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CLONING AND EXPRESSION OF RECOMBINANT POLYPEPTIDE FROM THE ERNS CODING REGION OF THE BOVINE VIRAL DIARRHOEA VIRUS IN *ESCHERICHIA COLI*

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Summary

Ekhtelat, M., M. R. Seyfiabad Shapouri, M. Ghorbanpoor Najafabadi, M. Lotfi & P. Mahmoodi Koohi, 2012. Cloning and expression of recombinant polypeptide from the Erns coding region of the bovine viral diarrhoea virus in *Escherichia coli*. *Bulg. J. Vet. Med.*, **15**, No 4, 236–245.

Bovine viral diarrhoea (BVD) is an economically important cattle disease with a worldwide distribution. Detection and elimination of animals persistently infected (PI) with bovine viral diarrhoea virus (BVDV) is essential for the control of BVD and eradication of BVDV. Usually, there are no pathognomonic clinical signs of BVDV infection. Diagnostic investigations therefore rely on laboratory-based detection of the virus, or virus-induced antigens or antibodies. Erns as an immunogenic protein of BVDV is genetically and antigenically conserved among different isolates and therefore, a candidate antigen for developing ELISA for serological studies or identification of PI animals. In this study, a segment of BVDV genome corresponding to the sequence coding for Erns was amplified using RT-PCR and cloned into expression vector pMalc2x, under the control of the *lac* promoter. After sequencing of the gene, the recombinant protein was expressed in *Escherichia coli* BL-21 and analysed by SDS-PAGE and western blotting. The strong promoter of vector pMalc2x allowed a high level of Erns expression. Based on our results it appears that this plasmid construct may be suitable for the production of Erns recombinant antigen and Erns specific antibodies to develop BVDV laboratory diagnostic assays.

Key words: bovine viral diarrhoea, Erns, pMALc2x, recombinant antigen

INTRODUCTION

Bovine viral diarrhoea (BVD) is one of the most important diseases of cattle worldwide (Gunn *et al*., 2005). The BVD virus (BVDV) is capable of producing a broad range of clinical signs, ranging from most often asymptomatic infection to severe acute disease with signs from the enteric, reproductive or respiratory organs. Bovine foetuses infected with BVDV between days 30 and 125 of gestation can develop immune tolerance against the virus and will be born persistently infected (PI) (Hilbe *et al*., 2007). PI animals are virus positive and antibody negative when they are infected with a sole genotype or subtype of BVDV

(Sandvik, 2005). These animals can be identified by combined use of serological and virological tests. PI animals constitute a major source of virus spread (Grego *et al*., 2007). Elimination of infected animals, especially PI animals is essential to control BVDV infections (Laureyns *et al*., 2010).

BVDV is a member of the *Pestivirus* genus in the *Flaviviridae* family. BVDV particles present the Erns in multiple copies on their envelope. Moreover, large amounts of a soluble form of Erns are shed into the medium by BVDV-infected cells (Grummer *et al*., 2001; Sandvik, 2005). This finding and the fact that Erns is genetically and antigenically conserved among different isolates (Ridpath, 2005) have permitted the use of Erns as a useful marker for identification of PI animals.

The most immunogenic proteins of BVDV (Bolin, 1993), including Erns (previously E0 or gp48) and E2 structural proteins and the non-structural NS3 protein have been prepared as recombinant proteins and applied to design enzyme immunosorbent assays (ELISAs) for the detection of specific antibodies in cattle sera (Chimeno Zoth & Taboga, 2006).

During the recent years, the impact of BVD on bovine health has gradually become more apparent, and control programmes aiming at eradicating BVD have consequently attracted increasing interest, compared to the BVD management by vaccination (Brock, 2003). Many European countries have initiated national and regional control and eradication campaigns for BVDV (Moennig *et al*., 2005). In Iran, the prevalence of BVDV antibodies in adult cattle is around 25% (Hemmatzadeh *et al.*, 2000; Morshedi *et al.*, 2004; Haji Hajikolaei & Seyfiabad Shapouri, 2007). It is therefore desirable to have a rapid, sensitive and reliable means of identifying infected animals for control and eradication of BVD. For this purpose, commercial antigen capture ELISA (AC-ELISA) kits, exploiting Erns-specific monoclonal antibodies (MAbs) were designed and used for identification of PI animals which constitute 1–2% of cattle populations in endemic areas (Houe, 1999; Sandvik, 2005).

