Genetic Variability of Bovine Viral Diarrhea Virus in the 5'-UTR in the Central Anatolia of Turkey

Baki Sarikaya[1], Ahmet Kürşat Azkur[1], Serkal Gazyagci[2] & Muhammet Eren Aslan[1]

ABSTRACT

Background: The genus Pestivirus in the family *Flaviviridae* comprises the members bovine viral diarrhea virus type 1 (BVDV-1), classical swine fever virus and border disease virus. The BVDV enveloped and the genome is a single-strand positive sense RNA molecule of approximately 12.3 kilobases in length. The genome is transcribed as a single open reading frame, flanked by 5' and 3' untranslated regions. Genetic typing of BVDV has usually been performed using sequences from the 5'-UTR, N^{pro} and E2 regions. BVDV is an RNA virus with a high genome variability having practical consequences on epidemiology, diagnosis and disease control. Genetic monitoring was suggested as the first step in BVDV control because genetic typing of BVDV shows evidence of an increasing number of variants. For this reason circulating genetic typing of BVDV is important update these data. Circulating BVDV in the field shows genetic and antigenic diversity. 5'-UTR nucleotide sequence analysis has been widely used for pestivirus genotype identification. To further characterize the BVDV, the nucleotide sequence of the 5'-UTR that represents a conserved region of the virus genome was analyzed in many studies. The purpose of the current study was to investigate genotypes of pestivirus were circulating in cattle populations in Central Anatolia Region of Turkey.

Materials, Methods & Results: Blood samples from 160 animals in randomly selected seven cattle dairy farms that lives with more than 1100 cattle, were collected between November 2009 and March 2010 from Kirikkale (n = 57), Corum (n = 50), Ankara (n = 21), Yozgat (n = 17), Kirsehir (n = 15) cities where are located in Central Anatolia region of Turkey. To detect BVDV in cattle, viral RNA was extracted from whole blood samples using QIAamp Viral RNA Kit and the 5'-UTR were targeted using RT-nested PCR accomplished with first round primers pair panpestivirus and with second round BVDV-1a, BVDV-1b and, BVDV-2 pooled blood samples, respectively. It was detected in second round of RT-nested PCR that BVDV-1a and, BVDV-2 rate are 0.625%, 7.5% in the cattle respectively but not BVDV-1b. Positive PCR amplicons were purified from agarose gel by using commercial DNA purification kit GeneClean III. Two panpestivirus positive PCR amplicons were sequenced using 326 primer. To determine genetic typing of circulating BVDV in the cities, two panpestivirus positive PCR amplicons were sequenced to found genetic diversity and all data were deposited in GenBank under accession numbers; BVDV/Turkey/Kirikkale/01 (HQ393488.2) and BVDV/Turkey/Kirikkale/02 (HQ393489.2). Gene sequences were compared to Mega 4.1 and ClustalW analyzing software.

Discussion: The BVDV has a world-wide distribution and causes significant economical losses especially on cattle farms. In this study, it was investigated genetic variability of BVDV subtypes by identifying the 5'-UTR nucleotide sequences of two panpestivirus amplicons from field samples. It was found that BVDV-1a and BVDV-2 in terms of BVDV epidemiology is genotyping, 0.625% and 7.5% using RT-nested PCR respectively. Genetic typing is important for the precise classification and molecular epidemiology of BVDV-1 and epidemiological information on currently epidemic viruses is also important for BVDV prevention and control. We suggest that vaccines should contain at least one strain of both species in Turkey. The study of genetic diversity of BVDV is useful for the understanding of pestivirus field locations as well as for epidemiological studies and planning future BVDV control and vaccination programs in Turkey.

Keywords: bovine, genotypes, pestivirus, BVDV subtypes, genetic variability, vaccination, Turkey.

B. Sarikaya, A.K. Azkur, S. Gazyagci, *et al.* **2012.** Genetic Variability of Bovine Viral Diarrhea Virus in the 5'-UTR in the Central Anatolia of Turkey.

Acta Scientiae Veterinariae. 40(1): 1013.

