



Isolation and identification of Bovine Viral Diarrhea virus among cattle and buffaloes in Kalubeya, Egypt (2013-2014)

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is the most prevalent infectious disease of cattle. It causes financial losses from a variety of clinical manifestations and is the subject of a number of mitigation and eradication schemes around the world. This study was designed for isolation and identification of BVDV in Kalubeya governorate. The study was carried out on 400 Buffy coat and tissue samples from cattle and buffaloes. Direct detection of BVDV antigen by antigen capture enzyme linked immunosorbent assay (AC-ELISA) showed positive results in 47.4% (95) and 10.5% (21) in cattle and 38% (76) and 6% (12) in buffaloes for buffy coat and tissue samples, respectively. Virus isolation (VI) on MDBK cell culture revealed negative results which subjected to indirect fluorescent antibody technique (FAT), revealed characteristic intracytoplasmic apple green fluorescence indicating presence of non-cytopathogenic strain of BVDV. Molecular detection using reverse transcription-polymerase chain reaction (RT-PCR) revealed the presence of specific PCR product at the correct expected size of the BVDV genotype I (190 bp).

Key words: BVDV, AC-ELISA, RT-PCR.

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1. INTRODUCTION

Bovine viral diarrhea (BVD) is one of the worldwide distributed viral diseases of livestock and wild animals (Lang et al., 2014). Broad nature, transmittance, and lack of treatment have made BVD a global pandemic, and one of the most significant cattle diseases in the world (Gunn et al., 2005). Bovine viral diarrhea virus (BVDV) is a positive single stranded RNA virus belonging to the Pestivirus genus of the Flaviviridae family (Darweesh et al., (2015). BVDV have two biotypes. The cytopathic (CP) biotype will damage tissue cultures and the much more common non-cytopathic (NCP) will not. Both biotypes can cause disease in cattle, however, greater than 95% of BVDV infections, all of the persistent infections, and the more severe forms of the disease are caused by the non-cytopathic biotype (Kummerer and Meyers 2000 and Kelling 2004). BVDV can be divided into two genotypes (BVDV-1 and BVDV-2) on the basis of antigenic and genetic differences, with each genotype containing both the CP and NCP forms. Both genotypes are divided into subtypes (Van den Hurk, 2000). BVDV-1 was classified into at least 11 genetic groups around the world based on the phylogenetic analysis of 5-untranslated region and

(5' UTR) and N-terminal protease fragment (N PRO) (Vileck et al., 2001). Antigen capture enzyme linked immunosorbent assay (AC-ELISA) and reverse transcription polymerase chain reaction (RT-PCR) are routinely used for the detection of BVDV antigen and nucleic acid among vaccinated and non-vaccinated cattle (Cornish et al., 2005 and Letellier and Kerhofs, 2003). These tests have high sensitivity and specificity for detecting BVDV in persistently infected animals. The aim of this study is the diagnosis and typing of BVDV among cattle and buffaloes in Kalubeya, Egypt.

2. MATERIALS AND METHODS

2.1. Bovine Viral Diarrhea Virus (BVDV):

BVDV NADL (genotype 1), a cytopathic strain was kindly obtained from the department of virology, Animal health Research Institute, Dokki, Giza. The virus had a titer of $10^6 \log_{10}$ TCID₅₀/ ml in MDBK cells. It was used as positive control in, antigen capture ELISA and RT-PCR detection methods for BVDV.

2.2. Samples:

A total of 800 sample were collected from suspected native breeds cattle and buffaloes at different localities at Kalubeya governorate (Benha, Toukh, Kalube and Shebin El-kanater) during 2013 and 2014.

2.2.1. *Buffy coat samples*

Buffy coat samples were collected from suspected cattle (No. =200) and buffaloes (No. =200) and subjected for detection of BVDV using antigen captured ELISA and RT-PCR and trials for virus isolation on cell culture.

2.2.2. *Tissue samples:*

Suspected tissue samples were collected from emergency slaughtered cattle (No. =200) and buffaloes (No. =200) and used for detection of BVDV antigen using antigen captured ELISA and trials for isolation on cell culture.

