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Bluetongue Research at a Crossroads: Modern Genomics Tools Can Pave the Way to New Insights

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Abstract

Bluetongue virus (BTV) is an arthropod-borne, segmented double-stranded RNA virus that can cause severe disease in both wild and domestic ruminants. BTV evolves via several key mechanisms, including the accumulation of mutations over time and the reassortment of genome segments.

Additionally, BTV must maintain fitness in two disparate hosts, the insect vector and the ruminant. The specific features of viral adaptation in each host that permit host-switching are poorly characterized. Limited field studies and experimental work have alluded to the presence of these phenomena at work, but our understanding of the factors that drive or constrain BTV's genetic diversification remains incomplete. Current research leveraging novel approaches and whole genome sequencing applications promises to improve our understanding of BTV's evolution, ultimately contributing to the development of better predictive models and management strategies to reduce future impacts of bluetongue epizootics.

INTRODUCTION

First described in South Africa at the turn of the twentieth century, bluetongue virus (BTV) remains an economically important, reemerging pathogen with substantial animal health impacts (1, 2). Its significance stems from its role as the etiologic agent behind recent devastating outbreaks of bluetongue disease among ruminants, coupled with its ongoing expansion into new regions. BTV belongs to genus *Orbivirus*, family *Reoviridae*, and is composed of 10 segments of double-stranded RNA (dsRNA) surrounded by a bilayered viral capsid. Transmitted by hematophagous midges in the *Culicoides* genus (Diptera: Ceratopogonidae), bluetongue disease is characterized by lameness and mouth lesions in ruminant species (2, 3). The disease was initially termed simply fever or epizootic catarrh but later was given the name bluetongue for its association with cyanosis of the tongue and mucous membranes (4–6).

Although it was suspected early on that BTV was an insect-transmitted disease, Du Toit (7) demonstrated that *Culicoides imicola* is a biological vector for the virus. Early attempts to control BTV via vaccination with an attenuated virus led to the discovery that multiple virus strains, or serotypes, exist (8, 9). Whereas animals exhibited lifelong protection against the specific strain used in vaccine preparations, it was soon discovered that these vaccines did not protect against all field isolates of BTV. Defined by the vertebrate host's immune response to the BTV VP2 outer capsid protein, viral serotype is still used today to classify strains of BTV. At least 29 serotypes of BTV have been described to date, with various serotypes circulating regionally (10, 11). Importantly, as early findings demonstrated, antibodies that develop in response to infection with one serotype of BTV are generally not cross-protective against other serotypes. Hence, the introduction of novel BTV serotypes even to enzootic areas poses a concern in terms of animal health risk.

Despite more than a century of research, significant knowledge gaps regarding BTV's ecology and evolution persist, particularly considering today's accelerating climate crisis (12, 13). The transformational advancement and widespread availability of whole genome sequencing and other novel sequencing technologies offer essential tools to explore and better understand the evolutionary dynamics of this segmented dsRNA arbovirus. These molecular approaches can offer a higher resolution of evolutionary patterns occurring within a multi-host transmission system, revealing potential reassortment events, emerging mutations across all genes, and intra-host diversity of the virus. In this review, we focus on what is currently known about BTV evolution in the context of numerous selection pressures and exogenous factors linked to arboviral evolution.

BLUETONGUE VIRUS TODAY: EXPANSION AND EPIZOOTICS

As of today, BTV has been detected on all continents except Antarctica (14). Its range is defined by the presence of competent vector species and is classically considered to exist between 35°S and

40°N (15). However, numerous reports in recent years indicate that this canonical distribution no longer captures the true range of BTV (1, 16, 17), likely reflecting viral evolution, vector range expansion, and animal movement, among other factors.

Several high-profile, economically devastating epizootics have occurred in the past 15 years, highlighting the ability of BTV to cause explosive outbreaks of disease with little warning. Perhaps most notable among these recent events was the BTV-8 epizootic that occurred in 2006 and 2007 in northern Europe, representing remarkable expansion of BTV to 58°N (18). BTV infection was associated with disease in sheep, as well as in other ruminant species that rarely demonstrate signs of BTV infection, such as cattle and goats (19, 20). Transplacental infection and transmission of BTV, usually a phenomenon confined to the use of live attenuated vaccine strains at certain points of gestation, was a common occurrence during this epizootic (21, 22). Collectively, animal losses, production declines, and trade restrictions resulted in a cost of more than €150 million in the initial years of the outbreak (23, 24).

Even in enzootic regions such as the United States, outbreaks of orbiviral disease have occurred frequently in the past two decades. In 2007, 2012, and 2015, BTV and epizootic hemorrhagic disease virus, a closely related orbivirus, caused significant disease in domestic and wild ruminants across the United States (25–28). Numerous reports worldwide indicate that nonenzootic serotypes are increasingly being identified in otherwise BTV-enzootic regions, although whether these strains become persistently established remains to be seen (29–31). In the United States, incursive BTV serotype 3 appears to have become widespread across the country, with recurrent detections in many states during the past decade (31).

VECTOR DISTRIBUTION AND ECOLOGY

Although BTV is occasionally transmitted horizontally and vertically in vertebrate hosts, the vast majority of BTV transmission occurs via the bite of an infected female midge of the genus *Culicoides* (32). Of ~1,400 species of *Culicoides* distributed worldwide (33, 34), only approximately 30 species have been implicated as BTV vectors (35), and even fewer have been confirmed as competent vector species by laboratory transmission studies (36). *Culicoides sonorensis* is the predominant vector in western North America, with *C. insignis* playing a lesser role given its geographic constraint to the southeastern portions of the United States (15). *C. insignis* is a key BTV vector in Central and South America and appears to be extending its range in the United States (37). In Africa and western Asia, *C. imicola* has been implicated as the major biological vector of BTV, whereas *Culicoides brevitarsis* and *Culicoides wadai* are known to transmit the virus in central and east Asia, as well as Australia (15, 38). In Europe, the *Culicoides obsoletus* complex is composed of several closely related species of *Culicoides* and is primarily responsible for BTV transmission (23). Numerous other *Culicoides* species may also be competent for various strains of BTV, but robust information on the distribution and competence of many of these species is lacking.

