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Molecular Diagnosis of Different Strains of Bovine Viral Diarhea(BVD)

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Abstract

In the present study, we reported the existence of BVDV type-1 in apparently healthy non vaccinated calves in Giza and Ismailia Governorates. Screening of 32 buffy coat samples has been conducted for the presence of BVDV using fluorescent antibody technique (FA) and RT-PCR for antigenic and genomic characterization. Results of RT-PCR based assay depend on primers located in the 5`untranslated region (5`UTR) revealed that 13 samples were positive. There were 12 BVDV positive samples by FA and RT-PCR were inoculated in MDBK cell culture and observed for three blind passages. There were 9 Cytopathic (CP) and 3 negative isolates of BVDV. Quantitative real-time qRT-PCR assay is a highly sensitive method for quantification and determination of BVDV genotypes, q RT-PCR can detect BVDV in two of five selected isolates depend on Pan Pesti Virus (PPV) probe which directed against the highly conserved 5 untranslated region (5UTR) of BVDV. This probe detects panpesti virus which may be classical swine fever or BVD. Sequencing of the two detected samples by qRT-PCR revealed that they are closely related to the first BVDV isolate in Egypt (Iman strain). Phylogenic analysis of the 2 original isolated viruses indicates clustering with other viruses of BVD-1. The present study reports the identification of BVDV genotype-1 in apparently healthy cattle population and the possible risk of prevalence of persistence infection (P.I.) animals in Egypt.

Key words: BVDV type-1 , Cytopathic, RT-PCR , calves .

Introduction

Bovine viral diarrhea virus (BVDV) is a pestivirus in the family Flaviviridae and is closely related to classical swine fever and Border disease viruses **(Donis 1995; Rhodes *et al*, 1999 and Heinz *et al*., 2000)**. Bovine viral diarrhea virus (BVDV) has world-wide distribution and causes various clinical syndromes in cattle including diarrhea, mucosal disease, reproduction disfunctions (abortion, teratogenesis, embryonic resorption, fetal mummification and stillbirth) and hemorrhagic syndrome **(Baker 1995; Coetzer and Tustin 2004; Passler *et al*. 2007)**. The immunotolerant cattle can shed the virus via secretion and excretion into the environment for a long time, thus being a major source of BVDV infections in herds **(Baker 1987).** Therefore, BVD with these economic losses, need to accurate and sensitive diagnostic methods for rapid identification and elimination of persistent carriers in the herds. These years several molecular methods like reverse transcriptase polymerase chain reaction (RT-PCR) are used extremely for detection of BVD viral RNA for diagnostic purposes **(Gilbert *et al*. 1999;Kim and Dobovi 2003).** BVDV isolates were usually typed based on comparison of genomic sequences from the 5'- untranslated region (5'-UTR), N (pro) and E2 region **(Vilcerk *et al*., 2005**). Viral genes in serum, nasal discharge and oral discharge were quantified with real-time polymerase chain reaction using SYBR Green by relative quantification method **(Tajima *et al*., 2008).**

In Egypt, BVDV-MD complex was considered as endemic disease before 1960 **(Baz, 1992). Hafez, 1975** isolated BVDV for first time from calf suffering from pneumoenteritis syndrome and the virus designated as IMAN-7912. BVDV was isolated from buffalo **(Mohamed,**

2002).

PCR was employed for the detection and diagnosis of BVDV **(Hussein 2001; Mohamed, 2002 and Hassanein, 2003)**.

The purposes of this current study were:

Detection of BVDV in field samples by FA and RT-PCR assay.

Application of qRT-PCR for confirmation of BVDV- isolates.

Genetic analysis of the BVDV strains circulates in clinically normal calves in different localities in Egypt using genotyping by sequencing of 5'-UTR region of BVDV.

