Egyptian J. Virol. 1, 421-435 (2004)

Phylogenetic Characterization of Some *Bovine viral diarrhea viruses* in Egypt

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Sixty seven buffy coat specimens, obtained from clinically suspected cattle at farms located in Belbees, El-Sharquia, were screened for BVDV. Only 9/67 of the tested specimens (13%) were BVDV positive by reverse transcription-polymerase chain reaction (RT-PCR). Whereas, virus isolation followed by immunostaining procedure in cell culture confirmed only 6/9 BVDVs that were all cytopathogenic. Direct sequencing of the RT-PCR amplicons revealed an extreme nucleotide sequence homology (≥98%) among all tested Egyptian isolates versus the local vaccinal strain. Sequence alignments showed variable identity (74% - 93%) of the Egyptian vaccinal strain versus reference laboratory and vaccinal strains of BVDV. Clustering and phylogenetic analyses suggested that these Egyptian vaccinal and field BVDVs are low-Kinetic variants of reference BVDV-1a, particularly the NADL, represented as a distinguished branch within the phylogenetic tree. Accordingly, BVDV-1a (NADL-like), is the dominant circulating genotype in Belbees, El-Sharquia, to date, and the local vaccine could likely induce an antigenically appropriate immune response. The present study offers a useful molecular epizootiological tool to group local BVDVs in a herd-specific manner at a geographic region, to recognize new virus introduction and thus to improve local vaccines and diagnostic assays for effective control policies.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a multi-systemic ubiquitous pathogen amongst cattle, associated with a variety of disease manifestations, exerting a worldwide economic impact and a threat to livestock industry (Baker, 1990 and Houe,1999). BVDVs have been segregated into two antigenically distinct genotypes, BVDV-1 and BVDV-2 that within both genotypes exist as one of two biotypes, cytopathogenic (cp) or non-cytopathogenic (ncp), (Ridpath *et al.*, 1994). Both viral biotypes, either separately or in combination, induce diseases that range from clinically mild to fatal such as mucosal disease. Primary post-natal BVDV infections, termed bovine viral diarrhoea (BVD), are usually subclinical (Baker, 1990 and Houe,1999). However, certain BVDV-2 strains induce severe form of acute BVD (Pellerin *et al.*, 1994). Discrimination between genotypes of BVDV by classical virological and serological means is cumbersome due to the antigenic relatedness of the viruses. BVDV is found as a frequent contaminant of commercial fetal calf serum. This leads to contamination of cell culture systems, viral stocks and biological products such as vaccines (Lohr *et al.*, 1983 and Bolin and Ridpath, 1998).

BVDV is a small enveloped RNA virus, currently classified in the genus *Pestivirus* that includes: BVDV-1, BVDV-2, *border disease virus* (BDV) and *classical swine fever virus* (CSFV) together with the genera *Flavivirus* and *Hepacivirus* within the family *Flaviviridae* (Van Regenmortel *et al.*, 1999). BVDV is represented by a single stranded RNA molecule of positive polarity and about 12.5 kilobases (Kb) in length. Viral gene expression occurs by translation of a single open reading frame (ORF) encoding a large polypeptide of about 4000 amino acids which is cotranslationally and post-translationally processed into mature viral proteins. The first encoded protein is a non-structural (NS) N-terminal proteinase (N^{pro}), followed by the structural proteins: the capsid protein C, the three envelope glycoproteins: E^{rns} (ribonuclease), E1 and E2 and the non-structural proteins: NS2, NS3, NS4A, NS4B, NS5A and NS5B (Collett *et al.*, 1988; Collett, 1992 and van Regenmortel *et al.*, 1999). The ORF is preceded by 360 to 385 nucleotides constituting the 5' untranslated region (5' -UTR), the most conserved region in the BVDV genome (DeMoerlooze *et al.*, 1993), which is found a favorite target for diagnostic polymerase chain reaction (Vilcek *et al.*, 1994; El-Kholy *et al.*, 1998 and El-Kholy 1999) and for phylogenetic analysis (Ridpath *et al.*, 1994; Baule *et al.*, 1997 and Vilcek *et al.*, 1999). However, genetic sequencing of the 5' -UTR of BVDV genome has revealed a high variability among the strains investigated (Pellerin *et al.*, 1994; Paton, 1995).

Based on genetic comparisons of E2 or NS3, BVDV genotype 1 has been subdivided into BVDV-1a (represented by reference strain NADL) and BVDV-1b (represented by reference strain Osloss (Pellerin *et al.*, 1994; Van Rijn *et al.*, 1997). Two further clusters, BVDV-1c and BVDV-1d, have recently been suggested, based on genetic data of the 5' UTR (Baule *et al.*, 1997). Bovine *Pestivirus* isolates mostly belong to the BVDV-1a, BVDV-1b and BVDV-2 group (Ridpath and Bolin, 1998; Jones *et al.*, 2001). However, it has been shown that BVDV isolates from the same herd are often indistinguishable genetically (Paton *et al.*, 1995). The BVDV cluster 1d was predominantly associated with field cases of respiratory disease in Africa (Baule *et al.*, 1997). A recent study indicated that BVDV could be clustered into not only BVDV-1a and BVDV-1b but also into 11 phylogenetic groups (Vilcek *et al.*, 2001). Moreover, BVDV-1b could potentially infect BVDV-1a vaccinated calves (Fulton *et al.*, 2002).