INTRODUCTION

The genus Pestivirus in the family *Flaviviridae* comprises the members bovine viral diarrhea virus type 1, 2 (BVDV-1,2) and border disease virus [4]. Pestiviruses can infect a wide range of animal species of the order artiodactyla. These viruses mainly infect cattle, sheep and pigs, but pestiviruses and anti-pestivirus antibodies have been identified in many other ungulate species [2,3,11,27]. BVDV causes also economic losses through decreased weight gains and milk production and abortion [11]. BVDV is present worldwide [10]. BVDV infection is widely spread among Turkey cattle with a serological prevalence ranging from 14.3% to 100% in unvaccinated animals [7,16]. There is no report about BVDV from Corum, Kirikkale, Yozgat and Kirsehir.

The BVDV enveloped and the genome is a single-strand positive RNA that is 12.3 kilobases in length. Genetic typing of BVDV has usually been performed using sequences from the 5'-UTR, N^{pro} and E2 regions and, many of these sequences are available from GenBank [3,6,25]. Genetic monitoring was suggested as the first step in BVDV control because genetic typing of BVDV shows evidence of an increasing number of variants [5]. For this reason circulating genetic typing of BVDV is important update these data. Circulating BVDV in the field shows genetic and antigenic diversity. On the basis of nucleotide sequence analysis the four pestivirus genotypes have been further subdivided into several subgroups [6,12]. To further characterize the BVDV, the nucleotide sequence of the 5'-UTR that represents a conserved region of the virus genome was analyzed in many studies [4,15,18,29]. The purpose of the current study was to investigate genotypes of pestivirus were circulating in cattle populations in Central Anatolia Region of Turkey.

MATERIALS AND METHODS

Sampling

Blood samples from 160 animals in randomly selected se ven cattle dairy farms that lives with more than 1100 cattle, were collected between November 2009 and March 2010 from Kirikkkale (two farms/n = 57), Corum (one farm/n = 50), Ankara (two farms/n = 21), Yozgat (one farm/n = 17), Kirsehir (one farm/n = 15) cities where are located in Central Anatolia region of Turkey. There aren't any clinical symptoms on cattle's and there haven't any vaccination against BVDV (data collected from farmers). All blood samples were obtained from left or right jugular vein of cattle by using vacutainer tubes with anticoagulant[1] and transported to the laboratory at ambient temperature. Total of 160 whole blood samples randomly pooled into 80 pools for testing and kept at -20ºC until analyzed.

RT-Nested PCR

Viral RNA was extracted from whole blood samples using QIAamp Viral RNA Kit[2] according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase[2]. Random primer (1.25 mM random primer)[3] was used in the first step of cDNA synthesis. For this purpose, 10 μL total RNA was combined with 1 μL (0.5 μg) random primer and preheated at 70°C for 10 min to denature secondary structures. The mixture was cooled rapidly, and 2 μL (100 mM) dNTPs, 5 μl 5X RT buffer, 1 μL 1 MMLV RT (200 IU/μl Promega)[4], and 6 μL H2O were added reaching to the total volume of 25 μL. The RT mix was incubated at 37°C for 90 min and stopped by heating at 94°C for 2 min. To optimize and accuracy for the detection of viral RNA extraction and cDNA synthesis, amplification of cDNA was performed with bovine cDNA encoding GAPDH primers by PCR. After PCR products were detected with 346 base pairs, on 1.5% agarose (Figure 1A). Amplification of cDNA was carried out in a total volume of 50 μL, under the following conditions: 5 μL cDNA, 25 mM Tris–HCl pH 8.9, 50 mM KCl, 3 mM MgCl$_2$, 2 mM of dNTP mix, 10 pmol of primers 324/326 and 3U Taq DNA polymerase[3]. The thermal profile used consisted of 32 cycles of the following; 94ºC for 45s, annealing at 56ºC for 45s and extension at 72ºC for 45s; and a final extension at 72ºC for 5 min. Second round RT- nested PCR was performed under same conditions with 1AF/1ABR, 1BF/1ABR and B5/B6 primer sets for 28 cycles (Table 1). Total of 160 whole blood samples randomly pooled into 80 pools before RT-nested PCR. Each pool was carried out first round RT-nested PCR with 324 and 326 primers. PCR amplicons from this stage were used as a new template in the second round RT-nested PCR that was performed using different primers pairs for each reaction (1AF/1ABR, 1BF/1ABR and B5/B6). When a pool showed positive signal for any primers pairs, both samples were carried out RT-nested PCR separately to type of BVDV. NADL (reference virus

strain) was using as positive control provided kindly by Central Veterinary Control and Research Institute (Etlik, Turkey). The PCR products were visualized using electrophoresis on 1.5% agarose gel containing ethidium bromide. Primers used for amplification of the 5'-UTR region of the pestivirus genome have been described previously (Table 1) [18,23,26].

