Bluetongue virus serotype 24 (BTV-24) in Israel: phylogenetic characterization and clinical manifestation of the disease

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Keywords
Bluetongue virus, Israel, Phylogenetic characterization, Serotype 24.

Summary
Bluetongue (BT), an arthropod-borne viral disease of ruminants, affects sheep most severely than other domestic animals. Bluetongue virus serotype 24 (BTV-24) is one of 26 known Bluetongue virus (BTV) serotypes. In this article, we present data of phylogenetic analysis of 9 viral genes (Seg1, Seg2, Seg3, Seg4, Seg5, Seg6, Seg8, Seg9, and Seg10) from 8 Israeli BTV-24 isolates and relate the genotype of the BTV-24 isolates to their phenotype with regard to clinical manifestations. The high level of genetic identity (> 99.6%) between Seg2, Seg4 and Seg5 in all 8 BTV-24 isolates indicated that these segments shared the same viral ancestor. Phylogenetic analysis of Seg1, Seg3, Seg5, Seg8, Seg9, and Seg10 revealed that the Israeli BTV-24 strains comprised 4 variants. Five of the viruses revealed high identity among all 9 segments, and represented variant 1. A second variant (BTV24/3027/6/10), isolated in 2010, showed significant variation from variant 1 in 3 gene segments (VP-1, VP-3, and NS-3 genes). A third variant (BTV24/3027/1/10) showed significant variation from variant 1 in 6 segments (VP-1, VP-3, VP-6 and NS-1, NS-2 and NS-3 genes), while a fourth variant (BTV24/2214/1/10) showed significant variation from variant 1 in 4 segments (VP-1, NS-1, NS-2 and NS-3 genes). These marked differences in sequence identity indicate that a high level of genetic reassortment is occurring between co-circulating BTV strains in Israel.

Caratteristiche genetiche e manifestazioni cliniche del sierotipo 24 del virus della Bluetongue (BTV-24) in Israele

Parole chiave
Caratterizzazione filogenetica, Israele, Sierotipo 24, Virus della Bluetongue.

Riassunto
La Bluetongue (BT) è una malattia virale dei ruminanti, trasmessa da artropodi, che colpisce gli ovini in maniera più grave rispetto ad altri animali domestici. Il sierotipo 24 del virus della Bluetongue (BTV-24) è uno dei 26 sierotipi riconosciuti del virus BTV. In questo articolo si analizzano i dati filogenetici di 9 segmenti del genoma virale (Seg1, Seg2, Seg3, Seg4, Seg5, Seg6, Seg8, Seg9 e Seg10) di 8 isolati di BTV-24 provenienti da Israele. L'analisi ha permesso anche di mettere in relazione il genotipo degli isolati BTV-24 con le manifestazioni cliniche ad esso correlate. L'alto livello di identità genetica (> 99,6%) dei Seg2, Seg4 e Seg5 in tutti gli 8 isolati BTV-24 ha dimostrato che questi segmenti hanno probabilmente un'origine comune. Le analisi filogenetiche dei Seg1, Seg3, Seg5, Seg8, Seg9 e Seg10 hanno mostrato che i ceppi di BTV-24 israeliani possono essere raggruppati in 4 varianti. La variante 1 include 5 dei ceppi che hanno presentato un'alta identità genetica nei 9 segmenti considerati. Una seconda variante (BTV24/3027/6/10), identificata nel 2010, ha mostrato una variazione significativa rispetto alla variante numero 1 in 3 dei segmenti genici analizzati (i segmenti che codificano la VP-1, la VP-3 e la NS-3). Una terza variante (BTV24/3027/1/10) ha mostrato un cambiamento significativo rispetto alla variante numero 1 in 6 segmenti genici (i segmenti che codificano: VP-1, VP-3, VP-6 e NS-1, NS-2 e NS-3). Una quarta variante (BTV24/2214/1/10) ha mostrato un cambiamento significativo dalla

variante 1 in 4 segmenti genici (i segmenti che codificano: VP-1, NS-1, NS-2 e NS-3). Queste differenze nella sequenza genica indicano il verificarsi di un alto livello di riassortimento genetico tra diversi ceppi di BTV circolanti in Israele.

