

## Original Research Article

# Production of a phage-displayed single chain variable fragment antibody against infectious bursal disease virus

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Sent for review: 1 September 2017

Revised accepted: 27 November 2017

### Abstract

**Purpose:** To develop specific single chain variable fragments (scFv) against infectious bursal disease virus (IBDV) via phage display technology.

**Methods:** Purified viruses were initially applied for iterative panning rounds of scFv phage display libraries. The binding ability of the selected scFv antibody fragments against the IBDV particles was analyzed by indirect enzyme-linked immunosorbent assay (ELISA) followed by blotting assays. Three-dimensional (3D) structure of the selected scFv antibody fragment and VP3 protein were predicted through *in silico* analysis. Structural characterization of the antibody-antigen complexes was carried out by computational docking analysis.

**Results:** The serological results obtained from the ELISA and blotting analysis showed that the selected clones produced specific scFv antibody fragments that were capable of effectively detecting infectious bursal disease (IBD) in the infected animal tissue. Biodiversity analysis by BstNI finger printing and nucleotide sequencing revealed that there was no major difference in nucleotide sequences of the selected clones. Further analysis demonstrated that this recombinant fragment of the antibody was able to bind to VP3 structural protein of IBDV with a molecular weight of ~30 kDa. Molecular docking results revealed that the binding energy of scFv to IBDV-VP3 was 545 kJ/mol.

**Conclusion:** The developed scFv antibody fragments possess great potentials for the diagnosis of IBD. The findings of the present study confirm the feasibility of using phage display technology for rapid production of antibodies against IBD diseases by applying naïve scFv libraries.

**Keywords:** Antibody, Molecular docking, Phage display technology, Single chain variable fragments

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## INTRODUCTION

Infectious bursal disease (IBD), commonly known as *gumboro*, is one of the most important

viral diseases in the poultry industry throughout the world [1]. The economic losses associated with IBD are caused by both increased rate of mortality and the immunosuppressive effects of

the disease on infected animals. The disease is caused by infectious bursal disease virus (IBDV), a double stranded (ds) RNA virus belonging to the genus *Avibirnavirus* under the family Birnaviridae. The virus is normally present within the bursa of Fabricius as well as other organs such as skeletal muscle, intestines, kidney, and spleen. Two serotypes of the virus have been isolated from chickens; serotype I is pathogenic with high antigenicity whereas serotype II is not pathogenic and is not followed by any clinical signs or immunosuppression [2].

*Gumboro* has no treatments or drugs and therefore, it is important to try to prevent the disease by observing strict hygienic and vaccination programs [3]. Nevertheless, application of vitamins as well as antibiotics can prevent subsequent infections [4,5].

Since *Gumboro* has been reported all around the world and that it spreads rapidly, constant monitoring and early detection of the disease is of crucial importance. This could be achieved based on clinical symptoms, flock history, and necropsy approaches. However, accurate detection can only be accomplished by applying diagnostic methods such as *in situ* hybridization, immunofluorescence, immunohistochemistry, RT-PCR, and enzyme-linked immunosorbent assay (ELISA) [2].

Nowadays, poly- and monoclonal antibodies play a major role in the detection of many kinds of diseases. Monoclonal antibodies have several advantages over their polyclonal counterparts including higher specificity and more accuracy. However, the production of monoclonal antibodies is usually more time-consuming and complicated compared with polyclonal antibodies. Recombinant antibody technology is an efficient approach for the production of target-specific monoclonal antibodies through the application of the phage display technology [6]. This technology has been employed for developing several monoclonal recombinant antibodies against a wide range of antigens [7-12]. Therefore, the present study was aimed at developing an IBDV-specific monoclonal recombinant antibody from the Tomlinson I antibody library by using the phage display technique.

## EXPERIMENTAL

### Source of virus

The virus isolate used in this study was a commercial IBDV vaccine provided by the Razi Vaccine and Serum Research Institute (RVSRI)

(Arak, Iran). This vaccine was an attenuated form of an Iranian isolate of IBDV emulsified in montanide oil. The pre-confirmed IBDV-infected (as positive control) and healthy (as negative control) chicken bursa tissues were also prepared and provided by the RVSRI.