Material and Method

Buffy coat samples:

A total of 32 blood samples on EDTA were collected from non vaccinated clinically normal calves representing 2 different Egyptian Governorates. Buffy coats were collected, resuspended in 1.5ml of MEM containing 40mg/liter gentamycin and stored at -70^0c until used. The samples were collected during 2008. The detailed data of the samples sources are represented in Table (1**).**

Table (1): Number of blood samples collected from cattle at some Egyptian Governorates.

Governorates	No of samples	Age (months)	Breed	Sex
Giza	12	8-18	Native	Male
Ismailia	15	2	Native	Female
Total	32			

Detection of BVDV antigen by direct fluorescent antibody technique (FAT):

Fluorescent antibody technique was applied on the 32 buffy coat samples according to **Fernelius, (1964) and Botton *et al.* (1998)** using procine anti-BVDV flourocine conjugate**. (Eappel organon , Tecknika Crop West Cheste)** was supplied by the ELISA unit and viral strain bank, Animal Health Research Institute.

Detection of BVDV by RT-PCR:

Extract RNA from buffy coat samples using **QIAamp ® Viral RNA Mini Sprin protocol** according to the manufacturer's instructions. At the same time isolate the RNA from stocks of tissue culture supernatant NADL, to be used as a positive control and the isolated RNA was eluted in 50 µl in DEPC-treated H2O and stored at -70^oC.

Conventional RT-PCR, Was performed using C. therm. Polymerase **One-Step RT-PCR System (Roche Diagnostic)** according to the manufacturer's instructions. Primers:

324: 5´-ATG CCC TTA GTA GGA CTA GCA-3´and

326: **5´-TCA ACT CCA TGT GCC ATG TAC-3´**) were used according to **Vilcek *et al.* (1994).** Final concentration of each primer was 0.3µM in reaction. Reverse transcription was performed with 5 µl of the total RNA at 60°C for 30 min, followed by a short incubation at 94°C for 2 min to inactivate the reverse transcriptase and to denature cDNA. Then the PCR consisted of 35 cycles of denaturation at 94°C for 30 s, annealing of primers at 55°C for 1 min and extension at 70°C for 1 min. The final step included extension at 70°C for 7 min and cooling to 4°C. PCR products were detected in 1.5% agarose gel in TAE (Tris-acetate-EDTA) buffer and visualized by ethidium bromide (0.5 µg/ml) staining.

Inoculation of positive BVDV samples on MDBK cell culture:

Twelve samples that revealed positive in both

FAT and RT-PCR were inoculated in BVDV-free MDBK cell line (200 ul from each buffy coat were used in inoculation). The cells were incubated for 2 hours-adsorption time at 37^0C. The non adsorbed inoculum was removed and 10ml of MEM containing 2% horse serum were added. The cells were incubated at 37^0C for 5 days. Non inoculated cells as negative control and cells inoculated with reference BVDV were included.

Real time RT-PCR for confirmation of BVDV isolation:

Five isolates selected and tested by real time RT- PCR at **Veterinary Laboratories Agency (VLA) in Wybrideg ,London,UK** as follows:

Quantitative RT-PCR was carried out using the **TaqMan® fluorogenic probe and Stratagen Mx3000/Mx3005 PCR system** according to **SOPs (standard operating procedure) of VLA laboratory. PPV TaqMan ® dual-labeled probe (5um) HPLC purified Sigma Genosys 5´ FAM -CTGATAGGGT-GCTGCAGAGGCCCACT- TAMRA 3´.** This probe is directed against the highly conserved 5 untranslated region (5UTR) of BVDV and was described by **McGoldrick *et al* (1998)** using probe termed Pan Pesti Virus (PPV) that detects CSFV, BVD&BVD-1. Probe (286-310). The primers used are: **A11**: **5´-AGT ACA GGG TAG TCG TCA GTG GTT CG -3´**and **A14**: **5´-CAA CTC CAT GTG CCA TGT ACA GCA G -3´**

324:**5´-ATG CCC TTA GTA GGA CTA GCA-3´**and 326: **5´-TCA ACT CCA TGT GCC ATG TAC-3´.** All primers are supplied by Sigma Genosys.