The majority of BTV vector species are closely associated with livestock production and are more abundant in livestock habitats than less intensively managed ecosystems (39). Wildlife habitats or captive wild game ranges are often managed adjacent to livestock or native ruminants (40, 41). Because many *Culicoides* species are opportunistic feeders (40, 42, 43), the proximity of multiple host species may have implications for viral evolution through passage between multiple vertebrate species. As a genus, *Culicoides* develop in an extremely wide range of moist and semiaquatic habitats, though preferences for specific habitats vary by species. For example, *C. sonorensis* prefers to oviposit on the mud edges of manure-enriched standing water, whereas *C. obsoletus* develops in composted manure and *C. brevitarsis* develops in intact dung pats (36). In livestock habitats, the presence of acceptable larval development sites is thought to be the limiting factor for *Culicoides*

populations. Animal agriculture tends to keep localized populations of midges in close contact with hosts, increasing vector–host contact and vectorial capacity. Emigration and immigration of individuals from outside populations, even from distances >1 km, is likely common (44, 45) and may introduce new BTV serotypes into habitats. Few data are available on midge movement between sylvatic and agricultural ecosystems, though there is some evidence that *Culicoides* will travel between different habitats, and wild ruminant hosts may potentially also be involved in maintaining certain serotypes in circulation (41, 46). Moreover, as climate change advances, the range of these vectors is expected to expand, as has been seen in North America and the Mediterranean (37, 47, 48).

Culicoides midges also serve as vectors for several other viruses with significant animal health impacts, such as epizootic hemorrhagic disease virus and African horse sickness virus. In particular geographic regions, circulation overlaps among these viruses (27). Different midge species may be driving transmission of each of these pathogens respectively; however, the interactions that occur when these viruses coinfect a single midge remain poorly characterized. Midges can be infected with more than one strain of BTV and may subsequently transmit more than one virus during blood feeding on a susceptible animal (49). Work from mosquito-borne disease systems, like *Aedes aegypti*– and *Aedes albopictus*–transmitted flavi- and alphaviruses (50), suggests that competent vector species can develop disseminated and transmissible infections of multiple, even closely related viruses (51). Our limited understanding of virogenesis in *Culicoides* comes entirely from work in *C. sonorensis*, whereas *A. aegypti* and *A. albopictus* are very closely related species in the same subgenus (*Stegomyia*) (52). *C. sonorensis* (subgenus *Monoculicoides*) is not as closely related to other key vector species in the *Avaritia* subgenus, like *C. imicola* or *C. obsoletus* (53). Importantly, our conclusions drawn from viral evolution studies using *C. sonorensis* as a model species may not apply to other *Culicoides* vectors. These potential interactions between coinfecting viruses and the vector's response to each virus represent a gap in current understanding of *Culicoides*-borne viruses.

VIRAL STRUCTURE, FUNCTION, AND REPLICATION

When considering the evolution of BTV, it is informative to first understand its genetic composition. Of the viruses belonging to family *Reoviridae*, those in the genus *Orbivirus* are vector borne. Bluetongue and other viruses in the family *Reoviridae* possess a relatively unique genome structure, composed of 10–12 linear segments of dsRNA depending on the viral species. The BTV virion is nonenveloped. Structural studies have demonstrated that BTV is composed of a bilayered capsid that surrounds 10 segments of dsRNA, which range from 833 base pairs (segment 10) to 3,955 base pairs (segment 1) (54). The dsRNA segments in the viral core are associated with three key structural proteins: the RNA-dependent RNA polymerase (VP1, encoded by segment 1), the viral capping protein (VP4, encoded by segment 4), and the RNA helicase (VP6, encoded by segment 9) (55). Viral entry is mediated by VP2 (segment 2) and VP5 (segment 6), the two outer capsid proteins. VP2 is the receptor-binding protein and enables clathrin-mediated endocytosis, whereas the pH-sensitive VP5 protein is responsible for membrane fusion in the late endosome that allows ejection of the BTV inner core particle into the cytoplasm (56, 57). The inner capsid, composed of VP7 (segment 7) and VP3 (segment 3), forms an icosahedral structure that is sensitive to the presence of magnesium and nucleoside triphosphate (NTP), which promotes a shift in arrangement that allows for the release of viral messenger RNAs into the cellular milieu, beginning the process of viral protein synthesis and replication (54, 58). Single-stranded positive-sense RNAs function as both the template for genome replication (i.e., the synthesis of the complementary, negative-sense RNA strand), as well as the translation of viral proteins (59). Nonstructural proteins NS1 (segment 5) and NS2 (segment 8) play an important role during viral translation and assembly:

NS1 forms microtubules and specifically enhances the translation of viral proteins, and NS2 is the key component of viral inclusion bodies (VIBs) that recruit viral genomic RNAs and proteins to facilitate viral particle assembly (60–63). NS3 (segment 10) and a truncated version of this same protein, NS3a, are associated with viral egress from the cell. NS3a is translated from a slightly downstream start site from that of the NS3 protein and is the major viral egress protein produced in *Culicoides* cells (59, 64). Viral egress is nonlytic in *Culicoides* cells and lytic in mammalian cells, but the exact mechanism by which this occurs is not fully understood. Some degree of nonlytic viral budding likely occurs early in infection in mammalian cells, as recent studies have suggested (59, 65).

