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Research Article

Oral and Faecal Viromes of New Zealand Calves on Pasture With an Idiopathic Ill-Thrift Syndrome

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Since 2015, an idiopathic ill-thrift syndrome featuring diarrhoea and, in some cases, gastrointestinal ulceration has been reported in weaned New Zealand dairy calves. Similar syndromes have been described in the British Isles and Australia, but investigations in New Zealand have yet to identify a specific cause. Notably, the viromes of affected calves remain understudied. We conducted metatranscriptomic analyses of oral and faecal viromes in 11 calves from a dairy farm in Taranaki, New Zealand, experiencing an outbreak of this syndrome. This included nine calves showing clinical signs. Our analysis identified 18 bovine-associated viruses across two DNA and three RNA viral families, including six novel species. Oral viromes were dominated by *Pseudocowpox virus*, which was detected in all calves with oral lesions. Faecal viromes were more diverse, featuring adenoviruses, caliciviruses, astroviruses and picornaviruses. *Bovine bopivirus*, from the *Picornaviridae* family and previously unreported in New Zealand, was significantly associated with calves showing oral lesions and diarrhoea, indicating a possible link to disease, though its role remains unclear. The diverse viral communities of the calves complicate the identification of a single causative agent. Importantly, no novel viruses were significantly associated with the syndrome, and the viromes closely resembled those found in cattle globally. These findings suggest the syndrome likely has a multifactorial origin involving nutritional, management and environmental factors rather than being driven primarily by known or novel viruses. Further, research across regions and seasons is recommended to clarify the role of viruses in idiopathic ill-thrift among New Zealand calves.

Keywords: *Bovine bopivirus*; calf; enteritis; idiopathic ill-thrift; New Zealand; *Pseudocowpox virus*; stomatitis; virus discovery

1. Introduction

Cattle (*Bos taurus*) play an important role in New Zealand's economy, and were first introduced in the early 1800s with imports from Australia and the United Kingdom [1]. Live cattle imports continued for many years from multiple countries, including Chile [1] and the United States [2]; however, no live cattle have been imported into New Zealand since 2013, and animal product imports are subject to strict biosecurity controls. New Zealand, is now the world's largest exporter of dairy, which contributes over USD$6 billion to its economy annually [3]. Dairy cows in New Zealand, rely predominantly on a grass-based diet, and are therefore,

bred on a seasonal basis to coincide the dietary requirements of milk production with suitable pasture growth [2].

An idiopathic ill-thrift syndrome in weaned dairy calves aged between 3 and 12 months has been reported in the United Kingdom and the Republic of Ireland (where it is termed 'summer scour syndrome') and Australia (referred to as 'upper alimentary tract ulcerative syndrome [UAUS])' between 2015 and 2019 [4–6]. The ill-thrift is accompanied by diarrhoea (scour) in a proportion of the calves, with a smaller proportion having severe erosive or ulcerative oral and oesophageal lesions [7]. Notably, the condition is unresponsive to empirical treatments and investigations have ruled out common parasitic, bacterial or viral agents as a cause [5]. The New Zealand syndrome observed during the same period has tentatively been referred to as 'calf ulcerative stomatitis (CUS)' given the presence of oral lesions in a proportion of the affected calves [8]. Outbreaks of the syndrome in New Zealand have been extensively investigated, and exotic diseases have been conclusively excluded in all cases on clinical and epidemiological grounds. Further to this, samples from a number of outbreaks have been submitted to the Animal Health Laboratory (New Zealand Ministry for Primary Industries) where molecular and serological diagnostics for foot-and-mouth Disease (FMD) have all returned negative results.

Multidisciplinary investigations involving faecal examination and culture, serology, molecular assays, virus isolation, necropsies, biopsies and histological or transmission electron microscopy examination of tissues have excluded likely endemic differentials, including parasitic gastroenteritis, coccidiosis, salmonellosis, bovine viral diarrhoea (BVD), calf diphtheria (*Fusobacterum necrophorum*) and malignant catarrhal fever. Investigations into the syndromes overseas have similarly excluded these differential diagnoses [6]. While poxviruses have been found in a number of cases with upper gastrointestinal tract lesions, including in New Zealand, these are considered to be secondary infections in immunocompromised calves. Furthermore, poxviruses in cows are rarely linked to severe disease [9]. In Australia, metatranscriptomics on oral swabs and oesopharyngeal samples from confirmed UAUS cases did not identify any novel or consistently associated viral agents [4]. Pre- and post-weaning calf feeding and management practices leading to poor rumen development and dysfunction have been proposed as playing a role [7]. These, along with environmental stressors and secondary opportunistic infections have led to the suggestion of a multifactorial aetiology [6, 10].

New Zealand has maintained freedom from many pathogenic diseases of cattle, such as foot and mouth disease virus (FMDV), and has successfully eradicated other pathogens, including anthrax (*Bacillus anthracis*) and bovine brucellosis (*Brucella abortus*) [11]. Other significant infectious diseases such as bovine tuberculosis (*Mycobacterium bovis*) and *Mycoplasma bovis* are controlled through various management and eradication programmes [12, 13]. Viral diseases of cattle are generally well studied and characterised globally due to the economic importance of these animals [14–17]. The emergence or introduction of infectious diseases remains a concern; however, New Zealand maintains a comprehensive biosecurity and surveillance system to ensure that cattle industries in New Zealand remain free of potentially exotic or detrimental disease outbreaks [18].

We applied metatranscriptomics to investigate oral and faecal samples collected from 11 calves in a mob of 100 on a dairy support farm in Taranaki, New Zealand, experiencing an outbreak of the idiopathic ill-thrift syndrome—the seventh outbreak on the farm in 8 years. Samples were collected from calves with and without oral lesions and/or diarrhoea, to characterise their viral communities and identify viruses potentially significantly associated with the syndrome.

2. Methods

2.1. Animal Ethics. Samples were collected as part of a disease investigation under the New Zealand Biosecurity Act 1993, so animal ethics approval was not required.

2.2. Sample Collection and Disease Characterisation. The July–August 2023 born, Friesian–Jersey cross dairy replacement heifers had been moved to the support farm from three dairy platforms from early November onwards and run in two mobs. On December 7, the farm manager observed that one mob of 100 calves had, as a whole, lost body condition despite being on good pasture and supplemented with concentrate feed. A greater proportion of the calves in this mob were observed to have faecal staining and/or matting on the perineum and/or tail compared to the other similar age mob on the farm. Over the course of 1 week, 14 calves from the affected mob were determined to be sick and draughted out by farm staff for closer examination and isolation in a hospital paddock. When examined, a number of these calves were noted to have oral lesions.

On December 15, an MPI incursion investigator visited the farm along with the farm veterinarian. Five healthy (non-hospital paddock) calves, as assessed by farm staff were selected for examination. Two of these calves (Y18 and Y14) had tongue lesions, while a third calf (Y52) had evidence of diarrhoea (tail matting). Six calves from the hospital paddock mob of 14 were also selected and presented by farm staff. All six calves had oral lesions. Calves had lesions on the tongue ($n = 5$), cheek ($n = 3$) and dental pad ($n = 2$). Calf W64 only had lesions on the dental pad. Five calves had tail matting or perineal staining. One calf (P18) had lesions on the external surface of the lower lip. Overall, three had oral lesions only, one had diarrhoea only, five had oral lesions and diarrhoea and two were normal on clinical examination Table 1.