The main objective of this study was to produce the Erns antigen of BVDV in an efficient bacterial expression system to design in future a local AC-ELISA for detecting PI animals.

MATERIALS AND METHODS

Virus and cell

The NADL (National Animal Disease Laboratory) strain of BVDV maintained in Razi Vaccine and Serum Research Institute was used as a reference BVDV. The virus was propagated in bovine turbinate (BT) cells, cultured in Doulbecco's modified Eagle medium (DMEM) supplemented with 5% equine serum. After observing widespread cytopathic effects of the virus, the cells were subjected to one round of freezing at –40 °C and thawing at room temperature. The virus was then harvested and stored at –40 °C until used for RNA extraction.

PCR primers

To synthesise the complementary DNA (cDNA) and for PCR amplification of the Erns gene, the complete sequence of the NADL strain of BVDV, with the accession number NC_001461 was extracted from Gene Bank and used to design the necessary primers. Primers, which could amplify the complete sequence of Erns gene, were designed by the Primer3 software. Sequences of the primers were:

5′GCCGGGATCCGAAAACATAAC ACAGTGGAACCT-3′ (forward) and 5′GCCGCTGCAGTCAAGCGTATGCT CCAAACCAC-3′ (reverse) with *Bam*HI and *Pst*I restriction sites (underlined).

RNA extraction and amplification of Erns gene by RT-PCR

RNA was extracted from the BVDV, propagated in BT cell line, using Tripure isolation reagent (Roche, Germany). Briefly, 1 mL of Tripure was added to 0.2 mL of virus and extraction was followed as per the manufacturer instruction. Finally, the RNA was dissolved in 20 µL of diethyl pirocarbonate (DEPC)-treated water. Five µL of extracted RNA were mixed with 1 µL (50 pmol) of Erns R primer and 6.5 µL of DEPC-treated water and incubated at 65 °C for 10 min. The RNA was rapidly placed on ice, before adding 4 µL of 5× RT buffer (Fermentas, Lithuania), 2 µL of 10mM dNTPs mix and 0.5 µL (20 U) of ribonuclease inhibitor (Fermentas, Lithuania). After a 5-min incubation at 37 °C, 1 µL (200 U) of M-MuLV reverse transcriptase (Fermentas, Lithuania) was added and reverse transcription was performed for 60 min at 42 °C. For amplification of the synthesised cDNA, 5 µL of cDNA were added to 45 µL of PCR mix [0.5 µL (2.5 U) of Taq DNA Polymerase (Cinnagene, Iran), 5 µL of 10× PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4), 1.5 µL of 50 mM $MgCl_2$, 1 µL of 10 mM dNTPs mix, 1 µL (50 pmol) of each primer and 35 µL water]. The reaction was run under 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min. There were two steps of 94 °C for 3 min before and 72 °C for 10 min after 35 cycles. RT-PCR products were analysed by electrophoresis on 1% agarose gel in TAE buffer containing ethidium bromide (0.5 µg/mL).

Directional cloning of the PCR product

Erns PCR product was purified from agarose gel using a gel DNA extraction kit (Bioneer, Korea) and digested with *Bam*HI and *Pst*I restriction enzymes, through the sites created by primer sequences. Digested DNA was purified again as described above and ligated between the *Bam*HI and *Pst*I restriction sites of pMALc2x vector. Ligation was performed with T4 ligase during an overnight incubation at 4 °C, followed by 10 min inactivation at 65 °C. The construct was transformed into *E. coli* BL-21 by the method of Chung *et al.* (1989). In brief, *E. coli* was cultured in Luria Bertani (LB) broth and incubated overnight. Then the overnight culture was diluted 1:100 in fresh LB broth and incubated at 37 °C with moderate shaking until its optical density (OD) at 600 nm reached 0.3–0.4. One hundred µL of this bacterial culture were added to an equal volume of a cold 2× Transformation and Storage Solution [LB broth pH 6.5 containing 20% (w/v) polyethylene glycol 3350, 10% (v/v) DMSO and 50 mM $MgCl_2$].

