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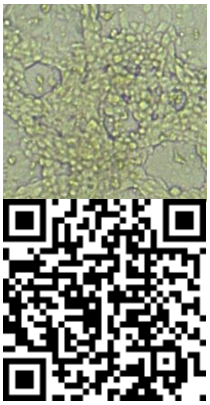

Development of a multiplex real-time PCR for the detection of BoHV-1 and BVDV in cryopreserved bovine semen

Desarrollo de una PCR en tiempo real multiplex para la detección de BoHV-1 y BVDV en semen bovino criopreservado

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ABSTRACT

In Mexico, artificial insemination constitutes one of the main reproductive biotechnologies applied in cattle farming, aimed at increasing herd productivity and genetic improvement. However, a large proportion of the semen used in this technique is processed in the field without strict sanitary control, which increases the risk of spreading infectious agents such as bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BoHV-1). To strengthen the detection of these pathogens, a multiplex real-time PCR assay with TaqMan probes was developed to simultaneously identify both viruses in cryopreserved bovine semen. Positive controls consisted of reference viruses replicated in MDBK (Madin Darby Bovine Kidney) cells, and negative controls included supernatants of non-infected cells and semen free of both viruses. Primers and probes were designed from sequences deposited in GenBank: the *E2* gene for BVDV and the *gB* glycoprotein for BoHV-1; additionally, the bovine *β-actin* gene of *Bos taurus* was used as a constitutive control. The primers showed amplification in endpoint assays and satisfactory results in real-time assays with positive controls. These findings establish a multiplex detection assay that will contribute to biosafety in artificial insemination programs, reducing the risk of BVDV and BoHV-1 transmission through semen.
Keywords: Artificial insemination, TaqMan, biosafety.

RESUMEN

En México, la inseminación artificial es una de las principales biotecnologías reproductivas en la ganadería bovina, empleada para incrementar la productividad y el mejoramiento genético del hato. No obstante, gran parte del semen utilizado se procesa a nivel de campo sin un control sanitario estricto, lo que incrementa el riesgo de diseminación de agentes infecciosos como el virus de la diarrea viral bovina (BVDV) y el herpesvirus bovino tipo 1 (BoHV-1). Para fortalecer la detección de estos patógenos, se

desarrolló una PCR en tiempo real multiplex con sondas TaqMan, capaz de identificar simultáneamente ambos virus en semen bovino criopreservado. Se emplearon controles positivos consistentes en virus de referencia replicados en células MDBK y, como controles negativos, sobrenadantes de células no infectadas y semen libre de los virus. Los cebadores y sondas se diseñaron a partir de secuencias depositadas en GenBank: el gen *E2* para BVDV y la glicoproteína gB para BoHV-1; adicionalmente, se utilizó el gen de *β-actina* de *Bos taurus* como constitutivo. Los ensayos demostraron amplificación específica en pruebas de punto final y resultados satisfactorios en tiempo real. Los hallazgos obtenidos permiten establecer una prueba diagnóstica multiplex que contribuirá a la bioseguridad en programas de inseminación artificial, minimizando el riesgo de transmisión de BVDV y BoHV-1 a través del semen.
Palabras clave: Inseminación artificial, TaqMan, bioseguridad.

INTRODUCTION

Bovine livestock health represents a global challenge, and its deficiency directly impacts economic losses for both producers and countries. In Mexico, the national agricultural survey indicates that most production units use artificial insemination with the purpose of improving herd genetics and reducing the transmission of reproductive diseases. However, not all commercialized semen comes from semen processing centers (CEPROSEM); much of it is processed at the field level, making it difficult to know its sanitary status with certainty and increasing the risk of spreading infectious agents that reduce productivity. Among these pathogens, the bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BoHV-1) stand out, both globally distributed, except in some European countries that have managed to eradicate IBR (Iscaro *et al.*, 2021; Rimayanti *et al.*, 2024; Bettini *et al.*, 2023). These infections cause considerable economic losses due to reduced fertility, abortions, neonatal death, and restrictions in the international trade of semen and embryos.

BVDV is an enveloped virus of the genus *Pestivirus* (family *Flaviviridae*), with a positive-sense single-stranded RNA genome (~15.5 Kb). The genome is packed in a protein capsid and surrounded by a phospholipid membrane that carries three glycoproteins (Erns, E1, and E2), key in viral entry and host immune response (Liu *et al.*, 2022). It presents two main genotypes (BVDV-1, low mortality; and BVDV-2, high mortality) and two biotypes (cytopathic, cp; and non-cytopathic, ncp) that differ in their effects on host cells (Al-Kubati *et al.*, 2021; Colitti *et al.*, 2019). Transmission occurs horizontally through respiratory secretions, saliva, semen, uterine secretions, placental fluids, feces, urine, and milk; or vertically, through embryo transfer or infection of pregnant females with the ncp biotype before 125 days of gestation, generating persistently infected (PI) animals, the main reservoirs of the virus (Larghi, 2018; Jia *et al.*, 2020). Infections may be subclinical or moderate and are associated with immunosuppression, respiratory, digestive, and reproductive problems. Highly virulent strains cause thrombocytopenia and ulcerative lesions in mucous membranes, while mucosal disease, present in PI animals, is characterized by ulcers in the nasal cavity, mouth, and digestive tract, watery diarrhea,

nasal discharge, salivation, and hoof lesions that cause lameness accompanied by fever (Bachofen *et al.*, 2010).

BoHV-1, the causative agent of infectious bovine rhinotracheitis (IBR), belongs to the family Herpesviridae, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. It has a linear double-stranded DNA genome and an icosahedral capsid surrounded by a lipid envelope that contains approximately 12 glycoproteins, including gB, gC, gD, and gE, which participate in viral entry, spread, and immune evasion. Subtypes BoHV-1.1 (mainly respiratory) and BoHV-1.2a/1.2b (mainly genital) are recognized, associated with different cellular tropisms and clinical manifestations (d'Offay *et al.*, 2016). Transmission can be horizontal (nasal, oropharyngeal, genital, and ocular secretions) or vertical (intrauterine infection or during birth). In males, the virus can be located in preputial and penile mucosa, even in semen donors testing negative by diagnostic tests, reflecting the possibility of false negatives. BoHV-1 can establish latent infection in sensory neurons of trigeminal and sacral ganglia, reactivating under stress or immunosuppression, generating recurrent infections, and contributing to the persistence of the virus in the herd (Chase *et al.*, 2017; Chothe *et al.*, 2018). The respiratory form of the disease is manifested by fever, cough, serous to purulent nasal discharge, and conjunctivitis, while the genital form causes vulvovaginitis, balanoposthitis, infertility, abortions, and stillbirths. Fetal infection can occur through the placenta or during birth, with a risk of neonatal mortality. Subclinical animals also represent a silent reservoir that favors the dissemination of the virus (Nettleton & Rusell, 2017).

