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## ■ Developing an Antiviral Drug Screening System for Anti-Bovine Viral Diarrhea Virus (BVDV) Therapies

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**ABSTRACT.** Bovine viral diarrhea virus (BVDV) is an economically important animal pathogen affecting cattle. Despite the use of vaccination, test and slaughter practices, BVD remains a serious problem of cattle breeding. This study was conducted in order to develop a cell line that expresses some of BVDV sub-replicons. BVDV-NADL NS3 and 5'UTR were cloned in pWPI-linker B lentiviral plasmid at the upstream of EGFP gene. Consequently, lentiviral vectors containing BVDV-NS3 and BVDV-5'UTR were produced by using the second-generation lentiviral packaging system. By these lentivectors, MDBK cells expressing BVDV-5'UTR and BVDV-NS3 partial fragments were prepared. The efficiency of the infection was evaluated by fluorescence microscopy, western blotting, and RT-PCR. The results indicated that the development of MDBK cell line expressing these transgenes provides a very sensitive antiviral drug screening system for anti-bovine viral diarrhea virus (BVDV) therapies.

**Keywords:** Packaging system, BVDV, Lentiviral vectors, Cell line.

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## INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens in cattle, causing economic losses all over the world. BVDV is an enveloped, positive sense, single stranded RNA virus that is classified in the genus pestivirus of Flaviviridae family (Li et al., 2010). BVDV can induce lifelong persistent infection in cattle populations (Li et al., 2010; Carmona et al., 2012), and it can lead to a severe disease involving the respiratory, enteric, reproductive, immune, and endocrine systems (Keyvanfar and Hemmatzadeh, 2000; Hemmatzadeh et al., 2006; Lambeth et al., 2007). This virus has two biotypes which are termed cytopathic (cp) and non-cytopathic (ncp). Furthermore, based on the difference in the nucleotide sequences, it has two genotypes: BVDV-1 and BVDV-2 (Spurgers et al., 2008; Khaliq et al., 2010; Li et al., 2010). Although commercial BVDV vaccines are available, its antigenic diversity is one of the reasons for the relative failure in vaccination programs. In addition, despite the extensive use of eradication and slaughter strategies after screening tests, the disease caused by BVDV remains prevalent in cattle herds all over the world (Wilson et al., 2005; Khaliq et al., 2010; Li et al., 2010).

BVDV genome is approximately 12 Kb in length and is flanked by a 3' and a 5' untranslated region (UTRs). The BVDV genome encodes a single open reading frame (ORF) with approximately 4000 codons (Houe, 2003; Henry et al., 2006; Zemke et al., 2010; Fan and Bird, 2012). This ORF encodes a poly-protein precursor which can simultaneously or post-translationally be cleaved by viral or cellular proteases to produce 12 or 13 proteins in the following order:

NH<sub>2</sub>-N<sub>pro</sub>-C-Erns-E1-E2-p7-NS2-NS3 (NS23)-NS4A-NS4B-NS5A-NS5B-COOH.

NS3 is known as a cp-BVDV marker with serine protease activity. Its cleavage sites in the genome of the virus are the following: NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B. Moreover, NS3 has RNA helicase and nucleoside triphosphatase activities. The expression of NS3 or NS23 is necessary for the formation of infectious virions. The

protease activity of NS3 can induce apoptosis in the infected cells (Xu et al., 1997; St-Louis et al., 2005; Pankraz et al., 2009).

The 5'UTR region with 370 b length, is the most conserved region of the viral genome, and it includes an internal ribosome entry sequence (IRES). The nucleotide sequence and secondary structures of 5'UTR is important for the translation of ORF, the regulation of gene transcription, and the expression of genes associated with the virulence of BVDV (Poole et al., 1995).

Considering the specific functional role of these two parts of BVDV genome, we produced a cell line, after the insertion of these parts, in order to evaluate the therapeutic strategies against this virus (Henry et al., 2006).

For that purpose, in this study, lentiviral vectors expressing NS3 and 5'UTR were produced by second-generation lentivirus packaging system, to infect Madin-Darby bovine kidney (MDBK) cells, permanently.

During their life cycle, lentiviruses can integrate their genomes into the host genome; therefore, they can be suitable tools for long-term expression of a transgene. Lentiviral vectors are modified so that only a limited number of lentiviral genes are required for the engineered viral vectors. Furthermore, the stable integration of viral DNA into the host genome can be provided by these essential genes. By this strategy, the desired recombinant gene can be expressed frequently, but the infectious virus cannot be produced. To create such a lentiviral vector, one of the newest methods is the cloning of the essential genes of lentivirus and the transgenes in three or four different plasmids. Consequently, the co-transfection of these plasmids can produce a lentivector in a host cell line.