Control measures for BVDV depend mainly on thorough screening of animals for BVDV infection periodically and prior to entering a herd, as well as vaccination. The primary aim of BVDV vaccination is the induction of fetal protection against a broad range of antigenically distinct strains and hence, BVDV carriers could be eliminated. So far, there are no BVDV vaccines, commercially available, that has been shown efficacious in prevention of antigenically homologous and heterologous BVDV infection (Reddy *et al.*, 1995). In Egypt, The genetic diversity amongst local and imported vaccinal strains of BVDVs versus the circulating field BVDVs remains unknown. That might be a threatening factor, leaving a vaccinated herd still susceptible to BVDV infection. Thus, there is a critical need to periodically distinguish the antigenic relatedness, on versatile molecular basis, among field BVDVs versus the local and imported vaccinal strains to reach a vaccine formulation that cope with this heterogeneity.

The study presented here in describes a start for genetic clustering of the 5' untranslated region (5' - UTR), based on their nucleotide sequences, among BVDVs currently circulating in Egyptian cattle in order to find out (i) how similar are field isolates to the currently available local vaccinal virus (Iman strain) and (ii) how the Egyptian local vaccinal strain compares with others from abroad, for epidemiological studies and effective control programs.

MATERIALS AND METHODS

Virus and cells

The Egyptian vaccinal BVDV: Iman strain (a cp BVDV), used in this study, was propagated and titrated on Madin Darby Bovine Kidney (MDBK) cell cultures. The MDBK cells were grown and maintained in minimum essential medium with Earle's salts (MEME) supplemented with 1% antibiotic-antimycotic solution and 10% bovine serum (BS). Both MDBK cells and BS were tested free of adventitious BVDV by indirect immunofluorescence (El-Kholy *et al*., 1998).

Virological samples:

Sixty seven bovine buffy coat specimens, each represented one suspected animal with signs of enteritis, either alone or with respiratory manifestations, were obtained from farms located in Belbees, El-Sharquiah province, to be screened for BVDV. The specimens were collected in the form of ethylene diamine tetra-acetic acid (EDTA)-anticoagulated blood (used for obtaining the buffy coat cells). All buffy coats were processed for BVDV isolation on MDBK cells and the clarified culture supernatants were examined by *In situ* immunoperoxidase staining and indirect immunofluorescence, using anti-E2 monoclonal antibody (MAb, kindly provided by Dr. Julia Ridpath, NADC, Ames, Iowa, USA) as a primary anti-BVDV antibody, as was described in El-Kholy *et al*. (1998).

Extraction of viral RNA

The total RNA was extracted directly from the buffy coat cells, diluted fourfold with RNase-free 10 mM PBS (pH 7.4) and from the BVDV Iman strain-infected MBDK cells using the guanidium isothio-cyanate method as has been described in El-Kholy *et al*. (1998). After being lysed in 10 volumes (Vol.) of denaturing solution (4M guanidium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5 % N-Lauroyl sarcosine and 0.1 M 2-mercaptoethanol), the lysates were mixed thoroughly with 0.1 Vol. of 2M sodium acetate (pH 4.0), 1Vol. of saturated phenol (pH 4.5), and 0.2 Vol. of chloroform/isoamyl alcohol (49:1; V/V), and incubated on ice for 15 min. The suspension was centrifuged for 20 min at 14,000 xg, 4°C and RNA was precipitated from the aqueous phase by addition of 1 Vol. of 100% isopropanol, incubation for 30 min at -20°C and centrifugation at 14,000 xg for 10 min at 4°C. The RNA pellet was washed in 75 % ethanol, dried at room temperature then, dissolved in RNAase-free water and stored at -70°C.

Amplification of viral RNA:

Primers:

The universal *Pestivirus* primers 324/326 complementary to the 5'UTR of BVDV genome were used for in vitro amplification of a 288 bp DNA fragment, in order to detect RNA of any BVDV (Vilcek *et al*., 1994).324 5' ATG CCC ATA GTA GGA CTA GCA 3'

(108-128). 326 5' TCA ACT CCA TGT GCC ATG TCA 3' (375-395).

Complementary DNA (cDNA) synthesis:

The cDNA was synthesized in a mixture consisting of: 10 µl of extracted RNA, 0.04 U (2 µl) of random hexamers (Pharmacia) and 8 µl RNase-free water. The mixture was heated at 65 °C for 10 minutes (for RNA denaturation) and immediately chilled on ice for 5 minutes. 48 U (2 µl) of RNA guard (Pharmacia), 5 µl of the first strand reaction buffer (Gibco, BRL), 200 µMol deoxynucleotide triphosphate mixture (dATP, dCTP, dGTP and dTTP), 200 U of Molony murine leukaemia virus reverse transcriptase (Perkin Elmer-Cetus) to a final volume of 25 µl. The reaction mixtures were incubated at 37 °C for 90 minutes and then heated in a boiling water bath for 5 minutes (for enzyme inactivation). The cDNA was used directly for PCR or stored at -70 °C.

Polymerase chain reaction (PCR):

Each reaction was applied in a total volume of 50 µl containing: 1 X PCR buffer (20 mMol Tris HCl pH 8.4 and 50 mMol KCl); 1.5 mMol $MgCl_2$; 0.2 mMol deoxynucleotide triphosphate mixture (dATP, dCTP, dGTP and dTTP); 50 pMol of each of 324 and 326 primers; 2.5 U *Taq* DNA polymerase (Promega); 4 µl of cDNA product and RNase-free (DEPC-treated) sterile double distilled water up to 50.0 µl. Then, resulting mixture was subjected to precised thermal profile in a programmable thermocycler as follows: 35 cycles: 94°C for 50 seconds-56°C for 50 seconds-72°C for 1 minute; One cycle: 72°C for 10 minutes.

The resulting PCR amplicons (10-15 µl) were analyzed on 1.5% agarose gel electrophoresis, visualized using ultraviolet transillumination after staining with ethidium bromide (Sambrook *et al.*, 1989).

Direct sequencing of PCR amplicons:

The PCR amplicons were sequenced in both directions with the PCR primers 324 and 326 using an ABI PRISM sequencing system, based on the incorporation of fluorescent-labeled dideoxynucleotide terminators.