Among the diagnostic methods available, viral isolation is considered the reference standard, although it requires confluent cell monolayers, a sufficient number of viral particles, and strict laboratory conditions, which makes it laborious. For BVDV and BoHV-1, recommended samples include nasal or ocular swabs, fresh blood, serum, plasma, semen, fetal organs, and lymphoid tissues rich in cells (Peyer's patches, ileum, spleen, thymus, lung, and liver); in the case of BoHV-1, fetal tissues and placenta are preferably used (Wellenberg *et al.*, 2001; Lin *et al.*, 2021). The detection of viral antigens allows the identification of conserved proteins on the virus surface more quickly and economically than viral isolation. In direct ELISA, the viral antigen is fixed to the plate and detected by an enzyme-labeled antibody; in indirect ELISA, antibodies present in the sample are detected, which bind to the antigen and are recognized by a secondary labeled antibody (Wang & Pang, 2024; Gutiérrez-Hernández *et al.*, 2021; Abad-Zavaleta *et al.*, 2016). Likewise, immunohistochemistry allows the localization of viral antigen directly in tissues, providing information about the distribution and replication of the virus.

Currently, molecular techniques based on PCR have shown greater sensitivity and specificity. For RNA viruses such as BVDV, the use of RT-PCR allows retrotranscribing the viral genome into cDNA and detecting genetic material with high precision, while real-time PCR facilitates quantification and rapid interpretation of results, applicable to various samples such as blood, serum, nasal and genital secretions, as well as semen (Lin *et al.*,

2021). In this context, it is necessary to develop molecular tests capable of simultaneously detecting BVDV and BoHV-1 in cryopreserved bovine semen. Although molecular protocols exist for the individual detection of these viruses, reports of multiplex assays specifically applied to semen in Mexico are scarce. This work proposes the development of a multiplex real-time PCR in order to strengthen biosecurity measures in artificial insemination programs and reduce the risk of transmission of these pathogens.

MATERIAL Y METHODS

Collection of biological samples

Thirty ejaculates and blood samples were collected from active bulls in the state of Jalisco, Mexico. The ejaculates were deposited in French straws of 0.25 mL at a concentration of 20×10^6 spermatozoa/mL and processed for cryopreservation with a non-penetrating lipoprotein-based diluent (soy lecithin). Subsequently, the straws were stored in liquid nitrogen at the National Center for Genetic Resources (CNRG), Tepatitlán de Morelos, Jalisco, Mexico. The blood samples were kept at 25 °C and sent to an external laboratory for complementary analyses.

Primer and probe design

The GenBank database was consulted to select specific molecular targets for each viral agent. For bovine viral diarrhea virus (BVDV), the *E2* gene was chosen, and for bovine herpesvirus type 1 (BoHV-1), the glycoprotein B (*gB*) gene. As an internal control, the *β-actin* gene of *Bos taurus* was selected. The sequences were evaluated in BioEdit to rule out unwanted homology and verified through phylogenetic analyses in MEGA7. Primer design was performed in Fast PCR, considering a maximum of 25 bases per oligo, fragments ≤150 bp, and annealing temperatures (Tm) in the range of 65 ± 5 °C. TaqMan probes were designed with ≤30 bases and different fluorophores (ROX and FAM), since the real-time PCR equipment does not allow simultaneous detection of two probes with the same fluorophore. Primers and probes were evaluated in OligoEvaluator™ to determine Tm, GC content, possible dimers, and secondary structures. The selected oligonucleotides were synthesized at T4Oligo (Irapuato, Mexico).

MDBK cell culture and infection with reference strains

The MDBK ATCC CCL-22 cell line, derived from calf kidney and with 130 passages, was used. The culture was established in minimum essential medium (MEM, Gibco® 1X) supplemented with 2.5% fetal bovine serum (FBS), in 25 cm^2 flasks (Corning®). Incubation was maintained at 37 °C with 5% CO_2. Subcultures were carried out every 2 days upon reaching ≥80% confluence, using trypsin (Gibco® 1:250).

MDBK cells were infected with reference strains of BVDV (ATCC-1561, noncytopathic) and BoHV-1 (ATCC-2181, cytopathic) to obtain positive controls. Infection with BVDV

was performed in 6-well plates, adding 20 µL of virus to confluent cultures in MEM and maintaining incubation for 48 h, with microscopic observation every 24 h. For BoHV-1, 20 µL of virus homogenized in MEM without FBS were inoculated in culture flasks with confluent cultures, incubating under the same conditions until cytopathic effect appeared.

Genetic material extraction

DNA extraction from cryopreserved semen was performed using the phenol-chloroform-isoamyl alcohol method (Green & Sambrook, 2012). Briefly, 1.0 mL of cryopreserved semen was thawed and centrifuged at 4,000 rpm for 10 min. The pellet was washed twice with PBS, resuspended in 200 µL of PBS, and 1.0 mL of lysis buffer was added and incubated under agitation. Subsequently, phenol:chloroform:isoamyl alcohol (25:24:1) was added, followed by purification with chloroform. The aqueous phase was precipitated with isopropanol and sodium acetate, incubated at -20 °C, and centrifuged at 4 °C. The pellet was washed with 70% ethanol, air-dried, and resuspended in 50 µL of 1X TE buffer, stored at -20 °C. For cell cultures, the medium was removed and the cells were detached with trypsin. After centrifugation, the pellet was homogenized in 750 µL of ZR Bashing Bead Buffer and processed with the Quick-DNA™ fecal/soil microbe miniprep kit (Zymo Research, USA) according to the manufacturer's instructions.

