Fecal virome characterization of dairy cows with winter dysentery reveals bovine torovirus, enterovirus F, a novel and recombinant enterovirus I, and a putative novel protoparvovirus

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Research Article

Keywords: winter dysentery, adult dairy cattle, virome, torovirus, enterovirus, protoparvovirus

Posted Date: July 25th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7116208/v1

Additional Declarations: No competing interests reported.

Abstract

Background

Winter dysentery (WD) is an acute diarrheal disease of adult cattle with a significant impact on milk production. Although bovine coronavirus (BCoV) is usually considered the primary etiological agent, other viruses may contribute to the disease. In this study, we investigated a WD outbreak in a bovine dairy herd in Uruguay through shotgun metagenomic sequencing and molecular virology techniques.

Results

Fecal samples from three affected cows were negative for common enteric pathogens, including BCoV, but two samples tested positive for bovine torovirus (BToV) by RT-qPCR. Virome analysis of pooled samples identified over 3,300 viral operational taxonomic units (vOTUs), predominantly bacteriophages, but also eukaryotic viruses from 11 families. Notably, we detected the complete genome of BToV (101/UY), closely related to a previously reported Uruguayan strain. Additionally, two enteroviruses were identified, including one divergent strain (MFB-556) that likely resulted from interspecies recombination between *Enterovirus idromi* and *Enterovirus fitauri*. A divergent protoparvovirus sequence, CPBI/UYU/2024, was also identified and confirmed by PCR and Sanger sequencing; phylogenetic and sequence identity analyses suggest it represents a putative new species within the genus *Protoparvovirus*.

Conclusion

These findings underscore the value of viral metagenomics for uncovering potential co-infections and novel viruses in diarrheic cattle with WD.

Background

Winter dysentery (WD) is a disease with a significant economic impact on the dairy industry due to reduced milk production in affected cows [1]. A study in Norway has quantified these losses at approximately 51 L of milk per cow from 7 days before to 19 days after the WD outbreak was reported, representing up to 15% reduction in milk yield per day [2], and in severe outbreaks, this figure may even double [1]. Moreover, decreased milk production may persist for several months after an outbreak [2, 3]. The main symptoms include the sudden onset of diarrhea (soft or liquid feces, ranging from dark green to black), occasionally accompanied by blood, as well as respiratory symptoms like nasal and ocular discharge and coughing [1–4]. Hyperthermia often precedes the onset of diarrhea, and some cows may also exhibit depression [1, 4]. These diarrhea episodes in adult cattle occur mainly during colder months, hence the name winter dysentery.

Despite its significant impact on production, the possible etiological agent(s) of WD (other than bovine coronavirus –BCoV-) are still largely unknown. Initially, the disease was associated with *Campylobacter jejuni* (formerly *Vibrio jejuni*), though later a viral origin was proposed [3, 5]. Currently, BCoV is considered the primary causative agent [2], although some reports have documented the detection of bovine torovirus (BToV) in adult cattle with diarrhea [6–8]. A study in the Netherlands showed higher incidence of BToV (but not BCoV) in herds affected by WD compared to controls, suggesting a potential etiological role for BToV [9]. Similarly, a Brazilian study suggested an association between BToV and WD outbreaks [10].

Given the limited understanding of WD and its possible viral etiologies that may cause and/or influence the disease, we sought to describe and characterize the viral diversity present in the feces of diarrheic cows during an outbreak of WD, and to molecularly characterize the bovine torovirus, two enteroviruses and a protoparvovirus identified.

Materials and Methods

Outbreak description, sample collection and initial laboratory testing

A spontaneous WD outbreak with 6% morbidity and no mortality was investigated in April 2024 in a herd of ~ 100 adult milking cows from a commercial dairy herd in the department of Colonia, Uruguay. Clinically, the affected cows had diarrhea occasionally containing blood, reduced feed intake and drop in milk production. Individual fecal and serum samples were collected from three of the six affected cows and submitted to the veterinary diagnostic laboratory to test for the main infectious and parasitic agents causing diarrhea in cattle, as part of a routine diagnostic investigation. Sampling procedures were conducted by a veterinarian and were reviewed and approved by the Ethics Committee on Animal Use of the National Institute of Agricultural Research of Uruguay (protocol number 2019.9). Fecal samples were initially cultured for *Salmonella* spp., analyzed by qPCR for *Mycobacterium avian paratuberculosis* [11], and specific RT-qPCRs for BCoV [12], BToV [13] and group A rotavirus (RVA) [14]. Serum samples were analyzed by RT-qPCRs for bovine viral diarrhea virus (BVDV) [15]. Additionally, fecal flotation (McMaster test) was conducted for nematode eggs and coccidia oocysts identification and semi-quantification, with a limit of detection of 40 eggs and oocysts per gram of feces (epg and opg, respectively) [16].

Shotgun metagenomics for virus detection

Fecal suspensions (10% v/v in PBS) were prepared from individual samples, which were then pooled to analyze the virome. The pooled sample was filtered through 0.22 µm membranes and treated with nucleases (RNase A and DNase I) to remove host cells, bacteria, and free genetic material, thereby enriching viral particles in the feces. Nucleic acids, both DNA and RNA, were extracted using the QIAamp Viral RNA Mini Kit (Qiagen), following the manufacturer's instructions, with the exception that the elution was performed with 30 µL of AVE buffer (Qiagen). Viral RNA was reverse-transcribed into copy DNA (cDNA) using the SuperScript III enzyme (Invitrogen), according to the manufacturer's instructions. The second strand DNA synthesis was performed using the Second Strand cDNA Synthesis Kit (Invitrogen),

resulting in double-stranded DNA representing the viral community. Thus, all the viral genetic material present in the pooled samples was finally obtained as double-stranded DNA. For a Shotgun Metagenomics sequencing approach, a library was prepared using the Nextera DNA XT library kit (Illumina) and sequenced (2 x 150bp) on a NovaSeq 6000 System (Macrogen, Inc).

