Iranian J Virol 2017;11(2): 36-42

Original Article

Polyclonal Antibody against Recombinant Nucleoprotein of the Influenza A Virus (H1N1); Production and Purification

Alikhani M[1,2], Behzadian F[2], Mehrbod [1], Khosravi Node F[1,2], Shokouhi Targhi H[1], Farahmand B[1*]

1. Influenza and other Respiratory Viruses Department, Pasteur institute of Iran, P.O. Box 13169-43551, Tehran, I.R. Iran
2. Department of Genetic Engineering, Research Center for Science and Biotechnology, P.O. Box 19395-1949, Tehran, I.R. Iran

Abstract

Background and Aims: Influenza is an acute respiratory illness that is caused by a virus belonging to Orthomyxoviridae family. This virus spreads rapidly every year in cold season and leads to morbidities and mortalities especially in adults and children, which causes billions of dollars of economic losses. Accordingly, development of a rapid, sensitive and inexpensive laboratory diagnosis based on antigen detection to distinguish this infection from other respiratory tract viruses is important. In addition, specific anti-influenza antibody production against influenza virus antigen is essential for basic and applied research programs. Influenza A virus nucleoprotein (NP) is a structural protein and a major component of the ribonucleoprotein complex. It has a high expression level during infection. NP consists of 498 amino acids with molecular weight of 57 KD. The aim of this study was to produce and purify polyclonal antibody against recombinant nucleoprotein of the influenza A virus.

Materials and Methods: Rabbit immunization was performed based on a specific program by NP purified recombinant antigen and Freund's adjuvant. Serum immunoglobulin was separated by ammonium sulfate and IgG purification was conducted by ion exchange chromatography (DEAE-cellulose). To evaluate the reaction between antigens and purified antibodies, SRID and ELISA serological tests were applied.

Results: The results obtained from SDS-PAGE and Western blot showed a dense band of purified NP. The results of ELISA confirmed an increase in NP antibody titer after one month. Antibody levels detection by ELISA showed a sensitivity of 1 to 50. In SRID, sedimentary areola was observed due to the interaction of NP antigen and antiserum. Western blot results were also positive for the NP protein.

Conclusions: The NP antigen purified in this study, as well as the produced and purified antibodies, had the ability to be used in serological tests to detect influenza A virus. It can also be used in basic research methods such as Western blot, immunohistochemistry and immunocytochemistry.

Keywords: antigen purification, polyclonal antibody production, antibody purification, Western blot.

Introduction

Influenza A virus is a member of the Orthomyxoviridae family with lipid coverage which originates from the cytoplasmic membrane of the host cell while germination [1]. The virus has a negative-sense single-stranded RNA with eight segments which code 11 proteins. This family consists of three types of A, B and C [2]. The type A virus is more seriously considered because of the pandemics and epidemisc [3,4].

[*] **Corresponding author:** Behrokh Farahmand
Influenza and Other Respiratory Viruses Department, Pasteur Institute of Iran, Tehran, Iran
Phone: +9821-64112183
Email: b_farahmand@pasteur.ac.ir.

The human population is always affected by influenza disease. The virus is transmitted rapidly and is always a threat to public health. According to World Health Organization (WHO), one of the most common causes of death worldwide is influenza epidemics. Annual epidemics contaminate 5-15% of the world population, from which the ones suffering from immune suppression will show chronic symptoms and may lead to about 500,000 deaths [5-8].

The NP gene is located in the section 5 of the genomic RNA of the virus with 1537 bp. The readable portion of influenza virus A / Puerto Rico / 8/34 (H1N1) NP nucleotide sequence is from 33 to 1529 and expresses a protein with 498 amino acids [9].

The influenza virus nucleoprotein forms protein scaffold of the spiral genome of the ribonucleoprotein complex and plays an important role in transcription of the RNA [10]. The primary function of NP is to pack the genome of the virus for transcription and reproduction. On the other hand, NP is associated with viral polypeptides and other components involved in the entry and release of the materials into the nucleus and a nuclear helicase RNA [11].

