Mansoura Veterinary Medical Journal 20:1 (2019) 22-26

Original Article *Virology*

Isolation and identification of Bovine Viral Diarrhea Virus (BVDV) from diseased cattle and buffaloes in Sharkia Governorate, Egypt

El-Kenawy, A. A.1, El-Zayat, M. I. 2, Saleh, M. A.2 and El-Tholoth, M.1

[1]Department of Virology, Faculty of Veterinary Medicine, Mansoura University, Egypt.
[2] Animal Health Research Institute, Sharkia, Egypt.

ABSTRACT

In the present study, 280 samples including whole blood, nasal swabs, rectal swabs and sera were collected from 70 diseased cattle and buffaloes suspected to be infected with BVDV in Sharkia Governorate, Egypt during the period from July 2013 to June 2014. Animals were suffering from fever, respiratory disorder with nasal lesions, diarrhea and abortion. A total of 210 samples including (70 nasal swabs, 70 rectal swabs and 70 heparinized blood from all animals) were used for isolation of BVDV that was carried out using MDBK tissue culture. Three passages were carried out for each sample. In addition 70 sera samples were used for direct detection of the virus using antigen capture ELISA (Ag-ELISA). The suspected BVDV in tissue cultures (after 3rd passage) was identified by indirect immunofluorescence antibody test (IFAT) and Reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, nucleotide sequence analysis of amplified 5`UTR gene of the identified BVD nucleic acid was done and compared with published reference sequences on GeneBank. The results of suspected virus isolation revealed that out of 210 examined samples, 12 (5.71%) samples showed clear CPE. The positive results percentage of Ag-ELISA showed that out of 70 examined sera samples, 11 (15.71%) samples were positive. The result of indirect IFA revealed that out of examined 70 samples, 11 (15.71%) samples were positive. Eleven selected samples that showed positive results with IFA were submitted to RT-PCR, and all submitted samples showed positive results of 5`UTR gene amplification. Sequence analysis of 5`UTR gene sequence identified in this study revealed that our isolate was closely related to Denmark BVDV strain (2005). In conclusion, both biotypes cytopathic and non cytopathic BVDV can be isolated from diseased cattle and buffaloes in Sharkia Governorate, Egypt. In addition, Ag-ELISA and IFA can be used for detection of BVDV with the same sensitivity. RT-PCR can be efficiently used for molecular identification BVDV. Further studies are required for better understanding genetic diversity among BVDV cirulating in egyptian farms based on full genome sequencing.

Keywords: Antigen captures ELISA, BVDV, IFA, RT-PCR.

1. INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an important cattle pathogen of a worldwide distribution. It can cause severe, if not fatal, infections and create huge economic losses (Brownlie 1991). BVDV has the ability to produce severe illness, death and cause a wide variety of sub-clinical and clinical infections manifested by immunosuppression, respiratory disease, and decreased reproductive performance (Brock 2004 and Radostits et al., 2007).

Bovine viral diarrhea (BVD) caused by RNA virus, genus *Pestivirus*, family *Flaviviridae*. In addition, the genus *pestivirus* includes two other important viruses, border disease virus (BDV) of sheep and classical swine fever virus (CSFV) which are closely related to BVDV (Heinz et al., 2000). There are two genotypes of BVDV, BVDV I strains and BVDV II strains depending on the sequenc of 5` UTR. Both genotypes may exist as one of two biotypes, cytopathic (CP- BVDV) and noncytopathic (NCP-BVDV), these biotypes can be differentiated by their effect in cell culture (Belknap et al., 2000 and Tautz and Thiel 2003). Infection with BVDV in the first trimester may result in stillbirth, resorbtion or abortion. Fetuses that survive will be born as persistent infected (PI) resulting in continuous shedding of the virus in their secretions and excretions into the environment (Houe et al., 1992).

The present study aimed to isolate field strains of BVDV from samples collected from diseased cattle and buffaloes suspected to be infected with BVDV in Sharkia Governorate, Egypt and identification of the virus using antigen capture ELISA (Ag-ELISA) and indirect immunofluorescence antibody test (IFAT) in prepared field samples and in tissue cultures after third passages. Confirmative molecular identification of BVDV was carried out using RT-PCR.