2.1.4. *Madine Darby Bovine Kidney (MDBK) cell line:*

A permanent cell line of MDBK was used all over the work for isolation of BVD. The cells were supplied by the Department of Virology, Animal Health Research Institute. Dokki, Giza.

2.3. *BVDV Antigen Test Kit/Serum plus ELISA Kit:*

IDEXX BVDV-Ag/serum plus is used for the detection of BVDV antigens in buffy coat, and tissue samples according to test protocol.

2.4. *Trials for BVDV isolation on MDBK cell culture:*

A total of 50 representative samples positive in antigen captured ELISA (16 lymph node, 8 Spleen, 4 intestines, 5 Lung and 17 Buffy) were used for isolation of BVDV on MDBK cell line through 3 blind passages and detection of cytopathic effect (CPE), (Marcus and Moll, 1968).

2.5. *Identification and biotyping of inoculated BVDV on MDBK cell line using indirect fluorescent antibody technique (FAT):*

Inoculated tissue and Buffy coat samples on MDBK cell culture were identified by indirect FAT (OIE, 1992).

2.6. *Reverse Transcription Polymerase-Chain Reaction (RT-PCR):*

RNA was extracted from pools of the samples containing the suspected viral isolates using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA), Cat. No.52904. The primer sequences was based on the sequence of BVDV 5' untranslated region (5' -UTR) gene as described by

Brian, (2007). The specific oligonucleotides primers were manufactured by Metabion international, Germany. P1 5' - GGGNAGTCGTCARTGGTTCG - 3' (forward primer). P2 5' - GTGCCATGTACAGCAGAGWTTTT - 3' (reverse primer). The primers were used to amplify a product of about 190 bp in length. The PCR products were loaded in 1.5 % agarose gel containing ethidium bromide with final concentration of 0.5ug/ml at 95 v for 30 min in 1X TBE buffer. Placed in the electrophoresis chamber and covered with electrolyte solution with allowing running the PCR product which could be visualized by the presence of marker (100-1000 bp, Qiagen) and using Gel documentation system (Biometra).

3. RESULTS

3.1. *Detection of BVDV antigen in buffy coat and tissue samples using antigen captured ELISA:*

It was observed that the percent of positive samples collected from cattle were 47.4% (95/200), 22% (11/50), 10% (5/50), 4% (2/50) and 6% (3/50) for buffy coat, lymph node, spleen, intestine and lung samples, respectively.

For buffalo samples, it was observed that BVDV antigen detection using ELISA showed that the percent of positive samples were 38% (76/200), 10% (5/50), 6% (3/50), 4% (2/50) and 4% (2/50) for buffy coat, lymph node, spleen, intestine and lung samples, respectively. These results were shown in table (1).

3.2. *Trials for Isolation and biotyping of BVDV on MDBK cell line:*

Isolation of BVDV from prepared 50 representative samples positive in antigen captured ELISA (16 lymph node, 8 Spleen, 4 intestines, 5 Lung and 17 Buffy coat) was tried on MDBK cell line through 3 blind passages with detection of CPE. No CPE was observed on inoculated cells that noncytopathic BVDV could be suspected, as shown in photos (1) and (2).

3.3. *Identification of BVDV using indirect FAT:*

Indirect FAT was adapted for detection of BVDV protein antigen in infected MDBK monolayer after propagation of samples using specific antisera against BVDV. It was showed that the number of positive isolated samples is 15 out of 50 distributed as (5/17), (6/16), (2/8), (1/4) and (1/5) for isolated samples from buffy coat, lymph node, spleen, intestine and lung, respectively (table 2). It was observed that specific yellowish green fluorescent granules emitted from the inoculated cell culture

showing no CPE indicating presence of non-cytopathogenic strain of BVDV (photo 4) as compared with normal control MDBK monolayer cells (photo 3).

3.5. Molecular detection and genotyping of BVDV isolate using RT-PCR:

The genomic RNA products extracted from suspected isolate and BVDV reference NADL strain were subjected to RT-PCR for genotyping using 5' untranslated region (UTR) specific primers of BVDV. Electrophoresis of the amplified products revealed the presence of specific PCR product at the correct expected size of the BVDV type I (190 bp) as shown in photo (5).