Sequencing of 5`UTR of BVDV:

The 5`UTR of the two isolates G4, 15 detected by qRT-PCR are sequenced. The sequencing procedures done in sequencing unit for VLA (Veterinary Laboratory Agency)Wybridge London UK according to SOPs of the lab using BigDye® terminator v3.1cycle sequencing kit protocol and 3130x1,16 capillary sequencing analyzer. The obtained sequences were analyzed using phylogenitic analysis was carried out using Mega u software **(Tamura *et al*., 2007).**

Results and Discussion

Bovine Viral Diarrhoea virus (BVDV) is a pathogen of worldwide importance for cattle, causing significant economic losses **(Brownlie, 1991)**. The immunotolerant cattle can shed the virus via secretion and excretion into the environment for a long time, thus being a major source of BVDV infections in herds **(Baker 1987).** the Persistently infected (PI) animals continuously shed virus and act as a major reservoir of infection for other cattle **(Paton *et al*., 1999)**.Therefore, BVD with these economic losses, need to accurate and sensitive diagnostic methods for rapid identification and elimination of persistent carriers in the herds. In past, the most common method was an isolation of virus in cell cultures but it was difficult, time consuming and lengthy process that requires experienced technicians. These years several molecular methods like reverse transcriptase polymerase chain reaction (RT-PCR) are use extremely to detection of BVD viral RNA for diagnostic purposes **(Gilbert *et al*. 1999; Kim and Dobovi 2003).**

Result of detection of BVDV in buffy coat samples using direct fluorescent antibody technique (FAT), utilized direct FA as herd screen test for detection of BVDV obtained results with degree of variability especially in the detection of persistent infected animals with NCP BVDV **(Bezek *et al*., 1988; Corapi *et al*., 1990;Trevorr and John, 1990 and Botton *et al*.1998)**. Out of 32 tested buffy coat samples, 12 samples were positive to BVDV whereas 20 samples were negative **(Table 2)**. The highest percentages of positivity were those samples from Ismailia. The positive infected cells for FA are shown in **photo (1)**. Moreover, the FA results represent a strong evidence of the presence of BVDV viable particles.

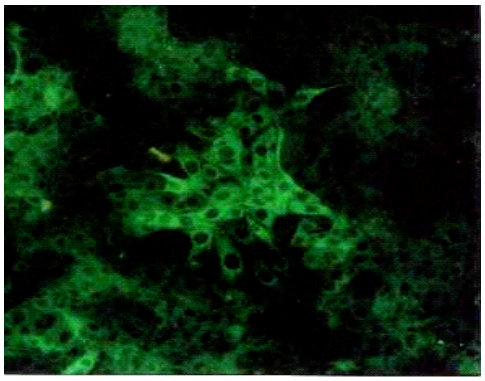


Photo (1) MDBK cells infected by BVDV local strain and stained by fluorescien conjugated anti-BVD serum showing specific diffused intracytoplasmic yellowish green fluorescent color (Mag. 200X).

Table (2) Results of detection of BVDV in buffy coat samples by direct fluorescent antibody technique (FAT).

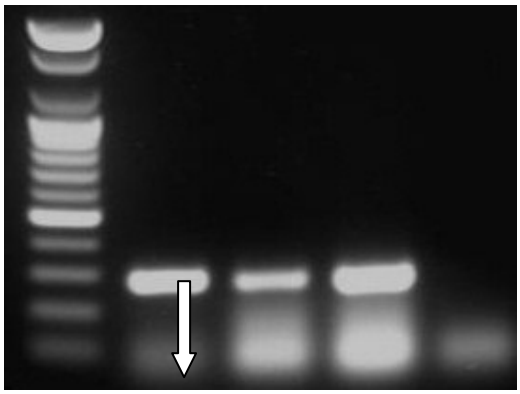
Governorates	NO of samples	No. of +ve samples by FA	No. of –ve samples by FA
Giza	12	4	8
Ismailia	20	8	12
Total	32	12	20

