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DETECTION OF BULLS PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS IN ASWAN PROVINCE, EGYPT

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ABSTRACT

This study was designed to detect the persistent infection with bovine viral diarrhea virus (PI-BVDV) among 114 bulls in Aswan Governorate. Sera samples were examined by ELISA and real time polymerase chain reaction (PCR) to detect BVDV antibodies and BVDV nucleic acid, respectively. The percent of infection was 23.7% (27/ 114) and 76.3% (87/ 114) were negative by ELISA technique, and 3.4% (3/ 87) for BVDV gene detection by real time PCR. Sensitivity of 89%and specificity of 96 % was reported using the commercially available ELISA kit for samples obtained from PI animals.

Keywords: ELISA, BVD, Persistent infection PI, PCR

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is included in the genus Pestivirus of the family *Flaviviridae*. BVDV occurs worldwide in cattle populations and causes economic losses, mainly due to its effects on health and reproduction (A. Khodakaram-Tafti *et al.,* 2016, Fray *et al.,* 2000, Byers *et al.,* 2011).

Pestiviruses are small enveloped RNA viruses of the *Flaviviridae* family; The genus includes classical swine fever virus, bovine viral diarrhea virus (BVDV) and border disease virus. The pestivirus genome consists of a single-stranded positive sense linear RNA that is approximately 12> 5 kb in size (Murphy *et al.,* 1999).

Two genotypes of BVDV (BVDV-1 and BVDV-2) were differentiated using serologically monoclonal and polyclonal antisera and molecular biology, subtypes of these two genotypes were also described (Vilcek *et al.,* 2001, Ridpath 2003).

BVDV-1and -2 infections are connected with bovine diseases, including subclinical infections, immunosuppression of acute diarrhea, respiratory diseases, reproductive disorders and mucosal diseases in persistently infected calves (Baker JC 1987).

The uncommon cytopathic (CP) biotype damages tissue cultures and the much more

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common non-cytopathic (NCP) does not. Biotypes apparently behave differently in vivo. Non-cytopathic strains have tropism for leukocytes, lymphatic organs and the respiratory tract, while CP strains are more restricted to the digestive tract (Bezek *et al*., 1994).

All PIs and more severe forms of BVD are caused by the NCP biotype (Birk *et al*., 2008; Neill *et al*., 2008).

Reproductive disorders caused by BVDV occur with a variety of clinical signs, including miscarriage, parturition and birth of persistently infected calves, depending on the stage of pregnancy at which the cow became infected (Walz 2010, Brigstocke 2010).

In the case of super infection with cytopathic (cp) strains, these persistent infected animals may develop mucosal disease (MD), a severe and fatal clinical form of BVDV infection. Infection in a healthy adult is usually subclinical and the main effects are decreased reproductive function and immunosuppression (Howard 1990).

Fetuses infected during the first trimester of pregnancy with non-cytopathic (NCP) strains may develop immunotolerance and become lifelong carriers of the virus. These permanently infected (PI) animals are a continuous source of the virus and play a major role in the spread of disease with a high prevalence of antibodies to BVDV, with more than 1-2% of newborn calves being PIs (Houe 1999).

BVDV can cause MD and chronic disease in PI animals (Evermann and Ridpath, 2002).

Animals with transient infections (TI) are responsible for up to 93% of all uterine infections that lead to the birth of PI calves (Wittum *et al.,* 2001).

The main route of transmission in infected herds is direct contact with the PI animal. Horizontal transmission of BVDV can be direct or indirect by inhalation or ingestion of virus-contaminated materials (Lindberg 2003).

If the cow is PI, her fetus will become infected. The virus has the ability to cause transplacental infection leading to different outcomes depending on the stage of pregnency in which the acute infection occurs, leading to fetal death, malformations, acute the neonate syndromes, immune tolerance and lifelong viral persistence (Peterhans *et al.,* 2003).

Other mechanisms of vertical transmission include: contaminated semen, embryo transfer, and contaminated modified live vaccines. Infected bulls may shed BVDV for a long time and cattle were infected after insemination with frozen semen from these animals (Bielanski *et al*., 2009).

