DISSERTATION

MOLECULAR SURVEILLANCE OF BLUETONGUE VIRUS: PREVALENCE, GENOMIC LANDSCAPE, AND IDENTIFICATION OF ADDITIONAL VIRUSES USING METAGENOMICS

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ABSTRACT

MOLECULAR SURVEILLANCE OF BLUETONGUE VIRUS: PREVALENCE, GENOMIC LANDSCAPE, AND IDENTIFICATION OF ADDITIONAL VIRUSES USING METAGENOMICS

Bluetongue virus (BTV) is an economically important arbovirus of wild and domestic ruminants with a global distribution. Vectored by *Culicoides* spp. biting midges, this virus can cause significant disease in susceptible hosts, primarily sheep, which is characterized by systemic vasculitis, edema, and ulceration. The cost of BTV outbreaks is high with losses associated with animal morbidities and mortalities, trade restrictions, and implementation of testing and control measures. Control of BTV is primarily through vector control and vaccination strategies but remains a global issue due to its segmented genome and broad antigenic range.

The genome of BTV is comprised of ten segments of double-stranded RNA that can undergo reassortment, or the production of a novel progeny virus consisting of genomic segments from both parental strains. Reassortment between different strains may increase genetic diversity, alter BTV transmission dynamics, and enhance its ability to spread to new regions. Segment 2, encoding for the outer capsid protein, is the primary antigenic protein for BTV and determines the strain, or serotype, of the virus. At present there are over 29 described serotypes of BTV that have limited cross-neutralization activity between serotypes which makes control by vaccination alone challenging due to this broad antigenic rage.

As this virus can undergo rapid evolution to change both its antigenic segment and others parts of the genome, active surveillance is needed to understand what serotypes circulate endemically in a region and when novel serotypes or genomes are introduced, as these have the potential to cause severe disease. The first aim of this work was to determine the prevalence of BTV in Colorado and surrounding states and which serotypes were circulating over the course of three years, 2021-2023. Using traditional molecular techniques, the prevalence of BTV-positive sheep and cattle was significantly higher in 2021 compared to 2022 and 2023. Additionally, cattle had significantly higher odds of detection compared to sheep. Five different serotypes (BTV-6, -10, -11, -13, and -17) were detected with the predominant serotype that was detected varying annually. These results highlight the ongoing transmission of multiple BTV serotypes within domestic livestock within the U.S. and provide updated information for which serotypes are present in this area.

The methods utilized within the first aim provide insight into the current epidemiological situation of BTV within the region but did not provide further information to the genomes of these viruses that are circulating or if reassortment is occurring within this viral population. With the decreased cost and accessibility of whole-genome sequencing, whole genome characterization of BTV is needed to understand reassortment as it is happening in the field. Utilizing samples collected from a single sheep site across the three years of surveillance performed in the first aim, whole-genome sequencing was applied to further characterize these strains and identify evidence of reassortment. Phylogenetic analysis found evidence of reassortment across all genomic segments relative to segment two while nucleotide-level analysis showed high within-serotype homology for most segments. These findings reinforce that reassortment is common among circulating endemic viruses and can involve any segment.

With next generation sequencing becoming the standard by which many viruses are characterized, our laboratory has generated extensive sequencing datasets for both vertebrates and invertebrate hosts of BTV and epizootic hemorrhagic disease virus (EHDV), a related orbivirus, directly from field collected samples. Using metagenomics, samples from wild and domestic ruminants and midges were investigated for the presence of additional viruses. Results from this study identified a variety of viruses that have been previously described and a few sequences that are novel. Recovery of a full genome of a mosquito-transmitted orbivirus, Skunk River virus, from a deer represents the first detection of this virus in a vertebrate host. Detection of bovine hepacivirus from a dairy cow represents the second time this virus has been documented in the U.S. Novel sequences recovered from deer samples include sequences from the genus *Coltivirus* and genus *Narnavirus*. Sequences recovered from midges included Black Hawk Lake virus, several partitivirus-like segments, and Totivirus nijyuroku. These findings demonstrate the utility of metagenomics in conjunction with sequencing for virus surveillance for further characterization and the discovery of putatively novel viruses.

Together, these findings provide an in-depth look at BTV and the wealth of information that can be obtained from field collected samples. This work provides a contemporary view of how much BTV and what strains were circulating during surveillance, the genomes of these viruses and reassortment as it occurs in nature, and the other viruses these animals may be co-infected with.

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CHAPTER 1 – INTRODUCTION

Introduction

The genus *Orbivirus* contains several arboviruses of veterinary significance, including bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), and African horse sickness virus (AHSV). Transmitted by *Culicoides* spp. biting midges, these viruses can cause substantial economic losses and severe disease in affected animal populations. Recent outbreaks of BTV and EHDV in Europe have drawn international attention, highlighting the persistent challenges and global burden posed by these emerging and re-emerging orbivirus species. Shared characteristics among these viruses, including vector-borne transmission and similar genomic architecture, mean that advances in understanding one virus may have broader implications for others. Additionally, increasing access to technologies such as metagenomic sequencing is rapidly expanding our capacity to investigate these pathogens and enabling a more comprehensive understanding of viral diversity.

BTV Structure, Function, and Replication

BTV and other viruses of the genus *Orbivirus* (order *Reovirales*, family *Sedoreovirdae*) have a bi-layered viral capsid that contains genome comprised of 10 linear segments of double-stranded RNA[1]. These segments encode for seven structural proteins (VP1-VP7) and three non-structural proteins (NS1-NS3) and are identified by the segment according to its size, with segment 1 being the largest[2]. VP1, encoded by segment 1, functions as the RNA-dependent RNA polymerase (RdRp)[3]. VP2 (segment 2) mediates host cell receptor binding[4,5], while VP3 (segment 3) forms part of the inner shell (T2)[6]. VP4 (segment 4) serves as the viral capping enzyme[7], and NS1 (segment 5) forms microtubules that enhance viral protein translation[8]. VP5

(segment 6) facilitates membrane penetration[9], and VP7 (segment 7) forms the middle shell of the core particle[10]. NS2 (segment 8) is involved in the formation of viral inclusion bodies[11,12]. Segment 9 encodes VP6, which has a role in RNA binding, helicase action, and RNA packing[13,14], and NS4, an interferon antagonist associated with immune evasion and virulence[15–17]. NS3/NS3a (segment 10) mediate viral egress[18,19]. The outer capsid of BTV consists of VP2 and VP5[20]. The core of the virus is composed of two major proteins, VP3 and VP7, and three minor proteins that make up the transcription complex, VP1, VP4, and VP6[20].

Replication of BTV starts with attachment of the virion to the host cell via VP2, likely to sialic acid on the host cell, followed by clathrin-mediated endocytosis of the viral particle[21,22]. Within the early endosome, the acidic pH is sensed by VP2, triggering a conformation change that results in dissociation of VP2 from the virion. This particle then traffics to the late endosome, where the lower pH induces a second conformational change associated with VP5 that allows for membrane penetration and release of the viral core into the cytoplasm[5,23,24].

The viral core, composed of VP3 and VP7, protects the dsRNA genome within the cytoplasm[6]. Inside the core particle, VP1 will synthesize positive-sense ssRNA transcripts with assistance from VP6. These transcripts are capped by VP4 before being extruded through pores in the core into the cytoplasm[6,7,14,25,26]. Viral mRNAs are subsequently translated by host ribosomes, and production of NS2 drives the formation of cytoplasmic inclusion bodies where positive-sense RNAs and newly synthesized structural proteins localize[12,27,28].

Viral assembly begins with formation of the subcore by the VP3 scaffold within which all ten genomic segments are synthesized inside the assembling viral particle[3,20,29]. VP7 is then added to the completed core particle, followed by VP5 and VP2 to complete the outer capsid[30]. NS3 mediates intracellular trafficking, and mature virions are released from the cell. The

mechanism of release differs between insect and mammalian cells[19,18,28,31,32]. Non-lytic release is more common in insect cells, in which the virus exits without causing immediate cell death, while cell lysis occurs more frequently in mammalian cells[19,28,32–34].

BTV Evolution

The evolution of BTV is complex and occurs through a combination of genetic drift, reassortment of its ten genetic segments, and the pressures placed on this process by the vertebrate/invertebrate transmission cycle[35–39]. Most RNA viruses evolve rapidly because they replicate using an RdRp that lacks proof-reading ability, resulting in high mutation rates and increased genetic diversification[40–42]. Therefore, each replication cycle introduces mutations into the viral genome, generating a heterogenous population of closely related variants centered around a consensus sequence, referred to as a quasispecies[43–46].

However, not every quasispecies will be successfully passed on to the next host and it is likely that these viruses undergo increased purifying selection during transmission between vertebrate and invertebrate hosts[47–49]. Yet when quasispecies are successfully passed on, these minor variants from the quasispecies virus population are amplified in the new host, becoming the new dominant viral consensus, what is known as the founder effect[35].

While the changes to the genome attributable to genetic drift are typically slow and incremental over time, BTV can also go through rapid evolution via a process called reassortment. Unique to segmented viruses, reassortment occurs when two or more related viruses co-infect a single cell, permitting exchange and co-packaging of genome segments during assembly, thereby generating progeny viruses that contain a mosaic of segments derived from each parental virus[50,51]. Despite the conditions that have to be met for reassortment to occur, such as co-infection of a host with two different strains of BTV, reassortment is frequently

reported from field isolates and is likely a major driver of genotypic and phenotypic change in BTV[52]. There is some evidence to support that this process can involve any segment while other studies have shown some associations between segments, suggesting that some segment reassortment is constrained by segments that must go together[52,53].

BTV Diversity

VP2, encoded by segment 2, and VP5, encoded by segment 6, form the outer capsid of BTV and are the most variable viral proteins[54]. However, the breadth of BTV diversity is largely driven by variability in VP2. VP2 mediates host cell attachment and viral entry and is therefore the primary antigenic determinant for neutralizing antibodies[4,55–57]. Prior to the development of advanced molecular assays, BTV strains were characterized using *in vitro* serum virus neutralization assays to determine serotype[58,59]. To date, there over 29 reported serotypes of BTV (BTV-1 through BTV-29)[60–66]. Diversity within individual serotypes has also been observed in the form of topotypes, which are geographically distinct viral lineages broadly classified as eastern (Asia and Australia) or western (Africa and the Americas)[67]. These lineages likely reflect long-term geographic separation and regional evolution of BTV strains. Substantial genetic variation has been observed within a single serotype, with nucleotide variation in segment 2 reaching ~21% between strains of the same topotype and up to ~32% between strains within the same serotype[67].

BTV in the Ruminant Host

BTV is traditionally considered as a disease of sheep, as this species most commonly develops clinical signs following infection. However, BTV has a broad host range that includes numerous wild and domestic ruminants as well as camelids[68–76]. After cutaneous deposition of BTV via the bite of an infected *Culicoides* spp. biting midge, the virus initially replicates in the

regional lymph node before disseminating systemically[77]. Due to its trophism for endothelial cells, mononuclear phagocytes, and lymphocytes, subsequent rounds of replication occur within other lymphatic organs including lymph nodes, spleen, bone marrow, and thymus[78–84]. As infection progresses, the virus can also be detected in additional tissues such as lung, tongue, muscle, heart, liver, kidney, and reproductive tissues where replication primarily occurs in endothelium[78,84]. During infection, BTV also associates with the invaginations of the erythrocyte membrane and can be detected for the lifespan of the infected red blood cell[85,86].

The clinical lesions and manifestations of BTV are associated with vascular mediated injury secondary to this virus's preference for endothelium[87–89]. Infected animals may have oral and muzzle erosions and ulcers, coronitis, facial edema, petechia and ecchymoses, and pulmonary edema[90,91]. In some severe cases, the tongue may become swollen and cyanotic ("bluetongue") and protrude from the oral cavity. Infected animals may present with fever, increased respiratory rates, inappetence, lameness, nasal discharge, excess salivation, and weakness[90]. In peracute cases, pulmonary edema will present as dyspnea, frothing from the nose and mouth, followed by death by asphyxiation[91]. In more chronic cases, secondary bacterial pneumonia can also result in the death of these animals. Many animals that become infected and experience mild disease will recover and clear the virus on their own. The viremia of infected animals can range from 27-54 days in sheep and goats and up to 63 days in cattle[92,93].

Clinical manifestations of BTV can vary depending on the host species, age, viral strain, and other host and environmental factors. Many infections are asymptomatic and may produce no observable clinical signs during the course of infection, including in some sheep[91,94]. Cattle and goats are typically considered to experience few or no clinical signs when infected with BTV[80,90,95].

In response to infection, animals will produce antibodies against the outer capsid proteins of BTV, VP2 and VP5, as well as the inner core protein, VP7[55,57]. However, it is only antibodies for VP2 that are neutralizing and will provide protection during subsequent challenge with the same serotype[56,96,97]. Protection against future infections becomes challenging when multiple serotypes are circulating in that region as there is limited cross-protection between serotypes[98–100]. Antibodies appear to be quite long lived, perhaps even lifelong in many animals, with evidence supporting their presence 4-6 years after infection and 5-8 years after immunization[101–103].

Transmission and The Vector

Although other methods of transmission have been described, including artificial insemination with infected semen, transmission via contaminated needles used for multiple injections, and vertical transmission from mother to fetus, BTV is predominantly transmitted via the bite of an infected female *Culicoides* spp. biting midge (Diptera: Ceratopogonidae)[104–109]. *Culicoides*, similar to mosquitoes, are poikilothermic and require semi-aquatic developmental habitats, which limits the range in which they can survive. Historically, the distribution of biting midges, and therefore BTV, has largely been between 40-50°N and 35-40°S[94,110]. In tropical regions *Culicoides* are active throughout the year, whereas their activity is seasonal in temperate climates[90,111–114]. Although more than 1,300 species of *Culicoides* have been described worldwide, only approximately 30 species have been identified as known or potential vectors of BTV[115,116]. In North America, *Culicoides sonorensis* is the predominant vector, with a geographic range primarily west of the Mississippi river[117,118]. Other species, including *C. stellifer*, *C. debilipalis*, and *C. insignis*, are also capable of transmitting BTV and are found primarily in the eastern United States[118].

BTV History and Distribution

BTV was first described in South Africa in the late 1800s and confirmed as the etiological agent of the disease in 1905[90]. Although *Culicoides* spp. were suspected to be the vectors responsible for transmission, this was not confirmed until 1944[104]. Since its initial description, BTV has been detected on every continent except Australia. In the last several years, the known distribution of BTV has expanded beyond its historically recognized range, with outbreaks reported at increasingly higher latitudes. Notably, cases detected in northern Europe, including Denmark, Norway, and Sweden in 2024, highlight a continued northward expansion of transmission risk. This expanding geographic distribution is likely associated with increasing temperatures in these regions, which facilitates midge survival and transmission of the virus[119,120].

The first descriptions of BTV in the United States occurred in 1948 in Texas and 1951 in California[121,122]. Today, BTV has been detected in nearly all contiguous U.S. states, with the exception of the Northeast, although cases have been reported in New York[123–125]. Occasional incursions of BTV have also been observed farther north, such as the Okanagan Valley, British Columbia and Chatham-Kent, Ontario in Canada[126–129]. However, the virus does not appear to be established in these regions. Historically, BTV-10, -11, -13, and -17 have been considered endemic in the U.S.[110,125,130] In 2022, the U.S. Department of Agriculture (USDA) updated its classification system for BTV, categorizing BTV serotypes as established, reported, or not reported. Established serotypes are defined as those that have been reported annually for at least two consecutive years in regions where vector incursions due to weather events are unlikely, or for which there is phylogenetic evidence of a virus reassortment with a previously established strain. As of 2026, the established serotypes in the U.S. are BTV-3, -6, -10, -11, -12, -13, and -

17[131,132]. The addition of BTV-3 and -6 are directly associated with work performed by our laboratory over the past decade[133,134].

Control of BTV

Available methods for controlling BTV infections are primarily limited to reducing animal exposure to infected midges, vaccination, and import/export testing. Although the application of pesticides can reduce host-feeding by midges, the required frequency of application, cost, potential development of resistance, and environmental impacts make this approach largely unfavorable[135,136]. Recent evidence indicates that pyrethroids, deltamethrin and ivermectin, do not prevent blood-feeding, but may reduce onward transmission[137]. Moreover, such interventions are generally restricted to captive ruminants and are not feasible for use in free-range or wild ruminant populations.

Vaccination for BTV control is associated with several practical challenges. Foremost among these is the extensive antigenic diversity of the virus, with more than 29 recognized serotypes and limited cross-protection among them, making the development of a single broadly protective vaccine extremely difficult. Although modified live virus (MLV) vaccines have historically been used, reassortment between vaccine strains and circulating field strains of BTV have been documented, along with evidence that midges can acquire and transmit vaccine-derived virus strains[138–140]. Due to these risks, recent outbreak responses have increasingly favored the use of inactivated vaccines[141,142]. Several novel vaccine platforms and technologies are currently under development. However, none of these have become commercially available.

Movement of infected animals or germplasm from BTV-endemic regions poses a substantial risk of introducing the virus into areas where it is not currently circulating, as well as introducing previously unreported serotypes into regions where they have not been historically

detected. Consequently, many countries require testing prior to the import or export of susceptible animals or germplasm. During outbreaks, similar principles have been applied, with regulatory authorities implementing movement restrictions to reduce the risk of viral dissemination and limit geographic spread[143].

Diagnostic Methods

Diagnostic tests to confirm the presence of BTV or antibodies generated following exposure to the virus are widely available. Serological assays are an important component of BTV diagnostics, and several commercial competitive enzyme-linked immunosorbent assays (cELISA) are commercially available. When information regarding the infecting serotype is required, virus neutralization assay can be performed. However, these culture-based methods are labor and resource intensive. Additionally, cross-reactivity among serotypes can complicate interpretation of neutralization results.

Traditional laboratory diagnosis of BTV has relied on virus isolation using susceptible cell lines (KC, CuVa, BHK21, Vero) or embryonated eggs, which remains the only method capable of detecting live virus[144]. However, virus isolation is resource, time, and labor intensive. In contrast, detection of BTV RNA via real-time polymerase chain reaction (RT-PCR) provides sensitive, specific, and rapid detection of BTV and is the diagnostics method of choice. Several protocols are widely available in addition to commercially available kits[144]. Serotyping can also be performed by RT-PCR using serotype specific primers and probes, but this can become costly given the large number of potential serotypes that must be evaluated. Moreover, initial attempts at serotype identification may fail if VP2 has diverged significantly from previously characterized sequences. Today, with the continued reduction in cost and increasing accessibility

of next-generation sequencing (NGS), this technology is increasingly being applied as part of BTV characterization.

Next-generation Sequencing for BTV

Utilization of next-generation sequencing for whole genome characterization of BTV is crucial for characterization of this virus as it can undergo reassortment. However, sequencing segmented genomes presents unique challenges as each segment must be individually amplified and sequenced. Sequence independent single primer amplification (SISPA) is one method used to overcome this challenge[145–148]. By incorporating primers that bind randomly across cDNA, SISPA enables simultaneous amplification of all genomic material, increasing the likelihood of recovering complete sequences from each segment in a single reaction. This feature is particularly useful for segmented viruses where conventional targeted amplification would require separate primers and reactions for each segment.

Modern sequencing approaches used to generate complete BTV genomes generally fall into one of two categories: short-read or long-read platforms. Commercial short-read platforms (100-300 bp), such as Illumina, were used to produce some of the first whole BTV genomes in 2012 and continue to be widely used today[149–151]. More recently, commercial long-read platforms (1-100 kbp), such as Oxford Nanopore Technologies (ONT), have been applied for whole genome sequencing of BTV[152–154]. In addition to the random SISPA primers, these long-read protocols also incorporate primers that are conserved for the terminal sequences of BTV segments to enrich for BTV-specific reads[152,155].

Metagenomics for Virus Detection

Sequencing all nucleic acids within a sample, as performed in metagenomic approaches such as SISPA, enables detection of genetic material from all organisms present in the sample

without prior knowledge of the target sequences. The resulting sequence data can then be compared against reference databases of nucleotide and protein sequences to identify organisms within the sample, including both known and previously undescribed viruses[156]. Application of metagenomic sequencing has substantially expanded our understanding of viral diversity, as nucleic acids can be sequenced directly from clinical or environmental samples without the need for prior isolation. This approach facilitates the detection of viruses that are difficult to propagate *in vitro* and avoids the selective pressures associated inherent to culture-based methods, which may bias the viral populations detected.

