

Molecular diagnosis and adaptation of highly virulent infectious bursal disease virus on chicken embryo fibroblast cell

Yogender Singh¹, Kalpana Yadav¹, Vinod Kumar Singh² and Rakesh Kumar³

1. Animal Biotechnology Centre, Nanaji Deshmukh Pashu-Chikitsa Vigyan Vishwavidyalaya, Jabalpur, Madhya Pradesh - 482004, India; 2. Division of Virology, Indian Veterinary Research Institute, Izatnagar, Bareilly-243122, India; 3. Division of Dairy Cattle and Breeding, National Dairy Research Institute, Karnal, Haryana-132001, India

Corresponding author: Yogender Singh, email: yogender_bly@yahoo.com

Received: 13-03-2014, **Revised:** 13-04-2014, **Accepted:** 19-04-2014, **Published online:** 25-05-2014

doi: 10.14202/vetworld.2014.351-355

How to cite this article: Singh Y, Yadav K, Singh VK and Kumar R (2014) Molecular diagnosis and adaptation of highly virulent infectious bursal disease virus on chicken embryo fibroblast cell, *Veterinary World* 7(5): 351-355.

Abstract

Aim: To collect Infectious bursal disease virus (IBDV) infected bursae based on postmortem findings of clinical samples followed by molecular confirmation and adaptation on chicken embryo fibroblast cell.

Material and Methods: Enlarged bursae were processed to make 10% suspension in phosphate buffer saline and were used for viral RNA isolation to carry out *VP2* gene fragment amplification using RT-PCR technique. Suspension was also used for adaptation of IBDV on chicken embryo fibroblast cells prepared from 11 day old chicken embryos. Infective titer of virus was calculated using Reed and Muench method.

Results: Fifteen dead birds suspected of IBD infection were collected from different local poultry farms of Jabalpur (Madhya Pradesh). After post-mortem, twelve enlarged bursae sample were used for total RNA isolation which was used for amplification of *VP2* gene. Out of twelve, eleven were found positive for *VP2* gene amplification. Virological characterization of PCR positive sample was done on chicken embryo fibroblast cell culture and various cytopathic effects like rounding of cells, cellular detachment and vacuolation were shown by five IBD field isolates after 48 hours on 4th passage. TCID₅₀ per ml of the adapted virus on 4th passage at 48 hours after infection was 1.46×10^7 .

Conclusion: *VP2* gene amplification using RT-PCR technique is a specific target for IBDV detection. Passaging of highly virulent IBDV field isolates in cell culture leads to attenuation of virus which can be exploited as a cell culture adapted vaccine candidate.

Keywords: chicken embryo fibroblast, IBD virus, molecular diagnosis, *VP2* gene.

Introduction

Infectious bursal disease (IBD) is a highly contagious disease of young chicken caused by infectious bursal disease virus (IBDV), characterized by immunosuppression and mortality generally at 3 to 6 weeks of age [1]. It is economically important to the poultry industry worldwide, due to increased susceptibility to other diseases and negative interference with effective vaccination. IBDV is a double stranded RNA virus with bi-segmented genome and belongs to the genus *Avibirnavirus* of family *Birnaviridae* [2]. There are two distinct serotypes of the virus, but only serotype 1 viruses cause disease in poultry and six antigenic types are identified by virus neutralisation test. Although viral antigen has been detected in other organs within the first few hours of infection, the most extensive virus replication takes place primarily in the bursa of fabricius. Activated dividing B lymphocytes that secrete IgM serve as target cells for the virus. Viral infection results in lymphoid depletion of B cells and the destruction of bursal tissues, leading to an increased

susceptibility to other infectious diseases and poor immune response to vaccines. *VP2* is considered to be the major host protective antigen and contains the major antigenic site responsible for eliciting neutralizing antibodies [3]. *VP2* induces virus neutralizing antibodies that protect chickens from IBDV [4]. It is responsible for antigenic variation [5], tissue culture adaptation and virulence [6].

Study of virological characterization and molecular diagnosis of IBD will strengthen the antiviral therapy and disease control. IBD virus can infect and grow on various primary cell cultures of avian origin. Commonly used cell lines are chicken embryo fibroblast (CEF), chicken embryo kidney, chicken embryo bursa, vero, baby hamster kidney etc. The virulence of IBDV is lost during the adaptation on CEF cell culture but antigenicity is retained [7].

