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Phylogenetic Comparison of the S3 Gene of United States Prototype Strains of Bluetongue Virus with That of Field Isolates from California

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To better define the molecular epidemiology of bluetongue virus (BTV) infection, the genetic characteristics and phylogenetic relationships of the S3 genes of the five U.S. prototype strains of BTV, the commercially available serotype 10 modified live virus vaccine, and 18 field isolates of BTV serotypes 10, 11, 13, and 17 obtained in California during 1980, 1981, 1989, and 1990 were determined. With the exception of the S3 gene of the U.S. prototype strain of BTV serotype 2 (BTV 2), these viruses had an overall sequence homology of between 95 and 100%. Phylogenetic analyses segregated the prototype U.S. BTV 2 strain to a unique branch (100% bootstrap value), whereas the rest of the viruses clustered in two main monophyletic groups that were not correlated with their serotype, year of isolation, or geographical origin. The lack of consistent association between S3 gene sequence and virus serotype likely is a consequence of reassortment of BTV gene segments during natural mixed infections of vertebrate and invertebrate hosts. The prototype strain of BTV 13, which is considered an introduction to the U.S. like BTV 2, presents an S3 gene which is highly homologous to those of some isolates of BTV 10 and especially to that of the vaccine strain. This finding strongly suggests that the U.S. prototype strain of BTV 13 is a natural reassortant. The different topologies of the phylogenetic trees of the L2 and S3 genes of the various viruses indicate that these two genome segments evolve independently. We conclude that the S3 gene segment of populations of BTV in California is formed by different consensus sequences which cocirculate and which cannot be grouped by serotype.

Bluetongue virus (BTV), an orbivirus from the family *Reoviridae*, is biologically transmitted by *Culicoides* spp. to wild and domestic ruminants. BTV infection occurs throughout most of the tropical and temperate areas of the world, in parallel with the distribution of its competent vector. Twenty-five serotypes have been recognized worldwide (6), five of which have been isolated in the United States (serotypes 2, 10, 11, 13, and 17) (35). The BTV genome consists of 10 double-stranded RNA segments that encode at least 10 viral proteins, 3 of which are nonstructural and 7 of which are structural. The structural proteins form an inner core consisting of VP1, VP3, VP4, VP6, and VP7, which is surrounded by an outer capsid composed of VP2 and VP5 (35). Genome segments L2 and M2 encode VP2 and VP5, respectively, and are the two most variable genes. Genome segment S3 encodes the third minor inner capsid protein, VP6. The S3 gene, like the genes encoding other core and nonstructural proteins, is conserved across all serotypes (7, 35).

The two-host cycle of arboviruses imposes a considerable evolutionary constraint on the viral population. Arthropod-borne viruses must be able to infect and replicate in two very different biological systems. They must infect and amplify in the insect vector and then be transmitted to the vertebrate host and produce high-titer viremias that ensure reinfection of the insect vector. The survival of the viral population obviously is dependent on a very delicate equilibrium between the virus and its environment. The potential for variation of the RNA genome of most arboviruses endows them with the ability to adapt to changes in the environment, or their hosts, by following different pathways of evolution (46). Genetic drift and gene reassortment are two mechanisms utilized by BTV to overcome the constraints imposed by its complex two-host cycle of infection. Reassortment of BTV genes in *Culicoides* spp., as well as in ruminants (31, 37, 38, 41), influences the epidemiology of BTV infection and bluetongue disease through the generation of new viral variants with novel antigenic and virulence characteristics (9, 44).

Phylogenetic analyses based on the complete or partial nucleotide sequences of conserved and variable BTV genes were carried out to determine genetic relationships between different serotypes of BTV (8, 11, 18, 22, 23, 26, 50). Characterization of the molecular epidemiology of the L2 gene of field isolates of two BTV serotypes in California has demonstrated that viral variants obtained in a restricted geographical region evolve via different and independent pathways (8, 11). It was also demonstrated that the BTV population was formed by a constellation of cocirculating variants, all of which are related to a prototype consensus sequence. Long-term sequence conservation and the replacement of one consensus sequence by another in one geographical area were observed, as also occurs with other arboviruses (8, 11).

