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Molecular Cloning and Expression of Nucleocapsid Gene of Chicken Infectious Bronchitis Virus Strain Massachusetts H120

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Abstract

Chicken infectious bronchitis virus (IBV) causes severe respiratory and renal dysfunction in chickens. In the present study, we used reverse transcription-polymerase chain reaction (RT-PCR) to amplify the nucleocapsid (N) protein gene of strain Massachusetts (Mass) H120 of IBV that is commonly used in the vaccine production in Iran. The PCR product with the expected size of 1.2 kb was cloned into a TA-vector and subsequently sub-cloned into expression vector pET-23a(+). The recombinant plasmids were transformed into competent BL21(DE3) strain of *E. Coli*. The recombinant N protein was expressed as a soluble fusion protein tagged at its C-terminal with 6 x His residues. In Western blot analysis, a convalescent serum from a chicken tested positive by a commercial IBV detection kit specifically reacted with the recombinant protein. The IBV negative chicken serum did not react with the recombinant protein. In a reciprocal experiment, antiserum raised against the purified fusion protein reacted with the corresponding proteins of strains H120 and 4/91. This is the first report of cloning and expression of the N protein gene cDNA of strain Mass H120 of IBV in a bacterial expression system. The potential uses of the cloned gene and the recombinant N protein have been discussed.

Keywords: Infectious bronchitis virus; Nucleocapsid; Expression

Introduction

Infectious bronchitis (IB) is an acute, highly contagious upper respiratory disease of chickens [1-3]. It has been well documented that chickens and commercially reared pheasants are the only natural hosts for IBV [4]. The major clinical signs include tracheal rales, sneezing and coughing. The disease causes a high mortality rate in young chickens when it is complicated with secondary bacterial infections such as *E. coli* and mycoplasma [1]. The disease may also involve kidney and oviducts [5]. When the reproductive tract is affected a drop in egg production and a decrease in egg quality are observed in the flock [6,7]. In Iran, the disease has been identified in chicken flocks with serological and virus isolation methods [8, 9]. Despite the wide use of live attenuated and inactivated vaccines to protect commercial chickens, IB still remains

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responsible for serious financial losses to the poultry industry of the country.

Infectious bronchitis virus (IBV) is the causative agent of IB [10]. The virus belongs to coronaviruses, a genus of the family Coronaviridae. Since the first report of the disease [2], many IBV serotypes have been identified [4]. Recently, it was demonstrated that there exist some newly emerging IBV isolates in Iran that can not be neutralized with the antiserum against the IBV Massachusetts strain [11,12]. Determination of the nucleotide sequence and restriction enzyme analysis of the spike protein gene have demonstrated that the new isolate circulating in Iran is a variant of strain 4/91 [11, Haqshenas *et al.*, submitted] that was reported in Europe for the first time [13,14]. The viral particles are enveloped with diameters ranging from 100 to 120 nm. The virion contains a large single stranded positive sense genomic RNA with a length of 27.6 kb [15]. The genome encodes three major structural proteins; the spike (S) glycoprotein (150-200 kDa), the integral membrane glycoprotein (M; 20-30 kDa) and the nucleocapsid phosphoprotein (N; 43-50 kDa) [16-18]. The N protein is an immunodominant antigen inducing cross-reactive antibodies in high titres whereas the S1 glycoprotein induces serotype-specific and cross-reactive antibodies [19].

In coronaviruses, N protein binds to the leader sequence of viral RNA through its RNA-binding domain [20,21]. It was recently shown that the amino and carboxyl domains of the IBV N protein interact with 3' genomic RNA [22]. During assembling of the structural viral proteins, N protein interacts further with M protein leading to the formation of virus particles [23]. N protein may also play a role in viral RNA synthesis [24].

In order to design a study to understand the molecular biology of IBV structural proteins, and to express the N protein, having N protein gene cloned into a plasmid vector will be a great asset. The cloned gene may be also used to generate recombinant N protein for the use in diagnostic kits [19,25]. Here, for the first time, we cloned the IBV N protein gene of the strain H120 and showed that the cloned gene can be used to generate recombinant N protein specifically reacting with IBV antiserum. Since the IBV vaccine strain used in vaccination of commercial broiler chicken flocks in Iran is strain H120, developing a detection assay based on a homologous recombinant protein would be valuable for the evaluation of vaccination. The results reported here provide the opportunity to work on the N protein gene and also to generate diagnostic methods based on the recombinant IBV N protein.