One hundred µL of the competent cells were added slowly to 5 µL of ligation reaction on ice and incubated for 30 min at 4 °C. In the next step, 900 µL of LB broth containing 20 mM glucose was added and the protocol was followed by 1 h incubation at 37 °C with moderate shaking. Bacteria were finally cultured on LB agar containing 100 µg/mL ampicillin and incubated at 37 °C overnight. Bacterial colonies which appeared on this medium were screened for the presence of recombinant plasmid (pMalc2x-Erns) by miniprep extraction of plasmids and restriction endonuclease analysis. It was expected that double digestion of recombinant plasmids results in isolation of a 680 bp DNA fragment, corresponding to

the expected size of Erns PCR product. To ensure that there is no mutation in the insert which could change the coding amino acids, three recombinant plasmids were sequenced from both directions with plasmid specific primers malE and M13 F-40 (New England Biolabs, USA). For sequencing, the plasmids were sent to Genfanavaran company, Iran.

Expression of MBP-Erns fusion protein

A bacterial colony which had no mutation in the Erns insert was selected for protein expression in *E. coli* BL-21 strain. Three mL of LB broth containing 50 µg/mL ampicillin was inoculated with the bacteria and incubated overnight at 37 °C. Thirty µL of the overnight culture were used to inoculate 3 mL of fresh LB ampicillin medium containing 2 mg/mL glucose and incubated at 37 °C with shaking for about 2.5 h until the OD_{600} reached 0.5. Then 1 mL of the culture was centrifuged at 13,000 rpm and bacterial pellet was suspended in 50 µL of SDS-PAGE sample buffer. Isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM was added to remaining bacterial suspension and incubation continued for another 3 h. Bacterial cells were harvested by centrifugation and suspended in SDS-PAGE sample buffer as described above. Both samples were electrophoresed in a 10% polyacrylamide gel and the expression of the expected protein was verified after the gel was stained with Coomassie brilliant blue.

Western blotting

The nature of polypeptide(s) presumed to correspond to MBP-Erns fusion protein was evaluated by Western blotting. To analyse the expressed protein by Western blotting, SDS-PAGE was repeated and separated protein bands were transferred to nitrocellulose membrane. The membrane was blocked with PBS-Tween (Phosphate Buffered Saline with 0.05% Tween20) containing 5% (v/v) equine serum for 2 h at room temperature, washed 3 times with PBS-Tween and incubated for 1 h with 1:100 dilution of a BVDV infected cattle serum in PBS-Tween, containing 2% equine serum. The membrane was washed again as above and incubated for 1 h with anti-bovine IgG conjugated with horseradish peroxidase (Sigma, USA), diluted 1:1000 in PBS-Tween containing 2% equine serum. After washing, the membrane was developed by 4-chloro 1-naphtol (Sigma, USA) and H_2O_2 (Harlow & Lane, 1988).

Purification of MBP-Erns fusion protein

Purification of the expressed protein (MBP-Erns) was carried out on a column of maltose-affinity chromatography based amylose resin according to the manufacturer's instructions (New England Biolabs, USA). In order to this matter in the first step, the purification was performed based on MBP's affinity to amylase. Then in the second step, the MBP-Erns protein was detached from amylose resin by using 10 mM maltose solution as a competitor with amylase.

RESULTS

RT-PCR resulted in amplification of a DNA band of 680 bp, corresponding to expected length of the Erns coding region (Fig 1). After purifying from agarose gel and digesting with *Bam*H I and *Pst* I restriction enzymes, amplified DNA was cloned into plasmid pMALc2x. The insert in 3 recombinant plasmids was sequenced from both directions. The results of sequencing confirmed the identity of DNA fragment as being amplified from the Erns

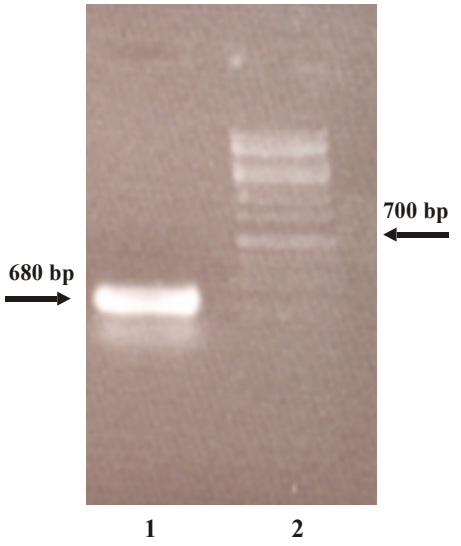

Fig. 1. Electrophoresis of PCR product. Lane 1: PCR product of Erns; lane 2: DNA size marker.

coding region of NADL strain of BVDV. A clone in which the Erns coding insert did not show any PCR induced mutation was selected for protein expression in *E. coli* BL-21 strain. Expression was induced by adding IPTG to a bacterial culture prepared as the instruction of pMalc2x expression system (New England Biolabs, USA).