Raw reads were quality-checked using FastQC [17] and filtered by quality and length (SLIDINGWINDOW:3:30 MINLEN:130) using Trimmomatic [18]. Reads with average Q < 30 and length under 130 bp were discarded. To get rid of any host sequence left behind after nuclease treatment, a host filtering step was done mapping trimmed reads to *Bos taurus* genome version ARS-UCD2.0 (GCF_002263795.3). Unmapped reads were *de novo* assembled using SPAdes with the metaviral module [19]. Scaffolds and unmapped reads were used as input for the Modular Viromics Pipeline (MVP) v.1.1.4 [20]. This user-friendly pipeline, written in Python, uses geNomad to identify viruses, proviruses, and plasmids [21]; CheckV for quality assessment, filtering and Average Nucleotide Identity clustering [22], and Bowtie2, Samtools and CoverM for abundance/coverage estimation [23–25].

Conventional PCRs for enterovirus and novel protoparvovirus detection

Two conventional PCR assays were developed to detect enteroviruses (EVs) and the novel protoparvovirus in individual samples. Based on sequences obtained from the metagenomic analysis, two primer sets were designed to target the novel bovine protoparvovirus and bovine enterovirus E, F and I (Table 1). Viral DNA and RNA were extracted from fecal suspensions using the QIAamp Viral RNA Mini Kit (Qiagen), following the manufacturer's instructions. For enterovirus detection, viral RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). PCR reactions were performed in 0.2 mL tubes using 12.5 µL of MangoMix (Bioline), 1 µL of 10 µM primer forward, 1 µL of 10 µM primer reverse, 1 µL of DMSO, 4.5 µL of nuclease-free water, and 5 µL of DNA or cDNA template (for protoparvovirus and enterovirus, respectively). Thermal cycling conditions for protoparvovirus were 95°C for 5 minutes, followed by 45 cycles of 94°C for 1 minute, 48°C for 1 minute and 72°C for 1 minute, and a final extension of 72°C for 10 minutes. For enterovirus, thermal cycling conditions were 95°C for 5 minutes, followed by 45 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, and a final extension of 72°C for 10 minutes. PCR products were visualized by electrophoresis in 2% agarose gel stained with GoodView (SBS Genetech). Positive samples were purified using Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer’s instructions, and both DNA strands were sequenced by the Sanger method at Macrogen Inc. (Seoul, South Korea).

Table 1
Primers used in conventional PCRs

Primer name	5'-3' sequence	Virus	Fragment length	Genomic region
BPPV-F	AAAAGATGCWTGGTGGCT	Bovine protoparvovirus	726 bp	NS1
BPPV-R	GCTTTRAAYTGGTTTACTTG			
EV EFI-F	GGGGAGTAGTCCGACTCCGC	Bovine enterovirus	170 bp	5'UTR
EV EFI-R	GGGGAAACAGAAGTGCTTGACC			

Phylogenetic analyses

Phylogenetic analyses were conducted for BToV, EVs, and the novel protoparvovirus. In all cases, the best-fit evolutionary model was selected for each dataset, and branch support was evaluated using the SH-aLRT method. All resulting trees were visualized with MEGA X [26].

For BToV, all complete torovirus genomes available in GenBank as of June 2025, regardless of host species, were downloaded. Sequences were aligned using Clustal Omega [27], and the phylogenetic tree was reconstructed with W-IQ-TREE [28].

For EVs, complete reference genomes from different types within species E, F, and I were retrieved (June 2025). Alignment was performed with ClustalW and phylogenetic tree was generated using nucleotide sequences of the complete genomes through the Galaxy platform [29]. In addition, amino acid sequences of the full-length polyprotein, as well as of the P1, P2, P3, and VP1 regions, were obtained, aligned with ClustalW, and analyzed phylogenetically in Galaxy platform [29].

For the novel protoparvovirus, the sequence identified as belonging to the *Parvoviridae* family was analyzed by BLAST (June 2025) using blastn (somewhat similar sequences) to retrieve sequences with the highest query coverage and percent identity. Alignments were performed with ClustalW and phylogenetic analyses were conducted at both nucleotide and amino acid levels using Galaxy platform [29].

Estimation of genetic distances

To assess genetic divergence, pairwise evolutionary distances were estimated using MEGA X [26] for the divergent viruses MFB-556 (Enterovirus I) and CPBI/UYU/2024 (Protoparvovirus), based on the nucleotide and amino acid sequences.

Recombination analysis

To further investigate potential recombination events in MFB-556, analysis was performed using RDP5 [30]. The dataset included the MFB-556 genome and reference sequences from enterovirus species E, F,

and I. Default parameters were used, and recombination events supported by multiple detection methods within RDP5 were considered as potential signals.

Viral isolation

Madin-Darby bovine kidney (MDBK) cells were cultured in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin, streptomycin, and amphotericin B). For viral adsorption, 1 mL of fecal suspension filtered through 0.22 µm membranes and treated with nucleases was used as inoculum, followed by incubation at 37°C with 5% CO_2 for 1 hour. After incubation, the inoculum was removed, cells were washed twice with MEM, and 5 mL of fresh MEM supplemented with 5% FBS was added. Cells were incubated for 3 days under the same conditions. Three consecutive passages were performed. For each passage, three freeze–thaw cycles were conducted, and 1 mL of lysate was used to infect the next passage, following the same procedure as the initial inoculation. Uninfected MDBK cells (mock-infected controls) were included in parallel to monitor for nonspecific cytopathic effects.

Results

Initial description of the outbreak and laboratory testing

All three samples were negative for *Salmonella* spp., *Mycobacterium avian paratuberculosis*, BVDV, BCoV, RVA, and coccidia oocysts. BToV was detected by qPCR in one sample with Ct = 20.5 and other with Ct = 38, while the other sample was negative. Fecal flotation revealed low numbers of nematode eggs (40 epg) in two of the cows; no eggs were identified in the other.