Oral and rectal swabs were collected from these 11 calves. In collecting swabs from calves with oral lesions, the lesions were vigorously swabbed. Swabs were placed in DNA/RNA Shield (Zymo Research), kept chilled and placed in a −20°C freezer within 6 h and transferred to −80°C freezer within 72 h. Under sedation and local anaesthetic lesions from calves Y17 and P18 were biopsied. Samples were fixed in 10% neutral formalin and processed routinely for histology by a commercial veterinary diagnostic laboratory.

2.3. RNA Extraction and Sequencing. Frozen oral and faecal swabs stored in DNA/RNA Shield were defrosted and, using

Table 1: Summary of clinical signs in the examined calves.

Clinical examination	Non-hospital paddocks	Hospital paddock	Total
Normal	2	0	2
Oral lesions only	2	1	3
Diarrhoea only	1	0	1
Oral lesions and diarrhoea	0	5	5
Total	5	6	11

sterile forceps, transferred to BashingBead lysis tubes (0.1 and 0.5 mm) (Zymo Research) filled with 750 µL of fresh DNA/RNA Shield. Samples underwent a total of 5 min of lysis in a Mini-BeadBeater-24 disruptor (Biospec Products Inc.) at 0.5–1.5-min increments, with 30 s on ice in between. About 200 µL of supernatant was transferred to a sterile microfuge tube and incubated with Proteinase K. Total RNA was extracted and purified following the ZymoBIOMICS MagBead RNA kit protocol (Zymo Research), including the optional DNase I incubation step to remove DNA contaminants. RNA was quantified using a NanoDrop Spectrophotometer (ThermoFisher) and each of the 22 samples (11 oral and 11 faecal) was subject to total RNA sequencing. The Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit (Illumina) was used for library preparation. Libraries were sequenced on the Illumina NovaSeq 6000 platform, and 150 bp paired-end reads were generated at the Australian Genomics Research Facility in Melbourne, Australia.

2.4. Transcriptome Assembly, Annotation and Abundance Estimation. Forward and reverse reads from the 22 sequencing libraries were trimmed using Trimmomatic (v0.38) [19] to remove Nextera paired-end adapters. Bases were cut if they fell below a quality of five using a sliding window approach or if below a quality of three at the beginning or end of the reads. Library quality was assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmed reads were then assembled de novo using MEGAHIT (v1.2.9) [20] to generate a metatranscriptome for combined oral samples and for combined faecal samples.

Assembled contigs were annotated using a sequence similarity-based approach. Contigs were annotated at the nucleotide level against NCBI's nucleotide (nt) database using the BLASTn algorithm [21] and at the protein level against the non-redundant (nr) protein database using DIAMOND BLASTx (v2.02.2) [22] with the 'more-sensitive' flag set. Both searches were run with e-value cutoffs of 1×10^{-5}. Contig abundances were estimated by mapping sequencing reads from each of the 11 individuals to the oral and faecal metatranscriptomes using Bowtie2 with default parameters [23]. The annotated protein outputs were merged into two tables, one for each sample type, containing the annotated contigs and abundances for those contigs in each calf. Read counts were standardised to reads per million (RPM) by first dividing contig counts in each library by their respective library size and multiplying these by 1 million.

2.5. Bovine Virus Discovery. Bovine-associated viruses were screened for manually by filtering contigs by those annotated as being from the viruses domain/superkingdom. Viral sequences with e-values $<1 \times 10^{-10}$ were inspected with additional BLASTn and BLASTp searches using the online BLAST tool to eliminate false positive hits and potentially endogenous viral elements (EVEs). Putative viruses were considered if their top BLASTp genetic matches were to known vertebrate host-infecting viruses or those known to infect cattle (*Bos*).

2.6. Phylogenetic Analysis of Bovine Viruses. RNA-dependent RNA polymerases (RdRps), DNA polymerases or hexon proteins (in the case of adenoviruses) from putative viruses identified in the calf metatranscriptomes were collated and organised by their proposed viral families for phylogenetic analysis. Viruses were placed into an evolutionary context using maximum-likelihood phylogenetic trees generated using IQ-TREE (v1.6.12) [24]. First, representative viral sequences from each genus in the viral families identified were collected from NCBI's Taxonomy database (https://www.ncbi.nlm.nih.gov/taxonomy) and amino acid translations of these were aligned with the bovine-associated viruses identified here using the L-INS-i algorithm in MAFFT (v7.450) [25]. Alignments were manually inspected to observe the alignment of conserved motifs and ambiguously aligned regions were trimmed using trimAL (v1.2) [26] with the 'automated1' flag set. Then, IQ-TREE was used to generate phylogenies based on these alignments with the ModelFinder 'MFP' flag set to select the best-fit model of amino acid substitution and 1000 ultrafast bootstrapping replicates [27]. The 'alrt' flag was also added to perform 1000 bootstrap replicates for the SH-like approximate likelihood ratio test [28]. The resulting phylogenetic tree was rooted at its midpoint and annotated in FigTree (v1.4.4) (http://tree.bio.ed.ac.uk/software/figtree/). Viruses were determined to be novel if they shared less than 90% amino acid identity with other known viruses across the analysed region or protein sequence.

2.7. Differential Viral Abundance Analysis. To determine which viral groups were more abundant in calves with oral lesions compared to calves without oral lesions, a differential abundance analysis was performed using the DESeq2 package in R (v4.11) with eight 'affected' calves and three 'unaffected' calves as controls (Table 1, Figure 1B). We also repeated the analysis between calves with (five) and without (six) diarrhoea and those with both key clinical signs (oral lesions and diarrhoea) (four) versus those with none or only one clinical sign (seven). Total virome abundances (as raw reads) of the calves were condensed at the genus level where possible, followed by family, order or as 'Unclassified Riboviria' (RNA viruses) or 'Unclassified DNA viruses' where more detailed taxonomic

