VP2 gene sequence analysis of some isolates of bluetongue virus recovered in the Mediterranean Basin during the 1998-2002 outbreak

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Summary

Since 1998, five serotypes of bluetongue virus (BTV), BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16, have been reported in countries surrounding the Mediterranean Basin. Preliminary data on the sequencing analysis of the VP2-genes of BTV isolates recovered during the 1998-2002 epizootic of BT in Italy, Greece and Israel were studied. The VP2-genes of the Italian BTV-2 and BTV-9, Greek BTV-4 and BTV-9, Israeli BTV-4 and BTV-16 and South African BTV-2, BTV-4, BTV-9 and BTV-16, together with those of their corresponding South African serotype reference and vaccine strains, were cloned and the sequences of their terminal ends determined. These sequences, as well as those of all BTV VP2-gene sequences currently available on GenBank, were used to compile a phylogenetic tree to determine the probable geographic origins of the BTV incursions into Europe. The Italian isolates included in this study were from different regions, animal hosts and years (2000-2002). The results demonstrated that sequencing of the terminal end of the VP2-gene of BTV can be used for topotyping. According to the phylogenetic analysis, the Italian BTV-2 and BTV-9 isolates were stable across all species, irrespective of geographic origin and year of isolation. The sequencing data of the Italian isolates were identical to those of a BTV-2 isolate from Corsica. There was 97% homology between the Italian and Corsican BTV-2 isolates and the BTV-2 vaccine and reference isolates from South Africa. Italian BTV-9 isolates were also identical to the Greek BTV-9 isolates (99% homology). Surprisingly these BTV-9 isolates had only 67% homology with the reference BTV-9 isolate from South Africa. Conversely, BTV-9 field isolates from Australia and elsewhere in Europe had 89% homology with the Italian isolate at the nucleic acid level. Greek and Israeli BTV-4 isolates were almost identical (98% homology) and shared a 90% homology with the BTV-4 South African reference and vaccine strains. Israeli BTV-16 and South African BTV-16 reference strains were also similar. From these results, it may be concluded that Italian and Corsican BTV-2, Israeli and Greek BTV-4, and South African and Israeli BTV-16 had a common origin. The Greek BTV-9 isolate had more than 99% homology with the isolates from Italy, indicating these isolates to have had a common origin. The European BTV-9 isolates, grouped as 'eastern isolates', were more similar to the Australian isolates than to the South African reference strains.

Keywords

Bluetongue – Gene sequence – Mediterranean – Phylogenetic analysis – Serotype – Viral protein– Virus.

Introduction

The Mediterranean Basin is currently experiencing a severe and long-lasting outbreak of bluetongue (BT) disease in sheep. Since 1998, BT has been reported in many European countries. In Italy, the first evidence of BT infection was reported in Sardinia in August 2000 and since then numerous outbreaks

have been described, including the regions of Sicily, Calabria, Basilicata, Puglia, Campania, Lazio, Tuscany, Abruzzo and Molise (2, 3). Five serotypes of the bluetongue virus (BTV): (BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16) have been reported from many of the countries surrounding the Mediterranean Basin (2, 3, 4). Several hypotheses have been formulated regarding the possible origin of these incursions into Europe, but little data are available. It is crucial to know more about the possible origin of the various BTV serotypes recovered in these countries in order to better understand the epidemiology of the infection for the implementation of more effective control measures in the future. This study provides preliminary data on the sequencing analysis of the VP2 genes of BTV isolates recovered during the 1998-2002 epizootic of BT in Italy, Greece and Israel (1, 6, 7).

Materials and methods

Virus isolates

A phylogenetic tree was compiled using the BTV VP2 gene sequences of the Italian, Greek, Israeli, South African BTV-2, BTV-4, BTV-9 and BTV-16 reference and field isolates; also included were all BTV VP2 gene sequences currently available on GenBank. Italian isolates were from different regions, animal species (cattle, goats, sheep and deer) and years (2000-2002). Table I lists the various isolates sequenced.

dsRNA extraction

The isolates of BTV were propagated in a 75 cm^2 flask containing a monolayer of BHK-21 cells. From each isolate, the total RNA was extracted using Tri-Reagent (MRC), following the protocol of the manufacturer. The ssRNA was then removed from the total RNA by precipitation with 2M LiCl. The dsRNA was purified from the supernatant using a Gel extraction Kit (Qiagen).