2. MATERIALS AND METHODS

2.1. Clinical specimens:

In the present study, 280 samples including (nasal swabs, rectal swabs, whole blood and sera samples) were collected from 70 clinically diseased cattle and buffaloes in Sharkia

Governorate, Egypt showed clinical signs believed to be BVD for BVDV isolation and identification. A total of 210 samples including (70 nasal swabs, 70 rectal swabs and 70 whole blood from all animals) were used for isolation of BVDV while 70 sera samples were used for direct detection of the virus using Ag-ELISA.

2.2. Standard BVDV:

the BVDV strain is NADL strain was propagated on MDBK tissue culture (virus titer $10^{5.6}$ TCID50). It was kindly obtained from Genome Department, Animal Health Research Institute, Dokki, Egypt. It was used as control positive in RT-PCR.

2.3. Preparation of collected samples

The collected nasal swabs and rectal swab were prepared according to Smithies and Modderman (1975), while blood samples were prepared for buffy coat and sera collection according to Duffell and Harkness (1985) before inoculation into MDBK tissue culture.

2.4. Inoculation of prepared samples on MDBK:

Propagation of field samples into MDBK tissue culture was carried out according to (Jewett et al., 1990). A continuous cell line of MDBK BVDV free was used for viral isolation in the collected samples. The cells were kindly obtained Veterinary Serum and Vaccine Research Institute, Abassia. It was grown with modified Eagle's minimum essential medium (EMEM) with Earl's balanced salt solution. Prescription flasks, either inoculated or control, were incubated in CO2 incubator at 37^{o}C for 1 hour, for the inoculum adsorption. Then maintenance media was added (10ml at each flask). Flasks were incubated at 37^{o}C with daily examination for recording the development of CPE. The inoculated plates were examined daily for 7 days for recording the cytopathic changes. After 7 days, the prescriptions were frozen and thawed several times then the suspension were passaged for 3 successive blind passages by the same way.

2.5. Detection of BVDV antigen in prepared sera samples by using Ag-ELISA

Ag-ELISA kit (IDEXX - Switzerland) was used for BVDV antigen detection. According to kit instruction and recent studies (Dubovi 2013; Hilbe et al., 2007 and Lanyon 2014), the preferred samples to be used in the techniques are fresh or frozen sera. Seventy serum samples representing each animal, were selected for detection of BVDV antigen. ELISA reader was blanked on air and the absorbance was read at 450 nm. Interpretation of the technique: when optical denisty value is equal or less than 0.3 sample considered negative, while when optical denisty value is more than 0.3 sample considered positive.

2.6. Identification of the suspected isolated virus using indirect IFA:

Indirect IFA was carried out according to Magar et al. (1988) with some modification in methodology. The technique was performed on 70 harvested tissue culture after third passage representing each animal using LabTech® slides. Growth media was added to tissue culture cell suspension and cells were distributed into sterile 16 micro wells LabTech slides. The LabTech® slides were closed and incubated at 37°C for 24 hr. After formation of 60 - 80 % cells confluence, 50 µl of isolated virus were inoculated in each microcell except first 4 wells were inoculated only with maintenance medium and left as negative control. Both Specific anti-BVDV polyclonal antiserum and fluorescein isothiocyanate (FITC)-labelled antibovine were diluted at 1:200 in PBS. Wells were examined for presence of intra-cytoplasmic greenish yellow fluorescence using fluorescent microscopy.

2.7. RT-PCR

It was carried out for molecular diagnosis of isolated BVDV. Identification of BVDV using RT-PCR was done according to Viljoen et al. (2005). Eleven harvested tissue culture after third passage showed positive results with IFA (including 4 samples show CPE and 7 samples did not show CPE during virus isolation) were submitted for further confirmation of BVDV depending on 5` UTR gene. Oligonucleotide primers designed by Vilcek et al., (1999) for amplification of 5` UTR were used.

