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Molecular Characterization of the Isolated Strains of Bovine Viral Diarrhea Virus

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Abstract: Infection with bovine viral diarrhea virus (BVDV) occurs globally and is imposing large direct and indirect productivity losses on the beef and dairy industries. During 2005, bovine viral diarrhea (BVD) was added to the OIE list of the diseases and at that time, BVD was already notifiable in seven EU countries. The target of this study was to isolate a new virus from died calf in a private farm suffered from BVDV infection and compared its molecular characterization with the different Egyptian strains of BVDV. An international reference strain (NADL: National Animal Disease Laboratory; Ames Iowa, USA), the Egyptian strains (Iman and Kenna) as cp-BVDV-I that isolated at 1975 and 1982, respectively and Behera-CP 58/99 that isolated from the milk and identified as cp-BVDV-II were cultured, Concentrated and purified. A new isolate was isolated with antigenic and genetic identification. The soluble proteins of the five strains were analyzed using SDS-PAGE and revealed that many apparently homologous proteins migrate heterogeneously. These differences suggest genetic variability in the respective regions of their genomes coding for these polypeptides. Concerning to the dendrogram analysis, the relatedness percentage between the different Egyptian strains and NADL strain was 53.49% and this supports the hypothesis that large discrepancies present among the Egyptian strains as well as the international reference one. From the results, there are high antigenic and genetic variation among the local strains and the international one. These developments alter the pressure for control and will probably result in important changes relating to national BVD control. Vaccine prepared from the local strains that include both genotypes must be use.

Key words: BVDV · Egyptian strains · Molecular characterization

INTRODUCTION

Bovine viral diarrhea virus (BVDV) infection is imposing large direct and indirect productivity losses on the beef and dairy industries. Severe financial losses incurred as reduced rates of ovulation, fertilization, embryonic survival, fetal survival, perinatal survival, milk production, respiratory disorders, abortion, congenital defects and growth retardation [1, 2].

Two distinct genotypes were existing, BVDV-I and BVDV-II, together with border disease virus (BDV), classical swine fever virus (CSFV) and Giraffe, constitute the genus *Pestivirus* of the family *Flaviviridae* [3]. BVDV-II was detected initially in cattle of North America [4, 5] and later sporadically in several other countries [6]. However, according to cell culture behavior, BVDV occurs in two biotypes, non-cytopathic (ncp) and cytopathic (cp) [7].

BVDVs are genetically variable, containing a single positive-stranded RNA of approximately 12.5 kilo base (kb) in length. The viral genome contains a single large open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR) [8, 9]. The ORF encodes a proximately 3988 amino acids and ultimately yields 11 to 12 final cleavage polyprotein products (NH2---N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B---COOH) through co- and post-translational processing by cellular and viral proteases [10 - 12].

There are two forms of infection associated with BVDV: acute or transient infection and persistent infection (PI). Acute infection is post-natal infection in an immunocompetent host. In contrast, PI only occur in utero infection of the developing fetus with ncp-BVDV prior to the development of immunocompetence [13].

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The target of this study was to isolate a new virus from died calf in a private farm suffered from BVDV infection and compared its molecular characterization with the different Egyptian strains of BVDV.

MATERIAL AND METHODS

Viral Strains and Cell Culture: An international reference strain (NADL: National Animal Disease Laboratory; Ames Iowa, USA), the Egyptian strains (Iman and Kenna) as cp-BVDV-I that isolated in 1975 and 1982, respectively [14, 15] and Behera-CP 58/99 that isolated from the milk and identified as cp-BVDV-II in 2002 [16], were cultured in Madin-Darby bovine kidney (MDBK) cells. The strains were titrated according to Reed and Muench [17] method.

Animals and Tissue Samples: Calves (n=120) in a private farm were suffered from bloody diarrhea, epistaxis, petechial and ecchymotic hemorrhages on mucous membrane. A case fatality was 44/120 (36.6%) that calves were death at age 5 days to 2.5 months. Post-mortem (PM) lesions on 3 died calves sent to the laboratory (one freshly died and the others died since 24 and 36 hrs) were mucosal hemorrhage on the internal organs, congested liver, lung and spleen, petechial hemorrhage on intestinal mucosa, lymphoid depletion and congenital anomalies of the central nervous system.

From the freshly died calf (13 days old), samples of mesenteric lymph nodes, spleen, lung, brain and liver were collected. The samples were prepared and inoculated on MDBK with daily examination for the cytopathic effects (CPE) development along 5-7 days for 3 passages as cited by George *et al*. [18].

