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ORIGINAL ARTICLE

Heterologous expression of Infectious bursal disease virus VP2 gene in *Chlorella pyrenoidosa* as a model system for molecular farming

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Abstract Infectious bursal disease is a highly contagious and immunosuppressive disease of chickens. Available vaccines induce some sort of stress during vaccination program in chickens. In the present study, an attempt was made to overexpress IBDV VP2 in edible algae. These algae expressing IBDV VP2 could be used to vaccinate animals in stress free environment. IBDV VP2 over expression was carried under CaMV35S promoter and the gene expression cassette was cloned into pART27 binary vector. IBDV expressing construct was transformed into *Chlorella pyrenoidosa* through *Agrobacterium tumefaciens* mediated gene transfer. Transformed *C. pyrenoidosa* cells were selected through PCR screening test. Further, expression of IBDV VP2 protein in transformed *C. pyrenoidosa* was confirmed by western blot analysis to ensure the production of IBDV VP2 protein. This study will provide a new platform to generate oral vaccine against IBDV by using *C. pyrenoidosa* as model platform.

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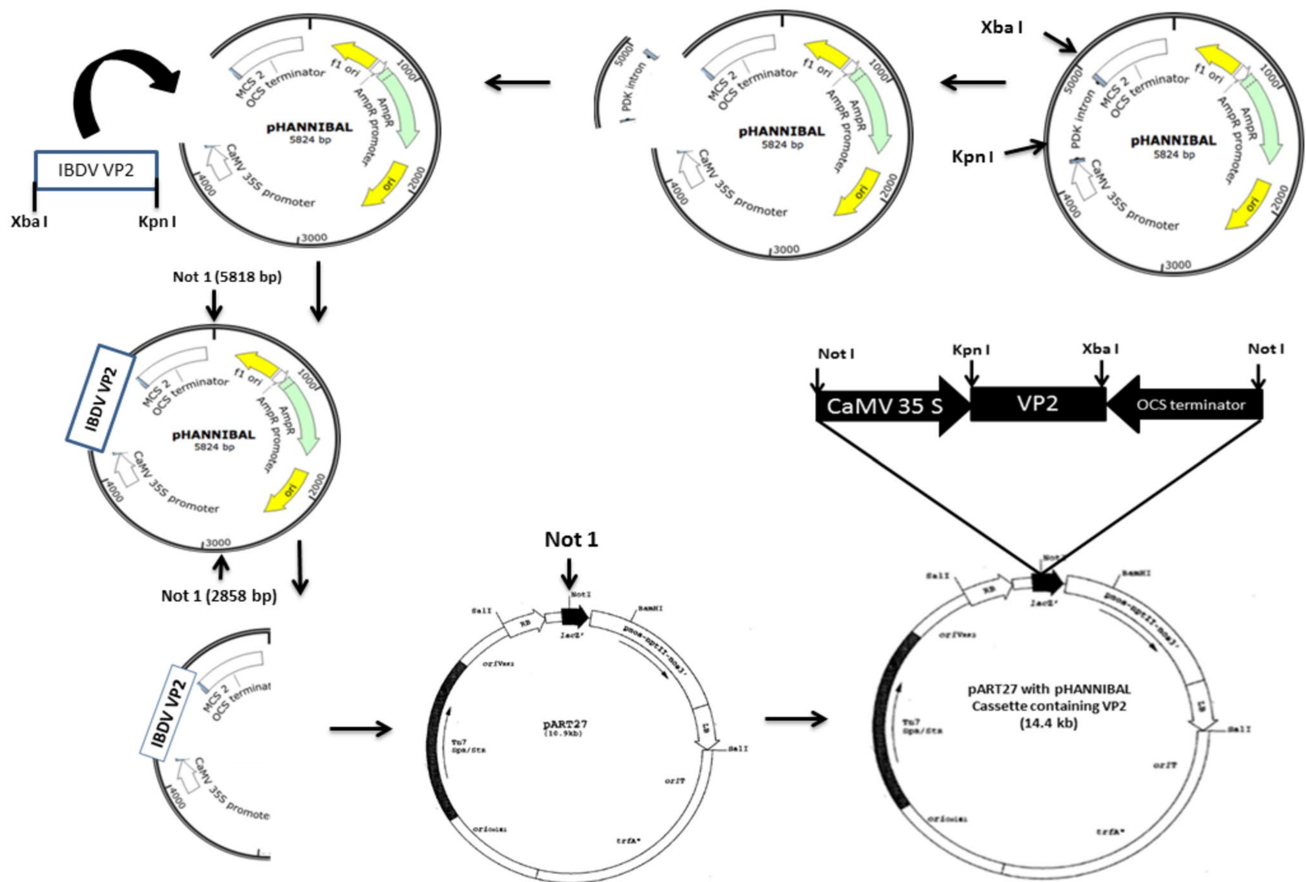
Graphical Abstract