Resuspension of the lypholized primers was performed by Tris/EDTA (TE) buffer making a 100 pmol/µL concentration. The primers were specific to detect 347 bp of UTR gene. Sequences of the used primers were as follow: sense primer 5`-AGCACTGGTGTCACTCTGTTGG-3` and antisense primer 5`-ACCATGGACGTCGAGTTAACCT-3`. RNA extraction was done using QIAamp extraction kit as described in the recommended manufacture's instructions. RT-PCR was done using Thermo Scientific Verso1-Step ReddyMix Kit. RT-PCR was done as the following: 1 cycle at 50 °C for 15 minutes (for cDNA synthesis); then 1 cycle at 95 °C for 2 min (Verso inactivation); followed by 35 cycles at 95 °C for 20 s, 50 °C for 45 s, and 72 °C for 1 min; and then a final cycle at 72 °C for 5 min. Amplicons of PCR reaction were visualized using agarose gel electrophoresis.

2.8. Sequencing of amplified 5`UTR gene of BVDV

Purification and extraction of RT-PCR products from the gel was done according to Viljoen et al. (2005) by using Wizard® SV Gel and PCR Clean-Up System kit according to manufacturer's instructions. RT-PCR products were submitted

to MACROGEN clinical laboratory, Korea for DNA sequencing using the same primer used in RT-PCR. Analysis of the obtained sequence data was done through (http://www.ebi.ac.uk/ Tools/msa/clustalo). Then the neighbour-joining (N-J) phylogenetic analysis was performed using the alignment output file. After that MegAlign (DNASTAR, Lasergene, Madison, WI, USA) was used to perform the calculation of divergence and identity per cents. Bioedit software v.7.0.0 was used for the molecular analysis for the sequence of amino acids.

3. RESULTS

3.1. BVDV isolation from collected samples on MDBK cell lines

Normal control MDBK appeared as confluent monolayer cells without any changes as shown in Figure (1). The virus induces cells rounding, aggregation in scattered area of the monolayer followed by cellular darkness and clusters formation as shown in Figure (2). Different samples (rectal swab, nasal swab, and buffy coat) of the same animal showed positive results (clear CPE). The percentage of positive results after 3rd passage were out of 210 examined samples, 12 samples showed clear CPE with percent of 5.71%. as shown in Tabel (1).

3.2. Ag-ELISA technique for detection of BVDV antigen

The results revealed that out of 70 tested sera samples, 11 samples showed positive results with percent of 15.71%. The positive sera samples include 4 samples that showed CPE during virus isolation (of the collected samples of the same animals) with percent of 5.71% and 7 samples that did not show CPE with percent of 10% as described in Table(1)

3.3. Indirect IFA technique for detection of BVDV antigen

The results were similar to Ag-ELISA, in which out of 70 harvested tissue culture after third passage, 11 samples showed positive results with percent of 15.71%. Positive results show green yellowish colour by flourescent microscope whlie negative results show dark green colour as shown in Figure (3,4). Results of identification of inoculated tissue culture suspension after third passage using IFA are described in Table (1).

3.4. Molecular identification of BVDV by RT-PCR depending on 5`UTR gene

Analysis of RT-PCR products obtained from amplification reaction of extracted RNA from harvested tissue cultures (after third passage) by agarose gel electrophoresis along with GeneRuler 100 bp DNA Ladder Marker was carried out and all samples (that showed positive results in IFA and submitted to RT-PCR) showed positive results of 5`UTR gene amplification with correct size (347bp) as shown in Figure (5).

3.5. Nucleotide sequencing analysis of amplified 5`UTR gene of BVDV

The alignments of the 5`UTR gene nucleotide sequences (347bp) of our BVDV isolate compared with other reference BVDV isolates on the Genbank. Phylogenetic tree pattern after alignments of the sequenced viruses and references BVDV was carried out as in shown in Figure (6). The phylogenetic tree pattern revealed that our isolate in this study was closely related to Denmark BVDV strain (2005) with percent identity of 96%.