Regarding of conventional RT-PCR, RT-PCR assays for detection of BVDV had demonstrated its potentiality and ability to amplify strains containing considerable genetic diversity and gathered a lot of information on studying the evolution and pathogenesis of the virus **(Vilcek *et al*., 1994; 2001; Sullivan and Akkina, 1995)**. The assay proved its usefulness in detecting BVDV in P.I. animals even in the presence of the virus and maternal antibodies **(Sandvik, 1999)**. Primers utilized in the RT-PCR assay has been previously showed its ability to detect any pestiviruses due to its location at highly conserved region of the genome **(Collett *et al*., 1989)**. Although several researchers described PCR assays depends on utilization of primers located at E^{rns}, E_1, P_{54} and P_{80}, these assays failed to detect all BVDV strains **(Vilcek *et al*., 1994; Tajima *et al*., 1995)**. 5`UTR and P_{80} regions are considered the highly conserved regions of pestiviruses genome **(Collett *et al*., 1989; Laamanan *et al*., 1997)**.

In the present study 5`UTR primers showed its ability to detect BVDV in the samples with constituent and district amplification product like previous work **(Hussein, 2001; Mohamed, 2002; Hassanein, 2003)**. The Conventional RT-PCR has been described for a rapid detection (**Hyndman *et al*., 1998; Renshaw *et al*., 2000)** of BVD virus. Of the 32 samples assayed by RT-PCR, 13 samples were positive and 19 were negative **(Table 3). Figure (2)** represent some of the positive samples in the RT-PCR assay. The amplified target revealed strong visible band (corresponding to the molecular weight marker specific bands), which confirmed primers, and fragment specificity. Cell control, serum and PCR control showed negative results.

Table (3) Results of RT-PCR for detection of BVDV.

Governorates	No. of samples	No. of RT-PCR +ve samples	No. of RT-PCR -ve samples
Giza	12	4	8
Ismailia	20	9	11
Total	32	13	19



ladder DNA marker Lane 1; sample G4,Lane 2; sample I5 Lane 3; NADL strain Lane 4 as control positive and negative sample Lane 5.

Result of BVDV isolation of positive samples by FA and RT- PCR on MDBK cells was carried out on three blind passages. The result of the 3rd passage was shown in **table (4). Photo (3),**the results were similar to observation of **(Hosney *et al*., 1996; Shehab *et al*., 2001 and Nawal *et al*.,2003)** in the form of cell rounding , shrinkage and vacule formation.

Table (4) Result, of the BVDV isolation in MDBK cells.

Code no	*Days post inoculation**				
	1st. day	2nd.day	3rd.day	4th. day	5th. day
1G	-	+	++	++++	
2G	-	-	+	++	+++
3G	-	++	+++	++++	
4G	-	++	+++	++++	
1I	-	+	++	+++	++++
2I	-	-	-	-	-
3I	-	-	+	++	+++
4I	-	-	-	+	++
5I	-	-	+	+	+++
6I	-	+	+++	++++	
7I	-	-	-	-	-
8I	-	-	-	-	-

