Pakistan J. Zool., vol. 50(3), pp 989-994, 2018. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2018.50.3.989.994

Cloning and Expression of Truncated Spike (S_{f200}) Glycoprotein of Infectious Bronchitis Virus (IBV) in *Escherichia coli*, and its Immunogenicity to Mice

Basit Zeshan[1,2,*], Mushtaq A. Saleem[2], Javed Iqbal Wattoo[2], Mohd Mokhtar Arshad[1] and Maizan Mohamed[1]

[1]*Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Kota Bharu 16100, Kelantan, Malaysia*
[2]*Faculty of Life Sciences, University of Central Punjab, Lahore, Pakistan*

Article Information
Received 25 August 2017
Revised 13 October 2017
Accepted 01 November 2017
Available online 19 April 2018

Authors' Contribution
BZ did experimental work. MMA and MM helped in data analysis. JIW and MAS helped during writing the manuscript.

Key words
IBV, Truncated spike glycoprotein, Infectious bronchitis virus Truncated S1, Sf200.

ABSTRACT

Complete S1 gene of the Infectious Bronchitis Virus (IBV) was amplified and cloned into transfer vector. Truncated S1 gene designated as S_{f200} (containing five antigenic sites located at 24–61, 291–398 and 497–543 amino acid residues of S1 glycoprotein) were amplified by overlap PCR, cloned into prokaryotic expression vector resulting pET-S_{f200} and confirmed the construct by sequencing. The recombinant plasmid was identified by restriction enzyme and sequencing analysis. The *in vitro* expression of the truncated protein was analyzed in *E. coli* with a molecular weight of 38kDa determined through SDS-PAGE and confirmed by Western blotting. The recombinant truncated protein was then purified from the culture media. The immunogenicity of the protein was studied in an animal experiment on mice, in which mice were injected subcutaneously. These findings suggest that the truncated S_{f200} expressed in the pET-32a (+) prokaryotic vector can be used as antigen to detect antibodies against IBV.

INTRODUCTION

Infectious Bronchitis Virus (IBV) belong to *Cornoaviridae* family is the etiological agent of Infectious Bronchitis (IB) disease in poultry. This disease is highly contagious and has economic significance due to decreased egg production and quality. The main clinical manifestations in affected birds are related to respiratory problems. However, it causes extensive damage to in various parts of the body including kidney and the oviduct (Cavanagh, 2003; Yu *et al*., 2001; Liu and Kong, 2004). In many reports, the nephropathic lesion have been observed in vaccinated flocks which strongly suggest that the currently used IB vaccination may not providing good protection (Cavanagh and Naqi, 1997).

The IBV encodes four major structural proteins, known as, envelope (E) protein, membrane (M) protein, nucleocapsid (N) protein and spike (S) glycoprotein. The spike glycoproteins are synthesized as precursor glycoprotein which post translationally cleaved into two subunits, S1 and S2. The S1 subunit plays an important role in the attachment of the virus to the host cell membrane as it carries receptors for the virus attachment. The neutralizing epitopes for induction of T cytotoxic lymphocytes as well as induction of antibodies for virus neutralization are also present in S1 (Kant *et al*., 1992; Koch *et al*., 1990). The amino acid sequence of S1 subunit is highly variable (Cavanagh *et al*., 1992) however, some studies have demonstrated that it was an excellent candidate for developing recombinant vaccines, monoclonal antibodies (mAbs) and polyclonal for developing the diagnostic reagents as compared to other structural proteins (Wang *et al*., 2002; Johnson *et al*., 2003). The expression of Spike gene has been reported in various prokaryotic and eukaryotic expression systems (Jain *et al*., 2017; Sepideh *et al*., 2012; Zhou *et al*., 2003).

In this study, keeping in view the antigenic mapping regions in S1 of IBV (as the complete S1 about 1650 base pairs (bp) is very difficult to be expressed in *Escherichia coli* (*E. coli*) we expressed truncated spike polypeptide antigen produced in *E. coli* designated as S_{f200} {(truncated S1 having five antigenic regions on amino acids residues (aa) 24–61 (S1D), (aa) 291–398 (S1CAB) and (aa) 497–543 (S1F)} construct was cloned into pET-32a (+) vector

* Corresponding author: dr.basit@ucp.edu.pk
0030-9923/2018/0003-0989 $ 9.00/0

and expressed in *E. coli* BL21. The polyclonal antibodies were developed in mice by using the recombinant S_{f200} protein inoculated into mice. The polyclonal antibodies were used to identify the recombinant adenovirus as well as *E. coli* expressed S_{f200} protein.