Virome characterization

To investigate the presence of other viral agents, the virome of the fecal pool was obtained. Sequencing generated a total of 76,4M raw reads, of which 99.9% did not map to the bovine genome. After *de novo* assembly 204,719 scaffolds were obtained, of which 14,794 were classified as viral. These viral scaffolds were clustered into 3,328 vOTUs. Based on predicted host association, 3,254 (97.8%) vOTUs were classified as prokaryotic viruses, 14 (0.4%) remained unclassified, and 60 (1.8%) were identified as eukaryotic viruses (Fig. 1a). The viral scaffolds included 958 complete genomes, 257 near-complete genomes (90.0–99.99%), 479 partial genomes (60.0–89.99%), 429 vOTUs representing 30.0 to 59.99% of genomes, and the remainder representing up to 29.99% viral genome coverage (Fig. 1b).

Bacteriophages

Three classes of bacteriophages were identified: *Caudoviricetes* (1,720 vOTUs), *Malgrandaviricetes* (1,533 vOTUs), and *Faserviricetes* (1 vOTU) (Fig. 1c). All detected *Malgrandaviricetes* belonged to the family *Microviridae* and the *Faserviricetes* belonged to the family *Inoviridae*.

Eukaryotic viruses

Among the eukaryotic viruses, 9 families were represented. The most abundant were *Smacoviridae* (36 vOTUs), followed by *Circoviridae* (8 vOTUs), *Geminiviridae* (4 vOTUs), *Nanoviridae* (3 vOTUs), *Picornaviridae* (2 vOTUs), and one vOTU each from *Genomoviridae*, *Endornaviridae*, *Solemoviridae* and *Tobaniviridae* (Fig. 1d). Additionally, two scaffolds were classified individually as members of *Tombusviridae* and *Parvoviridae* families.

Bovine torovirus

As mentioned, the 3 samples were analyzed individually by qPCR obtaining one positive result with Ct = 20.5, one with a Ct = 38 and one negative result. Consistent with the qPCR result, metagenomics assembly from the pooled samples recovered one scaffold comprising the complete BToV genome with a length of 28,281 nucleotides (nt) and high mean depth of coverage (137X). This genome contained the genes typically found in bovine toroviruses: ORF1a, encoding the replicase (genome positions 789 to 14,042); S, encoding the spike protein (positions 20,821 to 25,575); M, encoding the membrane glycoprotein (positions 25,604 to 26,305); HE, encoding the hemagglutinin esterase (positions 26,323 to 27,582); and N, encoding the nucleocapsid phosphoprotein (positions 27,625 to 28,116). In the phylogenetic analysis, the sequence obtained in this study, namely 101/UY, clustered closely with a previous sequence obtained by our group from a calf in Uruguay, as well as with most other bovine torovirus (excluding Breda virus) (Fig. 2).

Bovine enteroviruses

One complete and one near-complete EVs genome sequences were obtained, with lengths of 7,334 nt (MFB-556) and 7,190 nt (Soleuba-2066). Both EVs genomes were sequenced at high coverage depth with 510X and 92X, respectively. The genome organization of both viruses was consistent with that of other EVs, presenting a 5'UTR and a 3'UTR flanking the P1-P2-P3 coding regions. Taxonomic classification identified both sequences as members of the *Picornaviridae* family, *Enterovirus* genus, with Soleuba-2066 classified as *Enterovirus fitauri* species (EV-F6 type) and MFB-556 as a divergent enterovirus. Phylogenetic analyses confirmed the assignment of Soleuba-2066 to the EV-F6 type; however, the results for MFB-556 were inconclusive (Fig. 3). At the amino acid level, MFB-556 clustered with *Enterovirus idromi* (EV-I) sequences, based on VP1, P1, and the complete polyprotein, as did the nucleotide-based analysis of complete genomes. However, at the amino acid level for both P2 and P3 regions, MFB-556 grouped with *Enterovirus fitauri* (EV-F) sequences. Interestingly, in all analyses, it consistently clustered with a Chinese sequence, BEV9 (GenBank accession number ON986121), obtained in 2021. Genetic distance analyses revealed the same pattern (Table 2). Specifically, MFB-556 showed the closest relationship to BEV9, with pairwise distances ranging from 1.3 to 1.8. The lowest genetic distances to reference strains of different species (and types within them) were observed with EV-I for VP1 and P1, with EV-F5 for P2, and with EV-F6 for P3 and the complete polyprotein. It is also worth noting that, except for the P3 region (which is generally the least variable), Soleuba-2066 and MFB-556 showed considerable divergence in both phylogenetic and genetic distance analyses. These somewhat contradictory results suggested a potential recombination event, which prompted further

investigation. This additional analysis provided evidence supporting the recombination event between EV-I and EV-F (Fig. 4), possibly giving rise to a common ancestor of BEV9 and MFB-556, which may have achieved bicontinental distribution in Asia (China) and South America (Uruguay).

Table 2
Estimates of evolutionary divergence between MFB-556 and the different types within species E, F, and I. The number of amino acid differences per site is shown, and the lowest distance are highlighted in bold.