* after the3rd passage

(+) = Cell rounding and darkness (++) = Cell aggregation and shrinkage

(+++) = Vaculation (++++) = Cell detachment

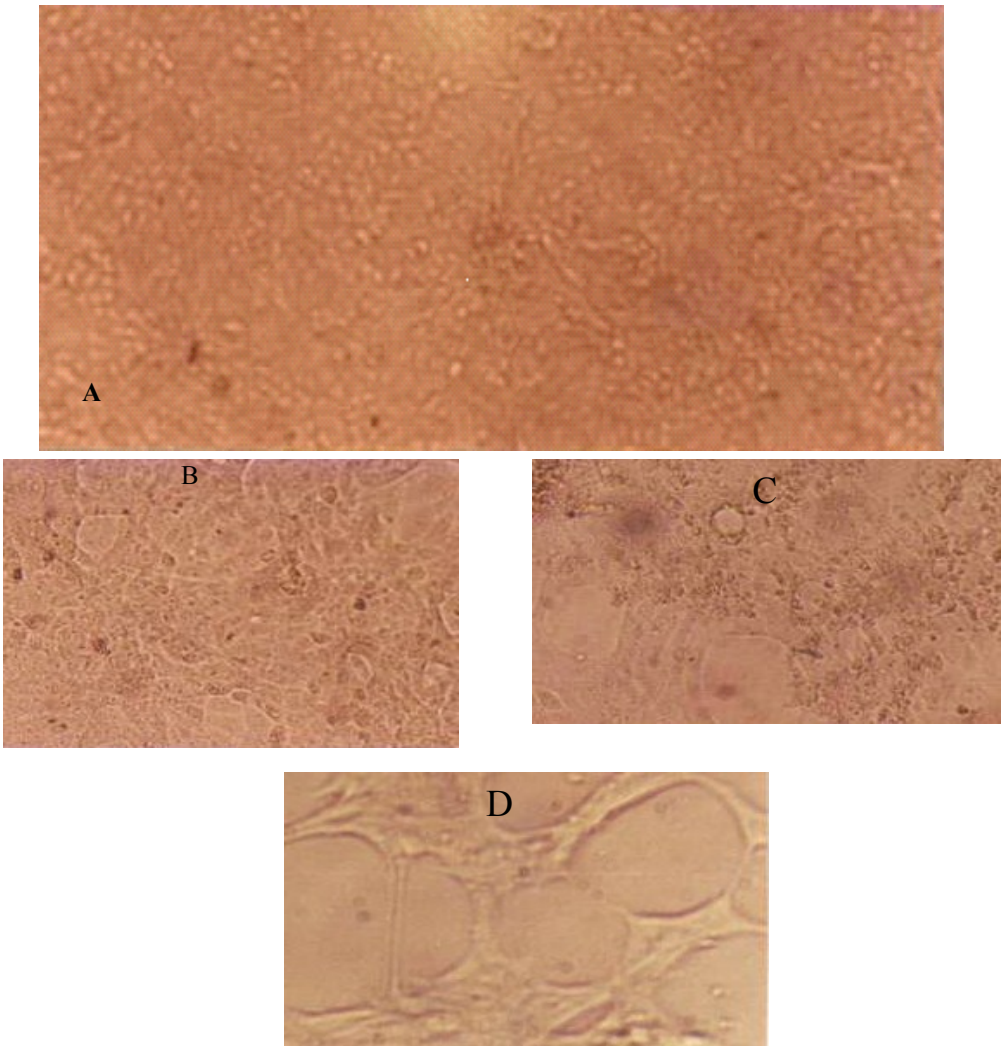


Photo (3) MDBK normal and infected with BVDV local isolates showing progressive CPE observed during 5 days post inoculation
(A) Normal non infected MDBK cells showed no CPE. (B) Cell rounding
(C) Cell aggregation and shrinkage (D) Vaculation

Result, of Real time PCR for BVDV confirmation after isolation revealed that the developed real-time PCR assay has some advantages compared to the conventional PCR; it is an important diagnostic tool yielding reliable and reproducible results, does not require post-PCR analysis (gel electrophoresis, hybridization), and the risk of cross contamination is limited **(Mackay, 2004). Bhudevi and Weinstock, 2001, 2003; Lettellier and Kerkhofs, (2003)** reported that the using of real-time PCR to quantify the viral RNA levels in BVDV-infected animals presents particular technical and biological challenges. The disadvantage of the real-time PCR assay compared to the conventional PCR might be the cost of the instrument.

The results obtained by qRT-PCR showed high agreement in the demonstration of genetic structures of BVD virus in five samples under testing. **Figure (4)** showed the result of qRT-PCR. The negative results of three samples may reflect that these samples not detectable by the used probe due to un successful virus isolation.