MATERIALS AND METHODS

Animals, virus and sera

A new highly virulent nephropathogenic IBV strain named CK/CH/XDC-2/2013 field strains of IBV Gene bank accession number KM213963 were isolated from the chickens showing typical signs and postmortem lesion of the disease. The virus was propagated into 9 days old specific pathogen-free (SPF) embryos. The eggs were harvested immediately if there was death of the embryo noticed by candling. The 50% Embryo infective dose (EID_{50}) was calculated by inoculating serial 10-fold dilutions of virus into SPF embryonated chicken eggs. Four-week-old SPF BALB/c mice were purchased local suppliers. The serum samples collected from the mice were stored at -20°C until used.

Total RNA extraction and cDNA synthesis

The total RNA was extracted from the allantoic fluid using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA pellet was dried, re-suspended and washed by ethanol. The pellet was suspended in diethyl pyrocarbonate (DEPC) treated water and processed immediately for subsequently cDNA preparations. For reverse transcription 3µl (0.5µg/ µl) total RNA was used at 37°C for 1h using 1µl Oligo (Oligo(dT) 10pmol/µl), 0.5µl dNTP (7.5Mm/ µl), 5X RT buffer and 0.5µl (200U/µl) of M-MLV Reverse transcriptase (Promaga, USA).

Amplification, cloning of S1 and selection of protein gene S_{f200}

Conventional PCR was performed in thermocycler (Eppendorff Mastercycler, USA). Three pairs of primers were designed to amplify complete S1 gene of IBV (Table I) and cloned into pMD18-T vector using 25µl total volume mixture containing 12.5µl PCR master mix, 3µl cDNA, 0.5µl (10 pmol) of each pair of primer (listed in Table I) and 8.5µl of ddH_2O. Three fragments of 150 bp, 351 bp, and 162 bp were amplified using primers (Table I). PCR Reaction was carried out at different annealing temperature and extension time depending on fragment lengths (Table I). Amplified products were visualized and photographed by digital Gel Doc Transilluminator imaging system (Hamamatsu, Japan) at 1% agarose gel electrophoresis.

Construction of plasmid pET-32a-S_{f200}

After amplification of these three fragments, they were ligated with overlap PCR. The PCR products were purified and sub-cloned into a prokaryotic expression pET-32a (+) vector (Novagen, USA). The recombinant expression plasmid was constructed as pET-S_{f200} and confirmed by sequencing analysis.

Expression of recombinant S_{f200} protein

The transformed *E. coli* BL21 containing the recombinant plasmid was propagated in LB broth containing ampicillin (0.1 mg/ml) overnight at 37°C with rapid shaking (160 rpm). In order to make the culture fresh, one milliliter of overnight grown bacterial was inoculated into 100 ml of fresh LB broth with ampicillin antibiotic at 0.1 mg/ml. When the OD_{600} reached 0.6, the culture was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) of 1 mM followed by further incubation for four hours on shaker. Under the optimized expression conditions, 100 ml of bacterial culture was collected and centrifuged at 12000 g for 30 min at room temperature. The resultant pellets were re-suspended with 1/10 volume of Buffer A (100 mm $NaH_2PO_4.2H_2O$, 10 mm Tris base and 8 m urea, pH 8.0) and then sonicated on ice.

Purification of recombinant S_{f200} protein

The fusion protein expressed by recombinant pET-32 α (+) vector contains His-Tag which is often used for affinity purification by Ni-NTA spin kit according to manufacturer's protocol (Qiagen). Briefly, the supernatant was collected after centrifugation at 12000 g for 30 min at 4°C, and slowly added onto Ni-NTA affinity chromatography column balanced with 8 ml Buffer B (100 mm $NaH_2PO_4.2H_2O$, 10 mm Tris-base and 8 m urea, pH 6.3). Buffer C (100 mm $NaH_2PO_4.2H_2O$, 10 mm Tris-base and 8 m urea, pH 5.9) and Buffer D (100 mm $NaH_2PO_4.2H_2O$, 10 mm Tris-base and 8 m urea, pH 4.5) with 4 ml each were used to elute the bound protein and the eluted solution was collected in 1 ml aliquot. The protein bands were separated by SDS-PAGE and the truncated protein was detected by Western Blot.