Table (1) Detection of BVDV antigen in cattle and buffalo samples from Kalubeya governorate using ELISA

Samples	No. of Samples	Cattle		Buffaloes		
		Positive No.	Samples %	No. of Samples	Positive No.	Samples %
Buffy coat	200	95	47.5	200	76	38
Lymph node	50	11	5.5	50	5	2.5
Spleen	50	5	2.5	50	3	1.5
Intestine	50	2	1	50	2	1
Lung	50	3	1.5	50	2	1

Table (2): Identification and biotyping of suspected BVDV on MDBK cell line using FAT

Samples	No. of samples isolated on MDBK cell line	No. of isolated samples positive using FAT
Buffy coat	17	5
Lymph node	16	6
Spleen	8	2
Intestine	4	1
Lung	5	1
Total	50	15

4. DISCUSSION

The complex and unique nature of BVDV continues to challenge infectious disease researchers, veterinarians, and the cattle industry. BVDV has recently been targeted for eradication in several national programs (Gilbert et al.; 1999). Because carriers are constantly viraemic and continually shed and maintain the virus in the environment, their identification and removal from the herd is an essential component of programs for the control and eradication of BVDV (Bitsch and Ronsholt, 1995).

Many tests are currently available for the accurate detection of BVDV from a variety of samples, including virus isolation (VI) of WBC lysates, tissues, or whole blood in cell culture; reverse transcription-polymerase chain reaction (RT-PCR); antigen capture enzyme-linked immunosorbent assay (ACE), followed by identification of the viral isolate by immunofluorescence assay is one of the most reliable diagnostic techniques. This assay is considered to be the "gold standard" for the

detection of BVDV (Misty et al., 2007). In this study BVDV antigen were detected by antigen captured ELISA test in cattle and buffaloes Buffy coat samples from different localities in kalubeya governorate, Egypt. The overall numbers of positive samples were 95 (47.4%) and 76 (38%) out of 200 total examined samples for each species, respectively. Our results were in agreement with that of (Mervat et al., 2008) who detected BVDV antigen in (30%) of buffy coat samples using ACE.

For detection of BVDV antigen in tissue samples of cattle, positive tissue samples were 22% (11/50), 10% (5/50), 4% (2/50) and 6% (3/50) for lymph node, spleen, intestine and lung samples, respectively. Positive tissue samples for buffaloes were distributed as 10% (5/50), 6% (3/50), 4% (2/50) and 4% (2/50) for buffy coat, lymph node, spleen, intestine and lung samples, respectively. Our results were in agreement with (Refaat et al., 2010) who detected BVDV antigen in 16 samples out of 54 samples tested by ACE ELISA. But disagreed with (Mervat et al., 2008) who reported that BVDV antigen were 40% (2/5) in spleen and 60% (3/5) in lymph nodes tissue samples.