Fetal infections during the first 120 days of pregnancy If the fetus survives an early infection, it always becomes PI (Uzal *et al.,* 2016).

The ability of the virus to cross the placenta during early pregnancy can result in the birth of permanently infected (PI) calves. Permanently infected animals are generally much more effective transmitters of BVDV than transiently or acutely infected animals because they are able to shed large amounts of virus throughout their lives and are considered the primary reservoirs for BVDV. Due to the nature of viral infections, there is no cure for a complete cure of the animal's viral infection. All control programs used in many countries of the world depend mainly on the detection of PI animals, their elimination and the prevention of their return to herds. Detection of PI animals at an early stage, especially shortly after birth, is a significant contribution to the implementation of BVDV control programs (A.Khodakaram-Tafti *et al.,* 2017).

Some PI calves can survive into adulthood, and if kept for breeding, their off spring is always PI, but often fails to survive. PI bulls can produce sperm of acceptable quality, but can be associated with infertility (Moennig *et al.,* 2005).

Reported a case of a bull that was strongly sero-positive and non-viremic but permanently excreted the virus in semen (Voges *et al.*,1998).

All PIs, and other severe forms of BVD are caused by the NCP biotype (Birk *et al.,* 2008; Neill *et al.,* 2008).

Pestiviruses have been shown to circulate widely in British sheep. Understanding the implications of this fact for the national BVD eradication system is crucial given the possibility that sheep could circulate BVD (Jennings *et al.,* 2018).

On mixed farms (bovine and ovine) with BVD on the possible spread to the ovine herd and, if possible, to prevent exposure of pregnant sheep to PI (Nikki Moore, case report, 2020).