Immunogenicity studies in mice

Generation of polyclonal antibodies

The purified recombinant pET-S_{f200} protein emulsified with Freund's complete adjuvant was injected into five BALB/c mice through subcutaneously (S/C) route using 26 gauge needle with an infectious dose of 50µg per mouse. The second booster dose was injected at 21 days S/C route. The last third booster dose of the antigen emulsified with Freund's incomplete adjuvant was injected at 40 days through intraperitoneal (I/P) route. The mice were

sacrificed after two weeks at days and the blood samples were collected.

SDS-PAGE and Western blot analysis

The molecular weight of the *E.coli* expressed S_{f200} protein was estimated by running it on SDS-PAGE electrophoresis (run through the stacking gel at 50 V. Then, the resolving gel was run at 100 V) followed by western blotting to in order to determine the binding properties and immunoreactivity with minor modifications of the method described by Zeshan *et al*. (2011) and Calandrella *et al*. (2001). Firstly the protein samples were boiled in water bath for five minutes and loaded to the SAD-PAGE 10% gel for 45 min. The bands were transferred to nitrocellulose membrane and probed using polyclonal antibodies raised in mice. Secondary antibody *i.e.* goat anti-mouse IgG conjugated with horseradish peroxidase (Boster Bio-Tech.) was used at the dilution of 1:3000 in PBS-T. Proteins were visualized using chemiluminescence luminol reagents (Thermo Scientific Super Signal West Femto Maximum Sensitivity Substrate). After developing the band, the membrane was washed with water to remove the substrate solution.

RESULTS

Cloning and sequence analysis of S1 gene

RNA was extracted from the allantoic fluid containing IBV virus and cDNA was constructed. Using set of primers (Table I), the complete S1 gene of IBV was amplified by RT-PCR and cloned into pMD-18 T vector resulting pMD-18 T-S1. The ligation was confirmed by restriction enzyme digestion and sequencing (Fig. 1). The proper open reading frame (ORF) of the gene was checked upon sequence analysis which was right followed by BLAST in NCBI, Gene Bank, which showed 99% similarity with IBV nephropathogenic strain isolated from Hebei, China.

Cloning of S_{f200} by overlap PCR and expression and identification of prokaryotic recombinant

Three fragments of 150 bp, 351 bp and 162 bp were amplified from pMD-18 T-S1 with the help of forward and reverse primers as shown in Table I. The PCR products were run at 1% agarose gel. The three fragments were joined together by overlap PCR with 660 bp size (Fig. 2). Proper ligation and of the overlapped fragments was confirmed by sequencing and restriction enzyme analysis using *BamH*I and *Xho*I (Fig. 3).

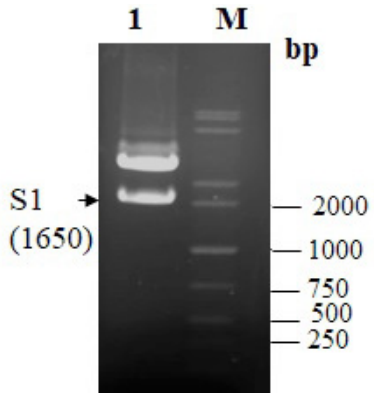

Fig. 1. Gel electrophoresis showing restriction enzyme digestion of complete S1 gene (1650bp approx.) after digestion with *Xho*I while 1kb DNA ladder is on the other side.

Table I.- Primer sequences, PCR Conditions and restriction enzyme sites complete S1 and truncated S1 gene fragments of field strain of IBV.

