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RESEARCH ARTICLE

Engineered Recombinant NDV-Fusion Protein and Its Polyclonal Antibodies Production

Maira Zahid[1], Asma Irshad[2], Sana Shakoor[1], Tahir Rehman Samiullah[1], Naila Shahid[1], Adnan Iqbal[1], Sana Tanveer[1], Shehla J Akram[3], M Azam Ali[1], Shafique Ahmed[1,4], Tayyab Husnain[1], Ahmad Ali Shahid[1] and Abdul Qayyum Rao[1]

[1]Centre of Excellence in Molecular Biology, University of the Punjab, 87-West Canal Bank Road Lahore, Pakistan
[2]University of Management and Technology Lahore Pakistan; [3]Akram Medical Complex Lahore, Pakistan
[4]Allied Health Sciences, The Superior College, Lahore, Pakistan
*Corresponding author: qayyumabdul77@yahoo.com

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ABSTRACT

Antibodies have important role in biological research and diagnosis. Polyclonal antibodies can be produced against more than one epitope of an antigen. Newcastle disease (ND) is one of the most devastating diseases that considerably effects the global poultry industry. Newcastle disease virus (NDV) possess Fusion (F) protein for the attachment and pathogenicity. In the present study TA plasmid having F gene was restricted by EcoR1 and it was ligated into pET30a expression vector. The F gene ligated pET30a was transformed in BL21DE3 expression strain. After the expression of NDV F protein, it was verified by the appearance of 67 kDa band on SDS-PAGE gel and western blot. The F protein was partially purified by the column of 70 kDa cut. The recombinant F protein was used for the generation of polyclonal antibodies. Two groups (experimental group and negative control group) of 3 months old rabbits were used. Two doses of recombinant F protein with interval of 15 days were injected subcutaneously for production of antibodies against the F protein. Antibodies were obtained from serum taken after scarifying the rabbits. Production of polyclonal antibodies was confirmed through Dot Blot Assay and ELISA. In this study maximum antibody titer was estimated to be 4.676 (OD at 450 nm) in case of using concentrated antibody. Produced polyclonal antibodies can be further purified in order to use as tools in biomedical and biochemical researches and diagnostic kits.

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INTRODUCTION

Cellular and chemical defense in the form of Lymphocytes and antibodies are generated by immune system of organism upon exposure to any stimulus. The response generated by immune system can also be classified as (i) cellular responses and (ii) humoral responses. The cellular response is generated in the form of T Lymphocytes while antibodies produced and secreted by B-lymphocytes (Plasma cells) are responsible for humoral responses (Hoffman *et al.*, 2016).

In cellular immune response upon exposure to stimuli, antibodies are produced by adaptive immune system as host proteins which are present in extracellular fluid (plasma) to neutralize the foreign particle/molecules. The binding occur between host protein and foreign antigen on the basis of their affinity is highly specific. This specific binding makes them to use in various applications of medical science and contribute directly or indirectly to various discoveries in the field of biology. In the field of therapeutics and diagnostics, antibodies are found to be involved in the improvement of health of organisms (Lipman *et al.*, 2005).

Plasma cells (specialized B lymphocytes) secrete the antibodies that are glycoproteins in nature. These glycoproteins have same domain in their structure that are present in many other proteins and are referred to as immunoglobulins. These immunoglobulins consist of four polypeptide chains; two heavy and two light chains. These two copies of both heavy and light chains have disulfide and noncovalent linkages. These linkages are responsible for the Y shape glycoprotein (Andrews *et al.*, 1997).

Generally, antibodies are classified as (i) monoclonal and (ii) polyclonal antibodies. When produced by single clone of B-lymphocytes with specific binding of single epitope antibodies are referred as monoclonal antibodies.

But when antibodies are produced by different clones of B-lymphocytes and are able to bind with different epitopes of same antigen then such antibodies are named as polyclonal antibodies (Seida, 2017). In 1891, for the very first time, Emil von Behring and Shibasaburo Kitasato used the polyclonal antibodies for passive immunization with specific antisera. Till now polyclonal antiserum is used in the treatment of various diseases. Unlike monoclonal antibodies, polyclonal antibodies identify and bind with multiple epitopes and responsible for broad spectrum protection against the antigen (Stiehm *et al.*, 2008).