Metagenomic approaches are particularly valuable for surveillance as viruses can be detected in asymptomatic hosts, aiding the discovery of novel viruses and improving our knowledge of host range and epidemiology of known viruses. These methods are also useful in investigations of diseases of unknown etiology, where metagenomic sequencing can help identify the etiologic agent. In cases where a pathogen is already known, sequencing data can inform analyses of genomic variation linked to pathogenicity or reveal additional coinfecting organisms that may influence disease outcomes. Integration of metagenomic sequencing into routine pathogen surveillance and diagnostic frameworks therefore represents an important tool for protecting both human and animal health[156–158].

Summary

BTV remains a virus of international concern due to its capacity to cause significant disease in animals and the substantial economic impacts associated with both endemic circulation and episodic outbreaks. Control of this virus is challenging because of its arboviral transmission cycle, broad antigenic diversity making it difficult to control via vaccines, and its capacity for rapid genomic evolution through segment reassortment. Consequently, active and

sustained surveillance of this virus is needed to establish contemporary baselines for BTV prevalence within diverse geographic regions.

The first aim of this work provides three consecutive years of BTV prevalence data from Colorado and surrounding states between 2021 and 2023, including identification of the serotypes detected during this period. In addition to surveillance, sequencing circulating BTV strains is necessary to characterize all ten genomic segments to identify potential reassortment as it is happening with circulating endemic strains and strains exotic to the region. The second aim of this work applied whole genome sequencing to characterize the genetic landscape of BTV from a single sheep flock across three years of sampling.

In addition to targeted sequencing efforts, integration of metagenomic analysis into surveillance and diagnostic workflows can facilitate the detection of additional viruses that may influence disease dynamics. Leveraging the diversity of orbivirus-positive vertebrate and vertebrate samples obtained through surveillance activities and diagnostic submissions, metagenomic analyses were performed to identify other viruses present within these samples. Some of the viruses detected represent putatively novel species, where others expand our understanding of the ecology and distribution of more recently described viruses.

Collectively, this work investigates viral ecology through the lens of BTV, integrating surveillance, genomic characterization, and metagenomic discovery to advance our understanding of BTV and other viruses circulating within natural systems.

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CHAPTER 2 – MULTI-YEAR PREVALENCE OF BLUETONGUE VIRUS IN DOMESTIC RUMINANTS IN COLORADO AND SURROUNDING STATES

Introduction

Bluetongue virus (BTV) is an orbivirus spread by *Culicoides* spp. biting midges that infects wild and domestic ruminants. Although infections can occur in a variety of species, clinical disease tends to be more severe in sheep compared to cattle and goats, which are frequently asymptomatic. Predominant clinical findings are associated with viral-mediated vascular injury that can manifest as pyrexia, facial and pulmonary edema, nasal discharge, oral ulceration, and coronitis[1–9]. Affected animals may be reluctant to eat or ambulate and have difficulty breathing[2,7,9]. Morbidity and mortality rates vary widely depending on the host species, age, and serotype of BTV. The economic impact of BTV epidemics is high with losses resulting from animal mortalities, production losses, implementation of diagnostics and control measures, and trade restrictions[10–12]. Control of BTV is primarily achieved through vector control or vaccination[13]. However, neither strategy will provide complete protection due to the complicated ecology of the virus and its ability to quickly obtain new antigenic segments through reassortment, a method of viral evolution unique to segmented viruses like BTV[13–17].

The genome of BTV is double-stranded RNA and consists of ten segments[18,19]. The outer capsid protein (VP2), encoded by segment 2, is the primary antigenic epitope for neutralizing antibodies and determines viral serotype[20–23]. To date, there are over 29 documented serotypes of BTV[24–32]. Serotype distribution varies geographically and multiple serotypes may co-circulate within endemic regions[33–35]. The segmented genome of BTV allows for reassortment to occur

when a host or vector is co-infected with two different serotypes, contributing to the extensive genetic diversity observed in BTV populations[14].

Historically, BTV has been detected between 35°S and 40°N with BTV-10, -11, -13, and -17 considered endemic in the United States[36–38]. However, incursions of BTV have been observed as far north as the Okanagan Valley, British Columbia, Canada (49°N)[39–41]. The transmission cycle of this virus is complex and greatly influenced by environmental factors including precipitation and temperature, in addition to movement of the virus via domestic livestock, wildlife, and midges[42–46]. At temperate latitudes, infections of vertebrate hosts are correlated with vector abundance and are therefore seasonal. In North America, the start of the BTV season can occur as early as July and ends in October or November[47,48]. Mechanisms for year-to-year maintenance of BTV in these temperate regions with periods of inactivity, known as overwintering, remain unclear[13]. The current presumption is that BTV is maintained throughout winter via the survival of infected adult midges, as transovarial transmission has not been demonstrated[49–51].

The first report of BTV in Colorado dates to 1953[33,52]. Historical reports support circulation of BTV-11 and BTV-13 in the state since the 1960s, followed by the first detections of BTV-10 and -17 in the 1970s[33,52–55]. More recently, BTV-3 and BTV-6 were detected for the first time in Colorado 2015 and 2020, respectively[56,57]. It remains unclear whether BTV-3 continues to circulate in Colorado, as passive surveillance in 2018 failed to identify any BTV-3 positive animals[58]. In contrast, BTV-6 was detected again in 2021, suggesting continued circulation in the state[56].

Investigation of BTV prevalence in locations likely to experience seasonal Culicoides immigration from warmer regions and that experience freezing winters is crucial to

understanding the epidemiology of this virus, how it is maintained year to year, the seasonality of infections, and how infection rates and the serotypes present could change over time. Colorado is a prime location to study BTV due to its central location within the continental United States, large populations of both domestic and wild ruminants, and a temperate climate with warm summers and winter temperatures that are frequently below freezing. This study describes the prevalence of BTV as determined by serology and RT-qPCR in a study of sheep and cattle during the BTV field seasons of 2021-2023 in Colorado and neighboring states.

Methods

Sampling design

Blood samples were collected across three years in Colorado and surrounding states during 2021-2023. Sites were selected based on the willingness of producers to participate in the study, availability of animals for sampling, and the number of animals. Sites were de-identified and labeled based on the primary species sampled: S = sheep, C = cattle, B = both, followed by a sequential number (e.g., S01, B02, C05).

In 2021, cross-sectional sampling took place at four sheep sites and four cattle sites in Northern Colorado (Larimer, Weld, and Boulder counties) in October or December 2021 (Figure 1). One sheep site, S01, had longitudinal collections performed in August, September, October, and December.

In 2022, monthly longitudinal sampling took place at four sheep sites and four cattle sites in Northern Colorado (Larimer, Weld, Boulder, and Washington counties) starting in June and ending in December (Figure 1). Monthly sampling was repeated in 2023 at four sheep sites and

four cattle sites in Northern Colorado (Larimer, Boulder, and Weld counties) starting in July and ending in December (Figure 1).

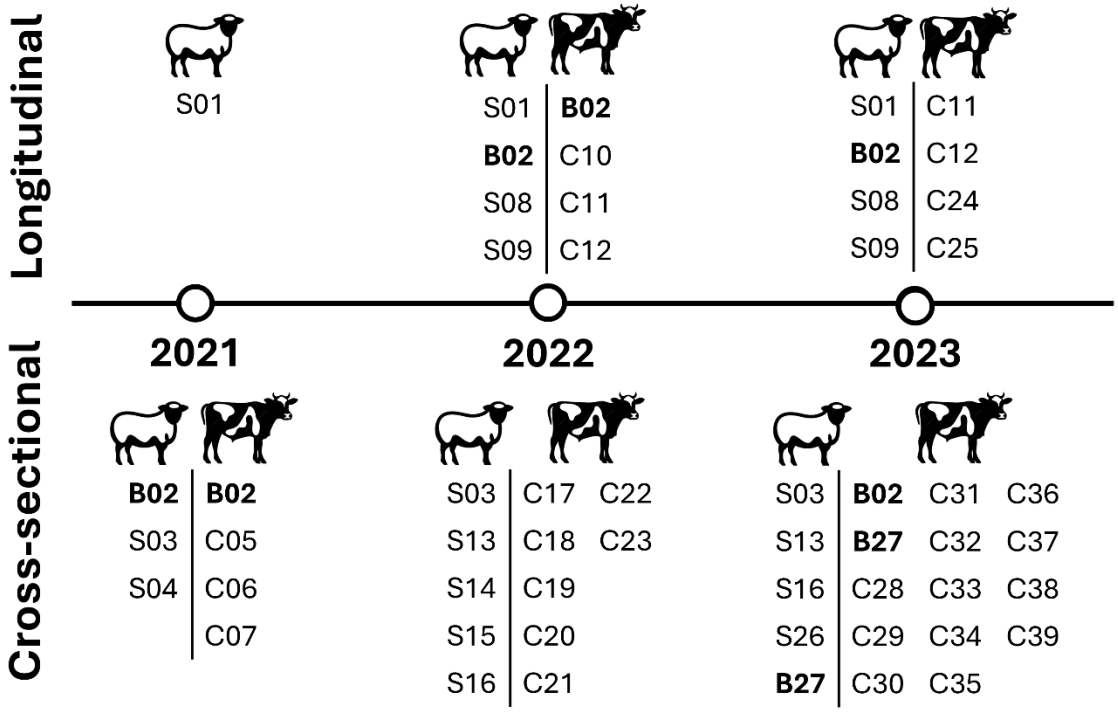

Figure 1 – Overview of collection sites and species sampled from during the 2021-2023 longitudinal and cross-sectional sampling periods. Sites in which both sheep and cattle were sampled from in the same year are bolded.

To complement the longitudinal sampling in 2022 and 2023, a larger cross-sectional study was conducted both years to enhance sampling coverage and increase the probability of detecting low-frequency serotypes. For 2022, cross-sectional sampling took place during a two-week period in October (10/13/2022 through 10/26/2022) in which an additional five sheep and seven cattle sites in Larimer, Weld, Washington, and Gunnison counties in Colorado and Scotts Bluff County in Nebraska were sampled (Figure 1). For 2023, cross-sectional sampling was conducted during a two-week period in October 2023 (10/04/2023 through 10/13/2023) in which an additional six sheep sites and fourteen cattle sites were sampled in Larimer, Weld,

Washington, Logan, Garfield, Montrose, San Miguel, Chaffee, and Kit Carson counties in Colorado and Carbon County in Wyoming (Figure 1).

Sample collection

All work was approved by Colorado State University's Institutional Animal Care and Use Committee (IACUC) with additional written approval from the producer. For the 2021 sample collections, 14-15 animals were randomly selected from the population at each site for sample collection. To account for animal attrition during the 2022-2023 longitudinal studies, this number was increased to 20-24 per site. For the 2022-2023 cross-sectional studies, 15-17 animals were randomly sampled. These cross-sectional collections also included animals at meat processing facilities. Sample collection was performed via jugular venipuncture in sheep and either jugular or coccygeal venipuncture in cattle using either a vacutainer system or needle and syringe. For sample collection at meat processing facilities, collection tubes were held under the carcass after the major vessels of the neck had been transected. Samples were collected in red top serum collection tubes for serum and EDTA tubes for whole blood. Red top tubes were centrifuged at 1,500 rpm for 5-10 minutes and the resulting serum was collected. Samples were maintained at 4^{o}C until analysis.

Serologic detection of antibodies against BTV

Serum samples were tested for the presence of BTV-specific antibodies using the commercial Bluetongue Virus Antibody Test Kit, cELISA v2 (VMRD, Pullman, Washington, USA). Briefly, serum samples and controls were diluted 1:2 in antibody-peroxidase and added to antigen-coated microplate wells. Plates were incubated for 30 minutes at room temperature, washed three times with distilled water, and incubated with substrate for 10 minutes. Stop solution was added and optical density was read at 630 nm. Samples with ≥ 60% inhibition were

considered positive, per the manufacturer's instructions. Seroprevalence during longitudinal sampling of individual animals was reported as positive if an animal had one positive cELISA result during the sampling period.

RNA extraction

Nucleic acids were extracted from whole blood using the Applied Biosystems™ MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions for whole blood samples with 200 µL sample input on the KingFisher96™ magnetic particle processor (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 200 µL of sample was combined with the magnetic bead mix and mixed by plate shaking, followed by addition of lysis/binding solution and additional mixing by plate shaking. Plates were processed on the KingFisher96™ magnetic particle processor. Wash steps included two washes with Wash Solution 1 and two washes with Wash Solution 2. Nucleic acids were eluted in 90 µL elution buffer. For tissue samples, the manufacturer's instructions for low-cell-content samples were utilized with 100 mg of sample input. Samples were processed as described above with recommended changes to reagent volumes as provided by the manufacturer's instructions.

BTV qRT-PCR

The presence of BTV RNA was assessed via a singleplex or duplex quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay to detect BTV segment 10. The singleplex assay to detect BTV was performed using primers (10 µM) and probes (10 µM) as described by Hoffman et al.[59] Briefly, 1 µL of each primer was added to 3 µL of template RNA was denatured for 5 minutes at 95 °C (Table 1)[26,60]. Reactions were prepared using half-reaction volumes of Invitrogen™ SuperScript™ III Platinum™ One-Step qRT-PCR Kit w/ROX (Thermo

Fisher Scientific, Waltham, MA, USA)[61]. Thermocycling was performed as follows: reverse transcription at 48°C for 30 minutes, initial denaturation at 95°C for 2 minutes, followed by 40 amplification cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. Samples were run and analyzed on an Applied Biosystems™ QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). A duplex qRT-PCR assay for detection of both BTV segment 10 and epizootic hemorrhagic disease virus (EHDV) segment 9 was developed by our lab by adapting a protocol previously described by Wilson et al. using primers and probes described by Maan et al.[62,63] Briefly, duplex qRT-PCR was performed using the Applied Biosystems™ Path-ID™ Multiplex One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) on an Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). First, 5 µL of template RNA was denatured for 5 minutes at 95°C, followed by the addition of 20 µL of master mix with primers and probes into the same tube. Thermocycling conditions were as follows: 48°C for 10 minutes (reverse transcription), 95°C for 10 minutes (denaturation), followed by 40 amplification cycles of 95°C for 15 seconds and 56°C for 45 seconds. Prior to implementation, the duplex qRT-PCR assay was internally validated against the simplex qRT-PCR assay using characterized BTV-positive samples, demonstrating comparable diagnostic sensitivity between assays. Ct values less than 40 were considered positive for both the singleplex and duplex qRT-PCR assays.

Table 1 – List of primers and probes used for pan-Bluetongue virus (segment 10) detection and serotype-specific detection targeting segment 2.

Primer/Probe Name	Sequence 5'-3'	Reference
Pan BTV F	TGGAYAAAGCGATGTCAAA	[59]
Pan BTV R	ACATCATCACGAAACGCTTC	[59]
Pan BTV Probe	ARGCTGCATTCGCATCGTACGC	[59]
BTV-2e F	GAGCATTTGTTGAAARGTTATG	[64]

BTV-2e R	GATATCRAAYGCGTACATYTCTG	[64]
BTV-2e Probe	CCAAGATGGCCGACATGACGTATC	[64]
BTV-2w F	GATGAYRYAARTAYTCTGAG	[64]
BTV-2w R	GYATCYYTTTCGAARTCRATTGTRAG	[64]
BTV-2w Probe	CATTCCATCCACCATCTATAATTTCCCC	[64]
BTV-3 F	GARCGGTTRTCRACGGAWGARG	[64]
BTV-3 R	TATCRTAAGCGTTATCTCCTARCYG	[64]
BTV-3 Probe	CYCCRCAGTTTCAYACAATACAGAGGAACCATC	[64]
BTV-6 F	GTCGATGTYACACAGTTGATCGT	[64]
BTV-6 R	TAGCACGTCTAATCGTTTCTATG	[64]
BTV-6 Probe	CACCTTGAYTCATCCACACTACGAAC	[64]
BTV-10 F	TATTRACWACWGAACCAAACCT	[64]
BTV-10 R	GYGARTTRATCCRTTTGTCAT	[64]
BTV-10 Probe	YCTTGGYNCGCGYTCTGAATTAGTATTYCCRCCY	[64]
BTV-11 F	GGATGCGYAYYTGAATATTAG	[64]
BTV-11 R	ATCTCTCCATGAGTTATTCGCA	[64]
BTV-11 Probe	YGTGCTCCCAAGTTATTTCGATCAATGGATCTAC	[64]
BTV-13 F	GGTGACGTYTATTATAAATTGCG	[64]
BTV-13 R	GGCGATCCARATCYCGWGG	[64]
BTV-13 Probe	CTTATATCCCTCACGTACGCTCCAYTCATACC	[64]
BTV-17 F	TGCTRAAAGAGATCAAATTTGTRCGG	[64]
BTV-17 R	ACTTGATCGTATCGTCAAACA	[64]
BTV-17 Probe	CCTCCCTCTGATGTTCCTTGTTCATGATAAC	[64]

BTV serotyping qRT-PCR

Serotyping of positive samples was performed using serotype-specific RT-qPCR utilizing the same reagents and reaction conditions as described above with serotype-specific primers and probes targeting segment 2 (Table 1)[64]. For samples collected in 2021, all samples underwent serotyping to test for the presence of 5 different serotypes: BTV-6, -10, -11, -13, and -17. Samples for which a serotype could not be determined were additionally tested for serotypes BTV-2e, -2w, and -3. For samples collected in 2022 and 2023, a modified testing scheme was

used in which all samples were tested for the presence of BTV-13, -11, -17, -6, and -10, in that order. Once a sample tested positive (Ct <40), no further additional testing was performed.

Cross-sectional sample analysis

All cross-sectionally collected samples from 2021, 2022, and 2023 were evaluated via BTV cELISA, qRT-PCR, and serotyping qRT-PCR as described above.

Longitudinal sample analysis

All samples collected from S01 sheep in 2021 were evaluated via BTV cELISA, qRT-PCR, and serotyping qRT-PCR. For all 2022 longitudinally sampled animals, BTV cELISA and qRT-PCR were performed on all samples collected from August to December. The sample with the lowest Ct value was selected for serotyping. For the 2023 longitudinally sampled animals, samples were first tested for BTV antibodies in July. All animals were tested for both BTV antibodies and RNA in October. Animals that tested positive by qRT-PCR in October were subjected to additional testing to determine the timing of infection and if they remained positive throughout the sampling period.

Statistics

Prevalence estimates are reported with 95% exact binomial confidence intervals calculated using the Clopper-Pearson method. A mixed-effects logistic regression model was used with species and years as fixed effects. Site and individual animal IDs were included as random effects to account for clustering within sites and repeated sampling between years of some animals. Type III Wald chi-square tests were used to assess the overall significance of species and year. Odds ratios and 95% confidence intervals were used to evaluate

significance at p-value < 0.05. All analyses were performed in R version 4.5.1 using the glmmTMB and car packages.

Results

Sampling Year – 2021

Blood was collected from a total of 58 sheep and 59 cattle across seven sites (Figure 1). B02 was the only site from which both sheep and cattle were sampled. At Site S04, 14 sheep were sampled; however, PCR and cELISA results were available for only 13 animals each due to missing whole blood and serum samples, respectively. BTV RNA and antibodies were detected at all sites, and all animals that tested positive for BTV RNA also tested positive for BTV-specific antibodies.

The combined average BTV RNA prevalence for both species was 27% (95% CI: 19-36%) (Figure 2A). At the species level, cattle and sheep tested positive for BTV RNA at 25% (95% CI: 15-38%) and 28% (95% CI: 17-42%), respectively. Sheep at site S01 had the greatest percentage of BTV RNA positive animals at 73% (95% CI: 45-92%). Overall, cattle sites ranged from 7-40% while sheep sites ranged from 7-73%.

Of the 31 positive animals, BTV serotypes -6, -11, and -17 were detected in both sheep and cattle samples (Figure 3). Samples were additionally tested for BTV-2w, -2e, -3, and -13, all were negative. In total, serotype was not determined for five samples. Most samples were identified as either BTV-6 (n=11) or BTV-11 (n=12), with only three samples identified as BTV-17. S01 and C06 were the only sites with multiple serotypes detected. Two different serotypes were identified in one animal, a sheep from S01, in which both BTV-11 and -6 were detected.