Introduction

Bluetongue virus (BTV) belongs to the genus *Orbivirus* within the family *Reoviridae* (Attoui *et al.* 2009). The virus is transmitted by haematophagous arthropod vectors and causes disease ranging from inconspicuous to fatal, in sheep, cattle, goats, deer, and wild ruminants. The BTV occurs in almost all continents where haematophagous arthropod vectors (*Culicoides* biting midges) are present (MacLachlan *et al.* 2009).

The genome of BTV consists of 10 double-stranded RNA (ds-RNA) segments, coding 7 structural (VP1-VP7) and 4 non-structural (NS1-NS4) proteins (Attoui *et al.* 2009, Belhouchet *et al.* 2011). In accordance with the antigenic and genetic structure of the VP2 protein, all BTVs are currently subdivided into 26 serotypes (Hofmann *et al.* 2008, Maan *et al.* 2012).

In Israel, Bluetongue (BT) was first observed in 1944. From 1964 to 2004, 5 serotypes (BTV-2, BTV-4, BTV-6, BTV-10 and BTV-16) were found to be circulating (Shimshony 2004), and from 2006 up to date, 8 serotypes (BTV-2, BTV-4, BTV-5, BTV-8, BTV-12, BTV-15, BTV-16, and BTV-24) were identified (Brenner *et al.* 2010, Brenner *et al.* 2011, Bumbarov *et al.* 2012).

Clinical signs attributed to BT include fever, anorexia, dysphagia, ulcerative and necrotic lesions of the oral mucosa, hyperaemia and oedema of the conjunctival mucosa, sore muzzle, hyperaemia of the teats and udder, haemorrhage, dehydration and lameness (Darpel *et al.* 2009, Elbers *et al.* 2008 a, b, c, Elbers *et al.* 2009, Eschbaumer *et al.* 2010, Pardon *et al.* 2010).

The aim of the present study was to characterize, through genetic sequencing and phylogenetic analysis, selected Israeli BTV-24 isolates from outbreaks occurred between 2008 and 2010, in order to assess the levels of reassortment and to investigate whether there are links between disease manifestations and the genetic properties of these viruses.

Materials and methods

Virus isolation and serotyping

Eight BTV-24 isolates, comprising 7 isolates from sheep and 1 from cattle, were selected from the virus collection at the Kimron Veterinary Institute for further characterisation (Table I). The methods used for virus isolation in embryonated chicken eggs and in tissue culture have been described previously (Brenner *et al.* 2010, Komarov and Haig 1952). We extracted RNA from the tissue culture supernatant and chicken embryo homogenates by means of the Invisorb Spin Virus RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany), QIAmp Viral RNA Mini Kit and RNeasy Mini Kit (Qiagen, Hilden, Germany). Virus detection was performed by conventional real time polymerase

Table I. *Clinical manifestations and genetic variations of Bluetongue virus serotype 24 (BTV24) isolated in Israeli flocks.*

Virus	Collection date	Host	Main clinical manifestations in flocks from which BTV was isolated	Phylogenetic groups									Genetic variant
				VP1	VP2	VP3	VP4	VP5	VP6	NS1	NS2	NS3	
BTV24/2305/08	Nov-08	Ovine	Classical BT symptoms	1	1	1	1	1	1	1	1	1	1
BTV24/2425/08	Nov-08	Ovine	Classical BT symptoms	1	1	1	1	1	1	1	1	1	1
BTV24/2755/1/10	Aug-10	Ovine	Red udder syndrome, agalactia	1	1	1	1	1	1	1	1	1	1
BTV24/2944/1/10	Nov-10	Ovine	No specific symptoms were reported besides abortions	1	1	1	1	1	1	1	1	1	1
BTV24/3258/1/10	Dec-10	Cattle	Abortion and bloody diarrhea	1	1	1	1	1	1	1	1	1	1
BTV24/3027/6/10	Nov-10	Ovine	Central nervous system symptoms Border disease- like	2	1	3	1	1	1	1	1	2	2
BTV24/3027/1/10	Nov-10	Ovine	Central nervous system symptoms Border disease- like	2	1	2	1	1	2	2	2	2	3
BTV24/2214/1/10	Sep-10	Ovine	Central nervous system symptoms Border disease- like	3	1	1	1	1	1	2	3	2	4