Keywords *Agrobacterium tumefaciens* · *Chlorella* · Edible vaccine · Infectious bursal disease virus (IBDV) · Microalgae · Transformation

Introduction

Infectious bursal disease virus (IBDV), a non-enveloped icosahedral dsRNA virus is an etiological agent of a highly contagious immunosuppressive disease called Gumboro disease. It affects young birds of 3–6 weeks causing severe losses in Poultry (Wyeth and Cullen 1976). IBDV has two serotypes 1 and 2, but only serotype 1 is pathogenic in chickens (Lukert and Saif 2003). Serotype 1 viruses replicate in the bursa of Fabricius. Presence of Antibodies or virus is found in other avian species without any signs of infection. Serotype 2 viruses are considered to be cosmopolitan when compared to serotype 1 and were detected in the respiratory tract of turkeys, cloacal swabs of ducks or in the bursae of Fabricius of chickens (OIE 2016). There is no report of clinical disease caused by infection with serotype 2 virus (Eterradossi and Saif 2013). Infection of IBDV leads to destruction of B-lymphocytes in the bursae of Fabricius, which ultimately results in infiltration of T-cells and immunosuppression. IBDV genome consists of two segments, Segment-A has two open reading frames (ORFs), ORF1 encodes a non-structural protein VP5 protein, whereas ORF2 encodes 115 kDa precursor polyprotein, which is post translationally modified into VP2, VP4 and VP3 (Mundt et al. 1995). Segment-B encodes RNA Polymerase (VP1) of 90 kDa size. Serotype specific antigenic determinants are located on VP2 which are key to induce neutralizing antibodies (Snyder et al. 1988).

As there is a thrust for new vaccination strategies to prevent viral epidemics, recombinant VP2 was expressed in a baculovirus system which conferred protection in young chickens against virulent IBDV upon vaccination (Vakharia et al. 1994). Apart from baculovirus, other heterologus systems like Fowlpox virus (Shaw and Davison 2000), Herpes virus (Perozo et al. 2009), Adenovirus (Perozo et al. 2008), *Escherichia coli* (Rong et al. 2007), *Pichia pastoris* (Pitcovski et al. 2003) and plant viruses (Chen et al. 2012) were also used for expression of IBDV VP2 protein. Traditional oral vaccines induce stress during administration and provide serotype specific

immunity but edible vaccines will provide stress free administration of vaccine and also immunization against specific virus.

