



# **Efficacy of Recombinant S1 Protein Expressed in *Pichia pastoris* in Chicken & Using for Detection Titer Antibodies against Avian Infectious Bronchitis by ELISA**

**Mahdi Ahmadi<sup>1</sup>, Khosrow Aghayipour<sup>1\*</sup>, Arash Ghalyanchi Langroudi<sup>2</sup>, Hossein Godarzi<sup>3</sup> and Masood Hashemzadeh<sup>4</sup>**

<sup>1</sup>Department of Genomics and Genetic Engineering, Razi Vaccine and Serum Research Institute (RVSRI), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

<sup>2</sup>Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

<sup>3</sup>Department of Avian Disease Research and Diagnosis, Razi Vaccine and Serum Research Institute (RVSRI), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

<sup>4</sup>Department of Research and Production of Poultry Viral Vaccine, Razi Vaccine and Serum Research Institute (RVSRI), Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

## **Authors' contributions**

This work was carried out in collaboration among all authors. Authors MA and KA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AGL, HG and MH managed the analyses of the study. Author MH managed the literature searches. All authors read and approved the final manuscript.

## **Article Information**

DOI: 10.9734/JPRI/2019/v29i230234

Editor(s):

(1) Dr. Mohamed Fathy, Professor, Department of Pharmaceutics, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

Reviewers:

(1) Wafaa Abd El-Ghany Abd El-Ghany, Cairo University, Egypt.

(2) Wuyi Liu, Fuyang Normal University, China.

Complete Peer review History: <http://www.sdiarticle3.com/review-history/50758>

**Original Research Article**

**Received 30 May 2019**  
**Accepted 13 August 2019**  
**Published 22 August 2019**

## **ABSTRACT**

Infectious bronchitis (IB) is an acute, extremely contagious upper respiratory disease in chickens. The objective of this study was expressed and using of recombinant S1 Protein serotype 793/B expressed in *pichia pastoris* in order to serodiagnosis against avian infectious bronchitis antibody. The complete S1 gene (1623bp) was cloned in PTZ57 plasmid and transferred to *E. coli* (Escherichia coli)-XL1blue bacterium. Next cloned to pPICZB vectors and transferred to *p. pastoris*(Km71H). Produced protein were visualized by SDS (sodium dodecyl sulfate) PAGE gel

\*Corresponding author: E-mail: drkhosrowaghaeipour@yahoo.com;

that was about with 62KDa. Finally, the S1 recombinant protein was used to coat 96 well plate to recognize antibodies in sera against live Infectious bronchitis virus (IBV 4/91). As a result, this recombinant S1 protein Antigen can be used in an enzyme-linked immunosorbent assay (ELISA) for recognizing antibody titer against IBV. This recombinant protein was evaluated by Elisa, using a panel of field sera for known IBV titer That in the commercial kits, the optical gain of positive control is  $0/741 \pm 0/007$  and the negative control is  $0/167 \pm 0/002$ , next the average Optical Density (OD) in the three farms were 2.585, 2.001, 1.657, resulting to the S/P ratio of was 4.212, 2.886, 2.484, respectively. The results of our effort for positive control and the negative control are  $0/9 \pm 0/010$ ,  $0/067 \pm 0/008$  respectively. The mean Optical Density of sera in 3 Flocks were showed 3.390, 2.737, 2.921, and the ratio of S/P 4.01, 3.242 and 3.46. These results showed that our outcomes are neared to Biochek kit. The results also showed that recombinant protein S1 was able to identify different kinds of infectious bronchitis virus serotypes in poultry. It can be used for commercial ELISA kit.

**Keywords:** IBV; ELISA; S1 glycoprotein; cloning; expression; serodiagnosis.

## 1. INTRODUCTION

Infectious bronchitis (IB) is an acute, extremely contagious, and economically important respiratory disease in poultries. It is caused by the IB virus (IBV), which is a significant respiratory pathogen that causes considerable economic losses in the commercial poultry industry worldwide. First, IBV was reported in poultry flocks of Iran in 1994 by Aghakhan et al [1]. The major clinical signs are included tracheal rales, sneezing and coughing. The IBV genome is a single-stranded, positive-sense RNA that is 27.6 kb in size. It is member of Gammacoronavirus, subfamily Coronavirinae, family Coronaviridae, Order Nidovirales [2,3,4,5]. It encodes four major structural proteins, named, glycosylated spike protein (S), membrane protein (M), phosphorylated nucleoprotein (N), and envelope protein (E), and 15 nonstructural proteins (nsp2–nsp16). Generally, nonstructural proteins are present in infected cells but not in the virus, and they only play a role in the process of virus infection and replication [6].