DNA sequence comparisons and phylogenetic analysis

Positive PCR amplicons were purified from 0.75% agarose gel by using commercial DNA purification kit GeneClean III[5] according to the manufacturer's instructions. Two panpestivirus positive PCR amplicons were sequenced using 326 primer [30]. Gene sequences were compared to Mega 4.1 and ClustalW analyzing software. The phylogenetic tree generated by using Multiple Sequence Alignment using the T-Coffee program and treewiew software. Sequences analyzing were carried out in the commercial company (Iontek, Istanbul, Turkey).

RESULTS

To detect BVDV in cattle, the 5'-UTR were targeted using RT-nested PCR accomplished with first round primers pair panpestivirus and with second round BVDV-1a, BVDV-1b and, BVDV-2 pooled blood samples, respectively. In first round RT-nested PCR expected PCR amplicons size as 288 base pairs were detected in two samples (Figure 1B). Second round PCR was carried out for each sample via type specific primers (Table 1). To detect subtype of BVDV, second round RT-nested PCR was carried out for each sample via type specific primers. Expected PCR amplicons size as 169 bp and 91 bp PCR amplicons were detected in blood samples (Figure 1B). It was detected in second round of RT-nested PCR that BVDV-1a and, BVDV-2 rate are 0.625% (1 positive sample in 160 samples), 7.5% (12 positive samples in 160 samples) in the cattle respectively but not BVDV-1b. BVDV1a positive samples comes from Kirikkale, BVDV-2 positive samples come from Corum (n = 8), Kirsehir (n = 2), Kirikkale (n = 2) cities.

To determine genetic typing of circulating BVDV in the cities, two panpestivirus positive PCR amplicons were sequenced to found genetic diversity and all data were deposited in GenBank under accession numbers; BVDV/Turkey/Kirikkale/01 (HQ393488.2) and BVDV/Turkey/Kirikkale/02 (HQ393489.2) that were taken from Kirsehir city.

HQ393488.2 and HQ393489.2 were compared with some BVDV sequences that are available from GenBank to generate phylogenetic tree (Figure 2). The results of the phylogenetic tree with bootstrap trials comparing the sequences with other pestivirus sequences for the 5'-UTR region are shown in Figure 2.
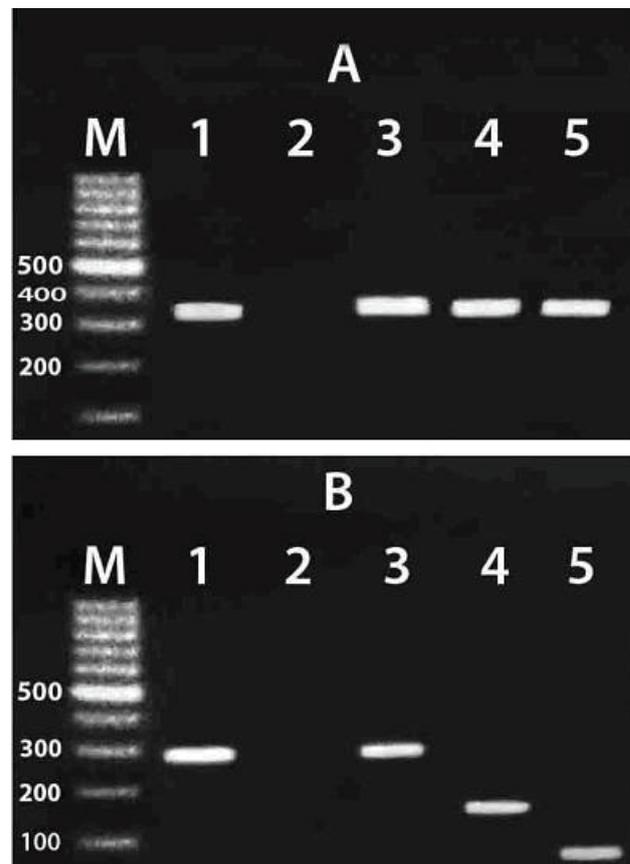