Total RNA was obtained with TRIzol® Reagent (Invitrogen, USA). For semen samples, 500 µL of semen diluted in cryoprotectant with soy lecithin were used, to which 1 mL of TRIzol® was added, following the manufacturer's protocol. For cell cultures (infected and non-infected, with >80% confluence), the content of one culture well was used, treated with 1 mL of TRIzol®. In both cases, the extracted RNA was stored at -70 °C. cDNA synthesis was carried out by reverse transcription with the SuperScript First-Strand kit (Invitrogen, USA) in a final volume of 21 µL, using 50 ng of RNA and the recommended concentrations of dNTPs, random hexamers, $MgCl_2$, DTT, RNaseOUT, SuperScript II RT, and E. coli RNaseH. Incubation conditions were applied according to the manufacturer's instructions.

The quality and integrity of DNA and RNA were verified by spectrophotometry (Nanodrop 2000, Thermo Fisher, USA), agarose gel electrophoresis, and amplification of bovine *β-actin* by endpoint PCR.

PCR standardization

With endpoint PCR, the detection of BVDV was performed by amplification of the *E2* gene, using 25 µL reaction mixtures that included 1X buffer, dNTPs (0.16 mM), $MgCl_2$ gradient (1.0-3.0 mM), specific primers (0.8 µM each), Taq polymerase (1.5 U), and control DNA (100 ng). As controls, cDNA from infected MDBK cells (positive) and DNA from healthy MDBK cells (negative) were used. The amplification conditions were: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 35 s, 58 °C for 45 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. For the detection of BoHV-

1, the *gB* gene was amplified under similar conditions, varying the $MgCl_2$ gradient (1.0-3.0 mM) and the annealing temperature (62-66 °C). The products were separated by electrophoresis in 2% agarose gel stained with SYBR Safe, using a 50 bp marker and 1X TAE buffer, and visualized in a photodocumenter. The amplicons corresponding to the expected fragments were sent for sequencing to Macrogen.

The standardization of simplex qPCR for BVDV detection evaluated two reaction conditions. In Option A, the mixture (20 µL) included TaqMan Fast Advance Master Mix 1X, 100 nM of each *E2* gene primer, 100 nM of *E2* gene probe, and 300 ng of DNA. The amplification protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 45 cycles (94 °C for 1 min and 67 °C for 30 s). In Option B, the mixture (20 µL) contained TaqMan Fast Advance Master Mix 1X, 300 nM of each *E2* gene primer, 180 nM of *E2* probe, and 100 ng of DNA. The cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 40 cycles (94 °C for 1 min, 64 °C for 45 s, and 72 °C for 30 s). For BoHV-1, the reaction (20 µL) included TaqMan Fast Advance Master Mix 1X, 300 nM of each *gB* gene primer, 180 nM of *gB* probe, and 100 ng of DNA. The amplification conditions were the same as Option B for BVDV. Positive and negative controls in all simplex assays were DNA from infected and healthy MDBK cells, respectively, and water for the no-template control (NTC).

Duplex qPCR assays were standardized for the simultaneous detection of each virus and an endogenous internal control, the bovine *β-actin* gene. For BVDV, the *E2* and *β-actin* genes were co-amplified. Reactions were carried out in triplicate in a volume of 20 µL, evaluating a gradient in the concentrations of oligonucleotides and probes. The following concentrations were tested: *E2* primers (300-900 nM), *E2* probe (180-600 nM), *β-actin* primers (360-900 nM), and *β-actin* probe (50-180 nM). For BoHV-1, the *gB* and *β-actin* genes were co-amplified. The reaction mixture (20 µL) was also optimized with concentration gradients: *gB* primers (300-900 nM), *gB* probe (300-500 nM), *β-actin* primers (100-300 nM), and *β-actin* probe (50-180 nM). In all duplex assays, positive controls (DNA gradients from infected cells, 100, 50, and 10 ng), negative controls (equivalent gradients of DNA from healthy cells), and water for the NTC were used. The cycling conditions for both duplex assays were identical: initial denaturation at 94 °C for 5 min, followed by 40 cycles (94 °C for 1 min, 64 °C for 45 s, and 72 °C for 30 s).

Blood analysis

The blood samples collected along with the ejaculates were analyzed by indirect ELISA for the detection of BVDV and BoHV-1. These analyses were conducted at LIVE Veterinary Immunology Laboratory SA de CV (Jalisco, Mexico) as a complementary validation method.

RESULTS

Cell cultures of the MDBK ATCC CCL-22 line were successfully established from the initial vial, generating eight flasks with the parameters defined in the methodology. The cells reached over 80% confluence within 24 h (Figure 1a). The replication of BVDV in confluent MDBK cells was carried out using the non-cytopathic strain ATCC-1561, with no morphological changes associated with infection observed in any of the cultures (Figure 1b). In contrast, infection with the BoHV-1 strain ATCC-2181 showed cytopathic effect, with morphological alterations detectable 42 h after inoculation (Figure 1c).

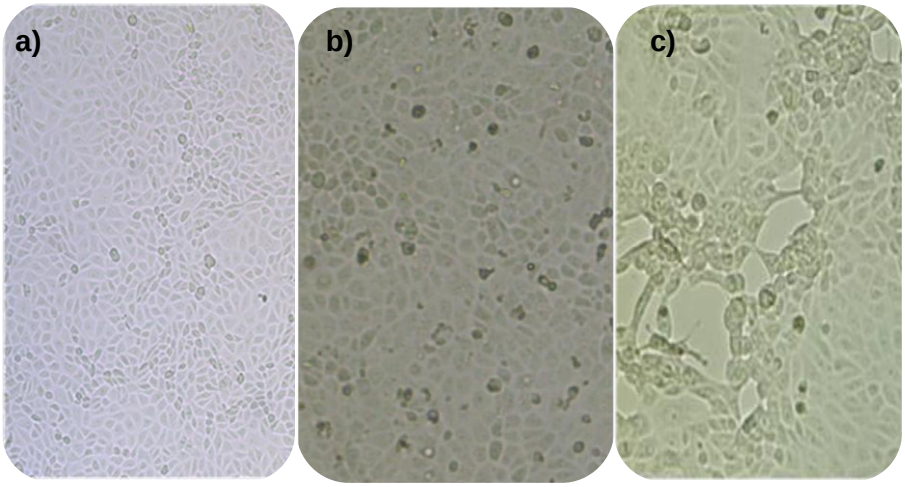

Figure 1. MDBK ATCC CCL-22 cell cultures observed under a Leica DFC295 inverted microscope with Leica S 40/0.45 camera. a) Culture of healthy cells with confluence >80%, 24 h after the start of incubation. b) Culture of cells with confluence >80%, infected with BVDV ATCC-1561 NCP, 24 h after infection. c) Culture of cells with confluence >80%, 24 h after infection with the BoHV-1 ATCC-2181 strain

Primer design and endpoint PCR

The designed primer and probe sequences (Table 1) showed homology greater than 90% with reference sequences in the GenBank database, confirmed by BLAST. Phylogenetic analysis corroborated their belonging to the same genotype and to the bovine species (*Bos taurus*), with no relation to other species.