Computer-assisted phylogenetic analysis:

The nucleotide sequences were aligned by the Clustal W (1.82) program of European Bioinformatics Institute (EBI, EMBL). Phylogenetic analysis was done using the PHYLIP and Treeview 32 (1.6.6) programs. All programs used in this study were accessed through their interactive web services. The resulted nucleotide sequences of field BVDVs were compared to the local vaccinal strain. The latter was then compared to reference laboratory and vaccinal strains of BVDV genotype 1: NADL (a cp BVDV-1a), Singer (a cp BVDV-1a), Osloss (BVDV-1b), NY-1 (ncp BVDV-1b), and 890 (BVDV-2).

RESULTS AND DISCUSSION

Nine out of 67 suspected bovine buffy coat samples analysed (13%) were BVDV positive by RT-PCR. The PCR amplicons were equally at the expected size of about 288 bp. Whereas, virus isolation on MDBK cells followed by immunodetection procedure (FA and IPS) detected only 6 cytopathogenic (CP) BVDVs (9%) that induced clear cytopathic effect in cultured cells (cell shrinkage, cytoplasm profusion, vacuolation and cell death). The two assays gave the same results for the 6 samples (i.e., concordance of 9 % and correlation of 69 %; r = 0.69).

The nucleotide sequences obtained in this study were clearly readable that revealed the presence of a single major viral species (isolate) in each sample analyzed. The sequence of 10 Egyptian BVDVs (9 isolates and a vaccinal strain) were compared in Figure (1) and Table (1) while, sequence of the Egyptian vaccinal strain (Iman), representing the sequence majority of Egyptian BVDVs, was compared with data from representative referenece strains of BVDV in Gen Bank (Fig.2 and Table 2).

Interestingly, the 9 Egyptian isolates had similar nucleotide sequence that differed only in 1 – 3 nucleotide substitutions. The nucleotide sequence alignment revealed extreme nucleotide homology (≥98%) among Egyptian BVDV isolates and with the local vaccinal strain (Table 1). The percentage nucleotide identity between the Egyptian BVDVs majority, represented by the local vaccinal strain (Iman), and the reference laboratory and vaccinal BVDV strains was 74% - 93% (Table 2). The highest nucleotide sequence identity, in the region analyzed, was observed between Egyptian BVDVs and the NADL strain (93%). While, the lowest sequence identity percentage (74%) was noticed between Egyptian BVDVs and strain 890 (a genotype 2 of BVDV). Also, Egyptian BVDVs were aligned to other reference BVDVs with variable nucleotide sequence percentages as follows: 92 % with Singer strain; 90% with NY-1 strain; and 81% with Osloss strain.

Phylogenetic analyses indicated that the local vaccinal strain (Iman), representing the majority nucleotide sequence of Egyptian BVDVs, belongs to BVDV genotype 1a. Thus, the Egyptian BVDV was grouped as a BVDV-1a in a distinguished branch within the phylogenetic tree, together with Singer and NADL strains (Fig. 3).

DISCUSSION

Bovine viral diarrhea (BVDV) is endemic in most bovine-raising countries, causing significant economic losses worldwide (Houe, 1999). Diagnosis of BVDV infections by classical virological assays is facing several problems because of the antigenic and genetic diversity existed among BVDVs (Paton, 1995). Viral isolation of BVDV must be confirmed by immunochemical staining to characterize the viral antigen that requires specific anti-BVDV polyclonal or monoclonal (MAb) antibody. In this study, indirect immunofluorescence (FA) and *in situ* immunoperoxidase staining (IPS), using anti-E2 MAb as a primary antibody, was inferior to PCR (r = 0.69). This could be attributed to poor growth of viruses in cell cultures or presence of toxic elements in the processed samples (Horner *et al.*, 1995). Although the E2 protein of BVDV contain the main neutralizing epitopes, yet, some viral strains react poorly with MAb prepared against E2 polypeptide of heterologous variants of BVDV (Reddy *et al.*, 1995 and Van Rijn *et al.*, 1997). Moreover, the FA and IPS are based on antigenic identification of viable viral particles while, the PCR detects viral RNA of both viable and non-infectious (dead or immature) viral particles (Lennette, 1992).

The 5'-UTR is the most conserved region of the BVDV genome that has a highly structured internal ribosome entry site (IRES) and has some possible markers of virulence (Le *et al.*, 1998 and Topliff and Kelling, 1998). Being involved in the regulation of genome replication and gene expression (Becher *et al.*, 2000), it has been widely used in taxanomy and epidemiological studies (Baule *et*