cDNA cloning

The cDNA of the BTV-2 (Italian), BTV-4 (vaccine) and BTV-9 (Italian and Greek) isolates were amplified by one step reverse transcriptase-polymerase chain reaction (RT-PCR) using primers based on the sequences of previous BTV-2 and nine Italian isolates and the BTV-4 reference isolate, respectively (Table II). For isolates that could not be amplified using these primers (isolates 8, 3C, 35C and 38C) their whole genome was amplified using the sequence independent dsRNA cloning method (5). The resultant VP2 gene amplicons were separated from other amplicons by gel electrophoresis, and purified using a gel extraction kit (QIAGEN). The purified full-length VP2 gene PCR products were cloned into pGEM-T easy (Promega), according to the specifications of the manufacturer. Positive clones were selected based on insert size and restriction analysis.

Table I
Field and vaccine strains of bluetongue virus used for VP2 gene sequencing

No.	NRV	Serotype	Year	Region	Province	Animal host
Italian isolates						
1	1155	2	2002	Sicily	ME	Goat
2	1156	2	2002	Sicily	ME	Sheep
3	1157	2	2002	Sicily	ME	Cattle
4	1322	9	2001	Calabria	CZ	Sheep
6	1617	2	2000	Sardinia	OR	Sheep
7	1853	9	2001	Basilicata	PZ	Sheep
8	1931	9	2001	Vaccine	RC	Sheep
9	2057	2	2000	Sicily	PA	Sheep
10	2060	2	2000	Calabria	RC	Sheep
11	2808	2	2001	Sardinia	CA	Sheep
12	2849	2	2001	Lazio	LT	Sheep
13	3559	2	2001	Sardinia	NU	Deer
14	3565	2	2001	Sicily	ME	Sheep
15	3820	2	2001	Calabria	NU	Sheep
16	3987	2	2001	Tuscany	GR	Sheep
17	4025	2	2001	Lazio	RM	Sheep
18	4031	2	2001	Lazio	VT	Sheep
19	5401	2	2001	Tuscany	SA	Cattle
20	8340	2	2000	Sardinia	CA	Sheep
Israeli isolates						
3C		16	1999	Beit Dagan		Sheep
35C		4	2001	Nahalal		Sheep
38C		4	2001	Hatzav		Sheep
Greek isolates						
BTV-4		4	2001			Sheep
BTV-9		9	1999			Sheep
South African isolates						
BTV-2, BTV-4, BTV-9, BTV-16 reference and vaccine strains						
5	1373	4	2002	Vaccine		Cattle
8	1931	9	2001	Vaccine		Sheep

NRV identification number for the IZSA&M Virology Department

ME	Messina	CZ	Catanzaro
OR	Oristano	PZ	Potenza
RC	Reggio Calabria	PA	Palermo
LT	Latina	NU	Nuoro
GR	Grosseto	RM	Roma
VT	Viterbo	SA	Salerno
CA	Cagliari	BTV	bluetongue virus

Table II
Primers used for the reverse transcriptase-polymerase chain reaction of full-length bluetongue virus VP2

BTV serotype	Forward primer sequence	Reverse primer sequence
BTV-2 (South Africa)	5′-GTTAAAACAGGATCGCGATGGATGAGC-3′	5′-GTAAGTTGAACAGATCGCGGACCTGC-3′
BTV-2 (Italy)	5′-GTTAAAACAGGATCGCGATGGATGAGC-3′	5′-GTAAGTTGAACAGATCGCGGACCTGC-3′
BTV-4 (South Africa)	5′-GTTAAAAAGAGTGTTCCACAATGG-3′	5′GTAAGTGTAAGAGGCCACAGGTCCG-3′
BTV-9 (South Africa)	5′-GTTAAAAGCTTTTCAGGATGGACG-3′	5′-GTAAGTGTAAGCTTCAGGAGTCCC-3′
BTV-9 (Italy)	5′-GTTAAAAGTTATCTAGGATGG-3′	5′-GTAAGTGTAAGCTCTAGGAGTCCC-3′

Sequencing and sequence analysis

Purified PCR-products were either sequenced directly using the forward type-specific primer for the corresponding serotype or each of the VP2 gene-clones were sequenced using M13 forward and reverse primers (Promega). Sequencing reactions were analysed with an Abi Prism® 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

Resulting sequences were subjected to BLAST analysis (NCBI). Phylogenetic analysis was performed using Dnaman (Lynnon Biosoft).