NP protein is an internal conserved protein [12] and is important in the pathogenesis of the virus. By recombinant production and purification of this protein, as well as polyclonal antibody production against it, diagnostic and differential tests can be developed which can be used at the time of the epidemic onset as well as basic research and development for prevention and treatment. Antibody production against this recombinant protein is one of the proposed processes in this field that has been considered in this research.

In this study, with the aim of producing polyclonal antibody against NP conserved protein for the rapid and accurate detection by inducing a humoral immune response in New Zealand rabbit [13], the sensitivity of the produced antibodies against NP antigen was examined. In fact, antibodies are proper tools used in research. Mammalian serum is an important and economical source for immunoglobulin, which is widely used in the diagnosis and treatment of diseases. Polyclonal antibodies are a mixture of monoclonal antibodies that are produced against different epitopes and bind to an antigen molecule with high affinity [14].

Methods

Expression and transformation optimization. Firstly, 500 ng of the recombinant plasmid pET28a-NP was transformed into E. coli BL21. In order to optimize the expression conditions, the transformed bacteria were separately induced by IPTG with 0.5 and 1 mM concentrations at 37 and 28°C. The samples were taken at 0, 1, 2, 3, 4, and 5 hr after induction and run on SDS-PAGE gel. Increasing the expression was evaluated by increasing the incubation time.

Antigen purification. Following optimization and confirmation of the NP protein expression, bacterial sedimentation was performed by Native extraction method using different buffers. The number of pulses and the power of sonication (Hielscher's, Germany) were 8 times each time 20 pulses per 0.5 second with a power of 85w. In order to purify the His-Tagged recombinant protein, the extraction suspension containing all the proteins of the bacteria and recombinant protein were passed from the Protino Ni-TED nickel column.

Antibody preparation. A white New Zealand rabbit weighing about 2 kg was immunized by NP antigen to produce polyclonal antibody against NP of influenza A virus. For this purpose, NP antigen was mixed with Complete Freund's adjuvant, so that a homogeneous gelatinous and milky emulsion was obtained. The emulsion was injected intramuscularly and subcutaneously into the rabbit. Further immunizations were performed to increase the immune response. Other injections were performed after 14 days using 0.5 mg NP antigen and Incomplete Freund's adjuvant. Seven days after each injection the blood samples were taken from the rabbit. Blood was collected in sterile tubes and placed in a

refrigerator for 1 hr. The samples were clarified by centrifugation at 1800 g for 10 min at 4°C.

Evaluation of polyclonal antibody by ELISA. The sera from rabbit were evaluated by ELISA which is more sensitive than SRID method. First, using checkerboard method, different dilutions of protein and antibody as antigen and antiserum, respectively were titrated, and then proper dilutions for ELISA were determined.

Immune response evaluation of by SRID method. For Single Radial Immunodiffusion (SRID), 1% agarose gel was prepared and poured into a small petri dish. For each ml of the gel, 10, 20 and 30 µl of antisera containing anti-NP were added. The wells were created with a diameter of 3.5 mm, 4.5 mm apart from each other. Regarding the antigen concentration, 10, 20 and 30 µl of the antigens were serially placed into the wells. After 24-48 hr keeping in a dark container for antigen penetration, it was placed in NaCl solution (0.9%) for 24 hr to remove un-reacted proteins. The gel was then completely dried by exposing to hot air flow. It was stained with Coomassie blue (0.5%) for 20-30 minutes and then de-stained to reveal the sedimentary areolas. After de-coloration, the areolas with different diameters were observed around the wells.

Antibody purification. In order to purify IgG, the serum was sedimented by adding 30% ammonium sulfate on ice using a magnetic stirrer with a very low circle. Following 30 min, the solution was centrifuged at 402 g for 30 min at 4°C. The precipitated immunoglobulin was dissolved in PBS 0.01 M and dialyzed for 16 hr to remove ammonium sulfate salt. The obtained immunoglobulin was placed on the DEAE-cellulose chromatography column with pH adjusted to 6.3 [16]. The specimens containing protein were identified by Bradford assay and ran on 10% polyacrylamide gel for purification confirmation. The Western blot was used to confirm the antibody function. The protein bands were transferred from polyacrylamide gel to membrane.