Bacterial cell samples were harvested before and after induction and analysed by SDS-PAGE. Protein profiles of induced and non induced bacteria revealed that at least two polypeptides of 62 and 66 KDa were expressed in the induced bacteria (Fig. 2). Considering molecular weights of 42 and 24 kDa for MBP and Erns respectively, MBP-Erns fusion protein was estimated to have an approximate molecular weight of 66 kDa. As a result, the fusion protein seems to be expressed in the bacterial host.

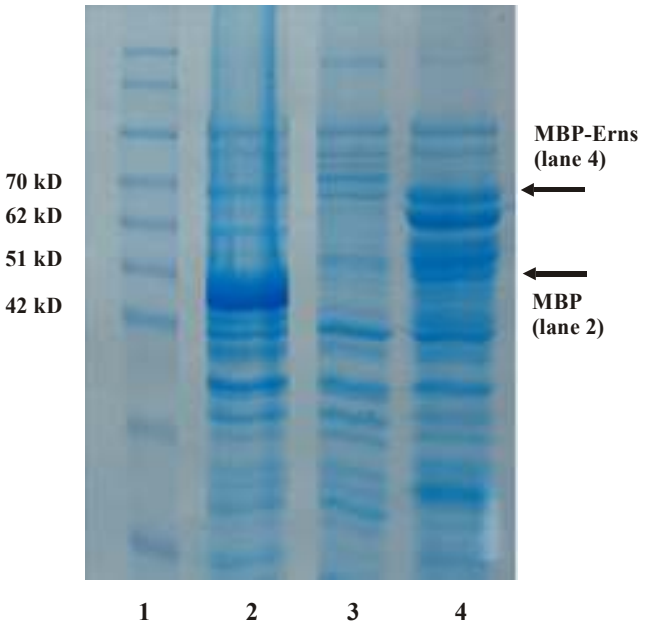

Fig. 2. SDS-PAGE analysis of bacteria expressing MBP-Erns fusion protein before (lane 3) and after (lane 4) induction by IPTG. Lane 2 indicates a bacterium expressing MBP. The molecular weight marker is shown in Lane 1.

To further analyse the protein expression, protein(s) expressed after induction were analysed by Western blotting, using a BVDV antibody positive bovine serum. In bacteria expressing the presumed MBP-Erns fusion protein, a major protein band of 66 kDa and multiple smaller bands, forming a smear like pattern, appeared after developing of nitrocellulose membrane in 4-chloro 1-naphtol (Fig. 3). This pattern indicated that the MBP-Erns fusion protein was subjected, in some extent, to proteolytic cleavage(s) in the bacterial host. This finding was confirmed by SDS-PAGE analysis of MBP-Erns fusion protein, purified on a column of amylose resin. The purified preparation of MBP-Erns revealed multiple protein bands of 66 to 52 kDa (Fig. 4). Therefore, all of the expressed and purified polypeptides seemed to be related to MBP-Erns fusion protein.

DISCUSSION

Acute individual infections of BVD can be monitored by antibody titration of paired serum samples (Weiss *et al*., 1994) in ELISA and virus neutralisation test (VNT). VNT, as the "gold standard" for antibody detection against BVDV, detects E2 specific antibodies (Edwards, 1990). This is a sensitive and specific assay but cell culture dependent and labour intensive. ELISA is more suited for screening large series of samples. So far, several BVDV-specific ELISAs have been developed for detection of both antibodies and viral antigens (Lindberg, 2003; Sandvik, 2005).