The present study aims at RT-PCR based detection and adaptation of IBDV field isolates.

Materials and Methods

Source of IBDV field isolates: Fifteen dead birds aged about 3 to 5 weeks exhibiting clinical signs suggestive of IBD were collected from different local poultry farms of Jabalpur, Madhya Pradesh, India. After post-

Copyright: The authors. This article is an open access article licensed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>) which permits unrestricted use, distribution and reproduction in any medium, provided the work is properly cited.



Figure-1a: Hemorrhages on chest muscles

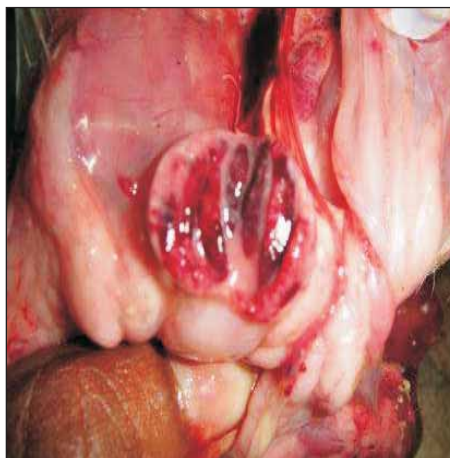


Figure-1b: Enlarged hemorrhagic bursa

mortem, twelve birds were found to show enlarged bursa which were collected aseptically and processed to make 10% suspension.

Processing of samples: Collected bursae were washed 3-4 times with sterile 1X Phosphate buffer saline (PBS). All samples were homogenized separately with pestle and mortar in chilled 1X PBS (0.01M, pH 7.4) to make 10% suspension. Processing was done in 3 repeated steps i.e. freezing-thawing for 3 times after 10 minutes interval. Homogenate was centrifuged at 7500 rpm for 20 minutes in refrigerated centrifuge. The supernatant was collected carefully and filtered through 0.22 μm syringe filter (Nalgene) and used for isolation of viral RNA or stored at -80°C until further use.

Isolation of viral RNA and cDNA preparation: Isolation of viral RNA from 10% bursal tissue suspension was done by TRIzol method as per manufacturer's instructions with slight modifications. RNA in each sample was quantified using Nanodrop ND-2000 spectrophotometer V3.5 (Thermo scientific U.S.A.). Three hundred nanograms of RNA from each culture was treated with DNase to remove genomic DNA and then used to produce cDNA using First-strand cDNA synthesis RT-PCR Kit (Fermentas, Life sciences, USA).

PCR amplification of VP2 gene of IBDV: PCR amplification of VP2 gene segment of 480 bp was done using published primers; IBDVP2F3 5'ACAGGC CC AGAGTCTACACCATAA3' and IBDVP2R3 5'ATC CTGTTGC CACTCTTTCGTAGG 3' [8] with the initial denaturation of 95°C for 5 minutes followed by 34 cycles of denaturation, annealing and extension at 95°C /30 seconds (sec), 59°C /60 sec and 72°C /30 sec, respectively and the final extension was carried out at 72°C /6 minutes. The PCR products generated were confirmed for their size in 2% (w/v) agarose gel in 0.5X tris borate EDTA (TBE) buffer as per the method of Sambrook and Russel [9] using horizontal submarine electrophoresis and staining with ethidium bromide.

Adaptation of IBDV field isolates on chicken embryo fibroblast cell: Chicken embryo fibroblast (CEF) cells were prepared from 11 day embryonated egg [10].

Confluent monolayer of CEF cells at 24 hours after sub-culturing was used for infection with field IBDV. The CEF cells were infected by 0.5 ml of filtered IBDV 10% suspension. The inoculum was spread uniformly over the monolayer 25mm² tissue culture flasks and was gently rocked every 15 min for 1 hour. Cultures were incubated at 37°C in 5% CO_2 . After 48 hour of incubation, cells were scrapped and inoculated on cells having 70-80% confluency. In such a way seven passages were done and monolayers examined twice daily for cytopathic effects (CPEs). Cell control with no inoculum and inoculation control with 0.5 ml sterile PBS were also incubated and observed in parallel to test samples.

