4) and solubilized with 1X PBS buffer containing 20 mM β–mercaptoethanol, 0.5 % SDS, 5 mM DTT. Recombinant VP6 protein (rVP6) was further purified by affinity chromatography using AF-Chelate-650 resin (Tosoh, Japan) and checked by the SDS-PAGE method. Protein concentration was measured by Bradford method.

2.2.2. Production of polyclonal antibody against rVP6 in rabbits

Rabbits were immunized with the rVP6 as follows: 250 µl of rVP6 (1 mg/ml) was emulsified with equal volume of Complete Freund's adjuvant for the first injection and Incomplete Freund's adjuvant for the booster injections with 2-weeks intervals. The first injection was performed intradermally and the booster injections were introduced intramuscularly. Before each injection, rabbits were bled and antibody titers were determined by an indirect ELISA. Rabbits were completely bled 2 weeks after final injection. Antiserum was

separated from the clot by decant method. Antibody was purified using Protein A sepharose bead column as the instruction of producer.

2.2.3. Western Blot

Western blot method was performed as follows: Proteins were separated by SDS-PAGE and then semi-dry transferred to the PVDF membrane. The membrane was blocked with 5 % skim milk for two hours and incubated with monoclonal anti-VP6 primary antibody overnight. After three washes with PBS-T buffer, the membrane was incubated with anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase for 2 hours. After 3 times of washing, the signal was developed by incubating the membrane with the substrate solution of NBT/BCIP.

2.2.4. ELISA

The optimal dilution of VP6 antigen in 50 mM carbonate buffer was coated in each well overnight at 4 °C. After 3 times of washing with PBS containing 0.05% Tween 20 (PBS-T), nonspecific sites were blocked with PBS containing 5% non-fat dry milk for 2 hours followed by 2 washes with PBS-T. Primary antibody (anti-VP6 monoclonal antibody, prepared rabbit anti-VP6 antibody) was added to each well and incubated for 2 hours at 37 ^{o}C. The plate was washed 5 times with PBS-T and incubated with secondary antibody conjugated with HRP for 1 hour at room temperature. After 5 times of washing, the TMB substrate solution was added to each well. After 20 minutes of incubation in a dark place, the reaction was stopped with 5% solution of sulfuric acid. Absorbance of wells was read by a plate reader at 450 nm.

2.2.5. Gel image analysis and statistics

The intensity of the protein band on the gel electrophoresis were analyzed and identified by software QuantyOne 4.6.9 (Bio-Rad, USA). % rVP6 = CrVP6/C_t × 100, which is the CrVP6 intensity of rVP6 target proteins, C_t is the total intensity of the protein band on a running lane.

2.2.6. Procedure for the LFA test

Standard virus sample was prepared in PBS buffer at 1.6 x 10^6 particles/ml. Three drops of prepared virus solution was loaded into the sample window of the LFA strip. The result was read after 3-5 min development.

3. RESULTS AND DISCUSSION

3.1. Preparation of recombinant VP6 antigen

3.1.1. Expression and purification of recombinant VP6 protein

The recombinant construct (pET22*::vp6)* encodes a fusion VP6 protein with a theoretical molecular mass of approximately 47 kDa. After induction with 0.1 mM IPTG in suitable conditions, recombinant VP6 protein was extracted and purified by the procedure described in section 2.2.1. The protein extract was checked by SDS-PAGE and showed the appearance of a strong band of VP6 protein on the electrophoresis pattern (Fig. 1). The capacity of VP6 production by *E. coli* BL21(DE3)/pET22*::vp6* in selected conditions was 0.24 mg/ml. This was

10-fold greater than that of [13]. The recovery efficiency of VP6 protein after affinity purification was 80.0 % (data not shown). Purity of recombinant VP6 protein extract was 90.7 %.