This animal was positive for only BTV-11 at the August and September collections but was positive for both BTV-6 and BTV-11 in October and December.

The seroprevalence of BTV-specific antibodies for 2021 was 56% (95% CI: 47-65%) for sheep and cattle combined. Individual sites ranged from 7-93% with a greater seroprevalence among cattle herds than sheep flocks at 64% (95% CI: 51-76%) and 47% (95% CI: 34-61%), respectively (Figure 2B). S01 and C05 had the highest percentage of positive animals, each at 93% (95% CI: 68-99.8%).

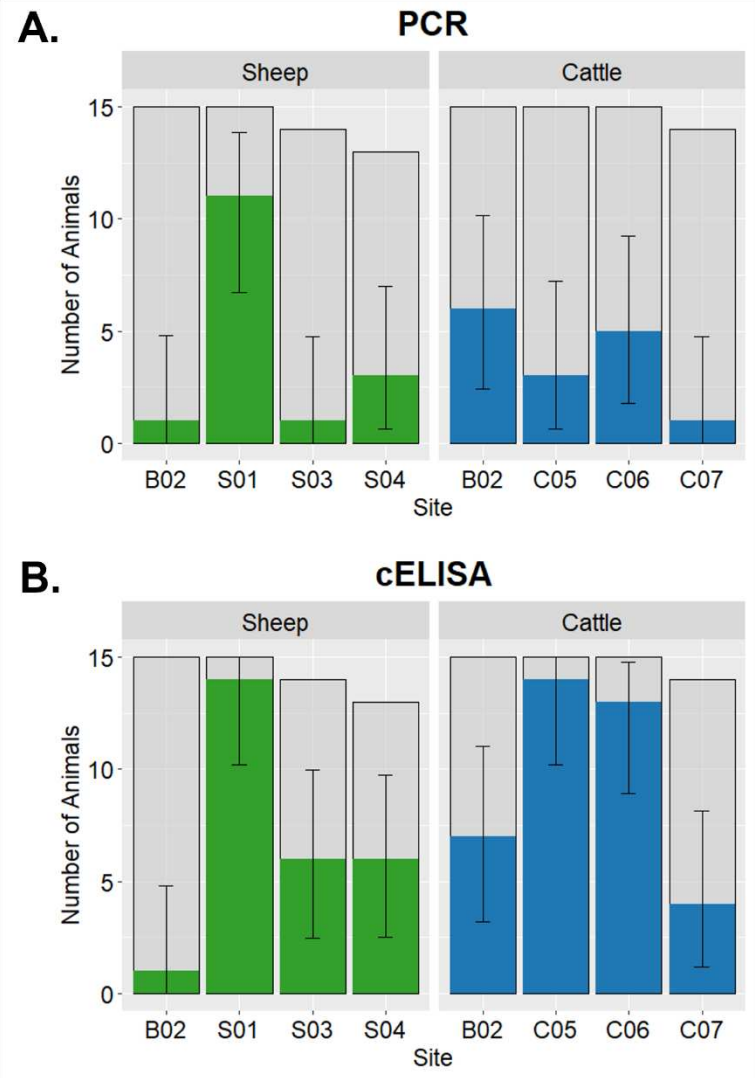

Figure 2 – BTV RNA and serology data for sheep and cattle sites in 2021. Data were collected cross-sectionally at all sites except S01, which was sampled longitudinally. Sheep are shown in green and cattle in blue. A. Number of animals positive via qRT-PCR for BTV. B. Number of animals positive via cELISA for BTV. Error bars are 95% confidence interval.

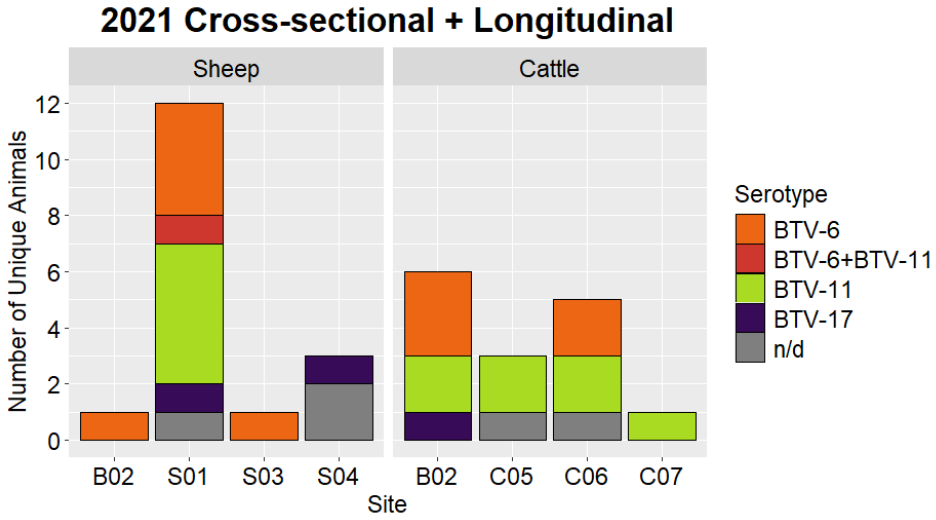

Figure 3 – Serotype of BTV positive animals by site from sheep and cattle in 2021. n/d = not determined.

S01 was the only site in 2021 for which longitudinal sampling took place (August through December). In August, 2/15 sheep were both PCR and cELISA positive while 5/15 sheep were cELISA positive but PCR negative. Three of the five PCR-negative, seropositive sheep never became PCR positive over the course of the study. Two subsequently tested PCR positive, with one becoming positive in September and the other in October. PCR positivity increased in September, with 8/14 sheep testing positive, followed by one additional sheep becoming positive in October. One sheep was PCR and cELISA negative in August and died in September prior to collections. Spleen was obtained from this sheep at necropsy and the presence of BTV RNA was confirmed and serotyped as BTV-11.

Sampling Year – 2022

Longitudinal

For the 2022 longitudinal collections, a total of 74 cattle and 75 sheep were sampled. Sampling was repeated at S01 and B02 (Figure 1). Sampling at C10 was limited to the months of July, September, and November. Sampling did not take place at B02 for sheep or cattle during the month of September at the request of the producer.

The combined average prevalence of BTV RNA in 2022 for both species was 28% (95% CI: 21-35%) (Figure 4A). Of the longitudinally sampled animals, 28% of cattle (95% CI: 19-40%) and 27% of sheep (95% CI:17-38%), tested positive for BTV RNA. Prevalence at cattle sites ranged from 0-75% while sheep sites ranged from 22-35%. B02 was the only location in which both sheep and cattle were sampled, making it the only location with mixed-species sampling. Similar to findings in 2021, a greater proportion of cattle (12/16) were BTV RNA positive as compared to sheep (4/18).

Longitudinal sampling allowed for the identification of time periods during which new BTV infections were occurring. For this analysis, samples were unavailable for C10 in August and October, and for B02 sheep and cattle in September. The first BTV-positive animal was identified at B02 in a single bovine in August, followed by 18 additional new positive animals in September at S01, S08, S09, C10, and C11. October had the greatest number of new positive cases with 19 animals at B02 in both sheep and cattle, S08, S09, and C11, followed by only two new detections in November at S01 and C10. Given the missing September data for B02 and October data for C10, it cannot be ruled out that some of these animals became positive at that time. Additionally, two sheep at S01 had not been sampled previously and were therefore included opportunistically. The producer reported clinical signs consistent with disease in one sheep prior to the September collection and in another sheep prior to the November collection. Both sheep tested positive for BTV RNA.

Of the 41 BTV RNA positive animals sampled in 2022, most samples were serotyped via PCR as BTV-13 (n=21), followed by BTV-11 (n=6), -10 (n=1) and -17 (n=3) (Figure 5A). Serotype was not determined for nine samples. B02 was the only location in which all of the above serotypes were detected; however, BTV-17 RNA was not detected in any sheep at this site. All sites that had BTV positive animals had multiple serotypes present, except for S01, in which only BTV-13 was detected.

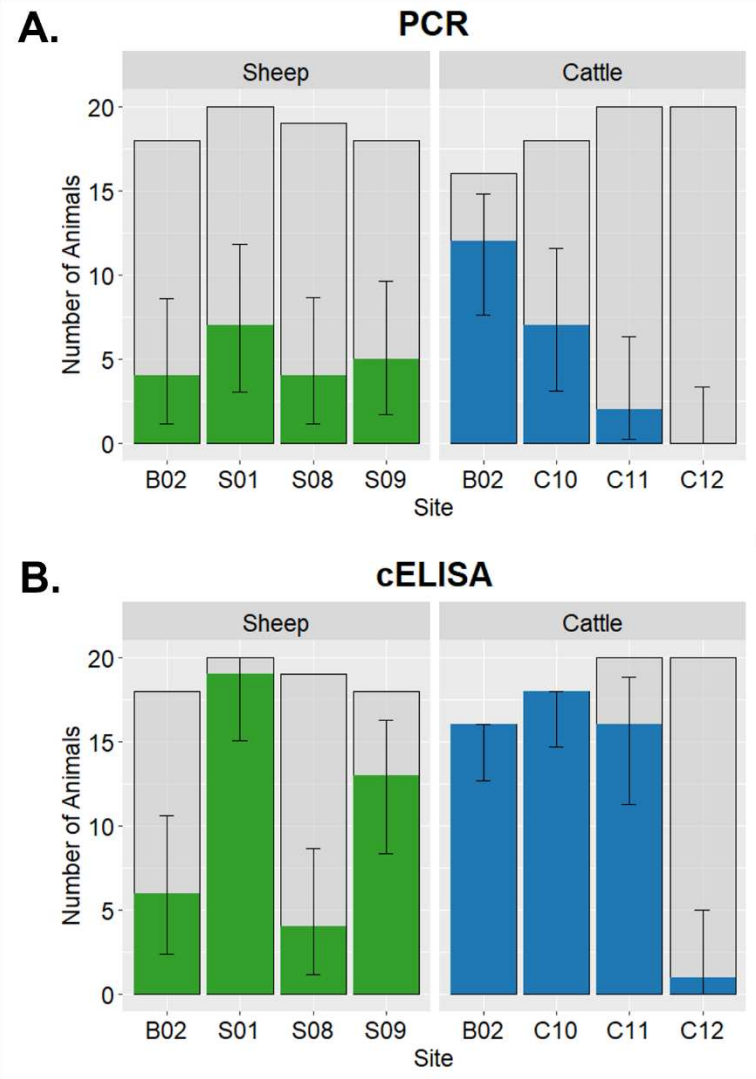

Figure 4 – BTV RNA and serology data for longitudinally sampled sheep and cattle sites in 2022. Sheep are shown in green and cattle in blue. A. Number of animals positive via qRT-PCR

for BTV. B. Number of animals positive via cELISA for BTV. Error bars are 95% confidence interval.

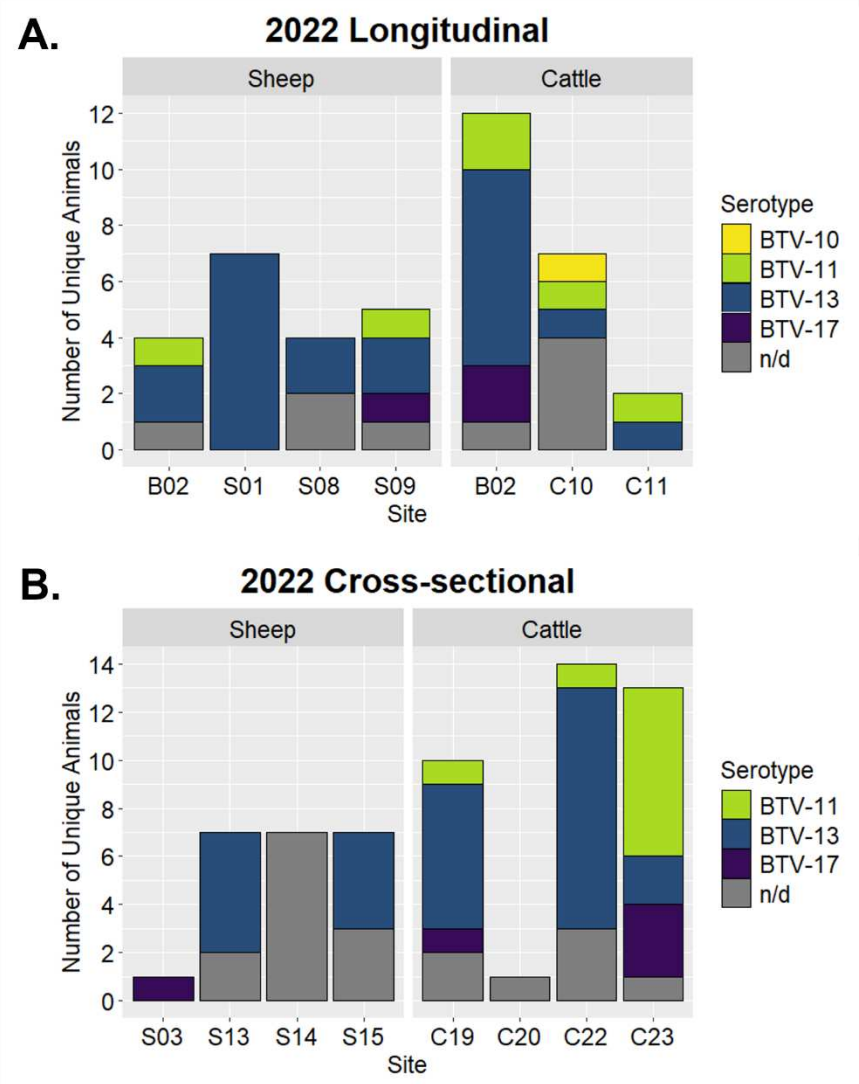

Figure 5 – Serotype of BTV positive animals by site from A. longitudinally and B. cross-sectionally collected sheep and cattle sites in 2022. n/d = not determined.

The combined average seroprevalence for the longitudinally sampled animals was 62% (95% CI: 54-70%) with 69% (95% CI: 57-79%) positive cattle and 56% (95% CI: 44-68%) positive sheep (Figure 4B). Seroprevalence at the site-level ranged from 5-100% in cattle and 21-95% in sheep. During this longitudinal study, almost all animals that tested positive via BTV

qRT-PCR subsequently developed an antibody response. One exception, a sheep at S08, was PCR positive in October and November but did not test positive via cELISA at any timepoint during the longitudinal sampling period. At the species level, 30% of both cattle (11/37) and sheep (10/33) had detectable antibodies at the beginning of the sampling period that later became BTV positive.

Cross-sectional

During the two-week cross-sectional collection period in October 2022, an additional 105 cattle and 76 sheep were sampled from 12 sites, including 11 new sites and one repeat site from 2021, S03 (Figure 1). One cattle site, C23, was the only location outside the state of Colorado, located in Scotts Bluff County, Nebraska.

The combined average positive detection of BTV RNA for both species was 33% (95% CI: 26-41%) with 36% (95% CI: 27-46%) of cattle samples and 29% (95% CI: 19-41%) of sheep samples testing positive (Figure 6A). Prevalence at the site level ranged from 0-93% in cattle herds and 0-47% in sheep flocks. Several cattle sites (B02, C17, and C18) had no detection of BTV RNA; in contrast, only a single sheep site (S16) was negative.

Serotypes detected from the 60 BTV positive cross-sectional samples mirrored the findings from the longitudinally sampled animals. The majority of samples were determined to be BTV-13 (n=27), with fewer detections of BTV-11 (n=9) and -17 (n=5) (Figure 5B). There were 19 samples in which serotype was not determined, including all seven positive sheep at S14.

The combined average seroprevalence of BTV antibodies in the cross-sectionally sampled animals was 51% (95% CI: 43-58%) with 61% (95% CI: 51-70%) of cattle and 37%

(95% CI: 26-49%) of sheep testing positive (Figure 6B). A number of sites (S14, S16, and C17) all had no detectable BTV antibodies. Additionally, S15, C19, C22, and C23 had over 90% of sampled animals test positive for antibodies. Of the 60 BTV RNA positive animals, 10 animals, seven of which were from S14, did not have positive antibody detections.

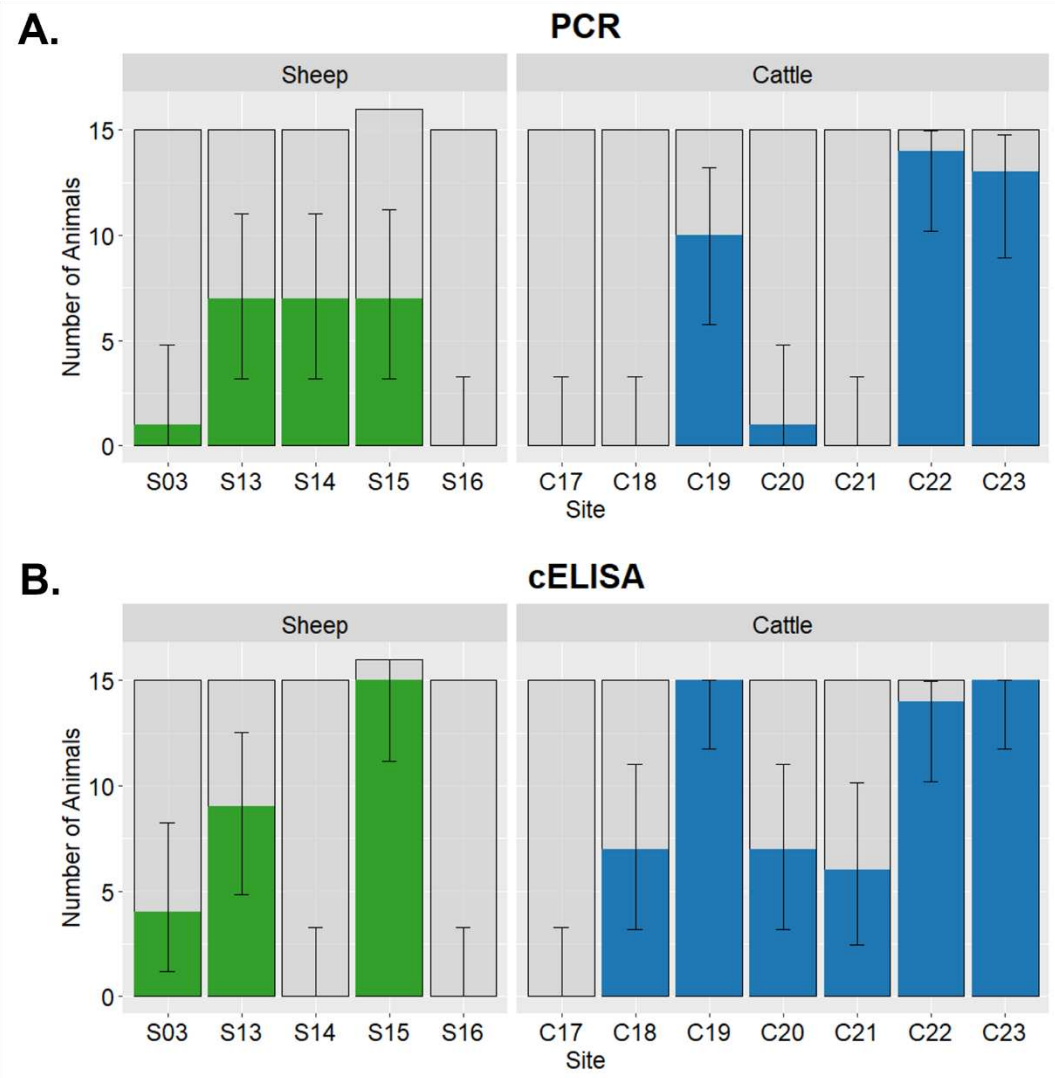

Figure 6 – BTV RNA and serology data for cross-sectionally sampled sheep and cattle sites in 2022. Sheep are shown in green and cattle in blue. A. Number of animals positive via qRT-PCR for BTV. B. Number of animals positive via cELISA for BTV. Error bars are 95% confidence interval.