	Polyprotein	VP1	P1	P2	P3
PV862188.1 Soleuba-2066	12.7	37.7	27.9	5.9	**1.1**
KP345887.1_Enterovirus_I	18.8	**30.0**	**20.2**	22.7	14.3
KP345888.1_Enterovirus_I	19.0	30.3	20.5	22.7	14.5
D00214.1_Enterovirus_E1	27.1	46.9	35.7	23.2	20.6
Enterovirus_E2	26.7	46.7	35.3	22.8	20.4
DQ092792.1_Enterovirus_E3	26.3	45.6	34.8	22.1	20.4
KU172420.1_Enterovirus_E5	26.8	45.6	34.7	22.8	21.7
DQ092770.1_Enterovirus_F1	14.6	38.1	28.2	7.1	5.4
DQ092795.1_Enterovirus_F2	14.4	38.2	27.7	6.9	5.4
DQ092794.1_Enterovirus_F3	14.4	40.8	29.8	4.3	5.2
AY462106.1_Enterovirus_F4	17.5	41.9	30.2	10.6	8.9
PQ415717.1_Enterovirus_F5	12.9	36.4	29.1	**2.6**	3.0
PQ415718.1_Enterovirus_F6	**12.6**	37.7	27.0	5.2	**2.2**
KU955844.1_Enterovirus_F7	16.0	42.6	32.3	5.0	6.6
LC038188.1_Enterovirus_F8	14.8	41.0	31.3	4.3	4.8
ON986121.1_BEV9/2021/CHN	**1.5**	**1.8**	**1.3**	**1.6**	**1.6**

Novel protoparvovirus

The parvovirus sequence obtained, CPBI/UYU/2024, was 980 nt in length, representing around 20% of the complete genome, and almost 50% of the NS1. Additionally, a scaffold of 1,779 nt in length was obtained comprising the complete NS1, although with low depth of coverage. To ensure a conservative and robust approach, the analyses were based on the 980-nt sequence. Nonetheless, the same analyses were also conducted using the complete NS1 sequence, and the results are shown in the supplementary material (Supplementary material 1). Within the *Parvoviridae* family, CPBI/UYU/2024 showed highest similarity to the recently described tusaviruses (genus *Protoparvovirus*) but exhibited high divergence (>

30%) at both nucleotide and amino acid levels, suggesting it may represent a novel species within *Protoparvovirus* genus (Table 3). Phylogenetic analysis supported this, placing the novel sequence with the tusavirus group but in a distinct clade, consistent with the divergence observed in the sequence identity analysis (Fig. 5).

Table 3
Evolutionary divergence between CPBI/UYU/2024 and related protoparvovirus

	p-distance (nt/aa)*
OL692339.2_Protoparvovirus_sp._strain_goat_tusavirus/KT-G5/2020/HUN	0.312/0.324
NC_075988.1_Tusavirus_1_strain_Tu491	0.315/0.324
OR734234.1_Ovine_tusavirus_strain_OTV/0215	0.325/0.324
PP083003.1_Protoparvovirus_incertum1_strain_NM21C6	0.327/0.324
OL692345.1_Protoparvovirus_sp._strain_sheep_tusavirus/TB7/2009/HUN	0.328/0.324
OL692343.1_Protoparvovirus_sp._strain_sheep_tusavirus/TB1/2009/HUN	0.328/0.324
OQ708493.1_Tusavirus_1_strain_GZ814	0.325/0.327
OL692346.1_Protoparvovirus_sp._strain_sheep_tusavirus/TB9/2010/HUN	0.327/0.324
OQ708494.1_Tusavirus_1_strain_GZ1068	0.326/0.333

*nt = nucleotide, aa = amino acid

Enterovirus and novel protoparvovirus PCR

Based on the virome analysis, two PCR assays were developed to detect enteroviruses and the novel protoparvovirus (tusavirus-like virus) in both individual samples and cell culture passages. All three individual samples tested positive for enterovirus, and two were positive for tusavirus-like virus. In addition, enterovirus was also detected in the cell culture passages, confirming successful viral isolation in MDBK cells. These results were confirmed by Sanger sequencing of the PCR products.

Sequence Data Availability

The nucleotide sequences obtained in this study have been deposited in GenBank under the following accession numbers: PV862187 (101/UY), PV862188 (Soleuba-2066), PV862189 (MFB-556), and PV872381 (CPBI/UYU/2024).

Discussion

The morbidity observed during the outbreak was 6%, which is within the typical range, as the percentage of affected animals in a herd usually ranges from 5–10%, although it can reach up to 50% within a few

days [1].

After ruling out the most common agents responsible for diarrhea in adult cattle, the presence of BToV was confirmed by RT-qPCR in two of the affected cows, one of which excreted the virus with a presumably high viral load based on the Ct value (20.5). This finding is particularly relevant, as although BToV has not been extensively studied, its presence has been associated with dysentery in adult cattle [8–10, 31]. This virus had previously been reported in Uruguay in cases of neonatal diarrhea, showing epidemiological similarities to BCoV [13]. In line with this, we now detected BToV in cases of diarrhea in adult cattle, in which BCoV is considered a possible etiological agent [2]. Through metagenomic analysis, the complete genome of BToV was recovered—representing the second complete genome reported in Uruguay and Latin America, and the fourth outside of Asia. This is noteworthy, as BToV detection has increased in recent years, mainly in cases of diarrhea but also in some respiratory infections, highlighting its potential role as a bovine pathogen. Understanding BToV evolution is therefore critical to mitigating its impact. Phylogenetic analysis showed that the genome clustered with the previously detected Uruguayan strain and Japanese strains, whereas all Chinese genomes grouped separately together with a recently reported sequence from Ethiopia.

To further explore possible viral etiologies associated with dysentery, we performed a virome analysis. Some of the eukaryotic viruses detected are commonly associated with plants (e.g., *Geminiviridae*, *Nanoviridae*, *Solemoviridae*, *Tombusviridae*), likely reflecting ingestion of forages by the cows - a common finding in humans eating vegetables [32], and even more common in herbivores such as cattle. Other detected viruses commonly infect fungi (*Genomoviridae*), or both plants and fungi (*Endornaviridae*). Members of the *Smacoviridae* family are suspected to infect animals, although association with methanogenic archaea has also been suggested [33]. In addition, 21 contigs corresponding to *Circoviridae* –a family of animal-infecting viruses–were detected. Circoviruses can cause a range of diseases, depending on the viral type, although some may be non-pathogenic [34, 35]. Most of these eukaryotic viruses are members of the phylum *Cressdnaviricota*, which includes small circular DNA viruses, typically encoding just two proteins, a genome replication initiation protein (Rep) and a capsid protein. These genomes are known or predicted to replicate by a rolling-circle mechanism and are grouped as circular Rep-encoding ssDNA (CRESS-DNA) viruses [36].

