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ORIGINAL RESEARCH

Genetic characterization of bovine viral diarrhea virus detected in backyard cattle farms in Mexico

Caracterización genética del virus de la diarrea viral bovina detectado en granjas de traspatio en México

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ABSTRACT

Infection by bovine viral diarrhea virus (BVDV) in cattle remains a source of significant economic losses for livestock producers. This virus is classified within the *Pestivirus* genus, including three main species: *Pestivirus A* (BVDV-1), *Pestivirus B* (BVDV-2), and *Pestivirus H* (HoBi-like pestivirus). Here, we performed a molecular epidemiological investigation aiming to evaluate the genetic diversity of BVDV in cattle from backyard farms in a municipality in Mexico named Tepalcingo, Morelos, with records of reproductive disorders. RT-PCR was conducted in 111 serum samples from affected cattle. Viral RNA was detected in 47.74% of the samples analyzed. Sequencing and phylogenetic analysis based on 5'UTR showed that the circulating subgenotype was BVDV-1a in all positive samples. These findings reveal the prevalence of BVDV in the

surveyed population; thus suggesting a possible association with the previous records of reproductive manifestation in the herd. However, further studies are needed to confirm BVDV as the causative agent. Additionally, our results represent a helpful tool for designing control and prevention strategies accurate to the current regional epidemiological situation. Moreover, obtained information from this type of epidemiological study will assist the implementation of biosafety measures on backyard farms with limited economic resources.

Keywords: bovine viral diarrhea virus, cattle, Mexico, pestivirus, phylogenetic analysis

RESUMEN

Las infecciones causadas por el virus de la diarrea viral bovina (VDVB) se mantienen como una causa importante de pérdidas económicas para los productores de ganado bovino. Este virus esta clasificado dentro del género *Pestivirus*, el cual incluye 3 especies: *Pestivirus A* (VDVB-1), *Pestivirus B* (VDVB-2), y *Pestivirus H* (HoBi-like pestivirus). Con la finalidad de evaluar la diversidad genética del VDVB, investigamos la epidemiología molecular del VDVB que circula en ganado de traspatio proveniente del municipio Tepalcingo, Morelos, Mexico. Se realizó RT-PCR en 111 muestras de suero de bovinos afectados. El RNA viral se detectó en el 47.74% de las muestras analizadas. La secuenciación y análisis filogenéticos basados en la región 5'UTR demostraron que el subgenotipo circulante es el VDVB-1a. Estos hallazgos evidencian la prevalencia del VDVB en la población evaluada; por lo tanto, sugiere una posible asociación con reportes previos de deordenes reproductivos en el hato. Sin embargo, se requieren estudios adicionales que lo confirmen. Adicionalmente, los resultados obtenidos representan una herramienta útil para el desarrollo de estrategias de control y prevención adecuadas a la situación epidemiologica regional actual. Así mismo, la información obtenida contribuirá a la implementación de medidas de bioseguridad en granjas de traspatio en Mexico.

Palabras clave: análisis filogenético, ganado bovino, México, pestivirus, virus de la diarrea viral bovina

1. INTRODUCTION

Bovine viral diarrhea (BVD) is a highly prevalent and economically important animal disease affecting ruminant populations globally (Brodersen, 2014). The viruses associated with BVD belong to three species within the genus *Pestivirus,* namely *Pestivirus A* (bovine viral diarrhea virus-1, BVDV-1), *Pestivirus B* (bovine viral diarrhea virus-2, BVDV-2), and *Pestivirus H* (HoBi-like pestivirus) (Simmonds *et al*., 2017). Currently, according to sequence comparison based on 5'UTR, at least 21 subgenotypes have been described for *Pestivirus A* (BVDV-1a to 1u), four *Pestivirus B* (BVDV-2a to 2d), and four for *Pestivirus H* (a-d) (Yesilbag *et al*., 2017).

Infection by these pestiviruses leads to various clinical presentations ranging from mild to severe, including respiratory disease, gastrointestinal disorders, and reproductive

failures. The latter represents the main factor for significant economic losses to the cattle industry due to reabsorption, abortion, stillbirths, congenital malformations, and the birth of weak calves or persistently infected animals (PI), along with infertility and poor conception rates (Grooms, 2004; Houe *et al*., 2006). Therefore, in several countries, BVD control programs focus on detecting and removing PI animals and implementing biosafety measures and vaccination protocols (Wernike *et al*., 2017).