Materials and Methods

Bacteria and Viruses

IBV strain Mass H120 was kindly provided by Iranian Veterinary Organization (IVO). DH5α and BL21(DE3) strains of *E. Coli* were kindly provided by Dr. Alireza Zomorrodipour, NRCGEB, Iran. Isolate Shiraz3.IBV (a variant of strain 4/91) was recently molecularly characterized in our lab (Haqshenas *et al.*, submitted to Iranian Journal of Veterinary Research).

RNA Extraction

The lyophilized IBV vaccine containing 2000 doses was reconstituted in 1 ml sterile distilled water. Allantoic fluid from an embryonated chicken egg uninoculated with IBV (kindly provided by Dr. Assasi, Shiraz University) was used as control negative sample. The total RNA was extracted with RNx-plus reagent (Cinagen, Iran) from 100 µl of the reconstituted IBV vaccine. This method of RNA extraction is based on phenol-guanidinium thiocyanate method. The RNA pellet was then dried at room temperature and resuspended in 12 µl of diethyl pyrocarbonate (DEPC)-treated deionized water. The extracted RNA was immediately used in reverse transcription reaction to generate the N protein gene cDNA.

cDNA Synthesis

To generate the N protein gene cDNA, we followed the method described earlier [26,27]. Using the N protein gene nucleotide sequence of strain H120 of IBV available in GenBank (accession no. AY028296), antisense primer (RIBV) was designed with OLIGO™ version 5.0 software (Cambio, UK). The primer sequence is available upon request from the authors. The primer was designed in-frame with the C-terminal His-tag sequence downstream of the polylinker site of the expression pET-23a(+) vector (Novagen, USA). To facilitate cloning of the final PCR product into the expression vector, a restriction site for *Xho*I was engineered in the primer sequence. The obtained RNA from the previous step was denatured at 65ºC for 3 min. RT was performed at 42ºC for 60 min with 1 µl of specific antisense primer (10 µM), 1 µl of AMV-reverse transcriptase (25IU/µl,; Cinagen, Iran), 1 µl of RNase inhibitor (40IU/µl; Cinagen, Iran), 4 µl of 5 x RT buffer, and 1 µl of 2.5 mM dNTPs. Finally, the reverse transcriptase was inactivated at 70°C for 10 min.

PCR

The primers for PCR were designed with OLIGO™ version 5.0 software. The primer RIBV used in the cDNA synthesis reaction was used as antisense primer

in the PCR. The *Nde*I restriction site was engineered in the sense primer (FIBV) sequence to facilitate cloning of the PCR product into the expression vector. PCR was performed on 10 µl of the RT reaction mixture containing 2 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM each sense and antisense primers and 2.5 IU Taq DNA polymerase (Cinagen) in a 50 µl reaction volume. The PCR thermal cycles, performed in a Corbett thermal cycler (Australia), included an initial incubation at 94°C for 4 min to separate RNA/DNA hybrids. This initial cycle followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 52°C for 40 sec, and extension at 72°C for 110 sec, with a final incubation at 72°C for 10 min. The resulting PCR products were analyzed on an 1% agarose gel and stained with ethidium bromide.

PCR Product Cloning

The amplified PCR products were purified from the agarose gel with High Pure PCR Product Purification kit (Roche Diagnostics GmbH, Germany) and ligated into plasmid pTZ57MCS (MBI Fermentas, UK) that contains one overhung "T" at its 3' ends to facilitate cloning a PCR product containing an "A" at its 3′ ends. Cloning of the PCR product into the vector was performed with T4 DNA ligase (Roche Diagnostics GmbH, Germany) by standard procedures [28]. The recombinant plasmids were transformed into competent DH5a cells prepared by calcium chloride method [28]. White colonies were selected and grown in LB broth supplemented with 100 µg/ml ampicillin. The cells carrying plasmids with the inserts were detected with PCR using primers FIBV and RIBV. The plasmids from positive cells were extracted and digested with *Nde*I (MBI Fermentas) and *Xho*I (MBI Fermentas). The resulting DNA fragments were separated on an 1% agarose gel. The DNA band with an approximate size of 1.2 kb was excised from the gel and purified as described above. Subsequently, the purified DNA was directionally cloned into expression vector pET-23a(+) (Novagen, USA) in which the target gene expression is driven by a T7 promoter upstream of the polylinker site. Finally, the construct, designated as pET-23a-NP, was transformed into competent *E. coli* strains DH5a and BL21(DE3). The latter has been engineered to produce T7 RNA polymerase. The cells harboring plasmid pET-23a-NP were detected with PCR and restriction enzyme analysis as described above.