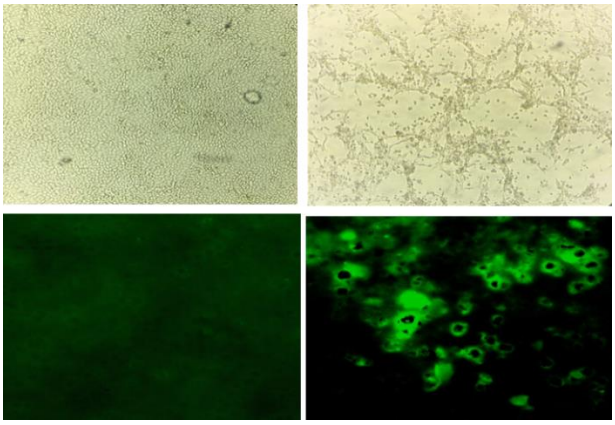

Figure 1. Confluent monolayer MDBK cells represent the negative control.

Figure 2. MDBK cells inoculated with prepared nasal swab showed CPE after third passage (rounding of the cells, cellular darkness and clusters formation(

Figure 3. MDBK cells represent the negative control by fluorescence microscope (X 200), (no immonoflourescent reaction)

Figure 4. MDBK cells inoculated with isolated virus after third passage show intracytoplasmic green yellowish colour by fluorescence microscope (X 200).

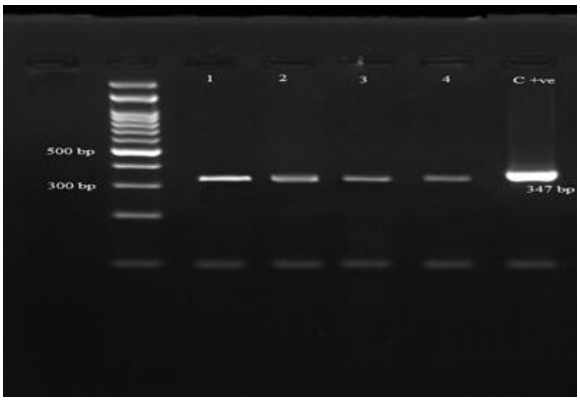

Figure 5. Gel electrophoresis of RT-PCR products of 5`UTR gene.
Lane 1-4: amplified products prepared from inoculated tissue culture after third passage

C+ve: control positive. Reference BVDV (NADL strain, v.titer 10 TCID50).

Table (1): Results of BVDV isolation, Ag-ELISA and IFA in collected samples

Animals	Age	Suspe -cted	+ve Virus isolation		+ve Ag-ELISA		+ve IFA	
			No	%	No.	%	No.	%
Cattle	< 6 months	26	1	3.8 %	4	15.4 %	4	15.4 %
	> 6 months	39	3	7.6 %	6	15.4 %	6	15.4 %
Buffaloe	< 6 months	2	0	0%	0	0%	0	0%
	> 6 months	3	0	0%	1	33.3 %	1	33.3 %
Total		70	4	5.71 %	11	15.7 %	11	15.7 %