In general, acute IBV infections can be diagnosed by detection of IBV virus (antigen) or the specific antibody response. The most common assays for routine virus detection are virus isolation (VI), immunofluorescence assay (IFA), immune peroxidase assay (IPA), polymerase chain reaction (PCR), and for antibody detection the haemagglutination inhibition (HI) test, agar gel precipitation test (AGPT), and enzyme linked immunosorbent assay (ELISA). The virus neutralization test (VNT) is rarely used for routine diagnosis because it is relatively expensive and laborious [7,8]. All coronavirus S glycoproteins consist of four domains; a signal sequence that is cleaved during synthesis; the ectodomain, which is

present on the outside of the virion particle; the transmembrane region responsible for anchoring the S protein into the lipid bilayer of the virion particle; and the cytoplasmic tail. The IBV S glycoprotein (1162 amino acids) is cleaved into two subunits, S1 (535 amino acids, 90-kDa) comprising the N-terminal subunit of the S protein and S2 (627 amino acids, 84-kDa) comprising the C terminal subunit of the S protein. The S1 subunit contains the receptor-binding activity of the S protein [9,10]. This glycoprotein can be inducing neutralization of antibodies for protecting against virus. There are six antigenic sites on S1 protein that are induced of response immune [11].

The ELISA assay is a convenient method for monitoring of both the immune status and virus infection in chicken flocks. Several commercial ELISA kits for IBV specific antibodies detection are already available. However, proliferation & purification of IBV is tiresome and expensive. Unlike full virions, recombinant proteins produced via gene engineering detect only specific antibodies to one certain protein and are safer, easier to produce and less expensive [12,13]. Actually the S1 glycoprotein has potential as an antigen for developing an ELISA diagnosis instrument.

In this study, we keep complete S1 gen(1623bp) for expression in *pichia pastoris*. The polyclonal antibodies were produced in SPF chickens, and it used for making ELISA kit and used evaluate response system.

## 2. MATERIALS AND METHODS

We got standard infectious bronchitis virus (IR/773/2001/B/793) from Razi Vaccine and Serum Research Institute (RVSRI).

## 2.1 Reverse Transcription (RT) and PCR (RT-PCR)

RNA virus was extracted with RNA X kit (Sina Gene Company) according to user manual and cDNA synthesis was performed by RT Kit (GeneAll Company). The program was run at 55°C in 60 min. Next it used as template for S1 gene amplification. The S1 gene was amplified pair of primer (Table 1), which had EcoR I and Xba I cut enzyme positions. The DNA of the gene was synthesized and multiplied using PCR kit (BIOfact kit) in thermal cycler (Applied Biosystem). The PCR program was done with initial denaturation step at 95°C for 5 min next 35 cycles of denaturation 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, next final extension at 72°C for 15 min. The final fragment length was 1623 bp.

## 2.2 Cloning, Expression and Purification of S1 Gene

After that amplicon was confirmed by gel electrophoresis, S1 gene was cloned in *pTZ57* plasmid and transferred to *E.coli*-XL1blue bacterium by T/A cloning kit (Ins T/A clone PCR product cloning kit, fermentase). Then the vector was extracted by a plasmid purification kit (Biofact Corporation) according to user manual. After that, S1 gene was cloned to the original non-secretive *pPICZB* vector (which it was already enzymatically digested by EcoR I & Xba I) of yeast *Pichia pastoris* by TA cloning kit according to user manual kit. The expression was initiated by 10µg *pPICZB* which was linear by SAC I(Thermo) for transfection to the host *Pichia pastoris* (Km71H). The *Pichia pastoris* were broken with acid-washed glass(0.5mm) and breaking buffer containing pmsf furthermore centrifuged at 3000 g for 15 minutes, and the liquid was removed and it was stored in -70°C (according to EasySelect™ *Pichia* Expression Kit). The protein purification was done by protein His Tag Ni-IDA column kit (MN kit Ni-IDA 2000) and the SDS PAGE test was performed to determine the expression of the 62 KDa. The

purified protein concentration was determined by the Bradford method at 595 nm wavelength.