Sampling Year - 2023

Longitudinal

During the 2023 longitudinal collections, a total of 74 cattle and 84 sheep were sampled. Sampling was repeated at the same four longitudinal sheep sites and only two of the longitudinal cattle sites (Figure 1).

There were fewer detections of BTV RNA from the 2023 longitudinally sampled animals with only eight positive animals in total, seven sheep and one bovine, for a combined average prevalence of 5% (95% CI: 2-10%) (Figure 7A). Prevalence ranged from 0-5% at cattle sites and 0-20% at sheep sites. Positive animals were detected at only three sites: S01, S09, and C11. Two animals first tested positive in September, while the remaining six first tested positive in October. Of these, one sheep from S01, had no September sample and thus may have become positive at that time. Of the eight positive samples, four serotyped as BTV-17, one as BTV-11, and one as BTV-13, while two could not be serotyped (Figure 8A). All positive sheep at S01 serotyped as BTV-17, C11 had a single detection of BTV-13, and S09 had detections of BTV-11 and -17, with two samples yielding no serotype result.

The combined average seroprevalence in 2023 was 46% (95% CI: 38-54%) with antibodies detected in 49% (95% CI: 37-61%) of cattle and 44% (95% CI: 33-55%) of sheep (Figure 7B). All animals that became PCR positive subsequently developed detectable antibodies. At the start of the season, only three sheep were seropositive, and each of these animals later became PCR positive.

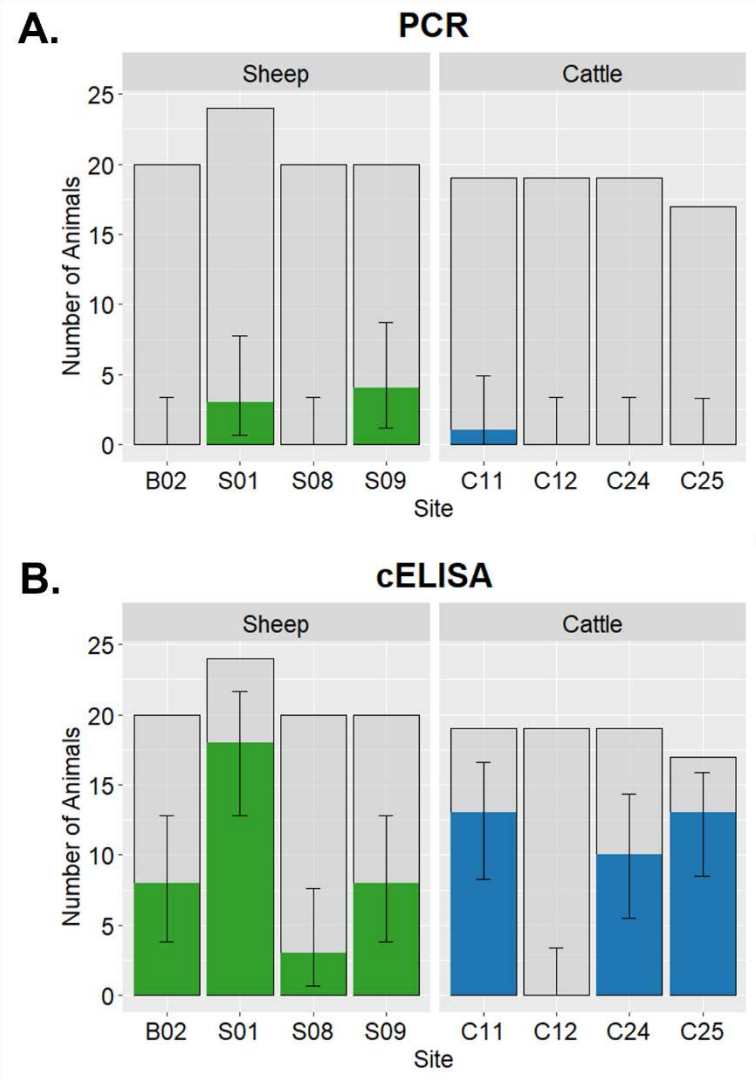

Figure 7 – BTV RNA and serology data for longitudinally sampled sheep and cattle sites in 2023. Sheep are shown in green and cattle in blue. A. Number of animals positive via qRT-PCR for BTV. B. Number of animals positive via cELISA for BTV. Error bars are 95% confidence interval.

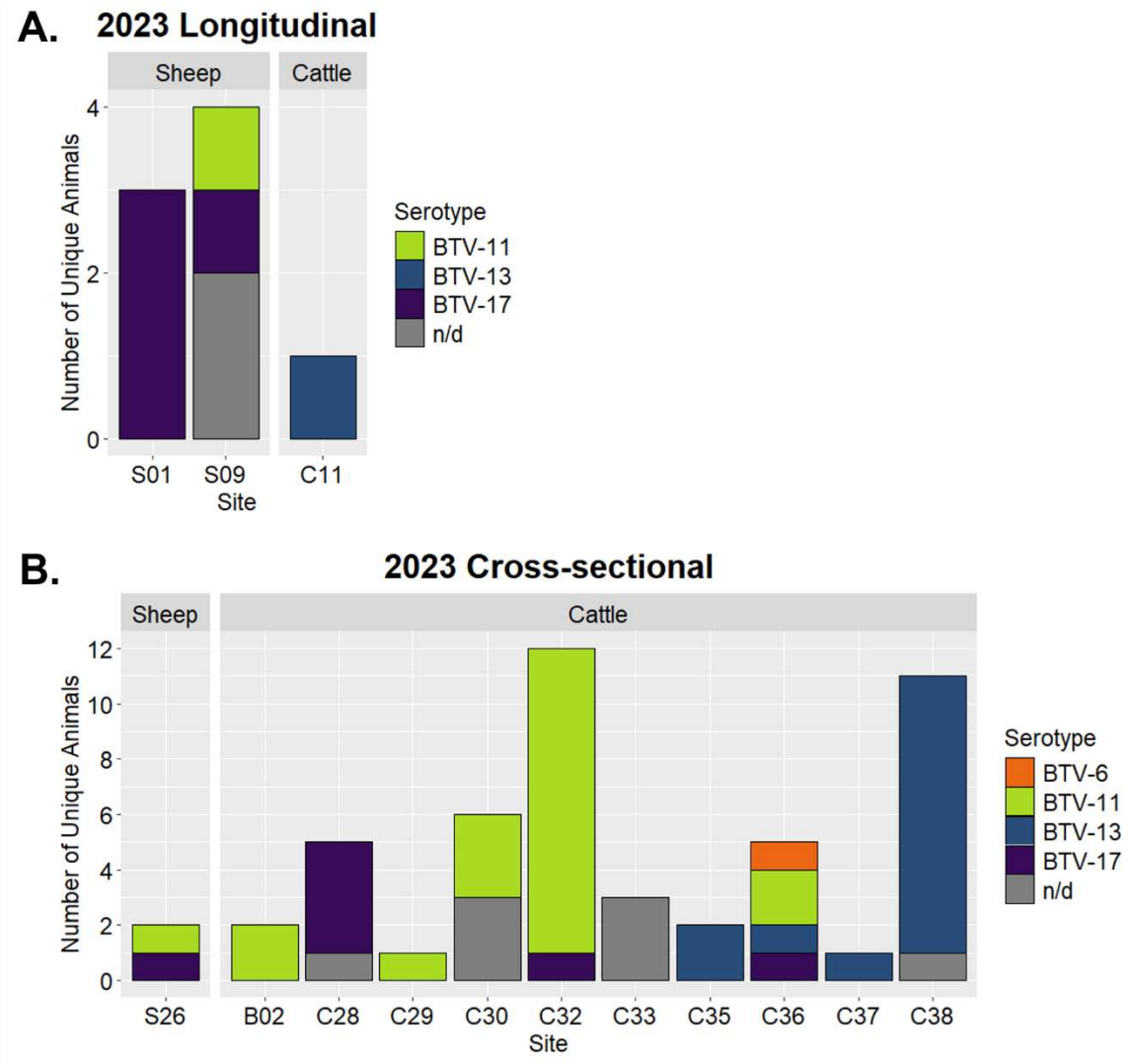

Figure 8 – Serotype of BTV positive animals by site from A. longitudinally and B. cross-sectionally collected sheep and cattle sites in 2023. n/d = not determined.

Cross-sectional

A total of 212 cattle and 77 sheep were sampled during the 2023 October cross-sectional sampling period from 15 new sites and four previously sampled sites (B02, S03, C13, and S16) (Figure 1). One new site, B27, had both sheep and cattle that were sampled from. C39 was the only site located outside of Colorado and was located in Carbon County, Wyoming.

The prevalence of BTV RNA among cross-sectionally collected animals was 17% (95% CI: 13-22%), with 23% of cattle (95% CI: 17-29%) and 3% of sheep (95% CI: 0-9%) testing positive (Figure 9A). This prevalence was higher than what was observed in the 2023 longitudinal cohort. At the site level, prevalence ranged from 0-75% in cattle herds and 0-13% in sheep flocks. Most of the sheep flocks had no detection of BTV RNA. Of the 14 cattle sites, four of these sites (B27, C31, C34, and C39) had no detection of BTV RNA. Additionally, sheep and cattle from B27 were all BTV negative. Although B02 cattle were not available for longitudinal sampling this year, samples were obtained from animals during the cross-sectional period and contained two positive detections. Four serotypes were detected from the 2023 cross-sectionally collected animals: BTV-6, BTV-11, BTV-13, and BTV-17 (Figure 8B). BTV-11 and -17 were identified from the two positive sheep, while BTV-6 (n=1), -11 (n=20), -13 (n=14), and -17 (n=7) were detected from the cattle samples. Serotype was not determined from eight samples. Most cattle sites had detection of one or two serotypes while S36 was the only site to have detection of four different serotypes.

The overall seroprevalence of BTV in the cross-sectionally collected animals was 51% (95% CI:45-57%) and was greater in cattle at 55% (95% CI: 48-62%) than compared to sheep at 39% (95% CI: 28-51%) (Figure 9B). Site level seroprevalence ranged from 0-67% in sheep flocks and 0-100% in cattle herds. Of the qRT-PCR positive animals, only six cattle from 5 different sites did not have paired positive serology.

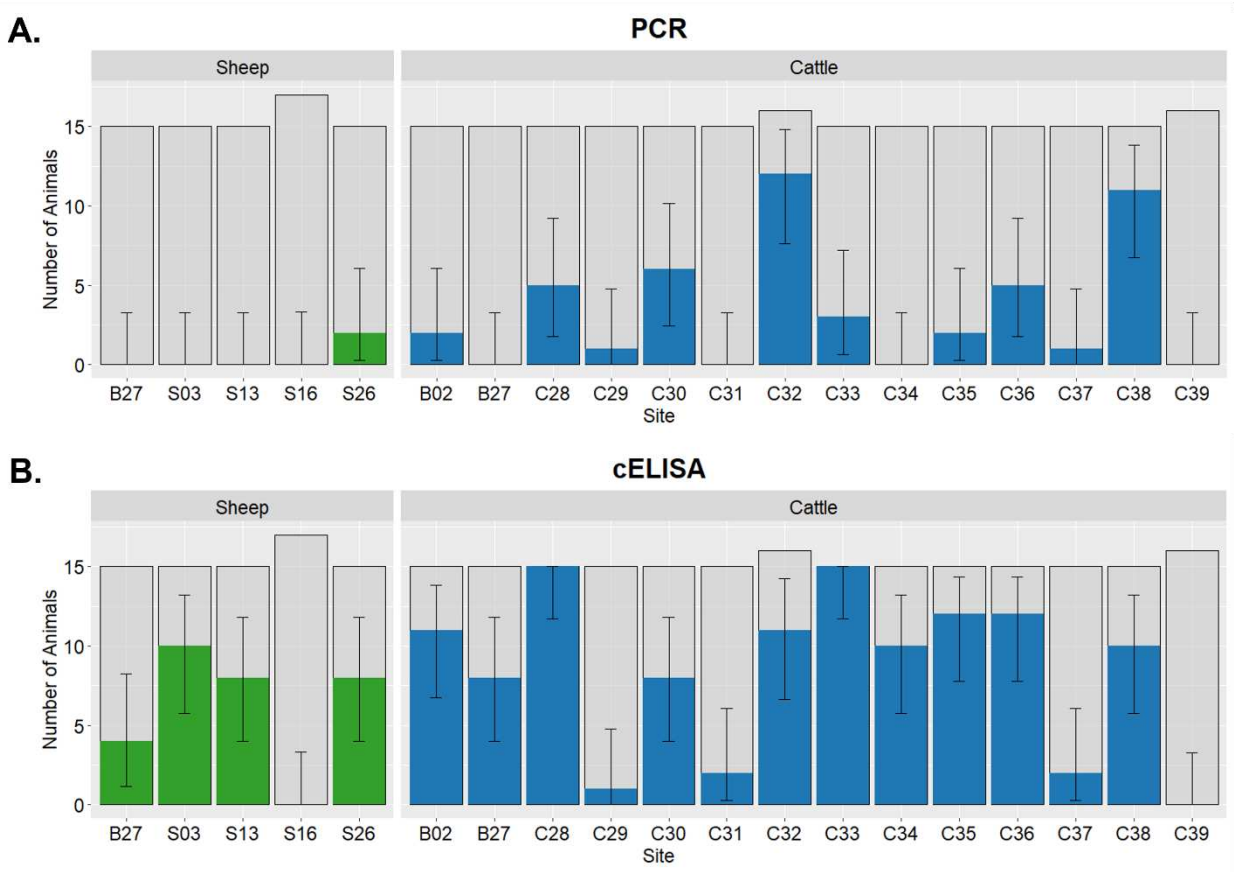

Figure 9 – BTV RNA and serology data for cross-sectionally sampled sheep and cattle sites in 2023. Sheep are shown in green and cattle in blue. A. Number of animals positive via qRT-PCR for BTV. B. Number of animals positive via cELISA for BTV. Error bars are 95% confidence interval.

Statistics

Overall, species ($\chi 2 = 10.29$, df = 1, $p = 0.0013$) and year ($\chi 2 = 27.70$, df = 2, $p < 0.0001$) were significant overall predictors in the model (Table 2). Cattle had significantly higher odds of being PCR-positive compared with sheep (OR = 4.49, 95% CI: 1.79-11.22, p = 0.0013). Relative to 2023, animals sampled in 2021 (OR = 6.32, 95% CI: 2.56-15.63, p <0.001) and 2022 (OR = 6.54, 95% CI: 3.18-13.46, p <0.001) had significantly greater odds of testing PCR-positive. Random effects indicated substantial heterogeneity among sites (variance = 4.33, SD = 2.08), while the variance attributable to repeated sampling of individual animals was small

(variance = 0.055, SD = 0.23), suggesting that differences among sites contributed more strongly to variability in BTV PCR positivity.

Table 2 – Results of multivariable analysis using mixed-effects logistic regression model and type III Wald chi-square tests to identify exposure variables associated with testing PCR-positive for BTV. Sheep are the reference species and 2023 is the reference year. OR = Odds ratio, CI% = Odds ratio confidence interval.

Factor	Level	n	OR	CI%	p-value	Type III p-value
Species	Sheep	369		Reference		0.0013
	Cattle	524	4.49	1.79-11.22	0.0013	
Year	2023	447		Reference		<0.0001
	2021	116	6.32	2.56-15.63	<0.0001	
	2022	330	6.54	3.18-13.46	<0.0001	

Discussion

This study characterizes the prevalence and serotype diversity of BTV in livestock in Colorado and surrounding areas over the course of three years. The prevalence of BTV RNA in domestic sheep and cattle was found to be 27% in 2021, 31% in 2022, and 13% in 2023. These findings are similar to detections reported in Colorado in 2007[65]. The difference in prevalence between years, notably the significantly higher odds of infection in 2021 and 2022, demonstrates the dynamic nature of BTV across years that is likely due to a variety of factors such as yearly weather conditions that impact the midge vector, immunity against the circulating serotypes, among others.

A total of five different serotypes of BTV (BTV-6, -10, -11, -13, and -17) were detected. Although multiple serotypes were detected each year, with many herds/flocks testing positive for multiple different serotypes, the predominant serotype detected each year changed. In 2021, the predominant serotypes detected were BTV-6 and -11, followed by BTV-13 in 2022, and BTV-11

and -13 in 2023. Co-circulation of different serotypes is a crucial prerequisite for coinfection in either vertebrate or invertebrate host and is required for reassortment events involving segment 2 to occur.

Reassortment, the process by which segmented viruses swap genetic material to create new progeny viruses, is a significant mechanism by which BTV can evolve[15]. Interestingly, co-detection of BTV-6 and -11 was found in a sheep that tested positive for BTV-11 at the August and September collections and was later positive for both BTV-6 and -11 in October and December. Although this animal had co-detection of two different serotypes, it is challenging to call this a true co-infection. BTV RNA can be detected in the blood months after infectious virus is no longer present[66,67]. However, co-detection of two distinct serotypes in a single animal two months after the initial infection suggests that re-infection can occur within a single BTV season when multiple serotypes are circulating, particularly given the limited cross-protection between these serotypes *in vitro*[68].

The detection of BTV-6 in multiple animals in 2021, and a single animal in 2023, is intriguing given the recent introduction of this serotype into the area[56]. Historical serotyping data supports the longtime circulation of BTV-10, -11, -13, and -17 in Colorado[33,52–55,58]. Animals exposed to BTV-6 likely had limited humoral protection due to the poor cross-protection offered between divergent BTV serotypes[68,69]. Notably, S01 is the same sheep flock from which the 2020 BTV-6 sheep sample was obtained[56]. The detection of this serotype the following year at the same site and others in the region highlights the persistence of this newly introduced serotype from 2020 to 2021. However, no BTV-6 positive animals were identified in 2022 and only a single BTV-6 positive bovine was detected in 2023. Some explanations for the observed decrease in BTV-6 detections after 2021 include poor transmission among vector populations or the

development of host herd immunity among previously exposed animals. This may also be the case for BTV-3, as no positive cases have been detected in Colorado since it was first reported in 2015[57]. Further work is needed to understand the maintenance of some serotypes in a region and the mechanisms that drive their persistence, re-emergence, or local extinction.

Despite efforts to serotype all BTV positive samples, serotype was not determined for 43 (22.6%) qRT-PCR positive samples, highlighting the potential of next generation sequencing (NGS) platforms for comprehensive serotype identification. Additionally, the characterization of a segmented viral genome using only a single genomic segment limits our understanding of how the remaining segments may be changing via reassortment. As evidenced by our group's evaluation of the 2020 and 2021 BTV-6 genomes, these viruses had likely already undergone reassortment with circulating established serotypes[56]. By applying NGS directly to field-collected matrices, we can obtain critical insights into viral genome structure and evolution while also serving as a powerful tool for detecting emerging and previously unreported serotypes, without the time and cost associated with viral isolation. Using Oxford Nanopore Technologies (ONT) long-read sequencing, our lab (unpublished data) has characterized four of these untyped samples as BTV-10. Analysis of the primers and probes sequences as described by Maan *et al*. and used for serotyping in this study revealed numerous mismatches between the forward primer and our field-derived sequences[64]. These mismatches likely reflect natural sequence drift within this serotype, further underscoring the advantages of sequencing-based approaches over qRT-PCR for BTV characterization.

Longitudinal sampling of animals for three years corroborates the previously described seasonality of BTV in Colorado with the first detections of BTV RNA occurring in August or September with most animals becoming positive by October or November. These data align with

previous reports on the seasonality of BTV in North America, as disease has historically been seen between the months of July through November[47]. This observed seasonal pattern is presumed due to the abundance of the predominant vector, *Culicoides sonorensis*, and has been shown to lag approximately 4.5 weeks behind the peak vector index, the point at which the abundance of BTV-infected female *C. sonorensis* was highest[48].

Overall seroprevalence for BTV in both sheep and cattle was 56% in 2021, 56% in 2022, and 49% in 2023. In general, longitudinal animals that became positive via qRT-PCR subsequently developed detectable BTV antibodies in response. Animals in which an antibody response was not detected following a positive qRT-PCR result were uncommon but were documented in two sheep in 2022, suggesting limitations in assay sensitivity or a failure to mount a detectable antibody response. In contrast, discordant results were also observed among several cross-sectionally sampled animals. Two sheep in 2022 and six cattle in 2023 were qRT-PCR positive but cELISA-negative, which is consistent with sampling early during the course of infection prior to seroconversion, failure to develop a detectable antibody response, or limitations in assay sensitivity.