Ethics statement. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee. This research was reviewed and approved by the Ethics Committee of Pasteur Institute of IRAN (Protocol Number IR.PII.REC.1395.82).

Results

Initially, the expression of recombinant protein in a volume of 10 ml was performed with three variables of time, temperature and concentration of Isopropyl β-D-1-thiogalactopyranoside (IPTG). Following electrophoresis and gel staining, the recombinant NP protein band was observed at a range of 57 kDa. After comparing the results, the best expression conditions were chosen at 28°C and 1 & 3 mM IPTG concentrations (Fig. 1).

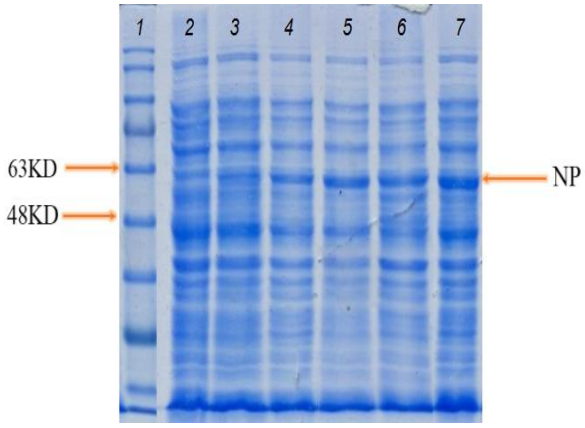

Fig. 1. The result of SDS-PAGE for optimizing the expression of NP in a volume of 10 ml culture medium at 37°C and IPTG concentration at 0.5 mM in different time points. Lane 1: protein marker (Sina Clone, Cat. No. PR911654), Lane 2: expression before induction, Lanes 3-7: expressions in 1, 2, 3, 4 hr and overnight after induction.

The Protino Ni-TED Nickel column was used to purify the recombinant NP protein containing His-tag. The results of purification are visible in Fig. 2. The results obtained from this method indicated that the produced

antigens have the ability to react with the recombinant NP protein.

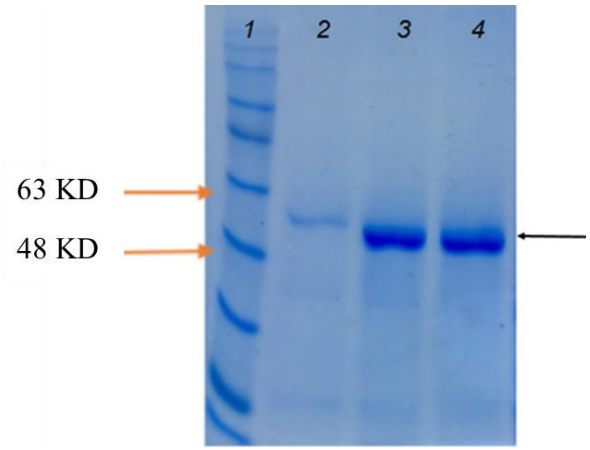

Fig. 2. The SDS-PAGE result of NP protein purification with nickel chromatography column at pH=8 and imidazole 250 mM. Lane 1: protein marker (Sina Clone), Lanes 2-4: fractions collected from the end of the column at the last stage (Elution).

After five times booster injection, ELISA with different dilutions of antigen and anti-serum was performed to confirm the immunization of the NP protein. The dilutions of 1:10000 and 1:100 were tested for NP antigen and antibody, respectively. The results showed ascending increase in antibody titer over the time (Fig. 3).

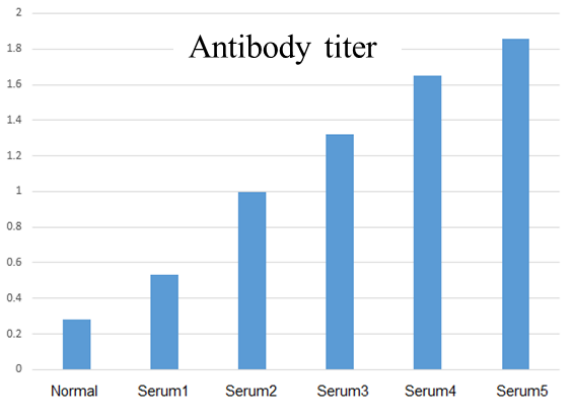

Fig. 3. The graph of ELISA results with NP antigen 1:10000 and antibody 1:100 dilutions. Normal (serum 0): serum before antigen injection, serum 1: 28 days after the first injection, serum 2: 14 days after the second injection, serum 3: 14 days after the third injection, serum 4: 14 days after the fourth injection, serum 5: 14 days after the fifth injection.