Table 1. Primer and probe sequences designed for the detection of BVDV and BoHV

Pathogen	Primer	Sequences (5' → 3')	# of bases	
Endpoint PCR				
VDVB	E2_Fw	AGCCAAGGGACCGGTACTTC	20	
	E2_Rv	CACCCAACAAGGCGACCACT	20	
BoHV	gB_Fw	CACGTGAACACCATGTTCAGC	21	
	gB_Rv	GCATCGAGTTCTCGATGAACAC	22	
β-actina bovina	Actb_Fw	ACTCGTACGTGGGGGATGAG	22	
	Actb_Rv	TCCATGTCGTCCCAGTTGGTG	21	
Real-time PCR	**Probe**	**Sequences (5' → 3')**	**Rep. 5'**	**Ext. 3'**
VDVB	E2_P	GGGGGTGGCAATATTGGTTCGACCTG	ROX	
BoHV	gB_P	TACGACTCGTTCGCGCTCTCGACC	ROX	TAMRA
β-actina bovina	Actb_P	GGGGGTGGCAATATTGGTTCGACCTG	FAM	

The functionality of the primers was validated by endpoint PCR, achieving the amplification of the expected 130 bp fragment of the *E2* gene in DNA from BVDV-infected cells, and of the 110 bp fragment of the *gB* gene under annealing temperature conditions of 62 °C and $MgCl_2$ concentration gradient (Figure 2).

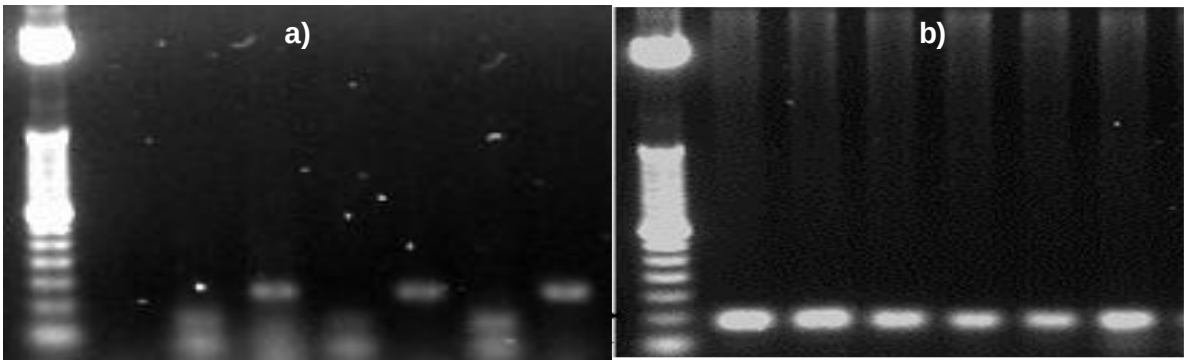

Figure 2. Amplification by PCR in 2% agarose gel with 50 bp molecular weight marker. a) *E2* gene, lane 1: NTC control; lanes 2, 4, 6: MDBK cells infected with 1 µL of the strain; lanes 3, 5, 7: MDBK cells infected with 20 µL of the strain. b) *gB* gene, lanes 1-5: Tm of 62 °C and $MgCl_2$ at 1.0, 1.5, 2.0, 2.5, and 3.0 mM, respectively; lane 6: Tm of 64 °C and 1.0 mM $MgCl_2$

Real-time PCR assay

In the real-time PCR assays for BVDV, the optimal parameters were obtained with 500 ng of *E2* primers, 360 ng of *E2* probe, together with 500 ng of *β-actin* primers and 180 ng of its corresponding probe, using 200 ng of DNA. In the positive controls, typical

amplification curves of the E2 and *β-actin* genes were observed, with Ct values >25 for *β-actin* and >30 for *E2* (Figure 3a). In the negative controls, only *β-actin* was amplified, whereas in the NTC controls no amplification was detected (Figure 3b).