## 2.3 Western Blot

Recombinant S1 protein was separated by 12.5% SDS-PAGE. Next it was electrotransferred to nitrocellulose membrane overnight at 40 V by mini protein 3 (BIORAD) at 4°C. The membrane was blocked in PBS-T buffer (contains BSA 2.5% & 0.1% Tween 20) by soaking in blotting buffer for 1 hour with shaking. Followed by incubating membrane in primary antibody(ab200537) anti-His tag (1:10000) for one hour then washing the blot three times with wash PBS-T buffer and incubated the blot in secondary antibody (goat anti rabbit ab205718) anti-His tag with horseradish peroxidase (1:10000) for one hour at room temperature. After that it was washed again with PBS-T buffer for removing the unbound antibodies and was developed by Add 10ul H2O2 (30%) to 10 ml of 0.05% DAB (3,3'-diaminodbenzidine) in PBS for 2-5 min.

## 2.4 Immunization and Field Serum

For providing protective properties study of this recombinant antigen as a candidate vaccine, three group of chicken were selected. Live poultry avian infectious bronchitis 4/91 vaccine (Nobilis-Holland) was inoculated via eye drops in SPF chicken at the age two weeks (from Razi Institute). The recombinant protein was also injected intramuscularly with montanide oil adjuvant in the second group, and the third group was without injected antigens it was considered as negative control (10 chickens). After that field sera were collected from 94 layer hens (at the age 22 weeks) that their antibody titer was also determined with commercial Bio-check CK119 IBV ELISA kit according to the manufacturer's instructions.

## 2.5 ELISA Assay

In-house ELISA system was development briefly, Recombinant S1 protein as an antigen

Table 1. Primer sequence with restriction enzyme

Gene	Sequence primer	Restriction enzyme	Size(bp)
S1	F:5' CG GAA TTC ATG TTG GGC AAA CCG CTT TTA CTA GTG R:5' GC TCT AGA CGT CTG GAG CGA CGT GTT CCG TTA G	EcoR I Xba I	1623

Source: Ahmadi et al., 2019 [22]

(approximately 50 ng/well) was coated to standard ELISA micro plates (Biomate plate 96 wells) using bicarbonate buffer at 0.1 M (pH:9.6). The coated micro plates were incubated for 18 h at 4°C. This was continued by a triple washing treatment with 10 mM PBS. Plates were coated with blocking through filling the wells with 150 µl of 2.5% BSA (Sigma-Germany). All steps were carried out by incubating of plates at room temperature for one hour. The 10 mM PBS/Tween 20 and 1% BSA buffers were used to prepare 1/500 dilutions of serum samples. One hundred µl of treated serum was transferred to a fresh coated ELISA plate. This was followed by incubation at room temperature for 30 minutes when the plate was washed for 5 times with washing buffer. The next step was adding 100 µL of Horse Radish Peroxidase (HRP) conjugate (anti chicken, abcam-UK) diluted by 10mM PBS, pH 7.2) to the wells and incubation for 30 min at room temperature followed by a washing phase. Afterward, 100 µl of Tetra-Methyl Benzidine (TMB) substrate was added to wells and the plate held for 15 min at room temperature while keep avoids from light. This was followed by adding 100 µl of stop solution (HCl 1 N) to the wells. Finally, the whole process was read and recorded by an ELISA reader (Biotech-Model TS 800) at 450 nm wavelength.