Identification of the Agent

Immunofluorescence Antibody (IFA) Technique: From the 3rd passage and after 48 hrs post-inoculation (Po.I.) of the 4th passage, the viral agent was identified using immunofluorescence antibody (IFA) technique as outlined by Bolin *et al*. [19]. Hyperimmune serum prepared in calf against NADL strain and fluorescence isothiocyanate (FITC) conjugated anti-bovine IgG were used to identify the inoculated tested virus.

Extraction of RNA: Intact RNA, from the inoculated tested isolate, NADL and Behera-CP 58/99 strains as positive control and mock infected cells as a negative control, was extracted using an acid guanidin-phenol-chloroform (AGPC) method as mentioned by Chomczynski and Sacchi [20].

Primers Design: According to NADL strain sequence published by Collett *et al.* [8], a primer set that amplify 288 (bp) within the 5' UTR for detection the viral genome was designated by Vilcek *et al.* [21]. The primer sequences were UTR1 5'….ATG CCC WTA GTA GGA CTA GCA…3' (108-128^{nts}) where W= A or T and UTR2 5'….TCA ACT CCA TGT GCC ATG TAC….3' (375-395^{nts}).

Also, Sullivan and Akkina [22] had designated the primers P1 5'…..AAC AAA CAT GGT TGG TGC AAC TGGT….3' (1424-1449^{nts}) and P2 5'… CTT ACA CAG ACA TAT TTG CCT AGG TTC CA….3' (2221-2250^{nts}) sequences that shared maximum homology with all ruminant pestiviruses. TS1 5'….TAT ATT ATT TGG AGA CAG TGA ATG TAG TAG CT…3' (1684-1716^{nts}), TS2 5'…. TGG TTA GGG AAG CAA TTA GG….3' (1802-1821^{nts}) and TS3 5'… GGG GGT CAC TTG TCG GAGG….3' (2027-2045^{nts}) sequences were type specific for BDV, BVDV genotype II and I respectively with the amplified products (P1 and P2) by using nested reverse transcription-polymerase chain reaction (nRT-PCR) technique.

Genomic Detection of BVDV Isolate: The reverse transcription of the extracted RNA was carried out using hexameric primer and molony murine leukemia virus-reverse transcriptase (MML-RT) enzyme according to the manufacturer (Promega, Madison, WI, USA). Amplification of the reverse transcript RNA within 5' UTR using primers UTR1 and UTR2 was carried out in 30 cycles as in Vilcek *et al*. [21].

Genotyping the BVDV Isolate by nRT-PCR Technique: In 35 cycles, the first amplification of the reverse transcript RNA using the primers P1 and P2 was done while the second amplification using the primers TS1, TS2 TS3 and amplified products of the first round (P1 and P2) was carried out in 25 cycles as described by Sullivan and Akkina [22].

Concentration and Purification the Viral Strains: For each strain, the viral suspension was collected, concentrated and purified using polyethylene glycol 6000 (PEG-6000) as described briefly by Kelling *et al.* [23].

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): The soluble proteins of the five strains were denaturated and separated in sodium dodecyl sulphate-12% polyacrylamide separating gel electrophoresis (SDS-PAGE) according to Laemmli [24].

Using mini-protein II electrophoresis cell (Bio-Rad) at 70 volts for 3 hrs, the soluble proteins were separated and matched with full-range rainbow molecular weight (M_r) marker (RNP800; Amercham Pharmacia-Biotech, UK Ltd., England) that range from 10 to 250 kilo dalton (KDa). The gel was stained using commassie brilliant blue R-250 quick method for 20 minutes and destain at room temperature in 5% methanol and 7.5% acetic acid with shaking for 30 minutes. The different fractions were quantified using Bio-Rad GS 700 imaging densitometer molecular analysis software.

Dendrogram Analysis: Dendrogram was constructed to reveal the relatedness percent between the different strains using gel proanalyzer version 4.5 cypermedica, USA.

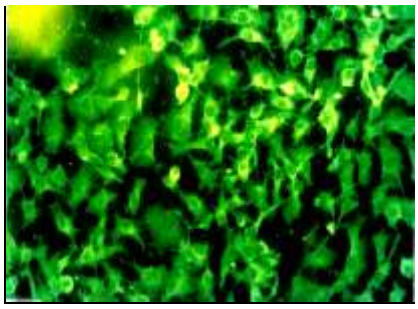

Fig. 1: BVDV-free MDBK cells inoculated with homogenized mesenteric lymph nodes, treated with anti BVDV hyperimmune serum and FITC conjugated anti-bovine IgG after 48 hrs Po.I of 4th passage. At nuclear pools and Perinuclear intra cytoplasm, fluorescence granules were seen.