Expression and Purification of the Recombinant Protein

The method that we followed to express the protein was basically as described earlier [29]. To determine the best time-point in which the maximum yield of protein is obtained, *E. coli* strain BL21(DE3) harboring the recombinant plasmids were grown in LB supplemented with 50 µg/ml ampicillin. After addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM to the culture, samples were collected at time-points of 0, 2, 3, 4, and 5 h. One culture was also induced with IPTG overnight at 30°C. Bacterial cells harboring a recombinant pET23a(+) plasmid expressing VP1 gene cDNA of foot and mouth disease virus (FMDV) was used as the control negative for our insert as well as a control to check our expression conditions. The crude bacterial lysates were separated on a 12% polyacrylamide gel containing 0.1% SDS and stained with Coomassie blue [30]. To determine the solubility of the expressed protein, 0.1 ml of overnight culture of the bacteria containing the recombinant plasmids were added to 10 ml LB supplemented with 50 µg/ml ampicillin. When the culture reached an OD/A600 of about 0.6, IPTG was added to a final concentration of 1 mM. The culture was incubated overnight in a shaker with a speed of 150 rpm at 30°C. The cells were harvested at 4000*g* for 10 min and the resultant pellet was resuspended in 1 ml of lysis buffer (50 mM NaH2PO4, 300 mM NaCl; pH 8.0). The cells were sonicated for 2 min (10 sec pulses with 1 min intervals) and the mixture was clarified by centrifugation at 10,000*g* for 30 min. The supernatant was recovered and stored at -70°C. The pellet was resuspended in 1 ml of phosphate-buffered saline (PBS, pH 7.4). Samples from the supernatant and the pellet suspension were analyzed SDS-PAGE as described above. The gel was then stained with Coomassie blue.

To preliminary purify the recombinant protein, the induced cells were lysed and the debris was pelleted as described above. To prevent non-specific binding of cellular proteins to the nickel, 10 mM imidazole was included in the lysis buffer. To 1 ml of the supernatant, 50 µl Ni^{2+}-NTA (Qiagen, USA) were added. After 10 min binding and one washing step with lysis buffer containing 20 mM imidazole, the protein was eluted in 200 µl of elution buffer (lysis buffer plus 250 mM imidazole) and 10 µl of the eluate were analyzed on a 12% polyacrylamide gel.

Preparation of Antibody against the Recombinant N Protein

To prepare antibody against the recombinant N protein of IBV, a 2-month-old New Zealand rabbit was subcutaneously inoculated with 200 µg of the recombinant protein thoroughly mixed with complete

Freund's adjuvant at day 0. The second dose (200 μg of the recombinant protein) was mixed with incomplete Freund's adjuvant and inoculated as described above. Two weeks after the second dose, the third dose was intravenously injected and the serum was examined for antibody against the N protein a week after the last inoculation. The animal was anesthetized and bled to death. The clotted blood was centrifuged at 4000 rpm for 10 min. The serum (45 ml) was recovered, aliquoted and stored at −20ºC.

Immunoblot Analysis

Positive and negative convalescent sera, tested with IDEXX IBV commercial kit (IDEXX, Westbrook, ME), were used to confirm the recombinant N protein. Following SDS-PAGE of the crude extract of the cells expressing the fusion N protein, the cells expressing VP1 gene of FMDV (as negative control), the purified recombinant N protein and vaccine strain IBV, the protein bands were trans-blotted onto a PVDF Western Blotting Membrane (Roche Diagnostics GmbH, USA) and blocked with 5% skimmed milk in TBS (10 mM Tris.HCl, pH 7.5; 10 mM NaCl) containing 5% normal rabbit serum for 1 h. The serum samples were diluted 1/500 in the blocking solution. The membranes were incubated with diluted positive and negative samples for 3 h. After washing with TBS-Tween buffer (0.05% Tween 20 in TBS), the membranes were incubated with a 1/2000 dilution of HRP-conjugated rabbit anti-chicken IgY (Sigma) at room temperature for 3 h. The strips were washed again as described and the immunocomplexes were detected using 4-chloro-1-naphthol (Merck, Germany) plus 0.01% H_2O_2 in 20% methanol. In a reciprocal Western blot experiment, the antiserum raised against the purified recombinant protein was used against the purified protein, the vaccine strain of IBV and isolate Shiraz3.IBV basically as described above.