### 3. RESULTS

In this disquisition, the agent gene amplified and visualized on agarose gel electrophoresis (Fig. 1). Subsequently, the products were ligated into *pTZ57* cloning, *PpICZB* vectors (Fig. 5) that were confirmed by restriction Enzymes *EcoR* I &

*Xba* I digestion (Fig. 2) and transferred to *E.Coli* *XI1blue*, and finally *pichia pastoris* competent cells. The recombinant produced proteins were visualized by SDS PAGE gel and western blot. The recombinant protein's size was 62 KDa (Figs. 3,4). Then recombinant S1 protein was coated in 96 wells microplate. Henceforth, the plate run by positive and negative samples obtained from specific pathogen free chickens. After checkerboard with control positive & negative. We determined the appropriate spot for the amount of protein coated by the positive and negative control. As a result, the positive control level is Fixed on 0/9 at 450nm wavelength. This plate used for detection of sera field. We tested 94 sera from farms. In the commercial kits, the optical gain of positive control is 0/741 ± 0/007 and the negative control is 0/167 ± 0/002 next the average ratio OD in the three farms were 2.585, 2.001, 1.657, and the ratio of S/P was 4.212, 2.886, 2.484, respectively. The results of this experiment for positive and negative control were 0/9 ± 0/010, 0/067 ± 0/008, respectively. The mean Optical Density (OD) of sera in three Flocks were showed 3.390, 2.737, 2.921, and the ratio of S/P 4.01, 3.242 and 3.46. These results showed that the outcomes of this study are very similar to Biochek kit (Fig. 6).

### 4. DISCUSSION

This survey was Succeeded in cloning and expression of recombinant S1-IBV (1623 bp) serotype 793/B (IR/773/2001/B/793) in *p.pastoris*. The S1 glycoproteins observed at 62 KDa in SDS-PAGE and then was confirmed with a western blot. For in vivo evaluation of this

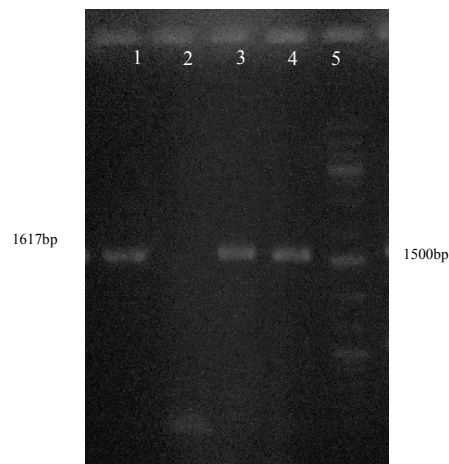
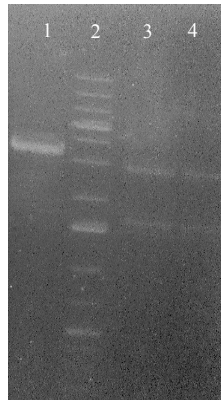
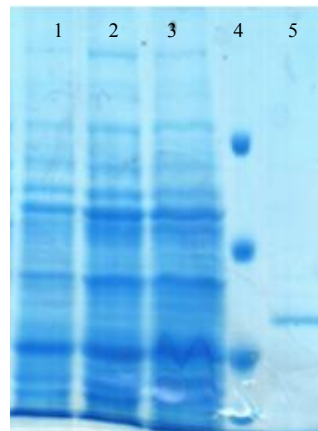


Fig. 1. Result PCR product of S1 gene IBv793/B (1617 bp) lane1,3,4 product, 2 negative and lane 5 ladder



**Fig. 2.** The result of the enzymatic digestion of Xba I and EcoR I, lane 1 vector non digest, lane 2 ladder and lane 3,4 vector digested



**Fig. 3.** Results extract and purified protein SDS-PAGE gel lane 1-5, respectively 48, 72, 96, ladder and bond S1 gene 62KDa