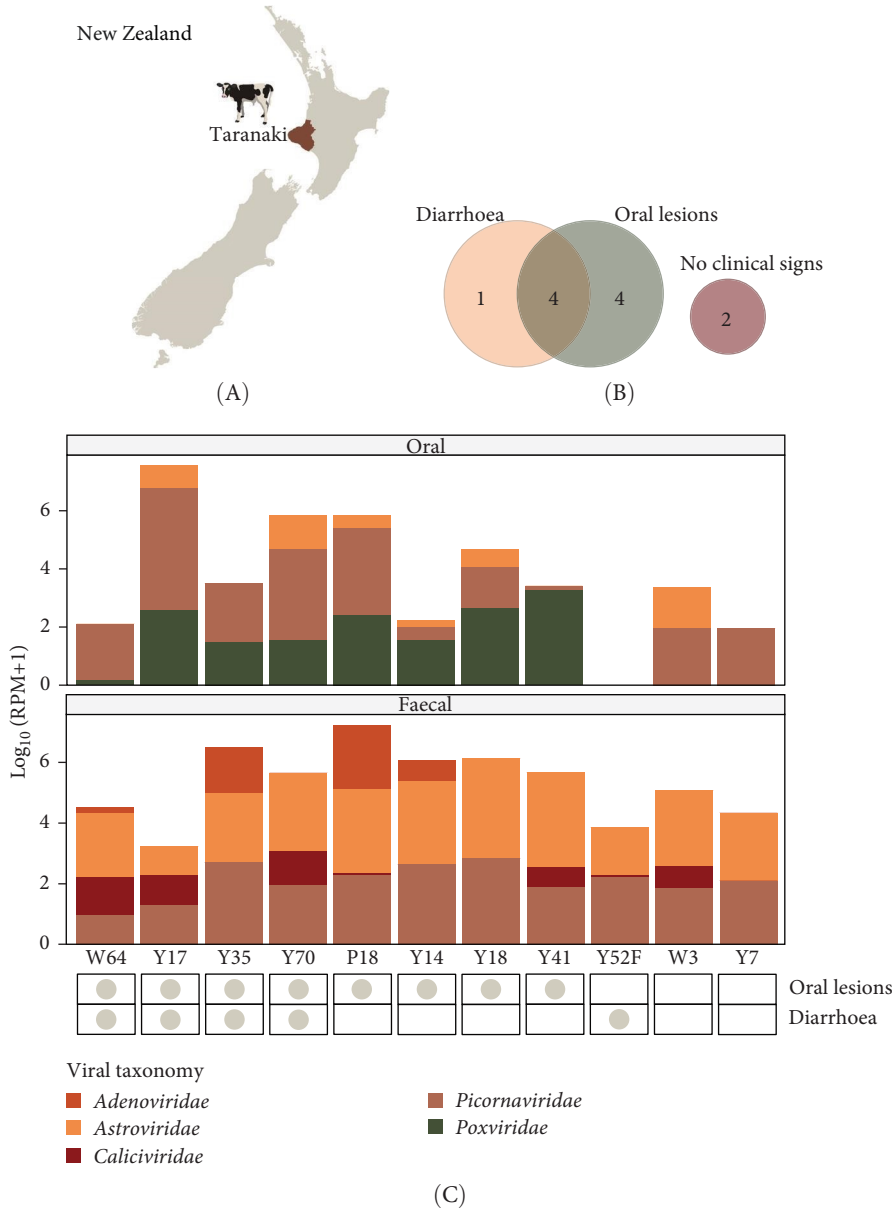

FIGURE 1: Summary of ill-thrift calves and abundance of mammal-associated viral families. (A) Map of New Zealand with Taranaki highlighted. (B) Venn diagram of the number of calves affected by ill-thrift. (C) Bar plot of $\log_{10}$-transformed RPM counts of viral families in the 11 calves. Viral families present in oral samples are shown in the top panel and those in faecal samples are shown on the bottom panel. A chart showing which clinical signs the calves were affected by is shown below.

information was not available. Viral groups were considered significantly more abundant in affected calves when the FDR-adjusted p-values (q-values) were <0.05. Viral abundance differences were considered in terms of their change in unaffected calves. The Benjamini–Hochberg (BH) multiple correction procedure was used to readjust p-values.

The initial categorisation of the calves into healthy (non-hostpital paddock) and unhealthy (hospital paddock) groups, when sampled by MPI in December 2023, was based on a diagnosis made by farm staff. However, the farm staff were unlikely to have carried out thorough oral examinations and were focused on removing the most severely affected calves to the hospital paddock. As such, we feel that recategorising the calves into affected and unaffected, based on the results of the clinical examination carried out by the veterinarians (Table S1), was justified to broadly compare the viromes of more severely affected calves to those without oral lesions.

3. Results

3.1. Histology of Oral Lesions. Histologically, oral lesions were characterised by multifocal areas of ulceration (Figure 2A,B), covered by crusts composed of neutrophils, karyorrhectic debris and fibrin (Figure 2C,E). There was hyperplasia (thickening) of the adjacent intact mucosa (Figure 2C), with variable degrees of ballooning degeneration of keratinocytes in the