Based on phylogenetic analysis, epidemiological surveys worldwide have reported the prevalence of predominant BVDV genotypes and subgenotypes from different regions (Yesilbag *et al*., 2017). Due to the recognition of genetic characteristics of the field, BVDV strains are crucial for the design of control or eradication programs and vaccine and diagnostic test design. For example, in a few regions of Mexico, subgenotypes BVDV-1a, BVDV-1b, BVDV-1c, and BVDV-2a in domestic cattle (Gómez-Romero *et al*., 2017), and BVDV-1b in captive deer (*Dama dama*) (Medina-Gudiño *et al*. 2022) have been reported.

Mexico is among the ten major bovine meat and milk producers worldwide (USDA, 2020; USDA, 2021). As a developing nation, cattle production systems based on backyard farming are a widespread cattle-keeping modality applied throughout the country (Gómez-Romero *et al*., 2021); therefore, they play a critical economic role. Specifically, in the southeast of Morelos state are located the municipalities with higher state livestock inventory; out of these, Tepalcingo municipality uses 70% of its territory for farming activities. A total of 631 traditional backyard farms are held where around 9,800 cattle have been raised (INEGI, 2013). Cattle are kept on small farms of 15 to 30 bovines. Population from Tepalcingo is considered under extreme poverty conditions; this dual-purpose cattle farming is the main economic activity performed (Hacienda, 2017).

The management and maintenance of backyard farms are characterized by reduced biosafety measures that may lead to the introduction and spread of infectious diseases. Therefore, in most cases, the epidemiological status of these farms is unknown. Hence, the identification of pestivirus in cattle from backyard farms in Mexico has not been described elsewhere. Because there is no information available regarding BVDV circulating in cattle from backyard farms in Mexico, this study aimed to detect and characterize the BVDV in domestic cattle from backyard farms in Tepalcingo municipality of Morelos state.

2. MATERIAL AND METHODS

2.1 Animals and serum samples

To assess the presence of subgenotypes of BVDV and accurately determine the genetic diversity of BVDV circulating in cattle from traditional backyard farms from Tepalcingo, Morelos, a total of 111 serums samples were collected in this study. Samples were collected from 2-year to 5-year-old crossbred beef and dairy production cows belonging to 4 production units with a recorded clinical history of abortions and infertility.

2.2 RNA extraction and RT-PCR

According to the manufacturer's instructions, total RNA was obtained from 400 µL serum aliquot of each animal using TRIzol reagent. For RNA extraction, each sample was mixed with 300 µl of TRIzol and incubated at room temperature for 5 min. Then 150 µl of chloroform were added to the tubes and vigorously shaken; the tubes were chilled in ice for 7 minutes, followed by centrifugation at 13,800 *g* for 20min. The aqueous phase was collected in new tubes, and 500 µl of isopropanol was added. All tubes were incubated at room temperature for 10 min and centrifuged at 18,800 *g* at four °C for 20 min. The resulting pellet was washed twice with 1 ml of 75% ethanol in RNase-free water. Next, the pellet was air-dried at room temperature for 10 min, dissolved in 20 µl of RNase-free water, and stored at -70°C.

The RNA obtained from serum samples and positive and negative controls were subjected to reverse transcriptase (RT-PCR). Further, BVDV NADL reference strain was included as a positive control, and supernatant of mock-infected BVDV-free MDBK cell line was used as a negative control. RT-PCR was conducted to amplify a fragment of 5'UTR using conditions previously reported (Mahony *et al*., 2005). Briefly, complementary DNA (cDNA) was synthesized in 20 µl volume using 50 ng of random primers, 10mM dNTPs, followed by incubation at 65ºC for 5 minutes, and immediately placed on ice. Then, 5X annealing buffer, 0.1 DTT, and 40U ribonuclease inhibitor were added and incubated at 37ºC for 2 min, followed by 200U M-MLV RT (Invitrogen) and incubation at 37ºC for 50 min, and at 70ºC for 15 min.