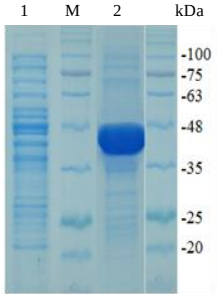

Figure 1. Electrophoresis pattern of VP6 protein extract. Lane 1, flow through fraction; lane 2, eluent fraction after eluted with 300 mM of imidazole; lane M, standard protein marker

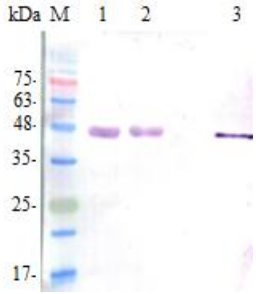

Figure 2. Western blot analysis of recombinant VP6 protein in total extract (lane 1) and purified extract (lane 2) and natural VP6 protein (lane 3). Lane M, protein ladder (Intron-Korea)

3.1.2. Characterization of recombinant VP6 protein

In the present study, recombinant VP6 protein was expressed in the form of inclusion body and dissolved in denaturation buffer. After purification, recombinant VP6 protein is soluble in the absence of denaturation agents (mercaptoethanol, DTT and SDS). To determine whether the recombinant VP6 protein was folded in native conformation, VP6 protein was checked by both immunological methods of Western blot (WB) and ELISA. For Western blot test, anti-VP6 monoclonal antibody (Abcam – USA) was used as primary antibody. The resulting pattern of WB shows appearance of a single signal band of approximately 47 kDa (Fig. 2) in both cases of total extract (lane 1) and purified extract (lane 2). The result shows specific anti-VP6 monoclonal antibody recognizes both recombinant and native VP6 proteins (extracted from rotavirus) (lane 3). This observation implicated that the conformation of recombinant VP6 protein was similar to native protein. Furthermore, the appearance of a single signal band implicated that recombinant VP6 protein in the extract was intact.

For further examination of recombinant VP6 protein conformation, both recombinant and natural VP6 proteins were checked by ELISA method using an anti-VP6 monoclonal antibody as primary antibody. The ELISA result shows the appearance of significant signal in both cases of natural and recombinant VP6 protein (Fig. 3).

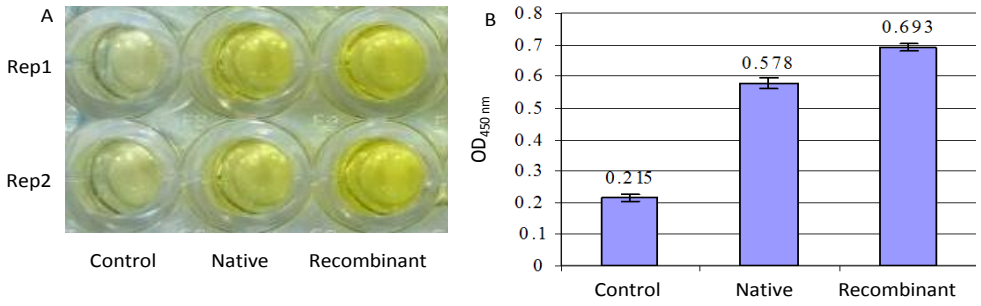

Figure 3. ELISA analysis of native and recombinant VP6 protein. Development of ELISA signal by adding TMB substrate (A) and the absorbance signal intensity of the plate wells coated with native and recombinant VP6 protein respectively (B).

Slight difference in signal intensity between coated native and recombinant VP6 protein may be due to incomplete extraction of native VP6 protein from rotavirus. The observed result indicated that anti-VP6 monoclonal antibody can recognize both recombinant and natural VP6 protein at the same level. From obtained results, it is clear that the conformation of recombinant VP6 protein is similar to that of native VP6 protein.

2.3. Preparation of polyclonal antibody against rVP6 protein

3.2.1. Production and purification of anti-rVP6 polyclonal antibody

Production of anti-rVP6 polyclonal antibody was performed by multiple immunizing the rabbits with the recombinant VP6 antigen. The ELISA assay was performed to determine the titer of the produced antibodies to rVP6. The highest level of anti-rVP6 polyclonal antibody was obtained after the 5th injection (data not shown).

In order to purify the anti-rVP6 polyclonal antibody, a protein A sepharose 4B column was used. The result of rabbit IgG purification using the protein A sepharose column showed that there were only two separate peaks on the chromatographic chart with retention times of 1.55 and 40.85 min, respectively (Fig 4. A). The first peak was present just after loading sample (1.55 min) indicating that proteins in this fraction were unbound proteins. The second peak was present just after adding the low pH glycine solution (pH 2.7) indicating that proteins in this fraction were protein A specific binding IgGs. The protein pattern of eluent fraction was analyzed by SDS-PAGE and showed the presence of two protein bands with size of approximately 50 and 25 kDa (lane 1.B) corresponding to the IgG heavy and light chain, respectively. Observed results demonstrated that IgG was successfully purified from serum. The amount of purified IgG was determined as 3.94 mg/ml serum. The relative purity of IgG antibody solution was approximately 97 %.