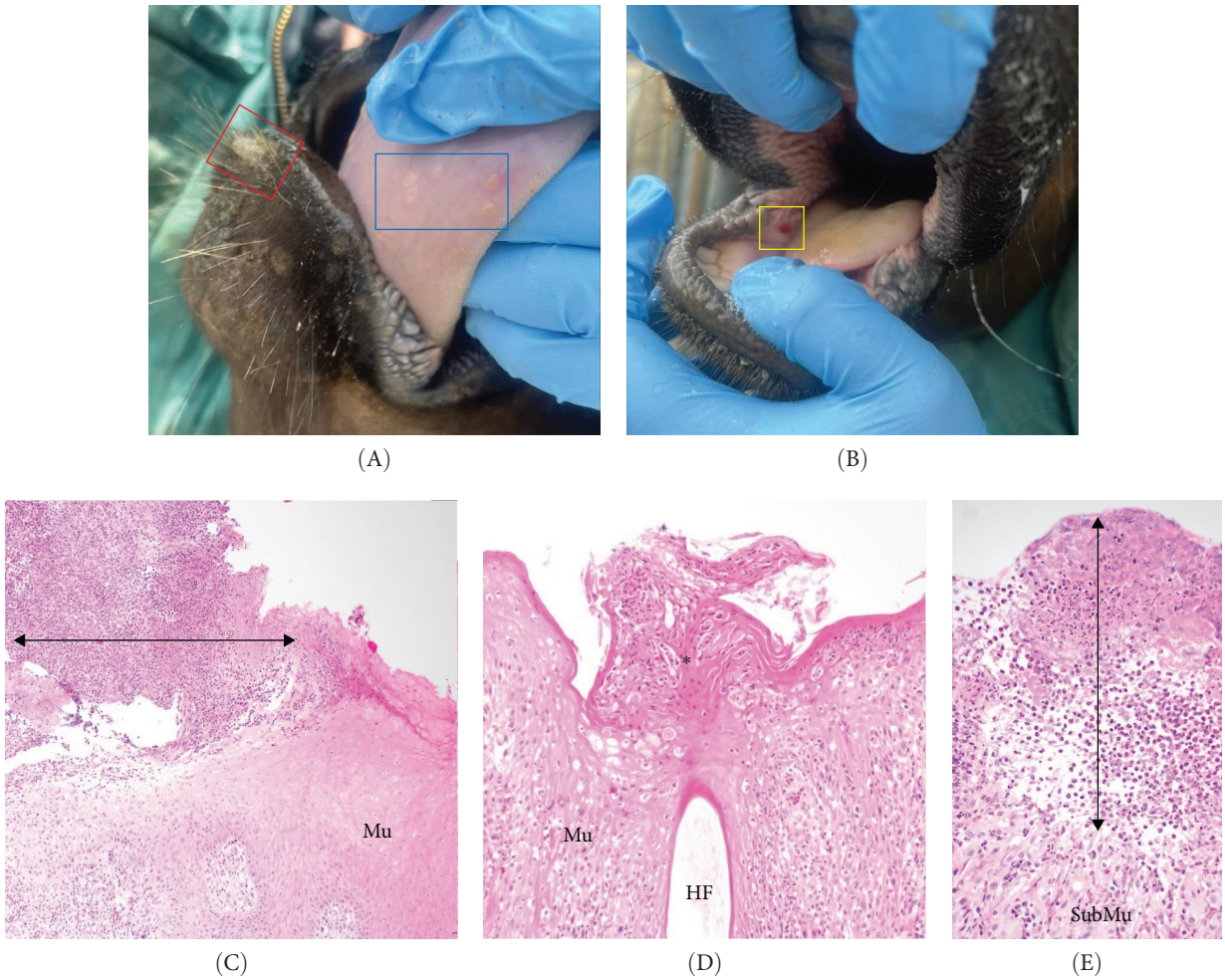

FIGURE 2: Clinical and histological appearance of oral lesions in calves with idiopathic ill-thrift. (A) Multifocal pale circular lesions on the underside of the tongue (blue box) and an area of thickened skin on the lower lip (red box). (B) A focal area of ulceration in the mucosa of the right lower cheek. (C) Histology of the tongue lesions in the blue box in image A. There is an area of ulceration (arrow) which is covered by a thick crust composed of degenerate neutrophils, fibrin and karyorrhectic debris. The adjacent mucosa (Mu) is hyperplastic. (D. Histology of the lip lesion in the red box in image A. The mucosa (Mu) is hyperplastic and there is marked ballooning degeneration of keratinocytes in the stratum corneum and spinosum. Overlying a hair follicle (HF), there is hyperkeratosis and sloughing keratocytes are admixed with small numbers of neutrophils (*). There are occasional hypereosinophilic, rounded keratinocytes (apoptotic cells), as shown in the inset. (E) Histology of the cheek lesion in the yellow box in image B. There is no identifiable mucosa as it is ulcerated and there are large numbers of neutrophils mixed with fibrin in its place (arrow). Inflammatory cells extend into the submucosa (SubMu) and are accompanied by loose connective tissue proliferation (granulation tissue).

stratum corneum and stratum spinosum (Figure 2D). Occasional rounded, hypereosinophilic keratinocytes were present in the intact mucosa, consistent with apoptotic cells (Figure 2D). There were no apparent viral inclusion bodies in the sections examined. These findings are consistent with previous cases of idiopathic ill-thrift in calves in New Zealand (H. Hunt, pers. comm.).

3.2. Sequencing and Total Viromes. Metatranscriptomes for each of the two sample types were assembled from all 11 calves, producing 381,570 oral-associated contigs and 2,834,178 faecal-associated contigs. Viruses accounted for 2%–23% of oral and 25%–91% of the faecal reads across the calves (Table 2). Overall, faecal samples contained a much higher proportion of viral reads than oral samples, likely owing to the high number of bacteriophage-associated sequences in faecal samples after depletion of host and bacterial rRNA during sequence library preparation. Viral reads accounted for 19,258–227,012 RPM in oral samples and 316,616–912,472 RPM in faecal samples (Table 2).

We identified mammal-associated viruses from two DNA viral families (*Adenoviridae* and *Poxviridae*) and three RNA viral families (*Astroviridae*, *Caliciviridae* and *Picornaviridae*) in the calf samples (Figure 1C). *Picornaviridae* was the most widespread family, with 91% of oral samples and 100% of faecal samples containing picornaviral sequences. Astroviruses were also present in all faecal samples. *Poxviridae* was the second most abundant family in oral samples and was only identified in calves with oral lesions. Adenoviruses and caliciviruses were also primarily found in faecal samples from affected calves but were only associated with 50% and 63% of affected calves, respectively.

Table 2: Calf sequencing library and metatranscriptome details and total virome abundances.

Calf ID	Oral reads	Faecal reads	Oral virome RPM	Faecal virome RPM	Total oral virome (%)	Total faecal virome (%)
P18	18,381,405	14,705,921	88,339	526,174	9	53
W64	12,838,378	13,216,843	19,258	316,616	2	32
Y14	15,485,106	12,354,419	135,156	346,992	14	35
Y17	14,220,387	11,868,221	78,066	912,472	8	91
Y18	16,365,398	13,717,264	139,326	292,950	14	29
Y35	13,484,657	14,014,061	87,765	362,560	9	36
Y41	18,880,150	11,767,740	66,553	254,106	7	25
Y70	32,056,352	12,210,744	94,466	378,581	9	3
W3	20,375,283	10,345,261	36,856	514,932	4	51
Y7	15,275,728	11,823,969	227,012	615,467	23	62
Y52	13,268,730	13,219,497	21,622	467,734	2	46

Six mammal-associated genera within the *Picornaviridae* (*Apthovirus* (*Bovine rhinitis* viruses), *Bopivirus*, *Enterovirus*, *Hunnivirus*, *Kobuvirus*, and *Parechovirus*) and one genus within the *Astroviridae* (*Mamastrovirus*) were identified across the samples. Of these, enteroviruses, kobuviruses and *Bovine rhinitis* viruses as well as mamastroviruses, were the most widespread and abundant across the samples, being found in both affected and unaffected calves (Figure 3 and Figure S1). *Pseudocowpox virus* (genus: *Parapoxvirus*), known to be infecting calves from Taranaki, was also identified only in calves affected by oral lesions at relatively high abundance (1.4–465 RPM in oral samples). The genera *Nebovirus* (family: *Calciviridae*), *Mastadenovirus*, *Bopivirus*, *Hunnivirus* and *Parechovirus* were either absent or present at very low abundances in unaffected calves. On average, calves harboured 7.2 viral genera (affected: 7.5 and unaffected: 6.3) across the two sample types (Figure 3 and Figure S1).

3.3. Phylogenetic Analysis of Bovine Viruses. To determine the evolutionary histories and novelty of the bovine-associated viruses identified in the calves, we estimated phylogenies using polymerase or hexon protein sequences from the identified species (Figure 4 and Table 3). Five viral families, represented by 18 viruses, could be assessed: the *Adenoviridae* ($n = 1$), *Poxviridae* ($n = 1$), *Astroviridae* ($n = 2$), *Caliciviridae* ($n = 1$) and *Picornaviridae* ($n = 13$). Of these species, six were presumed to be novel. The identified viruses are described in the following sections.

3.4. Oral Viruses. RdRp sequences from three viral species identified in oral samples could be analysed—*Pseudocowpox virus* (family: *Poxviridae*) and *Bovine rhinitis viruses A and B* (family: *Picornaviridae*; genus: *Apthovirus*) (Figure 4). The RPO147 subunit of the poxvirus identified in these samples shared 100% amino acid identity with *Pseudocowpox virus* (AHI44423.1) from cattle exhibiting oral lesions in Bangladesh [29], indicating the virus from the Taranaki calves is the same species. *Bovine rhinitis* viruses are common viruses of cattle that cause respiratory symptoms [30] and are established contributors to the bovine respiratory disease complex [31]. We identified viral sequences sharing at least 91% amino acid sequence identity with *Bovine rhinitis virus A* and *Bovine rhinitis virus B* from the *Aphthovirus* genus. The apthovirus polymerases from the calves shared 92%–97% amino acid identity with these rhinitis viral species (Table 3), suggesting they are the same viral species. *Bovine rhinitis virus B* was identified in 91% (10/11) of the oral samples, while *Bovine rhinitis virus A* was only found in three.