Due to the persistent lentiviral vector infection and its ability to enter different cell lines (Salmon and Trono, 2006), in the present study, we employed these vectors for the production of MDBK cell lines to express BVDV NS3 partial genes and BVDV 5'UTR. That system has been tested by our group for BVDV gene therapies (unpublished data).

## MATERIALS AND METHODS

### Cloning of *BVDV NS3* and *BVDV 5'UTR* in pWPI-Linker B lentiviral vector

#### 2.1.1- RT-PCR

BVDV was inoculated in Bovine testicular cell line (BT). BT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, America, Catalog No. 116- 12800) supplemented with 10% fetal bovine serum (FBS) (GIBCO, America, Catalog No. 106- 10270) and 1x penicillin streptomycin (Sigma, America Catalog No. 116-12800). Cultures were incubated for 24 hours at 37 °C with 5% CO<sub>2</sub>. Consequently, BVDV-NADL (NC-001461) from the Sekans animal health laboratory, Ankara University (a generous gift from Dr. Faraji) inoculated to BT cells. RNA was isolated 48 h after BVDV infection using QIAzol (QIAGEN, Germany, Catalog Number: 79306) according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed with TaqMan Reverse Transcription kit (Invitrogen, Germany, Catalog Number: 8080234N) according to the manufacturer's instructions. PCR was carried out using the primers designed for apart of *NS3* gene (3790-4000) and *5'UTR* (first 386 nucleotides (of BVDV-NADL (ac.no: NC00146). The sequences of primers were as follows: BVDV NS3 F: 5'-CGC CAC A GA TCT ACC GC AAG AGT ACT GG GGA A-3' and BVDV NS3 R: 5'-CGC CAC AGA TCT ACC CA TCA GTA GG TTA TAG TC-3' with *BglIII* restriction sites. BVDV 5'UTR F: 5'- CGC GGA TCC ACC A CTCGTAT ACG TAT TGG GC -3' with *BamHI* restriction site and BVDV 5'UTR R: 5'- GTT CCCC GG GC GT CCA TGT ACA CA GAG AT -3' with *SmaI* site. The PCR thermal cycle reactions were consisted of denaturation at 95 °C for 30 s followed by 30 cycles at 95 °C for 15 s, 68 °C (for NS3) and 69 °C (for 5'UTR) for 35 s and 72 °C for 40 s, followed by a final extension at 72 °C for 5 minutes. The positive and negative controls prepared from Animal Health Veterinary Laboratories Agency (AHVLA) (a generous gift from Dr. Steinbakh and Dr. Dastjerdi) were used in each test.

### Restriction enzyme digestion, ligation and colony PCR

The pWPI-Linker B lentiviral Plasmid had been prepared by the Research Center for Bio-Medical (IMIM) in Spain (a generous gift from Fabien Delaspre) and digested by *BamHI* (Roche, Germany, Catalog Number: 10220612001) and *SmaI* (Roche, Germany, Catalog Number: 10220566001) for cloning of 5'UTR. This plasmid had been also digested by *BamHI* for cloning of NS3. Therefore, 1 µl of 1 µg / µl plasmid, 1µl enzyme, 2 µl 10x enzyme buffer and nuclease-free water were used in a 20 µl reaction, after overnight incubation at 37 °C for single digestion and 20 °C for double digestion. Ligation reaction was performed with 2µl of T4 10x ligation buffer (Fermentas, Germany, Catalog Number: B69), 2µl of PEG-4000 50% (Fermentas, Germany), 1 µl of T4 DNA ligase (Fermentas, Germany, Catalog Number: EL0013), 150 ng of the inserting DNA and nuclease-free water up to 20µl. Then ligation products were transformed separately in the appropriate competent cell (Stbl4) by heat shock protocol. Briefly, 10 µl of ligation product was added to 100 µl of Stbl4, and the mixture was incubated for 30 minutes at 4 °C, 90 seconds at 42 °C and 5 min at 4 °C. Consequently, 900 µl of LB medium was added and shaken at 200 rpm for 45 minutes. After centrifugation at 6000 rpm for 3 min, the pellet was cultured in LB agar with 100 mg / ml ampicillin. Plates were incubated at 37 °C for 16-14 hours. In order to determine the presence or absence of the inserted DNA in plasmid constructs, 10 individual colonies from each plate were selected for PCR reaction and extraction of plasmid by using Mini preparation of DNA method. After electrophoresis in 0.8% agarose, the recombinant plasmids were sent for sequencing.