### Panning of phage display libraries

Specific scFv antibody fragments were isolated from naïve Tomlinson I scFv phage display libraries by applying the IBDV vaccine as described earlier [13]. IBDV particles were immobilized onto immunotubes (Nunc-Maxisorp, Denmark) followed by blocking with 2 % (v/v) skimmed milk and incubation with the phage suspension ( $\sim 10^{13}$  CFU). The bound phage particles were eluted from the surface of the immunotubes by adding 1 ml 100 mM triethylamine followed by adding 0.5 ml 1 M Tris, pH = 7.5. The eluted phage particles were then amplified in exponentially-growing *E. coli* TG1 and were purified by PEG precipitation. The panning step was repeated three times. After the third round of panning, individual clones expressing phages or soluble scFv antibody fragments were randomly selected and tested for binding specificity against IBDV by ELISA.

### Mini-induction of scFv

For low scale production of soluble scFv antibody fragment, after the third round of panning, ninety six colonies (HB2151 non-suppressor strain of *E. coli*) were randomly selected and cultured in a 96-well plate. Expression of scFv gene was initiated by addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG) to a final concentration of 0.5 mM followed by incubation at 30 °C overnight. The cells were isolated by centrifugation and supernatants were used for subsequent assays.

### Binding activity of scFv against

The specificity of produced scFv antibody fragments was analyzed by ELISA as described earlier [14]. Around 100  $\mu$ l of IBDV vaccine (50  $\mu$ g/mL) diluted in 1x PBS was loaded on ELISA plates (Maxisorp, Nunc, Denmark). Blocking step was performed by adding same amount of 1xPBS containing 2 % Marvel (2 % MPBS). About 100  $\mu$ L of expressed scFv antibody fragment were added into the wells and plates were incubated for 2 h at 37 °C. The specific binders were detected by using 100  $\mu$ l of 1:1000 diluted mouse anti-c-myc monoclonal antibody 9E10 followed by adding same amount of 1:10000 diluted horseradish peroxidase (HRP) conjugated to goat-anti-mouse polyclonal

antibodies. 100 $\mu$ l of ABTS substrate (2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)) was used for colour development at room temperature for 30 minutes. After that, the absorbance values at 405 nm (OD<sub>405 nm</sub>) was measured using a micro plate reader (ELx808 Absorbance Microplate Reader, Winooski, VT, USA). Colonies expressing scFv fragments with high binding ability against IBDV particles were selected and subjected to further analysis.

### Western blotting

For Western blotting, protein bands were initially separated on SDS-PAGE and transferred to PVDF nitrocellulose membrane. Blocking of membrane was performed with 1xPBS containing 2 % Marvel (2 % MPBS). The bacterially-expressed scFv fragment was used for detection. The banding of scFv antibody fragment to protein was revealed by 1:1000 diluted anti-*c-myc* 9E10 monoclonal antibody followed by 1:10000 diluted goat-antimouse IgG conjugated to alkaline phosphatase (GAM<sup>AP</sup>). Binding of antibody molecules to antigen was visualized by using a substrate containing nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

### Homology modeling

Three-dimensional (3D) structure of the IBDV (PDB ID: 2r18A, RCSB database) was retrieved. Tertiary structure of the anti-IBDV scFv was modeled by subjecting its amino acid sequences to the I-TASSER (<https://zhanglab.cmb.med.umich.edu/I-TASSER/>). This internet based server generates 3D models by collecting highest scoring and ranking of template models based on the target model amino acid sequence. The confidence score (C-score) used to estimate the quality of the I-TASSER-developed models is typically in a range of -5 to +2. Higher C-score values reveal the quality of a given model.

### Molecular docking

In order to investigate the mode of interaction between the IBDV 3D and the anti-IBDV scFv model, molecular docking was performed by BioLuminate module of the Schrodinger suite (version 1.9, Schrodinger, LLC, New York, NY). The possible interactions were analyzed using the Pymol software (The PyMOL Molecular Graphics System, version 1.3r1 edu, Schrodinger, LLC). The final model was selected based on the cluster size as described in the results session.

## RESULTS

### Phage display scFv selection

To obtain specific scFv recombinant antibody, iterative round of panning were performed by using around 10<sup>13</sup> cfu of recombinant phages. Upon the completion of every round of panning, the population of the eluted phages was evaluated. Table 1 tabulates the enrichment of IBDV-specific scFv antibody fragments achieved through the panning processes. A rising titer of the eluted phages throughout the panning rounds shows that the number and the specificity of the eluted phages were increased consequently.