S No.	Primer ID	Sequence	Enzyme site	Gene ID (Size)	PCR conditions
1	S1-F	5' GCG**CTCGAG**ATGTTGGGGAAGTCACTG 3'	*Xho*I	S1 (1650)	Denaturation: 94°C/45s
2	S1-R	5' CGC**CTCGAG**TTACATTTTGGTCATAGAA 3	*Xho*I		Annealing: 60°C/45s Extension: 72°C/60s
3	Sense 1	5' GCG**GGATCC**ATGGATAGTTATGTTT 3'	*BamH*I	S1D (150bp)	Denaturation: 94°C/45s
4	Anti 1	5' CACCACCTTTATTGCCTGCATTATT 3'	No site		Annealing: 56°C/30s Extension: 72°C/20s
5	Sense 2	5' CAGGCAATAAAGGTGGTGTTGATAC 3'	No site	S1CAB (351bp)	Denaturation: 94°C/45s
6	Anti 2	5' CCTCACAAGGCTGCGTCAATTCACC 3'	No site		Annealing: 53°C/30s Extension: 72°C/30s
7	Sense 3	5' TGACGCAGCCTTGTGAGGATGTTAA 3'	No site	S1F (162bp)	Denaturation: 94°C/45s
8	Anti 3	5' TAT**CTCGAG**TTACCTGGAACGACG 3'	*Xho*I		Annealing: 60°C/30s Extension: 72°C/20s

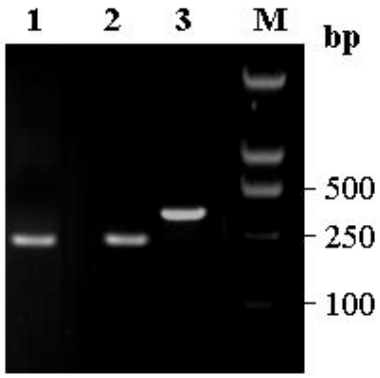

Fig. 2. Amplification of three fragments of S1 with the size of 150bp, 162bp and 351bp, respectively. Arrow indicate 1kb DNA ladder.

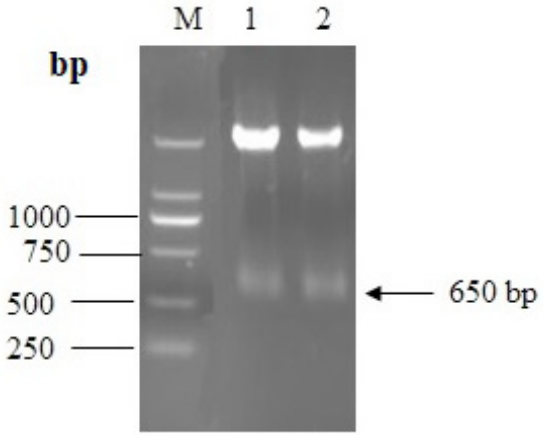

Fig. 3. Restriction enzyme analysis of pET-32a-S_{f200} after digestion with *BamH*I and *Xho*I (Lane 1, 3). 1kb DNA ladder is on the other side.

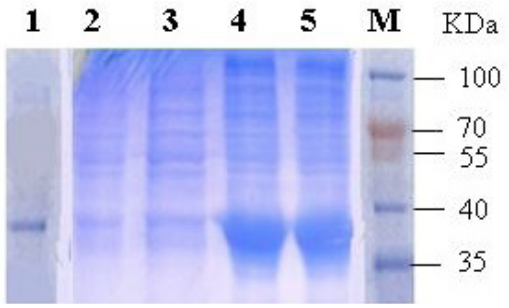

Fig. 4. SDS-PAGE of the Purified S_{f200} Lane (1) and the cell lysate of *E. coli* harboring the recombinant plasmid pET-32a-S_{f200} (38kDa approx.) after induction with IPTG containing *E. coli* BL21 (Lane 4), *E. coli* Rosetta strain (Lane 5), before induction with IPTG (Lane 2), pET-32a (+) vector control (Lane 3) while the proteins standards are showed on right side of panel.

