S10 segment sequence analysis of some Greek bluetongue virus strains

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Summary

Sequence analyses of the non-structural protein gene NS3/NS3A of eight Greek bluetongue (BT) virus (BTV) field isolates from the 1979 and 1999-2001 epizootics provide preliminary molecular data on the epidemiology of BT in Greece. These isolates from infected sheep belonged to serotypes BTV-1, BTV-4, BTV-9 and BTV-16. Phylogenetic analysis of the NS3/NS3A gene segregated these Greek isolates of BTV into two monophyletic groups. The first group was formed by all isolates of BTV-4; all were identical in their sequences, regardless of the area and year of isolation in Greece, and clustered with strains from Tunisia and Corsica. The isolates of BTV-1, BTV-9 and BTV-16 segregated into a second monophyletic group and clustered with Asian strains, showing a high homology (97-99%). From an epidemiological point of view, these preliminary results infer that one group of isolates is Mediterranean, whilst the second appears to be of Asian origin.

Keywords

Bluetongue – Epidemiology – Greece – Non-structural protein gene – Phylogenetic analysis – Topotypes – Virus.

Introduction

Bluetongue (BT) virus (BT) is the causative agent of BT, an arthropod-transmitted disease of wild and domestic ruminants. BTV is the prototype virus of the genus *Orbivirus* in the family *Reoviridae*. There are at least 24 serotypes known worldwide (5). Isolates belonging to four serotypes (BTV-1, BTV-4, BTV-9 and BTV-16) have been identified to occur in Greece thus far (authors, unpublished data).

The BTV double-stranded RNA genome consists of 10 unequal segments that are encapsulated by a double-layered icosahedral shell, and encodes 7 structural (VP1-VP7) and 4 non-structural (NS1, NS2, NS3/NS3A) proteins (11). The smallest genome segment, S10, encodes the proteins NS3/NS3A of 229/216 amino acid lengths. These two proteins mediate the release of BTV particles from infected cells (7). Therefore, they may play an important role in the transmission of the virus and its subsequent dissemination, thus playing a major role in the epidemiology of BT.

Different workers have published phylogenetic analyses of NS3/NS3A sequences of BTV. Comparison of strains from the United States of America (USA) and China showed that these segregated into different monophyletic clusters based on geographical location (1). Moreover, it was found that factors such as serotype, host species or year of isolation did not influence cluster formation (14).

BT occurs throughout the temperate and tropical regions of the world, in an area that parallels the distribution of the competent vector, *Culicoides* spp. (3). In Greece, BT occurred for the first time on the east Aegean island of Lesbos in the autumn of 1979 (8) and, following the application of a BT control programme, the island was declared officially free of the disease in 1991. New BT epizootics reappeared in the late 1990s (1998-1999) and during 2000-2001 not only on the East Aegean islands, but also in the north central, north-west, west and mainland Greece (authors, unpublished data). *Culicoides imicola* is considered to be the main vector species linked with BTV transmission in Greece, as is the case in most other European countries (10). The purpose of this

study was to investigate the epidemiology of BT in Greece, based on the phylogenetic analysis of the NS3/NS3A sequences of BTV isolates originating in different epizootics. Moreover, it was also considered of interest to study the extent of sequence variation in these Greek isolates and to compare them with those of other published strains (1, 14, 17) and on GenBank.

Materials and methods

Virus isolates

A total of eight BTV isolates belonging to four serotypes (BTV-1, BTV-4, BTV-9, BTV-16) were obtained from blood or spleen of naturally infected sheep, using the embryonated chicken egg (ECE) method and tissue culture techniques, and serotyped as described in the Office International des Épizooties (OIE) protocol (12). The isolates were stored at –70°C until further use. Details of all Greek isolates used in this study are shown in Table I. GenBank accession numbers of strains not otherwise published in the literature are provided in Fig. 1.

Table I
Details of the Greek field isolates used in this study

Virus	Serotype	Area	Year	Tissue	Species
GR79LS	BTV-4	Lesbos	1979	Spleen	Sheep
GR308/99RS	BTV-16	Rhodes	1999	Blood	Sheep
GR408/99ChS	BTV-9	Chalkidiki	1999	Blood	Sheep
GR395/99LS	BTV-4	Lesbos	1999	Spleen	Sheep
GR692/99EvS	BTV-4	Evia	1999	Spleen	Sheep
GR457/99PS	BTV-4	Pieria	1999	Spleen	Sheep
GR631/99MaS	BTV-4	Magnesia	1999	Blood	Sheep
GR15/01Gre	BTV-1	Grevena	2001	Spleen	Sheep

RNA extraction

Total RNA was extracted from BTV-infected cell cultures using the Trizol™ LS reagent (Invitrogen GmbH) in accordance with the instructions of the manufacturers. Each isolate was processed independently to minimise any sample-to-sample contamination. For each isolate, the amount of total RNA was measured in sterile RNAse free water, using the BioPhotometer (Eppendorf) and stored at –70°C.