al., 1997, El-Kholy *et al.*, 1998, EL-Kholy, 1999 and Jones *et al.*, 2001). The current work confirmed the possibility of genetic typing and clustering of BVDVs by direct sequencing a PCR-amplified fragment of 5'-UTR utilizing RNA extracted directly from archived buffy coat cells, obtained from EDTA anti-coagulated blood samples of BVDV-infected cattle. Use of buffy coat cells rather than viral cell culture isolates for viral sequence analysis eliminates the risk of contamination of collected samples with adventitious BVDV from fetal calf serum during cultivation of cell cultures (Lohr *et al.*, 1983 and Bolin and Ridpath, 1998). A fourfold dilution of the original samples in PBS improved the PCR-amplification, probably by reducing levels of the Taq DNA polymerase inhibitors (Horner *et al.*, 1995). The case histories of pneumoenteritis suggested that majority of the specimens tested were from BVDV-infected cattle, but this was not proven by repeated sampling and only 13% were BVDV-positive. The present results revealed that comparison of about 250 nucleotides of the 5'-UTR virtually identified field BVDVs and determined their phylogenetic type as well. All Egyptian BVDV isolates were closely related genetically and did come from herds located in the same geographical area. Previous studies showed that BVDV isolates could be grouped according to their herd of origin (Paton *et al.*, 1995 and Hamers *et al.*, 1998).The 324/326 primers flank 2 out of 3 variable regions within the 5' –UTR of *Pestivirus* genome (Deng and Brook, 1993). Although 5'-UTR does not encode antigenic epitopes, it revealed phylogenetic relationships amongst BVDVs, similar to other genomic regions that could have been analysed (Vilcek *et al.*, 1999). The similar nucleotide sequence, that differed only in 1 to 3 nucleotide substitutions, among Egyptian BVDV isolates and vaccinal strain indicated that the local vaccine could likely induce an antigenically appropriate immune response, particularly in this geographical area (Belbees, El-Sharquia). Stability of the 5'-UTR among field BVDVs likely reflects a high level of fitness of the genetic variants. However, new strains may be introduced to herds that already have active infection, although immunity derived from first exposure might partially reduce the chances of the new virus strain from becoming established. It has been reported that antigenically distinct BVDVs could infect BVDV-vaccinated calves due to difference in viral antigenic properties (Fulton *et al.*, 2002).

Clustering and phylogenetic analyses suggested that the Egyptian BVDVs (isolates and vaccinal strain) are low-Kinetic variants of reference BVDV genotype 1a isolated from other countries, particularly the NADL and Singer strains (nucleotide sequence homology were 93% and 92%, repectively). These BVDV strains could have been introduced during importation of foreign cattle breeds into Egypt by the 1970s, that is the case of causing international dispersion of some virus lineages. Recently, a new BVDV genotype II (BVDV 2) has been discovered in Canada and North America (Pellerin *et al.*, 1994 and Ridpath *et al.*, 1994); Japan (Nagai *et al.*, 1998); Europe (Wolfmeyer *et al.*, 1997); and Egypt (El-Kholy, 1999). The present analyses failed to provide any evidence for presence of BVDV genotype 2 (BVDV-2) in Belbees, despite a previous report of some isolates obtained from cattle in other geographical areas in Egypt, utilizing a different PCR-based genotyping rather than the current sequence analyses, during late 1990s (El-Kholy, 1999).

Table (1) Score table of multiple sequence alignment among Egyptian vaccinal strain (Seq. A) versus field isolates (Seq. B) of BVDV using CLUSTAL W (1.82) program

Seq. A Name	Len. (nt)	Seq. B Name	Len. (nt)	Score %
Vaccine_Eg	245	4_	245	98
Vaccine_Eg	245	9_	245	98
Vaccine_Eg	245	10_	245	98
Vaccine_Eg	245	12_	245	99
Vaccine_Eg	245	14_	245	98
Vaccine_Eg	245	24_	245	99
Vaccine_Eg	245	28_	245	99
Vaccine_Eg	245	35_	245	98
Vaccine_Eg	245	59B_	245	98

Seq. = sequence Len. (nt) = length in nucleotides Score (%) = sequence homology percent

Table (2) Score table of multiple sequence alignment among Egyptian vaccinal (Seq. A) versus reference vaccinal and laboratory strains (Seq. B) of BVDV using CLUSTAL W (1.82) program

Seq. A Name	Len. (nt)	Seq. B Name	Len. (nt)	Score (%)
Eg-vaccine strain	245	Osloss_Indian	269	81
Eg-vaccine strain	245	SINGER	248	92
Eg-vaccine strain	245	NY-1_NCP	247	90
Eg-vaccine strain	245	890_BVDV	248	74
Eg-vaccine strain	245	NADL	256	93

Seq. = sequence Len. (nt) = length in nucleotides Score (%) = sequence homology percent

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9_
AACAAGGGGGGTAGCAAAGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
10_
AACAAGGGGGGTAGCAAAGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
4_
AACAAGGGGGGTAGCAACGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
12_
AACAAGGGGGGTAGCAACGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
28_
AACAAGGGGGGTAGCAACGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
14_
AACAAGGGGGGTAGCAACGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
24_
AACAAGGGGGGTAGCAACGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
Vaccine_Eg
AACAAGGGGGGTAGCAACAGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
35_
AACAAGGGGGGTAGCAACGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
59B_
AACAAGGGGGGTAGCAACGGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT 60
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9_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
10_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
4_
AGCCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
12_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
28_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
14_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
24_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
Vaccine_Eg
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
35_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
59B_
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
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9_
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180
10_
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180
4_
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180
12_
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180
28_
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180
14_
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGTTCAGGTGAAAGCGGTTTAACCAAC 180
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24_
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGTTCAGGTGAAAGCGGTTTAACCAAC 180
Vaccine_Eg
ACGCCCAAAGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180
35_
ACGCCCAAGGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180
59B_
ACGCCCAAGGCACATCTTAACCTGAGCGGGGGTCGCTCAGGTGAAAGCGGTTTAACCAAC 180

********.**************************.************************

9_
CGTTACGGATGCAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTTTGCTACTAAAAATCT 240
10_
CGTTACGGATACAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTGTGCTACTAAAAATCT 240
4_
CGTTACGGATTCAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTTTGCTACTAAAAATCT 240
12_
CGTTACGGATACAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTTCGCTACTAAAAATCT 240
28_
CGTTACGGATACAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTTTGCTACTAAAAATCT 240
14_
CGTTACGGATACAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTTTGCGACTAAAAATCT 240
24_
CGTTACGGATACAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTTTGCTACTAAAAATCT 240
Vaccine_Eg
CGTTACGGATACAGCCTGATAGGGCGCTGCAGAGGCCCACTGTTTTGCTACTAAAAATCT 240
35_
CGTTACGCATACAGCCTGATAGGGCGCTGCAGAGGCCCACTGTATTGCTACTAAAAATCT 240
59B_
CGTTACGGATCCAGCCTGATAGGGCGCTGCAGAGGCCCACTGCTTTGCTACTAAAAATCT 240
               *******.**
*********************************....**.***********