The synthesized cDNA was sequentially submitted to PCR using the primers 5UTRfwd (5'-CTA GCC ATG CCC TTA GTA GGA CTA-3') and STARTrev (5'-CAA CTC CAT GTG CCA TGT AC AGC A-3'). The reaction mixes contained 1X Taq polymerase buffer, 1mM dNTPs, 1.5 mM MgCl2, 1.5 µM of each primer, and 1U of Taq DNA polymerase (Thermo Fisher Scientific). All PCR reactions were performed in 100 µl volume; PCR conditions were 94ºC for 5 min, 30 cycles consisting of 30 s at 94ºC, 30 s at 56.2 ºC, and 30 s at 72ºC followed by 7 min at 72ºC after the last cycle. Finally, the PCR products were visualized by electrophoresis in 1% agarose gel stained with GelRed Nucleic Acid®. Subsequently, PCR products were purified using the QIAquick Gel extraction kit following the manufacturer's indications.

2.3 Sequencing and phylogenetic analysis

The nucleotide sequences were generated in duplicate and in both directions at the Biotechnology Institute of the Universidad Nacional Autónoma de México using BigDye™ Terminator Sequencing Kit ABI PRISM® 3130xl Genetic Analyzer. Obtained nucleotide sequences were aligned using the Clustal W (Thompson *et al*., 1994) program with BioEdit software (Hall, 1999). Eighteen partial 5'UTR nucleotide sequences were deposited in GenBank under accession numbers: ON369375 to ON369392.

Phylogenetic analysis was based on the comparison of a 260-nucleotide region of the 5'UTR generated from the samples and reference strain. Phylogenetic inference was

performed in MEGA 7 (Kumar *et al*., 2016) with the maximum-likelihood method with the Kimura-2 parameter used as a substitution model (Kimura, 1980); bootstrap analysis was performed using 1000 replicates (Felsenstein, 1985)

3. RESULTS

To determine the genetic diversity of BVDV detected in cattle belonging to 4 production units distributed in Tepalcingo, Morelos (Fig. 1), serum samples were obtained and submitted to RT-PCR and sequencing. A total of 53 samples were positive for BVDV RNA using the primers 5UTR/START (Fig. 2).