**Table 1:** Enrichment of specific scFv antibody fragments to IBDV through three rounds screening of Tomlinson phage display library

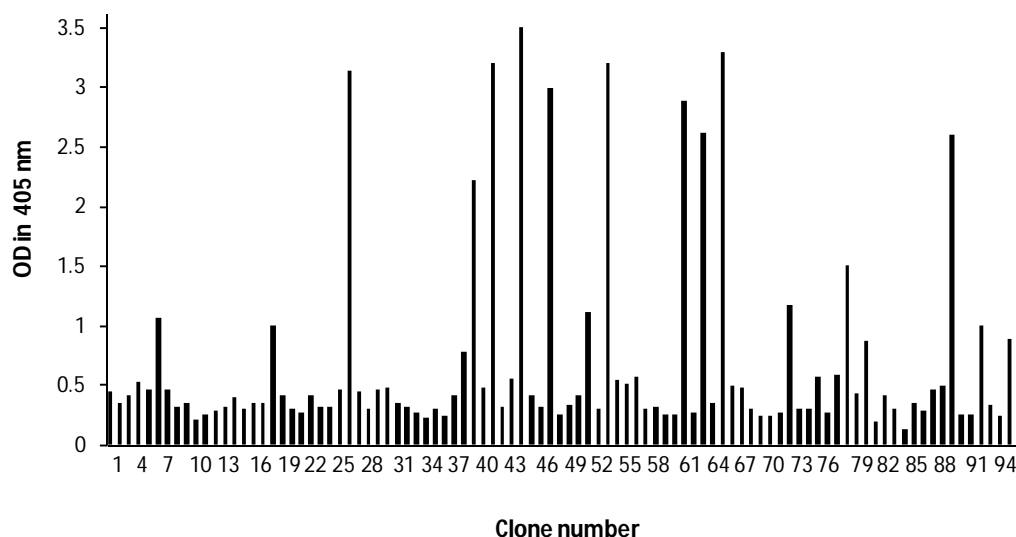
Round of panning	Input phage	Output phage
1	10 <sup>13</sup>	1.5 x 10 <sup>6</sup>
2	10 <sup>13</sup>	2.5 x 10 <sup>7</sup>
3	10 <sup>13</sup>	3.7 x 10 <sup>8</sup>

After performing panning procedure, around 96 colonies were randomly selected and expressed in bacterial host. Indirect ELISA was performed for analysis binding activity of expressed scFv fragments against IBDV. These results show that around 10 % of selected clones produce scFv antibody fragments with positive activity against the IBDV particles (Figure 1). These positive clones were then used for further analysis.

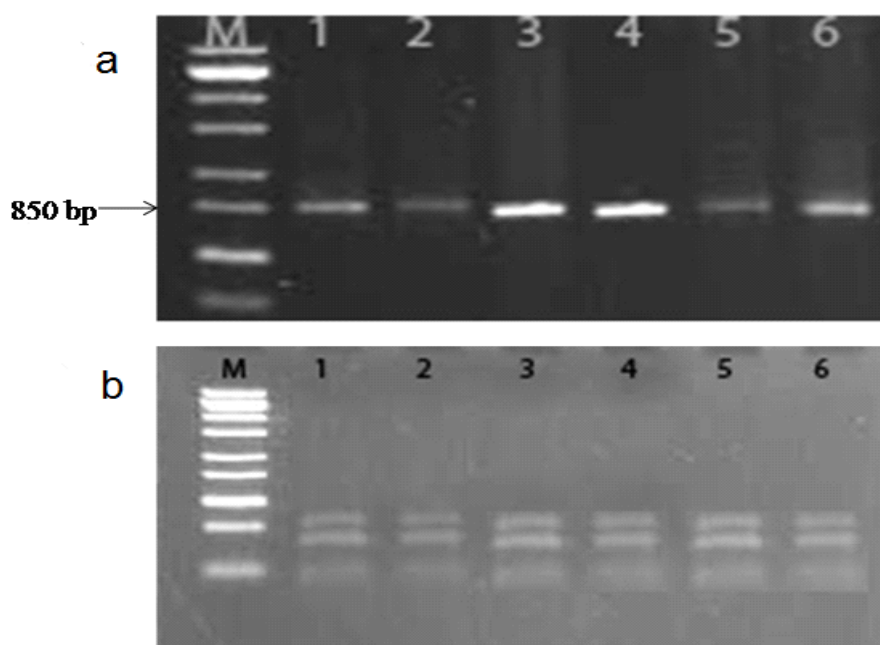
### PCR results and *Bst*NI fingerprint of individual phage clones

To determine the variability of the selected clones, the scFv genes were PCR amplified by using specific pHEN primers and were then subjected to *Bst*NI fingerprinting. All the analyzed clones showed an insert of about 850 bp (Figure 2A). Subsequent digestion patterns revealed only one type of band present in all samples (Figure 2B). These results proved the high similarity of the selected scFv genes.