Notably, in addition to the BToV complete genome, three other viruses infecting cattle were described. Two complete or near-complete genomes of distinct EVs and a partial genome of a parvovirus were detected. Identifying the precise causative agent of an infectious diarrhea outbreak in livestock is often challenging, not only due to the complexity of pathogen detection but also because many potential contributing pathogens and non-infectious predisposing or causative factors remain unknown or uncharacterized [37]. While this complexity poses a challenge, it also provides opportunities for a deeper understanding of disease dynamics and pathogen discovery. In this case, the detection of BToV is particularly compelling, as it has previously been implicated in dysentery outbreaks in cattle [8–10, 31], and may have played a role in this outbreak. However, given the inherent limitations of this observational study and the detection of other enteric viruses in the affected animals, whether BToV was causally

associated with the diarrhea outbreak we investigated remains uncertain. The potential involvement of EVs —particularly the novel EV-I— and the novel protoparvovirus in dysentery outbreaks should be further investigated.

Regarding EVs, their pathogenic role in cattle remains unclear. In this study, EVs were the only viruses consistently detected in all three diarrheic samples. Although this finding is far from conclusive, a causal or contributing role in the diarrheic condition cannot be ruled out. It is also possible that enterovirus infection increases susceptibility to diarrhea caused by other pathogens. In this regard, EVs were successfully isolated in MDBK cells, with cytopathic effects observed from the first passage and progressively increasing. By the third passage, the monolayer was completely detached within 12 hours post-inoculation. The virus was also detected in all passages by conventional PCR. This successful isolation opens the possibility of performing animal challenge experiments to further investigate the potential pathogenicity of these viruses under field conditions.

Phylogenetic analyses identified two distinct enteroviruses. One was classified as *Enterovirus fitauri* (EV-F) —specifically type F6— which has been previously reported in cattle. The other was a divergent enterovirus whose classification still needs confirmation. This strain does not meet all the criteria for inclusion within any currently recognized species. Depending on the genomic region analyzed, it could be classified either as *Enterovirus idromi* (EV-I) or *Enterovirus fitauri* (EV-F). Based on the P1 region (as well as VP1, which is commonly used for enterovirus classification), this divergent virus could be considered a new type of EV-I. However, analyses of the P2 and P3 regions clustered it within EV-F, suggesting a possible interspecies recombination event, which was further supported by recombination analysis. For recombination to occur, co-infection is necessary —a condition met in the present study– where animals were co-infected with EV-F6 and the divergent enterovirus. Another point worth noting is that, if this divergent virus is indeed a novel EV-I type, it would represent a new host record, as EV-I has thus far only been detected in dromedaries [38]. Interestingly, the EV-I sequences have now been detected in at least two individuals: dromedaries in the Middle East, and cattle in Uruguay (this study) and China (BEV9). These findings raise interesting questions about the cross-species transmission potential and the broader geographic distribution of *Enterovirus idromi*.

In 2014, a novel parvovirus was described in a child with unexplained diarrhea and was classified within the genus *Protoparvovirus* [39]. Since then, it has been sporadically detected in humans, generally at low prevalence [40–43], and more recently in goats and sheep [44–46]. Although we could not recover the complete genome, a partial sequence of the NS1 gene representing approximately 20% of the genome was obtained. Despite the high coverage of this region, concerns about the accuracy of the assembly could arise. To confirm the sequence and simultaneously assess the presence of the virus in the individual samples, primers targeting the NS1 region were designed, and PCR amplification confirmed its presence in 2 of the 3 samples. Phylogenetic analyses revealed substantial divergence. Although the sequence clustered with tusaviruses from humans, goats, and sheep, it likely represents a novel *Protoparvovirus* species. According to the current ICTV species demarcation criteria (https://ictv.global/report/chapter/parvoviridae/parvoviridae/protoparvovirus, last accessed July 2025),

members of a species must be monophyletic and share more than 85% amino acid sequence identity in the NS1. Although the full-length NS1 showed low depth of coverage, a limitation of this study, the partial sequence region (~ 50% of the full protein) showed > 30% divergence from known tusaviruses, suggesting the detection of a new *Protoparvovirus* species. Full genome sequencing would be ideal to confirm this classification.

A previous study did not detect tusavirus in 95 bovine fecal samples [44]. However, the primers used were designed based on tusavirus sequences from humans and goats, which—as shown in this study—are highly divergent. This likely limited the ability of detecting divergent strains. Further investigation is needed to better understand the prevalence and potential pathogenicity of this novel protoparvovirus in cattle.

The advent of viral metagenomics has significantly advanced our ability to identify pathogenic and non-pathogenic viruses and unravel the complexities of disease etiology. In many cases, it also provides insights into potential interactions among co-infecting pathogens. Determining whether a single virus or a synergistic interaction between multiple agents triggers disease is often the most challenging aspect. Nonetheless, identifying the viral repertoire present in affected animals is a necessary first step toward that goal. In this case, while BToV could have had a causative role, given its prior association with similar syndromes, the concurrent detection of other viruses —some of which may have contributed directly or indirectly to disease onset— should also be considered.