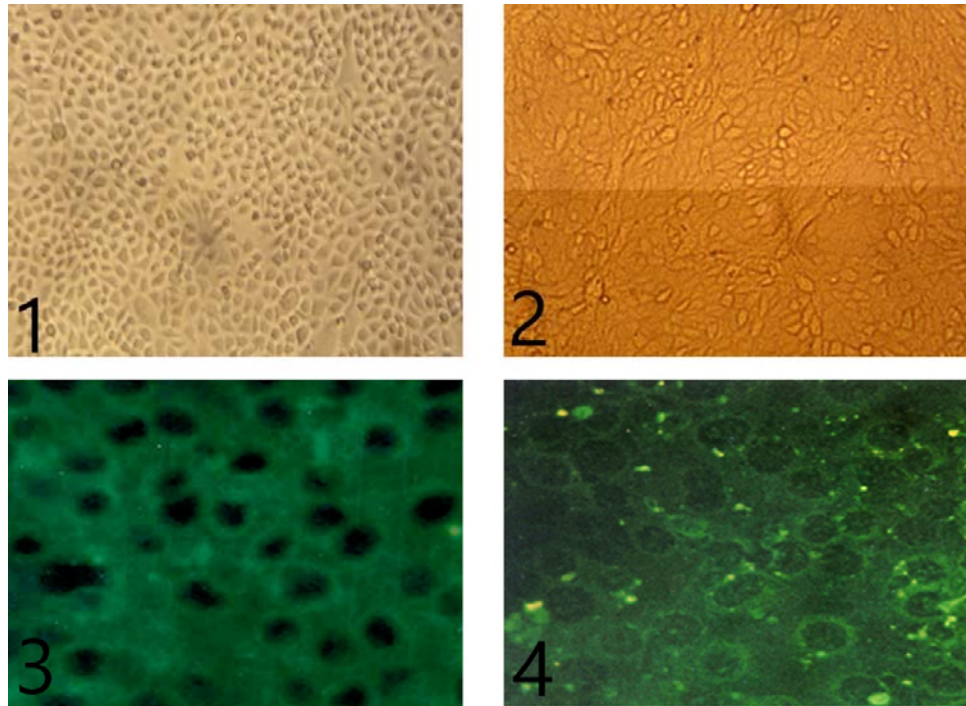


Photo (1): Normal control confluent monolayer MDBK cell line. Photo (2): Inoculated confluent monolayer MDBK showing no cytopathic effect may indicate NCP strain of BVDV. Photo (3): Negative indirect FAT on MDBK cell line under fluorescent microscope. Photo (4): Specific yellowish green fluorescent granules emitted from the normal inoculated cell culture indicating presence of non- cytopathogenic strain of BVDV.

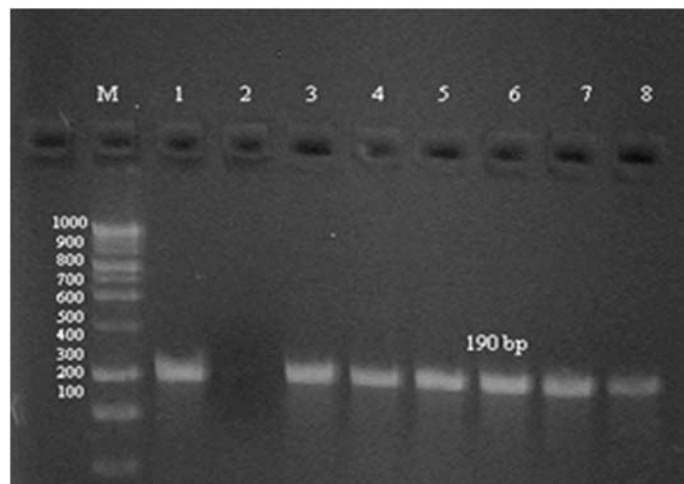


Photo (5): Electrophoresis of the amplified products for detection and genotyping of BVDV in serum samples. Specific PCR product at the correct expected size of BVDV type I gene (190 bp), in the same pattern with no differences between reference strain and detected field strain. M: Marker represents bands of molecular sizes of (100-1000bp). Lane 1: positive control (Reference BVDV NADL strain). Lane 2: negative control. Lane 3 to 8: detected BVDV field strain in buffy coat samples.

BVDV antigen was detected in cattle by Antigen-detecting ELISA in this study indicating circulated infection in the herd. In PI animals, BVDV antigen can be detected in sera during the whole life after maternal antibody has disappeared.

Though, a positive test result for BVDV antigen is likely to originate from PI animals (Sandvik, 2005). The Ag ELISA is a very robust, simple, cost-efficient diagnostic method; the test requires no cell-culture facilities and results are minimally

affected by prolonged storage (Saliki and Dubovi, 2004, Cleveland et al., 2006, Fux and Wolf, 2013).

Isolation of BVDV were applied. Buffy coat and tissue samples were inoculated onto MDBK cell culture and were examined for CPE. After 3 blind serial passages, samples were CPE negative suggesting a non-cytopathogenic BVDV (ncpBVDV) biotype. These results come in agreement with El-Bagoury et al., (2012) who isolated and biotyped a suspected BVDV from buffy coat on MDBK cell line.