Tissue culture Infective dose 50 (TCID₅₀): The infectivity of adapted IBDV to CEF cells was determined by calculating 50% end point, using Reed and Muench formula [11]. Ten fold of serial dilution of IBDV was prepared in PBS from 10^{-1} to 10^{-10} . A 96 well tissue culture micro titration plate was used to prepare CEF cell mono layer. A 100 μl of each virus dilution was added in each well of first row leaving last two wells as negative controls. The plate was incubated at 37°C for 1 hour to allow adsorption. Then 100 μl of prewarmed maintenance medium was added in each well and again incubated at 37°C in 5% CO_2 . The plate was observed twice daily for CPEs. The CPEs were stained with 1% crystal violet solution. The highest dilution of virus showing 50% CPEs was considered as end point to calculate TCID₅₀.

Results and Discussion

Post-mortem: Out of 15 birds collected, twelve showed post-mortem lesion of petechial hemorrhages on thigh muscles, chest muscles hemorrhages (Figure-1a), hemorrhagic enlarged bursa (Figure-1b), swollen kidney and erosions at the juncture of the proventriculus and gizzard which support the findings of Rathore *et al.* [12] and Islam and Samad [13]. It is

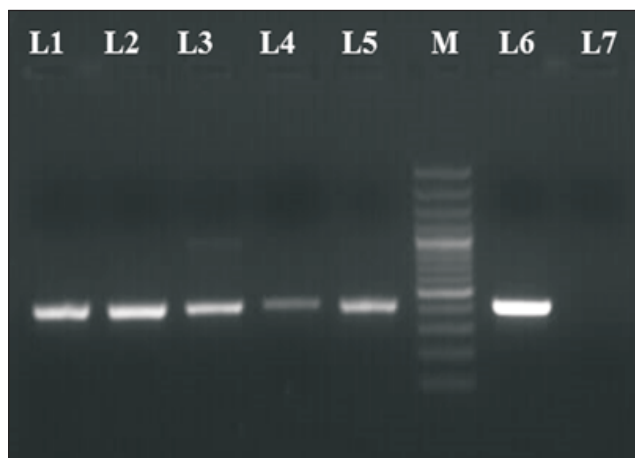


Figure-2: PCR amplification of VP2 gene fragment of IBDV Lane M: 100 bp plus DNA ladder (M/s MBI, Fermentas, Maryland, USA), Lane 1-5: Representative tissue samples Lane 6: Positive Control (IBD Vaccine) Lane 7: Non template control



Figure-3: CEF cells showing CPE, 48 hours post inoculation of IBDV on fourth passage indicated by arrows (black-plaque formation, yellow- rounding of cells, and red- clumping).

difficult to differentiate on the basis of symptoms as the clinical expression found in IBD is common with many other diseases [14]. Other viruses capable of causing similar clinical picture include chicken Infectious anemia, Mareks disease virus and avian reo virus [15]. Non-infectious causes include various mycotoxins, steroids therapy and poor nutrition.

RT-PCR amplification of VP2 gene: RT-PCR was used to detect and confirm IBDV in clinical field samples. Eleven samples were found positive by PCR amplification of VP2 gene out of the twelve samples. The target segment of VP2 gene is a hyper variable region and hence it may not amplify with a single primer set in every case. Distinct band of 480 bp (Figure-2) with different intensities were observed in agarose gel electrophoresis with suitable DNA ladder. Many other workers have verified the authenticity of PCR products by the size and nucleotide sequencing of the amplicons [16,17]. Recently, a study was carried out to investigate and characterize the nature of infectious bursal disease virus (IBDV) involved in the recurrent outbreaks in an experimental layer farm in Bareilly, Uttar Pradesh, India using direct tissue reverse transcription-polymerase chain reaction (RT-PCR) followed by nucleotide sequencing of VP2 gene [18]. Similar methods were used for detection and characterization of seven virulent IBDV in China

based on segment B of virus genome [19].