RESULTS

Immunofluorescence Antibody: Fluorescence granules were observed at nuclear pools and perinuclear intra cytoplasm in MDBK cells inoculated with homogenized mesenteric lymph nodes (Figure 1).

Genomic Detection of BVDV Isolate: Sharp bands (288 bp) were developed within the 5' UTR of the reverse transcript RNA in both NADL (a positive control) and new isolate while not in the mock infected MDBK cells (Figure 2).

Genotyping the BVDV Isolate Using nRT-PCR Assay: Amplification of the first round products (P1 and P2) with type specific primers TS1, TS2 and TS3 showed, the products (448 bp) were amplified in both Behera CP 58/99 as genotypeII positive control and the new isolate while a product (223 bp) was amplified in NADL strain as genotype I positive control (Figure 3).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): From figure 4 and table 1, the SDS-PAGE analysis of the soluble proteins for the five strains revealed that, NADL strain displayed only

Table 1: SDS-PAGE analysis of five strains of BVDV

Lanes:	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5		Lane 6	
Bands	KDa	%	KDa	%	KDa	%	KDa	%	KDa	%	KDa	%
1	133.72	43.7	150.32	34.2	149.15	24.12	130.63	50.4	158.76	30.9	250	5.33
2	93.65	2.29	80.762	7.67	84.658	6.1	80.762	5.19	79.148	5.43	160	5.8
3	48.536	21.6	44.837	13.2	41.012	5.55	45.735	8.79	67.149	7.949	105	6.51
4	37.514	1.46	34.587	10.3	38.645	5.78	34.724	10.2	46.65	18.6	75	8.56
5	32.596	2.6	30.358	2.07	31.582	2.82	30.599	2.33	38.264	13.3	50	9
6	29.324	4.19	28.772	7.47	30.238	11.25	28.992	1.13	28.663	7.178	35	5.76
7	27.805	2.19	27.076	5.3	28.992	7.86	27.7	1.97	26.668	2.46	30	15.9
8	25.191	5.5	24.705	5.93	27.282	11.24	26.466	2.6	24.125	14.2	25	14.1
9	16.693	16.5	21.169	4.33	26.166	2.94	25.479	8.7			15	20.5
10			17.926	9.54	25	3.22	22.465	8.75			10	8.5
11					22.465	10.71						
12					17.926	8.41						
Sum		100.0		100.0	100.0	100.0		100.0		100.0		100.0
In Lane		100		100	100	100		100		100		100

Lane 6= protein marker (RPN 800), Lane 5= NADL strain, Lane 4= Iman strain, Lane 3= Kenna strain, Lane 2= new isolate, Lane 1= Behera cp 58/99

Fig. 2: Agarose gel (1%) analysis of RT-PCR amplified products for genomic detection within 5' UTR. Lane M is 100 bp DNA ladder (Aβ gene, UK), lane 1 is a positive control (NADL strain), lane 2 is the amplified product of the new isolate (288 bp) and lane 3 is a negative control.

Fig. 3: Agarose gel (1%) analysis of nRT-PCR amplified products with type specific primers. Lane M is 100 bp DNA ladder (Aβ gene, UK), lane 1 is NADL genotype I (223 bp) and lane 2 is Behera- CP 58/99 genotype II as positive controls (448 bp), lane 3 is a negative control and lane 4 is the amplified product of the new isolate (genotype II, 448 bp).

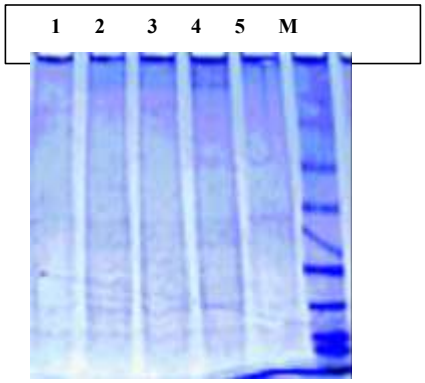

Fig. 4: SDS-PAGE analysis of the 5 strains. Lane M is the protein marker (RPN 800, Amersham Pharmacia-Biotech), lane 5 is the NADL strain, lane 4 is Iman strain, lane 3 is Kenna strain, lane 2 is the new isolate and lane 1 is Behera CP 58/99 strain.