Single-stranded viral RNAs (ssRNAs) are recruited to VIBs during the assembly process. BTV uses a highly ordered process to ensure all 10 segments are properly incorporated into each virion (59). Conserved untranslated regions (UTRs) at the 3′ and 5′ end of each segment are essential for mediating sequential recruitment of ssRNAs (66–69). The 3′ UTR of segment 10 is especially instrumental in this process and triggers the proper secondary conformational structures of subsequent segments to allow proper interactions (66). The smallest segments (segments 7–9) are recruited first, followed by segments 4–6, and finally by segments 1–3 (54). Once all 10 ssRNAs are incorporated in nascent virions, capsid assembly and synthesis of the complementary RNA strand for each segment may occur. BTV dsRNA replication, like that of other related viruses, is conservative (55, 70). After viral cores are equipped with a complete set of genome segments, the nascent BTV particle is released from VIBs following NS2 phosphorylation and acquires the VP2 and VP5 outer protein layer as it exits (54, 71).

BLUETONGUE VIRUS EVOLUTION

Although the field of viral evolution is a robust and ever-growing area of research, two main considerations are essential to discuss when specifically considering BTV. First, BTV's vector-borne nature plays an important role in its overall evolution because the virus must maintain its ability to replicate in both vector and vertebrate host species. Second, BTV's segmented dsRNA genome is a defining characteristic for its genetic diversification. Although many features of BTV are well-characterized, deficiencies exist in our understanding of BTV evolution that are underscored by the recent incursions of BTV into new regions and the introduction of novel serotypes into otherwise enzootic areas. These developments require an understanding of many contributing factors: environmental conditions, ruminant and vector ecology, host community structure, immunity in the vertebrate and invertebrate host, anthropogenic factors, and viral evolution.

Evolution of RNA Viruses: Underlying Concepts

BTV is transcribed via an RNA-dependent RNA polymerase, which, in contrast to DNA polymerase, does not possess proofreading ability (72). Thus, many RNA viruses demonstrate an inherently high mutation rate (73–75). The rapid generation of many progeny viruses with mutations in their genomes is believed to be an important part of the RNA virus' evolutionary repertoire (73, 76, 77). Replicating at very high rates, RNA viruses such as poliovirus (78), vesicular stomatitis virus (79, 80), and foot-and-mouth disease virus (81) generate massive populations of viral progeny that possess numerous low-frequency mutations. RNA viruses are often described as approaching the point of extinction or lethality because of their extreme mutation rate (82–85). However, RNA viruses are believed to preserve fitness by maintaining an optimal balance between mutation and viral population size, allowing for rapid adaptation to new fitness landscapes (73, 76, 86). This concept is captured by the terms viral quasispecies and mutant swarm, which are used to

characterize both the genetic diversity and the vast number of virions produced during infection (87).[1] Quasispecies theory posits that high mutation rates and viral swarm generation are evolutionarily beneficial for phenotypic plasticity. The ensemble of mutant viruses is considered in many ways to be its own evolutionary unit; selection pressures are exerted on the entire viral ensemble, with complementation, interference, recombination, and reassortment among viruses ultimately shaping the swarm's overall fitness (73).

Mesenteron escape barrier (MEB): barrier(s) to infection located between the epithelial cells of the midgut and the hemolymph

Mesenteron infection barrier (MIB): barrier(s) to infection that are located only at the epithelial level of the mesenteron

Several theories have been proposed to describe underlying mechanisms that may drive this phenomenon. Mutational robustness is one explanation for the presence of viral quasispecies. Whereas redundancy and gene duplication serve to reduce the effect of deleterious mutations in higher-complexity organisms, a single mutation in a viral genome may be lethal (88). Viral swarms may serve this same function, providing a rescue mechanism for high mutation rates (89, 90). Others have proposed that quasispecies allow virus populations to occupy neutral or flat fitness landscapes (91, 92). In this framework, populations with high, but narrow, fitness peaks (i.e., populations of virus that are highly fit but also highly similar) are less capable of withstanding mutation compared to broad, low peaks (i.e., populations of viruses that may be individually less fit but are also more diverse) (90).

Others have argued that high mutation rates in some RNA viruses, such as poliovirus, are the evolutionary by-product or fitness trade-off for rapid viral replication speeds (93). Most mutations are deleterious (85, 94, 95), so it stands to reason that an RNA polymerase capable of higher-fidelity replication would eventually evolve; however, this has not been demonstrated. Using a mutant poliovirus strain with an antimutator phenotype (conferred by a single mutation in its RNA-dependent RNA polymerase), Fitzsimmons et al. (93) demonstrated that this virus' reduced replicative fitness was directly related to its slower replication speed. They concluded that speedy replication, with lower rates of replication fidelity, may be a tolerable trade-off for poliovirus and other RNA viruses, because it is difficult "to be both fast and accurate" (93, p. 2).

Arbovirus Evolution

Arboviruses face significant challenges and bottlenecks during viral replication owing to the increased purifying selection exerted by the rigors of maintaining fitness in two disparate host systems, the vertebrate and invertebrate. The impact of bottlenecks on viral populations in insect hosts has been characterized using several different arboviruses, in which it was demonstrated that repeated bottlenecks shape viral populations during infection in the insect vector (96–99). Several anatomical and physiological bottlenecks have been identified within the arthropod vector that drive successive contractions and expansions in viral populations.