**Fig. 4.** Results western blot lane 1 ladder, 2 bond purified protein

Created with SnapGene®

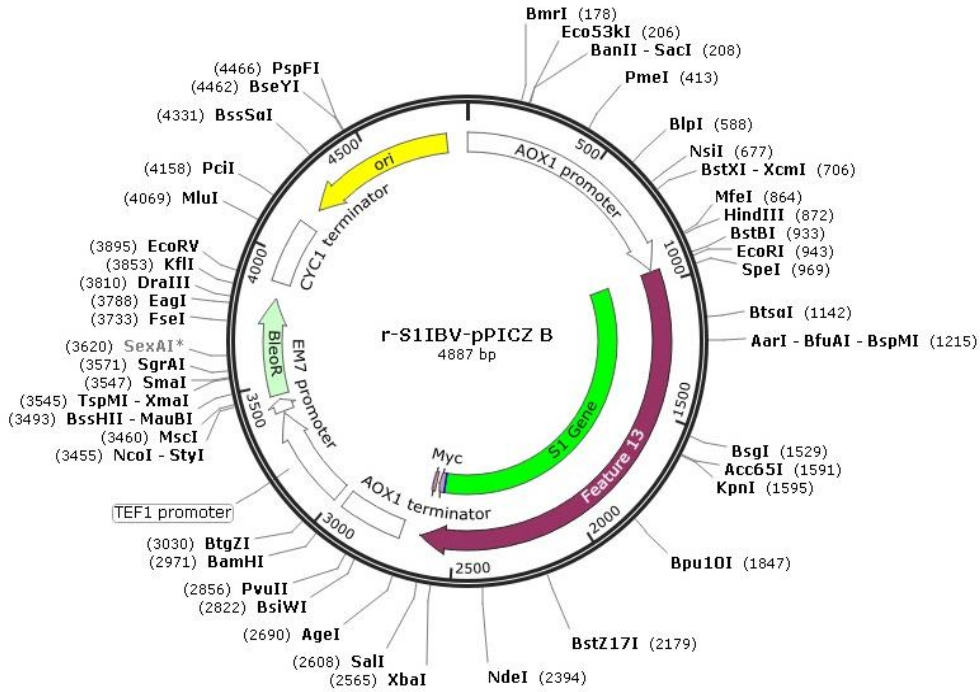


Fig. 5. Genetic map of plasmid pPICZB & S1793/B with 2 enzymatic cutting positions EcoR I & Xba I

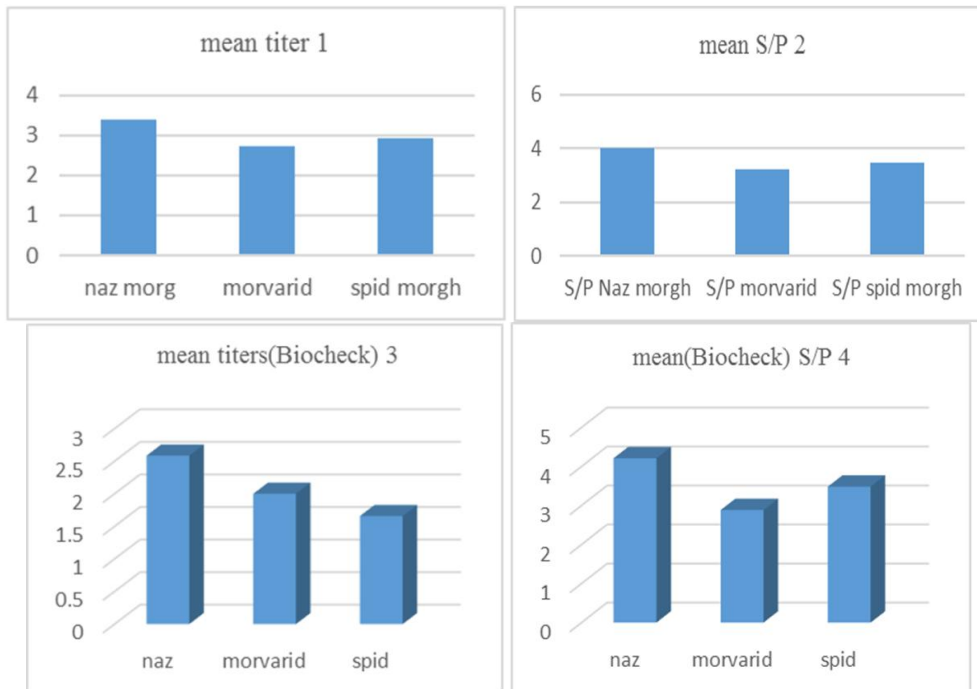


Fig. 6. Diagrams 1 & 2 means of titers and S/P between farms, 3 & 4 means titers and S/P with Biocheck kit