Different genes of the same virus may evolve at different rates. Different selection forces and evolutionary constraints are responsible for this differential pattern of evolution (47). The objectives of this study were to characterize the evolutionary relationships of the conserved genome segment S3 among field isolates of different serotypes of BTV obtained in California and, further, to define the evolution of variable and

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TABLE 1. Field isolates of BTV from California

Field isolate	Sero-type	Animal of origin	Year of isolation[a]	County	GenBank accession no.[b]
10O80V	10	Ovine	1980	Tehama	U55780
10B80Y	10	Bovine	1980	Tulare	U55778
10O80Z	10	Ovine	1980	Solano	U55781
10O90H	10	Ovine	1990	Mendocino	U55782
10O90Z	10	Ovine	1990	San Bernardino	U55783
10B90Z	10	Bovine	1990	Fresno	U55779
11UC2	11	Bovine	1981 (A)	Kern	U55786
11UC8	11	Deer	1981 (A)	Kern	U55787
11C81Z	11	Goat	1981 (B)	Kern	U55785
11O81Z	11	Ovine	1981 (B)	Kern	U55784
13B81X	13	Bovine	1981 (C)	Riverside	U55789
13B81V	13	Bovine	1981 (C)	Riverside	U55788
13B89Z	13	Bovine	1989	Fresno	U55791
13B89Y	13	Bovine	1989	Stanislaus	U55790
17C81W	17	Goat	1981 (D)	Kern	U55793
17O81Y	17	Ovine	1981 (D)	Kern	U55794
17B90Z	17	Bovine	1990	Fresno	U55792
17O90X	17	Ovine	1990	Kern	U55795

[a] Letters in parentheses indicate farms of origin.
[b] Prototype and VAC 10 GenBank accession numbers: BTV 2, U55799; BTV10, U55801; BTV 11, U55796; BTV 13, U55797; BTV 17, U55798; VAC 10, U55800.

conserved genes from the same virus isolates to better define the molecular epidemiology of BTV infection.

The S3 gene of 18 field isolates of BTV serotypes 10, 11, 13, and 17 from California and the U.S. prototype strains were characterized. The 1980 and 1981 isolates were obtained during an epidemiologic study (32). The 1989 and 1990 samples were originally isolated and serotyped in the California Veterinary Diagnostic Laboratory at the University of California at Davis. The origin of each isolate is listed in Table 1. The U.S. prototype strains of BTV have previously been described (34). The viruses were processed as described earlier (20, 29) and were propagated in BHK-21 cells (20). The commercially available modified live virus vaccine (VAC 10), derived from the original BTV 10 isolate (CA8) and attenuated by serial passage in embryonated chicken eggs (Poultry Health Laboratory, Davis, Calif.), was propagated once in BHK-21 cells. The viral RNA was phenol extracted, ethanol precipitated, and lithium chloride purified (10). Primers for PCR amplification and cycling sequencing of the S3 gene were designed from the published sequence of the U.S. prototype strain of BTV 10 (16). Two 15-mer oligonucleotide primers corresponding to nucleotide positions 1 to 15 and 1035 to 1049 were used to reverse transcribe and amplify the entire gene (1). The PCR products were gel purified. Both strands of the cDNA were sequenced by cycle sequencing using a commercial kit with a set of primers corresponding to nucleotide positions 247 to 266, 264 to 282, 381 to 400, 633 to 652, 759 to 778, and 847 to 864. The sequencing reactions were done as previously described (8, 11). Each sequencing reaction was repeated with PCR products from several different amplifications to control for artifacts.

The nucleotide sequence analyses of the S3 gene and the predicted VP6 protein of each virus were carried out with the Genetics Computer Group software, version 8.0 (17). The phylogenetic studies were done with the PHYLIP package, version 3.41 (13). The genetic distance between any two nucleotide sequences was calculated by using the DNADIST program on the basis of the Kimura two-parameter model (25) and the work of Jukes and Cantor (24). The phylogenetic analyses were carried out by utilizing a distance matrix method, based on the least-squares method (14) and implemented in the Fitch program of the PHYLIP package. The neighbor-joining method of Saitou and Nei (36) and the unweighted pair-group method with arithmetic mean, as implemented in PHYLIP, was also used (13). A bootstrap analysis with 100 replicates was done to assess confidence limits of the branch pattern (12). A value of >70% is considered significant (21).