Within invertebrates, such as mosquitoes and midges, the mesenteron escape barrier (MEB) and the mesenteron infection barrier (MIB) impede arboviral infection and dissemination (100–102). Whereas the salivary gland infection barrier and salivary gland escape barrier are well characterized in mosquitoes, evidence that these barriers are present in the *Culicoides* midge is lacking (100, 103). Another physical constraint within the vector is the small volume of blood ingested during blood feeding; *Culicoides* midges take a blood meal volume of approximately 0.1–1 μL (104). As a result, insects are exposed to a relatively low number of viruses when taking an infectious

[1] According to Domingo et al. (73, p. 46), "Viral quasispecies are currently defined as collections of closely related viral genomes subjected to a continuous process of genetic variation, competition among the variants generated, and selection of the most fit distributions in a given environment," and "viral quasispecies are the mutant distributions (also termed mutant swarms or mutant clouds) that are generated upon replication of RNA viruses, and some DNA viruses in infected cells and organisms."

blood meal. Additionally, the viral titer in the blood plays an important role, as higher titers are associated with a greater likelihood of successful infection of the vector (99). Forrester et al. (99) demonstrated that orally infecting mosquitoes with a high titer of Venezuelan equine encephalitis virus did not result in a significant bottleneck during midgut infection. However, when a more biologically relevant dose was used for oral infection, a very strong midgut infection bottleneck occurred. It is expected that only high-frequency variants would be likely to cross the midgut infection barrier in this circumstance unless a significant fitness advantage was conferred by an otherwise low-frequency mutant.

Müller's ratchet: the concept in evolution that any asexual population will develop permanent deleterious mutations over time within the population

Compounding the consequences of the physical barriers resulting in viral infection bottlenecks, a component of the arthropod host immune response, known as RNA interference (RNAi), has been documented in *Culicoides* midges (98, 105–108). RNAi mechanisms may further reduce viral fitness and are likely to contribute to genetic drift within the invertebrate host. Further exploration of this could be a fruitful topic for future research on vector–virus interactions of BTV and related viruses.

Host-Switching and Genetic Diversity

Preliminary work with BTV has demonstrated that some of the arboviral evolutionary features described above also apply to orbivirus evolution. Early on, various groups used electropherotype to characterize the genetic heterogeneity of BTV (109), but unfortunately these works are difficult to relate to our understanding of BTV evolution today owing to the relatively poor genomic resolution these early methods provided. With the advent of modern sequencing techniques, additional approaches to better understand BTV genetic diversification have become readily available. However, only a few studies have investigated how BTV's alternating host transmission cycle affects its evolution.

Initial work studied the impacts of host-switching on BTV evolution using *C. sonorensis* midges and domestic ruminants as a model system (110). This group found infrequent synonymous and nonsynonymous mutations that arose at different rates in two segments (segment 2 and 10) and concluded that founder effect and quasispecies generation were important features in the evolution of a single BTV strain. Importantly, however, this study used polymerase chain reaction (PCR), and errors can be introduced during reverse transcription, PCR, or cloning; thus, it is difficult to determine whether some of these putative BTV variants were artifactual (111, 112). Moreover, the true extent of viral quasispecies present in these experiments may have been masked by replication deficiencies of certain mutants, making them nondetectable by plaque assay.

Although other studies have suggested that propagation in cell culture or eggs results in viral attenuation of BTV secondary to purifying selection and a reduction in viral quasispecies, little work has been performed to understand how or whether purifying selection occurs during BTV host-switching (113, 114). In fact, virus isolation in non-native cell types, such as vertebrate cells or embryonated chicken eggs, likely enforces a dramatic bottleneck, possibly leading to a Müller's ratchet–like phenomenon, whereby severe bottlenecks are shown to increase the occurrence of deleterious mutations (81, 115, 116). However, these findings are difficult to relate to the overall evolution of BTV in its native hosts, because of the simplified nature of the culture experiments in which they took place.

Host-switching's impact on the evolutionary process across all 10 BTV segments, as well as whether each segment behaves similarly, is poorly characterized. Although direct experimental approaches have been applied to address the impact of alternate host transmission, various phylogenetic studies have indicated that BTV has a low overall mutation rate across its genome segments, consistent with other arboviruses (117, 118). This raises the possibility that some of the same mechanisms that drive slower rates of mutation in ssRNA arboviruses could be at play in

BTV's evolution as well. Interestingly, analysis of 4 segments across 290 BTV isolates revealed that BTV had lower substitution rates than many other arboviruses (mean rates of $\sim 0.5–7 \times 10^{-4}$ nucleotide substitutions per site, per year) (119). Others have found similar substitution rates and evidence of strong purifying selection among BTV isolates from Europe and Australia (117, 120, 121). This suggests that additional stringencies may affect BTV's genome stability, such as increased replication fidelity or stability, afforded by its double-stranded composition.

More recent efforts have focused on characterizing aspects of viral genetic diversity using whole genome sequencing across all 10 segments. Using insect cells to isolate a strain of BTV-8 from an experimentally infected sheep, Caporale et al. (122) found that virus sequenced from whole blood had more high-frequency single-nucleotide variants (SNVs) than the virus isolated onto invertebrate cells. Synonymous variants that were observed at high frequency in the virus sequenced directly from whole blood went to fixation when isolated and then propagated on invertebrate and vertebrate cells, respectively. When additional sheep were inoculated directly with either BTV-8 from whole blood or the cell-culture-isolated virus, those infected directly with BTV-8 from blood developed more severe disease, despite the blood having $\sim 100\times$ lower titer of BTV than propagated virus (122). Although these researchers suggested that the difference in disease phenotypes manifested in infected sheep may have been due to differences in the number of low-frequency variants, factors beyond the number of SNVs present in the viral populations could contribute to the difference in disease phenotypes observed in this case.