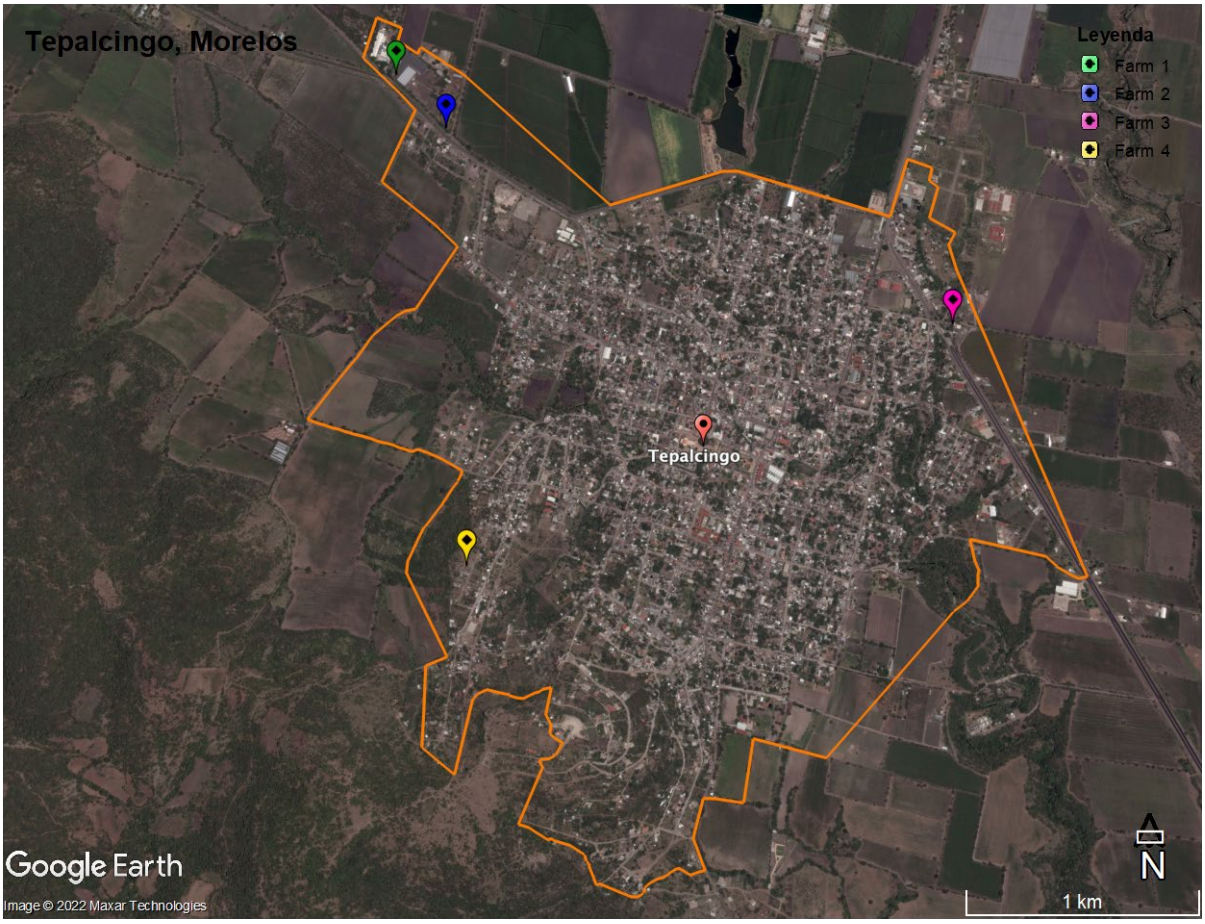

Fig. 1. Geographical distribution of the backyard farms included in this study. The location of farm 1 is indicated in green, farm 2 in blue, farm 3 in magenta, and farm 4 in yellow. Tepalcingo municipality of Morelos State is demarcated in the orange line. The map was drawn using Google Earth 2022 7.3.4. 8642. Elaborated on September 2nd, 2022.

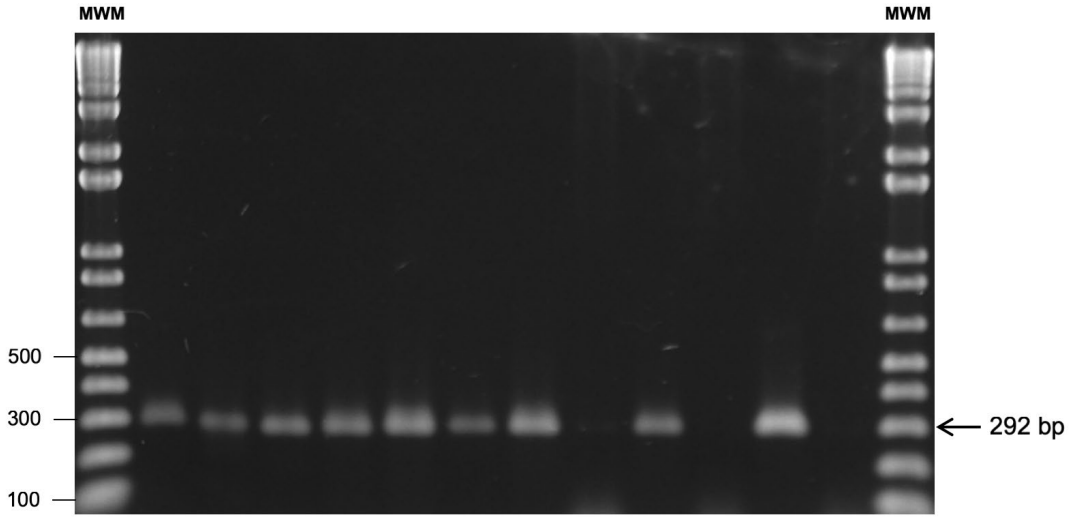

Fig. 2. RT-PCR products of BVDV electrophoresed on an agarose gel. Lane 1 and 14 contain molecular weight marker 1Kb Plus Ladder (Invitrogen). Lane 2-11 represents PCR products from animal serum samples in production unit 3. Lane 12 and 13 contain positive and negative controls, respectively. An arrow on the right indicates the amplified product of 292 bp.