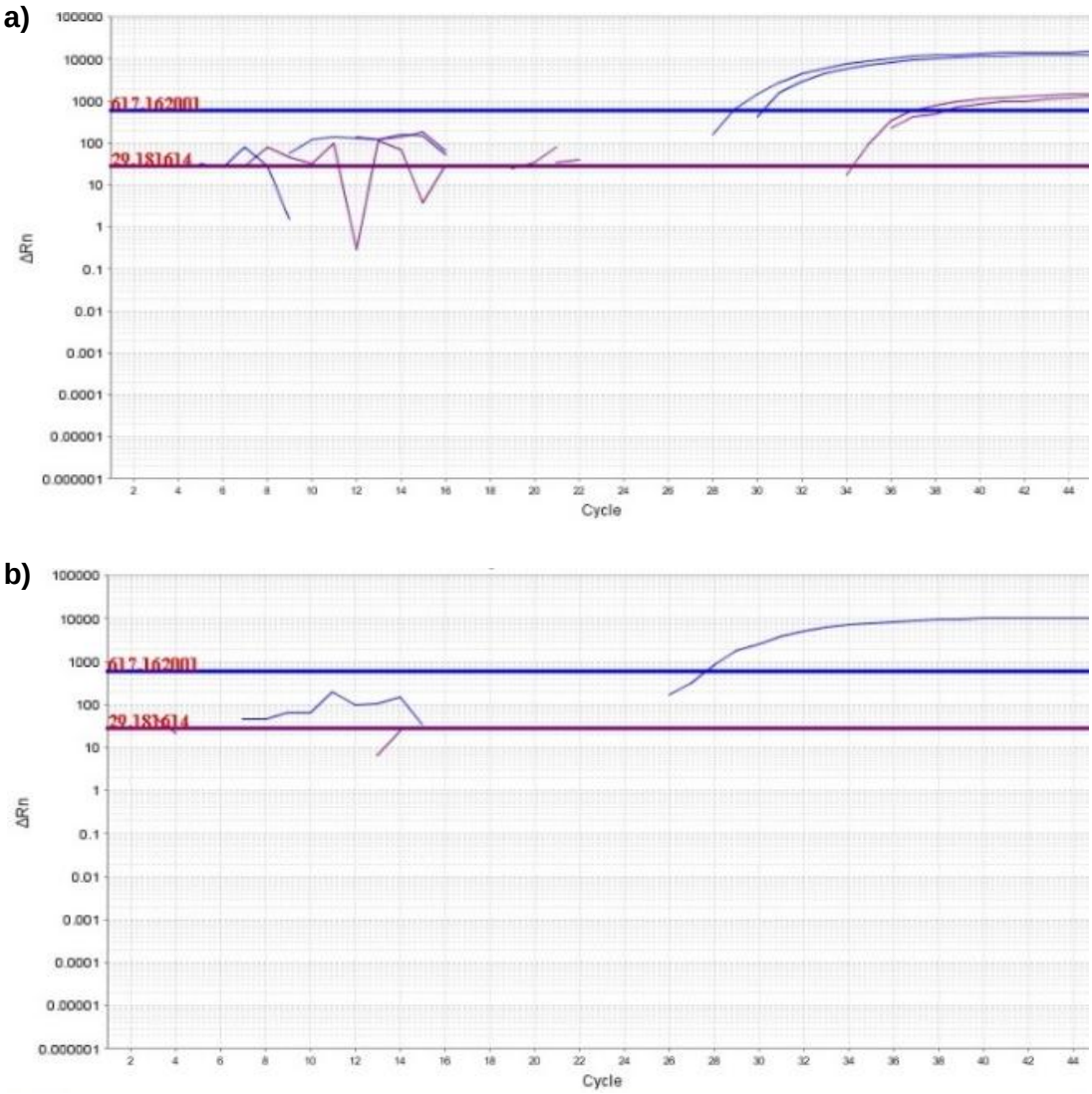

Figure 3. Amplification plots for the detection of BVDV. a) *E2* (purple) and *β-actin* (blue) genes in positive controls. b) *β-actin* (blue) in negative control

The analysis of DNA from cryopreserved bovine semen showed amplification of the *β-actin* gene in five samples, with Ct values >25, while the *E2* gene was not detected in any sample, with amplification observed only in the positive control (Figure 4, Table 2).

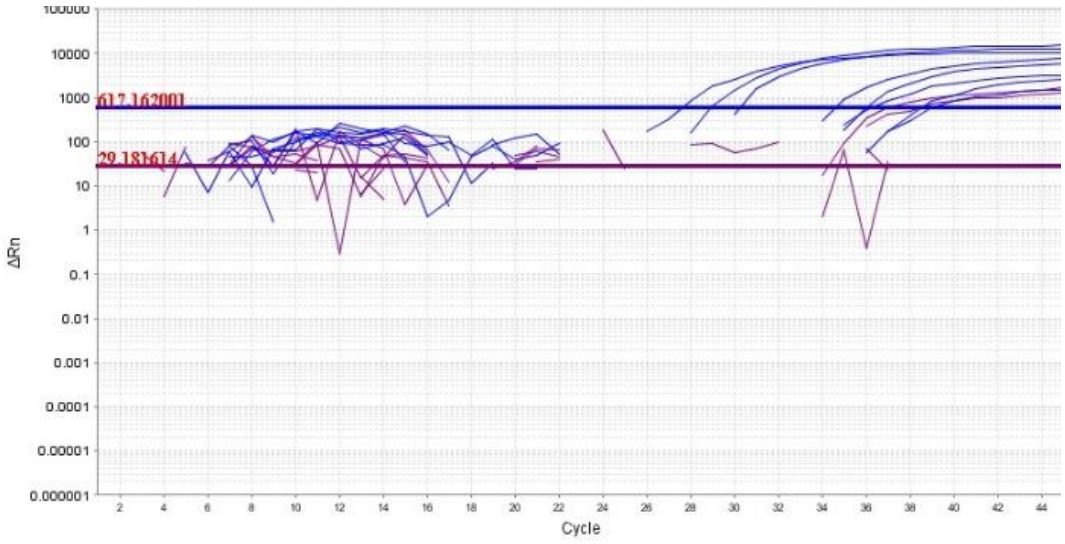

Figure 4. Amplification plots for the detection of BVDV in bovine semen. Amplification of the *E2* (purple) and *β-actin* (blue) genes

Table 21. Ct values (real-time PCR) for each amplified gene in bovine semen samples for BVDV detection and ELISA diagnostic results

Animal ID	DNA No.	Ct *β-actin*	Ct *E2*	ELISA Dx
2753	1	35.8	Not amplified	Negative
8215	2	34.6	Not amplified	Positive
9106	3	36.0	Not amplified	Negative
1622	4	35.9	Not amplified	Negative
x̄		35.58	-	
s		0.66	-	
C.V		1.84%	-	
CRBIVDVB	CP	29.0	35.2	-
CRBSI	CN	27.5	Not amplified	-
	NTC	Not amplified	Not amplified	-

CRBIVDVB: bovine kidney cells infected with BVDV; CRBSI: uninfected bovine kidney cells; x̄: mean; s: standard deviation; C.V: coefficient of variation

In the case of BoHV-1, optimal parameters were achieved with 600 ng of *gB* gene primers, 400 ng of its probe, 100 ng of *β-actin* primers, and 50 ng of its probe, using 20 ng of DNA. Positive controls showed amplification of the *gB* and *β-actin* genes with typical curves and Ct values >25 for *β-actin* and >20 for *gB* (Figure 5a). In the negative controls,

only *β-actin* was amplified, while no amplification was observed in the NTC controls (Figure 5b).