Furthermore, the sequencing results confirmed that there was no major difference in the nucleotide sequences of the selected clones. Three complementary determining regions (CDRs) and 4 framework (FW) regions were identified in each VH and VL fragments. The selected scFv antibody fragments had open reading frames encoding proteins with a molecular weight of 28 kDa. The FW and CDRs were determined according to the IMGT databases ([http://imgt.cines.fr/IMGT\\_vquest/](http://imgt.cines.fr/IMGT_vquest/)).



**Figure 1:** Indirect ELISA analysis for screening of the randomly selected clones from the Tomlinson I scFv library. After the third round of panning, the scFv antibody fragments expressed from selected clones was analyzed for binding activity against IBDV. The IBDV vaccine was coated on microtitre plates followed by blocking by 2% MPBS. The specific scFvs were detected by using 9E10 monoclonal antibody, and goat anti-mouse polyclonal antibody conjugated to horse radish peroxidase (GAM<sup>HRP</sup>) as secondary antibody. After 30 minutes the absorbance values at 405 nm (OD<sub>405 nm</sub>) was measured using a micro plate reader (ELx808 Absorbance Microplate Reader, Winooski, VT, USA)



**Figure 2:** (a) Analysis of the amplified fragment through PCR analysis of the IBDV binder clones from Tomlinson I phage display library. The PCR products were separated on a 1 % (w/v) agarose gel. 1:1Kb ladder 1-9: patterns of 6 selected clones reacting to IBDV. (b) Finger printing analysis of the IBDV binder clones selected from Tomlinson I phage display library. The genes encoding scFv fragments were initially amplified by using specific pHEN primers and amplified fragments subjected to *Bst*NI digestion. Samples were analyzed on a 2 % (w/v) agarose gel. 1-6: RFLP patterns of 6 selected clones, M: DNA ladder

### Serological analysis

To determine the specificity of the prepared scFv antibody fragments against the IBDV-infected chickens, the soluble scFv recombinant antibody expressed from the selected clones (scFv-c2,

scFv-E5, scFv-F1, scFv-F3 and scFv-F5) were further analyzed by the ELISA and Western blotting. The IBDV-infected chicken bursa was used as positive control. The ELISA results revealed that all selected scFv fragment antibodies has a high reactivity against IBDV

particles present in the vaccine and in the infected bursa (Figure 3). There was no cross reaction with the extract of healthy bursa nor with the BSA protein as negative control.

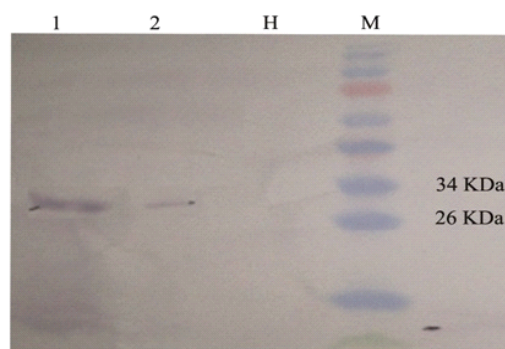
Complementary Western blot analysis re-confirmed the specificity of the selected scFv clone (scFv-c2) against IBDV (Figure 4). This finding revealed the presence of a specific antigen with a molecular weight of around 30kDa, strongly bound to the scFv monoclonal antibody. Furthermore, the positive response obtained in the Western blot analysis indicated that the corresponding epitope is a continuous type presented in both the vaccine and the native protein.