In biologics market, antibodies cover the largest proportion; it is reported that there is expense of more than 20 billion dollars every year to enhance immunity against different diseases with the 28 types of commercially available antibodies. An attempt was made in current study for polyclonal antibodies production of NDV F protein in rabbits as a part of study to use these antibodies with Gold nanoparticles for development of unique, sensitive, easy to use diagnostic tool of NDV outbreak in future.

MATERIALS AND METHODS

Cloning and transformation of F gene ligated pET30a into BL21DE3: The pET30a vector and TA plasmid having F gene were obtained from the Plant Transformation Lab, Centre of Excellence in Molecular Biology, Punjab University Lahore. In order to clone F gene into pET30a vector, EcoR1 restriction enzyme was used to produce overhangs in both TA plasmid having F gene and pET30a vector. DNA ligation kit (Thermoscientific cat # K1422) was used to ligate the F gene into pET30a vector as per manufacturer protocol.

Transformation of F gene in *E. coli* Rosetta strain: *E. coli*, BL21 DE3 cells were used for transformation of F gene ligated recombinant plasmid in pet 30a vector as described by Shahid *et al.* (2015). Confirmation of positive clones was done through restriction digestion with *EcoR1* enzyme and amplification by using the gene specific primers (NDVIF-F 5' AAGCACAACCGAAGGATTTG 3'; NDVIF -R 5'GCCGCTCAAACAGGAATAAA 3') after plasmid isolation with Gene Jet Plasmid Miniprep Kit (Cat. No. K0503). The amplification was visualized by resolving it on 0.8% Agarose gel.

Expression analysis of F-protein: Transformed cells were induced with 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by SDS-Polyacrylamide gel electrophoresis (PAGE) and Western Blot for the analysis of protein expressed in *E. coli* as described by Shahid *et al.* (2015). The *E. coli* expressed crude protein verified through western blot analysis was further subjected to its purification by using cut off column 70 kDa decided after close look on size of experimental protein as was done by Shahid *et al*. (2015).

Generation of NDV F protein antibodies in Rabbits: Two rabbits each of 3-months-old were taken for injection of NDV F protein and were named as experimental group while two others of same age were considered as negative control. The rabbits were acclimatized before injection by keeping them on experimental place for fifteen days and fed with normal protein diet. For injection total 250 µL of protein was mixed with 100 µL of complete adjuvant. The resultant milky white mixture was then injected into rabbits subcutaneously for first immunization. After 12-15 days, 800 µL of blood was taken from the ear of each rabbit and subjected to isolation of serum for the Dot Blot Assay used for the detection of polyclonal antibody in the serum of rabbit. Total 2 µL of protein (antigen) was added on the marked areas of nitrocellulose membrane. Blocking was done by using the 0.5% skim milk. After washing, 2 µL of serum was added on the marked area to be used as primary antibody. The membrane was incubated at room temperature for an hour after washing twice with 1X PBS. The membrane was subjected to BCIP/NBT solution dissolved in 20 mL of 1X PBS for color development. After 15 days of first injection, 250 µL of protein was mixed with 100 µL of incomplete adjuvant. The resultant milky white mixture was then injected into rabbits subcutaneously as booster dose or second immunization. All rabbits were dissected by following the SOPS devised by departmental ethical committee. The blood was obtained by puncturing the heart with the syringe. Serum was separated from the blood through centrifugation at 13,000 rpm for 5 minutes. Serum was used for the detection of polyclonal antibodies. Polyclonal antibodies against NDV F protein were detected by dot blot assay mentioned earlier and also by ELISA as described by Van-Earde *et al*. (2019). After second immunization both dot blot and ELISA were performed with different serum dilutions. The dilutions were prepared as: 2 µL serum into 998 µL PBS, 3µL into 997µL, 4µL into 996µL and 5µL into 995µL.