The SRID test was conducted to determine the interaction between antigen and antibody and also to determine the concentrations of antigen and antibody [15]. The result of this test (SRID) to determine the interaction between antigen and antibody is shown in Fig. 4. The sedimentary areolas were observed around the wells clearly.

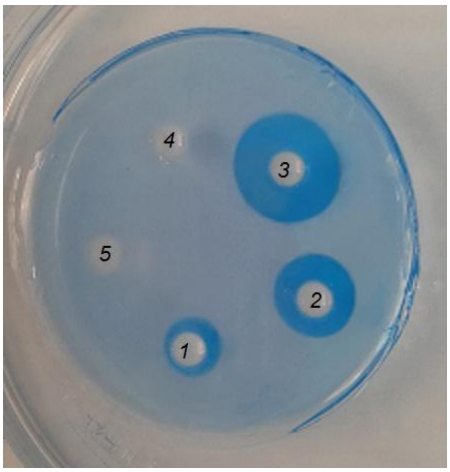

Fig. 4. SRID Results for protein and antibody reaction. 1) 10 µl of NP antigen (1 mg / ml); 2) 20 µl of NP antigen (1 mg / ml); 3) 40 µl of NP antigen (1 mg / ml); 4 and 5) negative control.

After confirmation of the antigen purity from the New Zealand white rabbit and isolation of the serum using ion exchange chromatography (DEAE-cellulose), IgG was purified from immunoglobulins.

The dialyzed immunoglobulin solution was poured onto the DEAE column, which was previously adjusted to pH=6.3 with a PBS buffer, and passed through the column according to the conditions stated above. Protein-containing specimens were determined by Bradford assay and electrophoresed on 10% polyacrylamide gel for confirmation of purification (Fig. 5).

The Western blotting was used to analyze the antibody reaction to NP protein, which indicates that the purified antibodies have the ability to react with NP protein. The result was shown in Fig. 6.

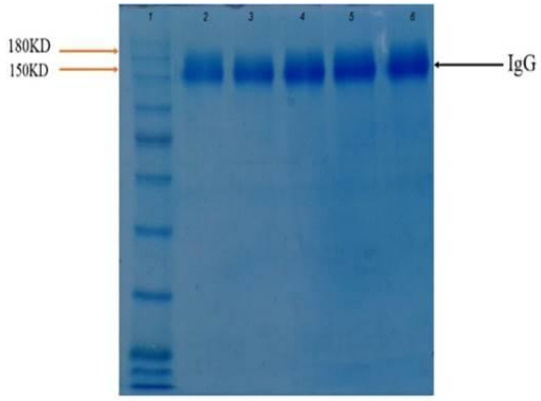

Fig. 5. SDS-PAGE results from antibody purification with a DEAE chromatography column. Lane 1: Protein marker (Sina Clone), Lanes 2 to 6: fractions collected from the end of the column at the last stage (Elution).

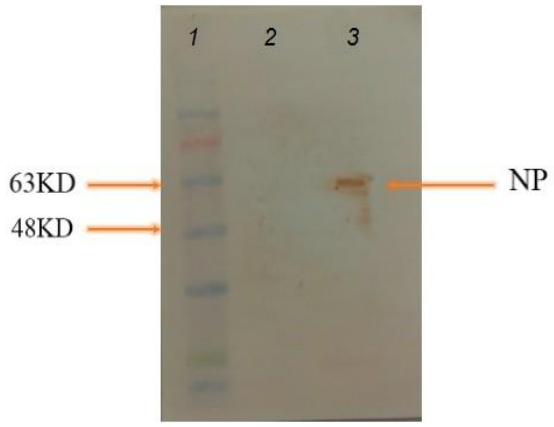

Fig. 6. Western blot of NP protein. Lane1: Protein Marker (Sina Clone), Lane 2: Negative Control, Lane