Results

Amplification and cloning of BTV VP2 cDNA

The full-length VP2 gene amplicons of all of the BTV-2, BTV-4 and BTV-9 isolates could be amplified in a one-step RT-PCR using primers based on sequences from previously cloned and sequenced BTV-2 and nine Italian isolates and the sequence from the reference strain of BTV-4 (Fig. 1). Only isolate 8 (BTV-9) could not be amplified using the primers against the Italian BTV-9. The whole

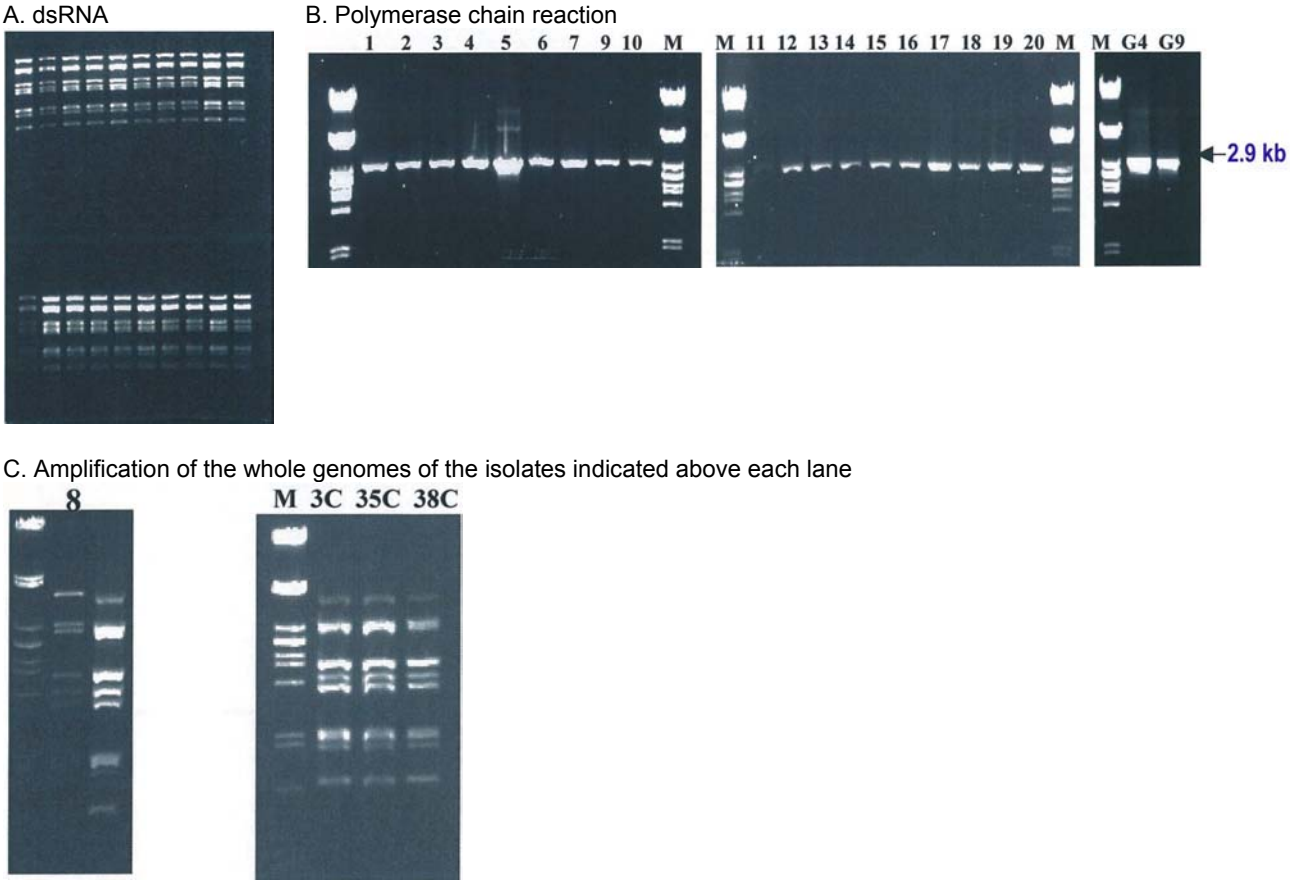

Figure 1
Agarose gel analysis of the dsRNA and polymerase chain reaction products of the Italian isolates

genome of this isolate and other isolates from Israel (3C, 35C and 38C) could be amplified using the method of Potgieter *et al.* (5) (Fig. 1). All the full-length BTV VP2 genes were cloned in the plasmid pGEM-T easy (Promega).