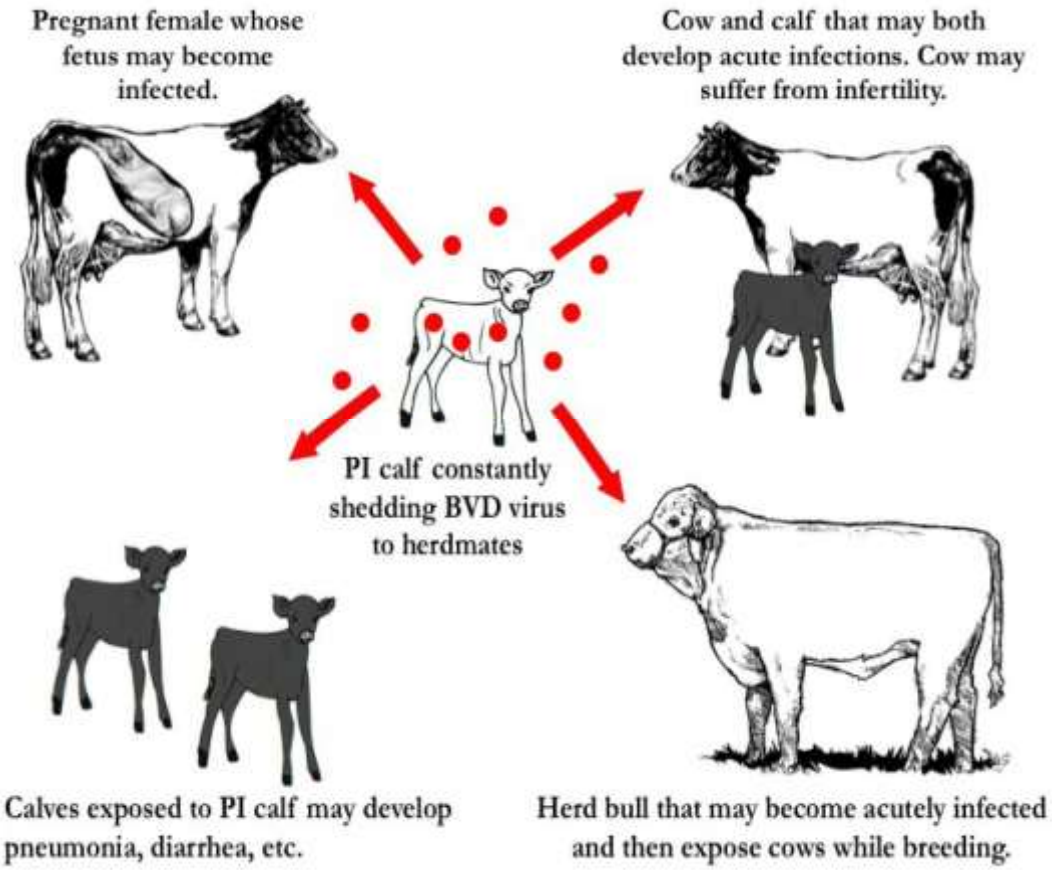

Fig. 1: PI cattle excrete the virus throughout the life being a source of infection for other animals in a herd (A. Khodakaram-Tafti *et al.,* 2017)

Serological methods such as enzyme-linked immunosorbent assay (ELISA) are commonly used to diagnose BVDV in clinical specimens (Fenton *et al.,* 1991; Entrican *et al.,* 1995).

The ELISA has good sensitivity, specificity and reliability for the detection of antibodies to BVDV; it is easily portable, economical and easy to implement (Belak and Ballagi Pordawy, 1993).

PCR is the recent method that facilitate the typing of BVDV at the genotype level directly from PI bovine blood samples (Letellier and Kerkhofs 2003, Baxi *et al.*, 2006, Aduriz G. *et al.,* 2015).

There is much evidence for detection of the BVDV genome by PCR in pooled bovine serum, fetal fluid, and seminal fluid (Kamil Saeed Intisar *et al.*, 2010).

Determine the genetic diversity of BVDV circulating among the cattle herds in Nineveh Province by PCR Assay, and this result could have significant implications for the epidemiology, diagnosis and control of the disease in Iraq (Sadam, D. Hasan and Kamal. M. Alsaad, 2018).

47 blood samples were selected for the target and tested positive by ELISA for BVDV antigen. BVDV positive samples were genotyped by PCR (Aduriz *et al.*, 2015).

46 blood samples were tested in EDTA and non-EDTA tubes from candidate bulls at artificial insemination centers. Blood serum samples were tested for antibodies to bovine viral diarrhea virus (BVDV) and leukocyte samples were tested for BVDV antigens by ELISA (Sibel Yavru *et al.*, 2013).

BVDV-1 infections were indeed present and the viruses were with genetic variations in Chinese goat herds. This information would be very useful for the prevention and control of BVDV-1 infections and consider as first report which investigate occurrence and genotyping of BVDV in goats in China (Li Mao *et al.*, 2016).

Using PCR to detect the molecular characterization and phylogenetic analysis of BVDV infection in dairy cattle herds in Fars province, Iran (A.Khodakaram-Tafti *et al.*, 2016).

Used a PCR test and some virological techniques to detect bovine viral diarrhea virus (BVDV) in clinical specimens to Elucidate their pathological effects on camel calves (JEHAN A. GAFER *et al.*, 2015).

The first report on the prevalence of bovine viral diarrhea virus in aborted fetuses of bovine, ovine, caprine, buffalo and camel by ELISA and PCR in Iran (Farhad Safarpoor Dehkordi, 2011).

The role of pestivirus, particularly bovine viral diarrhea virus (BVDV), in inducing respiratory infections in camels has been studied at four different sites in Sudan. Evaluation was performed by ELISA and positive samples were further confirmed by polymerase chain reaction PCR (Kamil Saeed Intisar *et al.*, 2010).

Statistical analysis of the data showed significant differences between ELISA and RT-PCR for virus detection in aborted fetuses (Farhad Safarpoor Dehkordi, 2011).

Our aim is detection of persistent BVDV infections in bulls by Real time PCR method in serologically negative serum sample for antibodies of virus.