RESULTS

Confirmation of pET30a_F Digestion: Digestion of recombinant pET 30a_F plasmid was confirmed through restriction digestion with *EcoR1*. The released band of 1662 bp when resolved on 0.8% agarose gel confirmed the successful ligation of F gene in pET30a vector as shown in Fig. 1.

Screening of *E. coli* strain Rosetta for pET30a_F Plasmid: For expression studies of F protein recombinant pET30a_F plasmid was transformed in *E. coli* (BL-21 DE3). Total 50µl and 100µl of transformed Rosetta having recombinant pET30a_F plasmid was spread on the plates supplemented with kanamycin and chloramphenicol. Visible transformed separated colonies were observed after overnight incubation at 37°C.

Confirmation of plasmid transformation through colony PCR: Colonies were further screened for recombinant pET30a_F plasmid through amplification by using gene specific primers. Amplification of 1662 bp product obtained determined the specificity of colony harboring desired plasmid when resolved on 0.8% agarose gel as shown in Fig. 2.

Determination of F Protein Molecular Weight through SDS PAGE: The specificity of F protein was also confirmed through western blot analysis. Appearance of

67kDA F protein on SDS PAGE determined the specificity of expressed protein in *E. coli* obtained after induction of 100mM IPTG. The invisibility of band on the specific size in control sample further confirmed the authenticity of expressed protein as shown in Fig. 3 (a).

Confirmation of His-Tagged Protein by Western Blot Analysis: Expression of His-tagged protein was further confirmed through Western Blot analysis by using positively charged nitrocellulose membrane, using polyclonal anti-His AP-conjugated antibodies (Santa Cruz, UK). Colour development with BCIP/NBT substrate confirmed the expression of the 67-kDa F protein after antigen antibody reaction.as shown in Fig. 3 (b).

Validation of Antibody against NDV F after First Immunization: The F protein is an immunogenic protein. After first immunization, immune-dot blotting assay confirmed the immunogenic response generated in rabbit. Dark purple dots in lane I-II confirmed the production of antibody in the rabbit as shown in Fig. 4a and 4b.

Confirmation of Antibody production through ELISA: ELISA was used to detect the antibody in different dilutions of serum. Color change after the incubation with BCIP/NBT, in wells of ELISA plate confirmed the presence of antibody in the serum as shown in Fig. 5a. Optical density was measured by spectrophotometer at 450 nm as shown in Table 1 and Fig. 5b.

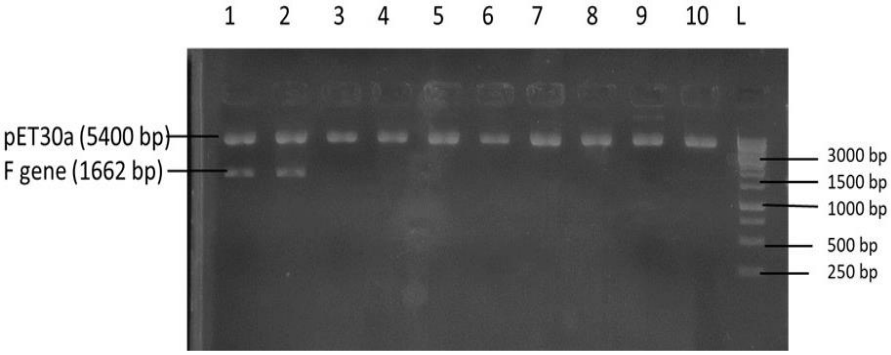

Fig. 1: Confirmation of digestion of pET30a plasmid (5400 bp) having F gene (1662 bp); Lane L: 1 Kb DNA Ladder, lane 1-10: digestion of recombinant pET30a plasmid having F gene, the released fragments at 1662 bp confirms the successful cloning of F gene in pET 30a vector.