9_              CTGCT 245
10_             CTGCT 245
4_              CTGCT 245
12_             CTGCT 245
28_             CTGCT 245
14_             CTGCT 245
24_             CTGCT 245
Vaccine_Eg      CTGCT 245
35_             CTGCT 245
59B_            CTGCT 245
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Fig. (1): Nucleotide sequences alignment in the 5′ - UTR of BVDV genomes: Egyptian vaccinal (Vaccine_Eg) and local field BVDVs from clinical specimens at Belbees, El-Sharquia: 4_Eg, 9_Eg, 10_Eg, 12_Eg, 14_Eg, 24_Eg, 28_Eg, 35_Eg and 54B_Eg. Numbers of the sequence indicate position in NADL strain (Collett et. al., 1988) which is at 130-374 nt. Dots mean that semi-conserved substitutions are observed and stars indicate homologous sequences in the alignment.

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Osloss_Indian     ----------------------------------------------------------
--
NY-1_NCP          ----------------------------------------------------------
--
Singer            ----------------------------------------------------------
--
NADL
AATGAGGGGGGTAGCAACAGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT  60
VaccineEg_Eg
AACAAGGGGGGTAGCAACAGTGGTGAGTTCGTTGGATGGCTTAAGCCCTGAGTACAGGGT  60
890_BVDV          ----------------------------------------------------------
--
Osloss_Indian     ----------------------------------------
GATGCCACGTTTACGAGGGC  20
NY-1_NCP          ----------------------------------------------------------
AGGGC   5
Singer            ----------------------------------------------------------
AGGGC   5
NADL              AGTCGTCAGTGGTTCGACGCCTTGGAA-
TAAAGGTCTCGAGATGCCACGTGGACGAGGGC 119
VaccineEg_Eg
AGTCGTCAGTGGTTCGACGCCTCGGAAATAGAGGTCTCGATATGCCACGTGGACGAGGGC 120
890_BVDV          ----------------------------------------------------
ACGAGGGC   8
*  *
Osloss_Indian     ATGCCCACAGCACATCTTAA--CCTGGAC----
GGGGGTCGTTCAGGTGAAAACGGTT-T  73
NY-1_NCP          ATGCCCACAGCACATCTTAA--CCTGAGC----
GGGGGTCGTTCAGGTGAAAACGGTT-T  58
Singer            ATGCCCAAAGCACATCTTAA--CCTGAGC----
GGGGGTCGCCCAGGTAAAAGCAGTTCT  59
NADL              ATGCCCAAAGCACATCTTAA--CCTGAGC----
GGGGGTCGCCCAGGTAAAAGCAGTTTT 173
VaccineEg_Eg      ACGCCCAAAGCACATCTTAA--CCTGAGC----
GGGGGTCGCTCAGGTGAAAGCGGTT-T 173
890_BVDV          ATGCCCACGGCACATCTTAA--CCCACGC----
GGGGGTTGCGTGGGTGAAAGCGCCATT  62
                    * ***     *****  *  *     *      * ** *      * *   *
*
Osloss_Indian     A-ACCAACCGCTACGAATA-CAGCCTGATAGGGTGCTGCAGAGGCCCAC--
TGTATTGCT 129
NY-1_NCP          A-ACCAACCGCTACGAATA-CAGCCTGATAGGGTGCTGCAGAGGCCTAC--
TGTATTGCT 114
Singer            A-ACCGACTGTTACGGATA-CAGCCTGATAGGGTGCTGCAGAGGCCCAC--
TGTTCTGCT 115
NADL              A-ACCGACTGTTACGAATA-CAGCCTGATAGGGTGCTGCAGAGGCCCAC--
TGTATTGCT 229
VaccineEg_Eg      A-ACCAACCGTTACGGATA-CAGCCTGATAGGGCGCTGCAGAGGCCCAC--
TGTTTTGCT 229
890_BVDV          C-GTGG--CGTCATGGACA-CAGCCTGATAGGGTGTAGCAGAGACCTGC--
TACTCCGCT 116
                          *        * *   * *        * *   ** *  *
***
Osloss_Indian     ACTGAATCTCTGCTGTACATGGCACATGGA 159
NY-1_NCP          ACTAAAAATCTCT----------------- 127
Singer            ACTAAAAATCTCT----------------- 128
NADL              ACTAAAAATCTCTGCTGTACATGGCAC--- 256
VaccineEg_Eg      ACTAAAAATCTCTGCT-------------- 245
890_BVDV          AGTAAAAACTCT----------------- 128
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Fig. (2): Nucleotide sequences alignment in the 5' - UTR of BVDV genomes : Egyptian vaccinal (Eg), NADL, Osloss, NY-1, Singer, and 890 with GenBank Accession numbers : AY690485, M31182, AY279528, L32879, L32875, L32886, respectively. Numbers of the sequence indicate position in NADL strain (Collett et. al., 1988) which is at 130-374 nt. Dashes mean that semi-conserved substitutions are observed or sequences are absent and stars indicate that the residues or nucleotides in that column are identical in all sequences in the alignment.