MATERIALS AND METHODS

Sample collection

Within 2018, a total of 114 bulls (about two years old, non-vaccinated against BVD) blood samples were randomly taken. Collected from El shalall slaughter house, Aswan. Specimens were transferred on ice to the Central Veterinary Research Laboratory at Aswan University, and separated serum were kept at −20°C till used. All examined animals were clinically healthy according to (Rosen Berger, 1979 and Radoiostits *et al.*, 2007)

Serological test

Indirect ELISA for BVDV antibody detection

All samples were tested using commercial Bio-X Diagnostics) BVDV ELISA KIT (GE&MBR genetic engineering and molecular biology Center, Assuite university, Egypt),in which microliter plates, the odd columns (1, 3, 5, 7, 9 and 11) are sensitized by the BVDV viral antigen and the even columns (2, 4, 6, 8, 10 and 12) by the cell lysate. The kit is based on the detection of the monoclonal antibodies specific to one of the antigenic determinants of BVDV virus. 100 µL of the diluted serum samples were added to each well. Then, 100 µL of diluted negative and positive controls were added into the appropriate wells and incubated for one hour at 21 ±3°C and washed (by wash solution three times). Following the final washing, the plate was slapped vigorously down on a bench top covered with clean absorbent paper. After washing, 100 µL of the diluted conjugate solution was added to each well and incubated for one hour at 21 ±3°C,and washed again then the 100 µL of chromogen (tetramethylbenzidine) was added to each well and the plates were incubated for 10 min at 21 ±3°C protected from the light and uncovered. The reaction was terminated by addition of 50 µL of stop solution to each well. Finally, the absorbance was monitored in ELISA reader at 450 nm .

Molecular

Detection of BVDV genome in the samples given negative result by ELISA, using conventional RT- PCR

In (Animal health research institute & Agriculture research center, Giza, Egypt)

RNA extraction

RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA). Cat. No.52904. The procedure was performed according to the company's instruction.

1- 560 µl of the prepared Buffer AVL containing carrier RNA were pipeted into a 1.5 ml microcentrifuge tube.

2- 140 µl of sample was added to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mixed by pulse-vortexing for 15 sec.

3- Viral particle lysis is complete after lysis for 10 min at room temperature (15–25°C).

4- Briefly the tube centrifuged to remove drops from the inside of the lid.

5- 560 µl of ethanol (96%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, briefly the tube centrifuged to remove drops from inside the lid. Only ethanol was used since other alcohols may result in reduced RNA yield and purity. In order to ensure efficient binding, the sample was mixed thoroughly with the ethanol to yield a homogeneous solution.

6- Carefully 630 µl of the solution from step 5 was applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. And centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp spin column then placed into a clean 2 ml collection tube, and discards the tube containing the filtrate. Each spin column was closed in order to avoid cross-contamination during centrifugation.

7- QIAamp Mini spin column opened, and step 6 was repeated.

8- QIAamp Mini spin column was opened, and 500 µl of Buffer AW1was added. Then samples were centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

9- QIAamp Mini spin column was opened, and 500 µl of Buffer AW2 was added, followed by centrifugation at full speed (20,000 xg; 14,000 rpm) for 3 min.

10- QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube. The collection tube containing the filtrate was discarded. Carefully the QIAamp spin column was opened and 60 µl of buffer AVE equilibrated to room temperature was added. Tubes were incubated at room

temperature for 1 min. Centrifuged at 6000 x g (8000 rpm) for 1 min. A single elution with 60 µl of buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini spin column. RNA elutes were preserved at -70°C.

Total RNA from bovine serum samples was isolated by using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The extracted RNA was eluted in 50 µl in DEPC-treated H2O and stored at -70°C.

Conventional RT-PCR assay

Conventional RT-PCR was performed using Polymerase One-Step RT-PCR System (Roche diagnostic) according to the manufacturer's instructions.Oligonucleotide sequence of primer according to (Vilcek *et al.*, 2001)

5-´ATGCCCTTAGTAGGACTAGCA-3´ and 5´-TCAACTCCATGTGCCATGTAC-3´) used for RT-PCR that gives a band at 288bp.