Table II. *GenBank accession numbers and genome fragment lengths of Israeli Bluetongue virus serotype 24 (BTV24).*

Designation	NS1 gene (bp)	NS2 gene (bp)	NS3 gene (bp)	VP1 gene (bp)	VP2 gene (bp)	VP3 gene (bp)	VP4 gene (bp)	VP5 gene (bp)	VP6 gene (bp)
BTV24/2214/1/10	JX889171 (786)	JX889172 (907)	JX889173 (781)	JX889174 (1929)	JN162682 (2816)	KM365243 (937)	JX889175 (903)	JX889176 (826)	JQ970464 (918)
BTV24/2755/1/10	JX889189 (786)	JX889190 (907)	JX889191 (729)	JX889192 (982)	JN162685 (2816)	KM365240 (933)	JX889193 (1918)	JX889194 (826)	
BTV24/2305/08	JX889177 (786)	JX889178 (907)	JX889179 (781)	JX889180 (1929)	JN162683 (2846)	KM365242 (877)	JX889181 (889)	JX889182 (826)	JQ970465 (918)
BTV24/2425/08	JX889183 (786)	JX889184 (907)	JX889185 (781)	JX889186 (1929)	JN162684 (2846)	KM365241 (927)	JX889187 (907)	JX889188 (826)	JQ970466 (918)
BTV24/3258/1/10	JX889212 (786)	JX889213 (907)	JX889214 (781)	JX889215 (1929)	JN162686 (2846)	KM365236 (935)	JX889216 (893)	JX889217 (826)	JQ970441 (918)
BTV24/3027/1/10	JX889201 (786)	JX889202 (907)	JX889203 (781)	JN546206 (1933)	JN546198 (331)	KM365238 (933)	JX889204 (900)	JX889205 (826)	JQ970468 (918)
BTV24/3027/6/10	JX889206 (786)	JX889207 (907)	JX889208 (781)	JX889209 (822)	JN546200 (332)	KM365237 (927)	JX889210 (903)	JX889211 (826)	JQ970469 (918)
BTV24/2944/1/10	JX889196 (786)	JX889197 (907)	JX889198 (781)	JX889218 (823)	JX889219 (942)	KM365239 (920)	JX889199 (904)	JX889200 (826)	JQ970467 (918)

chain reaction (RT-PCR) (Shaw *et al.* 2007) based on VP1, and by either real time RT-PCR based on NS3 (VIROTYPE BTV Plus; Labor Diagnostik, Leipzig, Germany) or VP1 genes (Shaw *et al.* 2007). The One Step RT-PCR kit (Qiagen, Hilden, Germany) was used for RT-PCR. The serotype of the virus was identified as BTV-24 by means of a serotype-specific, segment-2-specific RT-PCR, and was confirmed by nucleotide sequencing.

Sequencing

For sequencing analysis the VP1, VP2, VP3, VP4, VP5, VP6, NS1, NS2, and NS3 genes were amplified by RT-PCR with sets of primers specific for these segments, followed by nucleotide sequence analysis. The accession numbers of the nucleotide sequences and the fragment lengths are available in GenBank (Table II). Sequencing was carried out with a 3730 DNA Analyzer (Applied Biosystems, ThermoFisher, USA) at the Weizmann Institute of Science, Rehovot, Israel. The partial sequences of the 9 gene segments were analysed and compared by using the BLAST program (Altschul *et al.* 2009) and phylogenetic comparisons were performed with MEGA5 (Tamura *et al.* 2011).