Expanding on this work, Kopanke et al. (118) investigated how BTV's alternating-host transmission cycle impacts the occurrence of genetic variation within a single virus strain across all 10 segments. Whole genome sequencing was used to detect the occurrence and frequency of SNVs over successive passages in both insect and mammalian cells. The results showed that although low-frequency SNVs were present across passages of all cell culture conditions, the BTV genome remained highly genetically stable. This was true even when virus was passaged exclusively in invertebrate cells, which had been predicted to permit widespread genetic diversification. Additionally, compared to the other 9 segments, segment 10 demonstrated higher population complexity and divergence, regardless of cell culture condition. These findings suggest that evolutionary pressures may vary across BTV's 10 genome segments. Although the BTV genome remains remarkably stable when passaged in an in vitro system permissive to genetic diversification, further studies using in vivo models in native species are critical. Integrating these in vitro studies with deep sequencing provides a readily available method to query the extent of BTV evolution across all 10 segments.

Segmented Viruses and Reassortment

Building upon early electropherotype work, whole genome sequencing efforts over the past decade have provided increasingly strong evidence that reassortment may be a primary driver of BTV genetic diversification (121). Reassortment can occur when a cell is coinfected by more than one virus of the same species and is a characteristic feature of most segmented viruses (123). Viruses with segmented genomes, including those in the families *Orthomyxoviridae*, *Reoviridae*, *Picobirnaviridae*, and *Birnaviridae*, as well as the order Bunyavirales, have frequently been demonstrated to reassort, both in nature and experimentally (124–128). Although reassortment is a key feature of segmented virus evolution, our understanding of the mechanisms, constraints, and drivers of this phenomenon remains limited (**Figure 1**).

It has been suggested that reassortment may be akin to sexual reproduction, engaging fundamental evolutionary concepts of combining genetic material and contributing to accelerated adaptive fitness (129). In the context of Müller's ratchet, reassortment may be remarkably

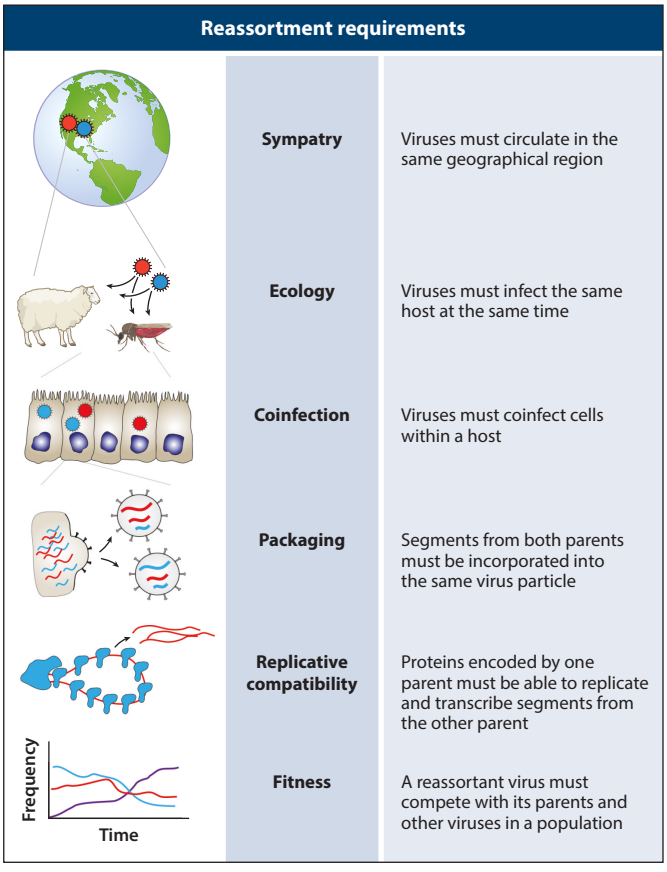

Figure 1

Reassortment has natural requirements that enable this viral phenomenon to occur. These requirements fall into three categories: location, host dynamics, and survival. Spatial aspects like sympatry and ecology (location) influence viral infection of the host. Molecular processes (host dynamics) within the host cell determine reassortment viability. Specifically, coinfection of the same cell with progeny virus having segments from both parents must result in viable virus. Finally, reassorted virus survival depends on competition with additional parental and nonparental viruses.

beneficial, particularly in small populations (129). The evolutionary origin of segmented viral genomes is incompletely understood, but beyond the potential gains from reassortment as a mechanism to introduce genetic variability in a population, there are fitness benefits to segmentation itself, such as improved viral stability and increased genome replication (130).

Reassortment among segmented viruses has been linked to significant consequences, including the occurrence of highly pathogenic hemorrhagic fevers (e.g., Ngari orthobunyavirus) and the origin of pandemic influenza viruses (131, 132). Moreover, analysis of influenza virus indicates that reassortment increases the likelihood of viral expansion into a new host (133). Reassortment is also a prominent feature among BTV field isolates, including those that seem to cause severe disease (134–136).

Characterization of bluetongue virus reassortment. The occurrence of reassortment has long been recognized among BTV isolates worldwide, and a suite of studies in the 1980s sought to

characterize reassortment between two enzootic North American strains in a variety of systems (in vitro, insect, and mammalian) (49, 137–141). Studies using electropherotype shifts in plaque-isolated progeny viruses following experimental coinfections as an indicator of reassortment established several important points. First, these studies demonstrated that BTV-10 and BTV-17, two of the virus serotypes used in these experiments, could reassort extensively in vertebrate cells, and that when viruses did not have equal multiplicities of infection (MOI), the virus with a higher MOI contributed more segments to reassortant progeny (139). Second, when *C. sonorensis* midges, cattle, and sheep were coinfected with different BTV serotypes to determine reassortment frequency in vivo following coinfection, reassortant viruses arose in all cases (49, 137, 140, 141). Notably, *Culicoides* seemed to support more robust levels of reassortment. Third, El Hussein et al. (138) found that when *Culicoides* coinfections were staggered by various lengths of time, reassortant viruses could be isolated from midges that had been infected with the second virus up to five days after the first virus was introduced. In the future, mathematical modeling could help integrate evolutionary processes observed in laboratory experiments and contribute to the overall knowledge of orbivirus epidemiology (see the sidebar titled Mathematical Modeling to Better Understand Reassortment) (**Figure 2**).