8 bands with M_r ranges from 24.125 to 158.76 KDa while the Egyptian strains displayed, 10 bands (22.465-130.63 KDa), 12 bands (17.926-149.15 KDa), 10 bands (17.926-150.32 KDa) and 9 bands (16.693-133.72 KDa) for Iman, Kenna, new isolate and Behera CP 58/99, respectively.

Dendrogram Analysis of Protein Fractions: Dendrogram analysis of electrophoritic bands for the five strains according to density and position of different bands showed, the homology between NADL and Iman strains was 57.14%, between Kenna and new isolate was 80%, between Kenna, new isolate and Behera CP 58/99 was 62.38% and finally between Kenna, new isolate, Behera CP 58/99, NADL and Iman strains was 53.49% (Figure 5).

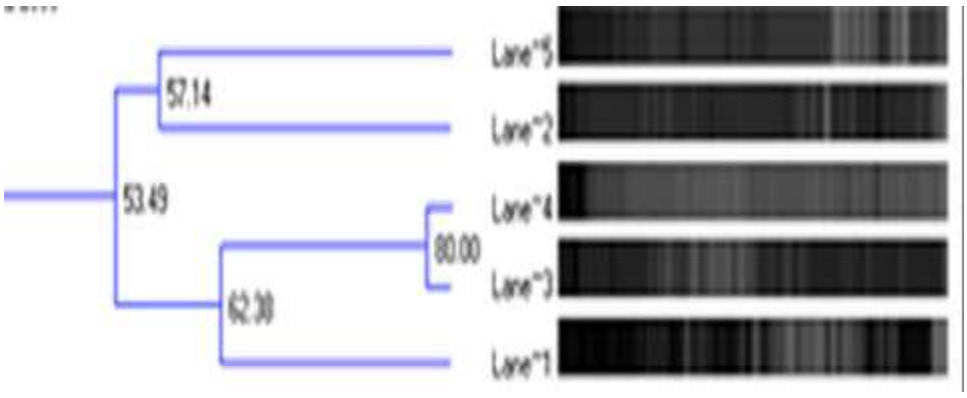

Fig. 5: Dendrogram analysis of SDS-PAGE of the five strains

DISCUSSION

There is a wide range of clinical presentations resulting from infection with BVDVs. With some severe acute BVD (SABVD), the gross lesions may be extensive. Died calf under study had mucosal hemorrhage on the internal organs, congested liver, lung and spleen, petechial hemorrhage on intestinal mucosa and congenital anomalies of the central nervous system as recorded previously by Corapi *et al.* [25] and Carmen *et al*. [26]. However, the lymphoid tissue is the principle site for the virus replication and spread. While clinically SABVD virus infections associated with lymphoid depletion, the depletion was seen with highly virulent ncp-BVDV strains by Ridpath *et al.* [27] as observed in this study.

It is difficult to generate polyclonal or monoclonal antibodies that differentiate BVDVs from BDV and CSFV but yet recognize all BVDV strains. BVDV specific antibodies can be classified into two functional groups. Antibodies to the viral glycoproteins (E0 and mainly E2) that neutralize the virus and cross react with different strains of BVDVs [28]. Conversely, the highly immunogenic non-structural protein NS2/NS3, which is essential for viral replication, is conserved antigenically between all pestiviruses [29]. Antibodies to the NS2/NS3 protein do not neutralize BVDVs but since they can be detected readily by other serological tests [30]. In the inoculated MDBK cells with mesenteric lymph nodes suspension, fluorescence granules were seen at nuclear pools and Perinuclear intra cytoplasm as recommended previously by Donis *et al.* [31], using hyperimmune serum prepared in calf against NADL strain (Figure 1).

The clearest system for differentiating pestiviruses is based on differences in genomic motifs. The most conserved portion of the pestivirus genome is located within the 5' UTR that about 70% of the nucleotides are participate in the base pairing to confirm a highly conserved secondary structure [5, 32]. The sequence homology within each genotype was over 86% - 93%, while only 75% between genotype I and II [33] and may reach to 95% [34]. Using a primer set flanked the region 108 to 395 within the 5' UTR as cited by Vilcek *et al.* [21], the reverse transcript RNA of the propagated isolate as well as the NADL strain as a positive control was amplified giving a sharp band of 288 bp (Figure 2).