Results

RT-PCR Amplification and Gene Cloning

Using RNx-plus reagent, total RNA was extracted from reconstituted IBV vaccine as well as from mock-infected allantoic fluid samples. To synthesize N protein gene cDNA, AMV-reverse transcriptase with optimal temperature of 42°C was used to overcome any possible secondary structure formation in the genomic RNA. Analysis of the PCR product on agarose gel revealed a very sharp band with an approximate size of 1.2 kb (Fig. 1). PCR amplification on the mock-infected sample did not generate any visible PCR product band. Nucleotide sequence analysis with the DNASTAR software (GATC Biotech AG, Germany) revealed no restriction sites for *Nde*I and *Xho*I in the N protein gene sequence. Therefore, these two restriction sites were engineered in the primers' sequences. Digestion of the PCR product with *Nde*I and *Xho*I further confirmed the absence of these two restriction sites in the N protein gene (data not shown). The PCR product was cloned into the TA vector. The recombinant plasmids were amplified in the bacterial cells. The insert was released from the purified recombinant plasmids with *Nde*I and *Xho*I and the insert was subsequently sub-cloned into expression vector pET-23a(+). The recombinant plasmids were amplified, purified, restriction digested (Fig. 1) and the nucleotide sequence of the entire insert was determined with automated cycle sequencing. Excluding the primer sequences, 6 nucleotide mutations were observed when we pairwise aligned the obtained sequence with the N gene sequence available in GenBank (Accession No. AY028296) (data not shown). Of these 6 mutations only one (position 928, G to A) caused substitution of an aspartic acid residue by an asparagine residue (position 310 in the depicted amino acid sequence).

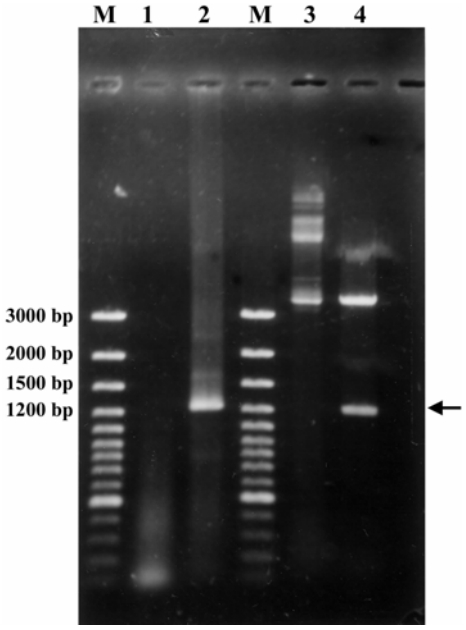

Figure 1. A: RT-PCR amplification and cloning of the N protein gene of vaccine strain of IBV into pET-23a(+). The PCR product has an approximate size of 1.2 kb. 1. mock-infected allantoic fluid, 2. Vaccine strain H120 of IBV. The PCR product was cloned into a pET-23a(+) expression vector (with an approximate size of 3.6 kb). 4. Undigested recombinant plasmid. 5. Recombinant plasmid digested with *Nde*I and *Xho*I restriction enzymes (4). The N gene has been pointed out by an arrow. GeneRulerTM 100bp ladder mix (Fermentas, UK) was used as DNA size marker (M)