Inoculated cell culture with no CPE was subjected to indirect FAT using specific antisera against BVDV revealed characteristic intracytoplasmic apple green fluorescence indicating presence of non-cytopathogenic strain of BVDV. This results agreed with Nahed et al., (2012) who used direct FAT in tissue samples (Lung, spleen, kidney, lymph node and liver) for identification of BVDV. Saliki and Dubovi (2004) refer to virus isolation as the 'gold standard' for BVDV diagnosis. While this is still the case today, the use of PCR has become increasingly common, with RT-PCR now being widely accepted as the standard for BVDV diagnosis. RT-PCR is often preferable to virus isolation as it is less time consuming, less expensive, not restricted to laboratories with cell culture facilities and is also highly sensitive (Kim and Dubovi, 2003, Givens et al., 2003).

The genomic RNA products extracted from examined Buffy coat samples and BVDV reference NADL strain were followed by RT-PCR for genotyping using 5' untranslated region (UTR) specific primers of BVDV. Electrophoresis of the amplified products revealed the presence of specific PCR product at the correct expected size of the BVDV type I (190 bp). From the present study, the samples from kalubia governorate belong to genotype 1. These results come in agreement with (El-Kholy et al., 2005 and Rabab, 2013) who identified and biotyped non-cytopathic BVDV-1 by RT-PCR. RT-PCR is one of the most sensitive methods for BVDV detection and capable even of detecting relatively low levels of virus shed during acute infections (Bhudevi and Weinstock, 2003). Finally, our study showed the prevalence of the BVDV genotype among cattle and buffaloes from different localities in Kalubeya governorate, Egypt and showed also the need to develop the suitable strategy to control the disease.

5. REFERENCES

- Bhudevi, B., Weinstock, D. 2001. Fluorogenic RT-PCR assay (TaqMan) for detection and classification of bovine viral diarrhoea virus. *Veterinary Microbiology* 83, 1–10.
- Bitsch V., Ronsholt, L. 1995. Control of bovine viral diarrhoea virus without vaccines. *Veterinary Clinics of North America: Food Animal Practice* 11, 627-40.
- Brian, J.Z. 2007. Multiplex Real-Time PCR in the Detection and Differentiation of Bovine Respiratory Disease Pathogens. The degree of Master of Science in Veterinary Science. Oregon State University.
- Cleveland, S.M., Salman, M.D., Van Campen, H. 2006. Assessment of a bovine viral diarrhoea virus antigen capture ELISA and a microtiter virus isolation ELISA using pooled ear notch and serum samples. *Journal of Veterinary Diagnostic Investigation* 18, 395–398.
- Cornish, T.E.; Van Olphen, A.L.; Cavender, J.I., Edward, J.M.; Jaeger, P.T.; Tool, O.D. 2005. Comparison of the ear notch immunohistochemistry, ear notch Antigen capture Elisa and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhoea virus. *J Vet Diagn Inves*, 17(2): 110-117.
- Darweesh, M.F., Rajput, M.K.S., Braun, L.J., Ridpath, J.F., Neill, J.D., Chase, C.C.L. 2015. Characterization of the cytopathic BVDV strains isolated from 13 mucosal disease cases arising in a cattle herd. *Virus Research*, 195:141-147.
- El-Kholy, A.A., Vilcek, S., Daoud, A.M. 2005. Phylogenetic characterization of some bovine viral diarrhoea viruses in Egypt. *Egyptian J. Virol.* 1, 421-435.
- Fux, R., Wolf, G. 2013. Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: A pitfall for BVDV eradication programs? *Veterinary Microbiology* 161, 13–19.
- El-Bagoury, G.F.; El-Nahas, E.M.; Khadr, K.A., Nawal, M. Ali. 2012. Isolation and genotyping of bovine viral diarrhoea virus field isolates from infected cattle in Kaluobia during 2011. *Benha veterinary medical journal*, 23(I): 6-10.
- Gilbert, S.A., Burton, K.M., Prins, S.E., Dereg, A.N.D.D. 1999. Typing of bovine viral diarrhoea viruses directly from blood of persistently infected cattle by Multiplex PCR. *J. Clin. Microbiol.*, 37: 2020-2023
- Givens, M.D., Heath, A.M., Carson, R.L., Brock, K.V., Edens, M.S., Wenzel, J.G., Stringfellow, D.A. 2003. Analytical sensitivity of assays used for detection of