ELISAs based on native BVDV antigens produced in cell culture are usually disadvantageous. This is due to the facts that BVDV produces low levels of proteins in cell culture and the virus is highly cell associated and difficult to purify. Recombinant proteins constitute an alter-

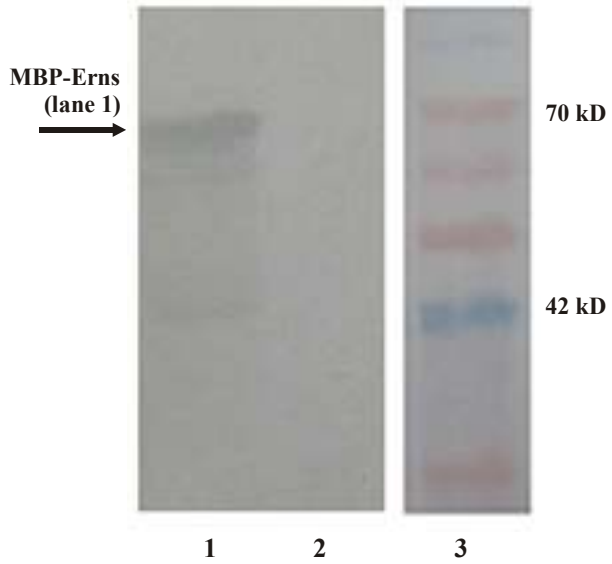

Fig. 3. Detection of recombinant protein by Western blot. Lane 1: a bacterium expressing MBP-Erns fusion protein); lane 2: bacteria expressing MBP (negative control); lane 3: protein molecular weight marker.

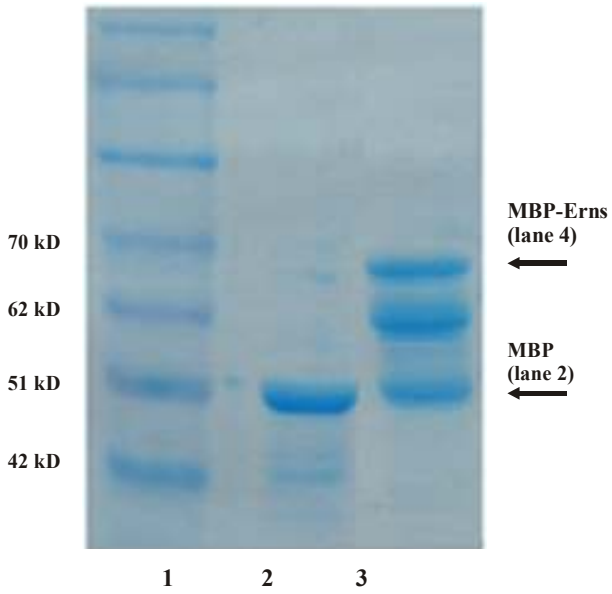

Fig. 4. SDS-PAGE analysis of purified MBP-Erns fusion protein. Lane 1: protein molecular weight marker; lane 2: purified MBP; lane 3: purified preparation of MBP-Erns fusion protein.

native source of viral antigens and several studies have demonstrated the ability of BVDV recombinant antigens to provide reliable detection of BVDV antibodies (Reddy *et al.,* 1997; Vanderheijden *et al.,* 1993).

To characterise the immune response of cattle to BVDV glycoprotein Erns, Kwang *et al.,* (1992) produced a recombinant insoluble Erns-GST (glutathione-S-transferase) fusion protein in *E. coli*. They found that antibodies to Erns were present in cattle, following vaccination with killed or modified-live virus as well as natural infection.

In a research by Reddy *et al.,* (1997), BVDV genes for Erns (gp48) and NS3 were expressed in *E. coli* and the recombinant proteins were used in ELISA to detect BVDV infection. The proteins which were expressed as GST insoluble fusion proteins were denatured by urea treatment, renatured by dialysis and purified on a column of glutathione. Statistical analysis showed a high degree of correlation between the reactivity of recombinants and natural antigens in ELISA using bovine sera. Moreover, monoclonal antiboies (Mabs) raised against BVDV-NADL strain reacted specifically with the recombinant Erns.

Chimeno Zoth & Taboga (2006) developed three independent ELISA tests based on Erns, E2 or NS3 antigens produced by baculovirus expression system. The three assays showed high levels of sensitivity and specificity, compared to the reference VNT test. However, Erns and NS3 ELISAs were able to detect anti-BVDV antibodies, undetectable by E2 ELISA and VNT. This is in agreement with the finding that Erns and NS3 proteins are more conserved among BVDV strains (Ridpath, 2003) and suggests that their inclusion in the design of a diagnostic test allows extending the spectrum of detectable antibodies.

