Short communication

Cloning and expression of VP2 gene of Infectious bursal disease virus in eukaryotic cells

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Abstract
Infectious bursal disease (IBD) is an economically important viral disease of chickens with worldwide distribution which suppresses the immune system of young chickens. VP2 is the major host-protective protein of infectious bursal disease virus (IBDV). The encoding region of VP2 protein was PCR amplified from a plasmid containing a cDNA fragment of large genomic segment of IBDV, strain D78. This region of 1356 bp was inserted into a eukaryotic expression plasmid, pCDNA4, under the control of human cytomegalovirus (hCMV) immediate early enhancer and promoter. Plasmid DNA was transfected into COS-7 cell line and transient expression of VP2 from the constructed plasmid was characterized by dot blotting with a polyclonal antibody to IBDV.
Keywords: Infectious bursal disease; IBDV; VP2; eukaryotic expression; pCDNA4; COS-7 cell line.

Infectious bursal disease (IBD), also popularly known as Gumboro disease, is an acute, contagious viral disease of chicken (Kibenge *et al*., 1988), caused by a double-stranded RNA virus of the *Birnaviridae* family (Murphy *et al*., 1995). The genome of IBD virus (IBDV) consists of two segments, designated A and B. Segment A contains two overlapping open reading frames (ORF). The larger ORF of the segment encodes a polyprotein consisting of VP2, VP4 and VP3. VP2 is a major capsid protein eliciting neutralizing antibodies. Segment B codes for a 97 kDa protein, designated as VP1, which represents the viral RNA-dependent RNA polymerase (Kibenge *et al*., 1991).

IBDV targets the lymphoid tissue of chickens, mainly the bursa of fabricious, causing severe bursal damage, and consequently suppresses the immune system. Thus, IBD is of major economic importance to the poultry industry. Following the appearance of so virulent strains of IBDV, it was evident that conventional IBDV vaccines could not protect chickens and therefore, less attenuated intermediate and hot vaccines were developed. These vaccines may have some pathogenic characters and induce moderate bursal atrophy. In addition, they might be able to revert to a virulent state (Tsukamoto *et al*., 1995; Lukert and Saif, 1997).

In order to circumvent the potential disadvantages of live vaccines against IBDV, many studies have been performed to develop more potent vaccines, based on recombinant DNA technology (Jagadish *et al*. 1988, Goudarzi *et al*., 2006; Shaw and Davison, 2000).

In the 1990s, an entirely new type of vaccine (DNA vaccines) was first described (Wolff *et al*., 1990;Robinson *et al*., 1993; Ulmer *et al*., 1993). These new vaccines used naked plasmid DNA to express foreign proteins in the host. DNA vaccines are specially modified bacterial plasmids that usually have an *Escherichia coli* origin of replication, an antibiotic resistant gene, eukaryotic promoter that drives the expression of the target gene, a target gene and a polyadenylation signal sequence. The target gene usually codes for an antigenic protein from a pathogenic infectious organism. Since these initial reports on this

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novel vaccine technology, DNA vaccines have been successfully used to immunize a number of different animal species against a multitude of infectious agents (Corr *et al*., 1996; Sakaguchi *et al*., 1996; Scholz *et al*., 1993; Fynan *et al*., 1993) DNA vaccines have also been successfully used in poultry to immunize against several pathogens (Robinson *et al*., 1993; Sakaguchi *et al*., 1996; Fynan *et al*., 1993).

E. coli strain TOP10F' was cultured routinely at 37 ºC in broth or on agar plates of LB medium supplemented, with 50 µg/ml ampicillin, if required . A plasmid DNA (pTZ57RVP2) containing the VP2 cDNA of IBDV, strain D78 (Goudarzi *et al*., 2006)) was used for PCR amplification of VP2 cDNA. A eukaryotic expression vector, pCDNA4, was applied for cloning and expression of VP2 gene in eukaryotic cells. COS-7, a fibroblast-like cell line, transformed by an origin-defective mutant of SV40 which codes for wild-type T antigen of SV40 was used in transfection studies. This cell line was cultured in Dolbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, maintained at 37°C and 5% CO_2.