Figure 1. To detect BVDV subtypes, RT-nested PCR was used for amplification of the 5'-UTR.
A: RT performed with GAPDH primer (346 bp). Lane M; DNA marker (100 bp, Fermentas, Lithuania), Lane 1; NADL, positive control, reference virus, Lane 2; negative control, Lane 3; Panpesti positive, Lane 4; BVDV-1a positive, Lane 5; BVDV-2 positive blood samples. B: PCR performed with 324/326 primer (288 bp), 1AF, 1BF/1ABR primer (169 bp) and B5/B6 primer (91 bp). Lane M; DNA marker (100 bp, Fermentas, Lithuania), Lane 1; NADL, positive control, reference virus, Lane 2; negative control, Lane 3; Panpesti positive, Lane 4; BVDV-1a positive, Lane 5; BVDV-2 positive blood samples.
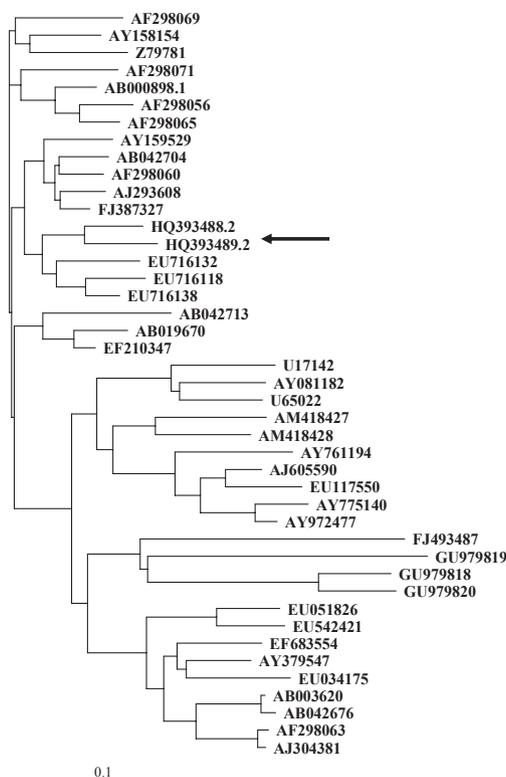
Figure 2. The phylogenetic tree for 5'-UTR of the pestivirus genome was generated by using Multiple Sequence Alignment using the T-Coffee program in Clustal W software and treewiew software. Sequences not generated by this study are derived from genbank excepted for sequences are HQ393488.2, HQ393489.2 (Arrow was shown these sequences).
BDV; AM418428, AM418427, U17142, U65022, FJ493487, AY081182, GU979820, GU979818, GU979819. BVDV-1; AB019670, AB042713, EU716138, EU716118, EU716132, AF298060, FJ387327, AB042704, AY159529, AJ293608, EF210347, AF298056, AF298065, AB000898.1, Z79781, AY158154, AF298069, AF298071. BVDV-2; AB042676, AB003620, AF298063, AY379547, AJ304381, EU034175, EF683554, EU051826, EU542421 CSFV; AJ605590, AY972477, EU117550, AY761194, AY7751.

Table 1. In this study, for reverse transcriptase nested polymerase chain reaction (RT-nested PCR) primers used for amplification of the 5'-UTR region of the pestivirus genome have been described previously**.**

Primers	Genotype	Sequence (5'-3')	Product (bp)	References
324	PanPestivirus	ATGCCCWTAGTAGGACTAGCA	288	26
326	PanPestivirus	TCAACTCCATGTGCCATGTAC	288	
1AF	BVDV-1a	TCGACGCCTTRRCATGAAGGT	169	23
1BF	BVDV-1b	TCGACGCTTTGGAGGACAAGC	169	
1ABR	BVDV 1a and b	CCATGTGCCATGTACAG	169	
B5	BVDV-2	ACTAGCGGTAGCAGTGAG	91	18
B6	BVDV-2	CTAGCGGAATAGCAGGTC	91	

DISCUSSION

The BVDV has a world-wide distribution and causes significant economical losses especially on cattle farms [9,16]. BVDV infection risk was reduced by year eradication program in all herds participating in the program [20]. It was reported that BVDV infection plays a master role in abortions problems when its incidence of abortion compared with other infections such as *Neospora caninum*, mycosis *Bacillus licheniformis* [1].