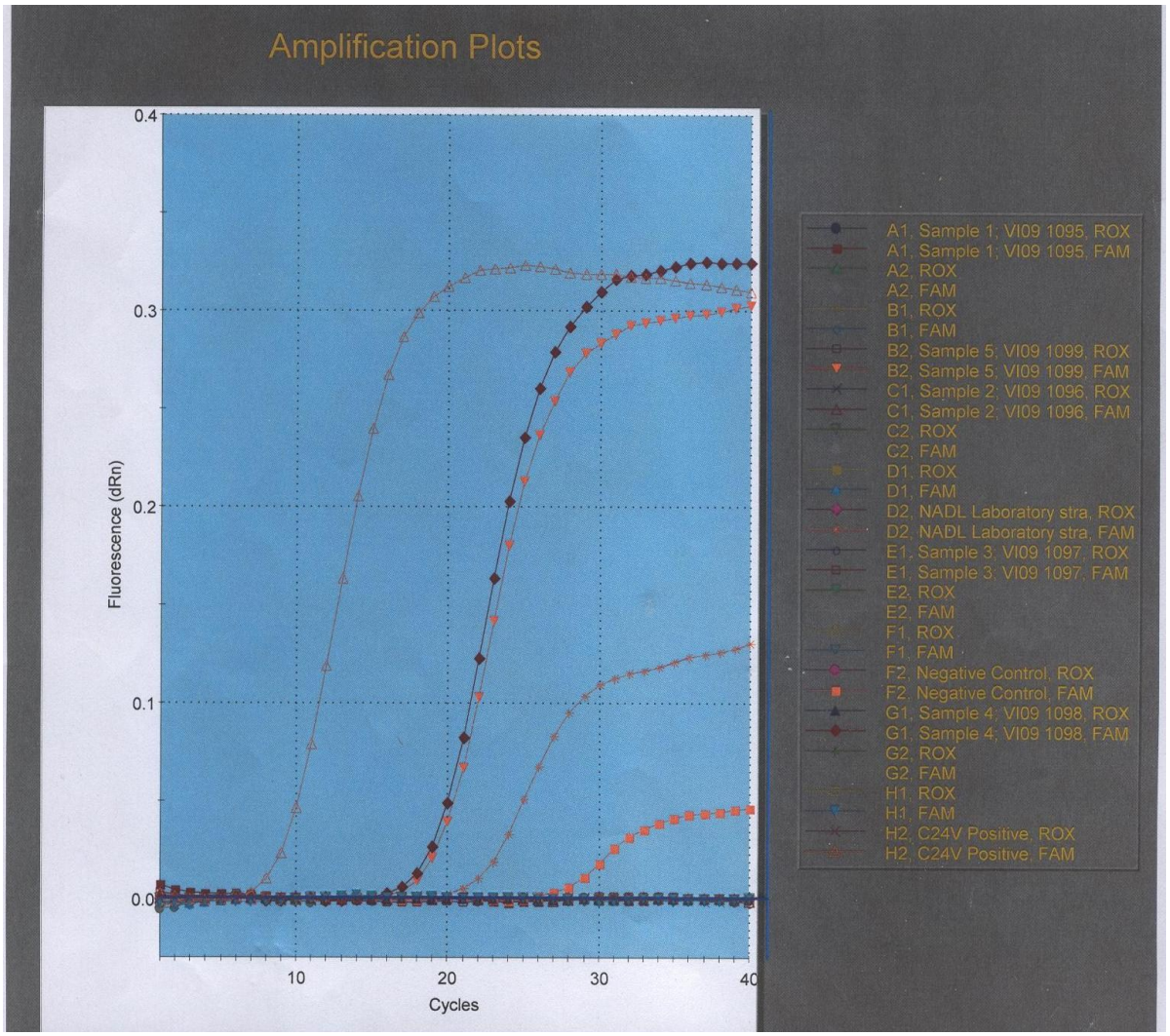


Figure (4) showed the result of qRT-PCR

Result, of Sequencing:

Sequencing of PCR products and phylogenetic analysis revealed that they belong to BVDV-1 group.Homology percentage of the two highly positive samples sequenced fragment to untranslated region is high among all known BVDV genomic sequences . Successfully used primers directed toward this region for RT-PCR Of RNA extracted and analyzed sequences using (NCBI) Blast for isolates G4 and I5. The nucleotide sequences are 388 and 384 base pair. The nucleotide sequences in the amplified region for assessment of variability in the amplified region **(Deng and Brock 1992 , Dubovi 1992 and Heid *et al*., 1996**). The comparison of these sequences with that of Iman strain revealed that sequences of these isolates are closely related to the original Egyptien BVDV isolate (Iman strain). Nucleotide sequence comparison with those of previously published pestivirus strains revealed great identity to BVDV type-1. The identity ranged from 92 to 100%. The homology inbetween the two isolates is 99%. The identity between the two isolates and Iman strain is 98%. The identity to NADL strain is 98%. **Figure (5)** showing the phelogenatic tree of isolate G4 and the phelogenatic tree of isolate I5 to the previously published pestiviruses.

In conclusion, the present study reports the identification of BVDV genotype-1 in apparently healthy cattle population and the possible

prevalence of persistence infection (P.I.) animals in 2 Egyptian Governorates. The real-time qRT-PCR assay is a highly sensitive method for quantification and determination of BVDV variability level. The isolated BVDV are closely related to Iman strain, the Egyptian virus isolated in 2002 . Further study to the undetected isolates using probe specified to BVDV-2 in qRT-PCR will be completed.