### Homology model building and validation

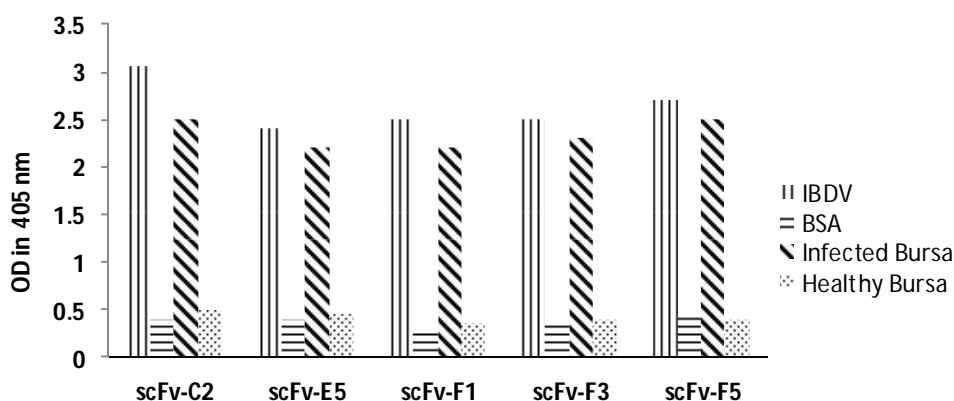
To predict the 3-D structure of the anti-IBDV scFv, the cognate amino acid sequence was applied to the I-TASSER server. The PDB file 5F72T was selected as the best template model based on the highest similarity (in the case of 5F72: 79 % identity) and C-score (in the case of 5F72T: 0.3) and least RMSD value (in the case of 5F72:  $5.9 \pm 3.7$  Å) (Figure 5A). The quality of the refined model was evaluated by a psi/phi Ramachandran plot using BioLuminate. Figure 5B shows that about 92 % of the amino acid residues were in the most favored regions and only 2 % were in the disallowed regions. Due to the lack of appropriate crystal structure in the (Glycine<sub>4</sub>Serine)<sub>3</sub> linker, the unfavorable residues were mainly located in this region of the model. These results indicated that the scFv model was suitable for the molecular docking study.

### Docking of IBDV with scFv

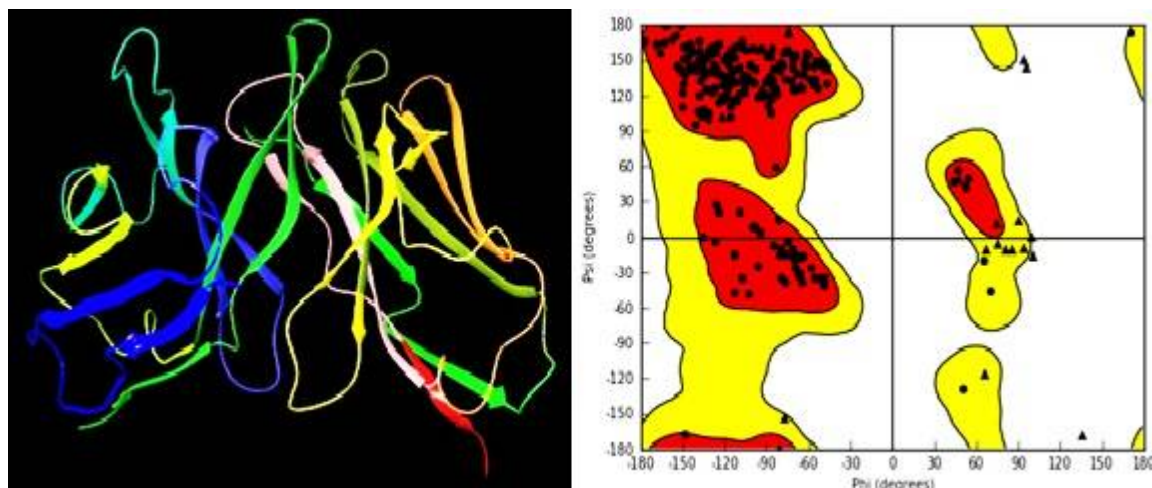
To visualize the interaction of the scFv with IBDV, the most stable form of the antigen, 2r18A model, was docked onto 5F72T scFv model using BioLuminate program. The suggested model with the lowest energy docking (among 30 models) was selected. According to the docking results, the binding energy of the scFv was estimated to be at  $-545$  kJ/mole when bonded to IBDV. The final configuration of the docking complex of the scFv-IBDV is shown in Figure 6 revealing the binding of the IBDV molecule to the gap between the VL and VH domains of the scFv.