The proportion of PCR products obtained represented a 47.74% of BVDV prevalence in the surveyed population (Table 1). Subsequently, 18 samples were submitted for further sequencing to determine the BVDV subgenotypes detected by performing alignment and phylogenetic analysis.

Production Unit	Number of samples	Number of positive RT-PCR	Percentage
Unit 1	50	22	44.00%
Unit 2	18	6	30.00%
Unit 3	14	8	57.14%
Unit 4	29	17	58.60%
Total	111	53	**47.74%**

Table 1. The number of RT-PCR positive samples per production unit. The number of evaluated serum samples and positive RT-PCR results per production unit is shown. The total and unit RT-PCR positivity percentage is indicated.

Our investigation describes a limited genetic diversity of BVDV in the analyzed population. The phylogenetic inference clustered all the amplified sequences in the BVDV-1a subgenotype (Fig. 3). Hence, BVDV-1a is the prevalent subgenotype

circulating in backyard farms from Tepalcingo, Morelos. The genetic analyses of the partial 5'UTR indicated that the sequences were highly similar and had a sequence identity of around 99.99% to reference strain BVDV-NADL. The partial 5'UTR sequences were submitted to GenBank under accession numbers ON369375 to ON369392. No sample sequences were clustered within BVDV-1b, 1c o 2a.

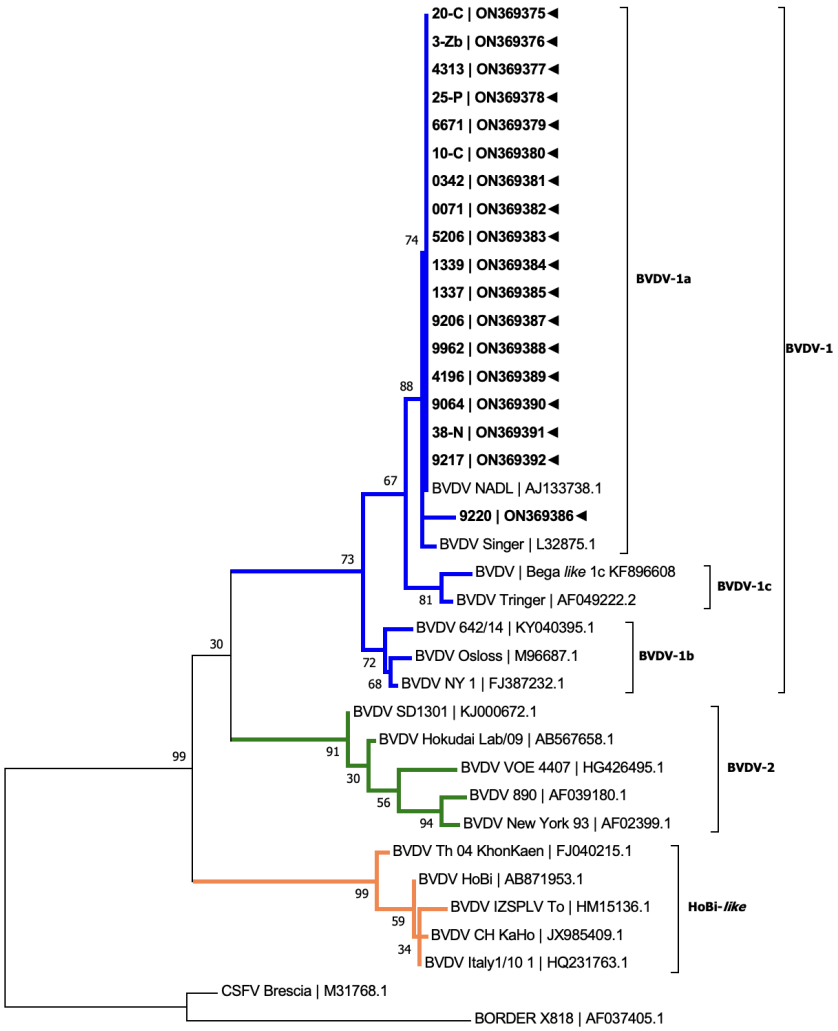

Fig. 3. Phylogenetic tree based on a partial sequence of 5'UTR. Molecular evolutionary genetic analyses were conducted on MEGA 7 using the maximum likelihood method. Distances were computed using the Kimura 2-parameter model. Evaluated sequences are shown with the assigned GenBank accession number in bold font and labeled with the symbol"◄". Reference sequences are identified by the GenBank accession number. The numbers in the nodes indicate bootstraps values.