The plasmid DNA pTZ57RVP2 was extracted from bacterial strain TOP10F' by Roche Plasmid Mini preparation Kit. The VP2 coding region of this plasmid was amplified by forward (5′-GCCGGAATTCATGACAAACCTGCAAGAT-3′) and reverse (5′-GCCGTCTAGAAACCTTATGGCCCGGAT-3′) oligonucleotide primers designed for cloning of the gene in pCDNA4 plasmid, downstream of a 6× Histidines tag sequence. The first ATG and the C-terminal stop codons of VP2 are underlined. For directional cloning, *EcoR*I and *Xba*I restriction sites were added at the 5′ ends of the forward and reverse primers, respectively. The cycling parameters were an initial denaturation at 100ºC for 5 min followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 58ºC for 1 min and extension at 72ºC for 1 min. The final cycle was followed by a long extension at 72ºC for 10 min. The amplified VP2 fragment (1365bp) and pCDNA4 vector were digested with *EcoR*I and *Xba*I and ligated after purification from a 1% agarose gel. The resulting DNA construct was designated as pCDNA4VP2 and used for transformation of competent TOP10F' *E. coli* cells. In order to confirm that no errors were introduced as a result of PCR amplification, complete sequence of VP2, inserted in pCDNA4 was determined by sequencing from two directions by vector specific primers and compared with the sequence of VP2 gene of IBDV, strain D78 (accession no. AF499929).

COS-7 cells were transfected by pCDNA4VP2 to verify protein expression. The cells cultured in 25cm^3 flasks were transfected with 20 µg plasmid DNA, using lipofect™ Transfection Reagent (Qiagene), as the manufacturer instructions. To verify expression, several clones were tested, due to the differences in expression levels of clones.

The cells were maintained as described above and zeocin was used for selection, 24 h post-transfection. The monolayers were then incubated for another 24 h to obtain maximum expression of VP2.

Twenty four hours after antibiotic selection, liopfected COS-7 cells were harvested by scraping, suspended in PBS and pelleted at 1200 *xg*. After three cycles of freeze-thaw to disrupt the cells, a short centrifugation was performed and the supernatant was used as antigen in dot blotting. Nitrocellulose membranes dotted with the antigen were blocked with 5% skim milk in PBS. VP2 protein was detected using a polyclonal chicken anti-IBDV serum (1:50 dilution), a goat anti-chicken IgG (H+L) peroxidase conjugate (1:30 dilution) and 4-chloro-1 Naphtol (Sigma, USA) peroxidase substrate. Polyclonal and conjugate sera have been produced by KPL company (USA). COS-7 cells, transfected with pCDNA4 were treated in the same manner and used as negative control.

The complete part of segment A encoding the VP2 protein of IBDV strain D78 was successfully amplified with designated primers, from the plasmid pTZ57RVP2. The amplified fragment was ligated to pCDNA4 vector and used for transformation of *Ecoli* strain TOP10F'. The identity of the insert was confirmed by sequencing. Alignment of the insert sequence with the original sequence of VP2 gene of IBDV strain D78 (AF499929) did not show significant differences (Fig. 1).

In order to determine the expression of VP2 in the eukaryotic system, pCDNA4VP2 construct was lipofected in COS-7 cells. The protein expression was detected by dot blotting, on the lysate of the cells, using a polyclonal anti-IBDV chicken serum. The strong reaction of antiserum with the lysate of cells lipofcted with pCDNA4VP2, compared to reaction with lysate of cells lipofected with pCDNA4 (the control), indicated that the protein was successfully expressed (Fig. 2).

IBDV still remains a serious problem for commercial broiler producers. Vaccination is the major tool for the prevention and control of IBD in the poultry industry, but the chickens vaccinated with conventional IBDV vaccines are not fully protected against chal-

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RT-PCR            ------------------------------------------------------------
RESULT.TXT   1    ACNGATTCGCCAGCTCTATACGACTCACTATAGGGAAAGCTTGCATGCAGGCCTCTGCAG    60

RT-PCR       1    ----------------------GCCGGAATTCATGACAAACCTGCAAGATCAAACCCAAC    38
RESULT.TXT   61   TCGACGGGCCCGGGATCCGATTGCCGGAATTCATGACAAACCTGCAAGATCAAACCCAAC    120

RT-PCR       39   AGATTGTTCCGTTCATACGGAGCCTTCTGATGCCAACAACCGGACCGGCGTCCATTCCGG    98
RESULT.TXT   121  AGATTGTTCCGTTCATACGGAGCCTTCTGATGCCAACAACCGGACCGGCGTCCATTCCGG    180

RT-PCR       99   ACGACACCCTGGAGAAGCACACTCTCAGGTCAGAGACCTCGACCTACAATTTGACTGTGG    159
RESULT.TXT   181  ACGACACCCTGGAGAAGCACACTCTCAGGTCAGAGACCTCGACCTACAATTTGACTGTGG    240

RT-PCR       160  GGGACACAGGGTCAGGGCTAATTGTCTTTTTCCCTGGATTCCCTGGCTCAATTGTGGGTG    220
RESULT.TXT   241  GGGACACAGGGTCAGGGCTAATTGTCTTTTTCCCTGGATTCCCTGGCTCAATTGTGGGTG    300