Conclusions

This study provides new insights into the viral etiology of a diarrhea outbreak in adult cattle, confirming the presence of BToV and uncovering additional viruses with potential pathogenic relevance. The detection of BToV—with a complete genome recovered— suggest its potential role in the outbreak, consistent with previous reports linking BToV to dysentery in adult cattle and adds valuable genomic data for future comparative studies. However, its precise role in the outbreak remains to be conclusively determined, especially in light of additional viral detections. Notably, two distinct enteroviruses—including a divergent strain potentially representing a new EV-I type—and a highly divergent tusavirus-like protoparvovirus were also detected. These findings expand the known diversity and host range of enteric viruses in cattle and suggest that viral co-infections may play a more significant role in disease outbreaks than previously recognized. The use of metagenomics was key to revealing this hidden virome, underscoring its importance as a tool for pathogen discovery and surveillance. Overall, the study contributes to a better understanding of bovine enteric diseases and highlights the need for continued investigation into the role of emerging and understudied viruses in livestock health.

Declarations

Ethics approval and consent to participate

Sampling procedures were reviewed and approved by the Ethics Committee on Animal Use of the National Institute of Agricultural Research of Uruguay (protocol number 2019.9).

Consent for publication

Not applicable

Availability of data and materials

The nucleotide sequences obtained in this study have been deposited in GenBank under the following accession numbers: PV862187, PV862188, PV862189, and PV872381. All data used to support the findings of this study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by the Uruguayan “Agencia Nacional de Investigación e Innovación” (ANII) under grant FCE_3_2022_1_172734, and the “Instituto Nacional de Investigación Agropecuaria” (INIA), grant PL_27.

Authors' contributions

MC conceived and designed the study, obtained the financial support, performed the virological analyses, analyzed the data, and wrote the manuscript. MJB-G performed the bioinformatics analyses, analyzed the data, and wrote the manuscript. LM performed the viral isolation. MLC, LS and AS performed the bacteriological and parasitological analyses. AL participated in the experimental design. MF, FG, NR-O and RC supervised parts of the experimental and/or laboratory work. FG obtained funding. All authors read, edited and approved the final manuscript.

Acknowledgements

MC and FG acknowledge support from ANII through “Sistema Nacional de Investigadores” (SNI). MC acknowledges support from PEDECIBA through “Investigador del área Biología”.

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Figures

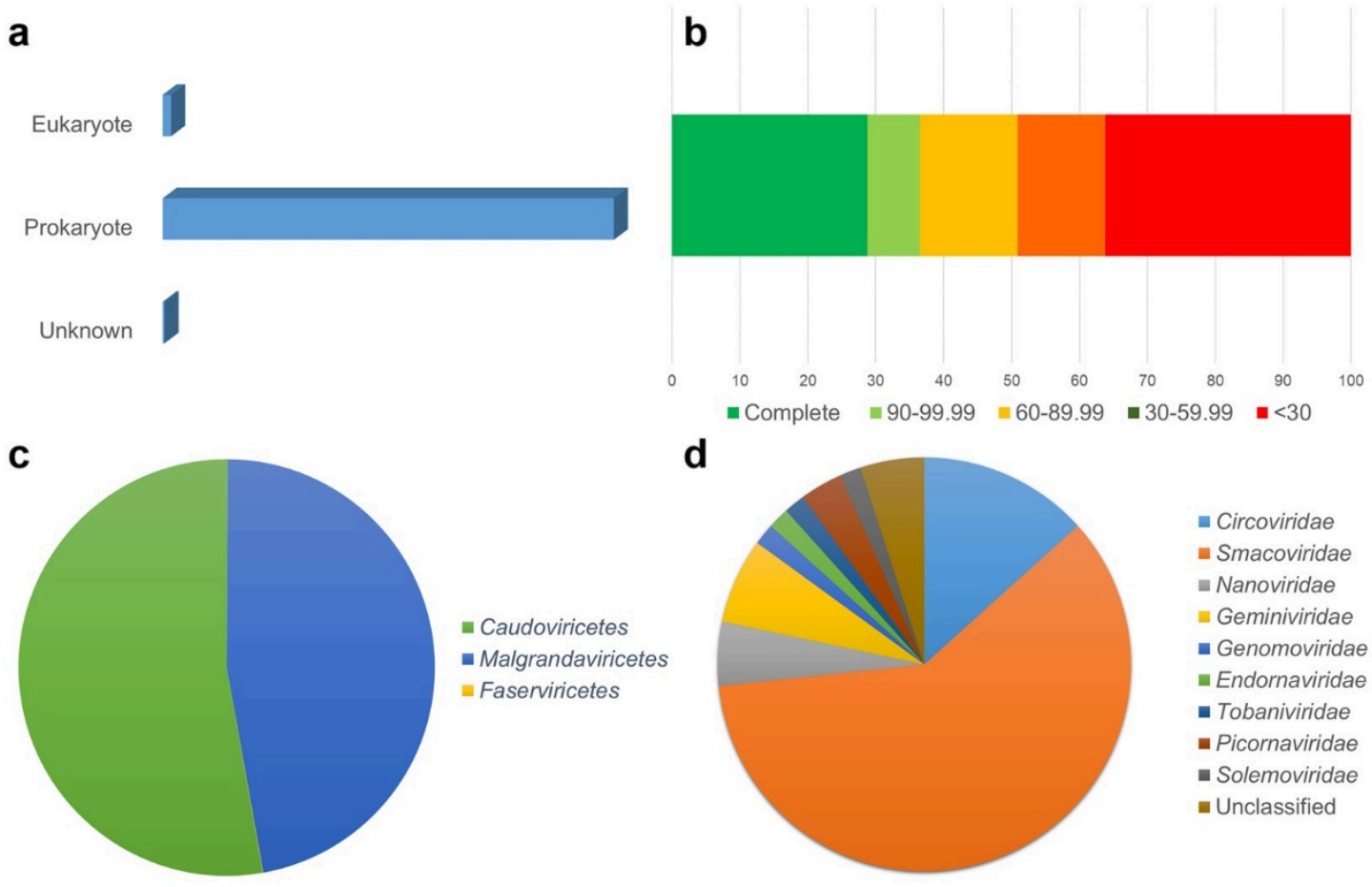

Figure 1

Description of the virome in the fecal pool of the diarrheic cows. a) Distribution of vOTU by host type. b) Genome completeness of detected vOTUs including complete genomes and the proportions of partial genomes across different coverage ranges. c) Abundance of vOTUs among prokaryotic viruses. d) Abundance of vOTUs among eukaryotic viruses.