Grego *et al.* (2007) prepared a baculovirus expressed Erns and applied it in ELISA to detect BVDV specific antibodies. Comparison of this ELISA with a commercially available competitive ELISA targeting anti-NS2/3 antibodies indicated a good correlation between both ELISA. Nevertheless, sera collected from cattle immunised with inactivated vaccines were positive in Erns ELISA but not in NS2/3 antibody ELISA. This is in accordance with the fact that animals immunised with inactivated vaccines may produce antibody only against structural proteins.

Overall, the results of studies on the application of recombinant Erns in ELISA outline this protein as an alternative candidate for detection of antibodies generated by natural infection or BVDV vaccination. On the other hand, Erns specific monoclonal antibodies are valuable tools in AC-ELISA for identification of PI animals. PI animals are highly infectious but often do not manifest their carrier status through obvious clinical symptoms (Ames, 2005). Identification of such animals is carried out by virus isolation, RT-PCR and viral antigens detection by immunohistochemistry and AC-ELISA. ELISA technology, although less sensitive, is better suited as a broad-based diagnostic tool for detecting BVDV antigens. In this regard, a commercial, easy to use AC-ELISA kit is available for detection of viral antigens in serum as well as whole blood and even skin biopsies of PI animals (Hilbe *et al.*, 2007). AC-ELISA microtitre plates are coated with MAbs specific for Erns (Sandvik, 2005).

Although recombinant Erns or its subunit have been previously expressed in prokaryotic and baculovirus systems, present study describes expression of the protein in *E. coli* with new primers and different vector, pMALc2x. This vector has a strong promoter and so its protein expression will be high compared to the other vectors. The rate of gene cloning into this vector and subsequent transformation was high and noticeable, which enables work with this vector to be done more easily than with the other vectors. The results of SDS/PAGE and western blotting showed that the transformed *E. coli* BL-21 expresses Erns considerably and after IPTG induction the amount of protein expression has increased because of the presence of *lac* promoter in pMAlc2x. The strong *lac* promoter of pMALc2x vector allows a high level of expression of the protein and the maltose binding protein tag which is added to the N terminus part of the molecule facilitate the purification of the recombinant protein by amylose resin. Moreover, MBP tag usually allows a soluble expression of eukaryotic proteins in *E. coli*. Purification of the MBP-Erns fusion protein without any treatment applied for insoluble inclusion bodies indicate that in this system the protein is expressed in a soluble form. The result of this study showed that Erns was successfully amplified, cloned and expressed in the prokaryotic system. Prokaryotic expression is still considered because it is simpler and less expensive than eukaryotic expression. Prokaryotic expression of proteins with pMAL-c2X plasmid is one of the most efficient expression systems and by exploiting this system to prepare Erns specific monoclonal antibodies and development of BVDV specific ELISAs, we have already produced a large amount of Erns recombinant antigen by this system. Based on our results it appears that this plasmid construct may be suitable for the production of Erns recombinant antigen and Erns specific antibodies to develop BVDV laboratory diagnostic assays.

REFERENCES

Ames, T. R., 2005. Bovine Viral Diarrhea Virus: Diagnosis, Management and Control. Blackwell Publishing, Oxford, UK, pp. 171–175.

Bolin, S. R., 1993. Immunogens of bovine viral diarrhea virus. *Veterinary Microbiology*, **37**, 263–271.

Brock, K. V., 2003. The persistence of bovine viral diarrhea virus. *Biologicals*, **31**, 133–135.

Chimeno Zoth, S. & O. Taboga, 2006. Multiple recombinant ELISA for the detection of bovine viral diarrhea virus antibodies in cattle sera. *Journal of Virological Methods*, **138**, 99–108.

Chung, C. T., S. L. Niemela & R. H. Miller, 1989. One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution (recombinant DNA). *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 2172–2175.

Edwards, S., 1990. The diagnosis of bovine virus diarrhea-mucosal disease in cattle. *Revue Scientifique et Technique (Office International des Epizooties)*, **9,** 115–130.

Grego, E., F. Uslenghi, M. Strasser, C. Luzzago, M. Frigerio, S. Peletto & S. Rosati, 2007. Development and application of an enzyme-linked immunosorbent assay for detection of bovine viral diarrhea antibody based on Erns glycoprotein expressed in a baculovirus system. *Journal of Veterinary Diagnostic Investigation*, **19**, 21–27.