Accurately determining the prevalence of BVDV infection in inidividual cattle or herds is problematic for a number of reasons. Herd sizes are relatively

large and testing every individual animal is neither logistically nor economically feasible. Therefore we did not prefer also to work each samples separately. Although testing strategies for pooled samples have been developed, they do not replace the need to obtain samples from each animal [17]. Therefore in the present study testing strategies for pooled samples have been used. In this study, it was investigated genetic variability of BVDV subtypes by identifying the 5'-UTR nucleotide sequences of two panpestivirus amplicons from field samples. It was found that BVDV-1a and BVDV-2 in terms of BVDV epidemiology is genotyping, 0.625% and 7.5% using RT-nested PCR respectively. However we got just two positive signal using panpesti primers. One should speculate why we found just two positive signal using first round RT-nested PCR. Clinical samples might be very low viral load and other we took all samples from cattle healthy appearance. It was reported that the PCR assay had a detection limit of 10 TCID50/0.025 mL of virus. For this reason second step RT-PCR assay would be useful for the typing of ruminant pestiviruses, particularly BVDV isolates from the diagnostic laboratory [8]. It was mentioned that when three techniques for the extraction of viral RNA from fetal fluids were compared to detect BVDV. The Qiagen kit was the most sensitive and reproducible and therefore adopted [13]. In the present study the Qiagen kit was preferred to use too. Although live BVDV can be detected using a virus isolation technique in which cultured cells are inoculated with test specimens, and then subjected to immunofluorescence or immunoperoxidase staining 3-5 days later. However, if the samples are suboptimal (e.g., attributable to autolysis), it is not the most sensitive test [11,16]. It was reported that the nested RT-PCR detected BVDV RNA in eight of the hundred fetal fluids screened, whereas BVD virus was isolated from only one sample [13]. The use of the nested RT-PCR may provide a more accurate picture of BVDV infection. Our findings from RT-nested PCR compatible with all of these reports too.

The 5'-UTR has highly conserved regions intercalated by three variable regions where nucleotide substitutions accounting for differences among pestivirus types are located. The 5'-UTR has been used widely in studies of evolution, epidemiology and taxonomy. Thus, 5'-UTR nucleotide sequence analysis has been widely used for pestivirus genotype identification [4,6,15,18,29].

In recent studies geographical distribution reported for BVDV subgroups in some European countries [27,28]. BVDV-1l reported was independently placed in different clusters and distinct from the Japanese subgenotypes [14,19,30]. It was reported that samples used were collected from seven provinces in western Turkey to detect different genetically typing came from all over Turkey with only five isolates originating from three western provinces (Bursa, Izmir and Edirne) included in the serological survey [30] . They also reported that the main subgroups of BVDV-1l circulating in Europe can also be found in Turkey. BVDV-2 infections have previously been detected in different European countries. The circulation of BVDV-2 has been reported in Turkey as well [20-22,30]. This widespread prevalence of virus variants may be explained by the intensive movements of cattle between farms within the same or different regions and by poor BVDV control procedures. In recent years, BVDV eradication programs have been used in a lot of countries [21]. These findings have important implications for vaccine development and immunization strategies. For the prevention of cattle from two genotypes of BVDV infection, it is necessary to develop effective BVDV vaccine using both BVDV-1 and BVDV-2 strains. Distinct patterns of geographical distribution have been reported for BVDV subgroups. Although BVDV-1a, 1b, 1c, 1d, 1f and 1h were found in some European countries, BVDV-1g is restricted to an area of middle Europe [24,28,30]. Our result showed that there is similar to genetic type of virus in the Central Anatolia.