Multiplicities of infection (MOI): the ratio of the number of virions to the number of cells

Although these prior studies provide an important starting point for investigating BTV reassortment, these experiments have several shortcomings. Polyacrylamide gel electrophoresis and related methods provide limited resolution for detecting subtle differences in the genome sequences of each segment. Thus, early studies were unable to distinguish the parental origin of several segments in reassortant progeny viruses, particularly for longer segments with indistinguishable migration, resulting in incomplete data. Variations in fitness and replication kinetics mean that slow-growing reassortants that produce smaller plaques may be missed or overgrown by plaques from relatively fast-replicating reassortants, potentially biasing detection of reassortment based solely on plaques.

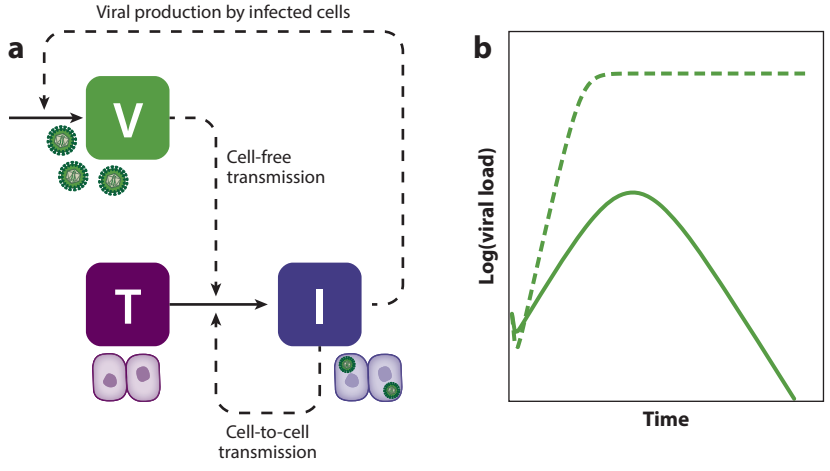

Figure 2

Mathematical modeling to better understand reassortment. (*a*) Typical target-cell limited model. Clearance of free virions, cell death, and cell replication are not shown here. (*b*) Typical viral load dynamics of a target-cell limited model. The solid line indicates typical dynamics of an acute infection, such as Zika virus in humans, and the dashed line illustrates typical dynamics of a chronic infection, such as bluetongue virus in *Culicoides*. Abbreviations: I, infected cells; T, target cells; V, free virions.

MATHEMATICAL MODELING TO BETTER UNDERSTAND REASSORTMENT

Certain within-host processes can be difficult to elucidate directly using in vivo models. In such cases, mathematical models can be powerful tools to complement in vitro and in vivo experiments and to make additional inferences from the data they generate (145). Examples of such processes that mathematical models have been used to infer include the in vivo replication and clearance rates of human immunodeficiency virus type 1 (HIV-1) (146), the relative roles that cell-to-cell and cell-free transmission play in HIV-1 infections (147), and the effect of the innate immune response and viral interference on Zika virus dynamics (148). Much of the effort in this field has been focused on dynamics of human viruses (146, 149–153), though several studies have explored the dynamics of malaria parasites in mosquitoes (154–157). For instance, by accounting for the processes that determine sporozoite density in mosquito salivary glands, Stopard et al. (157) showed that the extrinsic incubation period of malaria had greater variability than thought previously.

Adapting and extending these modeling approaches for bluetongue virus (BTV) in *Culicoides* could provide insight into the evolutionary and transmission dynamics of this pathogen. For instance, modeling could aid in inferring the rate at which reassortants emerge when *Culicoides* experience mixed BTV infections, or extrapolate from in vitro studies to estimate the frequency of reassortants in infectious blood meals.

The classical within-host model is known as a target-cell limited model (158) (**Figure 2**). This is a compartmental model, with compartments for target cells, infected cells, and free virions. Target cells are infected by free virions (cell-free transmission) or by infected cells (cell-to-cell transmission). Infected cells then produce new virions, and free virions are also cleared. Finally, cell death and replication are modeled if they are relevant on the timescale of infection, and cell death may occur at a higher rate in infected cells.

More recent in vitro work by Shaw et al. (142) using BTV-1 and BTV-8 found that reassortment between these two viruses was highly flexible, in that multiple combinations of genome segments resulted in viable reassortants. Some segment combinations demonstrated fitness disadvantages compared to others, highlighting that segment–segment, segment–protein, and protein–protein interactions affect the overall viability of reassortant BTV viruses. Although certain segments were detected more frequently than others in reassortant viruses, Shaw et al. (142) ultimately determined that reassortment could involve any combination of segments from the parental BTV-1 and BTV-8 viruses.

Shaw et al.'s (142) approach of generating mono-reassortants via reverse genetics represents a powerful way to study reassortment in a deliberate and well-controlled fashion. However, with 10 segments of genomic dsRNA and 1,024 possible reassortment combinations between two strains of virus, BTV poses a logistical challenge for this type of work. Compared to influenza, with just 8 segments and 264 possible segment combinations between two viruses, the number of segments present among viruses in family *Reoviridae* adds another level of difficulty to characterizing reassortment in these viruses. The complexity of these biological mechanisms could have an impact on each viral strain, potentially a reassortant strain, present in a viral swarm. Collectively, this could alter replication, kinetics, or even cell tropism within the vertebrate and invertebrate host. Therefore, novel techniques and holistic approaches for detection of reassortment are needed.