SDS-PAGE and Western blotting

After induction by 1 mm IPTG at 37°C for 4 h, the S_{f200} proteins were found in the inclusion bodies in cell lysate of *E. coli* BL21 (DE3) with the molecular weight of around 40 kDa, approx. which was consistent with the predicted values, and recognized by mouse anti-S1 polyclonal antibodies as well as the homologous chicken anti-IBV serum (Fig. 5). The expressed S_{f200} protein was purified, and their corresponding yields in bacteria culture were calculated to be determined by presence of 2.5 mg of pure S1 protein. Additionally, we also expressed the full-length of S1 protein or fragments of S1 protein in *E. coli*. Unfortunately, this protein can be expressed at very low level.

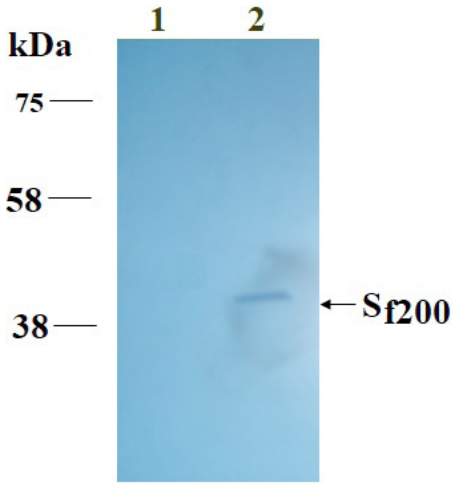

Fig. 5. Western blot analysis prokaryotic expressed S_{f200} in BL21 *E. coli* (Lane 2), while the proteins standards are showed on left side of panel.

DISCUSSION

Avian IBV is a significant pathogen of commercial poultry causing huge economic losses to the poultry industry worldwide. The severity of the disease is increased by the secondary bacterial infection leading to chronic complicated airsacculitis and nephritis. Diagnosis of IB is based on virus isolation or demonstration of viral nucleic acid and determination of of an ascending serum antibody response may also be useful. The S1 protein is a structural glycoprotein and is a key factor of virus neutralization and so is an excellent candidate for development of the novel IBV vaccines. S1 gene has also been used for development of ELISA kits (Gomaa *et al*., 2009; Hu *et al*., 2007; Loa *et al*., 2004).

In this study, S_{f200} protein S1 gene of field strain of IBV was expressed in *E. coli* BL21 strain at a higher

level using a prokaryotic pET32a (+) vector system. The expressed proteins can bind further with mouse anti-IBV serum, indicating that the recombinant S_{f200} protein is a potential valuable antigen for developing to detect antibody of IBV S1 protein and for an engineering subunit vaccine against IBV. The S1 region carries the receptor binding domain that defines tissue and host tropisms. Besides, it is responsible for serotype variability among IBV isolates (Sepideh *et al*., 2012). However, IBV S1 protein is characterized by presence of a signal peptide domain at the extreme 5′ end of the gene that was found to block successful cloning and expression of the complete S1 gene. To overcome this, a fragment of the S gene was selected for cloning and expression based on B cell epitope prediction (Larsen *et al*., 2006).

The observed molecular mass at 38 kDa of the expressed fusion S_{f200} protein is within the expected range. There are additional amino acids for the histidine tag in the C-terminal of the expressed fusion protein. These extra amino acids increase the molecular mass of expressed target protein.

Therefore, in future research, the specific location of amino acid residues involved in the epitopes needs to be further examined by mAbs as well as by using prokaryotic expression of S1 cDNA overlapping peptides. A preliminary ELISA method using the recombinant S1 protein as coating antigen could also be developed, which would have industrial application as well. A recombinant partial S1 protein has the potential to be used as a recombinant Ag in diagnostic kits in future.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Calandrella, M., Matteucci, D., Mazzetti, P. and Poli, A., 2001. Densitometric analysis of western blot assays for feline immunodeficiency virus antibodies. *Vet. Immunol. Immunopathol*., **79**: 261-271. https://doi.org/10.1016/S0165-2427(01)00265-3

Cavanagh, D., 2003. Severe acute respiratory syndrome vaccine development: Experiences of vaccination against avian infectious bronchitis coronavirus. *Avian Pathol*., **32**: 567-582. https://doi.org/10.1080/0307945031000162119 8

Cavanagh, D. and Naqi, S., 1997. Infectious bronchitis. In: *Diseases of poultry* (eds. B.W. Calnek, H.J. Barnes, L.R. McDougald and Y.M. Saif), 10th ed. London, pp. 511-526