3.5. Faecal Viruses. We identified two partial adenovirus hexon protein sequences across the faecal metatranscriptome, both clustering with mastadenoviruses (mammalian adenoviruses) from bovine, caprine and porcine hosts in their phylogeny (Figure 4). The hexon segments shared 86%–92% amino acid sequence identity with *Caprine adenovirus 2* (ABG22146.1). Adenoviruses are frequently found in the intestinal tracts of ruminants [32], and some adenoviruses can cause diarrhoeal or respiratory illness in calves [33]. The two non-overlapping hexon segments were recovered from the same two calves (Table 3) and are closely related, suggesting they represent different regions of the same viral species. We classified this virus as a novel adenovirus—Bos adenovirus 1—rather than *Caprine adenovirus 2* based on the longer (497 amino acids) segment being more divergent from known species and overlapping a less conserved genomic region (Table 3), along with observed phylogenetic incongruence between the two segments (Figure 4). Astroviruses were identified in all 11 faecal samples. Two astroviral RdRps fell into distinct clades and shared 97%–98% amino acid identity with astroviral proteins identified in yaks (WNO11820.1) and calves with diarrhoea in China (*Bovine astrovirus*) [34], therefore, representing previously known astroviruses. We propose the more descriptive name of Bos astrovirus 1 for the undefined *Astroviridae* sp. from yak (WNO11820.1) here. Three RdRp segments from neboviruses (family: *Caliciviridae*) were found in a small number of the faecal samples. These shared 88%–96% amino acid identity with other bovine neboviruses, which have been linked to diarrhoea in calves [35]. As all three sequences were closely related and found across the same individuals, they also likely represent different segments from a single, previously known viral species — *Bovine nebovirus*.

Finally, we identified 11 picornaviruses across the faecal metatranscriptomes falling within or close to the genera *Hunnivirus*, *Enterovirus*, *Bopivirus*, *Kobuvirus* and *Parechovirus*. We noted two hunnivirus species—one previously known, *hunnivirus A1*, originally identified in cow faecal samples

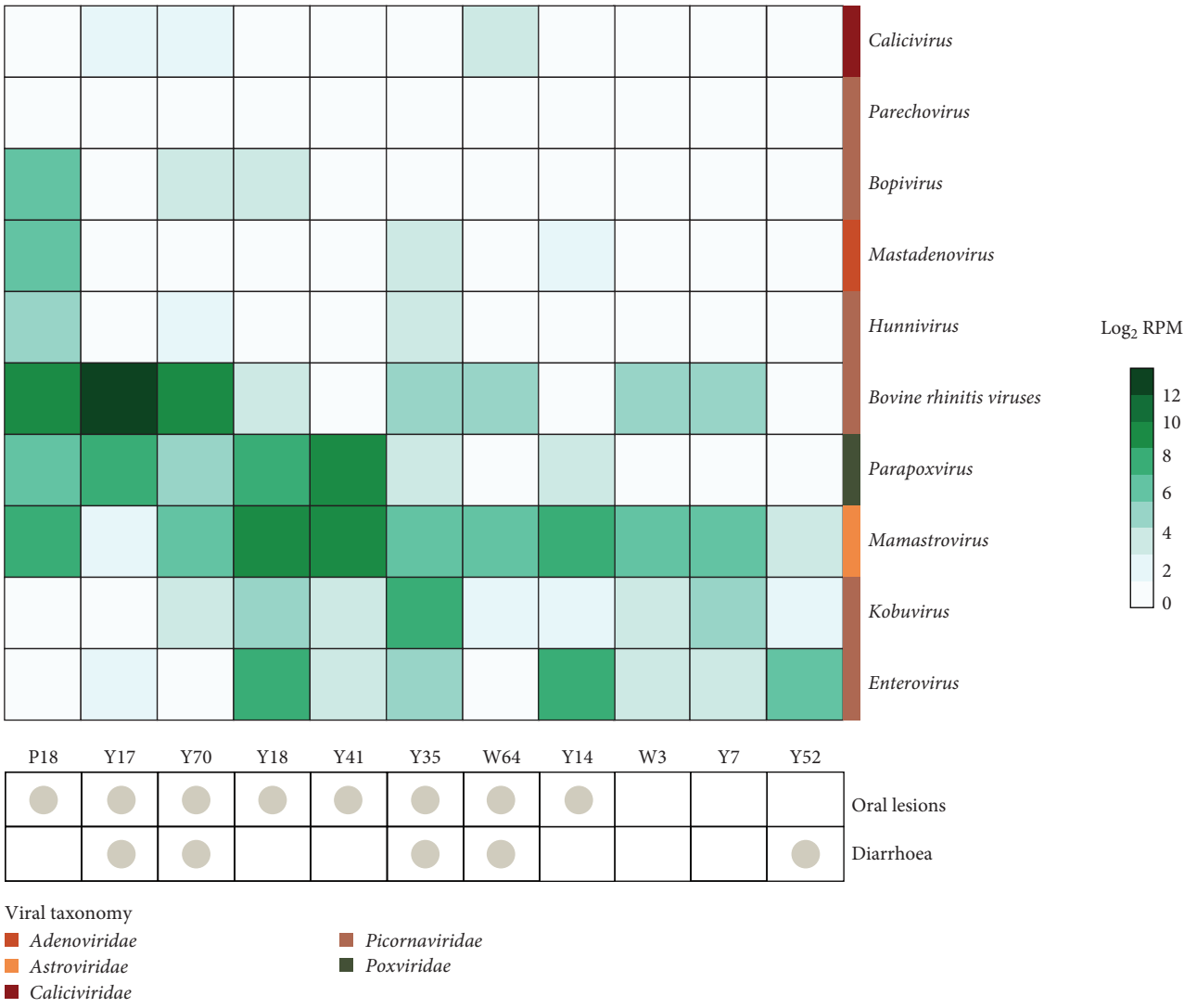

FIGURE 3: Clustered heatmap summary of bovine-associated viral genera and species abundances per sample. Abundances are shown as $\log_2$-transformed RPM with white indicating zero abundance and darker green indicating higher abundance. A chart showing which clinical signs the calves were affected by is shown below the heatmap.

from China (98% amino acid identity) and another slightly more divergent species we named Bos hunnivirus 1. Hunniviruses have been found in a range of mammalian species, including healthy and diarrhoeal cows [36]. Kobuviruses and *Enterovirus E* and *F* were detected in between one and 11 calves. All are common intestinal viruses with global distribution [37]. We also identified a previously known bopivirus, *Bovine picornavirus* (UVH30892.1). Viruses from the *Bopivirus* genus have been found in faecal samples from ruminants in Hungary, Italy, China and the USA [38]. Of the picornaviruses found in faecal samples, five were assumed to be novel viruses sharing less than 90% amino acid identity with known viruses (range: 39%–82%). These included the aforementioned novel hunnivirus, two bovine (*Bos*) picornaviruses falling basal to the *Hunnivirus* genus, a kobuvirus (Bos kobuvirus 1) and a bovine parechovirus, which was related to a virus isolated from faecal samples of cows from Japan [39].