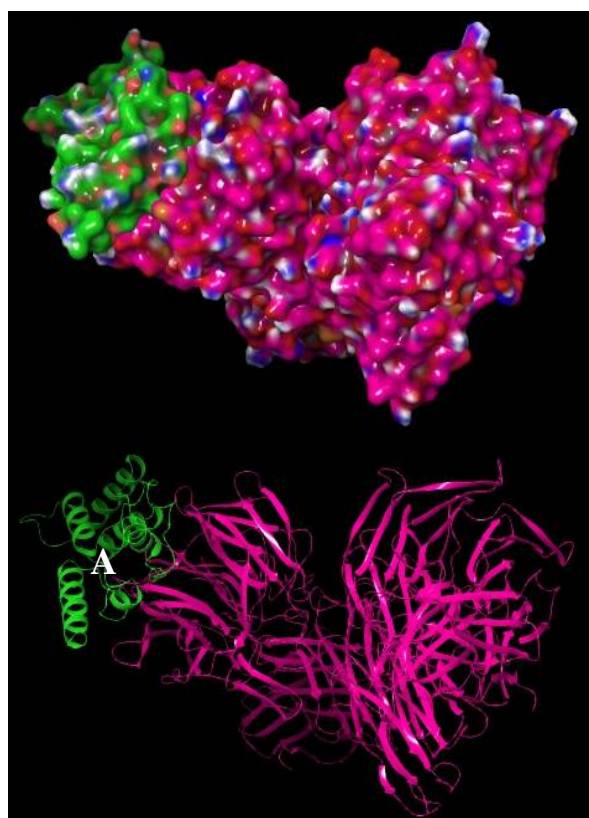
**Figure 4:** Western blot analysis of the bacterially-expressed scFv-c2 against IBDV-infected animal tissue. The purified IBDV vaccine and the extracts of the infected and healthy bursa were separated on a 12 % (w/v) SDS-PAGE gel. Proteins were blotted onto a nitrocellulose membrane and the bacterially-expressed scFv-c2 was used for detection. The banding was revealed by 9E10 monoclonal antibody and GAMAP antibody conjugated to alkaline phosphatase followed by staining with NBT/BCIP. 1: IBDV vaccine; 2: extract of infected bursa; H: extract of healthy bursa; M: Pre-stained protein marker



**Figure 3:** ELISA results showing the reactivity of the bacterially-expressed scFv fragments against IBDV antigens (vaccine and infected bursa). Binding of scFv fragments to cognate antigen was detected by using 9E10 monoclonal antibody, and goat anti-mouse polyclonal antibody conjugated to horse radish peroxidase (GAM<sup>HRP</sup>) as secondary antibody. After 30 minutes the absorbance values at 405 nm ( $OD_{405\text{ nm}}$ ) was measured using a micro plate reader (ELx808 Absorbance Microplate Reader, Winooski, VT, USA)



**Figure 5:** Three-dimensional homology model of anti IBDV scFv. (A) 3D model of scFv of IBDV. (B) Ramachandran plot of the scFv model



**Figure 6:** Three-dimensional binding poses of docking complex of the scFv for IBDV. The surface (A) and cartoon (B) 3D binding poses

## DISCUSSION

IBDV is among the major pathogens imposing high economic losses on the poultry industry worldwide. The present study strived to develop a recombinant antibody fragment against IBDV using molecular biology and genetic engineering. Based on the findings obtained herein, the recombinant antibodies could successfully be

used for efficient diagnosis of the diseases [11,15-18].

Phage display technology provides a straightforward approach for selection of specific recombinant antibody fragments with highest affinity for the target antigen from a huge number of variants in antibody libraries [19]. On that basis, in the present study, this technology was applied to isolate new antibodies against the IBDV virions from Tomlinson I naïve non-immunized antibody libraries. The isolated scFv antibody fragments were expressed in bacterial cells and were successfully used in typical diagnostic applications such as immunoblotting and ELISA.

Western blot data showed that the selected scFv antibody fragment strongly bound to a protein with molecular weight of 30 kDa (Figure 4). The IBDV capsid is mainly composed of VP2 and VP3 proteins. Hudson *et al* [20] showed that the IBDV encodes a 106 kDa polyprotein that is later processed to three mature proteins VP2 ( $50 \pm 52$  kDa), VP3 ( $30 \pm 32$  kDa), and VP4 ( $27 \pm 30$  kDa). It seems that the VP3 capsid protein of the IBDV with a molecular weight of 30kDa is the binding partner of the selected scFv fragment monoclonal antibody.