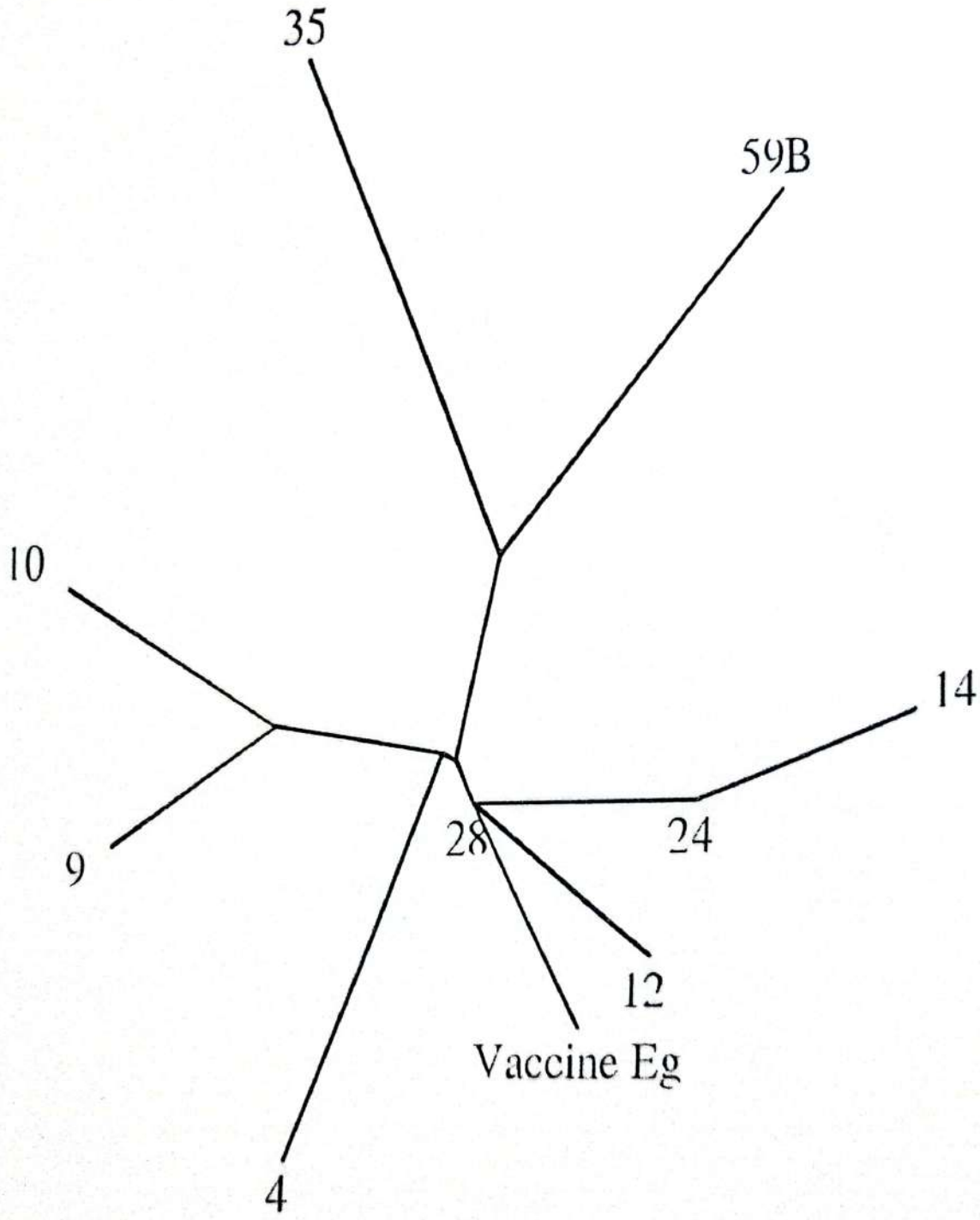

Fig. (3): Phylogenetic radial tree of Egyptian vaccinal (Vaccine_Eg) and local field BVDVs from clinical specimens at Belbees, El-Sharquia : 4_Eg, 9_Eg, 10_Eg, 12_Eg, 14_Eg, 24_Eg, 28_Eg, 35_Eg and 54B_Eg, generated from sequences analyzed in the 5'-UTR of BVDV genomes. The sequences were first aligned using Clustal W (1.82) program and the phylogenetic analyses were performed using PHYLIP package.