Grummer, B., M. Beer, E. Liebler Tenorio & I. Greiser Wilke, 2001. Localization of viral proteins in cells infected with bovine viral diarrhea virus. *Journal of General Virology*, **82**, 2597–2605.

Gunn, G. J., H. W. Saatkamp, R. W. Humphry & A. W. Stott, 2005. Assessing economic and social pressure for the control of bovine viral diarrhea virus. *Preventive Veterinary Medicine*, **72**, 149–162.

Haji Hajikolaei, M. R. & M. R. Seyfiabad Shapouri, 2007. Serological study of bovine viral diarrhea virus infection of cattle in Ahvaz. *Journal of Veterinary Research*, **62**, 21–26.

Harlow, E. & D. Lane, 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory (GSH), pp. 486–510.

Hemmatzadeh, F., G. Kojouri, R. Kargar Moakhar & M. Rohany, 2000. A serological survey on bovine viral diarrhea virus infection in Chahar Mahal Bakhtiary province. *Journal of the Faculty of Veterinary Medicine, University of Tehran*, **56**, 85–92.

Hilbe, M., H. Stalder, E. Peterhans, M. Haessig, M. Nussbaumer, C. Egli, C. Schelp, K. Zlinszky & F. Ehrensperger, 2007. Comparison of five diagnostic methods for detecting bovine viral diarrhea virus infection in calves. *Journal of Veterinary Diagnostic Investigation,* **19**, 28–34.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhea virus (BVDV) infections. *Veterinary Microbiology*, **64**, 89–107.

Kwang, J., E. T. Littledike, R. O. Donis & E. J. Dubovi, 1992. Recombinant polypeptide from the gp48 region of the bovine viral diarrhea virus (BVDV) detects serum antibodies in vaccinated and infected cattle. *Veterinary Microbiology*, **32**, 281–292.

Laureyns, J., S. Ribbens & A. D. Kruif, 2010. Control of bovine virus diarrhea at the herd level: Reducing the risk of false negatives in the detection of persistently infected cattle. *The Veterinary Journal*, **184**, 21–26.

Lindberg, A. L. E., 2003. Bovine viral diarrhea virus infections and its control. A review. *Veterinary Quarterly,* **25**, 1–16.

Moennig, V., H. Houe & A. Lindberg, 2005. BVD control in Europe: Current status and perspectives. *Animal Health Research Reviews*, **6**, 63–74.

Morshedi, A., A. L. Mahmoodian, B. Dalire Naghade & J. Hajizadeh, 2004. Serological survey of cattle infected with BVD virus by indirect ELISA and comparison of the use of milk ELISA and serum ELISA

in Urmia. *Journal of the Faculty of Veterinary Medicine, University of Tehran*, **59**, 227–231.

Reddy, J. R., Y. Kwang, O. Okwumabua, S. Kapil, T. M. Loughin, K. F. Lechtenberg, M. M. Chengappa & H. C. Minocha, 1997. Application of recombinant bovine viral diarrhea virus proteins in the diagnosis of bovine viral diarrhea infection in cattle. *Veterinary Microbiology*, **51**, 119–133.

Ridpath, J. F., 2005. Classification and molecular biology. In: *Bovine Viral Diarrhea Virus: Diagnosis, Management and Control*, eds S. M. Goyal & J. F. Ridpath, Blackwell Publishing, Oxford, UK, pp. 65–80.

Ridpath, J. F., 2003. BVDV genotypes and biotypes: Practical implications for diagnosis and control. *Biologicals*, **31**, 127–131.

Sandvik, T., 2005. Selection and use of laboratory diagnostic assays in BVD control programmes. *Preventive Veterinary Medicine*, **72**, 3–16.

Vanderheijden, N., L. D. Moerlooze, D. Vandenbergh, G. Chappuis, A. Renard & C. Lecomte, 1993. Expression of the bovine viral diarrhea virus Osloss p80: Its use as ELISA antigen for cattle serum antibody detection**.** *Journal of General Virology*, **74**, 1427–1431.

Weiss, M., C. Hertig, M. Strasser, H. R. Vogt & E. Peterhans, 1994. Bovine virus diarrhea/mucosal disease: A review. *Schweizer Archiv für Tierheilkunde***, 136**, 173–185.

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