The S3 genes of the five U.S. BTV prototype strains, VAC 10, and the 18 field isolates from California all are 1,049 nucleotides in length. All S3 genes present an initiation codon at nucleotide positions 16 to 18, and the termination codon of this open reading frame is at positions 1003 to 1005; thus, each gene encodes a protein of 329 amino acids. This first initiation codon is not in the optimal consensus sequence for initiation of translation (27). A second initiation codon at positions 28 to 30, as described for BTV 1 of South Africa (43), is present only in BTV 2, BTV 11, BTV 13, VAC 10, 11C81Z, and UC8, and this open reading frame encodes a protein of 325 amino acids. This initiation codon also is not in the optimal sequence context for initiation of translation, lacking a guanine (G) at position +4 but presenting a G at positions −3 and −6 (27). With the exception of UC2, 11O81Z, and BTV 17, the second initiation codon in the rest of the viruses is at positions 115 to 117. This is the strongest initiation codon according to the Kozak consensus sequence (27). The UC2 strain presents a possible initiation codon at positions 82 to 84, and 11O81Z presents one at positions 100 to 102. BTV 17 is the only virus in this study that presents a single open reading frame, which encodes the protein of 329 amino acids. The influence of multiple initiation codons in the S3 genes on the virus replication and adaptation is unknown. This could represent an adaptive response to selective pressures imposed by the vector and the vertebrate host.

Essentially identical phylogenetic trees were constructed by the neighbor-joining and Fitch and Margoliash methods (14) (Fig. 1). With the exception of BTV 2, which segregates in a different branch closer to BTV 1 from South Africa, all other viruses share the same hypothetical ancestor with a bootstrap value of 100%. The S3 gene of BTV 2 was the most divergent, with 10 to 11% divergence from the other viruses. The California field isolates, VAC 10, and the rest of the U.S. prototype viruses formed a closely related group with an overall sequence homology ranging from 95 to 100%. The calculated mean genetic distance of this group was 2.5%. With the exception of 11C81Z, these viruses are clustered in two principal groups, identified as A and B, which appeared in the bootstrap resampling analyses with frequencies of 86 and 81%, respectively. Different random seed numbers were used in this bootstrap analysis (13), and the resulting frequencies of these groups (A and B) were always more than 80%. It has been empirically demonstrated that bootstrap values of 70% or higher coincide with actual probabilities of more than 95% that a group is real (21). Therefore, the probability that groups A and B are real may be even higher than the bootstrap values would suggest (21). This analysis supports the concept that these two monophyletic groups represent distinct lineages of BTV genome segment S3 with two different direct immediate ancestors. This indicates that at least two different BTV gene S3 consensus sequences were circulating in the western United States between 1953 and 1990.

A striking characteristic of these two clades is that they include the S3 genes of viruses of different serotypes and different degrees of virulence and the S3 genes of viruses that were isolated in the same or different years and geographical regions. Group A is formed by most of the viruses isolated in California, with the exception of prototype BTV 13, which was