Protein Expression and Immunoblot Analysis

Computer analysis revealed that the fusion recombinant protein should have a molecular weight of about 46.5 kD (data not shown). Following transformation of the competent BL21(DE3) cells with the plasmid pET-23a-NP, expression of the N protein was induced with IPTG. Compared to the uninduced cells and the cells expressing VP1 of FMDV, two induced protein bands with approximate sizes of 50 kD and 43 kD were observed on the gel. When the samples collected from the IPTG-induced culture at various time points were examined on SDS-PAGE, a relative increase in the amount of induced protein bands was observed with time up to 5 h post-induction (Fig. 2). We also obtained a good yield of protein when the culture was incubated overnight at 30°C. Scanning of the gel showed that the samples taken at the time-points 5hrs and overnight contained the highest relative amount of the recombinant protein, about 61% and 59%, respectively (data not shown). The expressed recombinant fusion protein was subjected to Western blot analysis using a chicken convalescent serum tested positive with the IDEXX IBV commercial IBV detection kit. This antiserum reacted with the over-expressed protein bands (Fig. 3B). Negative control serum did not generate any signal at corresponding positions to our two bands of interest while it generated a few other signals in the lanes of crude lysates of positive and negative control lysates (Fig. 3A). Since most of chickens are positive for anti-*E. coli* antibody, these signals were likely related to the specific binding of *E. coli* proteins to the antibody against *E. coli* proteins. To confirm the results we performed this test with at least three positive and negative serum samples. This experiment verified that both protein bands were in fact related to IBV and the purified protein was highly pure. Further characterization of the recombinant protein demonstrated that the protein was mainly soluble in water (Fig. 2) as it was expected from computer analysis (data not shown). When the recombinant protein was preliminary purified, the protein with the approximate size of 50 kD remained as the major protein in the preparation (Fig. 2). This experiment also confirmed that the recombinant protein was expressed as a fusion protein tagged with 6 histidine residues. In the Western blot, performed for further confirmation of the protein, the antiserum raised in rabbit against the purified recombinant protein reacted with a same protein in the IBV vaccine as IBV-positive chicken antiserum did (Fig. 3B). Moreover, this antiserum reacted well with a protein of isolate Shiraz3.IBV banded at the same size position as the recombinant N protein. IBV Pre-inoculation rabbit serum did not generate any signal on the membrane.

Discussion

In the present study we amplified, cloned and expressed the N protein gene of the strain H120 of IBV. This is the first report of cloning and expressing of the N protein of this IBV strain that is regularly used in vaccine production in Iran.

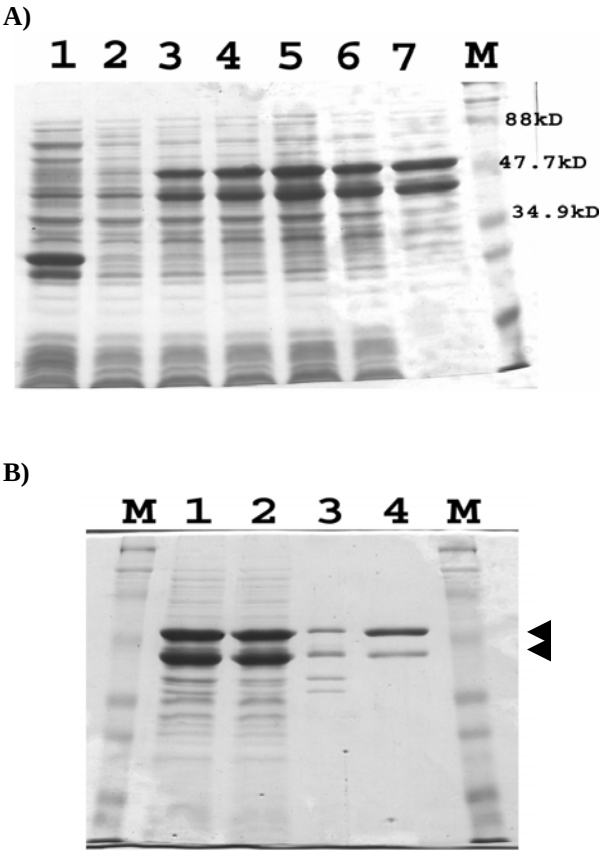

Figure 2. **A**: Expression of the N protein of the vaccine strain of IBV in BL21(DE3) cells. Following IPTG induction samples were taken at different time points of 0 (1), 2 (3), 3 (4), 4 (5), 5 (6) h at 37°C and overnight (7) at 30°C. A bacterial sample carrying the same plasmid expressing VP1 of FMDV (about 27 kd) was used as our control negative (1). **B**: Determination of the solubility of the protein and purification of the protein,1: lysate, 2: supernatant, 3: pellet, 4:purified protein. M. A pre-stained SDS-PAGE standards (Bio-Rad, CA) was use as the protein size marker. The protein samples were analyzed onto a 12% SDS-PAGE and the gel was stain with Coomassie blue stain. The interest bands have been pointed out by arrow heads.