Genetic typing is important for the precise classification and molecular epidemiology of BVDV-1 and epidemiological information on currently epidemic viruses is also important for BVDV prevention and control [10]. For this reason the study of genetic diversity of BVDV is useful for the understanding of pestivirus field locations as well as for epidemiological studies and planning future development of effective strategies of BVDV control and vaccination programs in Turkey. The available inactivated vaccines are safe, but their efficacy is not satisfactory. Eventhough to control BVDV infections, there is a need for better, safer vaccines [31]. We suggest that vaccines should contain at least one strain of both species in Turkey. The study of genetic diversity of BVDV is useful for

the understanding of pestivirus field locations as well as for epidemiological studies and planning future BVDV control and vaccination programs in Turkey.

SOURCES AND MANUFACTURERS

REFERÊNCIAS

1 Agerholm J.S., Willadsen C.M., Nielsen T.K., Giese S.B., Holm E., Jensen L. & Agger J.F. 1997. *Diagnostic studies of abortion in Danish dairy herds.* Zentralbl Veterinarmed A. 44(9-10): 551-558.

2 Azkur A.K., Gazyagci S., Aslan M.E. & Unal N. 2011. Molecular and serological characterization of pestivirus Infection among sheep in Kirikkale, Turkey. *The Journal of the Faculty of Veterinary Medicine, University of Kafkas.* 17(Suppl: A): 83-92.

3 Baker J.C. 1987. Bovine viral diarrhea virus: a review. *Journal of the American Veterinary Medical Association.* 190(11): 1449-1457.

4 Becher P., Orlich M. & Thiel H.J. 2000. Mutations in the 5' nontranslated region of bovine viral diarrhea virus result in altered growth characteristics. *Journal of virology.* 74(17): 7884-7894.

5 Ciulli S., Battilani G.M., Scagliarini A., Gentile A., Morganti L. & Prosperi S. 2008. Genetic typing of bovine viral diarrhoea virus: evidence of an increasing number of variants in Italy. *New Microbiologica.* 31(2): 263-271.

6 Deng R. & Brock K.V. 1993. 5' and 3' untranslated regions of pestivirus genome: primary and secondary structure analyses. *Nucleic acids research.* 21(8): 1949-1957.

7 Duman R., Yavru S., Kale M. & Avci O. 2009. Seroprevalence of viral upper respiratory infections in dairy cattle. *The Journal of the Faculty of Veterinary Medicine, University of Kafkas.* 15(4): 539-542.

8 Fulton R.W., D'offay J.M., Saliki J.T., Burge L.J., Helman R.G., Confer A.W., Bolin S.R. & Ridpath J.F. 1999. Nested reverse transcriptase-polymerase chain reaction (RT-PCR) for typing ruminant pestiviruses: bovine viral diarrhea viruses and border disease virus. *The Canadian veterinary journal.* 63(4): 276-281.

9 Fulton R.W., Ridpath J.F., Confer A.W., Saliki J.T., Burge L.J. & Payton M.E. 2003. Bovine viral diarrhoea virus antigenic diversity: impact on disease and vaccination programmes. *Biologicals.* 31(2): 89-95.

10 Houe H., Lindberg A. & Moennig V. 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. *Journal of Veterinary Diagnostic Investigation.* 18(5): 427-436.

11 Houe H. 1995. Epidemiology of bovine viral diarrhea virus. The Veterinary clinics of North America. *Food animal practice.* 11(3): 521-547.

12 Hurtado A., García-Pérez A.L., Aduriz G. & Juste R.A. 2003. Genetic diversity of ruminant pestiviruses from Spain. *Virus research.* 92(1): 67-73.

13 Hyndman L., Vilcek S., Conner J. & Nettleton P.F. 1998. A novel nested reverse transcription PCR detects bovine viral diarrhoea virus in fluids from aborted bovine fetuses. *Journal of Virological Methods.* 71(1): 69-76.

14 Jacková A., Novácková M., Pelletier C., Audeval C., Gueneau E., Haffar A., Petit E., Rehby L. & Vilcek Š. 2008. The extended genetic diversity of BVDV-1: Typing of BVDV-1 isolates from France. *Veterinary Research Communications.* 32(1): 7-11.

15 Jones L.R., Zandomeni R. & Weber E.L. 2002. Quasispecies in the 5' untranslated genomic region of bovine viral diarrhoea virus from a single individual. *The Journal of general virology.* 83(Pt9): 2161-2168.

16 Kalaycioglu A.T. 2007. Control of bovine viral diarrhea infections through vaccination. *The Journal of the Faculty of Veterinary Medicine, University of Kafkas.* 13(2): 103-108.