The developed scFv antibody fragments were shown to offer great potentials for diagnosis of *gumboro* disease. The findings of this study confirmed the feasibility of using the phage display technology for the rapid development of antibodies against IBD diseases by applying of naïve scFv libraries. In better words, the naïve non-immunized phage display libraries can be applied for the selection of specific antibody fragments elevating the need for animal immunization [13,21]. In the phage display

libraries, billions of peptides capable binding to different antigens are present on the surface of the bacteriophages. Screening and panning techniques will recover the specific antibody gene [22]. This technique is simple, cheap, and rapid and requires no special equipment. Moreover, because of elimination of the immunization stage from this technology, it does not involve ethical concerns related to animal use and scarification either [21].

The ability of the selected scFv antibody fragments to bind the corresponding antigens in both ELISA and blotting analysis confirm their specificity. The positive results obtained through the Western blot analysis confirmed the presence of the corresponding binding site (epitope) in the IBDV virions as a continuous epitope [23].

To the best of our knowledge, this is the first report on the construction of a monoclonal antibody against IBDV through non-immunized phage display libraries. Nevertheless, there are several reports on developing specific IBDV recombinant antibodies by applying chicken pre-immunized library and/or bacterial libraries. Zhang *et al* [11] described the application of bacterial display for isolation of scFv antibody fragments against IBDV from an antibody library via the VP2 protein. Sapats *et al* [24] developed two chicken scFv antibody fragments reacting with high virulent strains of IBDV. They argued that their method was 8-16 to times more sensitive for IBDV antigen detection than the mouse monoclonal antibody ELISA.

These serological results revealed that scFv could be used as highly specific and sensitive ELISA reagents for the detection of avian pathogens. In fact, the power of recombinant antibodies over previously described conventional methods, for fine antigenic differentiation and to provide a sensitive, specific, and simple platform has been well confirmed [24]. In another study, phage display technology was used for the isolation of a specific scFv antibody from the immune spleen cells of chickens immunized with a high virulent strain of IBDV [12].

Sapats *et al* [1] developed a number of scFv recombinant antibodies by biopanning of a phage-display libraries derived from the spleen of chickens immunized with an Australian strain of IBDV. These scFvs showed a significant potential for the discrimination of different IBDV strains and neutralizing capacity. Xu *et al* [10] used the bacteria display technology and produced a recombinant scFv antibody by flow

cytometry against VP2 protein from the chickens immunized with VP2 protein of IBDV. They obtained three scFv clones with different fluorescence intensity by random colony pick up after biopanning of the bacteria displayed antibody library. Subsequently, they investigated the feasibility of using the selected IBDV-specific scFv antibodies against the virus by the application of the isolated antibodies in Western blotting assay and ELISA.

Homology modeling and molecular docking are two appropriate computational methods to investigate protein-ligand interactions. Herein, the user-friendly internet based server 'I-TASSER' was used for homology modelling and generation of a reliable 3D structure. PDB file of 5F72T molecular model was used as input model in the antigen-antibody docking. Thirty models were generated by the BioLuminate program and the model with the highest rank was selected for the analysis. Docking results of the scFv-IBDV showed a binding energy of -545 kJ/mol.

The recombinant antibody fragments could also be used for developing more sophisticated diagnostic tools such as biosensors and nanobiosensors. For instance, Mechaly *et al* [25] applied scFv based biosensor for the efficient and specific detection of *B. anthracis* spores. Moreover, since the scFv antibody possesses a binding ability to antigens, thus it could be potentially used as unique molecule to be fused to other toxins/antigens in treatment procedures [26]. For instance, the anti-fAChR immunotoxin (scFv35-ETA) is a fusion of anti-fAChR Fab-fragment to *Pseudomonas exotoxin A* and has been used for the treatment of fatal neoplasm [27].

## CONCLUSION

The recombinant antibodies developed in the present study easily detect IBDV-infected animals and, therefore, can be applied for diagnostic purposes using conventional diagnostic platforms such as ELISA and dot blot assays. Furthermore, the developed antibodies can potentially be used for developing more sensitive diagnostic tools as well, for example, biosensors and nanobiosensors.

## DECLARATIONS

### Acknowledgment

The authors appreciate the financial support provided by Iran National Science Foundation (INSF) for this work.

## Conflict of interest

No conflict of interest is associated with this work

## Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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