IBDV field isolates in chicken embryo fibroblast cells: Out of 11 sample positive for VP2 gene amplification, only five IBDV field sample were found to adapt on chicken embryo fibroblast (CEF) cells and further confirmed positive by RT-PCR for VP2 gene segment (480 bp) amplification. Virus isolated from the field did not show changes in CEF cells; however virus showed CPE from 4th passage onwards on CEF cells and produced sequence of changes in CEF cells. The typical CPEs like rounding of fibroblast cells, cellular detachment and vacuolation were recorded at 48 hours post of inoculation (Figure-3). Khan *et al.* [7] recorded similar CPEs from third to seventh passage. Hossain *et al.* [20] found clear and optimum CPEs after 144 hour of incubation following infection. CPEs of egg adapted strain of IBDV in CEF cells, Vero cells and Baby hamster kidney (BHK) cells were observed following 36 to 40 hour of infection during 4th passage by Simoni *et al.* [21]. Mohammed *et al.* [22] used chicken mesenchymal stem cells for adaptation of virulent IBDV and observed CPE at 48 h post-infection (p.i.) in first passage, the CPE was characterized by rounding up of cells and monolayer detachment, intracytoplasmic brownish colouration was readily observed from 24 h p.i onwards [22]. The total infectious titer of CEF cell adapted IBDV (4th passage) was found to 1.46

$\times 10^7$ TCID₅₀/ml after 48 hours of infection. Kibenge *et al.* [23] also reported similar findings, when they observed growth pattern of five strains of serotype 1 and 2 and variant strains of IBDV in Vero cells. They found titers ranged from 6.85 to 8.35 log₁₀ TCID₅₀/ml in Vero cells after 48 hours of infection, while from 5.35 to 6.10 log₁₀ TCID₅₀/ml in CEF at 72 hours post-infection.

Conclusion

IBDV is the causative agent of a highly immunosuppressive Gumboro disease of the domestic fowl. Among the viral proteins, VP2 is the major structural protein and its hypervariable region (480 bp) is a good target for the molecular techniques applied for IBDV detection and inhibition assay. In the present study RT-PCR was used for the detection and confirmation of field isolate of IBDV in Jabalpur. VP2 gene sequences can also be used for genetic typing, tracing the origin and spread of virus. The epidemiological data thus generated can be used to design the control and eradication strategies. These field isolates were adapted in CEF cell lines and showed characteristic CPEs on 4th passage onwards upto 7th passage carried in the present study. Passaging of highly virulent IBDV field isolates in cell culture leads to attenuation of virus which can be exploited as a cell culture adapted vaccine candidate and mass production of test antigen for use in diagnostics.

Authors' contributions

VKS and RK collected the samples, carried out postmortem and processed the samples for virus and nucleic acid isolation. YS and KY isolated the nucleic acid and carried out RT-PCR and adaptation of virus. All authors participated in draft and revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful to Dr. Megha Kadam Bedekar, Scientist, Animal Biotechnology Centre for providing her able guidance to this work and for scientific review and editing. All authors are thankful to Director, Animal Biotechnology Centre, Nanaji Deshmukh Pashu-Chikitsa Vigyan Vishwavidyalaya, Jabalpur, India for funding the work and extending the full support while using facilities for the completion of the work.

Competing interests

The authors declare that they have no competing interests.