recombinant antigen, SPF chickens were immunized with S1 glycoproteins. The expressed and purified recombinant antigen was also used to develop an in house ELISA plate to detect anti-IBV antibodies. The glycoprotein S1 is known for immunogenic properties. It is well known that the glycoprotein S1 subunit induces humoral protecting immunity [14]. In previous studies, proteins have been expressed in the prokaryotic system, but Eukaryotic systems have the benefits such as packaging and glycosylation, which is not in the prokaryotic system or very low. Zehan et al. (2018) cloned & expressed the spike protein of IBV in *E. coli* which showed relatively acceptable immunogenicity in mice. They found that this protein can be used as antigen to detect antibodies against IBV [15]. Hsun-Lin and et al. (2012) they expressed S1 protein in prokaryotic system and applicate it for diagnosis of IBV antibodies (11). Wang (2002) was also used S1 spike polypeptide for ELISA. The results showed that S1 protein can be diagnosis antibodies against IBV (12). Song (1998) could clone and expressed S1 glycoprotein in Eukaryotic system by recombinant baculovirus. They indicated that S1 glycoprotein can induce immune response in chicken [16]. Finger (2018) was used N protein IBV for making ELISA and western blot. He indicated that N protein can also be used for the immunodiagnosis of avian infectious bronchitis (3).

The major IBV immunogenic protein is S1 glycoprotein that could induce antibody response against IBV [17,18]. The location of three overlapping antigenic sites on the IBV spike protein was similar to the location of antigenic sites on the spike protein of other coronaviruses [19]. There are eight antigenic regions on the spike protein (S2, G-H) that six of them are on the S1(S1, A-F) [20,21]. That main part of S1-f is conserved which is a proof for the cross-reactivity between the tested serotypes in antibodies based tests such as ELISA [12].

Generally, in chicken ELISA is used to diagnose disease and the antibodies titer of vaccinated chickens and for determining the next step of vaccination. Commercial kits use a logarithmic formula to determine the antibody titer, which is not possible manually. There is a parameter  $S/P(s/p = \frac{OD\ sample - OD\ negative\ control}{OD\ positive\ control - OD\ negative\ control})$  that is based on positive and negative control, and completely dependent on them. Based on the results obtained from the commercial kit, serum titers cannot be manipulated. Therefore, tried to

obtain best controls point with the changes in the absorption rate positive and negative control, so that when the formula is used, the results of the manufactured kit are made close to the commercial titer. Also, there is a S/P ratio in the software Biocheck. This is ultimately used in logarithmic formulas. For this reason, understood that if the results of serum titers are closed to the results of the commercial kit, So the final logarithmic results are similar. Positive control was placed based on OD = 0.9 at 450nm wavelength. After several times the checkerboard tests are obtained by manipulating the positive control. As a result, the final outcomes will be close to the commercial kit. Although the purpose of this study was not to achieve to the commercial kits, but tried to work on this issue. This study showed that recombinant protein S1 was able to identify different kinds of infectious bronchitis virus serotypes in poultry. These results are similar to the previous studies. Such as Wang [12] and Lin [11] Which made the ELISA kit using recombinant protein. In this research, S1glycoprotein cloned, expressed and used it for diagnosis of antibodies titer against IBV. Then. It was tested with 94 serums from three farms chickens which showed a close relationship with the result of the commercial Biocheck kits.

## 5. CONCLUSIONS

The results showed that recombinant protein S1 was able to identify serotypes different kinds of infectious bronchitis virus have poultry. It can be used for making commercial ELISA kit.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

As per international standard guideline the ethical approval has been collected and preserved by the authors.