### Lentiviral transduction of MDBK cells

The second-generation lentivirus packaging system (Addgene cat no. 12260 and 12259) was used to generate *BVDV NS3* and *BVDV 5'UTR* lentiviruses. Briefly, 293T cells (3×10<sup>6</sup> cells per 10 cm dish)

were co-transfected with a mixture of 4 µg of pMD2.G plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope, 7.5 µg of the psPAX2 packaging plasmid, 11 µg of the BVDV NS3 - pWPI-Linker B and BVDV 5'UTR - pWPI-Linker B, using calcium phosphate-DNA precipitate method. The following day, the medium was replaced with 7ml of DMEM (Invitrogen) and further cultured for 72h. The supernatant was collected 48h and 72 h after transfection, cleared by centrifugation (3000 rpm, 15 min) and it was kept in -70 °C until the infection of MDBK cells. For the titration of the produced BVDV NS3 and BVDV-5'UTR lentiviral particles, the 293T cells (3×10<sup>5</sup> cells per each well of a 6-well plate) were transduced by 1 ml of suspensions containing these lentiviral particles. Then, the cells were analyzed for eEGFP expression using fluorescent microscopy and the viral titer was calculated. Consequently, 10<sup>5</sup> trypsinized MDBK cells were infected in a 6-well plate by lentiviral vectors (MOI = 0.8), diluted in 1ml of DMEM medium supplemented by 10% FBS and polyberene (8 µg/ml). The medium was replaced with 7ml of DMEM (Invitrogen) 12h after the infection. MDBK cells were examined 48 h and 72 h after infection, by using a fluorescent microscope.

#### RT- PCR to validate infection of MDBK cells

To confirm the expression of *BVDV-NS3* and *BVDV-5'UTR* in MDBK cells, RNA was isolated from infected cells and following treatment with DNase, RT- PCR was carried out using the designed primers. The sequences of primers were as follows: BVDV NS3 F: CAT AGG TAG GCG TGA CCC AAC -3' and BVDV NS3 R: 5'- TCA GTG ACC CTC AGT GCT GC -3'. BVDV 5'UTR F: 5'- AGG GTA GTC GTC AGT GGT TC -3' with and BVDV 5'UTR R: 5'- AGG TTA AGA TGT GCT TTG GG -3'. The PCR materials and methods were the same with the ones described in section 2.1.1. The RT-PCR thermal cycle programs were consisted of denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 60 °C (for *NS3*) and 55 °C

(for *5'UTR*) for 30 s and 72 °C for 30 s, including a final extension step at 72 °C for 5 minutes. The positive (MDBK infected with BVDV) and negative (MDBK cells without any infection) controls were employed in each test.

#### Western blotting

For western blotting analysis, protein extracts were prepared from cell cultures in lysis buffer (50 mM Tris-HCl pH = 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 1 mM PMSF) for 20 min on ice. Protein extracts from positive and negative controls (MDBK cells infected with BVDV- NADL and MDBK cells without any lentiviral vector, respectively) were extracted too. All samples were vortexed for 10 min. Samples were centrifuged at 1000 g for 10 min at 4 °C. After Bradford and Lowry protein assay, 50 µg from the supernatant were added to sample buffer (Tris-HCl 62.5 pH = 6.8 mM, 15% SDS, 0.01% Bromophenol blue, 10% glycerol, mercaptoethanol 25%, water), boiled for 3 min and loaded in polyacrylamide Resolving [3.5 mL of Deionized water, 4.0 mL of 30% acrylamide:bis-acrylamide (29:1), 2.5 mL of 1.5 M Tris-HCl, 0.4% SDS, pH 8.8, 100 µL of 10% ammonium persulfate and 10 µL TEMED] and Stacking [2.1 mL of Deionized water, 0.63 mL of 30% acrylamide:bis-acrylamide (29:1), 1 mL of 0.5 M Tris-HCl, 0.4% SDS, pH 6.8, 30 µL of 10% ammonium persulfate and 7.5 µL TEMED] SDS - PAGE gels and then electrophoresed. After SDS-PAGE, proteins were transferred to PVDF membranes. Viral proteins were detected with a mouse anti *BVDV-NS3* monoclonal antibody (1:100) (Santa Cruz, America, Catalog Number: 101592sc) and rabbit anti-mouse IgG antibody conjugated with HRP (Santa Cruz, America, Catalog Number: 358914sc-) (1:10 000). As a positive cellular control, alpha tubulin was detected with primary anti alpha tubulin mouse monoclonal antibody (Sigma, America, Catalog Number: 9026T). To develop Western blot, we used an ECL detection system (Amersham, Piscataway, NJ) according to the manufacturer's directions.