Presence of antibodies in response to vaccination are unlikely but cannot be ruled out in this study. While a commercially available vaccine for BTV-10 was available from the Colorado Serum Company until approximately 2018, the longevity of BTV-specific antibodies is unknown and could be life-long in some animals[70–73]. However, when asked about vaccinating BTV, producers reported that they did not. Assessing the protective effect of antibodies from past exposure is challenging because current methods for determining serotype-specific responses are limited. At present, identifying the specific BTV serotype for which an animal has antibodies depends primarily on serum neutralization assays, a method that is both time-consuming and

resource-intensive given the extensive diversity of BTV serotypes. Of the 2022 longitudinally sampled animals, 30% of both sheep and cattle had detectable antibodies against BTV at the beginning of the collection season and still became PCR positive. This may indicate these animals had been previously exposed to a different serotype that was not cross-protective for the serotype they were infected with during this year. While overall seroprevalence provides insight into past exposure to BTV, it is challenging to say what level of protection is offered against future infection with the number of serotypes that can be circulating in any one year.

Collectively, these findings underscore the importance of sustained active BTV surveillance in both wildlife and domestic species for understanding the transmission dynamics and ecology of established serotypes, detecting unreported serotypes and changes in infection rates, and identifying emerging transmission patterns that could impact animal health, livestock production, and trade. Both wildlife and domestic species play key roles in BTV ecology, with certain species potentially serving as reservoirs for others that are more susceptible to infection. It remains unclear when and how new serotypes of BTV are introduced into the United States and interior states such as Colorado, how they disseminate across regions, and whether native Culicoides species can efficiently transmit all potential serotypes. Further research integrating surveillance, vector ecology, and viral genomics is needed to further characterize BTV ecology and evolution in the United States and to develop effective preparedness and response strategies.

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CHAPTER 3 – GENETIC LANDSCAPE OF BLUETONGUE VIRUS: INSIGHTS FROM A LONGITUDINAL SHEEP COHORT IN COLORADO

Introduction

Bluetongue virus (BTV) is an economically important arbovirus of wild and domestic ruminants with a global distribution. Vectored by *Culicoides* spp. biting midges, this virus can have significant health impacts in susceptible hosts, primarily impacting sheep through a variety of disease outcomes. Clinical disease is highly variable, ranging from asymptomatic to severe, including fever, coronitis and associated lameness, oral erosions, facial edema, and difficulty breathing[1–4]. Due to its significant animal health and trade implications, BTV is classified as a notifiable disease of international importance by the World Organization for Animal Health (WOAH)[5]. Outbreaks can result in considerable economic impacts when they occur and cause ongoing production losses in endemic regions[6–8]. Control of BTV remains challenging because of its vector-borne transmission cycles, broad host range, and limitations associated with vaccination strategies[9–11].

These ongoing control challenges are closely associated with the virus's genomic segmentation and antigenic diversity. The BTV genome is comprised of ten linear segments of double-stranded RNA[12,13]. Historically, BTV has been classified according to antigenic variation in VP2, the outer capsid spike protein encoded by segment 2, which is the principal determinant of viral serotype[14–17]. To date, over 29 serotypes of BTV have been documented[18–26]. Focus on genetic characterization of segment 2 has led to the production of RT-PCR assays to molecularly identify corresponding serotypes[27,28]. Importantly, it is presumed that all serotypes of BTV have the ability to undergo reassortment, or the production of viral progeny that contain genome

segments derived from coinfecting parental strains[29–32]. Whole-genome sequencing of BTV field isolates has demonstrated that natural reassortment occurs frequently and is a major mechanism by which BTV evolves[31–36]. Evaluation of all segments, therefore, provides the opportunity to understand reassortment events that may alter viral fitness, including virulence.

Despite extensive research on BTV, most whole-genome sequencing has primarily occurred opportunistically during cross-sectional studies or outbreak investigations on viral isolates. In contrast, investigating whole genomes directly from field-collected samples enables characterization of viral genetic diversity as it exists *in vivo*, including within-host quasispecies, mixed infections, reassortment between co-circulating strains, and selection pressures that occur during natural infection. Obtaining field isolates from non-clinical animals is rare, as is sequencing directly from whole blood that hasn't been passaged in culture or embryonated eggs[28,37–39]. Importantly, *in vitro* isolation can lead to population bottlenecks that alter quasispecies and introduce consensus-level genome changes[40–42]. For this reason, comparisons between uncultured, field-collected viruses within the same flock provide an opportunity to evaluate viral evolution, reassortment dynamics, and persistence under natural conditions.

This study examines the genetic landscape of BTV within a single sheep flock across three consecutive years using samples obtained during active surveillance following the introduction of a new serotype. Long-read sequencing was used to generate whole genomes and phylogenetic tanglegrams of each segmented compared to segment 2 were constructed alongside nucleotide-level comparisons between samples of the same serotype. This longitudinal approach provides insight into viral persistence, population turnover, and genomic diversification over time in an endemic setting. Moreover, to the best of our knowledge, this work represents the first longitudinal study to include whole genomes of BTV characterized from non-passaged isolates.

Methods

Sample collection and identification of BTV positive samples

Whole blood samples were collected from a single sheep flock in Northern Colorado during periods of active BTV transmission in 2021-2023. All work was approved by Colorado State University's Institutional Animal Care and Use Committee (IACUC) with additional written approval from the producer. Blood was collected in EDTA tubes and stored at 4°C until analysis. Nucleic acids were extracted from samples using the Applied Biosystems™ MagMAX™ CORE RNA/DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's simple workflow with automated processing performed on the KingFisher96™ magnetic particle processor (Thermo Fisher Scientific, Waltham, MA, USA). BTV-specific qRT-PCR for segment 10 and serotyping qRT-PCR for BTV-6, -10, -11, -13, and -17 were performed as previously described in Chapter 2. Up to five samples per serotype per year were selected for sequencing, with priority given to samples from animals that tested positive in multiple years.

Whole-genome sequencing using sequence-independent, single-primer amplification (SISPA) and Oxford Nanopore Technology

Samples were prepared for Nanopore sequencing utilizing methods previously described with some modifications[39,43]. Briefly, overrepresented rRNA from the host was depleted utilizing the NEBNext® rRNA Depletion Kit v2 (Human/Mouse/Rat) (New England Biolabs, Ipswich, MA, USA). Reverse-transcription, second strand synthesis, and amplification were performed as described with the inclusion of random and orbivirus specific-tagged primers (Table 1). A modification was made in the amplification step in which the number of amplification cycles was

reduced from 40 to 20 to avoid PCR jackpotting. Resulting amplicons were purified utilizing AMPure XP Beads for DNA Cleanup (Beckman Coulter Life Sciences, Brea, CA, USA) and eluted in 27 µL of DNase free water. Total dsDNA was quantified using a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared for sequencing using the SQK-NBD114.96 Native Barcoding Kit 96 V14 (Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. A final pooled library of 45 fmol was loaded onto a FLO-MIN114 R10.4.1 flow cell (Oxford Nanopore Technologies, Oxford, UK) and run on a GridION (Oxford Nanopore Technologies, Oxford, UK) using the super accurate basecalling (SUP) model.

Table 1 – List of primers used for cDNA synthesis and amplification.

Primers	Sequences 5'-3'
FR26RV-N (50 µM)	GCCGGAGCTCTGCAGATATCNNNNNNN
FR-BT_F (10 µM)	GCCGGAGCTCTGCAGATATCGTTAAAN
FR-BT_R (10 µM)	GCCGGAGCTCTGCAGATATCGTAAGTN
FR20RV (40 µM)	GCCGGAGCTCTGCAGATATC

Phylogenetic analysis

Genomic sequences were produced using a Nextflow pipeline developed by our lab, OrbiSeq (https://github.com/tdunham19/OrbiSeq), and the resulting sequences were analyzed in Geneious Prime v2025.2.1[44]. Multiple sequence alignments were generated using MUSCLE v5.1 in Geneious Prime and maximum likelihood trees were constructed using IQ-TREE v2.4.0. Ultrafast bootstrap support values were estimated using 1,000 replicates with the BNNI correction. Trees were midpoint rooted and branches shorter than 0.001 were converted to

polytomies. Tanglegrams were produced using R v4.5.1 with the phytools v2.5.2 and ape v5.8.1 packages. All comparisons were made against segment 2.

Genetic diversification

For each serotype, nucleotide alignments were generated separately for each genome segment in Geneious Prime using MUSCLE v5.1. A segment-specific consensus sequence was generated for each alignment using a majority rule threshold of 0%. Single nucleotide polymorphisms (SNPs) were identified for each sequence relative to the segment-specific consensus sequence using the "Find Variations/SNPs" function in Geneious Prime. SNPs were identified as either nonsynonymous or nonsynonymous. In cases where no single nucleotide reached majority consensus (e.g., ambiguous bases resulting from 50/50 splits), the position was treated as polymorphic. Functional classification for these positions were classified as synonymous if all nucleotide variants encoded the same amino acid and nonsynonymous if the variants resulted in different amino acids.

Results

Whole-genome sequencing

A total of 17 samples were sequenced from this flock spanning three consecutive years (2021-2023). Multiple serotypes were detected in 2021, consisting of BTV-6, BTV-11, and BTV-17. In contrast, only one serotype was detected in each subsequent year, with BTV-13 in 2022 and BTV-17 in 2023. Serotypes were initially determined by qRT-PCR and confirmed by sequencing. Several individual animals tested positive in consecutive years, each time with a different serotype, indicating repeated infection with genetically distinct viruses. Whole genome sequences were obtained from most samples (GenBank PZ528042-PZ528181; Table 2).

Complete genomes could not be recovered from three samples: S09 2021 (BTV-11), S02 2022 (BTV-13), and S01 2023 (BTV-17).

Table 2 – BTV positive sheep samples collected across three years of active surveillance from a single sheep flock. Serotype was first determined by serotype-specific qRT-PCR assays and confirmed via sequencing. Pan BTV Ct values were generated via qRT-PCR for BTV segment 10. *Samples for which complete viral genomes could not be recovered by sequencing.

Year	Sample ID	Serotype	BTV Ct
2021	S01 2021	BTV-6	27.93
	S02 2021	BTV-6	30.96
	S04 2021	BTV-6	31.67
	S08 2021	BTV-6	31.67
	S05 2021	BTV-11	28.83
	S06 2021	BTV-11	23.98
	S07 2021	BTV-11	28.15
	S09 2021*	BTV-11	36.30
	S03 2021	BTV-17	30.91
2022	S01 2022	BTV-13	29.48
	S02 2022*	BTV-13	33.92
	S03 2022	BTV-13	27.93
	S10 2022	BTV-13	28.22
	S11 2022	BTV-13	24.66
2023	S01 2023*	BTV-17	33.38
	S12 2023	BTV-17	30.94
	S13 2023	BTV-17	27.58

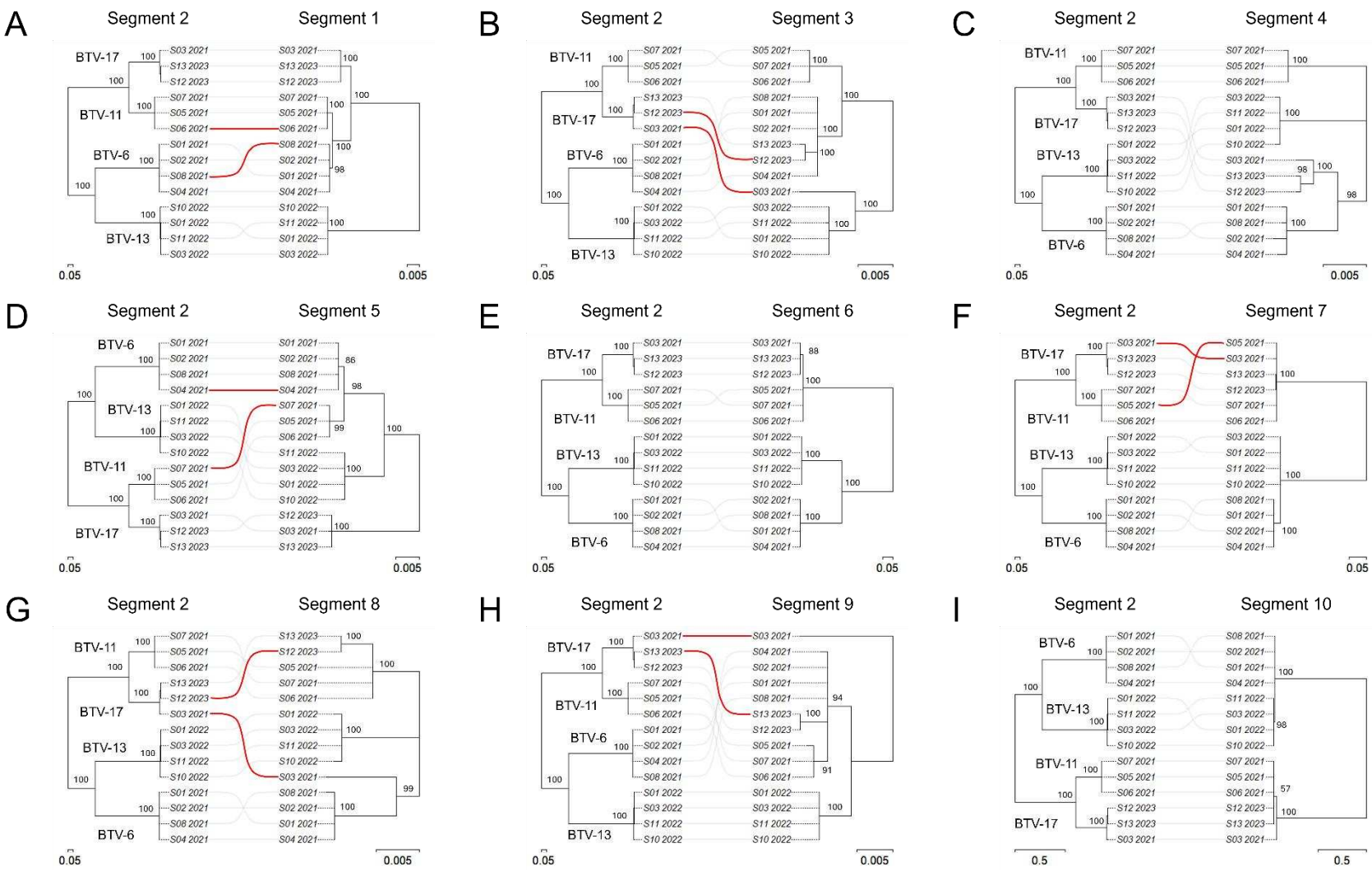

Figure 1 – Tanglegrams comparing phylogenetic relationship of BTV genome segments 1 through 10 against segment 2 (A-I). Maximum likelihood midpoint-rooted trees constructed using BTV nucleotide sequences from qRT-PCR positive sheep samples. Support values are shown for select nodes. Selected examples of phylogenetic discordance of pairs of samples consistent with reassortment are highlighted.

Phylogenetic analysis

The segment 2 phylogenetic tree is defined by two well-supported clades, with BTV-11 and -17 forming one clade and BTV-6 and BTV-13 forming the other, each supported by 100% bootstrap values at major nodes (Figure 1A). When compared against segment 2, all segments (Figures 1A-I) exhibited incongruent phylogenetic tree topologies consistent with reassortment. For example, samples S06 2021 and S08 2021 share high nucleotide identity for segment 1 (99.6%; Figure 1A), yet their segment 2 sequences only share 51.2% nucleotide identity, consistent with their classification as distinct serotypes and reassortment. However, reassortment was also evident among samples with high sequence similarity within segment 2. For instance, segment 2 sequences from S03 2021 and S13 2023 share 98.4% nucleotide identity, whereas their segment 9 sequences share only 97.4% nucleotide identity and occupy different positions within the segment 9 phylogeny (Figure 1H). The frequency of phylogenetic discordance observed across segments likely reflects a combination of historic and contemporary reassortment events.

Genetic diversification

To further characterize the genomes in this dataset, nucleotide-level comparisons were made between segments within the same serotype, which found a largely consistent pattern of high nucleotide identity. Comparisons among sequences within the same serotype revealed that several segments exhibited extremely high nucleotide identity. Among sequences from the same serotype, nine segment sequences were found to be 100% identical at the nucleotide level. For example, segments 3, 7, and 10 of the BTV-6 samples were identical across all animals. Overall, the BTV-11 samples exhibited the lowest level of genetic variation, with only six synonymous and five nonsynonymous single nucleotide polymorphisms (SNPs) detected across the entire

coding genome (Figure 2). Similarly, low levels of variation were observed for BTV-6 (16 synonymous, 6 nonsynonymous) and BTV-13 (18 synonymous, 5 nonsynonymous). In contrast, BTV-17 samples displayed the lowest overall nucleotide homology and the highest number of nonsynonymous SNPs across most segments. The two 2023 BTV-17 samples were highly similar to one another whereas the 2021 BTV-17 sample was quite different in comparison to these two samples. Across the full coding genome, the BTV-17 samples had the greatest number of both synonymous (n = 174) and nonsynonymous (n = 45) SNPs.

To determine whether genetic variation was distributed evenly across the genome, the total number of SNPs were evaluated by segment to identify segments that accumulated relatively high or low levels of variation. Segment 3 had the highest number of synonymous SNPs (n = 73), whereas segment 2 had the largest number of nonsynonymous SNPs (n = 20). In contrast, segments 5 and 6 had the fewest synonymous SNPs (n = 5 each), whereas no nonsynonymous SNPs were detected for segment 7. The 2021 BTV-17 sample frequently accounted for the majority of observed variation, particularly in segment 2 (30 synonymous, 16 nonsynonymous) and segment 3 (65 synonymous, 3 nonsynonymous). As compared to the other serotypes, increased numbers of SNPs were also observed for segment 8 (15 synonymous, 8 nonsynonymous), segment 9 (15 synonymous, 11 nonsynonymous), and segment 10 (22synonymous, 1 nonsynonymous) for the 2021 BTV-17 sample.

To determine whether specific SNPs were conserved across serotypes, SNP positions were compared across all the samples and serotypes. No nonsynonymous SNPs were shared across serotypes and only a single synonymous SNP, position 57 of segment 4, was detected in multiple serotypes across different samples. In addition, several samples within a serotype

exhibited polymorphic sites (50% one nucleotide, 50% a different nucleotide), most notably in segments 1, 2, and 3 of the BTV-13 samples.

Interestingly, all BTV-13 samples shared a unique nonsynonymous substitution in segment 10 affecting the stop codon. At position 708, an A/G → T substitution converts the stop codon to alanine, resulting in an extension of the open reading frame by three amino acids at the C-terminus of the BTV-13 segment 10 protein.

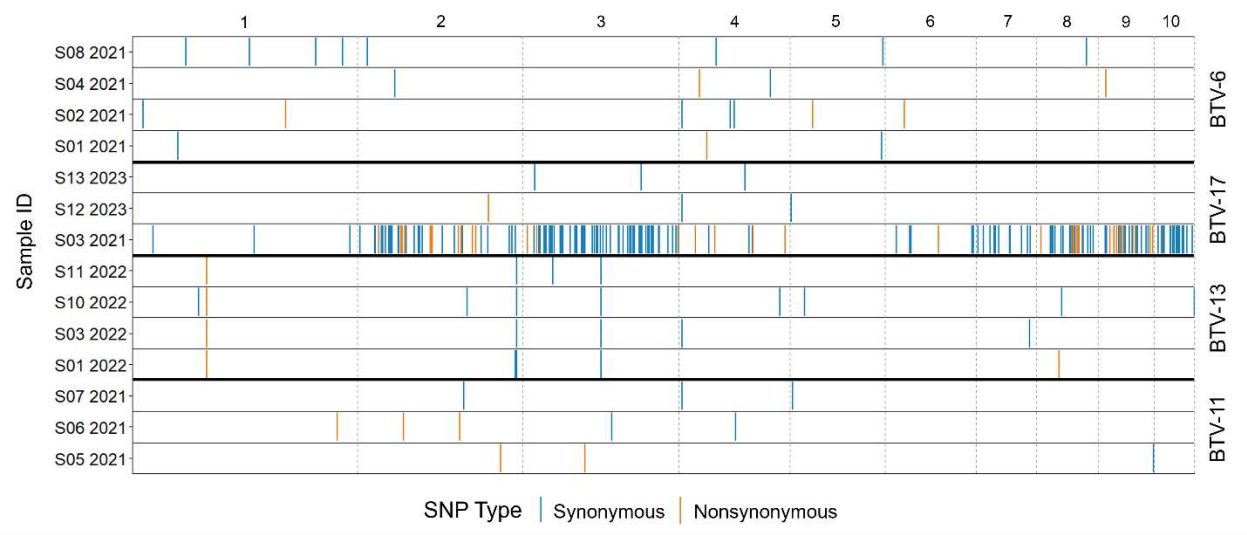

Figure 2 – Comparison of coding region nucleotide sequences within serotypes. Vertical lines represent the location of SNPs identified as nucleotide positions where individual viral sequences differ from a majority-rule consensus sequence generated from all samples within the same serotype across the concatenated BTV genome.