Mono-reassortants: viruses that have undergone one run-through of reassorting process and can be rescued through use of reverse genetics

Reverse genetics: genetic technique involving alteration of the genome that results in a specific phenotype

An alternative to using recombinant BTVs to study reassortment is to initiate experimental coinfections and use whole genome sequencing to track patterns in resulting progeny. Whether used for in vitro or in vivo studies, this tool can reveal global shifts in segment frequencies. Using two common strains of influenza to infect vertebrate cells, Zeldovich et al. (143) applied whole genome sequencing to track segment frequencies across several passages, ultimately revealing trends that echoed naturally arising influenza reassortant viruses. A similar approach has been

attempted for BTV, in which two enzootic strains of BTV were used to establish coinfections in vertebrate cells. By using whole genome sequencing, consistent trends emerged with regard to the distribution and frequency of certain segments across passages. These trends were largely in agreement with results from plaque isolation, although there were some exceptions, alluding to factors that may affect reassortment detection via plaque assay. In contrast to single-virus BTV studies, where viral population diversity remained relatively constant regardless of cell culture condition or passage history, it was found that population diversity in coinfected cell cultures increased across passages (144). These findings highlight the numerous complex factors that may limit or permit reassortment in segmented viruses, and arboviruses in particular, given the additional stringencies exerted by maintaining fitness in two divergent host systems.

Superinfection: any reinfection of a host with the same pathogen

Although this model has proved powerful for explaining overall within-host dynamics, extensions are usually necessary to explain specific processes. For instance, Best et al. (148) added terms to describe the innate immune response to Zika virus infection and viral interference with the response, finding that this fit experimental data better than models without these processes. Other models have added spatial structure to understand how cell arrangement influences dynamics (159). Still other models allow for cellular coinfection, either by increasing the number of compartments or by making assumptions about the distribution of cellular MOI (160–162). Within-vector models of malaria parasites take a different approach given the parasite's complex life cycle, instead using the different stages through which the parasite passes as their compartments (154–157).

Several refinements would be required to develop a model of BTV in *Culicoides*. For example, it may be necessary to incorporate barriers to infection, especially the midgut infection and exit barriers (MIB and MEB; see the section titled Arbovirus Evolution). There is evidence that in some cases just a few virus particles cross the MEB (99), and one study found that only ∼30% of midges infected orally result in disseminated infections (100). One way to model this would be to include separate stages for the midgut and the secondary tissues. If the aim were to understand the role of a founder effect in which virus genomes cross the MEB, then it may also be beneficial to include a stochastic element in the model. Because *Culicoides* are ectothermic, viral replication rates can be sensitive to ambient temperature, so it may be necessary to include temperature-dependent parameters (163). With these and other refinements, and combined with appropriate in vivo experimental work, models of this type could help further our understanding of BTV evolution in *Culicoides*.

Factors restricting reassortment. For successful reassortment to occur, several key features must be in place. First, coinfection of a single cell must occur. Second, this coinfection must occur within a time frame that permits superinfection or productive coinfection. For example, reassortment between influenza viruses can occur within the first three hours of a cellular infection, but thereafter cells become refractory to infection with a second virus (164). This may be due to several reasons, including viral-mediated destruction of receptors on the cell surface or host cell antiviral responses (123). Some studies indicate that viruses in family *Reoviridae* are relatively permissible to superinfection, with a prolonged window in which cells may be productively infected with more than one virus (165). El Hussein and colleagues' (138, 139) work indicates that this may be a feature of BTV coinfection.

Physical barriers within the cell may prevent productive reassortment. If the sites of viral replication are highly isolated within the cell, only limited interactions between different viruses may occur. The mechanism by which reassortment may occur during BTV coinfection is not well understood, particularly because BTV and related viruses have highly sequestered replication factories within the cell. Whether a trafficking mechanism exists to shuttle viral segments between

bluetongue VIBs is not known. A recent study with mammalian orthoreovirus demonstrated dynamic movement of viral factories within the host cell, which was mediated in part by microtubules (166). It is not known whether a similar mechanism might facilitate segment movement and reassortment among BTV strains.

Incompatibility between certain genome segments or their downstream proteins may preclude the generation of reassortant viruses. For instance, in rotaviruses, mismatches between the viral polymerase and capsid proteins appear to limit the spectrum of viable reassortant viruses (167). Our own work with in vitro BTV coinfections found that certain viral segments could disappear from the viral population over time, possibly owing to mismatches in segment–segment or segment–protein interactions (144). When BTV-2 and BTV-10 coinfections were established in vitro, BTV-10's segment 7 became essentially undetectable by whole genome sequencing after seven passages, despite being identical at the protein level to BTV-2's segment 7 (144).

Packaging signals, too, play an instrumental role in the likelihood of reassortment. Marshall et al. (168; see also 169) demonstrated that reassortment between engineered, near-identical influenza A viruses (differing by rare silent mutations in each segment) was highly efficient in an in vitro system, but when heterologous packaging signals (3′ and 5′ UTRs) were introduced on certain segments, reassortment was limited dramatically. Similar restrictions in reassortment mediated by incompatible packaging signals have been described for viruses in Bunyavirales (170, 171). Whereas 3′ and 5′ UTRs are quite conserved across BTV strains, additional packaging signals in the coding sequences may similarly restrict interstrain reassortment.