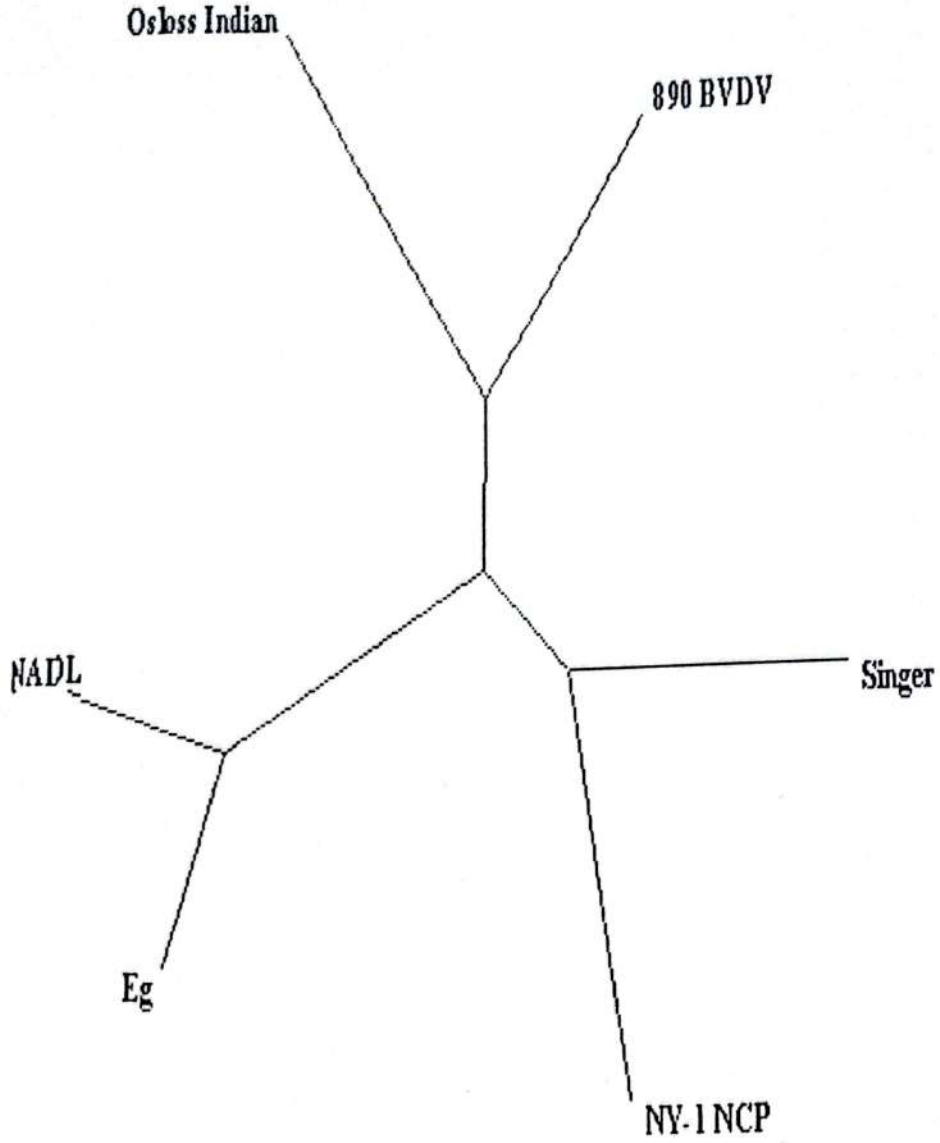

Fig. (4): Phylogenetic radial tree of Egyptian (Eg) and reference bovine *Pestiviruses* generated from sequences analyzed in the 5 -UTR of BVDV genomes. The position of the RT-PCR amplified fragment in the NADL strain (Collett et. al., 1988) is 130-374. Sequences for reference strains were taken from Gen Bank : NADL, Osloss, NY-1, Singer, and 890 with Accession numbers : M31182, AY279528, L32879, L32875, L32886, respectively. The sequences were first aligned using Clustal W (1.82) program and the phylogenetic analyses were performed using PHYLIP package.

Whether these earlier isolates were cell culture contaminants or actually circulating BVDV-2 in those other localities, that requires further re-examination of archived source materials, old virus stocks and animals in the same previous herds.

In conclusion, the present study revealed that BVDV-1a (NADL-like), is the dominant circulating genotype in Egypt, particularly in Belbees, El-Sharquia, to date. Also, direct sequencing of PCR products which were obtained by *in vitro* amplification of viral 5'-UTR directly from buffy coat cells provides an excellent molecular mean to group BVDVs in a herd-specific manner. Nevertheless, use of buffy coat cells for direct extraction of viral RNA rather than cell culture isolates for analysis of viral sequences eliminates the risk of cell culture contamination with adventitious BVDV during cultivation. The method offers a useful molecular epizootiological tool to recognize new virus introduction, to study BVDV infection in a geographic region and thus to help determining weakness in Control policies to improve local vaccines and diagnostic assays.

REFERENCES

Baker, J.C. (1990). Clinical aspects of bovine virus diarrhoea virus infection. In Bovine virus diarrhoea (J. Brownlie and M.C. Clarke, Eds). Rev. Sci. Tech. Off. Int. Epiz., 9 (1), 25-41.

Baule C.; Van Vuuren M.; Lowings J. P.and Belak S. (1997). Genetic heterogeneity of *Bovine viral diarrhea viruses* isolated in southern Africa. Virus Res., 52 : 205 – 220.

Becher, P.; Orlich, M. and Thiel, H. J. (2000). Mutations in the 5' nontranslated region of *Bovine viral diarrhea virus* result in altered growth characteristics. Journal of Virology 74, 7884-7894.

Bolin S. R. and Ridpath J. F. (1998). Prevalence of *Bovine viral diarrhea virus* genotypes in fetal bovine serum. J. Vet. Diagn. Investig., 10 : 135 –139.

Collett, M.S. (1992). Molecular genetics of *Pestiviruses*. Comp. Immunol., Microbiol. Infect. Dis., 15 (3) : 145 - 154.

Collett, M.S.; Larson, R.; Gold, C.; Strick, D.; Anderson, D.K. and Purchio, A.F. (1988). Molecular cloning and nucleotide sequence of the *Pestivirus* bovine viral diarrhoea virus. Virol., 165 : 191-199.

De Moerlooze, L.; Lecomte, C.; Brown-Shimmer, S.; Schmetz, D.; Guiot, C.; Vandenbergh, D.; Allaer, D.; Rossius, M.; Chappuis, G.; Dina, D.; Renard, A. and Martial, J. A. (1993). Nucleotide sequence of the bovine viral diarrhoea virus Osloss strain: comparison with related viruses and identification of specific DNA probes in the 5' untranslated region. Journal of General Virology, 74 : 1433-1438.

Deng R. and Brock K.V. (1993). 5' and 3' untranslated regions of pestvirus genome: Primary and secondary structure analyses. Nucleic Acids Res., 21: 1949 – 1957.

Fulton R. W.; Ridpath J. F.; Saliki J. T.; Briggs R. E.; Confer A. W.; Burge L. J.; Purdy C. W.; Loan R. W.; Duff G. C. and Payton M. E. (2002). *Bovine viral diarrhea virus* (BVDV) 1b : predominant BVDV subtype in calves with respiratory disease. Can. J. Vet. Res., 66 (3): 181 – 190.