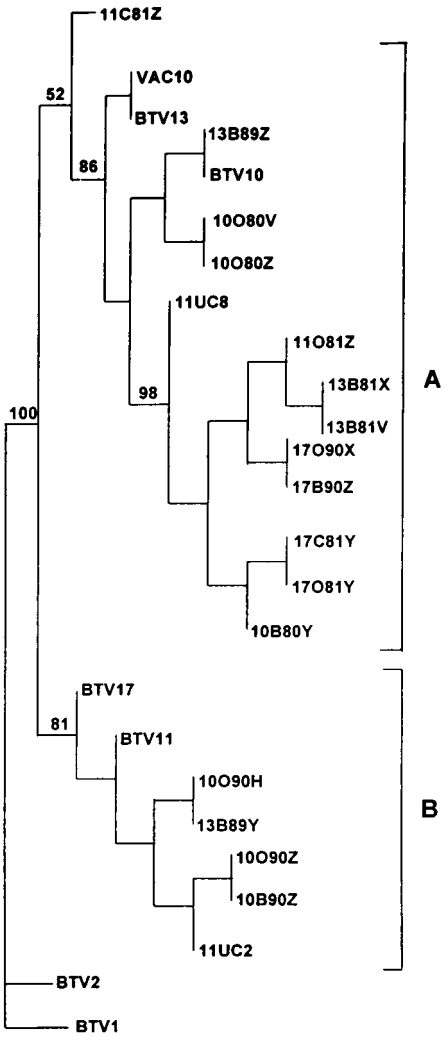

FIG. 1. Phylogenetic relationships of the S3 genes of U.S. prototype strains of BTV and California isolates. The tree was constructed by the Fitch-Margoliash method (14) with gene S3 of BTV 1 from South Africa as an outgroup. Numbers at the nodes indicate bootstrap confidence values (100 replicates) for the groups composed of viruses to the right of the node (12).

isolated in Idaho. It includes all the field isolates of serotype 17, most of those of serotypes 11 and 13, the BTV 10 prototype and 1980 BTV 10 field isolates, and VAC 10. Within this group are viruses isolated 37 years apart (BTV 10 and 17B90Z) and variants with different degrees of virulence such as 10O80V, obtained from a sheep without clinical disease, and 17O90X, from a sheep with severe clinical symptoms of bluetongue.

The S3 gene of the BTV 13 prototype strain showed the closest genetic relationships to prototype BTV 10, VAC 10, and the field isolates 10O80V, 10O80Z, and 13B89Z. The mean genetic distance between BTV 13 and these viruses was 0.58. Comparison of the prototype strain of BTV 13 with the rest of the viruses indicates that this virus, which was isolated in Idaho in 1967, is most related to VAC 10, as they present the shortest branch length. This result was corroborated by several methods, such as the Fitch-Margoliash method, neighbor joining, and the unweighted pair-group method with arithmetic mean, and by using different algorithms to calculate the corresponding genetic distances (13). The close genetic relationship between the S3 genes of these viruses was unexpected. Phylogenetic analyses of the L2 gene of serotypes of BTV from different parts of the world showed that U.S. BTV 13 did not segregate within the North America nucleotype. It was placed in a separate nucleotype together with serotypes 3 and 16, and it was considered an introduction from another geographical region (19). Similarly, BTV 2 in the United States was most likely an introduction (5). Unlike BTV 2, which showed a very restricted activity in this country, BTV 13 became endemic (33). Our data strongly suggest that U.S. BTV 13 became endemic in North America after its reassortment with existing endemic viruses. The acquisition by the newly introduced virus of genome segments from an endemic strain through reassortment likely gave this new variant a replicative advantage that facilitated its establishment and distribution. The close genetic distance and the similarities between the VP6 proteins of the U.S. prototype strain of BTV 13 and those of VAC 10, such as their sharing of a unique methionine at amino acid position 5, strongly suggest reassortment between these two viruses. VAC 10 is the vaccine currently used to vaccinate sheep in California, and it was derived from the first BTV 10 modified live virus vaccine. VAC 10 has been used extensively in the western United States since the initial BTV isolation in California (30, 45). It was shown that the original vaccine was transmitted from vaccinated sheep to unvaccinated animals by the vector *Culicoides variipennis*, causing disease in exposed sheep and new foci of infection (15). Opportunities for the reassortment of the original VAC 10 and BTV 13 strains are clearly evident, as occurs with other segmented RNA viruses (28).

Group B includes viruses obtained in other regions of the western United States over a period of 35 years. Field isolates of BTV 10 and 13 obtained in 1990 and 1989, respectively, and 11UC2, isolated in 1981, clustered with prototype strains of BTV 11 and BTV 17 obtained from Texas and Wyoming, respectively, in 1955 and 1967. The field isolate 11C81Z was more distantly related, showing average genetic distances of 3.4 and 4.1 with groups A and B, respectively. It associated only weakly with the other members of group A, and this association was not stable.

