Review

Bovine viral diarrhea virus (BVDV), currently classified as *Pestivirus bovis* (BVDV-1), *Pestivirus tauri* (BVDV-2), and *Pestivirus brazilense* (BVDV-3 or HoBi-like pestivirus), is one of the most serious pathogens causing drastic economic losses in the global livestock industry. This pathogen has an affinity for multiple organ systems, causing enteropathic and respiratory symptoms, reproductive disorders, and severe immunosuppression in cattle and other ruminants. It also contributes to the occurrence of persistently infected (PI) animals when infection occurs during pregnancy (average between 40 and 120 days of gestation). The strategic geographical location of the Thrace district in Turkey, which forms the direct land border with the European Union, makes this area a key epidemiological control point due to the intensive cross-border animal traffic and the import of cattle by sea from the Americas and Europe. The reviewed manuscript attempts to determine the molecular prevalence and genetic diversity of BVDV strains circulating in this strategic region between 2021 and 2023. To this end, the authors studied a cohort of 533 animals exhibiting clinical signs of respiratory or gastrointestinal disease.

Strengths of the manuscript

1. Sample size. Based on such a large study group (533 animals, including 450 from integrated farms and 83 from small family-owned farms), the work provides valuable data on the correlation between animal age and year of sample collection and infection prevalence, as illustrated using logistic regression analysis.

2. Identification of *Pestivirus brazilense*. The main scientific value of the work is the first identification of the circulation of this *Pestivirus* in the Thrace district. This discovery is of significant epidemiological significance, as this species, originally isolated in South America, demonstrates high pathogenicity and a degree of immune escape from the commonly used BVDV-1/2 vaccines. Demonstrating the presence of this pathogen at the border with the European Union redefines the cross-border risk profile for neighboring countries.

3. **11 new sequences**. Depositing this sequences in the GenBank database (PQ432872 – PQ432882) significantly enriches public reference resources.

Weaknesses of the manuscript

The most serious methodological and interpretative weaknesses of the manuscript include:

1. Severe selection bias. Sampling based solely on diseased animals (targeted sampling) drastically overestimates the overall positive rate compared to the general population. The authors draw conclusions about prevalence, forgetting that excluding healthy animals prevents an objective estimate of the virus's prevalence in the region.

2. Lack of longitudinal studies. Single-sample collection prevents the identification of PI animals, which are the epidemiological driver of BVDV epidemics in herds. Without re-testing positive animals after 3 – 4 weeks, it is impossible to distinguish PI from transiently infected (TI) individuals.

3. Laboratory procedure and its reproducibility. The diagnostic procedure described in the manuscript is based on the extraction of viral RNA from nasal and rectal swabs using a commercial kit, cDNA synthesis, and subsequent amplification using real-time RT-PCR and

conventional RT-PCR. Although the general outline of the methodology appears to be consistent with laboratory standards, the reviewer notes the limited reproducibility of the procedures in an independent laboratory setting. This concerns:

a. Unclear description of PCR inhibitor verification. The authors mention that potential PCR inhibitors were evaluated "as previously described" (Lines 136 – 137). This is an extremely imprecise statement. The lack of precise description of inhibitor controls undermines the reliability of negative results.

b. The absence of primer and probe sequences in the main text. The Authors have moved key data regarding primer and probe sequences for real-time RT-PCR, conventional RT-PCR for BVDV-1/2, and specific RT-PCR for HoBi-like pestiviruses to Supplementary Table 2. This information constitutes the methodological backbone of the work and must be included directly in the main text to ensure the scientific autonomy of the publication.

c. The absence of parameters for the control gene. It is indicated that “The real time RT-PCR control was performed by using beta-actin gene”(Lines 153 – 154), but the text completely omitted the primer sequences, reagent concentrations, and temperature conditions for this specific reaction. It is unknown whether this reaction was conducted in a duplex format with the screening reaction or as an independent single-phase assay.

d. The absence of volume specifications. To reproduce the procedure, it is necessary to provide the initial volume of the transport medium used for RNA extraction and the final elution volume of the nucleic acid. Differences in these parameters drastically modify the analytical sensitivity of the test.

e. Terminology inconsistencies in the documentation. In Supplementary Table 1, the authors They use the term "fecal swab," while in the main text and Supplementary Table 4 they consistently use the term "rectal swab." This requires standardization to maintain the rigor of the taxonomy of clinical samples.

4. Numbering of cited references. The References section contains a significant error: 72 references appear here, while only 70 are cited in the text – references 3 and 53 are continuations of references 2 and 52, respectively.

2. Yilmaz, H., et al., Genetic diversity and frequency of bovine viral diarrhea virus (BVDV) detected in cattle in Turkey. Comparative immunology, microbiology infectious diseases
3. 2012. **35**(5): p. 411-416.

52. Riitho, V., et al., Bovine pestivirus heterogeneity and its potential impact on vaccination and diagnosis. Viruses
53. 2020. **12**(10): p. 1134.

Most importantly, however, it is difficult to determine whether the references are cited correctly in the text.

Additional suggestions.

The *Pestivirus brazilense* strain (PQ432872) was detected in a rectal swab collected from an unvaccinated calf months in Kırklareli in 2023, demonstrating a Ct value of 27.85. The reviewer concluded that the text should clearly state that despite a targeted search for this pathogen using specific primers, the HoBi-like pestivirus remains rare in the studied population (1 positive swab in 533 samples tested).

In the reviewer's opinion, the Authors unnecessarily included statements regarding seroprevalence in vaccinated and unvaccinated cattle populations in the Discussion section (Lines 309–314). The reference to the presence of antibodies against BVDV makes little substantive sense, as the Authors focused their research on the pathogen and its genetic diversity, and did not conduct a single serological test aimed at antibody detection (e.g., ELISA or VN neutralization test).

I request that Authors adopt a consistent method for recording manufacturer data for reagents, commercial kits, and equipment used. For example, in one place, the Authors use the manufacturer's city and state name, omitting the country (line 134), while in another place, only the country of production is provided (lines 138 – 139, 151, 163) or they do not provide the place of production at all (lines 146 – 147, 160).

Conclusion

The manuscript provides extremely valuable epidemiological information, documenting for the first time the circulation of the pathogenic *Pestivirus brazilense* species in the European part of Turkey (Thrace), which is of significant importance to the veterinary border services of the European Union. However, the current version of the paper contains significant methodological gaps and terminological inconsistencies that require corrections. After carefully addressing the above comments and integrating the suggested corrections, the manuscript will constitute a highly valuable publication with a strong impact on regional programs for the eradication and control of infectious diseases in ruminants.