PCR Reaction Mix Volumes for one reaction (Qiagen, Valencia, Calif., USA)

Reaction Volume (uL) per Reaction Final Concentration

H2O 5 Ul
buffer 12.5 ul
Enzyme mix .5 ul
Primer (F) 1 ul 20 pmol/µl
Primer (R) 1 ul 20 pmol/µl
RNA product 5 ul

PCR Thermo cycling Conditions:

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. After the RT, PCR consisted of 35cycles 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

Analysis of the PCR products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) and using 100 bp DNA ladder (Fermentas, Thermo) to determine the fragment sizes. The gel was photographed by a gel decumentation system (Alpha Innotech, Biometra (Vilcek *et al.*, 2001)

RESULTS

BVDV antibody detection

Table 1 shows that 27 samples (23.7%) were positive for BVDV antibodies from total samples 114. In contrast the negative samples were 87(76.3%).

Table 1: Detection of BVDV antibodies in bulls sera in Aswan using ELISA during 2018.

Total no.	negative		positive		degree	of	positi	vety
	No.	%	No.	%	+	2+	3+	4+
114	87	76.3	27	23.7	0	12	3	12

BVDV gene detection

The results showed that 3 samples (3.4%) were positive.

BVDV gene was detected and 84 (96.6%) negative from total number 87 samples. The details are presented in Table 2.

Table 2: Detection of BVDV gene in bulls in Aswan using conventional RT-PCR (2018)

Total	positive		Negative	
	No.	**%**	No.	%
87	**3**	**3.4**	84	96.6

Fig.1)

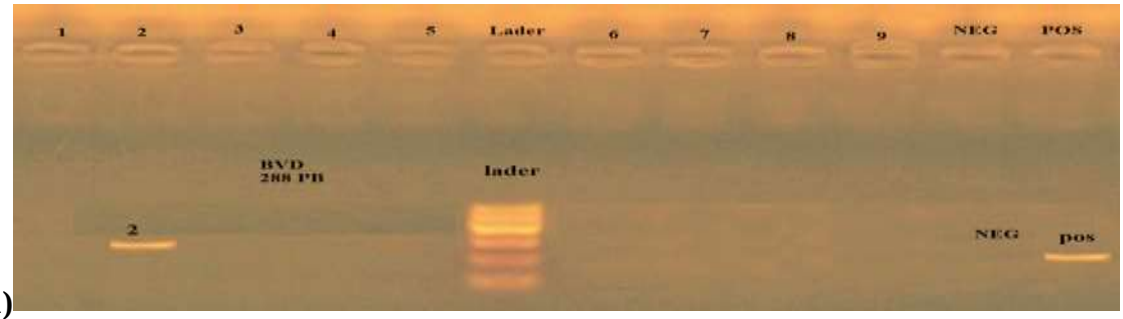

Figure (1)
Agarose gel electrophoresis 1.5% showing the PCR products of BVD virus. Lane ladder is 100bp DNA marker.
Lane (pos.): is control positive,
Lane (neg.): is control negative,
Lanes from1 to 10 are negative samples except lane (2) is positive giving band at 288bp

Fig.2)

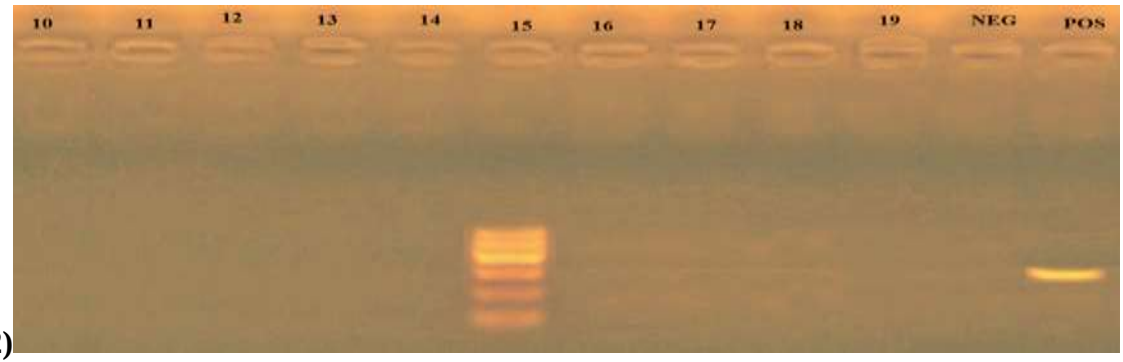

Fig.3)