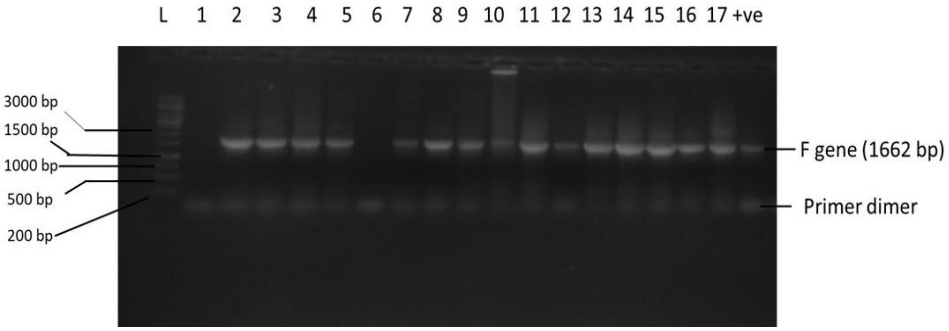

Fig. 2: Confirmation of presence of F gene (1662 bp) ligated in pET30a (5400 bp) transformed into Rosetta by PCR; The amplification of 1662bp F gene through gene specific primers in transformed Rosetta cells confirms the successful transformation of recombinant pET 30a in Rosetta cells. Lane L: 1 Kb DNA Ladder, lane 1-17: Amplification of 1662 bp F gene ligated in pET30a plasmid, +ive: positive control.

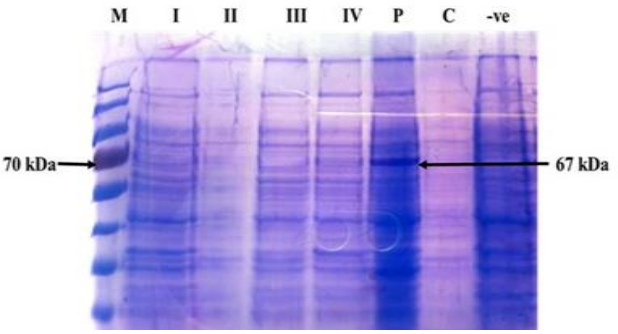

Fig. 3 (a): SDS- Polyacrylamide Gel Electrophoresis; The appearance of 67kDa F protein through SDS-PAGE Clearly showing the expression of F protein: Lane M: Pre-stained Protein Marker (Fermentas), Lane I-IV: Total cell lysates with different treatment, Lane P: Pellet dissolved in 1X PBS, Lane C: Transformed Rosetta without induction and Lane -ve: Rosetta culture as a –ve control.

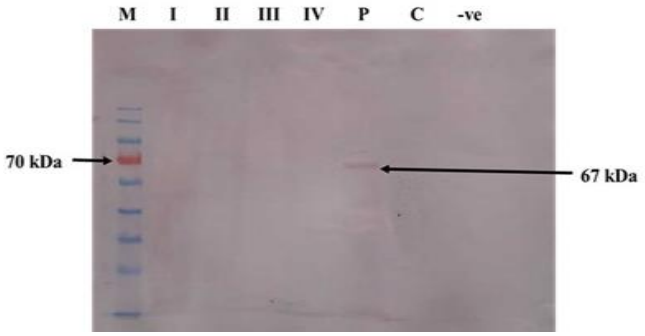

Fig. 3 (b): Western Blot analysis of F protein. The presence of 67 kDa in lane P (pellete) indicating the His-tagged F protein on nitrocellulose membrane by western blotting; Lane M: Pre-stained protein marker, Lane I-IV: Total cell lysates with different treatment, Lane P: Pellet dissolved in 1X PBS, Lane C: Transformed Rosetta without induction and Lane -ve: Rosetta culture as a –ve control.

Table 1: OD values of control and experimental rabbits' serum at 450 nm after ELISA in each well of 96 well-plate

Rabbits	2 µL: 998 µL (OD)	2 µL: 998 µL (OD)	Mean (OD)	3 µL: 997 µL (OD)	3 µL: 997 µL (OD)	Mean (OD)	4 µL: 996 µL (OD)	4 µL: 996 µL (OD)	Mean (OD)	5 µL: 995 µL (OD)	5 µL: 995 µL (OD)	Mean (OD)	Conc. Serum (OD)	Conc. Serum (OD)	Mean (OD)
Control (i)	0.011	0.013	0.012	0.018	0.019	0.018	0.021	0.026	0.023	0.036	0.042	0.039	0.066	0.069	0.067
Control(ii)	0.015	0.012	0.013	0.020	0.017	0.018	0.029	0.027	0.028	0.038	0.036	0.037	0.069	0.066	0.067
Exp I	0.692	0.595	0.643	0.513	0.573	0.543	2.132	2.232	2.182	2.692	2.952	2.822	4.231	4.321	4.276
Exp II	0.596	0.621	0.608	0.682	0.672	0.677	1.812	2.621	2.216	3.021	3.732	3.376	4.621	4.732	4.676