**RESULTS**

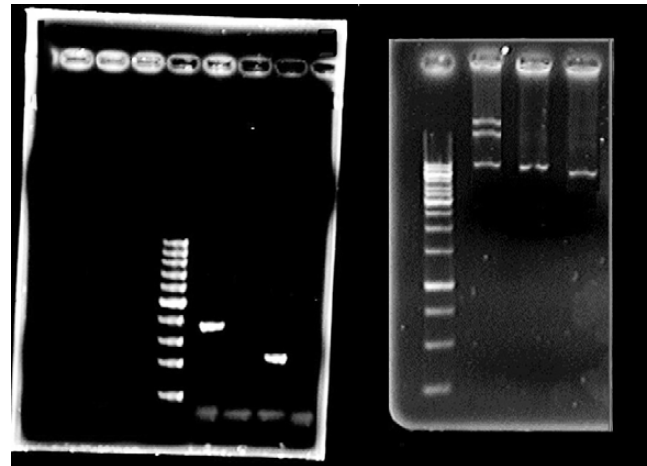
**RT-PCR**

The presence of *BVDV- 5'UTR* and *BVDV- NS3* fragments was detected using RT-PCR. Positive PCR products and positive control sample in the PCR test for detecting BVDV- NS3, were in the size of 223 bp, while the size of the *BVDV- 5'UTR* -specific band was 380 bp. The validity of pWPI- Linker B digestion was tested by electrophoresis in 1% agarose gel (Fig 1).

**Colony PCR**

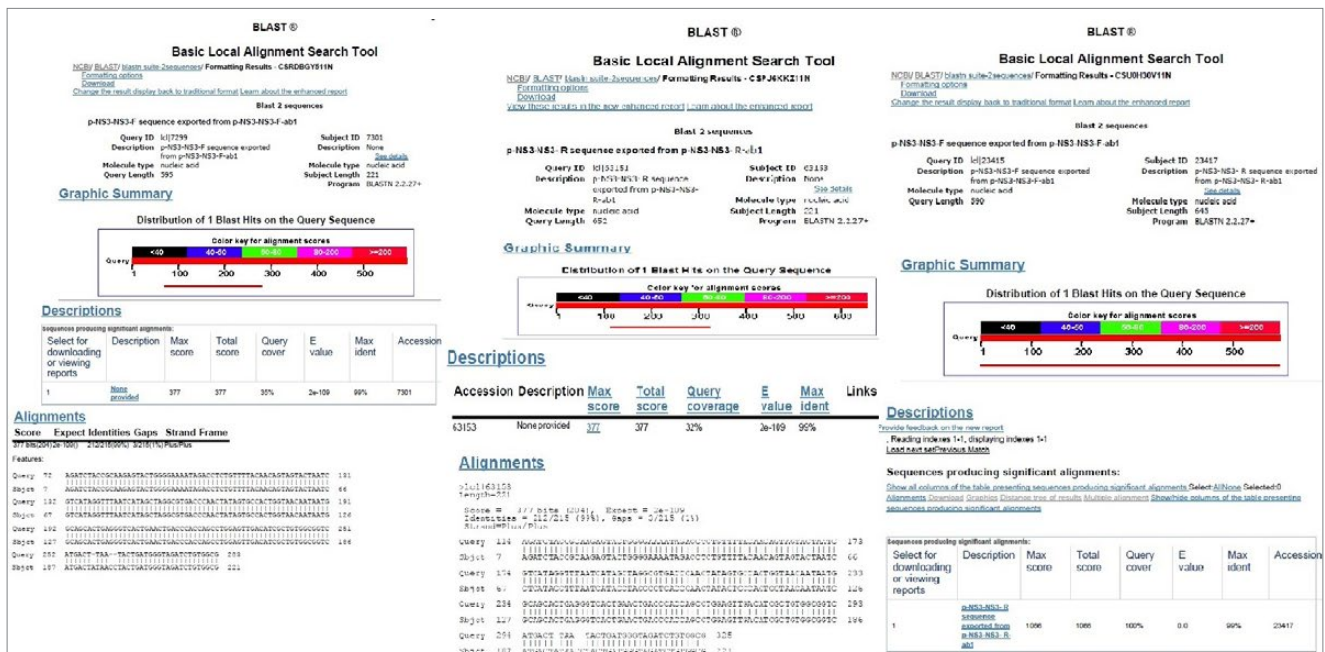
After the overnight incubation of the transformed *Stbl4* as well as the controls (positive and negative) on plates containing LB agar + ampicillin at 37 ° C, recombinant plasmid containing colonies and positive control colonies (containing pWPI-LinkerB plasmid resistant to ampicillin that confirmed in previous experiments) were observed, while in the negative control plate (only *Stbl4*) there weren't any colonies present.