Discussion

Since the polyclonal antibodies can be obtained in a short period of time (4-8 weeks) they are amongst the most important tools for diagnosis of influenza infection. These antibodies are not only used to determine the type of the virus in cell culture supernatants, but also used to detect viral antigens in the nasopharyngeal secretions. By launching specific immunoassays for the NP antigen, it is possible to differentiate influenza from other respiratory viruses such as parainfluenza, rhinovirus, adenovirus etc [17]. Also Chomel et al used anti-NP polyclonal rabbit antiserum for specific, sensitive and easier reading immunocapture ELISA test for diagnosis of influenza A and/or B in animal and human nasopharyngeal samples [17]. Avian influenza virus infection as critical step for control of virus subtypes and reducing the risk of pandemic human influenza was developed using ELISA method by polyclonal anti-NP [18-19].

Nucleoprotein is a multifunctional protein which is biologically important for the replication of viral ribonucleoprotein, the movement of the genome and its accumulation. The NP molecule is strongly conserved among the type A influenza strain and abundantly expressed during the infection. In mouse models it can be detected two days after infection. Due to NP's ability to confirm the immunogenicity of heterozygote subtypes, anti-NP antibodies through passive immunization in immature mice not only protect against infection but also produce immunity up to 1.5 years after infection. It is necessary to use IgGs produced against NP with a titer of 105 by injection to reduce the load of the virus. Mass production of anti-NP antibodies in different models for passive immunization is an ideal strategy for preventing the spread of influenza viruses [18].

In this study, in order to produce polyclonal antibody against influenza A virus nucleoprotein for different purposes application, the NP antigen was first expressed and optimized in the prokaryotic system and then purified. The results of SDS-PAGE showed that the obtained product was completely pure as compared to the other commercial products [9]. The New Zealand white rabbit was immunized with an optimized and purified antigen. This antigen was injected in a regular schedule with Freund's adjuvant to increase the stability and prolongation of the antigen release to the rabbit, subsequently blood sampling was done.

For different purposes, the suitability of antibodies is very important issue, thus each antibody doesn't have equal behavior in all assays. For example an antibody may work good in a Western blot analysis but fail in immunohistochemistry. Antibodies' nature depends on the epitope position on the 3D structure of molecule. This point must be considered as an important approach to study the proteins using immunoassay techniques. Because some immunochemical techniques such as; enzyme-linked immunosorbent assay (ELISA), immunonephelometry, SRID and Plasmon Surface Resonance are based on conformational epitopes.

In this study, the injectable suspension solution was a mixture of 400 µg of purified recombinant NP protein for the first injection and 200 µg for each booster (compatible to the usual dose range of 50 to 1000 µg of soluble protein administered to rabbits) and Freund's adjuvant [20]. The expressed recombinant NP protein in E.coli as prokaryotic system was successfully expressed and purified. The time-consuming recombinant NP protein purification steps are decisive for specific antibody production as a critical point for most of the immunoassays.

The antibody activity was evaluated by ELISA in collected serum. The antibody titer was comparable to that of the prominent companies' products such as GeneTex, ThermoFisher and Abcam. Antibody was then purified with ion exchange chromatography. Then indirect ELISA, one-way immunodefusion (SRID) and Western blotting were applied to evaluate the antibodies reactions. Secondary serological tests evaluated the antigen and antibody reaction through their binding effect (precipitation). SRID results showed clear areolas indicating the antibody response to the desired antigen.

Conclusion

The obtained information from this research indicated that purified NP antigen had the potential to induce immune response in rabbit, which leads to the production of NP polyclonal antibodies in rabbit. Purified antibodies against NP are able to be used in the diagnostic serological tests for the influenza A virus, as well as other safety applications such as Western blot analysis, immunohistochemistry and immunocytochemistry.

Acknowledgements

This work was funded by grant No 944 from Pasteur Institute of IRAN. We appreciate The Influenza and other Respiratory viruses Department.

Conflict of interest

The authors declare they have no conflict of interest.

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