Sequencing and phylogenetic analysis

Approximately 500 bp of the terminal ends of each of the VP2-genes of all the BTV isolates were sequenced. An analysis of each of these nucleotide sequences shows that all the cloned VP2 cDNAs were full-length copies of the VP2-genes of each isolate. Each gene contained the 5′GTT and TAC-3′ sequences. A BLAST (NCBI) analysis also confirmed the serotype of each of the VP2-genes. Phylogenetic analysis demonstrated that all the Italian BTV-2 isolates grouped together and no significant sequence changes were apparent. They were also identical to those of a BTV-2 isolate from Corsica. There was 96% homology between the European BTV-2 isolates and the BTV-2 South African reference and vaccine strains. The analysis of the Italian BTV-9 isolates showed clearly that these were almost identical to the BTV-9 isolates from 2001. More significant however was the fact that a BTV-9 isolate from Greece showed a very high homology (99%) with all the Italian BTV-9 isolates. Australian and European BTV-9 field isolates have 89% homology at the nucleic acid level but the two groups of isolates have only a 67% homology with the reference BTV-9 isolate from South Africa. Greek and Israeli BTV-4 were almost identical and grouped together with the South African BTV-4 isolates (90% homology). The BTV-16 isolate from Israel also grouped with the South African reference strain showing identical homology for the first 500 bp of the terminal end (Figs 2 and 3).

Discussion and conclusions

Results of this study demonstrated that the sequencing data analysis of terminal ends of VP2 genes of BTV can be used for topotyping (8). During the two years of the Italian outbreak, the isolates were stable despite originating from different animal hosts and from different regions; this would indicate that the outbreaks of BTV-2 in Italy, together with the Corsican outbreak, may have had a common source (Fig. 4).

The fact that the BTV-9 isolate from Greece has an homology of more than 99% with the isolates from Italy indicates that the outbreaks of this serotype in Italy may have originated in Greece. In addition, the recent isolates from Italy were almost identical to the isolates from the previous year, indicating their subsequent spread from a common source. All the

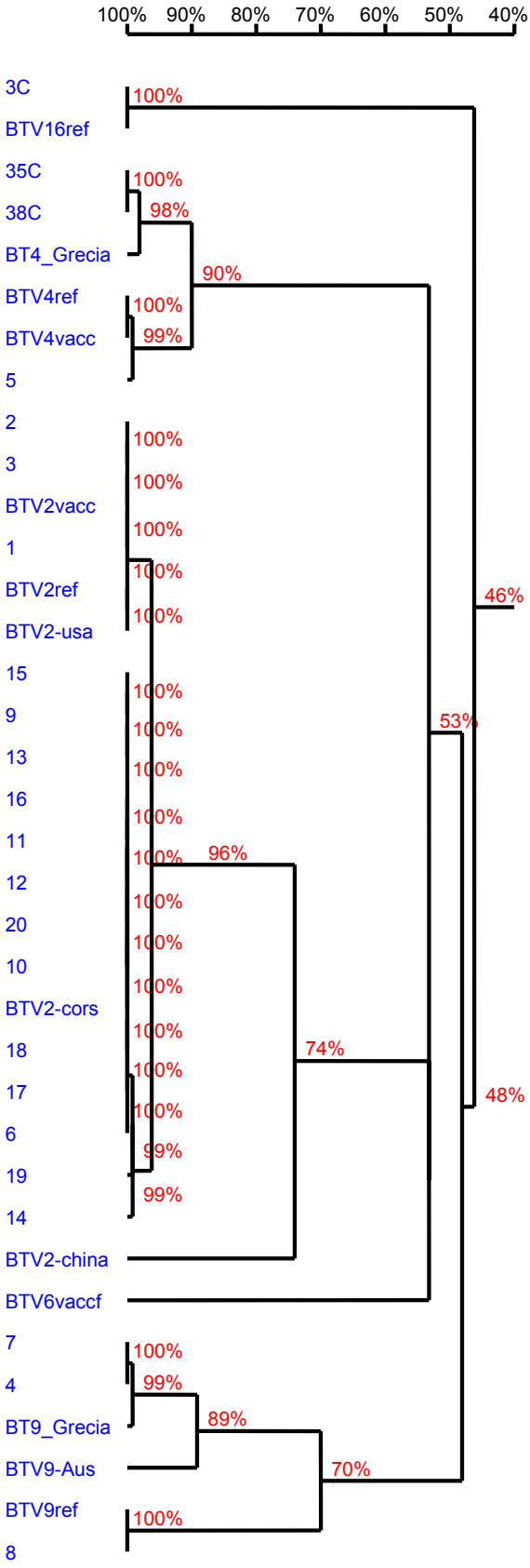

Figure 2
Homology tree based on bluetongue virus serotypes 2, 4, 9 and 16 VP2 gene sequences of Greek, Israeli, Italian and South African isolates compared to the Corsican, Australian, USA and Chinese BTV isolates of corresponding serotypes