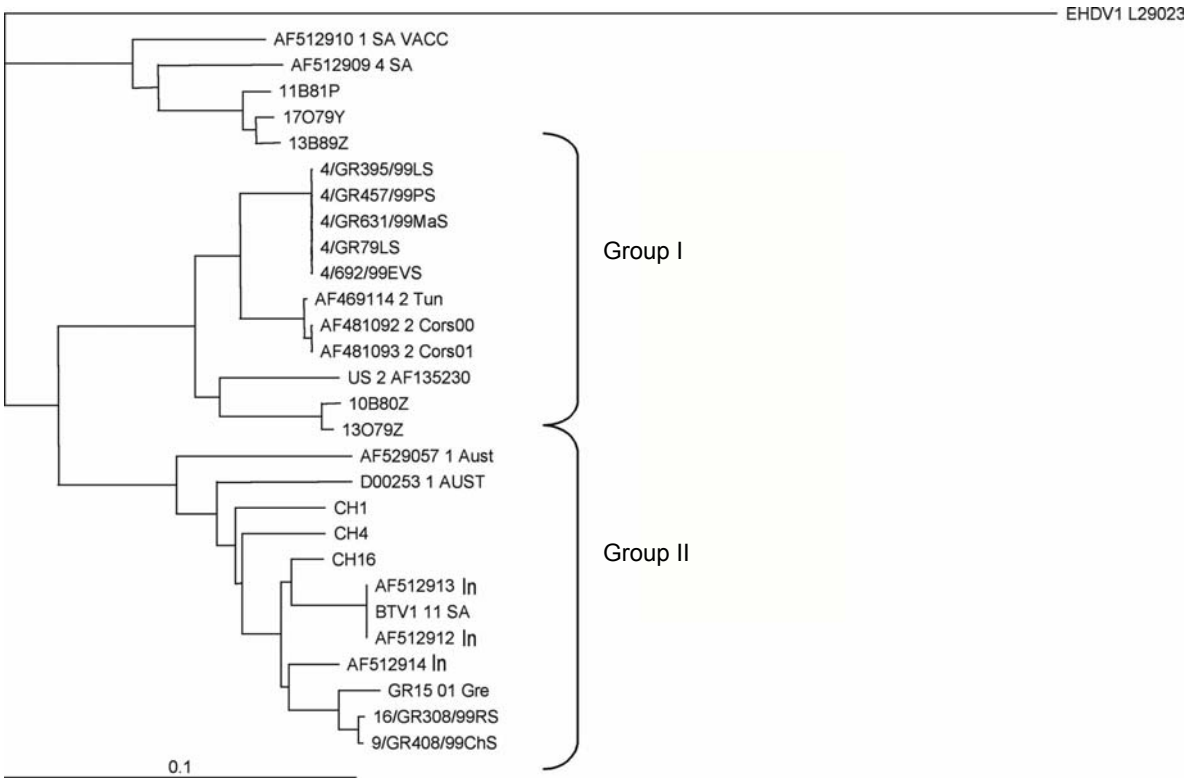

Figure 1
Phylogeny of the NS3/NS3A sequences of the Greek field isolates and other strains used for reference purposes in this study
The NS3 gene of EHDV-1 was included as the outgroup in the analysis
Branch lengths are indicative of the genetic distances between the sequences
Accession numbers not available from the bibliography (1, 14, 17) are provided in the Figure

Reverse transcription and amplification of the S10 segment

The primers were designed based on the sequences of the S10 segment published by Hwang *et al.* (6) and synthesised at MWG, Germany. The forward primer (F10P) identified nt 1-22 and the reverse primer (F10M) identified nt 798-822 on the S10 gene sequence. Both the reverse transcription and the PCR reactions were performed using the One Step RT-PCR kit (Qiagen GmbH, Germany) following the instructions of the manufacturer. Approximately 5 µg of total RNA was used for each reaction. The PCR product was run on a 1.7% agarose gel where the presence of a band (approximately above the 800 bp band on the 100 bp DNA ladder) indicated the amplified product.

Sequencing of the PCR product

The PCR amplification products were cleaned using the MinElute PCR Purification Kit (Qiagen GmbH) following which both strands were sequenced using the forward and reverse primers. The nucleic acids were analysed, aligned and a dendrogram created using the Clustal W Software program (16). The TreeView program of Page (13) was used to view and edit the dendrogram.

Results

Overall, among the Greek isolates, the NS3/NS3A sequence homologies ranged between 84-100%. The five isolates that belonged to serotype BTV-4 (GR79LS, GR395/99LS, GR692/99EvS, GR457/99PS and GR631/99MaS) presented 100% homology. The isolates that belonged to serotypes BTV-1, BTV-9 and BTV-16 (GR15/01Gre, GR408/99ChS and GR308/99RS, respectively), presented homologies of 97-99% in the NS3/NS3A sequences. The variation in the nucleotide sequences between the former and the latter group of isolates ranged between 15% and 16%.