It is well documented that proteins expressed *Inplanta* are capable of immunization against particular protein, when administered through parenteral routes or oral routes (Lucero et al. 2016). Production of protein-based pharmaceuticals is slowly drifting to plants and plant cell cultures from bacterial, fungal, and mammalian cell cultures (Twyman et al. 2005; Lico et al. 2012; Ghiasi et al. 2012; Merlin et al. 2014; O'Neill et al. 2008). A number of recombinant proteins have been produced in plants for instance, bovine trypsin in maize, TrypZean (Sigma-Aldrich); human lysozyme and lactoferrin in Rice (Yang et al. 2003; Hennegan et al. 2005); plant-based biopharmaceuticals in transgenic carrot and tobacco cells (Zimran et al. 2011; Van Dussen et al. 2013). Recombinant subunit vaccines are free from live pathogens and may contain part of the pathogen not the whole pathogen itself. Hence recombinant subunit vaccines are safer when compared to traditional vaccines. Various plants such as tobacco (Huy et al. 2016; Romero Maldonado et al. 2016), rice (Kim et al. 2016a, b), maize (Rosales Mendoza et al. 2017), potato (Rukavtsova et al. 2015; Kim et al. 2016a, b), alfalfa (Aguirreburualde et al. 2013), lettuce (Yiu et al. 2013), tomato (Gerszberg et al. 2015), carrot (Monreal-Escalante et al. 2016), peanut (Khandelwal et al. 2011) and soybean (Hudson et al. 2014) are used as platforms for production of edible vaccines and antibodies. Recently Habibi et al. (2017) reviewed optimized internal and external conditions needed for expression of recombinant proteins in plant systems. Optimization of Apart from plants and plant cells, recent studies on microalgae proved their efficacy for expression of recombinant proteins (León-Bañares et al. 2004). First plant based edible vaccine for chickens against NDV was developed by Dow AgroSciences LCC (Indianapolis, IN, USA). Successful *Agrobacterium* mediated transformation and expression of recombinant proteins in microalgal systems such as *Schizochytrium* species (Cheng et al. 2012) and *Chlamydomonas reinhardtii* (Kumar et al. 2004) paved a way of micro algal reactor for production of recombinant proteins. Previously Japanese flounder fish growth hormone gene and human growth hormone were expressed in *Chlorella ellipsoidea* (Kim et al. 2002) and *Chlorella* species (Hawkins and Nakamura 1999) respectively. Hence, *Chlorella* can be used as chassis for the production of recombinant viral proteins.

Expression of IBDV VP2 protein in *C. pyrenoidosa* will provide transient approaches for cutting edge research in stress free viral immunization for chickens. The present study was carried out to standardize the conditions for transient expression of IBDV VP2 gene in *C. pyrenoidosa* as a model expression system.

Materials and methods

Sample collection and total RNA extraction

Birds suspected for IBDV infection were collected from local poultry farms and were confirmed at Department of Veterinary Pathology, S. V. Veterinary University, Tirupati by pathological analysis. The characteristic hemorrhagic infected bursa were collected and stored at −80 °C freezer in buffered saline for further use. Total RNA was extracted from these samples using TRI Reagent (Ambion, Life Technologies, USA) following manufacturer's instructions and stored at −80 °C freezer.

PCR amplification, cloning and sequencing of IBDV VP2 gene

Complementary DNA (cDNA) was synthesized from isolated total RNA using RevertAid Reverse transcriptase enzyme (Thermo Scientific, USA) as per the manufacturer's protocol. PCR amplification of the full length VP2 gene was done using Forward primer (5′CCC*GGTACC*ATGACAAACCTGCAAGATCAAACCC-3′) and Reverse primer (5′AAA*TCTAGA*TTACCTTAGGGCCCGGATTATGTC-3′) with *Kpn*I and *Xba*I restriction sites. PCR amplification was standardized in a 50 µl reaction mix comprising of 1× *Taq* DNA Polymerase buffer (20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20.), 0.2 mM dNTPs, 10 pMol of each of Primers Forward and Reverse, 1.5 mM $MgCl_2$, 20 ng of cDNA as template and 2.5 U of Recombinant *Taq* DNA Polymerase (Thermo Fermentas, USA). Thermal cycling was programmed in Corbett Gradient Thermal Cycler (Corbett Research, USA) with initial denaturation of 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 2 min, and final extension at 72 °C for 10 min. Amplified products were resolved on a 1% agarose gel and documented by using gel documentation system (UVP, USA). The IBDV VP2 amplicon was eluted from agarose gel using QIAquick Gel Extraction Kit (QIAGEN, USA). The purified PCR fragments were cloned into pTZ57 R/T vector by using InsTAclone PCR cloning kit (Thermoscientific, USA) according to the manufacturer's protocol. Recombinant Plasmids were confirmed for presence of IBDV VP2 gene by PCR. Positive plasmid was sequenced at SciGenom Pvt. Ltd. India using M13 universal primers. Sequence homology analysis was done using BLAST Tool. Unrooted Neighbor joining phylogenetic trees were constructed using Mega 7 tool.