- bovine viral diarrhoea virus in semen samples from the Southeastern United States. *Vet. Microbiol.*, 96: 145-155.
- Gunn, G.J., Sattkamp, H.W., Humphry, R.W., Stott, A.W. 2005. Assessing economic and social pressure for the control of bovine viral diarrhoea virus. *Preventative Veterinary Medicine*. V. 72, 149-162.
- Kelling, C.L. 2004. Evolution of Bovine Viral Diarrhoea Virus Vaccines. *Veterinary Clinics Food Animal Practice*. 20: 115-129 .
- Kim, S.G., 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.
- Kummerer, B.M., Meyers, G. 2000. Correlation between point mutations in NS2 and the viability and cytopathogenicity of bovine viral diarrhoea strain Oregon analyzed with an infectious cDNA clone. *J. Virol* 74:390–400.
- Lang, Y.F., Gao, S.D., Du, J.Z., Shao, J.J., Cong, G.Z., Lin, T., Zhao, F.R., Liu, L.H., Chang, H.Y. 2014. Polymorphic genetic characterization of E2 gene of bovine viral diarrhoea virus in China. *Veterinary Microbiology*; 174(3/4):554-559.
- Letellier, C., Kerkhofs, P. 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhoea virus. *J. of Virol. Methods*. 114: 21-27.
- Marcus, S.J., Moll, T. 1968. Adaptation of BVDV to the Madin Darby Bovine Kidney cell line. *Amer. J. Vet. Res*; 29(4):817-819.
- Mervat, I. Ibrahim, Omayma, M. El-Desawy, Nelly, O. Ali, Samya, S. Abd El-Nabi 2008. Detection and isolation of bovine viral diarrhoea virus in cattle by using different virological methods. *Egypt. J. Comp. Path. & Clinic. Path.* Vol.21 No.3; 148-160.
- Misty A. Edmondson,1 M. Daniel Givens, Paul H. Walz, Julie A. Gard, David A. Stringfellow, Robert L. Carson, 2007. Comparison of tests for detection of bovine viral diarrhoea virus in diagnostic samples. *J Vet Diagn Invest* 19:376–381.
- Nahed, K.A., Eman, M.B., Wafaa, A.W., Hanaa A. Ahmed, 2012. Field studies of Bovine Viral Diarrhoea virus (BVDV) in El-Sharkia and Elkalubia Governorates. *An. Health Res. Inst. Assoc.*, pp. 428 – 436.
- OIE, 1992. Manual of diagnostic tests and vaccines of terrestrial animals (bovine viral diarrhoea) chapter 2.4.8, page 698-711.
- Rabab, T.M.H. 2013. Some virological, molecular and immuno-histopathological studies of bovine viral diarrhoea virus. M. Sc. Thesis, virology, faculty of veterinary medicine, Moshtohor, Benha University
- Refaat, M., Hala A. Salem, Jehan A. Gafer, Dardiri, M.A. 2010. Molecular, Virological and Pathological diagnosis of Bovine Viral Diarrhoea (BVD) virus infection in calves. *Egypt. J. Comp. Path. & Clinic. Path.* Vol. 23 No. 1. 32- 50.
- Saliki, J.T., Dubovi, E.J. 2004. Laboratory diagnosis of bovine viral diarrhoea virus infections. *Veterinary Clinics of North America – Food Animal Practice* 20, 69–83.
- Sandvik, T. 2005. Selection and use of laboratory diagnostic assays in BVD control programmes. *Preventive Veterinary Medicine* 72, 3-16.
- Van den Hurk, J. 2000. Health Management: Bovine Viral Diarrhoea. Accessed on line at [http://www1.agric.gov.ab.ca/\\$department/ddeptdocs.nsf/all/beef11736](http://www1.agric.gov.ab.ca/$department/ddeptdocs.nsf/all/beef11736)
- Vilcek, S., Paton, D.J., Burkovic, B., other authors 2001. Bovine viral diarrhoea virus genotype 1 can separated into at least eleven genetic groups. *Archives of virology*, 146, 99-115.