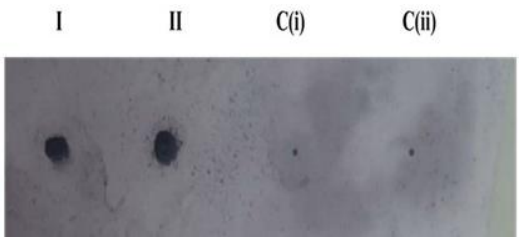

Fig. 4 (a): Confirmation of Antibody in rabbit serum by Dot Blot Assay Lane I: Serum of experimental rabbit 1, Lane II: Serum of experimental rabbit 2, Lane C(i): Serum of control rabbit 1 and Lane C(ii): Serum of control rabbit 2.

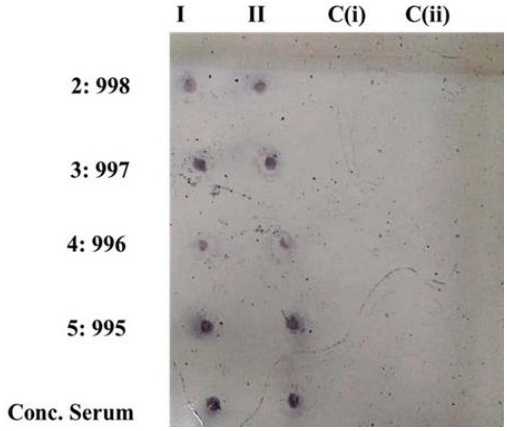

Fig. 4 (b): Confirmation of Antibody by Dot Blot Assay with different dilutions of rabbit serum. Lane I: Serum of experimental rabbit 1, Lane II: Serum of experimental rabbit 2, Lane C(i): Serum of control rabbit 1 and Lane C(ii): Serum of control rabbit 2.

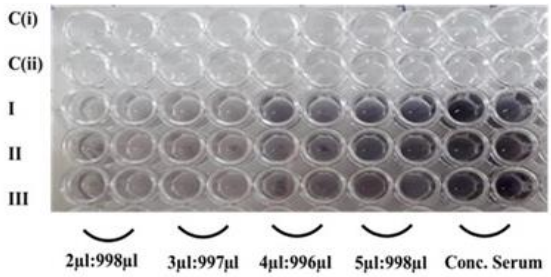

Fig. 5 (a): Confirmation of Antibody by ELISA in 96 well-plate with different dilutions of rabbit serum, purple color became darker when the conc. of serum increases (from left to right). Lane C(i): Serum of control rabbit 1 and Lane C(ii): Serum of control rabbit 2, Lane I: Serum of experimental rabbit 1, Lane II: Serum of experimental rabbit 2.

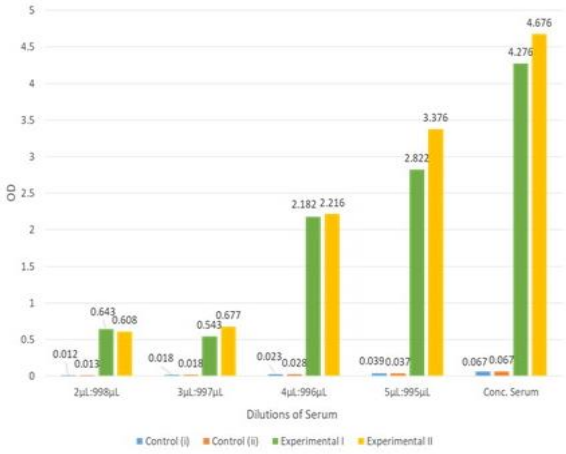

Fig. 5 (b): Graphical representation of optical density at 450 nm after ELISA.