The presence of *BVDV- 5'UTR* and *BVDV- NS3* fragments was detected in transformed colonies and



**Fig 1.** amplification of BVDV-NS3, BVDV – 5'UTR and digestion of pWPI-Linker B: Right: RT-PCR for amplification of BVDV-NS3 and BVDV – 5'UTR after inoculation of the virus into BT cell line: left to right: negative control for NS3, negative control for 5'UTR, 5'UTR band (380bp),Gene ruler(100bp), NS3 band(223bp). Left: Single and double digestion of pWPI-Linker-B: left to right: double digestion of pWPI-Linker B, single digestion of pWPI-Linker B, pWPI-Linker B, Gene ruler(1Kb)

positive control samples by using PCR (BVDV- NS3 bands were in the size of 223 bp, while the size of the BVDV- 5'UTR -specific band was 380 bp)



**Fig 2.** Alignment of the NS3 sequence derived from sequencing with the desired amplicon.Left: Single and double digestion of pWPI-Linker-B: left to right: double digestion of pWPI-Linker B, single digestion of pWPI-Linker B, pWPI-Linker B, Gene ruler(1Kb)

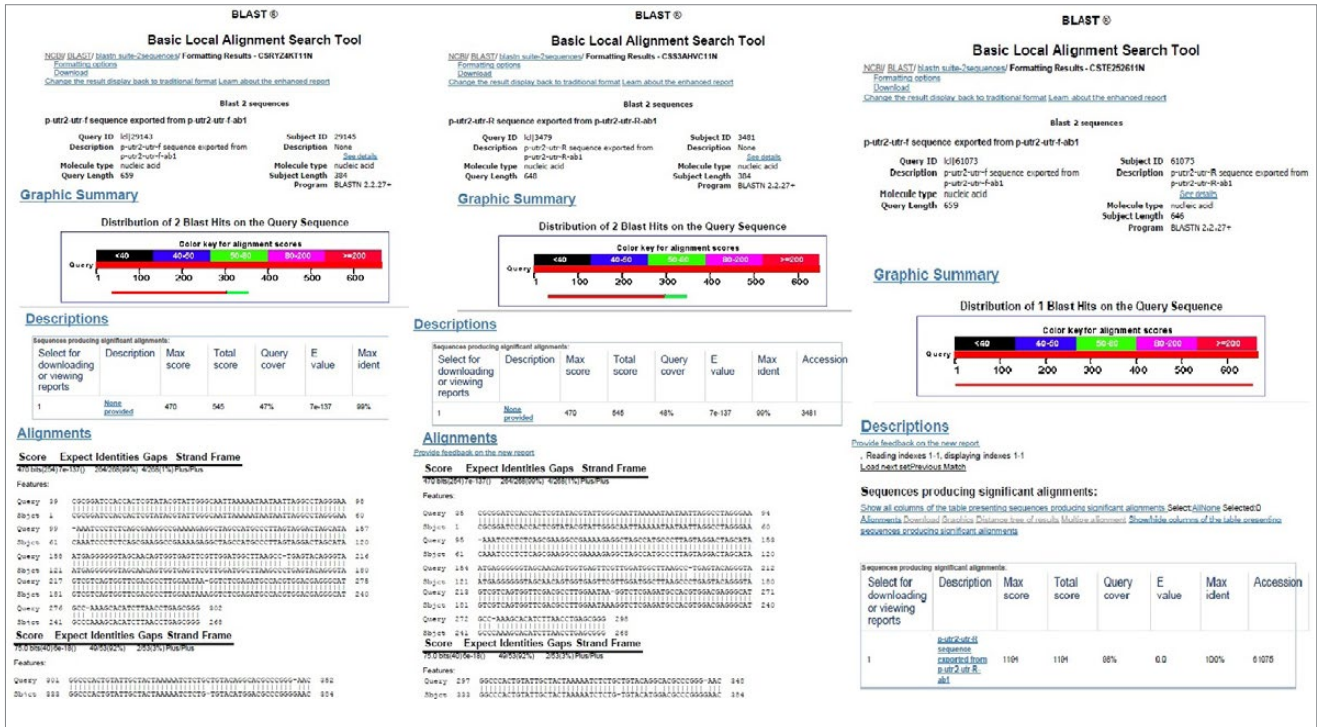


Fig 3. Alignment of the 5'UTR sequence derived from sequencing with the desired amplicon.