Sullivan and Akkina [22] had developed nRT-PCR assay to identify and distinguish between 3 genotypes of pestiviruses, BDV, BVDV genotype I and II. The use of primer homologous to a conserved region of the pestiviruses genomic sequence ensures that all strains of pestiviruses will be amplified (826 bp in the first round of amplification). Type specific primers have yield DNA products unique size characteristic for each of the 3 pestviruses. The new isolate was identified as genotype II that a sharp band of DNA (448 bp; TS2 and P2) was amplified as well as in Behera CP 58/99 (as genotype II positive control) while a sharp band of DNA (223 bp; TS3 and P2) was amplified in NADL strain genotype I positive control (Figure 3).

The single ORF of the BVDV genome is encoding a polyprotein of 3988 amino acids with an expected M_r 449 KDa [8]. SDS-PAGE analysis of soluble proteins of the five strains revealed that many apparently homologous proteins migrate heterogeneously. This suggests genetic variability in the respective regions of their genomes coding for these polypeptides (Figure 4). However, the reference strain, NADL, has only 8 bands while Iman and new isolate have 10 bands. Moreover, the Kenna strain has an excess polypeptide bands with M_r 22.465 and 17.392 KDa that not present in Iman and new isolate, while the Behera CP 58/99 has only 9 bands (Table 1). Concerning to the dendrogram analysis, the relatedness percentage between the different Egyptian strains and NADL strain was 53.49% and this supports the hypothesis that large discrepancies present among the Egyptian strains and the international reference one (Figure 5).

From the results, there are high antigenic and genetic variation among the local strains and the international one. These developments alter the pressure for control and will probably result in important changes relating to national BVD control. Vaccine prepared from the local strains that include both genotypes must be use.

REFERENCES

1. Houe, H., 2003. Economic impact of BVDV infection in dairies. Biological, 31: 137-143.
2. Viet, A.F., C. Fourichon and H. Seegersm, 2006. Simulation study to assess the efficiency of a test-and-cull scheme to control the spread of the bovine viral diarrhea virus in a dairy herd. Preventive Veterinary Medicine, 76: 151-166.
3. Fauquet, C.M., M.A. Mayo, J. Maniloff, U. Desselberger and L.A. Ball, 2005. Family Flaviviridae. In: Fauquet, C.M., M.A. Mayo, J. Maniloff, U. Desselberger and L.A. Ball, (Eds.), Virus Taxonomy-Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA, USA.