References

- Rashid, M.H., Chuniy, X., Islam, M.T., Islam, M.R., Zheng, and Yongchang, C. (2013) Comparative epidemiological study of infectious bursal disease of commercial broiler birds in Bangladesh and China. *Pak. Vet. J.*, 33(2): 160-164.
- Luque, D., Saugar, I., Rejas, M.T., Carrascosa, J.L., Rodriguez, J.F. and Caston, J.R. (2009) Infectious bursal disease virus: ribonucleoprotein complexes of a double-stranded RNA virus. *J. Mol. Biol.*, 386: 891-901.
- Delgui, L., Ona, A., Gutierrez, S., Luque, D., Navarro, A., Caston, J.R. and Rodriguez, J.F. (2009) The capsid protein of infectious bursal disease virus contains a functional alpha 4 beta 1 integrin ligand motif. *Virology*, 386: 360-372.
- Kumar, S., Ahi, Y.S., Salunkhe, S.S., Koul, M., Tiwari, A.K., Gupta, P.K. and Rai, A. (2009) Effective protection by high efficiency bicistronic DNA vaccine against infectious bursal disease virus expressing VP2 protein and chicken IL-2. *Vaccine*, 27: 864-869.
- Heine, H.G., Haritou, M., Failla, P., Fahey, K. and Azad, A. (1991) Sequence analysis and expression of the host protective immunogen VP2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type 1 strains. *J. Gen. Virol.*, 72: 1835-1843.
- Olivier, E., Cyril, L.N., Michel, A., Xavier, A., Kristen, M.O., Olivier, G., Didier, T., Hermann, M., Mohammed, R. I. and Nicolas, E. (2013) Both genome segments contribute to the pathogenicity of very virulent infectious bursal disease virus. *J. Virol.*, 87(5): 2767-2780.
- Khan, M.A., Hussain, I., Siddique, M., Rahman, S.U. and Arshad, M. (2007) Adaptation of a local wild infectious bursal disease virus chicken embryo fibroblast cell culture. *Int. J. Agri. Biol.*, 9: 925-927.
- Barlic, M.D., Zorman, R.O. and Grom, J. (2002) Detection of infectious bursal disease virus in different lymphoid organs by single-step reverse transcription polymerase chain reaction and micro-plate hybridization assay. *J. Vet. Diagn. Invest.*, 14: 243-246.
- Sambrook, J. and Russel, D.W. (2001) Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, New York. p460-473.
- Cunningham, C. H. (1973). A laboratory guide in virology. Burgess Publishing Co. Minnesota. 7th Edn. p155-169.
- Reed, L. J. and Muench, H. (1938) A simple method for estimating fifty percent end points. *Ame. J. Hyg.*, 27: 493-497.
- Rathore, S., Kumar, A., Thakur, P., Kumari, N., Meena, M. and Nimje, P. (2013) Pathological Study of infectious bursal disease in broilers of Haryana. *J. Immunol. Immunopathol.*, 15(1): 62-65.
- Islam, K. M. D. (1999) Adaptation of reovirus on Vero cell line. B.Sc. Thesis, Biotechnology Discipline, Khulna University, Khulna, Bangladesh.
- Engstrom, B., Eriksson, H., Fossum, O. and Jansson, D. (2003) *Fjaderfasjukdomar*, Sveriges Veterinärmedicinska Anstalt.
- Sharma, J.M. (2003) Immunity and Immunosuppressive Diseases of Poultry, Convention notes *American Association of Veterinary Medicine/World Veterinary Poultry Association*.
- Tao, Q., Wang, X., Bao, H., Wu, J., Shi, L., Li, Y., Qiao, C., Yakovlevich, S.A., Mikhaylovna, P.N. and Chen, H. (2009) Detection and differentiation of four poultry diseases using asymmetric reverse transcription polymerase chain reaction in combination with oligonucleotide microarrays. *J. Vet. Diagn. Invest.*, 21: 623-632.
- Amara, A., Akeila, M. and Khalil, S. (2011) Detection and isolation of infectious bursal disease virus from the field. *Alexandria Journal of Veterinary Sciences (AJVS)*, 34 (1): 185-197.
- Barathidasan, R., Singh, S. D., Kumar, M. A., Desingu, P. A., Palanivelu, M., Singh, M. and Dhama, K. (2013) Recurrent outbreaks of infectious bursal disease (IBD) in a layer farm caused by very virulent IBD virus (vvIBDV) in India: pathology and molecular analysis. *South Asian Journal of Experimental Biology (SAJEB)*, 3(4): 200-206.
- Yu, F., Qi, X., Yuwen, Y., Wang, Y., Gao, H., Gao, Y., Qin, L. and Wang, X. (2010) Molecular characteristics of segment B of seven very virulent infectious bursal disease viruses isolated in China. *Virus Genes* 41: 246-249.
- Hossain, S.K.A., Uddin, S.N., Rahman, M.S., Wadud, A. and Khan, M.H. (2006) Propagation of infectious bursal disease

- virus (IBDV) in chicken embryo fibroblast cells. *J. Biol. Sci.*, 6: 146-149.
21. Simoni, I.C., Custodio, R.M. and Fernandes, M.J.B. (1999) Susceptibility of cell lines to avian viruses. *Rev. Microbiol.*, 30: 9-13.
22. Mohammed, M. H., Hair-Bejo, M., Omar, A. R. and Aini, I. (2012) Replication of very virulent infectious bursal disease virus in the chicken mesenchymal stem cells. *J. Adv. Med. Res.*, 2(1): 1-7.
23. Kibenge, F. S. B., Dhillon, A.S. and Russell, R.G. (1988). Growth of serotype-I and II and variant strains of infectious bursal disease virus in Vero cells. *Avian Dis.*, 32: 298-303.