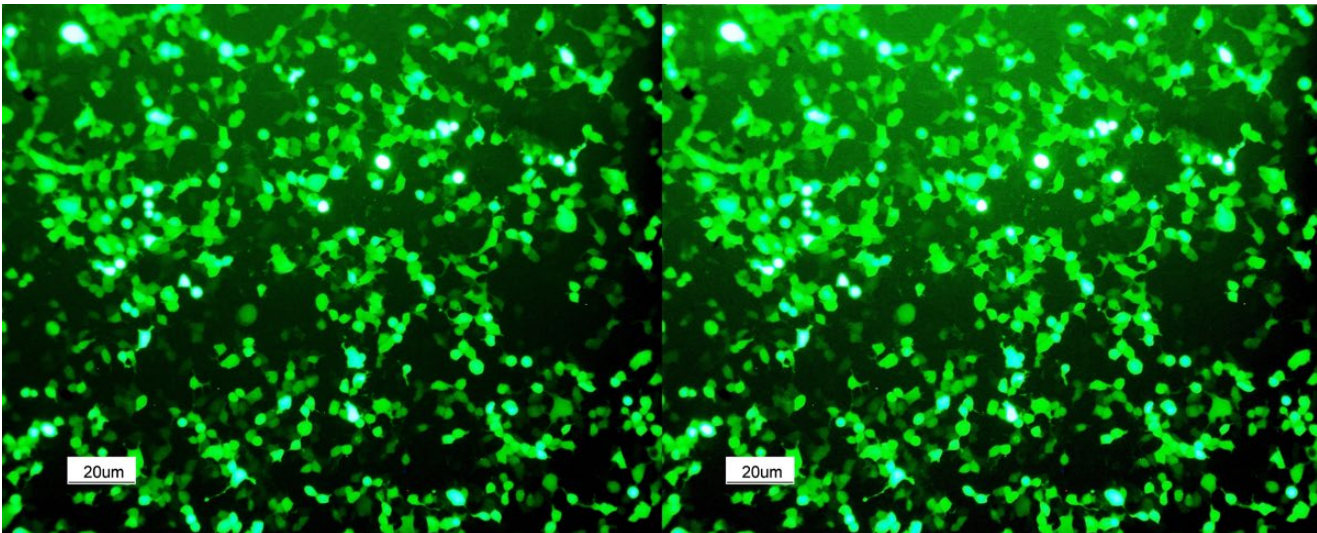


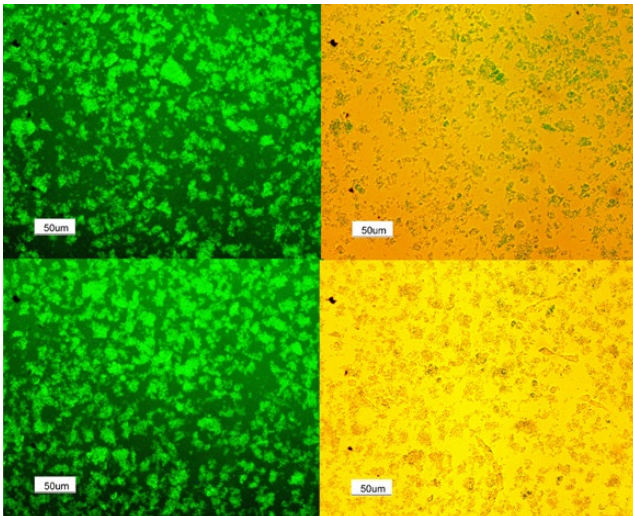
Fig 4. transfection of 293T cells with the lentiviral plasmids expressing the BVDV NS3 and BVDV 5'UTR Right: EGFP levels observed after transfection of pWPI-Linker B-5'UTR in a second-generation lentiviral packaging system. Left: EGFP levels observed after transfection of pWPI-Linker B- NS3 in a second-generation lentiviral packaging system.

After plasmid extraction from positive colonies, the validity of plasmid extraction and the presence of BVDV- NS3 and BVDV- 5'UTR fragments in the recombinant plasmids, were confirmed by digestion and electrophoresis. Sequence analysis of the recombinant plasmids revealed the presence of

BVDV- NS3 and BVDV- 5'UTR (Figs 2 and 3).

**entivirus production and infection**

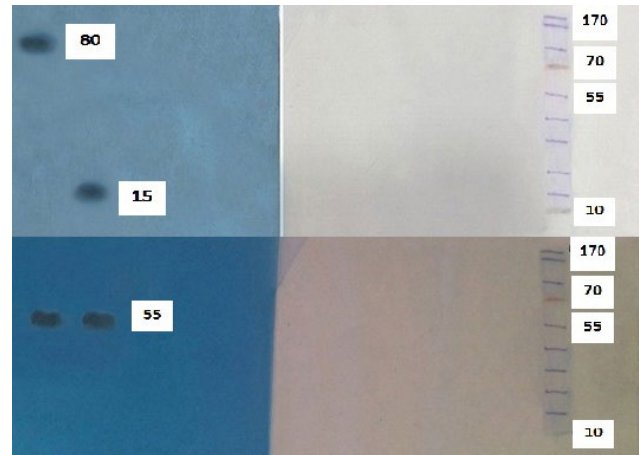
72 hours after transfection, the percentage of EGFP expressing 293 T cells was approximately 80-70%. That percentage suggested that the transfection effi-



**Fig 5.** Infection of MDBK cells with lentiviral vectors expressing BVDV NS3 and BVDV 5'UTR:  
Images above: Right: EGFP expression after infection with lenti-vector carrying BVDV NS3 - Left: The same picture by using light microscopy.  
Images below: Right: EGFP expression after infection with lenti-vector carrying BVDV 5'UTR - Left: The same picture by using light microscopy.