Discussion

The endemic nature of BTV in the United States, combined with the circulation of multiple serotypes, the capacity to evolve through reassortment, primary characterization based on segment 2, and the absence of sustained active field surveillance creates conditions that facilitate largely undetected viral evolution. Targeted sequencing of samples collected through active surveillance over a three-year period from a single sheep flock provides insight into the

genetic landscape of these circulating viruses, enabling assessment of variation between individual animals, viral turnover, and patterns of evolution over time without added cost and time of viral isolation.

The surveillance period during which these samples were collected is notable in that it coincided with the detection of a previously unreported serotype in this region, BTV-6, first reported in Colorado in 2020[35]. The flock examined in this study was the source of the BTV-6 2020 Colorado sheep genome sequence (GenBank accessions OR917943-OR917952)[35]. That virus was identified as a reassortant involving segments from endemic serotypes, raising the possibility that BTV-6 was introduced into the region prior to 2020 and subsequently reassorted locally. Historical samples from Colorado support the long-term circulation of BTV-10, -11, -13, and -17 over the last 50 years[45–49]. More recent sequencing projects from our laboratory have identified additional serotypes not previously reported in this region, including BTV-3 from a 2015 cattle sample and the previously mentioned BTV-6 in a sheep[35,36]. Both of these viruses exhibited evidence of reassortment with endemic serotypes.

This study identified the presence of four BTV serotypes circulating within a single sheep flock (BTV-6, -11, -13, and -17) across three years of active surveillance. Multiple serotypes (BTV-6, -11, and -17) were detected concurrently in 2021, whereas only a single serotype was detected in each subsequent year, BTV-13 in 2022 and BTV-17 in 2023. The co-circulation of multiple serotypes within a single sheep flock during a single transmission season highlights the potential for co-infection and subsequent reassortment in either the vertebrate host or vector. The absence of detectable multi-serotype circulation in later years may suggest a shift in the local transmission landscape or reflect the smaller number of BTV-positive sheep available for analysis.

Several animals (S01, S02, and S03) were BTV-positive across multiple years, indicating repeated infection events. The serotypes detected in subsequent years for individual animals are consistent with *in vitro* cross-protection studies that utilized segment 2 nucleotypes (A-K) to classify serotype cross-protection[50]. These studies suggest that antibodies generated in response to a given serotype may exhibit cross-protective immunity against other serotypes within the same nucleotype, whereas protection less likely following exposure to a serotype of a different nucleotype. The serotypes detected in subsequent years for individual animals in this study are consistent with these observations, as BTV-6 belongs to nucleotype C, BTV-11 and -17 belong to nucleotype A, and BTV-13 belongs to nucleotype B. In endemic systems where multiple BTV serotypes circulate over time, such serial infections likely occur frequently and contribute to both viral turnover and selective constraints on which serotypes are able to successfully re-establish infection in a partially immune host population.

Phylogenetic analysis of these samples revealed reassortment events not captured by serotype-based classification alone. The segment 2 phylogenetic tree was defined by two well-supported clades, with BTV-11 and -17 forming one clade and BTV-6 and BTV-13 forming the other. When compared against segment 2, incongruent phylogenetic tree topologies consistent with reassortment were observed for all segments, highlighting the genomic exchange that has occurred among co-circulating viruses in this flock. These findings suggest ongoing genomic exchange via reassortment rather than the continued circulation of clonal lineages. Reassortment is widely recognized as a common occurrence of BTV when multiple serotypes are co-circulating and allows for segments that encode determinants of transmission, host interaction, and virulence to be exchanged independently of segment 2, effectively dissociating serotype from clinical phenotype[32,51]. These results show that reassortment can be detected at the flock

level within a three-year timespan, highlighting that genomic diversification can occur locally and remains undetected without longitudinal and whole-genome surveillance. As a result, whole-genome characterization of viruses associated with clinical disease is essential to identify genotypes that may contribute to changes in disease pathogenicity or altered transmission dynamics, particularly in endemic systems where multiple serotypes co-circulate.

Overall, homology across samples was high, with most detected SNPs being synonymous. Although up to three serotypes were detected in the flock in a single year, viruses of each serotype exhibited high sequence homology, consistent with circulation of a single virus per serotype. In contrast, the 2021 BTV-17 sample had the highest number of both synonymous and nonsynonymous SNPs of any sample in the dataset when compared to the two 2023 BTV-17 samples, particularly for segments 2, 3, 8, 9, and 10. This increased nucleotide-level disparity was reflected in the corresponding phylogenetic trees, in which segments 3, 8, and 9 of the 2021 BTV-17 sample clustered separately from the 2023 BTV-17 samples. These differences suggest the detection of two distinct BTV-17 viruses. The observed divergence in tree topology and decreased nucleotide identity of some segments indicates that reassortment likely occurred in one of these viruses, although the timing of this event cannot be determined from the current data.

Although the segment 10 coding sequences of the BTV-13 samples were nearly identical to one another, it was observed that these sequences were uniformly nine base pairs longer than the coding sequences from other serotypes. This difference was identified as a shared A/G → T substitution at position 708 of segment 10 that altered the terminal stop codon, resulting in an extension of the open reading frame by three amino acids at the C-terminus of the segment 10 protein. This finding is unique, as segment 10 is generally highly conserved across global isolates of BTV and encodes for the nonstructural proteins NS3 and NS3a which play key roles

in virus maturation, intracellular trafficking, and viral egress[52–54]. The C-terminal domain of NS3 has been shown to interact with both VP5 (segment 6) and the outer capsid protein VP2 (segment 2), although the precise locations of the VP5 and NS3 interactions remains undefined[53–55]. Therefore, it is unknown if the observed C-terminal extension would affect NS3/NS3a function, but this substitution represents a notable deviation from the typically conserved structure of segment 10. Furthermore, BLASTp analysis of the amino acid sequence against the NCBI protein database returned no published sequences that contained the additional three terminal amino acids. This feature appears to be unique among BTV segment 10 sequences and has not been previously reported.

Overall, longitudinal whole-genome sequencing of BTV from a single sheep flock over three consecutive years revealed genetic evolution that could be a product of reassortment across genome segments alongside high nucleotide identity within serotypes. Phylogenetic analysis found evidence of reassortment across all genomic segments relative to segment 2. Nucleotide-level analysis showed high within-serotype homology for most segments with occasional nonsynonymous SNPs observed for most samples. Direct sequencing of field-collected samples via active surveillance provides insight into BTV evolution as it occurs in nature while avoiding potential biases introduced with viral isolation *in vitro*. Collectively, these findings reinforce that reassortment is common among circulating endemic viruses and can involve any segment. At the same time, the high homology among same-serotypes samples within a year highlights the introduction and maintenance of individual viral lineages within this flock. Expanded surveillance and sequencing efforts that include wild and domestic ruminants, as well as midges, across a broader geographic area are needed to fully characterize the genomic landscape of BTV

as it occurs across different hosts and regions with the goal of linking genomic variation to phenotypic outcomes and informing strategies for disease control.

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CHAPTER 4 – METAGENOMIC DETECTION OF ADDITIONAL VIRUSES IN ORBIVIRUS-POSITIVE HOSTS

Introduction

Orbiviruses (order *Reovirales*, family *Sedoreoviridae*) comprise a genus of double-stranded RNA (dsRNA) viruses with a wide range of hosts that include wild and domestic ruminants as well as arthropod vectors. Among these, bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are of substantial economic importance for their capacity to cause clinical disease and production losses in susceptible agricultural animals.[1] Both viruses are transmitted by *Culicoides* spp. of biting midge.[2,3]

In recent years, next-generation sequencing (NGS) has allowed for molecular characterization of BTV and EHDV, particularly during outbreak investigations. Application of NGS as part of orbivirus investigation enables accurate serotype determination, full genome recovery, and detection of reassortment, a key evolutionary mechanism in segmented RNA viruses that facilitates rapid genetic diversification.[1] Metagenomic next-generation sequencing (mNGS) provides an unbiased, sequence-independent approach to detect known, divergent, and previously uncharacterized viruses.[4,5] Unlike targeted assays, mNGS does not require prior knowledge of viral sequences, allowing for the identification of unknown viral communities within a sample. Over the last decade, advances in sequencing technologies and reductions in cost per sample have increased accessibility. Integration of mNGS into routine surveillance frameworks has emerged as a critical component towards viral discovery and characterization of host-associated viromes.[6–8]

Ongoing whole-genome characterization efforts for BTV and EHDV in our laboratory have generated extensive sequencing datasets from both vertebrate and invertebrate hosts. These datasets provide an opportunity to apply retrospective metagenomic analyses to evaluate for the presence of additional viruses in orbivirus-positive samples. Here, we present an exploratory, retrospective mNGS analysis of sequencing data to investigate the presence of other viruses in BTV- and EHDV-positive hosts.

Methods

Samples

Whole blood in EDTA and tissue samples were collected as part of active surveillance or submitted as diagnostic samples (Table 1). All active surveillance collections were approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC). Species represented include domestic cattle, domestic sheep, llama, alpaca, bighorn sheep, white-tailed deer, mule deer, and reindeer. Tissue samples consisted of spleen, lung, lymph nodes, or bone marrow. Whole female blood-fed midges were field caught in 2021 and morphologically identified as *Culicoides sonorensis*. All samples tested positive for BTV or EHDV RNA via quantitative reverse transcription polymerase chain reaction (qRT-PCR) either by our laboratory, by the Colorado State University's Veterinary Diagnostic Laboratory (Fort Collins, CO), or by the Washington Animal Disease Diagnostic Laboratory (Pullman, WA).

Table 1 – BTV/EHDV-positive host species, sample type, and number of samples utilized for Nanopore-based metagenomic analysis via CZ ID.

Species	Sample Types (n)
Bison (*Bison bison*)	Spleen (1)
Domestic cattle (*Bos taurus*)	Whole blood (11), lung (1)
Biting midge (*Culicoides sonorensis)*	Whole midge (6)

Llama (*Lama glama*)	Lung/spleen pool (1)
Mule deer (*Odocoileus hemionus*)	Whole blood (8), spleen (6)
White-tailed deer (*Odocoileus virginianus*)	Whole blood (3), lung (2), marrow (2)
Bighorn sheep (Ovis canadensis)	Spleen (5), lung (1), marrow (1)
Domestic sheep (*Ovis aries*)	Whole blood (20), spleen (1), lung (1)
Reindeer (*Rangifer tarandus*)	Lymph node/lung/spleen pool (1)
Alpaca (*Vicugna pacos*)	Lung (1)

Nucleic acid extraction

Prior to nucleic acid extraction, approximately 100 mg of tissue was homogenized in 1 mL of PCR water in a Lysis Matrix I tube (MP Biomedicals). Nucleic acid extraction was performed on whole blood and tissue samples using the MagMAX™ CORE RNA/DNA Kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's simple workflow with automated processing performed on the KingFisher96™ magnetic particle processor (Thermo Fisher Scientific). Individual midges were rinsed three times with 70% EtOH on a sterile petri dish prior to complete homogenization in a Lysis A column (MP Biomedicals). Homogenates were extracted using the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems, Thermo Fisher Scientific) using the manufacturer's instructions for low-cell-content samples. Eluted nucleic acids were then treated with the RNA Clean & Concentrator™ (Zymo Research) according to the manufacturer's protocol.

Whole-genome sequencing using SISPA and Oxford Nanopore Technology

Prior to library preparation, nucleic acids extracted from midges were treated with TURBO™ DNase (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol, with the exception that DNase activity was terminated by moving straight into a 1.8x RNAClean (New England Biolabs) bead clean rather than with the supplied stop solution.

Samples were then treated with the NEBNext® rRNA Depletion Kit v2 (Human/Mouse/Rat) (New England Biolabs) according to the manufacturer's protocol. RNA from midges and vertebrate samples was reverse-transcribed and amplified for Nanopore sequencing using a sequence-independent, single-primer amplification (SISPA) approach with enrichment for orbivirus sequences as previously described.[1,9] For vertebrate tissues and whole blood, a modification was made in the amplification step in which the number of amplification cycles was reduced from 40 to 20 to reduce PCR jackpotting. Sequencing libraries were prepared using the SQK-NBD114.96 Native Barcoding Kit 96 V14 (Oxford Nanopore Technologies) according to the manufacturer's instructions. Final pooled libraries were loaded onto a FLO-MIN114 R10.4.1 flow cell (Oxford Nanopore Technologies) and run on a GridION (Oxford Nanopore Technologies).

Viral sequence identification

Metagenomic analysis was performed on raw sequencing data using CZ ID (https://czid.org), a cloud-based, no-code platform enabling advanced long read metagenomic analysis.[10] The CZ ID pipeline begins with data pre-processing where reads are filtered for quality, length, and complexity using fastp[11], followed by removal of host and human reads with minimap2.[12] The remaining reads are then assembled *de novo* with metaFlye to generate contigs.[13] These contigs are queried against the National Center for Biotechnology Information (NCBI) nucleotide (NT) database using minimap2 and the non-redundant protein (NR) database using DIAMOND.[14] Reads that do not assemble into contigs are also queried against the NT database with minimap2. Both contigs and unassembled reads are assigned taxonomic identifiers (taxids), based on the taxid(s) of the best aligning nucleotide or protein sequence(s).

The output results for this dataset were filtered to retain viral taxa classifications only. Taxa classifications with NT or NR read counts greater than 1,000 were selected for downstream analysis. For viruses classified based on nucleotide sequence identity, reads were mapped to a reference genome with the highest number of reads aligned, as identified by CZ ID, using OrbiSeq (https://github.com/tdunham19/OrbiSeq), a Nextflow pipeline that uses iterative mapping to generate new virus draft consensus sequences.[15] For segmented viruses, reference datasets were generated using all available GenBank sequences for each segment, and alignments were performed using OrbiSeq. All assemblies were manually reviewed and refined in Geneious Prime (v2.17.0) and extended through several iterations using OrbiSeq. If initial alignments to the reference identified by CZ ID were of low quality, a preliminary consensus sequence was generated from the mapped reads and used to identify a more appropriate reference using the NCBI nucleotide database using BLASTn (v2.17.0), after which read mapping and consensus generation were repeated using the updated reference.[16] Final consensus sequences were compared against the NCBI nucleotide database.

For viruses initially identified based on protein sequence homology, CZ ID-generated contigs were inspected using the NCBI translated blast feature (BLASTx). When multiple contigs were available, alignments were performed using Geneious Prime to generate a draft consensus sequence. Reads were subsequently mapped to the draft sequence using OrbiSeq and assemblies were iteratively extended when possible. Final consensus sequences were compared against the NCBI protein database.

Three tissue samples were not successfully analyzed using CZ ID. According to CZ ID technical support, the pipeline failed during the nucleotide alignment step due to insufficient memory allocation and no resolution was available at that time.

Phylogenetic analysis

Sequences used to generate phylogenetic trees were selected from previous publications in which they were described. If no such publications were available, sequences were obtained from the BLASTx results. Nucleotide and amino acid sequences were aligned using MUSCLE (v5.1) in Geneious Prime.[17] IQ-TREE (v2.4.0) was used to generate maximum likelihood trees.[18] Branch support was assessed using 1,000 ultrafast bootstrap replicates and 1,000 SH-aLRT replicates. Phylogenetic trees were visualized using Figtree (v1.4.4, https://tree.bio.ed.ac.uk/software/figtree/). Trees were midpoint rooted.

Virus genome annotation

Potential viral open reading frames (ORFs) were predicted in Geneious Prime using built-in parameters (minimum size: 400; genetic code: standard; start codons: ATG). Predicted ORFs were evaluated by comparison to related viruses using BLASTx and queried against the NCBI Conserved Domain Database via CD-Search to identify conserved functional domains.[19]

Results

A total of 41 whole blood samples, 25 tissues, and 6 midges were evaluated for the presence of viral coinfection with BTV or EHDV using mNGS. Across the dataset, several whole or partially coding-complete viral genomes were recovered (Table 2).

Table 2 – Summary of viral sequences identified by metagenomic sequencing in BTV/EHDV-positive hosts. The most similar related sequence percent identity is reported as either nucleotide or amino acid sequence identity.

Host Species (ID)	Sample Type	Location (Year)	Virus	Most Similar Related Sequence	Most Similar Related Sequence GenBank Accession	Most Similar Related Sequence Percent Identity (nt/aa)	Average Coverage Depth	Length (nt)
Mule deer (A)	Spleen	Pueblo County, CO (2021)	Skunk River Virus	Skunk River virus isolate SkRV-IA07 VP1	MK100569.1	98.9%(nt)	516	3,990
				Skunk River virus isolate SkRV-IA07 VP2	MK100571.1	99.3% (nt)	710	2,346
				Skunk River virus isolate SkRV-IA07 VP3	MK100570.1	97.0% (nt)	529	2,855
				Skunk River virus isolate SkRV-IA07 VP4	MK100572.1	99.0% (nt)	611	2,010
				Skunk River virus isolate SkRV-IA07 NS1	MK100573.1	99.3% (nt)	630	1,857
				Skunk River virus isolate SkRV-IA07 VP5	MK100574.1	97.6% (nt)	1073	1,638
				Skunk River virus isolate SkRV-IA07 VP7	MK100576.1	91.7% (nt)	187	1,180
				Skunk River virus isolate SkRV-IA07 NS2	MK100575.1	96.8% (nt)	495	1,257
				Skunk River virus isolate SkRV-IA07 VP6	MK100577.1	96.7% (nt)	415	957

				Skunk River virus isolate SkRV-IA07 NS3	MK100578.1	99.6% (nt)	704	776
Domestic cattle	Whole blood	Weld County, CO (2021)	Bovine hepacivirus	Bovine hepacivirus isolate MARC/2019/60	OR543978.1	89.8% (nt)	333	8,784
White-tailed deer	Whole blood	Larimer County, CO (2024)	Colorado deer associated narnavirus	Shache tick virus 1 isolate XJ1L/2023	XQH03072.1	62.4% (aa)	1283	2,898
Mule deer (B)	Whole blood	Larimer County, CO (2024)	Horsetooth deer virus	Kundal virus strain MCL-13-T-316 segment 1	YP_010086008.1	68.1% (aa)	856	4,306
				Gierle tick virus isolate NT89 VP2	QYV43107.1	67.2% (aa)	341	3,564
				Gierle tick virus isolate NT89 VP3	QYV43108.1	54.4% (aa)	332	3,501
Midge (A)	Whole midge	Larimer County, CO (2021)	Totivirus nijyuroku	Totivirus nijyuroku isolate MrV40L	MN603497.1	98.7% (nt)	11,707	4,594
			Black Hawk Lake virus	Black Hawk Lake virus isolate BHLV-2020 RNA-dependent RNA polymerase	OM743939.1	99.8% (nt)	6,204	1,712
			Black Hawk Lake virus	Atrato Partiti-like virus 1 strain Mati 1738-218 segment 2	QHA33905.1	53.9% (aa)	47,774	1,401
			Partitivirus-like virus	Atrato Partiti-like virus 1 strain Mati 1755-173 segment 4	QHA33835.1	53.3% (aa)	49,486	1,380
Midge (B)	Whole midge	Adams County, CO (2021)	Partitivirus-like virus	Atrato Partiti-like virus 1 strain Mati 1755-173 segment 2	QHA33833.1	60.6% (aa)	1,785	1,491
			Partitivirus-like virus	Atrato Partiti-like virus 1 strain Mati 1755-173 segment 4	QHA33835.1	53.3% (aa)	49,486	1,380

nt = nucleotide, aa = amino acid

Skunk River virus

Ten genome segments of Skunk River Virus (SkRV; family *Sedoreoviridae*, genus *Orbivirus*) were recovered from a BTV-positive adult female mule deer spleen collected in Pueblo County, Colorado, in October 2021 (GenBank PZ565313.1- PZ565322.1). All recovered segments were coding complete and share high nucleotide homology with the reference sequences (Table 2). Phylogenetic analysis of the T2 subcore shell protein, encoded by SkRV segment 2 (VP3), demonstrated that the recovered sequence clustered with the original SkRV-IA07 isolate with strong branch support. Pairwise nucleotide identity between the two VP3 sequences was 97.0% (Figure 1).