4. Pellerin, C., J. van den Hurk, J. Lecomte and P. Tussen, 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology, 203: 260-268.
5. Ridpath, J.F., S.R. Bolin and E.J. Dubovi, 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology, 205: 66-74.
6. Vilcek, S., B. Durkovic, M. Kolesarova, I. Greiser-Wilke and D. Paton, 2004. Genetic diversity of international bovine viral diarrhea virus (BVDV) isolates: identification of a new BVDV-1 genetic group. Veterinary Research, 35: 609-615.
7. Yesilbag, K., C. Forsterb, B. Bank-Wolf, Z. Yýlmaz, F. Alkand, A. Ozkuld, I. Burgud, S.C. Rosales, Heinz-Jurgen and T.M. Konig, 2008. Genetic heterogeneity of bovine viral diarrhea virus (BVDV) isolates from Turkey: Identification of a new subgroup in BVDV-1. Veterinary Microbiology, 130: 258-267.
8. Collett, M.S., R. Larson, C. Gold, D. Strick, D.K. Anderson and A.F. Purchio, 1988. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. Virology, 165: 191-199.
9. Deng, R. and K.V. Brock, 1992. Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. Virology 191: 867-879.
10. Collett, M.S., V. Moenning and M.C. Horzinek, 1989. Recent advances in pestivirus research. Journal of General Virology, 70: 253-266.
11. Rumenapf, T., G. Unger, J.H. Strauss and H.J. Thiel, 1993. Processing of the envelope glycoproteins of pestiviruses. Journal of Virology, 67: 3288-3294.
12. Tautz, N., K. Elbers, D. Stoll, G. Meyers and H.J. Thiel, 1997. Serine protease of pestiviruses: determination of cleavage sites. Journal of Virology, 71: 5415-5422.
13. Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhea virus infection. The Veterinary Clinics of North America Food Animal Practice, 11: 425-445.
14. Baz, T.I., 1975. Isolation, characterization and serological studies on BVD-MD virus in Egypt. Thesis (Ph. D), Microbiology Department, Faculty of Veterinary Medicine, Cairo University.
15. Baz, T.I., 1982. Isolation of BVD-MD virus from new born calves suffering from intermittent diarrhea and severe unthriftiness. Agriculture Research Review, 60: 175-188.
16. Abd El-Hafeiz, Y.G.M., 2002. Bovine viral diarrhea virus (BVDV): Molecular-based diagnostic approach and isolation of cytopathic and non cytopathic strains genotype 11 from cow milk. Thesis (Ph. D), Virology Department, Faculty of Veterinary Medicine, Cairo University.
17. Reed, L.J. and H. Muench, 1938. A simple method of estimating fifty percent end points. American Journal of Hygiene, 27: 493-497.
18. George, V.G., J.C. Hierholzer and E.W. Ades, 1996. Cell culture, In: Mahy, B.W.J. and H.O. Kangro (Eds.), Virology methods manual. Academic press limited, London NW1, pp: 3-23.
19. Bolin, S.R., P.J. Matthews and J.F. Ridpath, 1991. Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. Journal of Veterinary Diagnostic Investigation, 3: 199-203.
20. Chomczynski, P. and N. Sacchi, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry, 162: 156-159.
21. Vilcek, S., A.J. Herring, J.A. Herring, P.F. Nettleton, J.P. Lowings and D.J. Paton, 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three geno-groups using polymerase chain reaction and restriction endonuclease analysis. Archives of Virology, 136: 309-323.
22. Sullivan, D.G. and R.K. Akkina, 1995. A nested polymerase chain reaction assay to differentiate pestiviruses. Virus Research, 38: 231-239.
23. Kelling, C.L., J.E. Kennedy, L.C. Stine, K.K. Rump, P.S. Paul and J.E. Partridge, 1990. Genetic comparison of ovine and bovine pestiviruses. American Journal of Veterinary Research, 51: 2019-2024.
24. Laemmli, U.K., 1970. Structural proteins during the assembly of the head of bacterophage T4. Nature, 227: 680-685.
25. Corapi, W.V., R.D. Elliott, T.W. French, D.G. Arthur, D.M. Bezek and E.J. Dubovi, 1990. Thrombocytopenia and hemorrhages in veal calves infected with bovine viral diarrhea virus. Journal of American Veterinary Medical Association, 196: 590-596.
26. Carmen, S., T. van Dreumel, J. Ridpath, M. Hazlett, D. Alves, E. Dubovi, R. Tremblay, S. Bolin, A. Godkin and N. Anderson, 1998. Severe acute bovine viral diarrhea in Ontario, 1992-1995. Journal of Veterinary Diagnostic Investigation, 10: 27-35.

27. Ridpath, J.F., J.D. Neill, M. Frey and J.G. Landgraf, 2000. Phlogenetic, antigenic and clinical characterization of type 2 BVDV from North America. Veterinary Microbiology, 77: 145-155.
28. Brock, K.V., 1995. Diagnosis of bovine viral diarrhea virus infections. The Veterinary Clinics of North America Food Animal Practice, 11: 549-561.
29. Collett, M.S., 1992. Molecular genetics of pestiviruses. Comparative Immunology, Microbiology and Infectious Diseases, 15: 145-154.
30. Sandvik, T., 1999. Laboratory diagnostic investigations for bovine viral diarrhea virus infections in cattle. Veterinary Microbiology, 64: 123-134.
31. Donis, R.O., W. Corapi and E.J. Dubovi, 1988. Neutralizing monoclonal antibodies to bovine viral diarrhea virus bind to the 56 k to 58 k glycoprotein. Journal of General Virology, 69: 77-86.
32. Ridpath, J.F. and S.R. Bolin, 1997. Comparison of the complete genomic sequence of the border disease virus, BD31, to other pestiviruses. Virus Research, 50: 237-243.
33. Kim, S.G. and E.J. Dubovi, 2003. A novel simple one-step, single tube RT-duplex PCR method with an internal control for detection of bovine viral diarrhea virus in bulk milk, blood and follicular fluid samples. Biological, 31: 103-106.
34. Ridpath, J.C. and S.R. Bolin, 1995. The genomic sequence of a virulent bovine viral diarrhea virus (BVDV) from the type II genotype: detection of a large genomic insertion in a noncytopathic BVDV. Virology, 212: 39-46.

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