**Fig 6.** RT-PCR to confirm the expression of BVDV 5'UTR and BVDV NS3 in MDBK cells:  
Left to right: MDBK-NS3, positive control: BVDV NS3, negative control for NS3, Gene ruler (100bp), MDBK 5'UTR, positive control: BVDV 5'UTR, negative control for 5'UTR



**Fig 7.** Western blot to confirm the expression of BVDV NS3 in MDBK cells:  
Top row: BVDV-NS3 band with the size of 80 kDa (left) and MDBK-NS3 serine protease as much as approximately 15 kDa (right)  
Bottom row: The 55 kDa band corresponding to alpha Tubulin in negative controls, positive controls (BVDV-NS3) and test sample (MDBK-NS3) (left to right)

ciency was appropriate for infection. By fluorescence microscopy observation, efficiency was also proved to be excellent (more than 90% EGFP expression). Thus, the MDBK cells infected with BVDV- NS3 - lentivector and BVDV- 5'UTR- lentivector were considered as cell clones expressing BVDV NS3 and BVDV 5'UTR (Figs 4-5).

#### RT- PCR to validate infection of MDBK cells

The expression of *BVDV- 5'UTR* and *BVDV- NS3* was detected in MDBK cells infected by lentiviral vectors expressing these transgenes and positive control (MDBK cells infected with BVDV) using RT-PCR tests (*NS3* band: 87bp and *5'UTR* band: 68bp) (Fig 6).

#### Western blotting

Viral product was detected with rabbit anti mouse IgG against *BVDV-NS3* (1:100). The specific band related to selected sequence of *NS3* was approximately 15 KD. As loading control, alpha tubulin was detected with a mouse monoclonal antibody (1:1000) and its specific band was 55 KD (Fig 7).

## DISCUSSION

BVDV establishes a persistent infection in cattle populations worldwide, often resulting in significant economic impacts. Although the control measures for eradication, namely selective test and slaughter as well as vaccination, are widely used, BVDV remains prevalent due to its diversified antigenicity. The efficiency and the specificity of RNAi are attractive therapeutic characteristics that may prove useful for the development of antiviral therapies (Lambeth et al., 2007; Zemke et al., 2010; Jordao et al., 2011). Constant changes in the antigenic regions of BVDV structure, particularly C, Erns, E1, and E2 antigens and the formation of scape mutants that evade the host immune response, have made the production of an effective vaccine difficult. The majority of BVDV vaccines are designed against the previously mentioned glycoproteins and proteins, and this is a crucial factor for the medium or low success to control or eradicate the disease (Meyers and Theil, 1996; Lambeth et al., 2007; Zemke et al., 2010; Jordao et al., 2011; Ni et al., 2012).

The aim of this study was to provide a suitable cell line expressing the genes of BVDV in order to evaluate the new antiviral treatment strategies, demonstrate their effectiveness and satisfy the methods for controlling or treating BVDV.

Gene therapies performed with the aim of antiviral treatments against Flaviviridae were targeted at almost all regions of the viral genome; however, due to the conservation of the 5'UTR sequences during the viral evolution and the critical tasks of NS2-3, NS3, NS5A, and NS5B, these parts have received more attention. Considering the previous studies with respect to the inhibitory efficacy of targeting each of these Flaviviral genomic sequences, NS3 and 5'UTR were selected for the cloning into lentiviral plasmid (Lv et al., 2003; Randall et al., 2003, Randall et al., 2004; Lambeth et al., 2007; Gamlen et al., 2010).

Moreover, NS3 and 5'UTR were selected because of their conservation during virus evolution and due to their specific biological roles in cell proliferation,