Construction of pART27 binary IBDV VP2 expression vector and transformation

IBDV VP2 gene was released by digesting TA clone of IBDV VP2 with *Kpn*I and *Xba*I restriction enzymes. pHANNIBAL Gateway cloning vector (provided by Prof. Indranil Dasgupta, University of Delhi, South Campus) was also digested with *Kpn*I and *Xba*I which resulted in release of the PDK intron. The *Kpn*I and *Xba*I digested IBDV gene product was ligated into *Kpn*I and *Xba*I digested pHANNIBAL gateway cloning vector to construct the IBDV VP2 expression cassette. This expression cassette was subcloned into pART27 binary vector using *Not*I digestion of both pHANNIBAL (IBDV-VP2) and pART27 (provided by Prof. Peter Waterhouse, Sydney). This recombinant plasmid was then transformed into *Agrobacterium tumefaciens* strain LBA4404 using $CaCl_2$ method (Jefferson et al. 1987). Positive *Agrobacterium* colonies were confirmed by VP2 PCR and *Not*I restriction digestion. *A. tumefaciens* harbouring the pART27 vector with IBDV VP2 cassette containing CaMV35S promoter was used for transformation into *C. pyrenoidosa*. The cloning strategy is represented as graphical abstract.

Agrobacterium mediated transformation of *C. pyrenoidosa*

Cultivation and maintenance of C. pyrenoidosa

Chlorella pyrenoidosa was obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratories (NCL), Pune, India. The culture was maintained in liquid and solid media containing Algae culture broth (ACB) (Himedia, Mumbai, India). Cultures were grown under continuous fluorescent light with 50 ± 5 µmol m^{-2} s^{-1} at 25 °C and agitated thrice a day for 1 min at 180 rpm.

Transformation of C. pyrenoidosa and selection of transformants

Transformation was carried out by co-cultivating *A. tumefaciens* harbouring recombinant IBDV VP2 pART27 clone with *C. pyrenoidosa* as reported by Kumar et al. (2004) and San Cha et al. (2012) with slight modifications. *A. tumefaciens* was, grown to OD of 0.5–1.0 in YEB medium at 600 nm. Cells were harvested by centrifugation at 1500×*g* and resuspended in ACB medium containing 100 µM acetosyringone (Sigma) to make it to final density of 0.5 OD at 600 nm. The bacterial suspension (~200 µl) was added on to the thin layer of *Chlorella* cells grown on solid ACB agar plates and co-cultivated for 3 days at 25 °C in dark. After co-cultivation, the algal cells were harvested and washed twice with liquid ACB broth containing cefotaxime (Himedia) by centrifugation at 1500×*g* for 5 min and resuspended in liquid ACB medium. The resuspended *Chlorella* was inoculated onto solid ACB medium containing kanamycin and cefotaxime and incubated at 25 °C under continuous fluorescent light with 50 ± 5 µmol m^{-2} s^{-1} for 15 days.

Total DNA was extracted from the selected transformants of *C. pyrenoidosa* grown in of ACB broth containing cefotaxime and Kanamycin according to Koboyashi et al. (1998). Total RNA was isolated from cell pellet of the algal culture using TRI Reagent (Ambion, Life Technologies). Complementary DNA (cDNA) was synthesized from isolated RNA using RevertAid Reverse Transcriptase (Thermo Scientific) as per the manufacturer's protocol. PCR was performed for the isolated total DNA and cDNA using IBDVVP2 specific primers.