## ACKNOWLEDGEMENTS

This study was supported by Razi Vaccine and Serum Research Institute (RVSRI).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Mehragha M, Seyfi Abad Shapouri MR. Expression of nucleocapsid gene of chicken infection bronchitis Virus Strain massachuset H120; Iranian Veterinary Journal. 2018;4(2):94-103.
2. Akrami H, Hedayati A, Farshian M, and Haqshenas G. Development of a recombinant protein-based dot-blot hybridization assay for the detection of antibody to1 chicken infectious bronchitis virus. Iranian Journal of Veterinary Research. 2013;14(4):350-353.
3. Finger PF, Pepe MS, Dummer LA, Magalhães CG, de Castro CC, Hübner SO, et al. Combined use of ELISA and Western blot with recombinant N protein is a powerful tool for the immunodiagnosis of avian infectious bronchitis. Virol J. 2018;15(1):189.
4. Kanwal B, Channo AA, Kalhoro NH, Soomro H, Korejo NA, Tauseef S. Prevalence and clinical pathology caused by infectious bronchitis virus in poultry birds at Sindh, Pakistan. J. Vet. Med. Anim. Health. 2018;10(9):231-236.
5. Mahzounieh M, Karimi I, Bouzari M, Zahraei Salehi Tand Iravani S. A serological survey for detection of avian infectious bronchitis virus antibodies in domestic village chickens in Esfahan, central Iran. Iranian Journal of Veterinary Research, University of Shiraz. 2006;7(2): 89-9.
6. Yan L, Hu J, Lei J, Shi Z, Xiao Q, Bi Z, Yao L, et al. Novel protein chip for the detection of antibodies against infectious bronchitis virus. BMC Veterinary Research. 2018; 14(284):1-9.
7. De Wit JJ. Detection of infectious bronchitis virus. Avian Pathol. 2000;29(2):71-93.
8. Onen EA, Ozgur Y. Investigation of infectious bronchitis virus strains by real time RT-PCR and S1 gene sequencing in broilers. J Vet Sci Med Diagn. 2017;6(4): 2-6.
9. Armesto M, Evans S, Cavanagh D, Abu-Median A, Keep S, Britton P. A recombinant avian infectious bronchitis virus expressing a heterologous spike gene belonging to the 4/91 Serotype, PLoS One. 2011;6(8):e24352.
10. Ahmadi M, Aghayipour M, Ghalyanchilangeroudi A, Hashemzadeh M, Alii M. Expression of S1protein from infectious bronchitis virus (IBV) serotype 793/B in Pichiapastoris. Medical Science. 2019;23(98):615-621.
11. Lin KH, Lin CF, Chiou SS, Hsu AP, Lee MS, et al. Application of purified recombinant antigenic spike fragments to the diagnosis of avian infectious bronchitis virus infection; Appl Microbiol Biotechnol. 2012;95:233–242.
12. Wang CH, Hong CC, Seak JC. An ELISA for antibodies against infectious bronchitis virus using an S1 spike polypeptide. Vet Microbiol. 2002;85(4):333-42.
13. Zhang DY, Zhou JY, Fang J, Hu JQ, Wu JX, Mu AX. An ELISA for antibodies to infectious bronchitis virus based on nucleocapsid protein produced in Escherichia coli, Vet. Med. – Czech. 2005; 50(8):336–344.
14. Meir R, Krispel S, Simanov L, et al; Immune responses to mucosal vaccination by the recombinant S1 and N proteins of infectious bronchitis virus. Viral immunology. 2012;25(1):55-62.
15. Zeshan B, Saleem MA, Wattoo JI, Arshad MM and Mohamed M. Cloning and expression of truncated spike (Sf200) glycoprotein of infectious bronchitis virus (IBV) in *Escherichia coli* and its immunogenicity to mice. Pakistan J. Zool. 2018;50(3):989-994.
16. Song CS, Lee YJ, Lee CW, Sung HW, Kim JH, et al. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by arecombinant baculovirus. J Gen Virol. 1998;79(4):719-23.
17. Cavanagha D, Davis PJ, Cook JKG, D. Kant LI, Koch G. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol. 1992;21(1): 33-43.
18. Yuan Y, Zhang ZP, He YN, Fan, WS, Dong ZH, Zhang LH, Sun XK, Song LL, Wei TC, Mo ML. Protection against virulent infectious bronchitis virus challenge conferred by a recombinant baculovirus co-expressing S1 and N proteins. Viruses. 2018;10(7):1-14.
19. Kant A, Koch G, Van Roozelaar DJ, Kusters J, Poelwijk FAJ, van der Zeijst BAM. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus



- glycopolypeptide; J Gen Virol. 1992;73(Pt 3):591-6.
20. Koch G, Kant A, Cook JKA, Cavanagh D. Epitopes of neutralizing antibodies are localized within three regions of the S1spike protein of infectious bronchitis virus. International Symposium of Infectious Bronchitis, world veterinary poultry association, Rauschholzhausen. 1991;154–161.
21. Moore KM, Jackwood MW, Hilt DA. Identification of amino acids involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus; Arch Virol. 1997; 142(11):2249-56.

---

© 2019 Ahmadi et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

*Peer-review history:*  
*The peer review history for this paper can be accessed here:*  
<http://www.sdiarticle3.com/review-history/50758>