4. DISCUSSION

Despite BVD being considered an endemic disease with nationwide distribution, information concerning the genetic diversity of BVDV in the national bovine population remains limited. Therefore, BVD control and preventive strategies are not mandatory; hence, control activities are based only on voluntary procedures depending on the BVDV knowledge of cattle handlers. Further, vaccine application, biosafety measures, and monitoring are essential tools for disease prevention, although most backyard farm keepers are not aware of the importance of their application. Accordingly, evaluating the genetic diversity of BVDV circulating in traditional backyard production units is crucial. Therefore, RT-PCR positive samples from backyard cattle were subjected to 5'UTR sequence-based genotyping.

The phylogenetic inference showed that analyzed samples clustered within the BVDV-1a subgenotype with a closer relationship to the BVDV NADL strain. BVDV-1a and BVDV-1b were the first subgenotypes reported in the early 1990s (Ridpath *et al*., 2010). Currently, BVDV-1b is considered the most predominant subgenotype, followed by BVDV-1a, which has been reported as the most frequently detected subgenotype globally. In 2017, a study performed by Yesilbag *et al*. recorded that the available sequence data of BVDV-1a comprises around 20.8% of the published sequences of total BVDV isolates at that time (Yesilbag *et al*., 2017). Moreover, this subgenotype has been detected in cattle from the US and Canada (Gilbert *et al*., 1999).

Subgenotype 1a is considered endemic to Mexican cattle populations. Previous studies demonstrated that the 1a subgenotype was the second most commonly detected BVDV in the cattle population surveyed (Gómez-Romero *et al*., 2017). Additionally, this subgenotype has been found as a contaminant in commercial veterinary vaccines, fetal bovine serum, and cell lines used in research and vaccine production in Mexico (Gómez-Romero *et al*., 2021). These findings indicate a strong spreading of BVDV-1a nationwide; therefore, its prevalence and circulation amongst livestock in Mexico. In addition, despite these latest BVDV reports, the epidemiological status of BVDV is not entirely defined.

Epidemiological studies have shown that various subgenotypes predominate in different geographic regions; thus, the BVDV subgenotypes detected per region can vary. For instance, during a serosurveillance assay in cattle from six regions, the mix of circulating BVDV was different among states (Gómez-Romero *et al*., 2017). Therefore, this highlights the importance of further phylogenetic analysis on BVDV to provide relevant insights on relatedness among BVDV subgenotypes due to the constantly changing scenario (Yesilbag *et al*., 2017).

Considering that owners of these backyard animals do not use BVDV vaccination as a prevention tool, mainly due to misinformation regarding commercial products and the advantages of their use and limited biosafety measures applied, it is common to find BVDV-1a as an endemic virus. Hence, the reported reproductive disorders that occurred in these backyard farms possibly be associated with BVDV infections. These can have a

direct impact on reproductive parameters such as fertility and conception rates, embryotic death, the birth of weak animals, and congenital malformations leading to early death in calves (Revell *et al*., 1988; Grooms *et al*., 1996; Fray *et al*., 2000). However, additional assays need to be performed to confirm BVDV as the causative agent of reproductive failure in the evaluated herd.

BVDV infection can play an important role in non-vaccinated animals with a record of reproductive failure; however, other reproductive pathogens must be considered for an accurate diagnosis. Here, we highlighted the fact that naïve cattle have been exposed to an unknown BVDV source, and natural BVDV infection in backyard cattle has been detected. Nevertheless, the source of infection cannot be determined; therefore, special emphasis on BVDV testing, monitoring, recordkeeping, animal trade, and farm keeper's training is necessary to mitigate the BVDV impact. Furthermore, this investigation revealed the presence of ongoing BVDV infections in all the production units. Consequently, viral dissemination among farms has been maintained may be due to the presence of PI animals as these animals are highly efficient for BVDV spreading (Brodersen, 2014). In this context, the presence of PI animals is considered the outcome of fetal infections and represents the main source of BVDV shedding in susceptible animals, as a consequence, reproductive failure, abortions, stillbirth, and mummifications might happen (Ridpath,2010). The latter has significant implications for trade and cattle mobilization activities. Therefore, the spreading of BVDV-1a to other regions may occur.