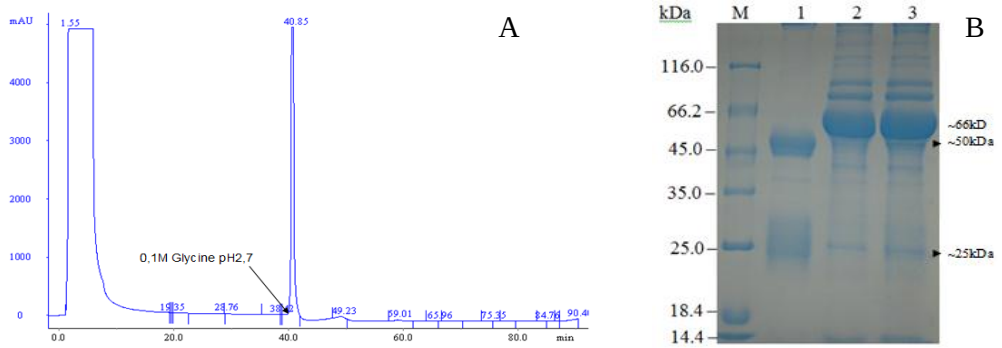

Figure 4. Affinity purification of anti-rVP6 polyclonal antibody using Protein A – Sepharose beads. Chromatographic chart (A) and protein pattern (B) of purified IgGs from rabbit. M, protein marker; 1, eluted IgG fraction (peak 2); 2, Flow through (peak 1); 3, whole serum.

3.2.2. Characterization of anti-rVP6 polyclonal antibody

Similar to VP6 antigen, property of anti-rVP6 polyclonal antibody (pAb-VP6) was also characterized by both immunological methods of western blot and ELISA. In western blot experiment, recombinant (crude and purified extract) and natural VP6 proteins were blotted on the nitrocellulose membrane at lane 1, 2, 3 respectively of the Figure 5. The pure generated pAb-VP6 was used as the primary antibody. The western blot result (Fig. 5B) shows that anti-rVP6

antibody recognizes both recombinant and native VP6 protein (lane 1, 2 and 3, respectively). The appearance of a single signal band on the western blot pattern (Fig. 5B) implicated that the generated pAb-VP6 bound specifically to rotavirus VP6 protein.

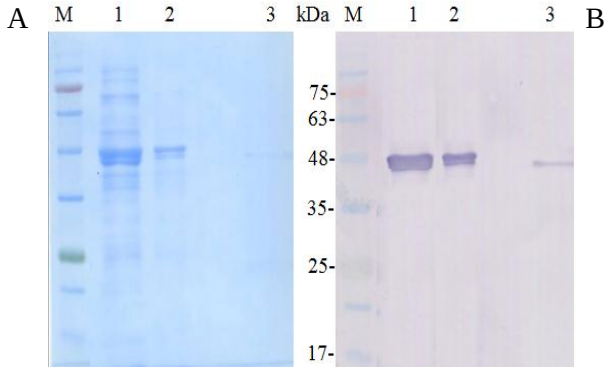

Figure 5. Characterization of pAb-VP6 by Western blot method. Ink staining of blotted nitrocellulose membrane (A) and Western blot (B). Lane 1-3, total protein from the extract of *E.coliBL21(DE3)/pET22b::vp6*, purified VP6 and total protein from rotavirus extract, respectively.

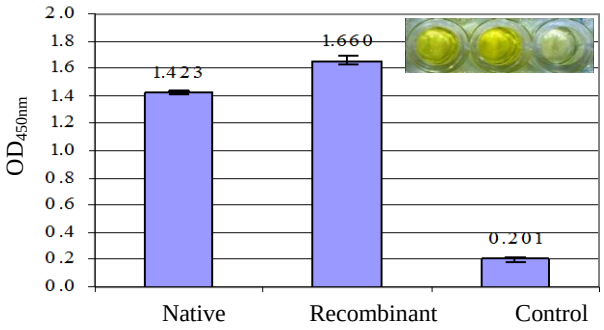

Figure 6. ELISA analysis of anti-rVP6 polyclonal antibody used as primary antibody. Different types of target proteins (native from rotavirus extract, recombinant VP6 from *E. coli*) and PBS (control) were coated on the bottom of plate wells, respectively.