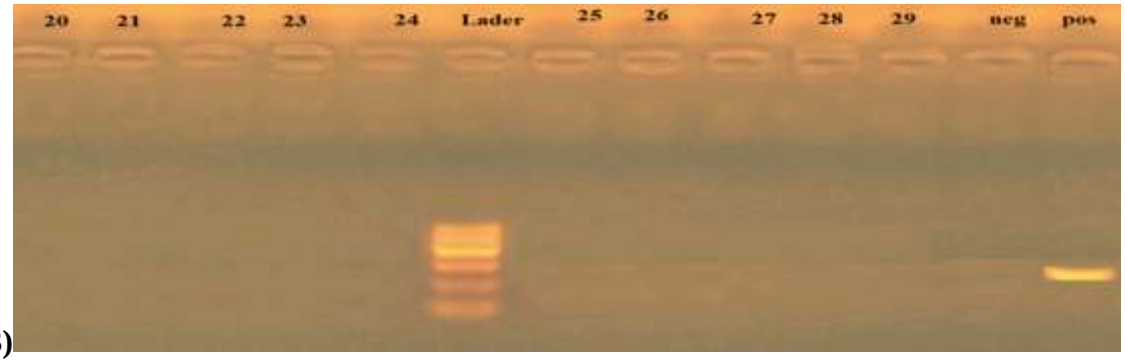

Figure (2) and (3)
Agarose gel electrophoresis 1.5% showing the PCR products of BVD virus. Lane ladder is 100bp DNA marker.
Lane (pos.): is control positive,
Lane (neg.): is control negative,
Lanes from11 to 29 are negative samples

Statistical analysis

The Statistical analysis in this study was done by using the golden-standard test

test	Diseased confirmed by PCR	No diseased confirmed by PCR	total
ELISA +ve	True +ve 24	False +ve 3	27
ELISA -ve	False –ve 3	True –ve 84	87
total	27	87	114

Sensitivity of ELISA is 24/27 = 89%
Specificity of ELISA is 84/87= 96%
Positive predictive value of ELISA is 24/27 = 89%
Negative predictive value of ELISA is 84/87 = 96

DISCUSSION

BVDV occurs worldwide and causes significant economic losses to livestock, mainly due to its impact on reproduction.

In this study, PI- BVDV has been detected in 3 (3.4%) out of 87 samples (serologically negative for BVD antibodies) collected from El shalall slaughter house, Aswan. In a previous study in aborted fetuses (bovine, ovine, caprine, buffalo and camel), (15.96%) and (18.49%) were positive for BVDV by ELISA and PCR. These results indicate that PCR is significantly faster and more accurate than ELISA for the identification of BVDV (Farhad Safar poor Dehkordi, 2011). With the agreement of our results.

In Turkey, 46 bovine serum and leukocyte samples were studied for the detection of BVDV antigens and anti-BVDV antibodies, (17.3%) 8 of the 46 bulls were detected seropositive, while 38 were seronegative. (6.5%) 3 of 46 bulls were detected for BVDV antigen positive, while 43 of 46 bulls were negative. Two of the 3 bulls detected for antigen positive had no antibodies to BVDV and 1 of 3 bulls detected as positive for antibodies and positive for antibodies (Sibel Yavru *et al.*, 2013), 2 (4.3%) positive antigen from 46 samples is relatively similar to our results which is 3 (3.4%) positive antigen from 87 samples.

Tested by ELISA for antigen capture, BVDV antigen was detected in 16 cattle from 400 serum samples tested (4%) and 8 of 12 farms (66.6%) were infected with BVD virus, indicating that BVDV infection is widely present of dairy herds in Fars province and suggested that cows with persistent infection may be a source of BVDV infection in dairy cattle in the area (GH-Farjani Kish *et al.,* 2013), this positive percentage is relatively similar to our results.