3.6. Differentially Abundant Viral Groups in Calves With Oral Lesions. To determine which viral groups may be more abundant and significantly associated with more severely affected calves (those with oral lesions, with or without diarrhoea), we performed a differential abundance analysis on oral and faecal viromes, including all viruses identified even if they were likely to be from dietary or environmental sources (Figure 5). A total of 148 viral taxonomic groups were present in the oral viromes (Figure 5A) and 234 in the faecal viromes (Figure 5C). Of these, 25 viral groups were found to be significantly more abundant in oral samples from affected calves (Figure 5B). Only the *Parapoxvirus* genus is associated with infections in mammalian/bovine species (q-value $= 1.2 \times 10^{-8}$). In faecal viromes, on the other hand, five viral groups were found to be significantly more abundant in affected calves and another five more abundant in unaffected calves (Figure 5D). Again, only one viral genus that was significantly

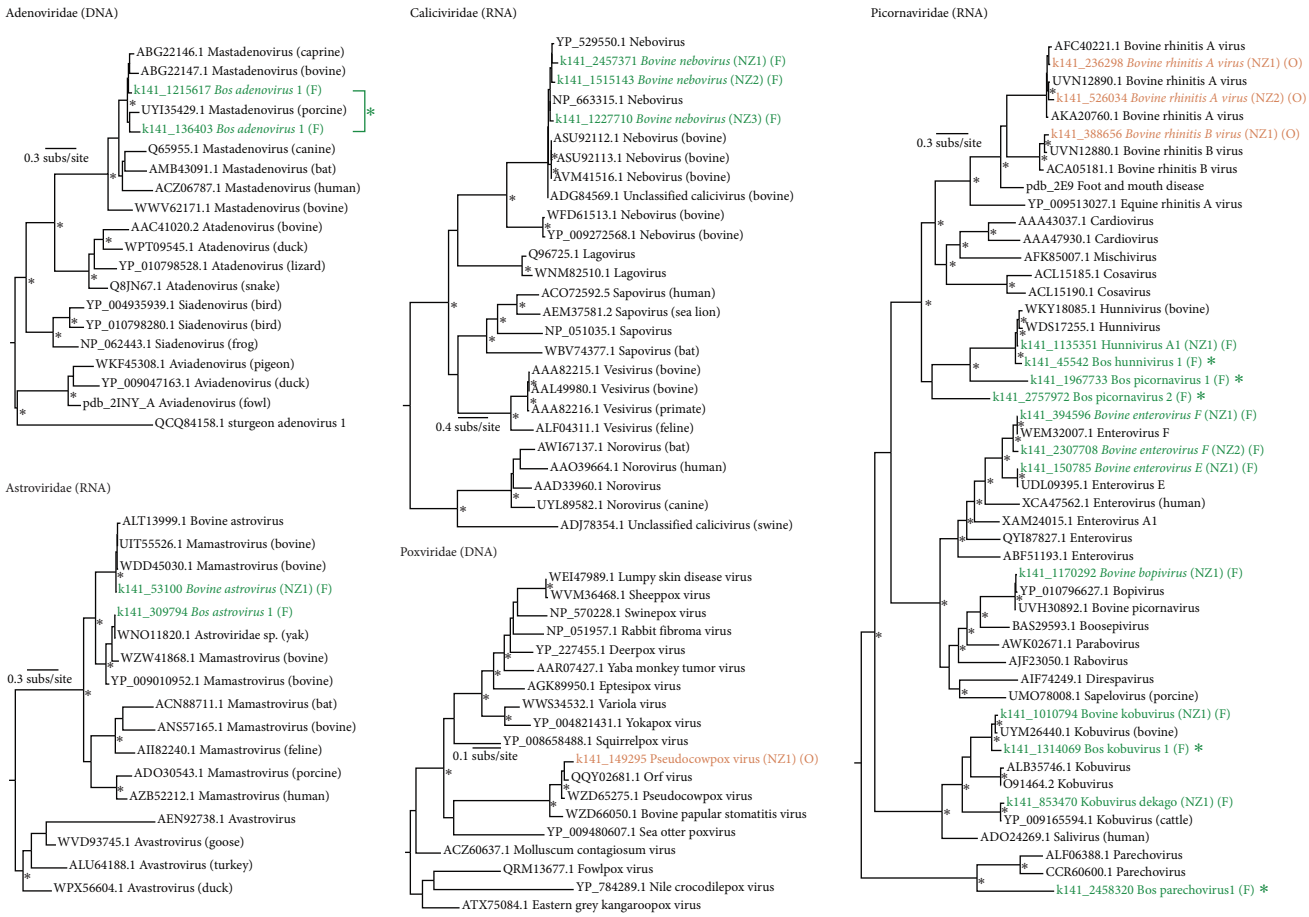

FIGURE 4: Maximum likelihood phylogenetic trees of bovine-associated RNA and DNA viruses present in New Zealand, calves based on DNA and RNA polymerase sequences, or hexon proteins (for viruses in the *Adenoviridae*). Viruses identified in oral samples (O) are indicated in orange and viruses identified in faecal samples (F) are highlighted in green. Viral proteins sharing less than 90% amino acid identity with their closest relative are indicated by large asterisks. Trees are rooted at their midpoints and node UF-bootstrap values ≥95% are indicated by small asterisks at the node.

more abundant in affected calves, *Bopivirus*, which is known to infect mammals (cows) (q-value = 0.03). Other viral groups significantly different between the two groups of calves included bacteriophages, such as those from the *Fiersviridae* and plant-infecting virus genera including *Gammacarmovirus* and *Tombusvirus*, likely associated with calves through dietary or environmental sources. Importantly, no bovine- or vertebrate-associated viruses were found to be significantly more abundant in the oral and faecal viromes of calves affected by diarrhoea compared to those without, or in those of calves with multiple clinical signs as opposed to only one or none (S2).

4. Discussion

In this investigation, we characterised the viral communities of poorly performing weaned dairy calves with clinical disease from a dairy farm in the Taranaki region of New Zealand (Figure 1A), as well as calves without any clinical signs, to identify a potential viral aetiology. Overall, oral and faecal viromes consisted of a total of 18 bovine-associated viruses that could be placed at the species level across five DNA and RNA viral families. Coinfections were common, with all calves carrying multiple viral species. In general, the viromes of calves closely mirrored those of calves overseas [40] and did not contain any notifiable exotic pathogens. Oral viromes contained polymerase sequences from *Pseudocowpox virus* and *Bovine rhinitis virus* genotypes A and B. *Pseudocowpox virus* is usually associated with lesions on the udders of cows but is one of a few poxviruses that can cause oral lesions in cattle and has zoonotic potential [41]. The virus is widespread and has previously been identified on this farm. We found *Pseudocowpox virus* to be significantly associated with affected calves, being found in all eight calves with oral lesions at relatively high abundance (up to 465 RPM) and none of the unaffected calves. Reports of viral involvement in lesions seen overseas, however, have been inconsistent and the observed lesions are generally more severe than would be expected for typical poxvirus outbreaks [6]. In addition, the main microscopic features of the oral lesions in the current outbreak included erosion and ulceration, whereas poxviral lesions are characterised by epithelial proliferation and hyperkeratosis [42]. *Bovine rhinitis* viruses are common causes of respiratory disease in cattle worldwide [30, 43] and have also been found to be carried asymptomatically [17]. These picornaviruses are part of the

Table 3: Summary of top blastx viral hits for the bovine viral proteins identified in the calves.