SUMMARY

Improvements in sequencing methods and bioinformatics analysis provide an extraordinary opportunity to investigate long-standing questions in BTV evolution and ecology. In this review, we have discussed known evolutionary traits and some of the underlying mechanisms driving BTV evolution. Clearly, many questions regarding the factors that drive or constrain BTV genetic diversification remain unanswered. Related work with other vector-borne and segmented viruses provides a compelling backdrop for further investigation of BTV. The increased application of whole genome sequencing and other novel sequencing platforms promises to build upon earlier work, setting the stage for improved understanding of bluetongue and related viruses. By applying recent advancements in technology in a variety of experimental approaches (applied fieldwork, in vitro systems, and in vivo models), investigators may better characterize the relative impact of host-switching, reassortment, and environmental conditions on the overall genetic diversification of BTV.

Although progress has been made in using molecular tools to study BTV, many critical and basic evolutionary gaps exist, especially within the context of virus–host interactions and evolutionary processes during coinfection. Despite the widespread and frequent occurrence of reassortment in BTV, the ecological context in which these within-host events translate into significant between-host transmission is poorly understood. Vector species with heterogeneous host feeding preferences add complexity to an already complicated system. Within this context, a possibility exists for exchange of genetic material between pathogens and increased adaptive fitness through reassortment. This sharing of genetic material can facilitate the emergence of strains with epizootic potential.

Multifaceted systems are difficult to study owing to the logistics, animal models, and vector colonies required (**Figure 3**). To fully investigate viral evolution, future work should explore the multitude of factors that affect virus–vector–host interactions. The mechanistic unknowns of BTV evolution and reassortment following coinfection and the RNA- or protein-based interactions that permit or limit generation of successful reassortant progeny also warrant further investigation.

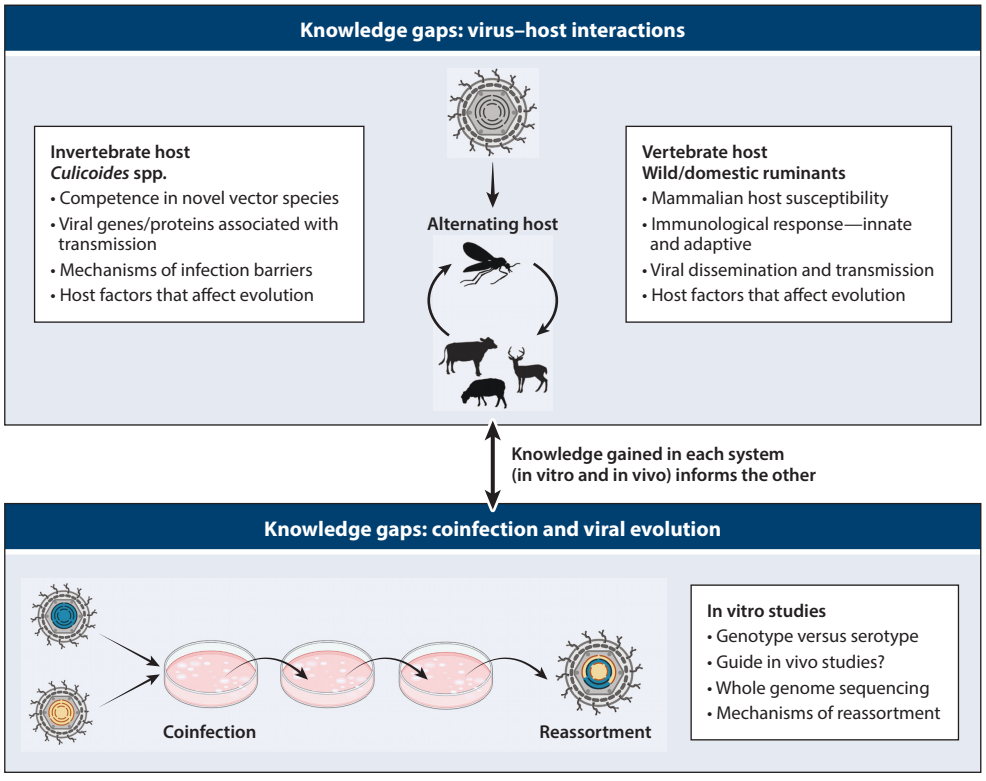

Figure 3

Knowledge gaps in bluetongue virus (BTV) virus–host interactions and evolutionary dynamics. Invertebrate and vertebrate hosts have unique BTV infection dynamics that have implications for virus evolution and transmission. Elements of invertebrate host BTV infection and vertebrate host BTV countermeasures remain undefined and may be contributing factors to viral evolution. Comparably, the role of vertebrate host immunity in maintaining BTV fitness and diversity has yet to be characterized, and investigations of virus–host interactions in the context of alternating host systems could bridge further understanding of BTV viral evolution. BTV coinfection and reassortment are other facets of BTV evolution that necessitate further examination. Specific stringencies that BTV's alternating host transmission cycle impose on reassortment remain relatively unexplored. In vitro coinfection studies in a relaxed system can result in population diversity through reassortment; however, in vitro studies are the initial step in guiding future investigations and in vivo studies. Portions of this figure were created with BioRender.com.

More broadly, the roles that host immunity, RNAi, host community structure, coinfection with other viral species, and microbiome composition play in shaping BTV's evolution are open to exploration. A refined understanding of the factors that drive or constrain BTV evolution will facilitate improved predictive models and preventive strategies with the intent of mitigating the risk of future bluetongue disease outbreaks.

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AUTHOR CONTRIBUTIONS

Conception and design: J.K., M.C., J.L., E. McDermott, S.C., J.R.R., T.A.P., C.K.M., M.S., and C.M. Writing and editing of original draft preparation: J.K. and C.M. Critical revision and

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Errata

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