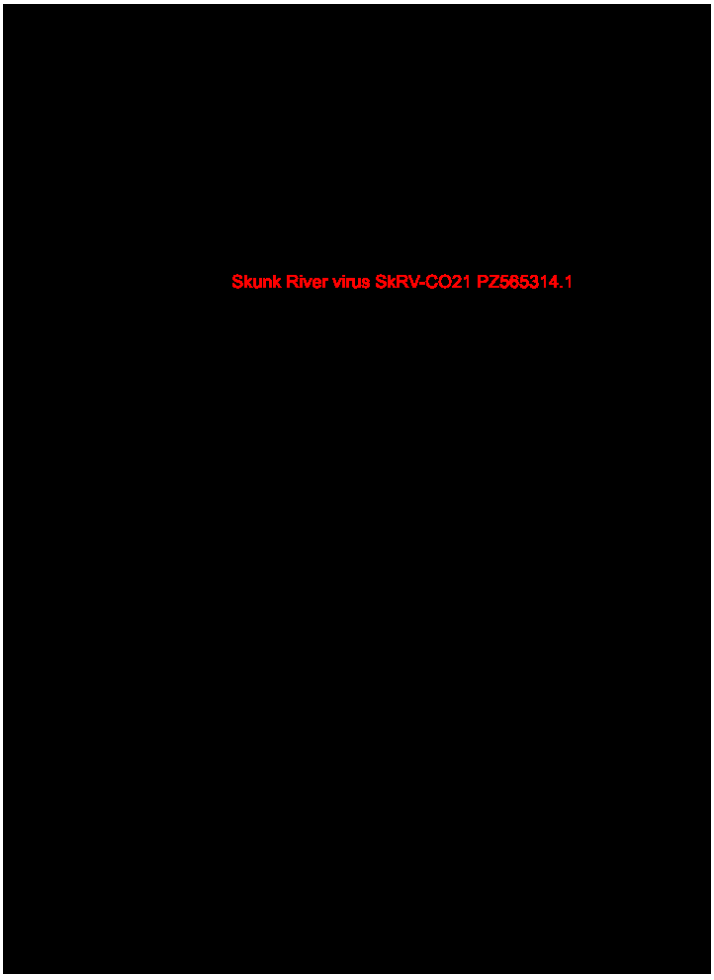

Figure 1 – Midpoint rooted maximum likelihood phylogenetic tree of nucleotide sequences encoding the T2 subcore shell protein coding of Skunk River virus (VP3) and other orbivirus species. Support values for select branches are indicated. Vector associations were defined by Tangudu et al. 2019.[20]

Bovine hepacivirus

One complete genome of bovine hepacivirus (BoHV; family *Flaviviridae*, genus *Hepacivirus*) was recovered from whole blood obtained from a BTV-positive adult female dairy cow in Weld County, Colorado, in December 2021 (GenBank PZ565323.1). The genome shares 89.8% pairwise identity with Bovine hepacivirus isolate MARC/2019/60 (GenBank OR543978.1) (Table 2). Phylogenetic analysis demonstrated that the recovered sequence clustered within the BoHV genotype 1, subtype E, with samples from China and United States (Figure 2).

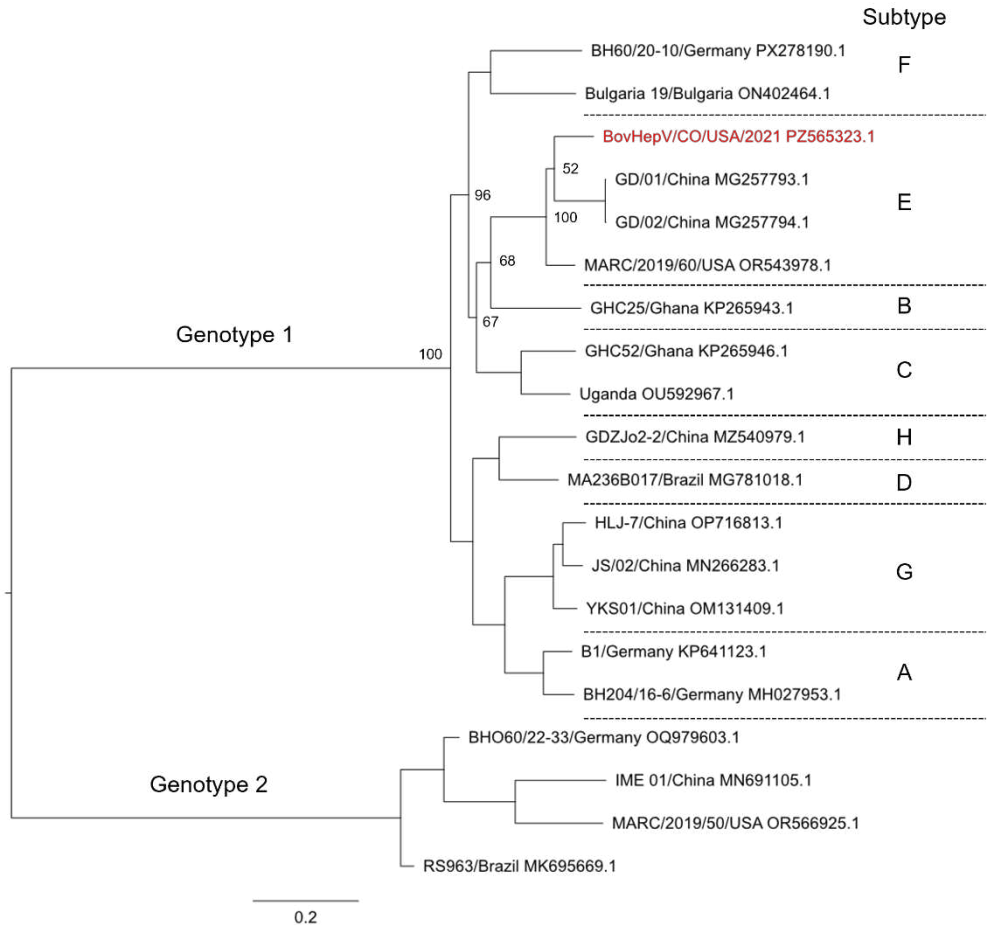

Figure 2 – Midpoint rooted maximum likelihood phylogenetic tree of bovine hepacivirus coding nucleotide sequences. Support values for select branches are indicated. Genotypes and subtypes as defined by Breitfeld et al. 2022.[21]

Colorado deer associated narnavirus

A sequence with homology at the amino acid level with Shache tick virus 1 (unclassified *Riboviria*) was recovered from whole blood of an EHDV-positive adult female white-tailed deer captured in Larimer County, Colorado, in September 2024 (GenBank PZ565324.1). This sequence is 2,898 bp in length and shares 62.4% identity with the reference amino acid sequence (GenBank XQH03072.1). The ORF spans 2,847 bp and includes the conserved domain for the *Narnaviridae* RdRP (cd23177). Phylogenetic analysis groups this virus and Sache tick virus 1 in a clade with Qingyang Narna tick virus 1 of family *Narnaviridae* (Figure 3). Both reference

sequences were obtained from ticks in China. Based on these findings, we proposed the name Colorado deer-associated narnavirus for this virus.

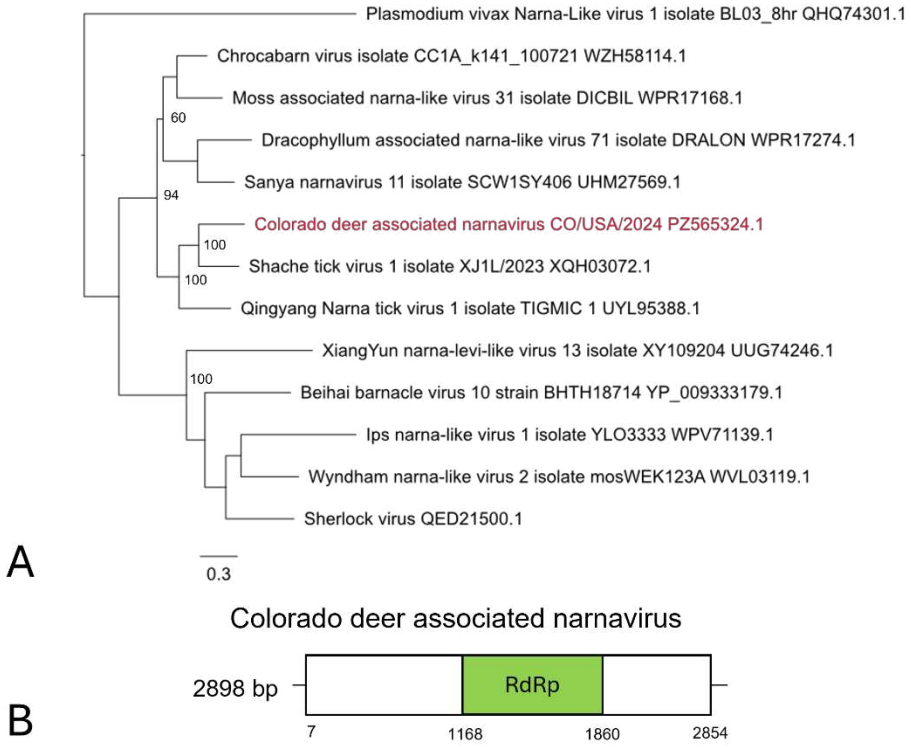

Figure 3 – A. Midpoint rooted maximum likelihood phylogenetic tree of Colorado deer associated narnavirus and related virus amino acid sequences. Support values for select nodes are indicated. B. Genome structure of Colorado deer associated narnavirus. The conserved domain for *Narnaviridae* RdRp (cd23177) is indicated in green.

Horsetooth deer virus

Three genome segments with amino acid homology to coltiviruses were recovered from whole blood of a BTV-positive adult male mule deer captured in Larimer County, Colorado, in December 2024. The first sequence (GenBank PZ565325.1) is 4,306 bp in length, appears to be coding incomplete at the 5' end, and shares 68% amino acid identity with segment 1 of Kundal virus strain MCL-13-T-316 (GenBank YP_010086008.1) which encodes the RdRp (VP1). No conserved domains were detected. Maximum likelihood phylogenetic analysis demonstrated that

the recovered sequence belongs to a coltivirus clade (Figure 4) where it clustered with Gierle tick virus, Jeddah tick coltivirus, Yanbian Reovi tick virus 1, and *Reoviridae* sp. isolate TGMIC 1. All closely related sequences were obtained from ticks.

A second partial coltivirus-like sequence (GenBank PZ565326.1) was recovered from the same deer sharing 67% amino acid identity with Gierle tick virus isolate NT89 VP2 (GenBank QYV43107.1). This sequence is 3,564 bp in length, appears to be coding incomplete at the 5' end, and had no detectable conserved domains. A third partial sequence (GenBank PZ565327.1) was recovered from the same sample sharing 54% amino acid homology with Gierle tick virus isolate NT89 VP3 (GenBank QYV43108.1). This sequence is 3,501 bp in length, incomplete at the 3' end, and contains the conserved domain for *Reoviridae* outer capsid protein VP3-like proteins (pfam18965). The proposed name for this virus is Horsetooth deer virus.

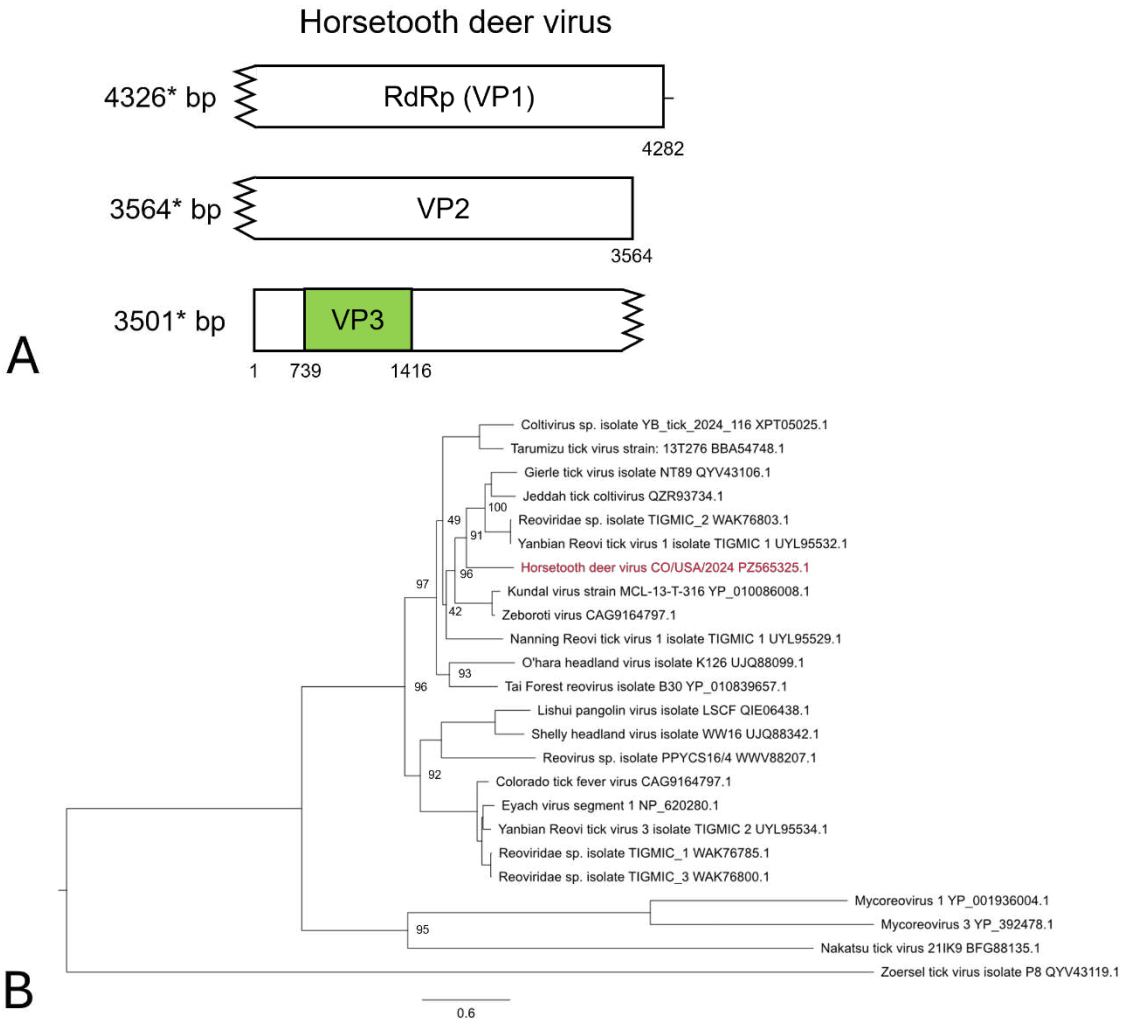

Figure 4 – A. Genomic structure of the three Horsetooth deer virus segments. Jagged edges indicate incomplete genome ends. The conserved domain for *Reoviridae* outer capsid protein VP3-like protein (pfam18965) is indicated in green. *Incomplete ORF. B. Midpoint rooted maximum likelihood phylogenetic tree of the RdRp (VP1) of Horsetooth deer virus and related virus amino acid sequences. Support values for select nodes are indicated.

Black Hawk Lake virus

A coding-complete RNA-dependent RNA polymerase (RdRp) sequence of Black Hawk Lake virus (BHLV, unclassified *Riboviria*) was recovered from a BTV-positive female midge, midge A, trapped at a multi-species urban livestock site in Larimer County, Colorado, in 2021 (GenBank PZ565328.1). The recovered sequence shares 99.8% nucleotide identity with the

reference isolate Black Hawk Lake virus BHLV-2020 (GenBank OM743939.1) and is the only closely related nucleotide sequence available in GenBank (Table 2). To assess broader evolutionary relationships, phylogenetic analysis of related amino acid sequences demonstrates that both the reference BHLV sequence and the sequence identified here are related to Atrato Partiti-like virus 1 strain Mati, originally isolated from *Mansonia titillans* mosquitoes (Figure 5A).

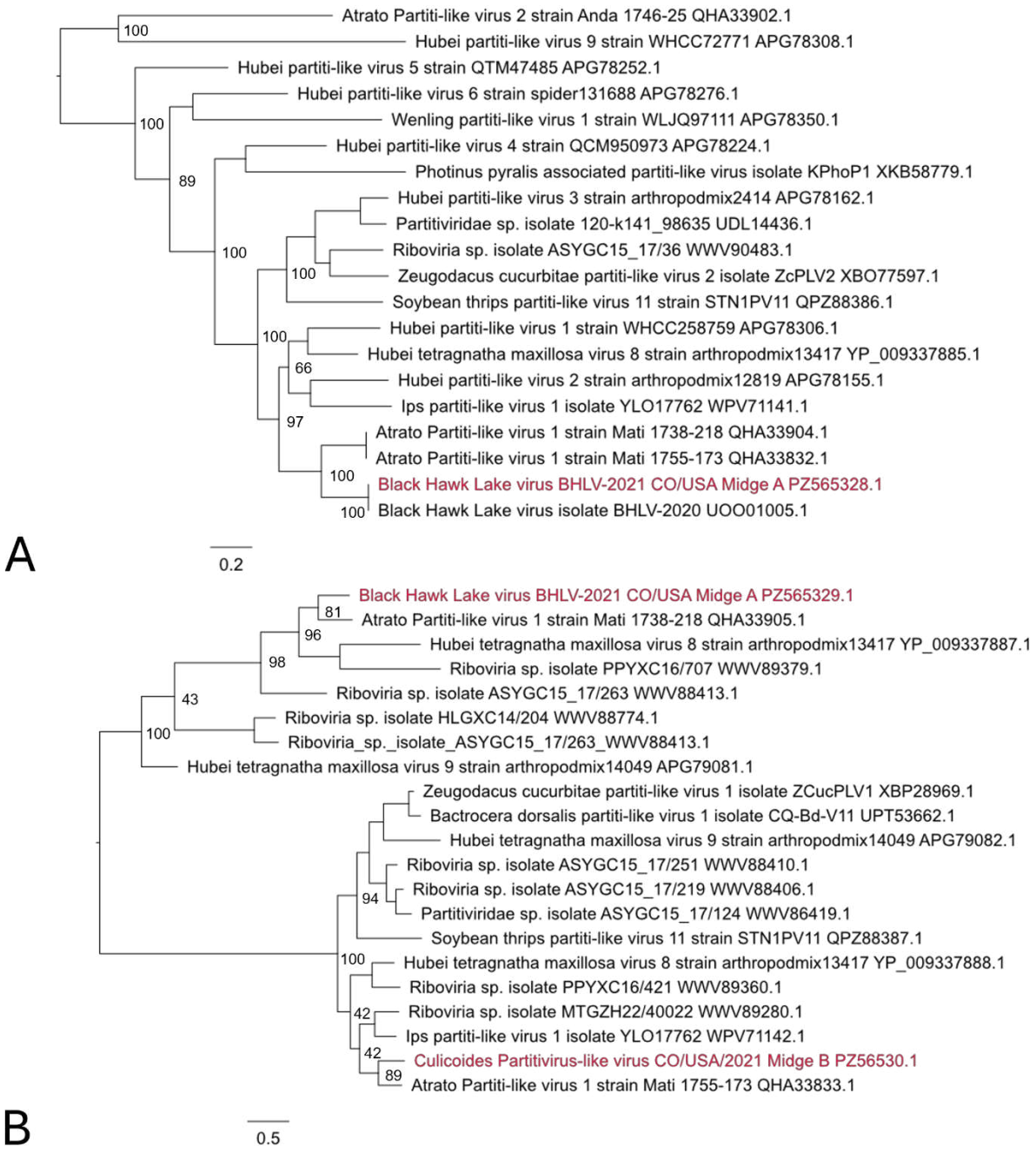

Figure 5 – A. Midpoint rooted maximum likelihood phylogenetic tree of Black Hawk Lake virus and related virus amino acid sequences. Support values for select nodes are indicated. B. Midpoint rooted maximum likelihood phylogenetic tree of the Black Hawk Lake virus and partitivirus-like virus capsid segments recovered from two different *Culicoides sonorensis* midges. Support values for select nodes are indicated.