Virus	Calf ID	GenBank accession	Blastx hit	Blastx virus	Amino acid identity to closest known virus (%)
Bos adenovirus 1	P18, Y35	PQ583780	ABG22146.1	*Caprine adenovirus* 2	92.35*
Caprine adenovirus 2 (NZ2) Bos adenovirus 1	P18, Y35	PQ583781	ABG22146.1	*Caprine adenovirus* 2	85.71*
Bovine astrovirus (NZ1)	P18, W64, Y14, Y17, Y18, Y25, Y41, Y70, W3, Y7, Y52	PQ583782	UIT55526.1	*Bovine astrovirus*	97.17
Bos astrovirus 1	P18, W3, Y7	PQ583783	WNO11820.1	*Astroviridae sp.*	98.48
Bovine nebovirus (NZ1)	W64	PQ583784	WEY07549.1	*Nebovirus sp.*	87.93
Bovine nebovirus (NZ2)	W64	PQ583785	YP_213937.1	Calicivirus isolate TCG	93.45
Bovine nebovirus (NZ3)	W64, Y70	PQ583786	WBW30788.1	*Bovine nebovirus*	95.69
Psuedocowpox virus (NZ1)	Y41	PQ583787	AHI44423.1	*Pseudocowpox virus*	100
Bovine rhinitis A virus (NZ1)	P18, Y35, Y70	PQ583788	AFC40221.1	*Bovine rhinitis A virus 2*	97.14
Bovine rhinitis A virus (NZ2)	P18, Y35, Y70	PQ583789	WDS52675.1	*Bovine rhinitis A virus*	92.63
Bovine rhinitis B virus (NZ1)	P18, W64, Y14, Y17, Y18, Y25, Y41, Y70, W3, Y7	PQ583790	UVN12880.1	*Bovine rhinitis B virus*	91.68
Hunnivirus A1 (NZ1)	P18, Y14, Y17, Y35, Y70, W3, Y7	PQ583791	WDS17252.1	hunnivirus A1	97.95
Bos hunnivirus 1	P18, Y17, Y35	PQ583792	WKY18085.1	Bovine hunnivirus	82.45*
Bos picornavirus 1	P18, Y14, Y17	PQ583793	ULF99738.1	*Picornavirales sp.*	47.47*
Bos picornavirus 2	P18	PQ583794	NP_740368.1	Equine rhinitis B virus 1	58.14*
Bovine enterovirus F (NZ1)	P18, W64, Y14, Y18, W3, Y52	PQ583795	WEM32007.1	*Enterovirus F*	97.36
Bovine enterovirus F (NZ2)	P18, W64, Y14, Y17, Y18, Y25, Y41, Y70, W3, Y7, Y52	PQ583796	WEM32009.1	*Bovine enterovirus type 2*	93.79
Bovine enterovirus E (NZ1)	Y14, W3, Y52	PQ583797	UDL09395.1	*Enterovirus E*	97.80
Bovine bopivirus (NZ1)	P18, Y18, Y70	PQ583798	UVH30892.1	*Bovine picornavirus*	98.24
Bovine kobuvirus (NZ1)	P18, W64, Y14, Y17, Y18, Y25, Y41, Y70, W3, Y7, Y52	PQ583799	UYM26440.1	*Bovine kobuvirus*	93.34
Bos kobuvirus 1	W3	PQ583800	QKZ93188.1	*Kobuvirus bejaponia*	74.33*
Kobuvirus dekago (NZ1)	Y35, Y7, Y52	PQ583801	YP_009165594.1	Kobuvirus cattle/Kagoshima-2-24-KoV/2015/JPN	94.88
Bos parechovirus 1	P18, Y14, Y18, Y25, Y41, Y70, Y52	PQ583802	XBH23987.1	Rousettus bat picornavirus	39.26*

* denotes a novel virus species.