The analysis of genetic sequences obtained in this study demonstrated the circulation of a monophyletic BVDV origin associated with subgenotype 1a. Furthermore, this phylogenetic data indicate that BVDV-1a is the predominant subgenotype in the surveyed population. In contrast, a similar study performed by Shi *et al*. showed a broader genetic BVDV diversity, detecting several subgenotypes of BVDV-1 genotype and HoBi-like viruses (Shi *et al*., 2020). Similarly, despite the scarce information in Mexico regarding BVDV genetic diversity, previous studies on cattle from six regions of Mexico revealed that BVDV-1c was the most common subgenotype detected, followed by 1a, 1b, and 2a, representing a unique combination of BVDV circulating in North America (Gómez-Romero *et al*., 2017). Additionally, genetic analysis of BVDV detected as a contaminant in fetal bovine serum exhibited consistent results, where the presence of 1a, 1b, and 2a subgenotypes was evidenced (Gómez-Romero *et al*., 2021). Interestingly, the BVDV sequences generated in these previous studies share a genetic identity estimated at 97.3% and 90.2% based on the 5’UTR, respectively. Thus, there is a high similarity between Mexican BVDV-1a distributed nationwide. Due to the high conservation level, the 5’UTR has been used for the genetic classification of BVDV into genotypes and subgenotypes (Yesilbag *et al*., 2017). Despite that, genetic segregation based on the comparison of the sequences of this region is an adequate tool for genetic characterization, variability throughout the full genome should be considered to confirm the genetic relatedness among further BVDV isolates from this geographical region and others within the country.

One of the limitations of this study was the number of samples used for sequencing. Hence, additional BVDV-positive samples of cattle from Tepalcingo, Morelos require further analyses to determine BVDV genetic diversity to accurately describe all BVDV subgenotypes and thus the epidemiological status of this region. In addition, the implementation of diagnostic assays with a higher potential for viral detection, such as real-time PCR, should also be considered for future studies. Despite these limitations, our results encourage monitoring the BVDV diversity and prevalence of BVDV in backyard farms in Mexico. To date, there is no sufficient data on BVDV epidemiology and distribution in the country.

Due to the economic relevance of backyard farms in Mexico, further studies are being implemented on the animal population in this region to understand the BVDV dynamics, evolution, and variability of the 1a subgenotype. Moreover, our findings demonstrate the urgency of training the farm keepers and implementing control programs that rely on continuous testing, vaccination, and establishment of biosafety strategies Additionally, biosafety measures and vaccination protocols have been suggested to the farm keepers to mitigate BVD impact on animal health (Fulton *et al*., 2020), and as a consequence in the economy of farm owners. Our survey exhibited a low level of genetic diversity; however, the change in the BVDV subgenotypes prevalence should not be ruled out.

In Mexico, no national mandatory BVD prevention and control programs have been implemented to date. Thus, the information provided by this study promotes the development and establishment of rapid and reliable diagnostic tests that allow the detection of national field BVDV strains. Consequently, effective surveillance should be performed in the region. Likewise, analyses of circulating BVDV subgenotypes will contribute to the improvement of vaccines; since the formulation of an efficacious vaccine must correspond to the antigenic profile of the viruses present in the region where the vaccine is going to be used.

There is ongoing interest in BVD control measures by backyard farm owners and keepers. The use of accurate diagnostic tools and genetic characterization, such as phylogenetic analyses, are a helpful tool for identifying the endemic subgenotypes in a population, BVDV dissemination to other regions, and remerging or reintroducing new BVDV subgenotypes. The latter has contributed to diagnostic screening since the BVDV-infected population represents a constant risk factor in viral spreading.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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