17 Kennedy J.A., Mortimer R.G. & Powers B. 2006. Reverse transcription polymerase chain reaction on pooled samples to detect bovine viral diarrhea virus by using fresh ear-notch-sample supernatants. *Journal of Veterinary Diagnostic Investigation.* 18(1): 89-93.

18 Letellier C., Kerkhofs P., Wellemans G. & Vanopdenbosch E. 1999. Detection and genotyping of bovine diarrhea virus by reverse transcription-polymerase chain amplification of the 5' untranslated region. *Veterinary Microbiology.* 64(2-3): 155-167.

19 **Minami F., Nagai M., Ito M., Matsuda T., Takai H., Jinkawa Y., Shimano T., Hayashi M., Seki Y., Sakoda Y., Sugiura K. & Akashi H. 2011.** Reactivity and prevalence of neutralizing antibodies against Japanese strains of bovine viral diarrhea virus subgenotypes. *Comparative Immunology Microbiology and Infectious Diseases.* 4(1): 35-39.

20 **Obritzhauser W., Klemens F. & Josef K. 2005.** BVDV infection risk in the course of the voluntary BVDV eradication program in Styria/Austria. *Preventive Veterinary Medicine.* 72(1-2): 127-132.

21 **Oguzoglu T.C., Muz D., Yilmaz V., Alkan F., Akça Y. & Burgu I. 2010.** Molecular characterization of Bovine virus diarrhea viruses species 2 (BVDV-2) from cattle in Turkey. *Tropical Animal Health and Production.* 42(6): 1175-1180.

22 **Pratelli A., Martella V., Cirone F., Buonavoglia D., Elia G., Tempesta M. & Buonavoglia C. 2001.** Genomic characterization of pestiviruses isolated from lambs and kids in southern Italy. *Journal of Virological Methods.* 94(1-2): 81-85.

23 **Ridpath J.F. & Bolin S.R. 1998.** Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. *Molecular and Cellular Probes.* 12(2): 101-106.

24 **Toplak I., Sandvik T., Barlic-Maganja D., Grom J. & Paton D.J. 2004.** Genetic typing of bovine viral diarrhoea virus: most Slovenian isolates are of genotypes 1d and 1f. *Veterinary Microbiology.* 99(3-4): 175-185.

25 **Ståhl K., Benito A., Felmer R., Zuñiga J., Reinhardt G., Rivera H., Baule C. & Jorge Moreno-López. 2009.** Genetic diversity of bovine viral diarrhoea virus (BVDV) from Peru and Chile. *Pesquisa Veterinaria Brasileira.* 29(1): 41-44.

26 **Vilcek S., Herring A.J., Herring J.A., Nettleton P.F., Lowings J.P. & Paton D.J. 1994.** Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Archives of Virology.* 136(3-4): 309-323.

27 **Vilcek S. & Nettleton P.F. 2006.** Pestiviruses in wild animals. *Veterinary Microbiology.* 116 (1-3): 1-12.

28 **Vilcek S., Paton D.J., Durkovic B., Strojny L., Ibata G., Moussa A., Loitsch A., Rossmanith W., Vega S., Scicluna M.T. & Paifi V. 2001.** Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. *Archives of Virology.* 146(1): 99-115.

29 **Wolfmeyer A., Wolf G., Beer M., Strube W., Hehnen H.R., Schmeer N. & Kaaden O.R. 1997.** Genomic (5'UTR) and serological differences among German BVDV field isolates. *Archives of Virology.* 142(10): 2049-2057.

30 **Yesilbag K., Förster C., Bank-Wolf B., Yilmaz Z., Alkan F., Ozkul F., Burgu I., Cedillo Rosales S., Thiel H.J. & König M. 2008.** Genetic heterogeneity of bovine viral diarrhea virus (BVDV) isolates from Turkey: Identification of a new subgroup in BVDV-1. *Veterinary Microbiology.* 130(3-4): 258-267.

31 **Zemke J., Konigv P., Mischkale K., Reimann I. & Beer M. 2010.** Novel BVDV-2 mutants as new candidates for modified-live vaccines. *Veterinary Microbiology.* 142(1-2): 69-80.