Cavanagh, D., Davis, P.J., Cook, J.K., Li, D., Kant, A. and Koch, G., 1992. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. *Avian Pathol*., **21**: 33-43. https://doi.org/10.1080/03079459208418816

Jain, S.K., Jain, H. snd Bedekar, M.K. 2017. Expression of immunogenic S1 gene of infectious bronchitis virus from field outbreak in *Eukaryotic* cells. *J. Anim. Res.,* **7**: 27-32. https://doi.org/10.5958/2277-940X.2017.00005.5

Johnson, M.A., Pooley, C., Ignjatovic, J. and Tyack, S.G., 2003. A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. *Vaccine*, **21**: 2730-2736. https://doi.org/10.1016/S0264-410X(03)00227-5

Kant, A, Koch, G., van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A.J. and van der Zeijst, B.A.M., 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. *J. Gen. Virol*., **73**: 591-596. https://doi.org/10.1099/0022-1317-73-3-591

Koch, G., Hartog, L., Kant, A. and van Roozelaar, D.J., 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: Correlation with biological functions *J. Gen. Virol*., **71**: 1929-1935. https://doi.org/10.1099/0022-1317-71-9-1929

Larsen, J.E., Lund, P.O. and Nielsen, M., 2006. Improved method for predicting linear B-cell epitopes. *Immunol. Res.,* **2**: 1-7.

Liu, S. and Kong, X., 2004. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian Pathol*., **33**: 321-327. https://doi.org/10.1080/0307945042000220697

Sepideh, G., Hosseni, S.D., Zolfaghari, M.R. and Masoodi, S., 2012. Expression of S1 glycoprotein gene of infectious bronchitis virus (IBV) in *Escherichia coli*. *Afri. J. Microbiol. Res*., **6**: 3139-3143.

Wang, C.H., Hong, C.C. and Seak, J.C., 2002. An ELISA for antibodies against infectious bronchitis virus using an S1 spike polypeptide. *Vet. Microbiol*., **85**: 333-342. https://doi.org/10.1016/S0378-1135(01)00525-9

Yu, L., Wang, Z., Jiang, Y., Low, S. and Kwang, J., 2001. Molecular epidemiology of infectious bronchitis virus isolates from China and Southeast Asia. *Avian Dis*., **45**: 201-209. https://doi.org/10.2307/1593029

Zhou, J.Y., Wu, J.X., Chen, L.Q., Zheng, X.J., Gong, H., Shang, S.B. and Zhou, E.M., 2003.

Expression of immunogenic S1 glycoprotein of infectious bronchitis virus in transgenic potatoes. *J. Virol*., **77**: 9090-9093. https://doi.org/10.1128/JVI.77.16.9090-9093.2003

Zeshan, B., Mushtaq, M.H., Wang, X., Li, L. and Jiang, P., 2011. Protective immune responses induced by *in ovo* immunization with recombinant adenoviruses expressing spike (S1) glycoprotein of infectious bronchitis virus fused/co-administered with granulocyte-macrophage colony stimulating factor. *Vet. Microbiol.,* **148**: 8-17. https://doi.org/10.1016/j.vetmic.2010.08.003

Hu, J.Q., Li, Y.F., Guo, J.Q., Shen, H.G., Zhang, D.Y. and Zhou, J.Y., 2007. Production and characterization of monoclonal antibodies to *poly*^{100}S1 protein of avian infectious bronchitis virus. *Zoon. Publ. Hlth.,* **54**: 69-77. https://doi.org/10.1111/j.1863-2378.2007.01030.x

Gomaa, M.H., Yoo, D., Ojkic, D. and Barta, J.R., 2009. Use of recombinant S1 spike polypeptide to develop a TCoV-specific antibody ELISA. *Vet. Microbiol.,* **138**: 281-288. https://doi.org/10.1016/j.vetmic.2009.04.010

Loa, C.C., Lin, T.L., Wu, C.C., Bryan, T.A., Hooper, T. and Schrader, D., 2004. Expression and purification of turkey coronavirus nucleocapsid protein in *Escherichia coli*. *J. Virol. Methods,* **116**: 161-167. https://doi.org/10.1016/j.jviromet.2003.11.006