survival, and virulence of the virus. In addition, for stable and long-term expression of the desired genes in the target cells, the lentiviral vector was utilized for transduction. Transfection of plasmid vectors has also been used by researchers to induce gene expression in the target cells, but with this method, the duration of gene expression is about 4-10 days. Lentiviral vectors are specific types of retroviruses that can infect both dividing and non-dividing cells because their pre-integration complex (virus "shell") can get through the intact membrane of the nucleus of the target cell. Lentiviruses can be used to provide highly effective gene therapy, as they can change the expression of their target cell's gene for up to six months. They can also be utilized for non-dividing or terminally differentiated cells, such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, muscle and liver cells, to which the previous gene therapy methods could not be applied. Employing lentiviral vectors (LVs) offers multiple advantages for gene therapy, because they encompass the efficient delivery, the ability to transduce the proliferating and resting cells, the capacity to integrate into the host chromatin to provide the stable long-term expression of the transgene, the absence of any viral genes in the vector, and finally the absence of interference from pre-existing viral immunity. In addition, LVs can transfer large nucleotide sequences (3000 bp). As a result, they are preferred to other viral vectors such as adenoviral vectors, retroviral vectors, and AAVs. Moreover, due to the lower frequency of integration at or near the cellular proto-oncogenes, the possibility of mutagenesis and carcinogenesis as a result of gene induction by lentiviral vectors, is low (KuÈmmerer et al., 2000; Haga et al., 2006; Henry et al., 2006; Salmon and Trono, 2006; Tiscornia et al., 2006).

The genetic and antigenic diversity of BVDV is a major concern for the development and the efficacy of current vaccines. Thus, anti-BVDV drugs might be an alternative strategy to control BVD. Over the past decade, *in vitro* anti-BVDV therapies have been conducted on cell systems

with either sub-genomic replicon or infectious viral particle of BVDV as the target. The BVDV sub-genomic replicon contains only non-structural regions, and the anti-viral therapeutic intervention of the structural region of BVDV cannot be studied. As for the infectious viral particles of BVDV, the time-consuming, labor-intensive, quantitative reverse transcriptase PCR methods have to be applied. A screening tool targeting both structural and non-structural proteins of BVDV and supporting high through-put anti-BVDV drug screening can be a monocistronic reporter virus with an enhanced green fluorescent protein (eEGFP) as a marker. The positive-oriented, single-stranded RNA viruses can be reconstituted, using reverse genetics strategy that provides a powerful tool for investigation of many aspects of the viral life cycle and pathogenesis. Numerous recombinant viruses and sub-genomic replicons have been generated by Flaviviridae family from viral cDNA clones and have been extensively employed as tools for antiviral drug screening. As for BVDV, efforts have been made by a few research groups to achieve a stable, easy-handling, reverse genetics system. Over the past decade, several milestones have been reached in the development of cell-based generation of recombinant BVDV. For example, the employment of a Bacterial artificial chromosome (BAC) vector stabilizes the viral genome during the construct replication in bacteria. It was previously found that BVDV is a suitable viral vector for the expression of heterologous proteins such as eEGFP when inserted between viral Npro and capsid proteins. In the study done by Fan and Bird in 2012, a stable, reverse genetic system was created on the basis of an infectious BAC cDNA clone (pBSD1) that has an NCP BVDV strain SD-1 background. A recombinant reporter BVDV was produced, stably expressing eEGFP protein as inserted between viral NS3 and NS4A proteins. Despite the huge advantages of monocistronic reporter BVDV for screening anti-BVDV drugs, this screening system has some disadvantages and limitations as well. First, it

takes time to design and produce this screening system accurately, and great bioinformatics and biotechnological knowledge are essential to prepare it. The selection of an appropriate insertion site for the marker gene is critical for the development of a recombinant reporter BVDV for antiviral drug screening, because the genotype and the phenotype of the recombinant virus should not be impaired by eEGFP or any other reporter gene insertion. Furthermore, the reporter gene must not affect the replication of the recombinant virus, which shows a same level of RNA and viral protein expression as the parent virus; in addition, the peak yield of progeny virus of the recombinant should not be less than the parent virus. The replication kinetics of the recombinant reporter BVDV and that of the parent virus should be similar. The reporter gene expression and its fluorescent signal intensity are correlated with the replication of the recombinant reporter BVDV. In many cases due to weaknesses in the design of monocistronic reporter virus, the fluorescence intensity is less than the amount approved for careful screening of antiviral drugs. Moreover, owing to the diversity and abundance of genetic changes that occur in the genome of RNA viruses, it is necessary to have the valid parent virus or its cDNA clone in order to produce the reporter virus (Kuemmerer et al., 2000; Wilson et al., 2005; Carmona et al., 2012; Fan and Bird, 2012). Because of the limitations and difficulties mentioned in the design and the production of recombinant monocistronic viruses, lentiviral vectors were utilized to induce the expression of target genes of BVDV.

## CONCLUSIONS

Therefore, in this study, MDBK cells expressing BVDV NS3 and BVDV 5'UTR were produced, using lentivector transduction. The results indicated that the development of MDBK cell line expressing these transgenes, acted very sensitive for anti-BVDV therapies screening. That cell line has been tested by our group for gene therapies based on RNAi against BVDV (unpublished data).

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. ■

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