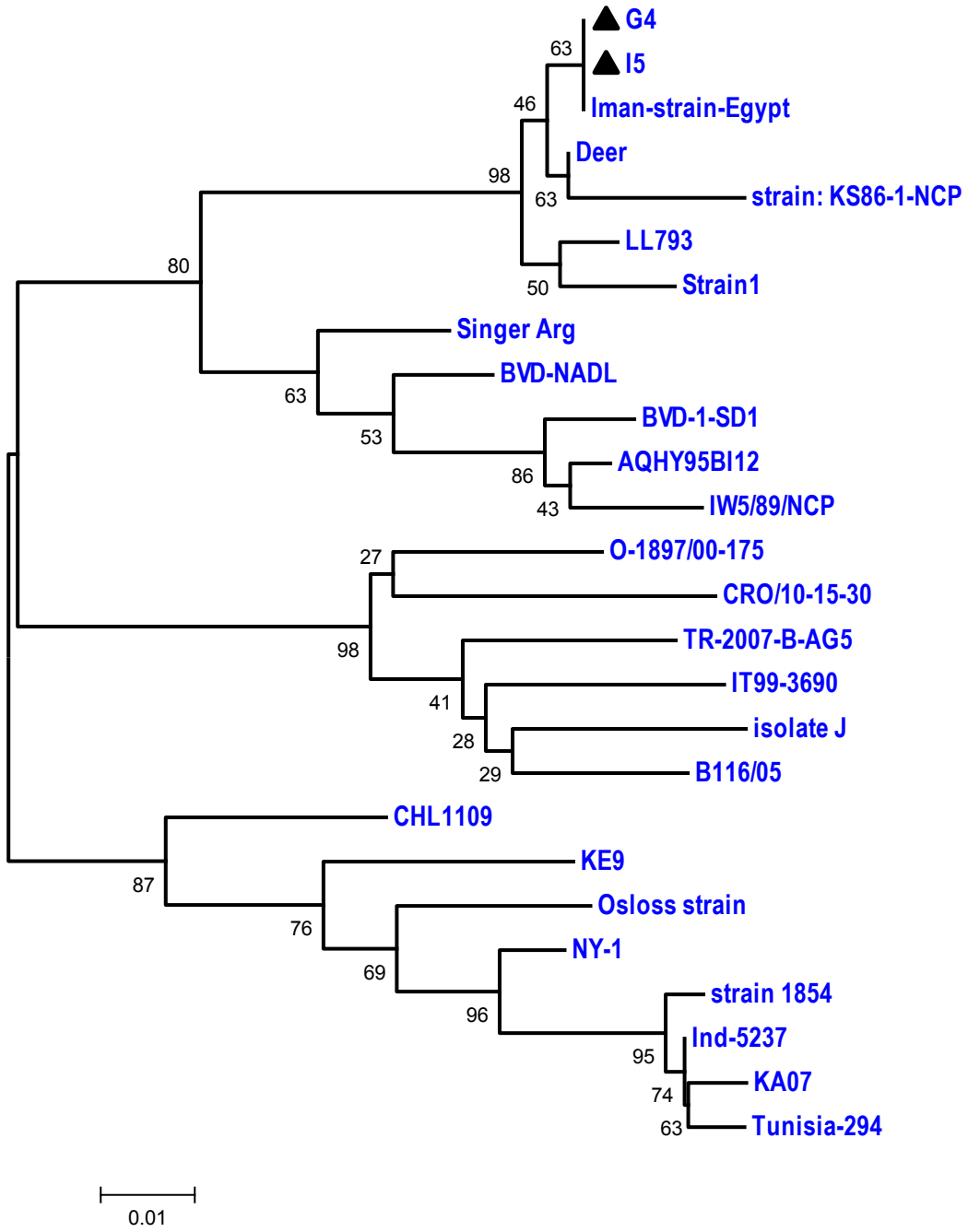


Figure (5): Phylogentic analysis of BVD viruses indicate that the 2 Egyptian viruses G4 and I5 were very closly related to the original Egyptian virus (Iman starin) and they were belong to genotype BVD-1. The tree was constructed using MEGA 4 software **(Tamura *et al.*, 2007)**

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التشخيص الجزيئى للعترات المختلفة لفيروس الاسهال الفيروسى البقرى

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الملخص العربى

فى هذه الدراسة تم تسجيل وجود فيروس الاسهال البقرى الفيروسى فى الماشية من النوع الأول فى ابقار سليمة ظاهريا وعجول غير محصنة فى محافظتى الجيزة والاسماعيلية.بعمل مسح لوجود الفيروس فى 32 عينة دم على مانع للتجلط باستخدام اختبار الفلورسنت ومعرفة الخواص الجينية للفيروس باستخدام اختبار البلمرة المتسلسل. باستخدام اختبار البلمرة المتسلسل RT-PCR المعتمد على البادئ الواقع عند 5`UTR نتج عنه 12 ,13 عينة ايجابية باختبار الفلورسنت والبلمرة المتسلسل على التوالى.تم حقن 12 عينة ايجابية بالاختبارين على خلايا الزرع النسيجى ومتابعتها ثلاث تمريرات متتالية,وجد ان 9 عينات كانت ممرضة CP و 3 عينات غير ممرضة NCP للخلايا. تم التعرف على الفيروس فى عينتين من المعزولات باستخدام اختبار البلمرة المتسلسل الحقيقى الكمى معتمدا على مجس Pan Pesti Virus (PPV)probe . التتابع الجينى فى عدد 2 معزولة للفيروس والتى تم اختبارها باستخدام اختبار البلمرة المتسلسل الكمى اظهر ان التتابع الجينى للمعزولتين يتبع التتابع الجينى للمعزولة المصرية فيروس الاسهال البقرى الفيروسى فى الماشية(العترة ايمان Iman strain) مقارنة التتابع النيكلوتيدى مع ما تم نشره عن عترات Pestivirus وأوضحت النتائج عدم وجود اختلاف مع العترات BVDV Genotype 1 وذلك باختبار التتابع الجينى. أوضحت الدراسة وجود هذه العترة فى ابقار سليمة وامكانية حدوث العدوى الدائمة PI فى المحافظات التى تمت فيها الدراسة. اختبار البلمرة المتسلسل الحقيقى الكمى اختبار حساس لتقدير مدى التغيرات التى تحدث فى فيروس الاسهال البقرى الفيروسى.