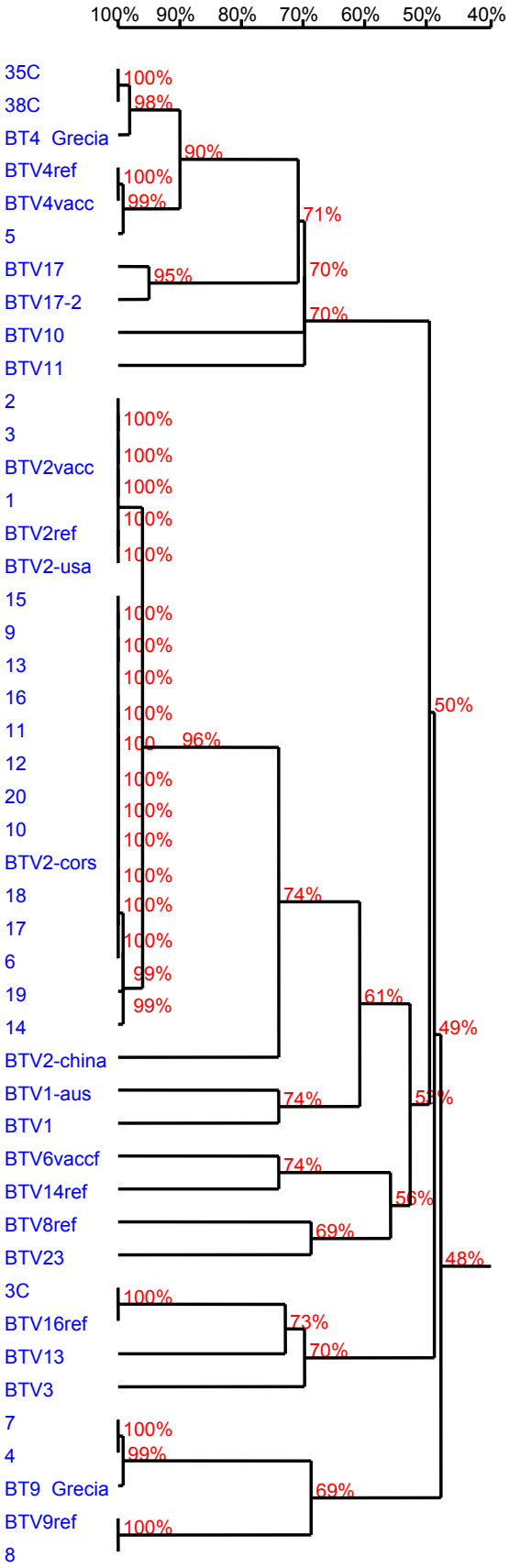

Figure 3
Homology tree based on bluetongue virus VP2 gene sequences of Greek, Israeli, Italian and South African isolates including sequences currently available on GenBank

Figure 4
Hypothetical geographic origin of two bluetongue virus incursions into Italy, based on analysis of the BTV VP2 gene

BTV-9 isolates are together referred to as 'eastern isolates' since they were more similar to the Australian isolates than to the South African reference strain.

The BTV-4 isolates from Greece and Israel were very similar with a 98% homology, demonstrating a close relationship. These isolates, as a group, had only a 90% homology with the South African reference and vaccine strains, indicating that outbreaks of these 'western isolates' cannot be linked to the South African vaccine strain.

However, the Israeli BTV-16 isolate had a 100% homology with the South African reference and vaccine strains showing it to be definitely a 'western isolate'. Whether this isolate can be linked to vaccination cannot be excluded but will only be confirmed if studies on other BTV gene segments are performed, such as on S8 and S10, which encode for NS2 and NS3, respectively.

It is recommended that more comprehensive sequencing studies of field isolates of BTV circulating in countries bordering the Mediterranean Basin be conducted to determine virus movement(s) and source(s) of BT epizootics in Italy. Not only should the VP2 genes be fully sequenced but studies on other BTV gene segments encoding for VP3, VP5, NS2 and NS3 should be conducted to obtain a more complete epidemiological 'picture'.

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