Figure 2

Phylogenetic analysis of bovine torovirus. The maximum likelihood tree is shown, with the sequence obtained herein labeled with a blue circle and the previously obtained sequence from Uruguay labeled with a blue triangle. Host species are shown on the right.

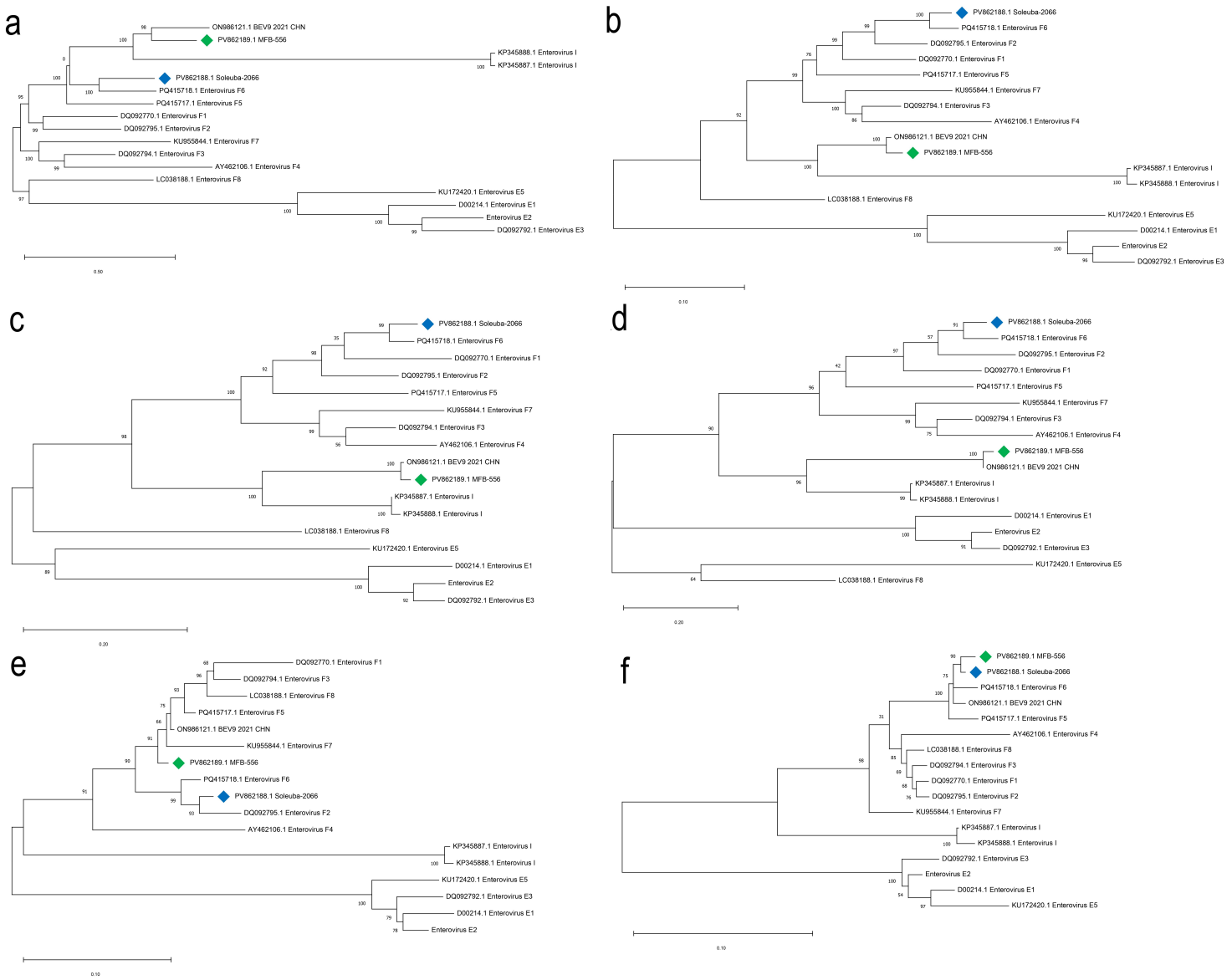

Figure 3

Phylogenetic analyses of enteroviruses. Maximum likelihood trees based on (a) complete genome, (b) polyprotein, (c) P1, (d) VP1, (e) P2, and (f) P3 are shown, constructed using full-length nucleotide sequences (a) and amino acid sequences (b–f). In all analyses, reference sequences representing the different types within species E, F, and I were included. The sequences obtained in this study are highlighted with green and blue diamonds (MFB-556 and Soleuba-2066, respectively).