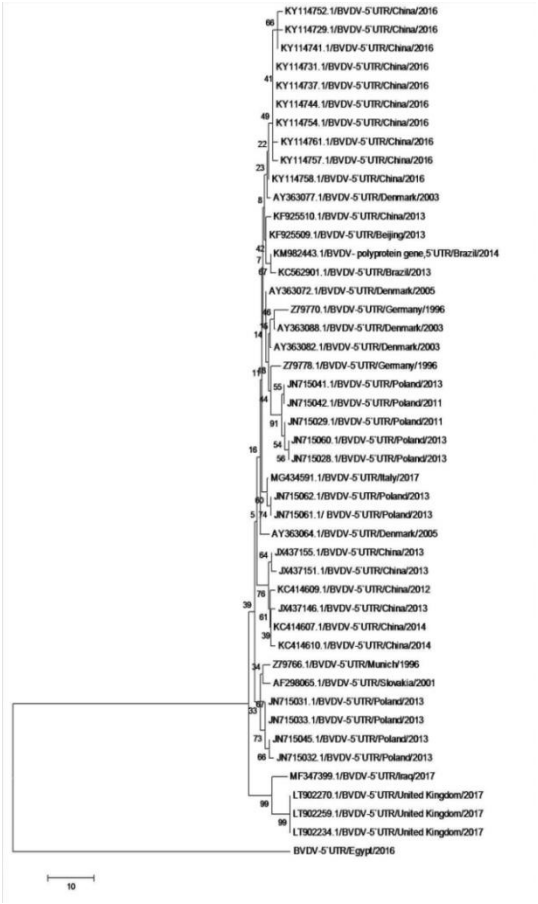

Figure 6. Phylogenetic tree virus in this study and other references BVDV retrieved from GenBank.

4. DISCUSSION

In this study, 5.6 trials for isolation, identification and molecular characterization of BVDV in collected field samples from 70 diseased animals suspected to be infected with BVDV in Sharkia Governorate were carried out. A total of 280 samples were collected including heparinized blood, nasal swabs, rectal swabs and sera from each animal. Animals were suffering from fever, respiratory disorder, abortion and diarrhea. Such clinical findings similar to that described by Brock (2003) and Radostits et al. (2007).

For BVDV isolation and propagation of field samples, MDBK cells free from BVDV latent infection were used. A total of 210 samples including (70 nasal swab, 70 rectal swaps and 70 buffy coat) were prepared then subjected for virus isolation via MDBK cell lines for three successive blind passages. Out of 210 examined samples, 12 samples showed clear CPE with percent of 5.71%. These results agree with Tsuboi and Imada (1998) who isolated the virus from infected animals with a percent of 7%. Prescription flasks that did not show CPE not considered negative for BVDV as it may contain NCP-BVDV so further confirmation was done using indirect IFA and RT-PCR in addition to using Ag-ELISA on collected sera samples. The low percentage of infected animals in the current study may be due to increased awareness of BVDV eradication along with simultaneous vaccination in many farms.

The results of Ag-ELISA showed that out of 70 sera samples, 11 samples were positive with percent of 15.71%. These results agree with Bechmann (1997); Bottcher et al. (1993); Hilbe et al. (2007) and Torstein and Johan (1995) who recorded the presence of BVDV in 15%-20% of examined samples by Ag-ELISA. The results differ from virus isolation results because Ag-ELISA is more sensitive in detection of BVDV and able to detect both biotypes either cytoparhic or non-cytopathic.

For further identification of BVDV antigen, IFA was carried out. The technique was performed on 70 inoculated tissue culture suspension after third passage representing each animal, using LabTech® slides. The results of IFA showed that out of 70 samples, 11 samples were positive with percent of 15.71%. These results agree with Gardiner et al., (1983); Dubovi (1990) and Shar et al. (1991) who recorded positive results with percent 16.2% in samples examined by IFA for BVDV identification.

Results showed by Ag-ELISA and IFA revealed that both techniques are more sensitive than virus isolation in detection of BVDV. Sensitivity of Ag-ELISA and IFA in virus detection is equal while Ag-ELISA technique is considered superior over IFA because ELISA is more rapid, protocol of the technique is more easier, the ability to do large numbers of samples simultaneously, results are computerized (compared to IFA that depend on visualization of result by microscope) and it overlook infectious virions when the amount of antigen is small (Saliki and Dubovi 2004).

Analysis of the results of virus isolation, Ag-ELISA and IFA in the current study showed that the presence of CP strains within BVD-suspected animals were 5.71% and NCP strains were 10%. These results agree with Baker and Houe (1995) and Saliki et al. (1997) who recorded that the presence of NCP strains in clinical infected animals is more than CP strains. Increase in Prevalence of NCP over CP biotypes usually attributed to the capability of non-cytopathic strains to cross placenta with production of many PI animals (Brownlie et al., 1989).