DISCUSSION

NDV infection poses a serious threat to poultry industry and lead to huge economic losses all around the globe (Hameed *et al*., 2015). Fusion protein of NDV is main contributor in the virulence of virus (Nylund *et al.*, 2008; Kim *et al.*, 2013). The polyclonal antibodies are currently used in vaccines and diagnostic tests for infectious diseases (Kumar *et al.*, 2011). Current diagnostic tests are based on clinical sign symptoms, isolation of viral strains and serological testing. Clinical sign symptoms are important in diagnosis but this type of diagnosis could result into the confusion because many bacterial and viral diseases have same sign and symptoms (Tariq *et al.*, 2018; Abbas *et al.*, 2019). Differential diagnostic testing on the basis of virus isolation is very conventional method in ND diagnosis but it takes a lot of time. In serological testing antibodies are the key players in determining the sensitivity of the assay including the ease and efficacy like ELISA (Bello *et al.*, 2018). Polyclonal antibodies have applications in diagnostic and therapeutic because they possess properties like multi-epitope binding (Ascoli and Aggeler, 2018).

Antibody based vaccines are considered as an effective remedy against toxins, extracellular pathogens, viruses and control of intracellular pathogens. This paradigmatic shift has tremendous applications in immunology as well as vaccine design and development. For immunology the observation that antibody can protect against intracellular pathogens has led to the discovery of new mechanisms of antibody action. For vaccine design the humoral immunity can be effective in protection means that the knowledge acquired in more than a century of antibody studies can be applied to make new vaccines against the intracellular pathogens (Casadevall, 2018).

Keeping in view the fact about antibody and its relation to be used as vaccine in current study an attempt was made to generate polyclonal antibody against F protein of NDV as was done by Stills (Stills, 2012). The *Ecoli* expressed F protein of 67KDa was isolated by using method described by (Shahid *et al.*, 2015; Kang *et al*., 2016; Li *et al*., 2016; Batumalaie *et al*., 2018). The protein was verified by using antigen antibody reaction on nitrocellulose membrane and polystyrene plate as was described by Vera *et al*. (1999). Two group of rabbits acclimatized to be used for immunization each comprised of two rabbits namely control and experimental. The Experimental Rabbit were immunized with expressed protein along with complete and incomplete adjuvant as previously described (Leenaars *et al*., 1999; Vera-Cabrera *et al.*, 1999) for immunogenic response in Rabbit against NDV F protein. The immunogenic response generated in the form of polyclonal antibody as was described by Nutt *et al*. (2015). The experimental animal, the site of injection, amount of antigen and age of the animal are the important factors for *in vivo* antibody production (Leenaars *et al.*, 1999). The rabbits were scarified by following the ethical principles devised by institutional committee and blood was obtained as was done by Donovan and Brown (Donovan and Brown, 2006). A similar case of generating antibody against Sato NDV was reported by Putri *et al.* (2018) and also reported the followed procedure of antibody generation (Putri *et al.*,

2018). The serum obtained from the blood was subjected to antigen antibody reaction on Nitrocellulose membrane in the form of dots called dot blot immunoblot assay to determine the presence of antibodies produced as was reported (Folitse *et al.*, 1998). Quantification of antibodies produced was done through antibody antigen reaction on polystyrene plate called ELISA plate. Maximum antibody titer was estimated to be 4.676 in case of using concentrated antibody as was seen in Table 1, while minimum was obtained in 1/5 dilution that was 0.608 respectively. Similar types of results are also reported by Boysen *et al*. (2019) while studying mouse IgG. The current study is part of an effort to use these antibodies with gold nanoparticles for diagnostic of NDV infestation which otherwise is complex and expensive in country like Pakistan.

Author contributions: MZ: Performed experiment and assist in article writeup; AI: Supervised experiment; SS: Assist during experimentation; TRS: Performed experiment; NS: Designed experiment; AI, ST, SJA, MAA and SA: Assisted in experimentation; TH, AAS and AQR: Supervised experiment.

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