El-Kholy A. A. (1999). Comparative polymerase chain reaction assays for detection and genotyping of bovine viral diarrhoea viruses in Egypt. Proc. 5th Sci. Cong., Egyptian Society for Cattle Dis., 28 – 30 Nov., 1999 : 164 – 170.

El-Kholy, A.A.; Bolin, S.R.; Ridpath, J.F.; Arab, R.M.H.; Abou-Zeid, A.A.; Hammam, H.M. and Platt, K.B. (1998).Use of polymerase chain reaction to simultaneously detect and type *bovine viral diarrhoea viruses* isolated from clinical specimens. Rev. Sci. Tech. Off. Int. Epiz., 17 (3) : 733-742.

Hamers, C.; Lecomte, C.; Kulcsar, G.; Lambot, M.and Pastoret, P. P. (1998). Persistently infected *cattle stabilize bovine virus diarrhoea virus* leading to herd specific strains. Vet. Microbiol., 61: 177-182.

Horner, G.W.; Tham, K.M.; Orr, D.; Ralston, J.; Rowe, S. and Houghton, T. (1995). Comparison of an antigen capture enzyme linked assay with reverse transcription polymerase chain reaction and cell culture immunoperoxidase test for the diagnosis of ruminant *Pestivirus* infections. Vet. Microbiol., 43 (1) : 75-84.

Houe, H. (1999). Epidemiology features and economical importance of *bovine virus diarrhoea virus* (BVDV) infections. Veterinary Microbiology, 64 : 89-107.

Jones, L. R.; Zandomeni, R. and Weber, E. L. (2001). Genetic typing of *Bovine viral diarrhea virus* isolates from Argentina. Veterinary Microbiology, 81: 367-375.

Le, S. Y.; Liu, W. M. and Maizel, J. V.Jr (1998). Phylogenetic evidence for the improved RNA higher-order structure in internal ribosome entry sequences of HCV and *Pestiviruses*. Virus Genes, 17 : 279-295

Lohr, C.H.; Evermann, J.F. and Ward, A.C. (1983). Investigation of dams and their offspring inoculated with a vaccine contaminated by *bovine viral diarrhoea virus*. Vet. Med. Small Anim. Clin., 78 : 1263-1266.

Nagai, M.; Sato, M.; Nagano, H.; Pang, H.; Kong, X.; Murakami, T.; Ozawa, T. and Akashi, H., (1998). Nucleotide sequence homology to *Bovine viral diarrhea virus 2* (BVDV 2) in the 5'-untranslated region of BVDVs from cattle with mucosal disease or persistent infection in Japan. Vet. Microbiol., 60 : 271 276.

Paton D.J. (1995). *Pestivirus* diversity. J. Comparat. Pathol., 112 : 215 -236.

Paton, D.J.; Carlsson, U.; Lowings, J. P.; Sands, J. J.; Vilcek, S. and Alenius S. (1995). Identification of herd-specific bovine viral diarrhoea virus isolates from infected cattle and sheep. Vet. Microbiol., 43 : 283 – 294.

Pellerin, C.; Van Den Hurk, J.; Lecomte, J. and Tijssen, P. (1994). Identification of a new group of *bovine viral diarrhoea virus* strains associated with severe outbreaks and high mortalities. Virol., 203 : 260-268.

Reddy, J.R.; Xue, W.; Rivera, S. and Minocha, H.C. (1995). Antigenic differences between a field isolate and vaccine strains of *bovine viral diarrhoea virus*. J. Clin. Microbiol., 33 (8) : 2159-2161.

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

Ridpath, J.F.; Bolin, S.R. and Dubovi, E.J. (1994). Segregation of *bovine viral diarrhoea virus* into genotypes. Virol., 205: 66-74.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning : a laboratory manual. Cold Spring Harbour Laboratory Press, Plainview, New York, 6 (13) : 6-19.

Topliff, C. L. and Kelling, C. L. (1998). Virulence markers in the 5' untranslated region of genotype 2 *Bovine viral diarrhea virus* isolates. Virology, 250 : 164-172.

Van Regenmortel, M.H.V.; Fauquet, C.M.; Bishop, D.H.L.; Carstens,

E.B.; Estes, M.K.; Lemon, S.M.; Maniloff, J.; Mayo, M.A.; Mc Geoch, D.J.; Pringle, C.R. and Wickner, R.B. (1999). Virus taxonomy. Classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Virol. Division, International Union of Microbiological Societies. Academic Press, San Diego, CA, USA, pp.867-872

Van Rijn, P.; Van Gennip, H. G. P.; Leendertse, C. H.; Bruschke, C. J. M.; Paton D. J.; Moormann R. J. M. and Van Oirshot J. T. (1997). Subdivision of the *Pestivirus* genus based on envelope glycoprotein E2. Virology, 237 : 337 – 348.

Vilcek S.; Alenius S.; Paton D.J. and Belak S., (1999). Genetic clustering of *bovine viral diarrhoea viruses* in cattle farms. The Veterinary Journal, 158 : 33- 38.

Vilcek S.; Herring A. J.; Herring J. A.; Nettleton P. F.; Lowings J. P. and Paton D. J. (1994). *Pestiviruses* isolated from pigs, cattle and sheep can be allocated into three genogroups using polymerase chain reaction and restriction endonuclease analysis. Archives of Virol., 136 : 309 – 323.

Vilcek S.: Paton D. J. and Durkovic B. (2001). *Bovine viral diarrhoea virus* genotype 1 can be separated into at least eleven genetic groups. Arch. Virol., 146 : 99–115.

Wolfmeyer A.; Wolf G.; Beer M.; Strube W.; Hehnen H. R., Schmeer N. and Kaaden O. R. (1997). Genomic (5' – UTR) and serological differences among German BVDV field isolates. Arch. Virol., 142 : 2049 – 2057.