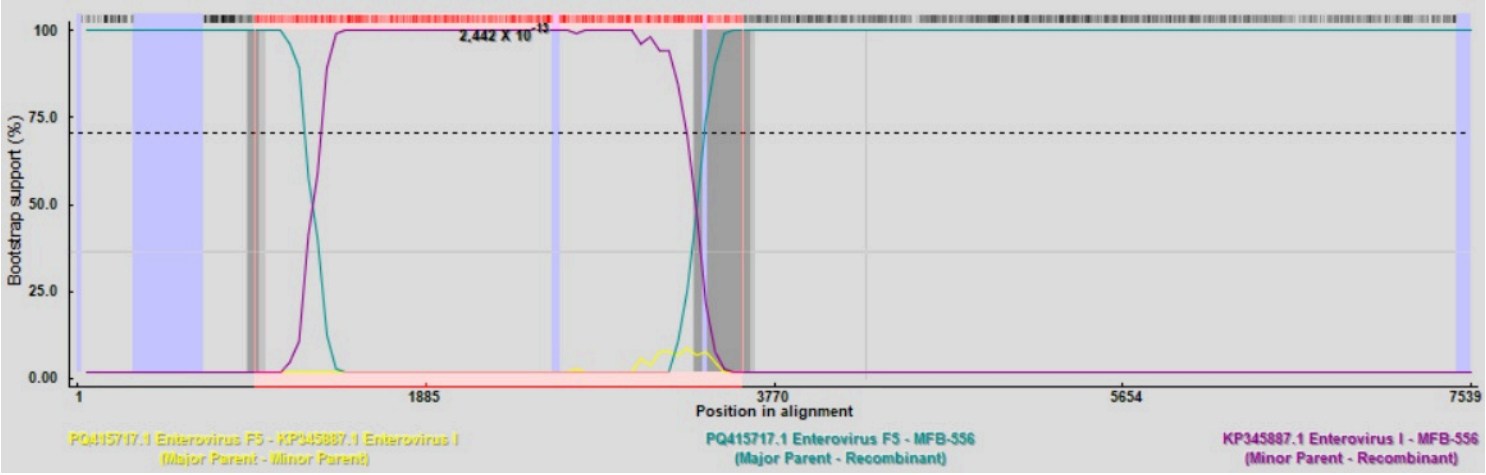

Figure 4

Recombination analysis of MFB-556. Bootscan results generated with the RDP5 program is shown. The MFB-556 sequence and all reference sequences for the different types of enterovirus E, F, and I were used as dataset and default parameters.

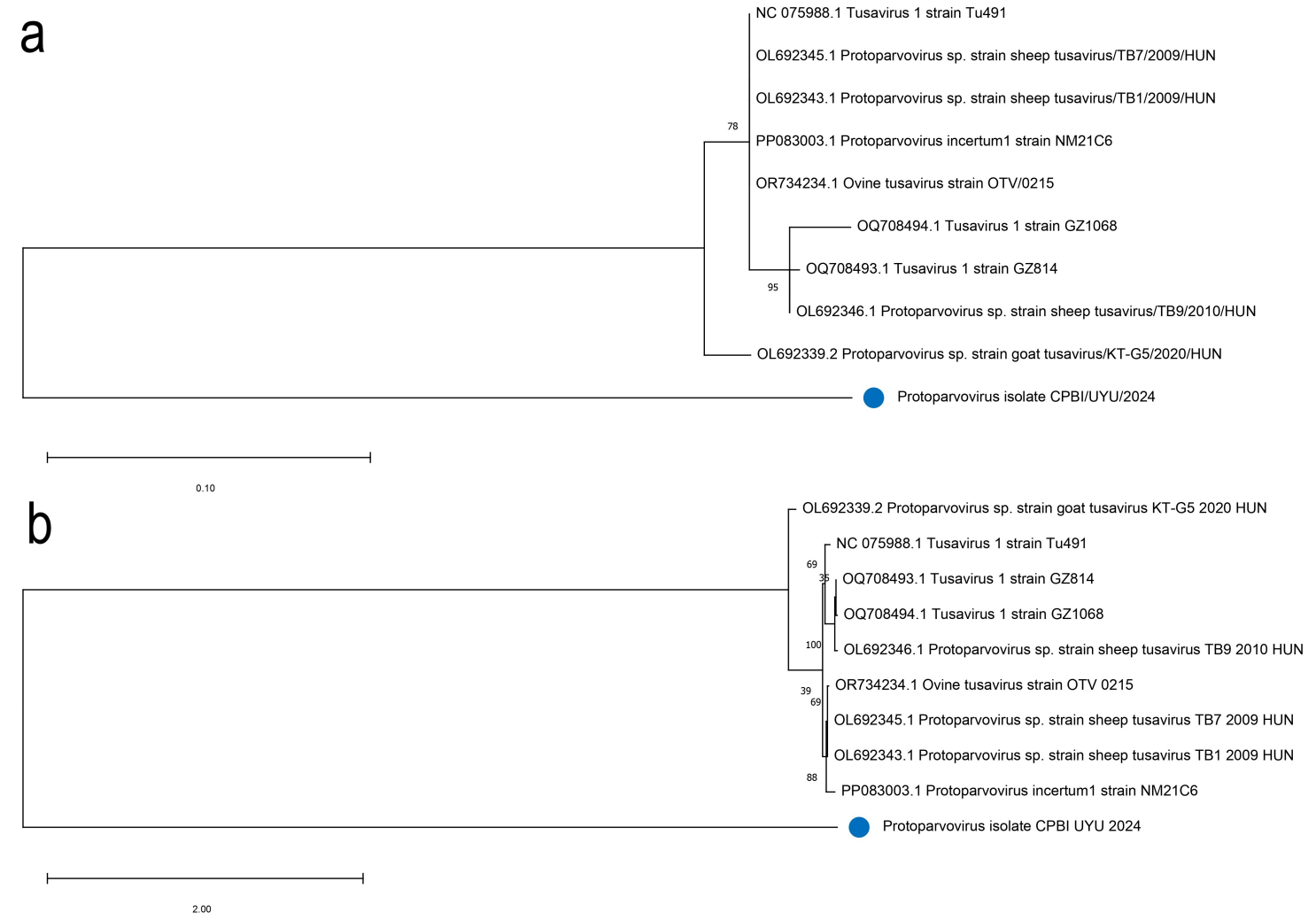

Figure 5

Phylogenetic analysis of CPBI/UYU/2024 and the closest related protoparvoviruses. Maximum likelihood trees based on amino acid (a) and nucleotide (b) sequences. aLRT values and scale bars are shown for both trees. The sequence obtained in this study is highlighted with a blue circle.

Supplementary Files

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