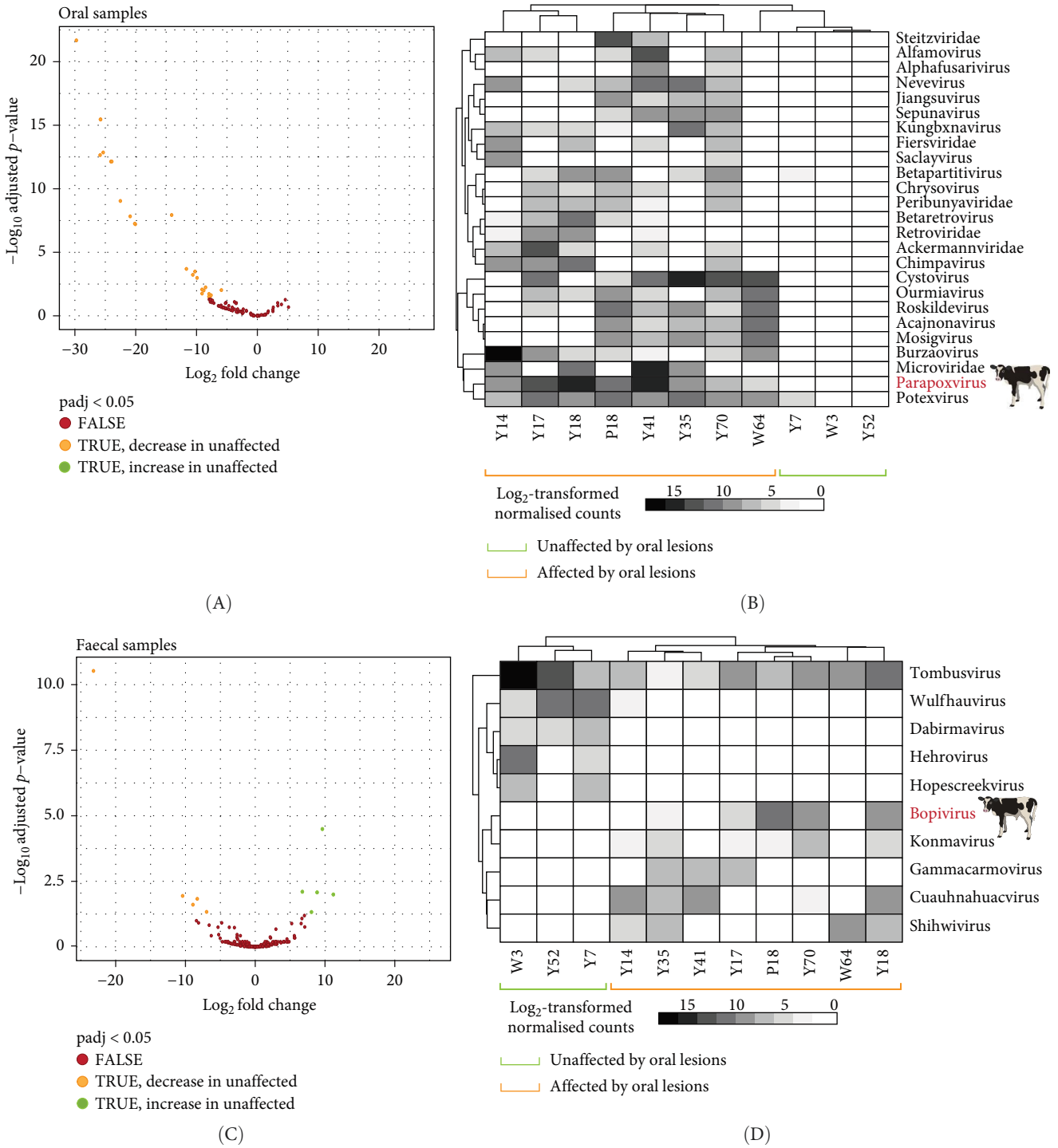

FIGURE 5: Significantly differentially abundant viral groups in the full viromes of calf oral and faecal samples were identified using DESeq2 between calves with and without oral lesions. (A and C) Volcano plots showing significantly differentially abundant viruses ($p$adj < 0.05) in oral (A) and faecal (C) samples (A,C). Viruses significantly more abundant in unaffected samples are represented by green dots and those significantly more abundant in affected calves are orange dots. (B and D) Clustered heatmaps showing the significantly differentially abundant viral groups as log2-transformed normalised viral counts in oral swab (B) and faecal swab (D) samples (B,D). White squares indicate no virus detected in a sample, darker grey indicates increased viral abundance. Mammal-associated viral genera are highlighted in red and shown by a cow symbol.

same genus, *Aphthovirus*, as FMDV [30], however, they have not been associated with oral lesions and were identified in calves with and without clinical signs of disease.

Diarrhoea is one of the most common causes of morbidity and mortality in calves, with viruses frequently being involved [37]. Faecal viromes of these Taranaki calves were more diverse

than oral viromes, containing adenoviruses, caliciviruses, astroviruses and picornaviruses. Astroviruses and picornaviruses, including enteroviruses, hunniviruses and kobuviruses, are extremely prevalent in bovine faecal samples and are frequently associated with, or causative of, diarrhoea [16, 37, 44]. Coinfections are common and can be involved in more severe disease. For example, bovine astrovirus infections and coinfections are common but typically do not result in clinical disease on their own; rather, when coinfected with other enteritically-transmitted viruses can lead to severe diarrhoea and more extensive astroviral infections [40, 45]. Coinfections were also prevalent in the Taranaki farm, with all 11 calf faecal viromes containing at least four viral species. Overall, however, no bovine-specific viruses were significantly more abundant in calves with diarrhoea than those without, regardless of oral lesion status. This suggests total viral burdens could potentially be associated with disease and increased severity rather than the syndrome being the result of a single agent.

Of note, we found that bovine picornavirus (bopivirus), in the genus *Bopivirus*, was also significantly associated with calves affected by oral lesions with or without diarrhoea. Bopiviruses and related ovipi- and gopiviruses from ovine (sheep) and caprine (goats), respectively, are a relatively new set of enteric viruses found in domestic livestock and wild deer [46, 47]. Bovine bopivirus has not been previously described in New Zealand, making this the first report of this virus here. It is entirely plausible that bovine bopiviruses have been present in New Zealand for sometime, and have been found during this present study due to the more comprehensive genomic methods used. Bovine bopiviruses have only recently been described worldwide, being found in Asia, Australia, Europe and the USA to date [38, 46–48]. Furthermore, their role in disease pathogenesis is yet to be elucidated [46], making it difficult to interpret the clinical significance of their presence in these calves. While it is difficult to disentangle primary or multifactorial causes of diarrhoea in the presence of coinfections, bopiviruses have been posited as a contributing factor to other cases of acute gastroenteritis [48]. Although no single enteric virus was found consistently across all affected calves, it is possible that the bopivirus found here could play an important role in this disease.

Importantly, no notifiable exotic viral agents were identified. The five novel picornaviruses and novel Bos adenovirus 1 we detected were also not associated with the syndrome and were closely related to other common bovine-associated faecal viruses. These may represent viruses that have undergone divergence since *Bos taurus*' introduction to New Zealand. In particular, a high diversity of RNA viruses in bovine samples is not unusual [49]. Virome investigation of affected calves in Australia similarly ruled out a novel viral aetiology [4].

Seasonal differences in pathogen diversity and abundance have been observed [50–52] and similar patterns could be explored in New Zealand dairy calves, especially given the seasonal nature of breeding. A key limitation of this study was the small sample size, with all samples being collected from a single dairy support property in December (early southern summer). Additionally, some calves were initially misclassified as showing no clinical signs, reducing the number of truly healthy or unaffected calves available for comparison in our analyses. To validate the viral findings reported here and better understand potential contributing factors to disease, future metagenomic exploration should be expanded to include additional time points and multiple farms across New Zealand. Baseline virome assessments of healthy calves would also be valuable for characterising the normal viral diversity of dairy herds and supporting efforts to associate specific viruses with clinical signs or disease outbreaks.

Calf viromes in New Zealand are largely consistent with those identified overseas with little divergence and primarily harbour common faecal- and oral-associated viral species. We determined *Pseudocowpox* virus to be associated with the oral lesions seen in affected calves from Taranaki. The poxvirus is known to be both a primary pathogen and a secondary invader of damaged tissue, and although this is an important finding, further work is required to better understand the pathogenesis of oral lesions in cases of idiopathic ill-thrift in calves. In addition, the enterically associated bopivirus was significantly associated with affected calves and has been linked to diarrhoea in other populations and species. However, calf faecal viromes contained several viral species linked to gastroenteritis that have not been posed as direct causative agents, and therefore, the contribution of any one or combination of these viruses to their condition should not be overstated.

Data Availability Statement

Sequencing reads are available on the NCBI Sequence Read Archive (SRA) under the BioProject accession (PRJNA1164330) and virus sequences are available under the GenBank accessions (PQ583780 PQ583802). Full R scripts and additional viral sequence data can be found on GitHub: https://github.com/maybec49/NZ_Calf_Viromes.

Disclosure

A preprint of an earlier version of this manuscript is available at https://www.biorxiv.org/content/10.1101/2024.12.18.629268v1 [53].

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information 1. Table SI: Clinical presentations summary of the sampled dairy calves.

Supporting Information 2. Figure S1: Bipartite networks showing calves and their co-infecting viral genera or species. Railway network showing individual calves and their co-occurring viral groups (top). Weighted network showing co-occurring viruses and their combined abundances in oral and faecal samples in RPM (bottom). Calves unaffected by oral lesions are highlighted in green and calves affected by oral lesions are highlighted in red.

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