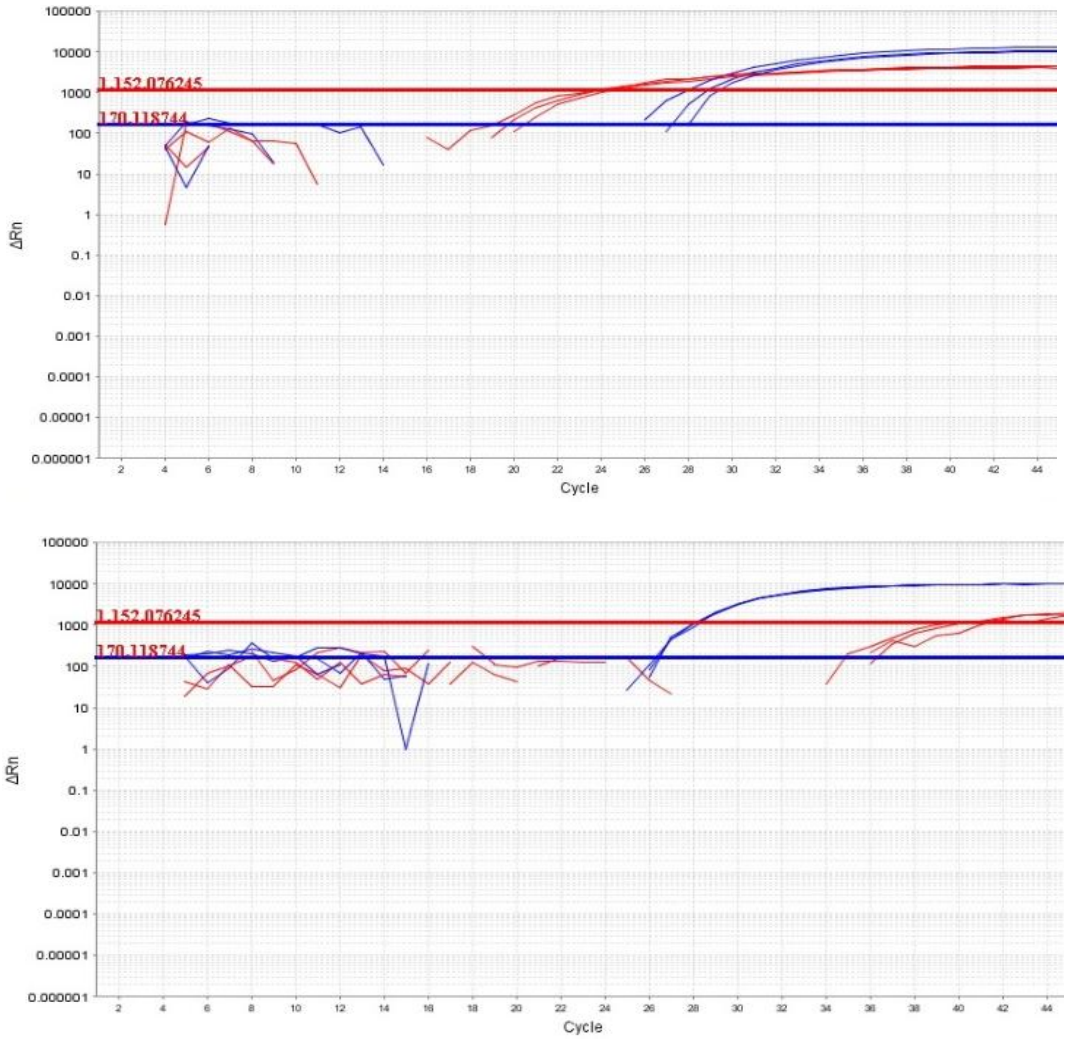

Figure 5. Amplification plots for the detection of BoHV-1. a) *gB* gene (red) and *β-actin* (blue) in positive controls. b) *β-actin* gene (blue) in the negative control

The analysis of DNA from cryopreserved bovine semen showed amplification of *β-actin* in five samples, with Ct values >25, while the *gB* gene was not detected in any sample, with amplification observed only in the positive control (Figure 6, Table 3).

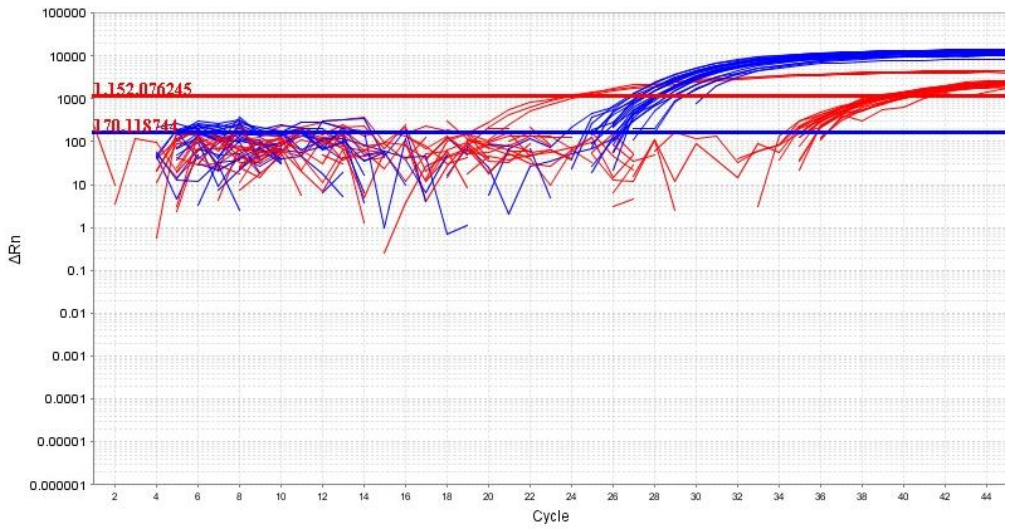

Figura 6. Amplification plots for the detection of BoHV-1 in bovine semen. Amplification of the *gB* (red) and *β-actin* (blue) genes

Table 32. **Ct values (real-time PCR) for each gene amplified in bovine semen samples for BoHV-1 detection and ELISA diagnostic results**

Animal ID	DNA No.	Ct *β-actin*	Ct *gB*	ELISA Dx
8659	1	25.1	Not amplified	Negative
8215	2	33.3	Not amplified	Positive
Censo	3	26.5	Not amplified	Negative
Censo	4	27.5	Not amplified	Negative
Censo	5	25.6	Not amplified	Negative
9106	6	26.3	Not amplified	Negative
5742	7	25.9	Not amplified	Positive
6711	8	26.6	Not amplified	Positive
1622	9	23.4	Not amplified	Positive
8659	10	25.3	Not amplified	Positive
$\bar{x}$		26.55	-	
s		2.61	-	
C.V		9.84%	-	
CCRBIBoHV		28.0	24.0	-
CCRBSI		26.1	Not amplified	-