RT-PCR       221  CTCACTACACACTGCAGAGCAATGGGAACTACAAGTTCGATCAGATGCTCCTGACTGCCC    280
RESULT.TXT   301  CTCACTACACACTGCAGGGCAATGGGAACTACAAGTTCGATCAGATGCTCCTGACTGCCC    360

RT-PCR       281  AGAACCTACCGGCCAGTTACAACTACTGCAGGCTAGTGAGTCGGAGTCTCACAGTGAGGT    340
RESULT.TXT   361  AGAACCTACCGGCCAGTTACAACTACTGCAGGCTAGTGAGTCGGAGTCTCACAGTGAGGT    420

RT-PCR       341  CAGGCACACTTCCTGGTGGCGTTTATGCACTAAACGGCACCATAAACGCCGTGACCTTCC    400
RESULT.TXT   421  CAGGCACACTTCCTGGTGGCGTTTATGCACTAAACGGCACCATAAACGCCGTGACCTTCC    480

RT-PCR       401  AAGGAAGCCTGAGTGAACTGACAGATGTTAGCTACAATGGGTTGATGTCTGCAACAGCCA    460
RESULT.TXT   481  AAGGAAGCCTGAGTGAACTGACAGATGTTAGCTACAATGGGTTGATGTCTGCAACAGCCA    540

RT-PCR       461  ACATCAACGACAAAATTGGGAACGTCCTAGTAGGGGAGGGGGTCACCGTCCTCAGCTTAC    520
RESULT.TXT   541  ACATCAACGACAAAATTGGGAACGTCCTAGTAGGGGAGGGGGTCACCGTCCTCAGCTTAC    600

RT-PCR       521  CCACATCATATGATCTTGGGTATGTGAGGCTTGGTGACCCCATTCCCGCAATAGGGCTTG    580
RESULT.TXT   601  CCACATCATATGATCTTGGGTATGTGAGGCTTGGTGACCCCATTCCCGCAATAGGGCTTG    660

RT-PCR       581  ACCCAAAAATGGTAGCCACATGTGACAGCAGTGACAGGCCCAGAGTCTACACCATAACTG    640
RESULT.TXT   661  ACCCAAAAATGGTAGCCACATGTGACAGCAGTGACAGGCCCAGAGTCTACACCATAACTG    720

RT-PCR       641  CAGCCGATGATTACCAATTCTCATCACAGTACCAACCAGGTGGGGTAACAATCACACTGT    700
RESULT.TXT   721  CAGCCGATGATTACCAATTCTCATCACAGTACCAACCAGGTGGGGTAACAATCACACTGT    780

RT-PCR       701  TCTCAGCCAACATTGATGCCATCACAAGCCTCAGCGTTGGGGGAGAGCTCGTGTTTCAAA    760
RESULT.TXT   781  TCTCAGCCAACATTGATGCCATCACAAGCCTCAGCGTTGGGGGAGAGCTCGTGTTTCAAA    840

RT-PCR       761  CAAGCGTCCACGGCCTTGTACTGGGCGCCACCATCTACCTCATAGGCTTTGATGGGAACG    820
RESULT.TXT   841  CAAGCGTCCACGGCCTTGTACTGGGCGCCACCATCTACCTCATAGGCTTTGATGGGAACA    900

RT-PCR       821  CGGTAATCACCAGGGCTGTGGCCGCAAACAATGGGCTGACGACCGGCACCGACAACCTTT    880
RESULT.TXT   901  CGGTAATCACCAGGGCTGTGGCCGCAAACAATGGGCTGACGACCGGCACCGACAACCTTA    960

RT-PCR       881  TGCCATTCAATCTTGTGATTCCAACAAACGAGATAACCCAGCCAATCACATCCATCAAAC    940
RESULT.TXT   961  TGCCATTCAATCTTGTGATTCCAACAAACGAGATAACCCAGCCAATCACATCCATCAAAC    1020

RT-PCR       941  TGGAGATAGTGACCTCCAAAAGTGGTGGTCAGGCAGGGGATCAGATGTCATGGTCCGCAA    1000
RESULT.TXT   1021 TGGAGATAGTGACCTCCAAAAGTGGTGGTCAGGCAGGGGATCAGATGTCATGGTCGGCAA    1080

RT-PCR       1001 GAGGGAGCCTAGCAGTGACGATCCATGGTGGCAACTATCCAGGGGCCCTCCGTCCCGTCA    1060
RESULT.TXT   1081 GAGGGAGCCTAGCAGTGACGATCCATGGTGGCAACTATCCAGGGGCCCTCCGTCCCGTCA    1140

RT-PCR       1061 CGCTAGTGGCCTACGAAAGAGTGGCAACAGGATCCGTCGTTACGGTCGCTGGGGTGAGCA    1120
RESULT.TXT   1141 CGCTAGTGGCCTACGAAAGAGTGGCAACAGGATCCGTCGTTACGGTCGCTGGGGTGAGCA    1200