Confirmation of IBDV-VP2 expression by western blot

Initially algal cells containing recombinant gene (IBDV VP2) were pelleted out and lysed using liquid nitrogen in a ceramic motor. Protein re-suspension buffer (50mM Tris, 400 mM NaCl, 0.1% Tween 20 and 0.1% PMSF) was added to lysed cells to extract total proteins. The resultant homogenate was centrifuged at 12,000×*g* at 4 °C for 15 min to remove debris and supernatant was used for IBDV VP2 protein analysis. Protein concentrations were determined by Bradford method (Bradford 1976). Similarly, proteins were extracted from empty vector transformed *Chlorella* were considered as control. These protein samples were used for identification of recombinant IBDV VP2 protein by Western blotting.

Total proteins isolated from algae were subjected to SDS-PAGE followed by electro-blotting onto nitrocellulose (NC) membrane (Hybond, GE healthcare life sciences) using Semi-dry Transfer Blotter (Amersham; GE healthcare life sciences) according to manufacturer's instructions. After transfer, membrane was blocked with PBS containing 5% skimmed milk and further incubated with anti IBDV antiserum at 1:500 dilution in PBS containing 5% skimmed milk for overnight at 37 °C. After two washes with 0.1% PBS-T for 5 min each, the membrane was then incubated with secondary antibody Anti-Chicken IgY (IgG)-Alkaline Phosphatase in rabbit (Sigma) at 1:5000 dilution in PBS and incubated for 1 h at 37 °C. After three washes with PBS-T, the membrane was developed by adding BCIP/NBT (Thermo Scientific) until the bands became visible.

Results and discussion

During the present study, IBDV VP2 gene was expressed in *C. pyrenoidosa* for its use as a potential oral vaccine

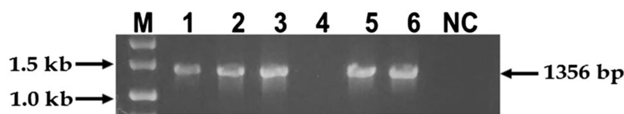


Fig. 1 Identification of IBDV infected birds from suspected field isolates using PCR. *Lane M* Marker; *Lanes 1–6* IBDV suspected samples; *Lane NC* negative control

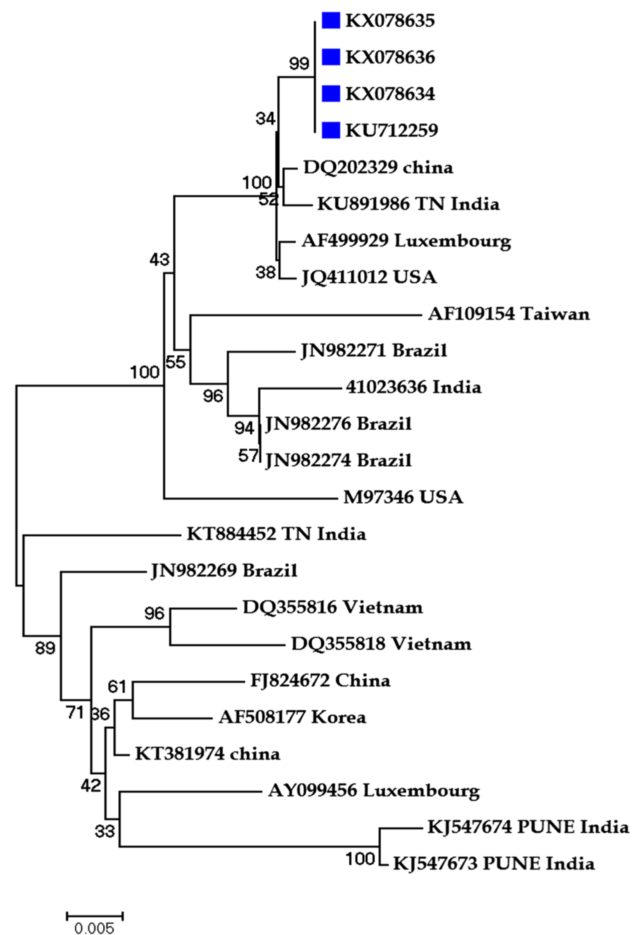


Fig. 2 Phylogenetic analysis of cloned VP2 sequence along with other VP2 sequences available in GenBank