CCRBIBoHV: bovine kidney cells infected with BoHV; CCRBSI: bovine kidney cells not infected; $\bar{x}$: mean; s: standard deviation; C.V: coefficient of variation

DISCUSSION

BVDV and BoHV-1 are viruses that cause high morbidity in cattle. Although mortality associated with BoHV-1 has been estimated between 0 and 15%, BVDV mortality can reach or exceed 25%, with notable increases in cases of mucosal disease (Grünberg, 2021). In Mexico, reported seroprevalence for BoHV-1 exceeds 50% in central and peninsular regions, while for BVDV it ranges from 30 to 76.5% across different areas of the country, with records of 100% in the south (Gutiérrez-Hernández *et al.*, 2021). These data reflect the wide distribution of both viruses and explain their impact on cattle productivity, as consequences include abortions, infertility in females and males, and general reduction in performance (Rimayanti *et al.*, 2024). Males represent one of the main disseminators, either following acute infections or in a persistently infected state in the case of BVDV. In these animals, the seminal vesicle and prostate gland are affected, compromising the sanitary quality of semen (Oguejiofor *et al.*, 2019). Therefore, it is essential to implement specific diagnostic tests in both fresh and cryopreserved bovine semen (Rimayanti *et al.*, 2024).

Various methods have been used for the detection of these pathogens, including ELISA, PCR, and recombinase polymerase amplification (RPA), applied to samples of blood, serum, nasal and anal swabs, tissues, and feces (Hou *et al.*, 2017; Jiang *et al.*, 2024). However, the commercialization of cryopreserved semen is associated with the transfer of samples that in most cases undergo only basic sanitary analyses, increasing the risk of disease dissemination. In this context, cryopreserved semen should be considered a priority sample for diagnosis. Although RPA offers rapid results (<40 min), real-time PCR remains the technique with the highest specificity (≈100%) and sensitivity (~97%) for detecting BoHV-1 in bovine semen. Indeed, PCR has been identified as the ideal method for detecting both pathogens in this type of sample (Chatterjee *et al.*, 2016; El-Mohamady *et al.*, 2020; Jiang *et al.*, 2024).

In the present study, the selected genes (*E2* for BVDV and *gB* for BoHV-1) are associated with virulence factors and have been successfully used in diagnostic assays. Additionally, the bovine *β-actin* gene was incorporated as an endogenous control to ensure that amplifications corresponded to bovine genetic material. Any sample with Ct values >25 for *β-actin*, >30 for *E2*, and >20 for *gB* was considered positive. These parameters are consistent with the study by El-Mohamady *et al.* (2020), who established Ct <40 for the detection of BVDV and BoHV-1, and with Soltan *et al.* (2015), who set Ct >30 for *E2*. In this work, amplification of all three genes was achieved in positive controls and only *β-actin* in negative controls, confirming the reliability of the system. Real-time PCR assays showed 100% negative samples for both BVDV and BoHV, in contrast to positive results obtained by ELISA, suggesting possible false positives in the latter technique. These findings indicate that the analyzed bovine semen does not constitute a source of virus dissemination under the conditions of this study, although the absence of detection

should be interpreted cautiously due to the limited number of samples. Therefore, future efforts should focus on expanding sampling and validating molecular protocols for bovine semen to strengthen biosecurity measures in artificial insemination programs and germplasm banks.

CONCLUSIONES

The dissemination of BVDV and BoHV through cryopreserved bovine semen poses a risk to cattle health and productivity. The results of this study demonstrate that the employed methodology is a useful tool for detecting these pathogens in seminal material, contributing to strengthened epidemiological surveillance and potentially supporting strategies aimed at their control and eradication in Mexico.

REFERENCES

ABAD-ZAVALETA J, Ríos-Utrera A, Rosete-Fernández JV, García-Camacho A, Zárate-Martínez JP. 2016. Prevalencia de rinotraqueítis infecciosa bovina y diarrea viral bovina en hembras en tres épocas del año en la Zona Centro de Veracruz. *No. Scien.* 8(16):213-227. https://novascientia.lasallebajio.edu.mx/ojs/index.php/novascientia/issue/view/16

AL-KUBATI AAG, Hussen J, Kandeel M, Al-Mubarak AIA, Hemida MG. 2021. Recent advances on the bovine viral diarrhea virus molecular pathogenesis, immune response, and vaccines development. *Front. Vet. Sci*. 8:e665128. https://doi.org/10.3389/fvets.2021.665128

BACHOFEN C, Braun U, Hilbe M, Ehrensperger F, Stalder H, Peterhans E. 2010. Clinical appearance and pathology of cattle persistently infected with bovine viral diarrhoea virus of different genetic subgroups. *Vet. Microbiol*. 141:258-267. https://doi.org/10.1016/j.vetmic.2009.09.022

BETTINI A, Stella M, Precazzini F, Degasperi M, Colorio S, Tavella A. 2023. Infectious bovine rhinotracheitis post-eradication program in the autonomous province of Bolzano, Italy: a retrospective study on potential bovine herpesvirus type 2 Cross-Reactivity. *Animals*. 13(22):e3502. https://doi.org/10.3390/ani13223502

CHASE CCL, Fulton RW, O'Toole D, Gillette B, Daly RF, Perry G, Clement T. 2017. Bovine herpesvirus 1 modified live virus vaccines for cattle reproduction: Balancing protection with undesired effects. *Vet*. *Microbiol*. 206:69-77. https://doi.org/10.1016/j.vetmic.2017.03.016

CHATTERJEE A, Bakshi S, Sarkar SN, Mitra J, Chowdhury S. 2016. Bovine herpes virus-1 and its infection in India-a review. *Indian J. Anim. Health*. 55(1):21-40. https://www.ijah.in/archive_article/50

CHOTHE SK, Sebastian A, Thomas A, Nissly RH, Wolfgang D, Byukusenge M, Mor SK, Goyal SM, Albert I, Tewari D, Jayarao BM, Kuchipudi SV. 2018. Whole-genome sequence analysis reveals unique SNP profiles to distinguish vaccine and wild-type strains of bovine herpesvirus-1 (BoHV-1). *Virology*. 522:27-36. https://doi.org/10.1016/j.virol.2018.06.015