Results

Clinical manifestations of BTV-24

BTV24/2305/2008 and BTV24/2425/2008 were isolated in November 2008 from 2 sheep in a flock exhibiting severe BT symptoms, and which lost about 50% of the adult sheep – approximately 2,000 out of 4,000. Although most of the animals showed some degree of illness, mortality occurred in pregnant animals or ewes after lambing. The most frequent manifestations were fever, anorexia, ulcerative and necrotic lesions of the oral mucosa, hyperaemia and oedema of the conjunctival mucosa, sore muzzle, hyperaemia of the teats and udder, haemorrhage, swollen head, and lameness. Most of the diseased animals exhibited only 1 or 2 of these lesions or signs.

BTV24/2755/1/2010 was isolated in October 2010 from sheep in a flock that exhibited the red/rough udder syndrome and agalactia related to BTV-24. Neither mortality nor other clinical signs, apart from abortions, were reported.

BTV24/2944/1/2010 was isolated in November 2010 from sheep in a flock that did not exhibit BT-attributed symptoms other than abortions.

BTV24/3258/1/2010 was isolated in December 2010 from bovines exhibiting clinical signs of both the Bluetongue/Epizootic haemorrhagic disease (EHD) (Brenner *et al.* 2011), and 5 primiparous heifers out of 500 milking cows died. Typical signs of EHD, such as sharp reduction in milk production, fever, weakness and stiff gait, serous/purulent nasal discharge, excessive salivation, nasal and lip redness, with cyanosis and erosions of the tongue, as well as petechiae on the tips of the lingual and buccal papillae (Yadin *et al.* 2008) were observed in many of the cows in this farm. The affected lactating cows exhibited other clinical signs which could be associated with *Orbivirus* infection, such as a sharp drop in milk production and marked loss of body weight. Five of the 20 diseased animals were culled because of mastitis.

BTV 2214/1/2010 was isolated in September 2010 from sheep in a flock in the Upper-Middle

Table III. *Nearest identity of Israeli isolates of Bluetonge virus serotype 24 (BTV24) with BTVs from the GenBank.*

Virus	Identity according to BTV gene (strain-percentage-genetic group)								
	Seg1/VP1	Seg2/VP2	Seg3/VP3	Seg4/VP4	Seg6/VP5	Seg9/VP6	Seg5/NS1	Seg8/NS2	Seg10/NS3
BTV24/2305/08	BTV15/ ISR2006/11- 99.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV2/ TUN2000/01- 95.7%-3rd gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/ Israel/1047/10- 99.6%-1st gg*	BTV12/75005/ S.A-96.7%- 1st gg*	BTV4/ ISR2008/02- 99.8%-1st gg*	BTV4/ ISR2008/02- 1st gg*
BTV24/2425/08	BTV15/ ISR2006/11- 99.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV2/ TUN2000/01- 95.7%%- 1st gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/ Israel/1047/10- 99.6%-1st gg*	BTV12/75005/ S.A-96.7%- 1st gg*	BTV4/ ISR2008/02- 99.8%-1st gg*	BTV4/ ISR2008/02- 1st gg*
BTV24/2755/1/10	BTV15/ ISR2006/11- 99.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV2/ TUN2000/01- 95.7%-1st gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/ Israel/1047/10- 99.6%-1st gg*	BTV12/75005/ S.A-96.7%- 1st gg*	BTV4/ ISR2008/02- 99.8%-1st gg*	BTV4/ ISR2008/02- 1st gg*
BTV24/2944/1/10	BTV15/ ISR2006/11- 99.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV2/ TUN2000/01- 95.7%%- 1st gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/ Israel/1047/10- 99.6%-1st gg*	BTV12/75005/ S.A-96.7%- 1st gg*	BTV4/ ISR2008/02- 99.8%-1st gg*	BTV4/ ISR2008/02- 1st gg*
BTV24/3258/1/10	BTV15/ ISR2006/11- 99.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV2/ TUN2000/01- 95.7%-1st gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/ Israel/1047/10- 99.6%-1st gg*	BTV12/75005/ S.A-96.7%- 1st gg*	BTV4/ ISR2008/02- 99.8%-1st gg*	BTV4/ ISR2008/02- 1st gg*
BTV24/3027/6/10	BTV16/1974/4/12 Isr-99.9%-2nd gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV2/1858/13 Isr- 97.5%- 3rd gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/ Israel/1047/10- 99.6%-1st gg*	BTV12/75005/ S.A-96.7%- 1st gg*	BTV4/ ISR2008/02- 99.8%-1st gg*	BTV-2IT (L)- 97.2%-2nd gg*
BTV24/3027/1/10	BTV16/1974/4/12 Isr-99.9%-2nd gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV8/1206/10 Isr- 99.9%- 2nd gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/Turkey/1999- 98.3%-2nd gg*	BTV8/ NET2006/04- 99.4%-2nd gg*	BTV8/ NET2006/04- 99.4%-2nd gg*	BTV-2IT (L)- 96.5%-2nd gg*
BTV24/2214/1/10	BTV4/2196/10 Isr- 99.2%%-3rd gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV2/ TUN2000/01- 95.7%-1st gg*	BTV-2IT (L)- 98.8%-1st gg*	RSArrrr/24/ S.A-ref-95%- 1st gg*	BTV4/ Israel/1047/10- 99.6%-1st gg*	BTV24/ NVSL053191- 97.9%-2nd gg*	BTV22/84/184 S.A-3rd gg*	BTV-2IT (L)- 97.2%-2nd gg*