Partitivirus-like virus segments

Several sequences were recovered from multiple midge samples that share homology at the protein level with different Atrato Partiti-like virus (family *Partitiviridae*, unclassified *Partitiviridae*) segments. The first sequence (GenBank PZ565329.1), obtained from the same midge previously described for BHLV, midge A, is 1,401 bp in length and shares 54% amino acid identity with segment 2 of Atrato Partiti-like virus 1 strain Mati 1738-218 (GenBank QHA33905.1), which encodes a putative capsid protein (Table 2). A single 1,317 bp ORF was detected with no conserved domains identified (Figure 5B).

A second coding complete partitivirus-like sequence was recovered from a BTV-positive female *Culicoides sonorensis* midge, midge B, trapped at a mixed species wildlife site in Adams County, Colorado, in 2021 (GenBank PZ565330.1). This sequence is 1,491 bp in length and shares 61% protein identity with segment 2 of Atrato Partiti-like virus 1 strain Mati 1755-173 (GenBank QHA33833.1), also a putative capsid protein (Table 2). A single 1,464 bp ORF was detected with no conserved domains identified (Figure 5B).

Two additional partitivirus-like sequences were recovered from the two BTV-positive midges previously described: midge A (GenBank PZ565331.1) and midge B (GenBank PZ565332.1). Both sequences are 1,323 bp in length and have 53% protein identity with segment 4 of Atrato Partiti-like virus 1 strain Mati 1755-173 (GenBank QHA33835.1), which encodes a hypothetical protein 2 (Table 2). These sequences are almost identical except for a single synonymous single nucleotide polymorphism (SNP) at position 141. A single 1,320 bp ORF was detected, and no conserved domains were identified.

Totivirus nijyuroku

A coding complete genome of Totivirus nijyuroku isolate MrV40L (family *Orthototiviridae*, genus *Totivirus*), a totivirus associated with *Malassezia restricta* yeast, was also recovered from midge A (GenBank PZ565333.1). The genome shares 98.7% pairwise identity with the reference nucleotide sequence (GenBank MN603497.1).

Discussion

Metagenomics is a powerful tool for studying novel, emerging, and previously uncharacterized viruses. Although these samples were originally selected based on BTV and EHDV status, metagenomic sequencing permits broader characterization of non-target viral sequences present within the dataset. Accordingly, we leveraged sequencing data generated from BTV- and EHDV-positive samples to evaluate the presence of additional viral reads in these samples. This dataset, comprised of 72 individual samples from *Culicoides sonorensis* midges and a variety of vertebrate hosts, revealed several known viruses as well as several putatively novel viral sequences.

The first virus recovered from the vertebrate samples in this dataset was Skunk River virus (SkRV), detected in a mule deer spleen collected in Colorado. SkRV is an orbivirus related to other mosquito- and tick-associated orbiviruses, such as Big Cypress orbivirus (BCPOV) and Sathuvachari virus (SVIV). Coding-complete genomes were obtained for all ten segments with high sequence identity (91.7-99.6%) to the single reference genome. Prior to this report, SkRV had only been documented once, when it was isolated from *Aedes trivittatus* mosquitoes collected in Iowa as part of the statewide arbovirus surveillance program.[20] This represents the first detection of SkRV in a vertebrate host and suggests that this virus may infect ruminants via

mosquito-mediated transmission. Similarly, BCPOV was originally isolated from *Psorophora columbia* mosquitoes in Florida and subsequently isolated from a white-tailed deer spleen three years later.[22,23] Further studies are needed to determine the host range of SkRV and assess its pathogenic potential in deer or other vertebrates.

The second virus identified from this dataset was bovine hepacivirus (BoHV), a virus related to hepatitis C virus in humans, that was first described in cattle from Ghana and Germany in 2015.[24,25] This virus was reported for the first time in U.S. cattle in Missouri in 2019.[26] The sequence recovered in this study originated from a dairy cow sampled in Colorado in 2021 and shares 89.8% pairwise identity with the genotype 1 virus previously detected in the United States. The route of transmission for BoHV remains unknown but is likely through contact with infected blood (re-use of needles during vaccination, rectal palpation, etc.).[21,24] A recent study in Germany identified BoHV genotypes 1 and 2 as the causative agents of severe clinical signs in a dairy herd using metagenomic analysis and PCR, reporting respiratory signs, fever, and increased mortality in this herd over a three-year period; however, the virus was also detected from animals without clinical signs.[27] Furthermore, the host range of BoHV has been expanded to include deer and wild boar.[21,28] Additional research is needed to determine the prevalence, pathogenic impact, and potential infection in other species for BoHV.

Several putatively novel viruses were also identified within this dataset from vertebrate samples based on protein-level homology to previously characterized viruses. The first of these was recovered from a white-tailed deer sampled in 2024. This sequence is ~3 kbp in length and shares 63% amino acid identity with Shache tick virus 1, an unclassified ribovirus identified from *Hyalomma* spp. ticks collected in China.[29] Phylogenetic analysis revealed this virus to be in clades with other narnaviruses identified from ticks. Members of the genus *Narnavirius* (family

Narnaviridae) are positive-sense RNA viruses traditionally associated with fungal hosts.[30] To date, there is no evidence that members of the family *Narnaviridae* infect vertebrate hosts. Although narna-like viral sequences have been detected in vertebrate-associated metagenomic datasets, these viruses are suspected to originate from parasitic organisms rather than the vertebrate host itself. For example, studies have identified narnaviruses from apicomplexan species that infect vertebrates, including a trypanosome species from a gecko as well as well-characterized pathogens such as *Plasmodium vivax* and *Toxoplasma gondii*.[31,32] Apicomplexan parasites are commonly found in deer and may be a plausible source of this putative narnavirus.[33–35] The phylogenetic placement of this virus with other tick-associated narnaviruses raises the alternative possibility that this virus originated from an arthropod source. Several recent tick virome studies have reported narnaviruses.[36–38] Additional investigation is needed to determine the true host of Colorado deer associated narnavirus.

Several genomic segments corresponding to the RdRp (VP1), VP2, and VP3 of a virus with amino acid homology to tick-associated coltiviruses were recovered from a white-tailed deer collected in 2024. Coltiviruses are members of the order *Reovirales* and possess genomes composed of 12 segments of dsRNA.[39,40] These viruses are typically transmitted by ticks and mosquitoes to vertebrate hosts. Phylogenetic analysis of the RdRp segment demonstrated that the recovered sequence clusters within a clade of tick-associated coltivirus. Colorado tick fever virus, a related *Coltivirus*, infects a broad range of vertebrate species, including deer, and is primarily transmitted by the Rocky Mountain wood tick (*Dermacentor andersoni*).[41,42] Although only three genomic segments were recovered in this study, phylogenetic analysis of the RdRp segment and the amino acid similarity to other known coltiviruses suggests that the detected sequences represent a putatively novel *Coltivirus* or coltivirus-like virus we have named

Horsetooth deer virus. The absence of additional sequences recovered from this sample may reflect low viral abundance, uneven genome representation, or sequence divergence from reference databases.

Several viral sequences were also identified from the midge samples within this dataset. The first virus recovered was Black Hawk Lake virus (BHLV), an unclassified member of the realm *Riboviria*, for which only a single genomic segment encoding the RNA-dependent RNA polymerase (RdRp) has been described. This sequence was reported here was recovered from a *Culicoides sonorensis* midge, midge A, trapped in Colorado in 2021. The reference BHLV sequence was obtained from *Culex pipiens* mosquitos in 2020.[43] Phylogenetic analysis at the protein level reveals that BHLV shares homology with Atrato Partiti-like virus 1 strain Mati, isolated from *Mansonia titillans* mosquitoes. These results suggest that BHLV is a partitivirus or partitivirus-like virus. Members of the family *Partitiviridae* possess bipartite or tripartite double-stranded RNA (dsRNA) genomes with one segment encoding the RdRp and the other encoding the capisid[44]. Tripartite genomes have been reported for other insect associated partitiviruses, such as Galbut virus from *Drosophilia melanogaster*.[45] A recent metagenomic study of midges trapped in Mexico reported the discovery of two novel partitivirus RdRp sequences without identification of additional genome segments.[46] Although CZ ID detected reads mapping to BHLV from a second midge, midge B, the number of reads was insufficient for downstream analysis.

In addition to the BHVL RdRp sequence, three partitivirus-like virus segments encoding two capsid proteins and a related protein were recovered from female *Culicoides sonorensis* midges trapped in Colorado. In some partitiviruses, additional dsRNA segments have been detected.[44,45,47,48] The functional role of these segments remains unclear; however, it has been

suggested that these segments may influence host physiology or modify virus-host interactions.[49–52] To date, no capsid protein has been described for BHLV. Given the phylogenetic placement of the BHLV RdRp sequences among other partitivirus-like viruses, it is plausible that one of the capsid encoding sequences identified represent the capsid segment for BHLV. Notably, no additional partitivirus-like virus RdRp sequences were recovered from this dataset, and both the BHLV RdRp sequence and capsid sequence were detected within the same individual midge, midge A. This co-occurrence supports the hypothesis that the capsid segment identified from midge A corresponds to BHLV, whereas the second capsid protein from midge B may belong to a related partitivirus-like virus for which the RdRp segment was not recovered. Additionally, two partitivirus-like sequences with homology to segment 4 of Atrato Partiti-like virus 1 strain Mati 1755-173 were recovered from both midge A and midge B that had almost 100% nucleotide identity. These sequences may represent an additional genomic segment associated with BHLV or a different partitivirus-like virus.

Finally, a consensus genome covering 99.7% of the Totivirus nijyuroku isolate MrV40L reference was also recovered from a female *Culicoides sonorensis* midge. This specific totivirus isolate was recovered from *Malassezia restricta* yeast and has no other known hosts.[53] As this specific virus is primarily associated with fungal hosts, the detection of this virus from a midge may be a result of yeast colonizing the midge gut or ingested as part of a blood meal. While totiviruses are generally viruses associated with different fungi species, recent evidence has emerged that arthropods, including midges, may also be hosts for these viruses.[46,54–59]

Viral co-infections are common in natural systems and can alter pathogen replication, host immune responses, and disease outcomes via synergistic or antagonistic virus-virus interactions.[60–62] However, there is almost no information regarding co-infection of BTV/EHDV

and other viruses and what impact this may have on the vertebrate or midge host. A spectrum of clinical disease can be observed in BTV/EHDV infected ruminant hosts ranging from subclinical infection to death. Infection with BTV has been shown to impair adaptive immune responses, as transient suppression of T-cell activation and disruption of B-cell antibody generation has been observed in BTV infected animals.[63–65] This immunomodulatory effect creates a window of reduced immune competence that can allow secondary infections to be established more readily. More work is needed to understand how co-infection with other viruses can impact the disease course in BTV/EHDV infected hosts.

Identification based solely on mNGS warrants cautious interpretation and requires additional validation to confirm the presence of these viruses. Several limitations are associated with genome identification using this approach alone. Contamination represents a major concern and may generate false-positive detections. External contamination may originate from personnel, laboratory air, reagents (the "kitome"), or the laboratory environment.[66] Internal contamination can also occur during sample preparation and sequencing, including cross-contamination between samples, index switching, and artifacts.[66] Additional analytical artifacts may arise during genome assembly. De novo assembly of metagenomic reads may generate chimeric contigs when reads from related genomes or repeated regions are incorrectly merged during assembly, potentially resulting in erroneous genome reconstruction.[67] False negatives may occur in mNGS datasets. Differences in sample matrices can affect nucleic acid extraction efficiency and low biomass samples with low viral load may yield insufficient sequencing depth for reliable detection.[66] In addition, viral discovery using mNGS is inherently constrained by the sequences available in reference databases, as viruses lacking closely related sequences may

remain undetected or be misclassified. This limitation can be partially mitigated by searching based on amino acid homology in addition to nucleotide similarity.

Currently, the gold standard for confirming viral sequences identified via mNGS remains targeted molecular validation, such as PCR or Sanger sequencing. In the absence of these confirmatory assays, several analytical indicators can increase confidence in metagenomic detections. These include presence of reads dispersed across the whole genome, high sequencing coverage, and read count exceeding predefined thresholds to distinguish true positives.[66] In addition, inspection of read alignments and manual curation of assembled contigs can help identify and correct potential assembly artifacts, such as misassembles or chimeric regions. The analytical approaches utilized in this study, including the application of read thresholds, observation of reads distributed across the genome with reasonable coverage, and manual evaluation and correction of assembled contigs, support the likelihood that the detected viral sequences represent genuine signals rather than artifacts.

NGS datasets represent an important resource for virus surveillance and characterization across vertebrate hosts, arthropod vectors, and other organisms. Here, we utilized datasets generated during active orbivirus surveillance to identify multiple additional previously described or putatively novel viruses through metagenomic analysis. Several of these findings expand current understanding of arbovirus diversity and highlight the complexity of viruses transmitted between arthropods and vertebrates. The analytical frameworks used in this study, including CZ ID and BLAST-based workflows, demonstrate how viral genome discovery can be incorporated into targeted sequencing projects with accessible bioinformatic tools. Although additional work is required to determine host range, transmission dynamics, and pathogenic relevance, these findings highlight the value of metagenomic approaches for viral discovery

while underscoring that sequence-based detection alone should be interpreted cautiously and used to guide targeted downstream investigations.

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CHAPTER 5: CONCLUDING REMARKS

Although more than a century has passed since the first scientific report on BTV, it persists as an important pathogen of ruminants worldwide. In the words of Gibbs and Greiner, "Understanding the epidemiology of viruses that spread directly from animal to animal is often described as a game of chess; with BT, the involvement of arthropod vectors transforms the challenge into a 3 dimensional game!"[1]. Building on this analogy, the epidemiology becomes even more complex, akin to a four-dimensional game, as the virus's broad antigenic range and capacity for genomic reassortment adds an additional layer of complexity that continues to challenge researchers. Investigations conducted both in the laboratory and in the field continue to expand our understanding of this virus and inform strategies to protect animal health. While *in vitro* studies have yielded significant insights into BTV biology, they cannot fully replicate the complexity of natural systems. Studying BTV in the field, where environmental conditions, vector dynamics, the variety of vertebrate hosts, and immune interactions simultaneously influence virus transmission and evolution, providing critical context that cannot be captured through controlled laboratory approaches alone.

The first aim of this work utilized both longitudinal and cross-sectional surveillance methods in sheep and cattle from 2021-2023 to provide contemporary insights into BTV prevalence and serotypes circulating in Colorado and surrounding states. The timing of this work was particularly notable, as it coincided with the recent introduction and detection of BTV-6 in 2020, a serotype that had not been detected outside of Florida since 2009. BTV-6 was one of the predominant serotypes detected in 2021. Although it was initially hypothesized that BTV-6 would become established in the region and continue to circulate and reassort with the

circulating endemic strains in subsequent years, BTV-6 was not detected from any animal in 2022 and only a single detection was made in 2023. These findings underscore the complexities of BTV circulation in natural systems, where limited transmission among vector populations may have contributed to its absence in sequent years. There is evidence that vector competence varies among *Culicoides* species and by BTV serotype, including the major vector of BTV in Colorado and surrounding states, *Culicoides sonorensis*[2,3].

Another notable finding from this aim was the significant decrease in BTV detections in 2023 compared with 2021 and 2022. Additionally, the predominant serotype detected differed each year. This pattern is likely multifactorial, reflecting a combination of influences including herd immunity, population turnover, and environmental factors affecting vector dynamics. Temperature has been shown to have significant impacts on *Culicoides* longevity, reproduction rates, timing of emergence, and virus replication rates[2–5]. Other environmental factors, such as soil moisture, rainfall, and relative humidity, have also been shown to be important for larval habitat suitability and adult survival. Identifying regional climate patterns that contribute to increases in *Culicoides* populations would support the development of predictive models for years in which BTV prevalence may be increased, enabling earlier producer outreach and implementation of measures to reduce animal exposure.

Serotyping of BTV-positive samples via qRT-PCR, as was utilized in the first aim to characterize these samples, is laborious and resource intensive because primer and probe sets specific to each serotype must be used. Notably, serotype was not determined for 43 (22.6%) qRT-PCR positive samples collected across the three years of surveillance. Sequencing data not included within this body of work suggests that some of these samples are a strain of BTV-10 that is not detectable using the primer and probe set that has long been utilized by our

laboratory[6]. Using these sequencing data, updated primer and probe sets should be developed and evaluated for this unique strain of BTV-10. Additionally, a number of these samples remain untyped. Future sequencing efforts of these samples may reveal additional instances of sequence drift of the presence of novel serotypes not historically detected in the region.

Although BTV is well established as endemic in Colorado and multiple serotypes circulate are known to circulate within this region, relatively little is known about the whole genomes of these viruses present, as serotyping via PCR only gives insight into VP2 of these viruses. The second aim of this work investigated the genetic landscape of BTV in the context of a single sheep flock across three years of active surveillance. To our knowledge, this represents the first instance in which longitudinal sequencing of samples collected from the same animals has been documented. The results from this aim found evidence of reassortment in all segments of the genome, underscoring the extent to which reassortment occurs in field conditions. Additionally, high sequence homology was observed between samples of the same serotype from the same year. High sequence homology was observed among samples of the same serotype collected within a given year. However, comparisons of the same serotype between different years showed numerous synonymous and nonsynonymous changes. These observations may reflect reassortment events or the circulation of a different BTV-17 strain.

As sequencing of field collected samples becomes more accessible, expanding these analyses to include a larger number of samples of the same serotype across years and host species would provide valuable insight into within-serotype diversity and the evolutionary rates of BTV. Furthermore, although reassortment is recognized as a common and widespread feature of BTV ecology in regions where multiple serotypes are co-circulating, limited work has been done to identify which genomic segments or mutations contribute to difference in clinical

disease. Because reassortment has been proposed as a mechanism by which BTV can acquire segments associated with increased pathogenicity, there remains a critical need to determine which genomic factors influence virulence. Future sequencing efforts should therefore compare genomes associated with asymptomatic versus clinically affected animals to identify the genomic changes linked to altered pathogenicity.

Finally, the third aim of this work highlights the utility of integrating metagenomic sequencing into targeted pathogen sequencing projects. By leveraging the metagenomic sequencing datasets generated while sequencing BTV- and EHDV-positive samples, several additional viruses were detected from both vertebrate and arthropod hosts. In most cases, further study is required to determine the host range and the virus's potential role in disease. The sequences obtained from this work have been made publicly available via GenBank to support future detection, comparison, and characterization of these viruses. Additionally, continued work towards characterization of Black Hawk Lake virus in *Culicoides sonorensis* and investigating associated virus-virus interactions may be particularly valuable, as partitiviruses have been proposed as potential biocontrol agents in other arthropods, particularly mosquitoes[7].

The research presented here provides a comprehensive examination of regional prevalence, the genetic landscape of circulating strains, and additional viruses detected during three years of active surveillance of BTV. Through a combination of traditional molecular techniques and sequencing approaches, the circulating BTV serotypes were identified and whole-genome data were generated to yield deeper insight into viral diversity. Metagenomic sequencing of *Orbivirus*-positive samples also resulted in the recovery of sequences from several additional viruses, including a few that may represent putatively novel species. Collectively, this work illustrates how integrating sequencing into a surveillance framework enhances understanding of

the virus of interest while simultaneously enabling the detection of other viruses present in both vertebrate and arthropod hosts. This integrated approach highlights the value of pairing molecular diagnostics with sequencing strategies to advance viral surveillance, characterization, and discovery.

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