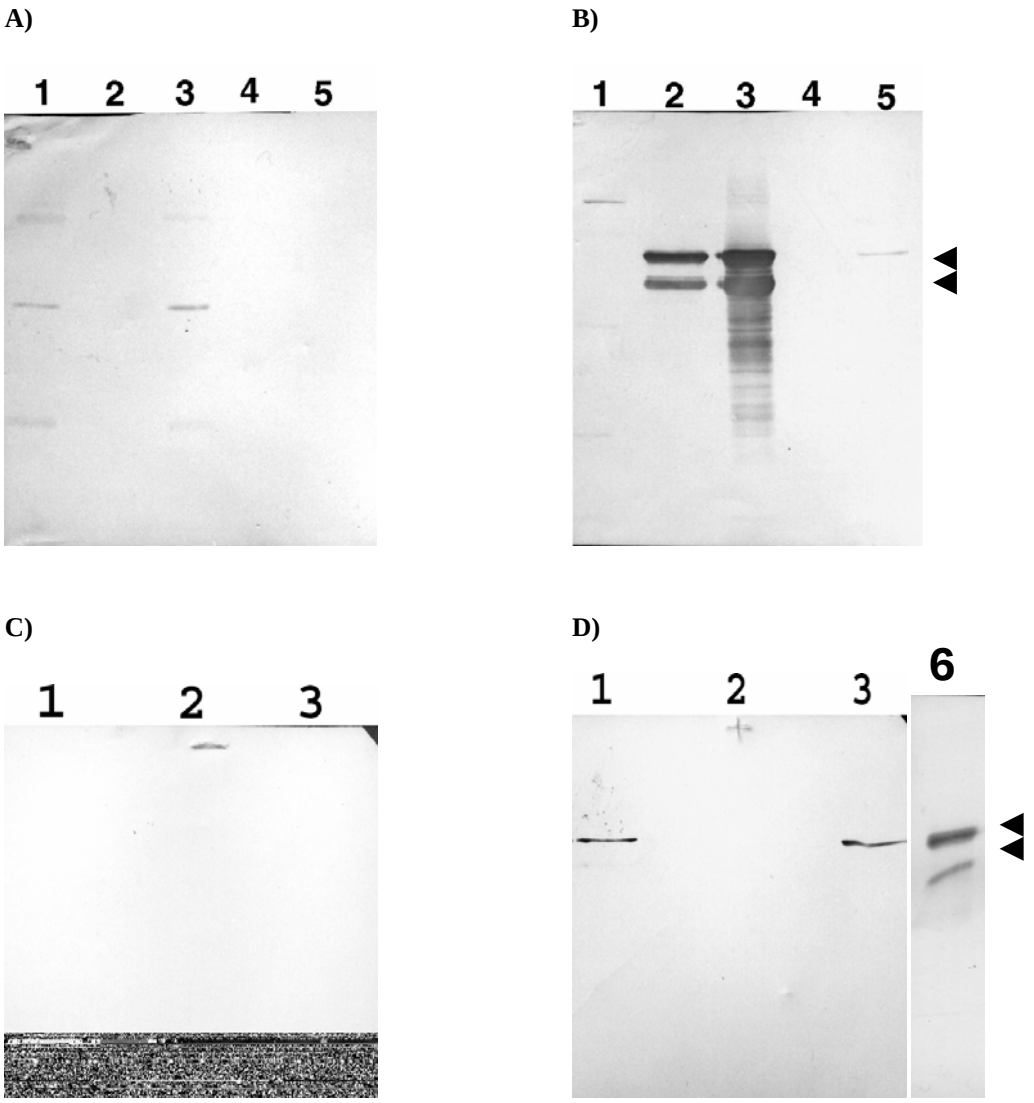

Figure 3. Western blot analysis of the recombinant fusion protein. IPTG-Induced bacterial cells containing pET-23 with a FMDV VP1 gene cDNA insert (A1,B1), purified (A2,B2,C1,D1) and unpurified (A3,B3) recombinant fusion N protein (2), mock-infected allantoic fluid (A4,B4,C2,D2)and allantoic fluid inoculated with the IBV vaccine strain (A5,B5,C3,D3) and allantoic fluid inoculated with isolate Shiraz3.IBV (D6) were analyzed with SDS-PAGE and transferred onto a PVDF membrane. A and B membranes were either reacted with IBV-negative (A) or IBV-positive (B) chicken sera. C and D membranes were either reacted with pre-inoculation (C) or hyper-immune rabbit anti-recombinant protein (D) sera. The bands of interest have been pointed out by arrow heads.