Phylogenetic analysis separated these isolates into two main clusters (Fig. 1): one that consisted of isolates belonging to serotype BTV-4 and the other that included isolates belonging to serotypes BTV-1, BTV-9 and BTV-16 (referred to as Group I and Group II, respectively, for the purpose of this study). Group I isolates clustered closely with the Corsican and the Tunisian strains and, at some distance, with the Group A United States field isolates of Pierce *et al.* (14) and the United States prototype strain of serotype BTV-2. On the other hand, Group II isolates clustered with the Asian strains (Indian, Chinese and Australian). The South African strain of BTV-1 used in this analysis also fell into this cluster.

Discussion

The objective of this study was to examine the sequence variation of the NS3/NS3A gene of Greek isolates and to investigate the epidemiology of BT in Greece during the epizootics of 1979 and 1999-2001 by molecular and phylogenetic analyses of this gene.

The NS3/NS3A gene is considered to be one of the conserved genome segments of BTV as the proteins encoded by this gene tolerate little variation (14). However, variation of up to 20% in nucleic acid sequence and up to 10% in amino acid sequence has been reported in certain cases (6, 14, 17). In this study, variation of 15% to 16% was observed among the Greek isolates and therefore is in agreement with the above reports.

The results of the present work divided the eight Greek BTV field isolates into two distinct groups on the basis of their NS3/NS3A gene sequences. All isolates of serotype BTV-4, regardless of the year and the area of isolation, were included in Group I and were identical in their sequences. Group II consisted of isolates of three different serotypes (BTV-1, BTV-9 and BTV-16) with homologies in the nucleic acid sequences of 97-99%. Our finding regarding 100% identity in the NS3/NS3A sequences among the Greek BTV-4 isolates from the 1979 and 1999 epizootics indicate that this serotype of BTV must have existed in the neighbouring areas during the past 20 years, without significant mutations so confirming the resistance of this gene to selective environmental pressure. It is noteworthy that two of these isolates, GR79LS and GR395/99LS, came from the same island (Lesbos), with a gap of 20 years, even though an eradication programme was implemented following upon the 1979 epizootic and, after extensive serological surveillance, Greece was declared free of the disease in 1991.

Phylogenetically, the isolates of Group I clustered closely with the serotype BTV-2 strains from Tunisia and Corsica and more distantly to the United States Group A strains of Pierce *et al.* (14) that included serotypes BTV-2, BTV-10 and BTV-13. This suggests that in spite of different origins and serotypes, all viruses in this cluster might have a common ancestor as has also been reported by others (17). On the other hand, the isolates of Group II were closely related to the Asian group, thereby indicating their origin from the countries of the East. Interestingly, the Greek BTV-4 isolates did not fall into the same cluster as the Chinese BTV-4 strain. This would mean that, despite being the same serotype, these two strains have different origins with regard to the NS3/NS3A gene.

Sequence analysis of a conserved gene, such as NS3/NS3A, may be used to assign a BTV isolate as a 'topotype' in a given geographic region, regardless of its serotype (4). Accordingly, it may be said that the isolates of Group I and Group II form two different BTV 'topotypes'.

Previous studies have shown that the BTV populations that circulated in a particular geographic region form one or two monophyletic groups (1, 14, 17). This is likely to be the consequence of the co-evolution of the virus populations and the specific insect vector that occurs in each particular region (3). In this study, the two distinct monophyletic groups that were identified could be related to the occurrence of different *Culicoides* vector species in Greece. As in other Mediterranean countries, *C. imicola* is the common BTV vector in Greece and it has been identified on the islands of Lesbos, Rhodes and Chios and on mainland Greece (2, 18). *Culicoides obsoletus* (*C. obsoletus*) is also suspected to be a potential vector of BTV transmission in different European countries (9, 18). Moreover, it has recently been reported that a member of the *C. obsoletus* species complex is co-involved with *C. imicola* in the transmission of BTV in Italy but that in Bulgaria and the neighbouring territories of the Former Yugoslav Republic of Macedonia, Serbia, Montenegro and Croatia, only *C. obsoletus* is involved (15). Therefore, it is possible that the presence of these two species of *Culicoides* has contributed to the formation of the two different monophyletic groups of BTV in Greece.

In conclusion, based on the NS3/NS3A sequence analysis, the various populations of the four serotypes of BTV identified in Greece during the epizootics of 1979, 1999 and 2001 are separable into two monophyletic groups. From an epidemiological aspect, it may be inferred that one group is related to other Mediterranean strains and that the other is of Asian origin.

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