In agreement with our results, many authors reported the presence of antibodies to BVDV in the blood serum of unvaccinated camels without signs of severe symptoms or lesions (Alfaleq *et al.,* 2007; Raoofi *et al.,* 2010)

PCR results detected BVDV in the serum of one clinically normal camel. This can be explained by information on permanently infected calves, which may show signs of chronic diseases; however, they may also appear clinically normal (Grooms *et al.,* 2002 and Jehan A. Gafer *et al.,* 2015), but similar evaluations have not been well described for PI alpaca because most were diagnosed immediately before death due to their normal appearance (Carman *et al.,* 2005 Mattson *et al.,* 2006; Foster *et al.,* 2007 and Byers *et al.,* 2009).

In PI calves, PCR is a reliable diagnostic method at any age and PCR has been

shown to be able to detect BVDV even in the presence of maternal antibodies to BVD, which affect ELISA results (Goyal, 2005; Sandvik, 2005), agreeing with our results.

Of the 236 goat serum collected from six areas in Jiangsu Province, China, BVDV-1 was identified in 29 (12.29%) samples by RT-PCR. BVDV-1 infections occurred with / without clinical signs (Li Mao *et al.,* 2016), this result higher than our result may be due to a difference in the species and test used and the study area.

At initial screening, 16 out of 400 (4%) blood samples were successfully taken from 12 herds of industrial dairy products with a previous history of diarrhea, abortion, or parturition and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) on a buffy coat. In the next step, 8 out of 100 samples (8%) of positive blood samples were taken from infected farms three weeks later, which were subsequently tested by ELISA and RT-PCR. these findings emphasize the importance of monitoring BVDV infection in cattle and suggest the detection and elimination of PI animals for the control and eradication of BVDV (Khodakaram Tafti *et al.,* 2016).

A total of 494 ear notch were taken from cattle in five different areas of Nineveh Province, Iraq and tested by multiplex PCR analysis. The overall prevalence of BVDV genotypes was 13.96% (69 of 494) (Sadam Hasan and Kamal Alsaad, 2018), this result is higher than our result due to the difference in sample and test type.

The agreement between serum ELISAs and PCR in BVD detection was reported to be 100% (Hill *et al.,* 2007).

100% sensitivity and 99.6% specificity were reported using a commercially available ELISA kit for samples obtained from PI animals (Kennedy *et al.,* 2006, Edmondson *et al.,* 2007), our result showed a sensitivity of 89% and a specificity of 96%.

Animal trafficking is one of the main risk factors for the entry of BVDV infection (Houe 1999) and can also be a source in these cases.

Control programs based solely on testing and killing of PI animals were launched in the Nordic countries of Europe several years ago (Houe *et al.,* 2006 and Aduriz *et al.,* 2015).

Regarding diagnostic tests, two tests using antigen ELISA and PCR showed perfect agreement, so both appear to be adequate for the detection of BVDV infections in blood samples (Aduriz *et al.,* 2015).

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التعرف على الثيران دائمة الاصابه بفيروس الاسهال الفيروسى البقرى فى محافظة اسوان ، مصر

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صممت هذه الدراسة للكشف عن استمرار الإصابة بفيروس الإسهال الفيروسي البقري (PI- BVDV) بين ١١٤ ثورًا في محافظة أسوان. تم فحص عينات الأمصال بواسطة ELISA وتفاعل البلمرة المتسلسل في الوقت الحقيقي (PCR) للكشف عن الثيران السلبية مصليًا للأجسام المضادة لـ BVDV ومولدات المضاد (antigen) BVDV، على التوالي. كانت نسبة الإصابة ٢٣,٧٪ (١١٤/٢٧) و ٧٦,٣٪ (١١٤/٨٧) كانت سلبية بتقنية ELISA ، و ٣,٤٪ (٨٧/٣) للكشف عن الجينات BVDVعن طريق PCR في الوقت الحقيقي. تم الإبلاغ عن حساسية ٨٩ ٪ وخصوصية ٩٦٪ باستخدام مجموعة ELISAالمتاحة تجارياً للعينات التي تم الحصول عليها من حيوانات.PI