in the future. Initially bursae from the symptomatic birds were collected and confirmed pathologically. IBDV symptoms included hemorrhages on bursa and thigh muscles (Fig. S1a, b). Reports reveal that the IBDV infected birds become propagators of the virus (Ramirez-Nieto et al. 2010).

A set of primers were designed to clone the major host-protective immunogen of IBDV i.e. the VP2 itself (Brandt et al. 2001). Besides VP2 has the determinants for cell tropism with at least two neutralizing epitopes and is recognized by neutralizing antibodies (Azad et al. 1987; Becht et al. 1988; Fahey et al. 1989; Heine and Boyle 1993). VP2 induces virus-neutralizing Abs that protect susceptible chickens from IBDV. An IBDV VP2 gene product of 1356 bp was amplified from total RNA isolated from IBDV infected chickens (Fig. 1). The amplicon was cloned into PTZ57R/T vector using InsTA cloning Kit and transformed into *E.coli* DH5-α. Positive colonies harbouring IBDV VP2 amplicon were selected by colony PCR. Recombinant plasmids isolated from the clones were sequenced at SciGenome Pvt. Ltd. India.

Analysis of nucleotide sequence revealed ~95% homology with reported VP2 sequences at nucleotide level. Cloned VP2 sequences obtained from IBDV infected chickens were deposited in GenBank with Accessions KU712259, KX078636, KX078635 and KX078634. Unrooted Neighbor joining tree constructed revealed that DQ202329.1 and KU891986.1 were more closely related to the sequences of study (Fig. 2). KU891986.1 was submitted from Tamil Nadu, which is geographically closer to Tirupati and hence may have evolved due to its prevalence in Tirupati region.

The cloned amplicon of IBDV VP2 was released from PTZ57R/T vector by digesting with *Kpn*I and *Xba*I enzymes (Fig. S2). IBDV VP2 digested amplicon was cloned into pHANNIBAL vector between CaMV35S promoter OCS terminator upon digestion with *Kpn*I and *Xba*I enzymes (Fig. S3a). The IBDV VP2 gene expression cassette was released from pHANNIBAL by *Not*I restriction digestion (Fig. S3a).

Expression cassette of pHANNIBAL was ligated into *Not*I RE digested pART27 (Fig. S3b). Ligation of pHANNIBAL expression cassette to pART27 was confirmed by RE digestion. pART27 is a commonly used plant expression vector for introduction of transgene into plants through *A. tumefaciens* (Gleave 1992). *A. tumefaciens* harbouring the pART27 with IBDV VP2 cassette was confirmed by colony PCR and used for transformation into *C. pyrenoidosa. Chlorella pyrenoidosa* as a chassis for production of IBDV VP2 provides an opportunity to produce IBDV VP2 protein in large amounts and also for stress free administration of vaccine against IBDV. Besides, *C. pyrenoidosa* is a rich source of proteins and fatty acids, which provide better nutrition values to their diet when combined and hence used as a single cell protein. Cefotaxime was used to eliminate the *Agrobacterium* present after post transformation. Cefotaxime, Kanamycin concentrations used in the present study was selected, based on the previous studies of Cha et al. (2012) and Kumar et al. (2004). Initially, PCR was performed to confirm T-DNA integration to resuspended *C. pyrenoidosa* genome using IBDV VP2 gene specific primers.