Despite the conserved nature of the BTV S3 gene, only two isolates in each monophyletic group presented identical genes. Those in group A were 13B81V and 13B81X, isolated from the same ranch, and those in group B were 10B90Z and 10O90Z, obtained in different counties.

Phylogenetic trees obtained with the variable L2 gene and the conserved S3 gene of the same field isolates of serotypes 10 and 17 and the U.S. BTV prototypes presented different topologies (data not shown). We have previously reported that the L2 genes of isolates of BTV 10 and 17 segregate into two distinct monophyletic groups reflecting their serotypes (8, 11). In contrast, the S3 genes segregate into groups defined by consensus sequences and include viruses of different serotypes in the same monophyletic group. The analyses of the topology of this tree also support the concept of reassortment between BTV 13 and the original vaccine. These results clearly show that the L2 and S3 genes of BTV reassort in nature and that they evolve independently. Similar results have been previously observed in the genus *Reovirus* (4). The phylogenetic analyses of the nucleotide sequence of *Reovirus* gene S2 from different serotypes showed that there was no correlation between the sequence diversity of this gene and the viral serotype (4).

The analyses of the genetic characteristics of different members of the BTV gene S3 population circulating in the western United States show that this population is formed by variants that cannot be clearly defined on the basis of serotype, but they can be grouped according to different consensus sequences that cocirculate in nature.

Reassortment likely is responsible for the lack of any direct relationship between S3 gene sequence and virus serotype. Reassortment of BTV gene segments occurs in nature and undoubtedly is a driving force leading to diversification and adaptation of BTV, as also occurs with other arboviruses (2).

Reassortment events that increase the frequency of a particular BTV S3 gene over another have been experimentally demonstrated during mixed infection of cattle (40). In animals simultaneously inoculated with BTV 11 and BTV 17, 89% of the viral progeny were reassortants and all of them contained genome segment S3 from the parental BTV 11 strain. This study also showed the preferential replication of BTV reassortants over both parental viruses. This mechanism would allow the rapid distribution of a particular variant within the virus population that confers on each of its members a replicative advantage ensuring progeny that are best adapted to the prevalent conditions.

Our data show that viruses belonging to different monophyletic groups, such as the pair 11UC2 and 11UC8 and the pair 11C81Z and 11O81Z, were isolated from the same ranch and during the same month. The cocirculation of different lineages of the same gene has been previously described for influenza B and C viruses (3, 49). This phenomenon, in BTV, might reflect selective pressures imposed by the vertebrate or invertebrate hosts and underscores the complexity of BTV epidemiology.

The epidemiology of BTV infection is strongly influenced by agricultural practices and trade. The active movement of animals throughout the western United States facilitates the sudden introduction of new viruses into a particular region. These events have been documented in California at least in two instances (39). Such introductions of novel viruses increase the variability of the BTV gene pool in an area and facilitate modification of the local virus population through reassortment as it occurs in other segmented viruses (47).

The vector also contributes to the genetic diversity of BTV. The competence of *Culicoides* spp., as well as their ability to transmit BTV, is under the control of diverse genetic factors. Individual insects within a vector species can differ in their competence (42). The principal vector of BTV in the United States, *Culicoides variipennis* var. *sonorensis*, does not form a genetically homogeneous population even in a restricted geographical region. Genetic differences between *Culicoides* populations from temporary and permanent breeding sites, only a few hundred meters apart, have been demonstrated (42). The mixed populations of vertebrate hosts and insect vectors that exist in a given area could create different ecological niches that favor the maintenance of different variants of a consensus sequence of BTV genes.

It has been proposed that the evolution of RNA viruses in a constant adaptive environment follows a landscape model in which numerous fitness peaks, representing dominating master sequences, are joined by adaptive valleys. Genetic plasticity and variability allow a virus to move from one peak through the valleys to the next peak (46, 48). As with other insect-borne viruses, BTV likely evolves according to this hypothetical model.

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