RT-PCR can be efficiently used for detection of BVDV depending on amplification of the 5`UTR gene. This finding of molecular identification of virus is in accordance with Vilcek et al. (1999) who confirmed BVDV infection and characterized the molecular properties of BVDV isolate using primers designed to amplify a specific segment of 5`UTR gene. RT-PCR is often more preferable for BVDV detection because it is less time consuming, less expensive, highly sensitive and aviod time required for virus isolation using tissue culture (Kim and Dubovi 2003 and Givens et al., 2003).

Furthermore, molecular characterization of the 5`UTR gene of our isolate was conducted to compare their nucleotide sequences with references available in the GenBank. The result obtained from sequence analysis of the chosen BVDV sample showed that our isolate in this study was closely related to Denmark BVDV strain (2005) and with percent of identity of 96%. Further studies is required for better understanding genetic diversity among BVDV cirulating in egyptian farms based on full genome sequencing.

Conclusion

Both biotypes of BVDV (cytopathic and non cytopayhic) in the collected samples from diseased cattle and buffaloes can be isolated and differentiated using MDBK tissue culture. Ag-ELISA and IFA can be used for detection of BVDV and sensitivity of both techniques in virus detection is equal. RT-PCR depending on 5`UTR gene can be efficiently used for detection of BVDV. Nucleotide sequence analysis of amplified 5`UTR gene of the identified BVD nucleic acid was carried out.

5. REFERENCES

Baker, J.C. and Houe, H. (1995): Bovine viral diarrhea virus. Clinics of North America food animal practice.

Bechmann, G. (1997): Serological investigation in diagnosis of viral infection drived from cattle and sheep. Dtsch Tierarzt Wochenr. 104(8):321 -4.

Belknap, E.B., Collins, J.K., Larsen, R.S. and Conrad, K. (2000): Bovine Viral diarrhea virus in New World came lids." J. Vet. Diag. Invets.; 12 (6): 568-570.

Bottcher, J., Gottschalk, E., Greiser-Wilke, I., et al. (1993): Diagnosis of bovine virus diarrhea by two enzyme- linked immunosorbent assays. Rev Sci Tech 12:461–469.

Brock, K.V. (2003): The many faces of bovine viral diarrhea. Vet. Clin. North Am. Food An. Pract. 20 (1) 1-3.

Brock, K.V. (2004): Strategies for the control and prevention of bovine viral diarrhea virus (BVDV) infections. Vet. Microbiol, 64:135-144.

Brownlie, J. (1991): The pathways for bovine viral diarrhea virus biotypes in the pathogenesis of disease. Arch virol. 3 (sup), 79.

Brownlie, J., Clarke, M.C. and Howard, C.J. (1989): Experimental infection of cattle in early pregnancy with a cytopathic strain of bovine virus diarrhea virus. Res. Vet Sci. 46:307–311.

Dubovi, E.J. (1990): The diagnosis of bovine viral diarrhea infections. Vet. Med. 85: 1133-1139.

Dubovi, EJ. (2013): Laboratory diagnosis of bovine viral diarrhea virus. Biologicals; 41:8–13.

Duffell, S.J. and J.W. Harkness (1985): Bovine viral diarrhea virus (BVDV) – mucosal disease (MD) of cattle. Vet. Rec. 117, 240-245.

Givens, M.D., Heath, A.M., Carson, R.L., Brock, K.V., Edens, M.S., Wenzel, J.G. and Stringfellow, D.A. (2003): Analytical sensitivity of assays used for detection of bovine viral diarrhea virus in semen samples from the Southeastern United States. Vet. Microbiol., 96: 145-155.

Gardiner, A.C., Nettlon, P.F. and Barlow, R.M. (1983): Virology and immunology of a spontaneous experimental mucosal disease – like syndrome in sheep recovered from clinical border disease. J. comp path. 93: 463-469.

Heinz, F.X., Collett, M.S., Purcell, R.H., Gould, E.A., Howard, C.R., Houghton, M., Moormann, R.J.M., Rice, C.M. and Thiel, H. J (2000): Family Flaviviridae. In: 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., Mcgeoch, D.J., Pringle, C.R., Wickner, R.B. (Eds.), Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of viruses. Academic press, San Diego, Pp. 859-878.