After 15 days growth of *C. pyrenoidosa*, total DNA, RNA and protein were isolated and checked for IBDV

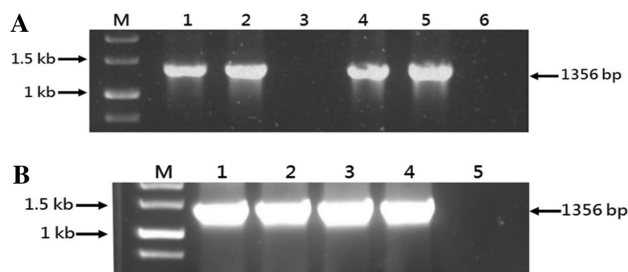


Fig. 3 **a** Detection of VP2 gene in transformed *C. pyrenoidosa* colonies. *Lane M* marker, *Lanes 1–5* PCR confirmations for VP2 gene in transformed colonies. *Lane 6* PCR confirmation for VP2 gene in pART27 empty vector transformed colonies (Negative control). **b** Semiquantitative RT-PCR confirmation for VP2 mRNA in VP2 transformed *C. pyrenoidosa. Lane M* marker, *Lanes 1–4*: Lane 5: negative control

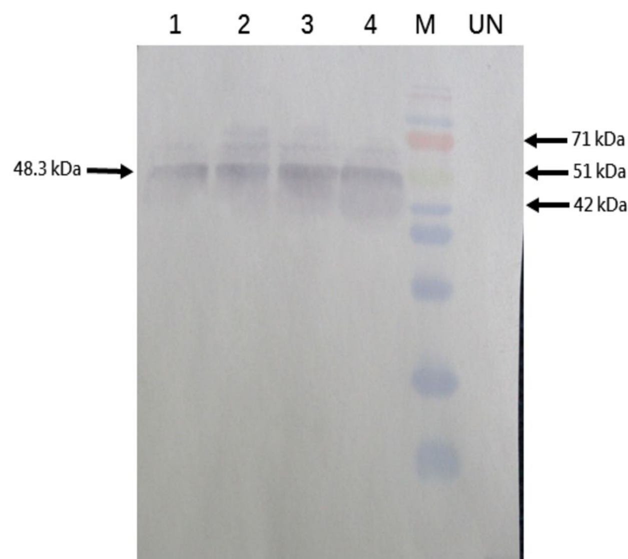


Fig. 4 Western Blot for VP2 protein in total proteins isolated from transformed *C. pyrenoidosa* containing VP2 gene *Lane M* Puregene 4 color prestained protein ladder, Genetix; Proteins isolated from PCR and RT-PCR positive transformed algal colonies (*Lane 1–4*) and negative control (*Lane C*)

VP2 and protein expression using RT-PCR and western blot analysis respectively. RT-PCR amplification of the transformed *C. pyrenoidosa* total RNA and PCR amplification of the total DNA revealed a band of 1356 bp length (Fig. 3a, b). Western blot analysis revealed 49 kDa band which is specific to IBDV VP2 protein (Fig. 4). 49 kDa protein identified corresponds to IBDV VP2 protein as the molecular weight of the protein calculated was similar, which is calculated by using *In silico* softwares. Confirmation of IBDV VP2 by western blot proves that *C. pyrenoidosa* as a promising candidate for production of transgene which are off pharmaceutical and economical importance.

Conclusion

Expression of IBDV VP2 protein in *C. pyredinosa* via *Agrobacterium* mediated transformation will provide transient approaches for cutting edge research in stress free IBDV immunization for chickens. *C. pyrenoidosa* as model for molecular farming for heterologous expression of IBDV VP2 protein could be exploited as edible vaccine. Further, this study will provide foundation for the development of edible vaccine against IBDV using algae as protein expression platform.

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Compilance with ethical standards

Ethical approval The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals and approved by the Institutional Animal Ethical Committee, Committee for the Purpose of Control and Supervision on Experiments on Animals (2003). Sri Venkateswara University, Tirupati (Regd. No. IAEC/No438/01/a/CPCSEA dt: 04.03.2002)

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