Properties of pAb-VP6 investigated by ELISA method. The generated antibody was used as the primary antibody. Native VP6 protein from rotavirus extract and pure recombinant VP6 protein from *E. coli* extract were coated on the bottom of plate wells. The ELISA result showed that the significant signals were observed with both cases of coated native and recombinant VP6 proteins (Fig. 6). The obtained results demonstrated that the generated polyclonal antibody is specific to rotavirus VP6 protein. This antibody is quality enough in purity and specificity for several applications.

2.4. Preliminary application of anti-rVP6 polyclonal antibody in the development of the lateral flow immunnoassay test strip

The lateral flow immunoassay test strip (LFA) has been well-established diagnostic tool. The format in LFA is based on the interaction of antigen and antibody on the surface of the nitrocellulose membrane. In this study, the generated anti-rVP6 polyclonal antibody was used to conjugate with gold nanoparticles to generate the conjugate pad and include in the LFA strip.

The Figure 7 shows the appearance of two significant signal bands (T-line and C-line) in case of positive sample and only single band (C-line) in case of negative sample. This result indicated that polyclonal antibody included in the conjugate pad of LFA strip recognized and captured rotavirus when it moves through the conjugate pad of strip to generate signal band at T-line position. Obtained results indicated that the generated anti-rVP6 polyclonal antibody is suitable for development of the lateral flow immunoassay test strip.

Figure 7. Generation of the lateral flow immunoassay test trip using the anti-rVP6 polyclonal antibody. (+) LFA strip was tested with positive sample, (-) LFA test strip was tested with negative sample (PBS buffer). Experiments were duplicated.

4. CONCLUSIONS

The present study has successfully produced pure recombinant rotavirus VP6 protein. The recombinant VP6 protein was highly immunogenic in rabbits. The generated polyclonal antibody was specific to the VP6 protein of rotavirus and could be used to the development of lateral flow immunoassay test trip for detection of rotavirus.

Acknowledgement.The research funding from Ministry of Education and Training (Grant number: B2014-01-66) and Basic project of HUST (No. T2016-PC-032) was acknowledged.

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TÓM TẮT

NGHIÊN CỨU TẠO KHÁNG THỂ ĐA DÒNG KHÁNG PROTEIN KHÁNG NGUYÊN VP6 TÁI TỔ HỢP CỦA VIRUS ROTA

Do Thi Thu Ha[1], Ngo Thu Huong[2], Nguyen Dang Hien[2], Truong Quoc Phong[1, *]

[1]Trường Đại học Bách khoa Hà Nội, 1 Đại Cồ Việt, Hà Nội
[2]Trung tâm Nghiên cứu sản xuất văcxin và sinh phẩm y tế, 418 Vĩnh Hưng, Thanh Trì, Hà Nội

[*]Email: *phong.truongquoc@hust.edu.vn*

Virus rota là nguyên nhân phổ biến nhất gây tiêu chảy ở trẻ nhỏ và trẻ em dưới 5 tuổi. Protein VP6 của virus rota được xem là kháng nguyên quan trọng trong phát triển văcxin và các công cụ chẩn đoán. Kháng thể đơn dòng kháng kháng nguyên VP6 có thể được sử dụng để tạo ra kit chẩn đoán dựa vào phương pháp sắc ký miễn dịch. Trong nghiên cứu này, chúng tôi trình bày kết quả nghiên cứu tạo kháng thể đa dòng kháng protein VP6 tái tổ hợp của virus rota. Protein VP6 tái tổ hợp đã được tổng hợp mạnh trong *E. coli* ở điều kiện thích hợp. Đặc tính của protein VP6 tái tổ hợp đã được xác định bằng phương pháp Western blot và ELISA. Protein VP6 tái tổ hợp tinh sạch đã được sử dụng làm kháng nguyên để gây miễn dịch trên thỏ. Protein VP6 tái tổ hợp được xác định là một kháng nguyên mạnh. Kháng thể kháng protein VP6 đã được tinh sạch từ huyết thanh thỏ sử dụng cột sắc kí Protein A. Tính đặc hiệu của kháng thể được xác định bằng

Western blot và ELISA. Độ sạch của kháng thể đạt được là 97 %. Hiệu suất tổng hợp kháng thể là 3,94 mg/ml huyết thanh. Hàm lượng và chất lượng của kháng thể thu được đảm bảo yêu cầu để phát triển các kit chẩn đoán.

Từ khóa: tiêu chảy, kháng thể đa dòng, tái tổ hợp, virus rota, protein VP6.