RT-PCR       1121 ACTTCGAGCTGATCCCAAATCCTGAACTAGCAAAGAACCTGGTTACAGAATACGGCCGAT    1180
RESULT.TXT   1201 ACTTCGAGCTGATCCCAAATCCTGAACTAGCAAAGAACCTGGTTACAGAATACGGCCGAT    1260

RT-PCR       1181 TTGACCCAGGAGCCATGAACTACACAAAATTGATACTGAGTGAGAGGGACCGTCTTGGCA    1240
RESULT.TXT   1261 TTGACCCAGGAGCCATGAACTACACAAAATTGATACTGAGTGAGAGGGACCGTCTTGGCA    1320

RT-PCR       1241 TCAAGACCGTCTGGCCAACAAGGGAGTACACTGACTTTCGTGAAATACACTTCATGGAGG    1300
RESULT.TXT   1321 TCAAGACCGTCTGGCCAACAAGGGAGTACACTGACTTTCGTGAAATACACTTCATGGAGG    1380

RT-PCR       1301 TGGCCGACCTCAACTCTCCCCTGAAGATTGCAGGAGCATTCGGCTTCAAAGACATAATCC    1360
RESULT.TXT   1381 TGGCCGACCTCAACTCTCCCCTGAAGATTGCAGGAGCATTCGGCTTCAAAGACATAATCC    1440

RT-PCR       1361 GGGCCATAAGGTTTCTAGACGGCAATCTAGATGCTTCGCNGGC    1402
RESULT.TXT   1441 GGGCCATAAGGTTTCTAGACGGC--------------------    1463
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Figure 1. Alignment of sequence of VP2 gene, obtained in this study, with the sequence of VP2 gene of IBDV D78 strain (accession no. AF499929).

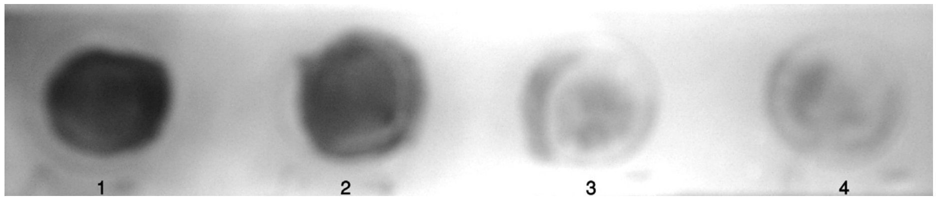

Figure 2. Dot blotting results of transfected cell lysates. Lysate of cells transfected with the constructed expression vector (1 and 2) and the cells transfected with the vector without insert (negative control) (3,4).

lenge with the very virulent strains of IBDV. Therefore, a safer and more efficacious vaccine to control IBD is essential.

Attempts have been made to generate subunit vaccines, by expression of VP2 or virus polyprotein in *Ecoli* (Jagadish *et al*., 1988) and yeast (Pitcovski *et al*., 2003; Goudarzi *et al*., 2006). Recombinant fowlpox viruses containing the genetic material of IBDV have also been developed and tested (Shaw and Davison, 2000).

In recent years several groups have tried the DNA vaccination approach targeting the polyprotein or VP2 of IBDV (Chang *et al*., 2001; Fodor and Fodor, 1999; Hsieh *et al*., 2007; Mahmood *et al*., 2006). DNA vaccination as an alternative approach to vaccinate and protect chickens against IBD has been pursued with some positive and promising results (Hsieh *et al*., 2007, Tang and Johnston, 1992). Application of CpG-ODN as DNA vaccine adjuvant (Mahmood *et al*., 2006), use of bacteria for efficient delivery of DNA vaccine (Mahmood *et al*., 2007; Li *et al*., 2006) and boosting the immune system by a killed vaccine, after using the DNA vaccine (Hsieh *et al*., 2007), all have been shown to enhance the efficacy of IBD DNA vaccines.

Efficacy of IBD DNA vaccine may also depend to other features, like amount of the expressed protein and duration of in vivo expression. In this study, we decided to use pCDNA4 as a vector for expression of IBDV VP2, because it was specifically designed for protein expression in eukaryotic cells but so far, has not been used in IBD DNA vaccine constructions. The plasmid pCDNA4 which is about 5.3 kb contains the CMV promoter, ampicillin resistance gene for selection in *E.coli*, zeocin resistance gene for selection in eukaryotic cell lines and an upstream histidin tag for protein purification.

Based on the results of dot immunoblotting of cells transfected by pCNA4VP2, the construct expresses the protein VP2 of IBDV, but further characterizations are needed to show its usability for *in vivo* expression and immunogenicity of VP2.

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