Hilbe, M., Hanspeter, R., Ernst, P., Michael, H., Marlies, N., Christoph Egli, Christian, S., Kati, Z. and Felix, E. (2007): Comparison of Five Diagnostic Methods for Detecting Bovine Viral Diarrhea Virus Infection in Calves. Journal of Veterinary Diagnostic Investigation Vol 19, Issue 1, 2007.

Houe, H., Pedersen, K.M. and Meyling, A. A. (1992): computerized spreadsheet model for calculating total annual losses due to bovine viral diarrhea virus infection in dairy herds and sensitivity analysis of selected parameters. In: Proceedings of the 2nd ESVV symposium on ruminant pestiviruses, Annecy, France, 1992 PP. 179-184.

Jewett, C.J., C.L. Kelling, M.L. Frey and A.R. Doster (1990): Comparative pathogenesis of selected bovine viral diarrhea virus isolates in genotobiotic lamb. Am. J. Vet. Res.Vol. 51, No., 10. 1640-1644.

Kim, S.G. and Dubovi, E.J. (2003): A novel simple one-step single-tube RT-duplex PCR method with an internal control for detection of bovine viral diarrhoea virus in bulk milk, blood, and follicular fluid samples. Biologicals 31, 103–106.

Lanyon, SR. (2014): Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. J Vet Diagn Invest; 26:783–787.

Magar, R., Minocha, H.C., Montpetit, C., Carman, P.S. and Lecomte J. (1988): Typing of cytopathic and noncytopathic bovine viral diarrhea virus reference and Canadian field strains using a neutralizing monoclonal antibody. Can J Vet Res. 1988 Jan; 52(1):42-5.

Radostitis, O.M., Gay, C.C., Hinchcliff, K.W. and Constable, P.D (2007): Veterinary medicine- A Textbook of the diseases of cattle, horses, sheep, pigs and goats, 10th (ed) Saunders El Sevier Printed in Spain: Pp: 1248-1276.

Saliki, J.T. and Dubovi, E.J. (2004): Laboratory diagnosis of bovine viral diarrhea virus infections. Vet Clin North Am Food Anim Pract 2004; 20: 69-83. 14.

Saliki, J.T., Fulton R.W., Hull, S.R. and Dubovi, E.J (1997): Microtiter virus isolation and enzyme immunoassays for detection of bovine viral diarrhea virus in cattle serum. J. Clin. Microbiol. 35:803–807.

Shar, A.F., A., Dulac, G.C., Dubucc, et al., (1991): Comparative evaluation of the fluorescent antibody test and microtiter immunoperoxidase assay for detection of bovine viral diarrhea virus from bull semen. Can. J. Res. 55: 91 – 93.

Smithies, L.K. and Modderman, E. (1975): Bovine viral diarrhea virus in commercial fetal calf serum "normal" and aborted fetuses. In

proceddings of the annual meeting of American Association of veterinary laboratory Diagnosticins, Portland OR, P. 113.

Tautz, N. and Thiel, H.J. (2003): Cytopathogenicity of pestiviruses: cleavage of bovine viral diarrhea virus NS2-3 has to occur at a defined position to allow replication. Arch. Virol. 148:1405–1412.

Torstein, S. and Johan, K. (1995): Evaluation of an Antigen-Capture ELISA for Detection of Bovine Viral Diarrhea Virus in Cattle Blood Samples. Journal of Veterinary Diagnostic Investigation. Vol 7, Issue 1, 1995.

Tsuboi, T. and Imada, T. (1998): Bovine virus diarrhea virus replication in bovine follicular epithelial cells derived from persistently infected heifers. J. Vet .Med. Sci. 60 (5):569-572.

Vilcek, S., Paton, D.J., Lowings, P., Bjorklund, H., Nettleton, P. and Belak, S. (1999): Genetic Analysis of BVDV and other Pestiviruses at the 5`end and 3` end of the Genome. Virus Genes 18:2, 107±114, 1.

Viljoen G.J.; Nel L.H. and Crowther J.R. (2005): Molecular diagnostic PCR hand book. IAEA