COLITTI B, Nogarol C, Giacobini M, Capucchio MT, Biasato I, Rosati S, Bertolotti L. 2019. Compartmentalized evolution of bovine viral diarrhoea virus type 2 in an immunotolerant persistently infected cow. *Sci Rep*.9:e15460. https://doi.org/10.1038/s41598-019-52023-w

D'OFFAY JM, Eberle R, Fulton RW, Kirkland PD. 2016. Complete genomic sequence and comparative analysis of four genital and respiratory isolates of bovine herpesvirus subtype 1.2b (BoHV-1.2b), including the prototype virus strain K22. *Arch*. *Virol*. 161:3269-74. https://doi.org/10.1007/s00705-016-3026-1

EL-MOHAMADY RS, Behour TS, Rawash ZM. 2020. Concurrent detection of bovine viral diarrhoea virus and bovine herpesvirus-1 in bulls' semen and their effect on semen quality. *Int. J. Vet. Sci. Med*. 8(1):106-114. https://doi.org/10.1080/23144599.2020.1850197

GREEN M, and Sambrook J. 2012. Molecular Cloning: A Laboratory Manual. 4th Edition, Vol. II, Cold Spring Harbor Laboratory Press. New York. ISBN: 978-1-936113-42-2.

GRÜNBERG W. 2021. Bovine viral diarrhea and mucosal disease complex. MSD Manual Veterinary Manual, Germany. https://www.msdvetmanual.com/infectious-diseases/bovine-viral-diarrhea/bovine-viral-diarrhea-and-mucosal-disease-complex

GUTIÉRREZ-HERNÁNDEZ J, Palomares-Resendiz G, Hernández-Badillo E, Leyva-Corona J, Díaz-Aparicio E, Herrera-López E. 2020. Frecuencia de enfermedades de impacto reproductivo en bovinos de doble propósito ubicados en Oaxaca, México. *Abanico Vet*. 10:e114. https://doi.org/10.21929/abavet2020.22

HOU P, Wang H, Zhao G, He C, He H. 2017. Rapid detection of infectious bovine *Rhinotracheitis* virus using recombinase polymerase amplification assays. *BMC Vet. Res*. 13:e386. https://doi.org/10.1186/s12917-017-1284-0

ISCARO C, Cambiotti V, Petrini S, Feliziani F. 2021. Control programs for infectious bovine rhinotracheitis (IBR) in European countries: An overview. *Anim. Health Res. Rev*. 22:136-146. https://doi.org/10.1017/s1466252321000116

JIA S, Huang X, Li H, Zheng D, Wang L, Qiao X, Jiang Y, Cui W, Tang L, Li Y, Xu Y. 2020. Immunogenicity evaluation of recombinant *Lactobacillus casei* W56 expressing bovine viral diarrhea virus E2 protein in conjunction with cholera toxin B subunit as an adjuvant. *Microb*. *Cell Fact*. 19:e186. https://doi.org/10.1186/s12934-020-01449-3

JIANG L, Zhang G, Wang P, Niu X, Liu Q, Zhang S, Gao W, Li Y. 2024. Simultaneous detection of bovine viral diarrhea virus (BVDV) and bovine herpesvirus 1 (BoHV-1) using recombinase polymerase amplification. *Sci. Rep*. 14:e10169. https://doi.org/10.1038/s41598-024-56869-7

LARGHI M. 2018. Comparative study in the control of bovine viral diarrhea. *Anim*. *Health Res. Rev*. 19:125-133. https://doi.org/10.1017/S1466252318000129

LIN J, Chen RH, Yang MJ, Zhu YM, Xue F. 2021. Isolation and molecular characterization of bovine herpesvirus 4 from cattle in mainland China. *Arch. Virol*. 166(2):619-626. https://doi.org/10.1007/s00705-020-04896-w

LIU CY, Guo H, Zhao HZ, Hou LN, Wen YJ, Wang FX. 2022. Recombinant bovine herpesvirus type I expressing the bovine viral diarrhea virus E2 protein could effectively prevent infection by two viruses. *Viruses.* 14(8):e1618. https://doi.org/10.3390/v14081618

NETTLETON P, Russell G. 2017. Update on infectious bovine rhinotracheitis. *In Pract*. 39(6):255-272. https://doi.org/10.1136/inp.j2226

OGUEJIOFOR CF, Thomas C, Cheng Z, Wathes DC: 2019. Mechanisms linking bovine viral diarrhea virus (BVDV) infection with infertility in cattle. *Anim. Health Res. Rev*. 20(1):72-85. https://doi.org/10.1017/S1466252319000057

RIMAYANTI R, Khairullah AR, Lestari TD, Moses IB, Utama S, Damayanti R, Mulyati S, Raharjo HM, Kusala MKJ, Raissa R, Wibowo S, Abdila SR, Fauzia KA, Yanestria SM, Fauziah I, Siregar JE. 2024. Infectious bovine rhinotracheitis: Unveiling the hidden threat to livestock productivity and global trade. *Open Vet. J.* 14(10):2525-2538. https://doi.org/10.5455/OVJ.2024.v14.i10.3

SOLTAN MA, Wilkes RP, Elsheery MN, Elhaig MM, Riley MC, Kennedy MA. 2015. Circulation of bovine viral diarrhea virus-1 (BVDV-1) in dairy cattle and buffalo farms in Ismailia Province, Egypt. *J. Infect. Dev. Ctries*. 9(12):1331-1337. https://doi.org/10.3855/jidc.7259

WANG Y, Pang F. 2024. Diagnosis of bovine viral diarrhea virus: an overview of currently available methods. *Front*. *Microbiol*. 15:e1370050. https://doi.org/10.3389/fmicb.2024.1370050

WELLENBERG GJ, Verstraten ER, Belak S, Verschuren SBE, Rijsewijk FAM, Peshev R, Van-Oirschot JT. 2001. Detection of bovine herpesvirus 4 glycoprotein B and thymidine kinase DNA by PCR assays in bovine milk. *J. Virol*. *Methods*. 97:101-112. https://doi.org/10.1016/s0166-0934(01)00341-x