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, *Eco*RI 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 *Eco*RI 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^{TM} 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).

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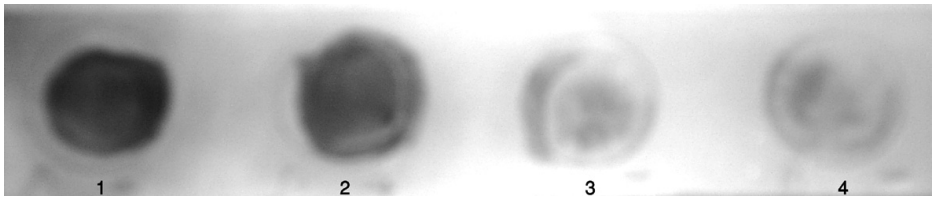

Figure 2. Dot blotting results of transfected cell lysates. Lysate of cells transfected with the constructed expression vector (1 and 2) and the cells transfected with the vector without insert (negative control) (3,4).

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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