Like other countries, vaccination with live attenuated and inactivated virus is performed in Iran to protect layer and broiler chicken flocks. However, the disease has remained as a serious problem for poultry industry in the country and causes significant financial losses for the poultry farmers. In fact, in all over the world chicken infectious bronchitis has a significant economic impact in broilers due to production losses (poor weight gains, condemnation at processing and mortality) and in laying birds, due to suboptimal egg production and downgrading of eggs [4].

In our study, the recombinant N protein appeared as two over expressed protein bands on SDS-PAGE as well as in Western blot hybridization. Reaction of the two protein bands using IBV positive serum confirmed that the both bands contained proteins related to IBV. Appearance of our recombinant fusion protein as two protein bands was not surprising since Zhou *et al.* (1996) had the same results when they expressed the N protein of the Gray strain of IBV in a bacterial

expression system [31]. They speculated that the smaller proteins originated either from proteolysis or premature termination of translation. Moreover, the N protein of isolate Shiraz3.IBV (when it was used in high amounts) also appeared in the Western blot analysis as two bands with the same approximate sizes of the recombinant protein bands. Therefore, we believe that the smaller protein band is, in fact, the truncated form of the N protein.

To further confirm that the over-expressed protein is the recombinant N protein of IBV, we raised antiserum against the recombinant N protein and demonstrated that it well reacted with the N proteins of IBV vaccine strain and isolate Shiraz3.IBV as a variant of strain 4/91. This experiment also revealed that N proteins of strain 4/91 and vaccine strain contain common antigenic epitopes.

The results of the present study are valuable tools to develop a detection kit as well as to construct chimer viruses carrying the N protein of IBV. Originally, it was believed that S1 protein is the only protein responsible for the induction of a protective immunity against virulent IBV strains [32,33] and N protein does not play any role in the induction of immune responses of chickens to protect them against infection [19]. Recently, it was shown that the inoculation of chickens with a recombinant fowl poxvirus expressing C-terminal N protein protected the chickens against challenge [34]. Thus, the N protein gene that we cloned is now available to construct a chimer virus expressing a protein identical to the N protein of vaccine strain of H120.

In order to monitor the response of chickens to the IBV vaccination, determination of the obtained antibody titer is routinely used by veterinary laboratories. To determine the antibody titer against IBV, a number of serological tests based on whole virus [35], S1 protein [36], and recombinant N protein [25] have been reported. Since the N protein shares 94-99% identity among different strains [37,38] our recombinant protein is a good candidate to be used in diagnostic tests to evaluate a vaccine trial (as a homologous protein) as well as a field IBV infection (as a heterologous protein). One means to design a detection assay based on a protein homologous to the IBV vaccine strain is to use purified viral particles [35]. Virus purification is a very expensive procedure and requires expertise and sophisticated facilities. The recombinant protein which we expressed in this study is one of the highly immunogenic IBV structural proteins [25] and consists the most identical amino acid sequence to the vaccine strain of H120. On the other hand, in this paper we have shown that the N protein of vaccine strain and isolate Shiraz3.IBV (a variant of strain 4/91) are closely antigenically related. Therefore, we believe that our recombinant protein can be used in diagnostic tests to detect chicken flocks infected with Shiraz3.IBV isolate.

By using IBV negative antiserum we showed that the His-tag located at the C-terminal of the fusion protein does not bind non-specifically to chicken IgY as we demonstrated this fact elsewhere [39,29].

In conclusion, we believe that a serological detection assay based on our recombinant protein would be one of the best choices to evaluate a vaccination trial against IBV and to sero-diagnose the diseased chickens. We are currently in process of optimizing a dot-blot assay and an ELISA based on the recombinant N protein in our laboratory.

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