gg* = genetic group.

Jordan Valley (South Israel), which showed severe nervous-like manifestations similar to those associated with border disease (BD). Lambs aged 4 to 6 months were affected. All the 20 affected lambs died. The affected animals were tested for BD by ELISA Ag (Bovine Viral Diarrhoea Antigen Test Kit/ Serum Plus-IDEX, Liebefeld-Berne, Switzerland) and found negative.

Two BTV-24 strains (BTV24/3027/6/2010 and BTV24/3027/1/2010) were isolated in a flock in Northern Israel, in which BD like or food intoxication/ enterotoxaemia were initially suspected because the affected lambs exhibited severe nervous-like manifestations, such as body tremors, stiff gait, recumbency. Some died, probably because of starvation. Subsequently, samples from weak and dead animals were found positive for BTV by RT-PCR.

Genetic analysis of BTV-24 strains isolated in Israel

Eight BTV-24 viral isolates from 7 clinically affected sheep and 1 affected bovine were selected for genetic characterization. Nine segments (Seg1, Seg2, Seg3, Seg4, Seg5, Seg6, Seg8, Seg9, and Seg10) of each viral genome were partially sequenced and the generated sequences were then compared with one another, as well as with related sequences in GenBank (Table III).

Segment 2 encoding outer coat-protein VP2

Phylogenetic comparative analysis of all BTV-24 Seg2 nucleotide sequences available in GenBank showed that the 8 Israeli isolates clustered as single group (Figure 1, Table III). The percentage difference between the 8 Israeli isolates did not exceed 1%, indicating that all the isolates were descended from a common ancestor. The Israeli isolates were approximately 95% identical to the prototype BTV-24, South Africa reference isolate (BTV-24RSArrrr/24/S.A-ref) and showed 87.7-89% identity with 3 other BTV-24 viruses (BTV24/06.1, BTV24/09.01 and BTV24/11/01), which had been isolated in Martinique (France) in 2006 and 2009, and in French Guyana in 2011.

Segment 6 encoding outer coat protein VP5

Phylogenetic comparative analysis of all BTV-24 Seg6 nucleotide sequences showed that the 8 Israeli isolates clustered in a single group (Figure 1, Table III). The percentage identity among the Seg6 sequences from the Israeli isolates was > 99%, indicating that all the isolates were descended from a common ancestor. It is noteworthy that the Israeli isolates were approximately 95% identical to the prototype BTV-24 South African strain (BTV-24RSArrrr/24/S.A-ref), but showed an even closer percentage identity (98%) with BTV-4 isolates from the Mediterranean region.

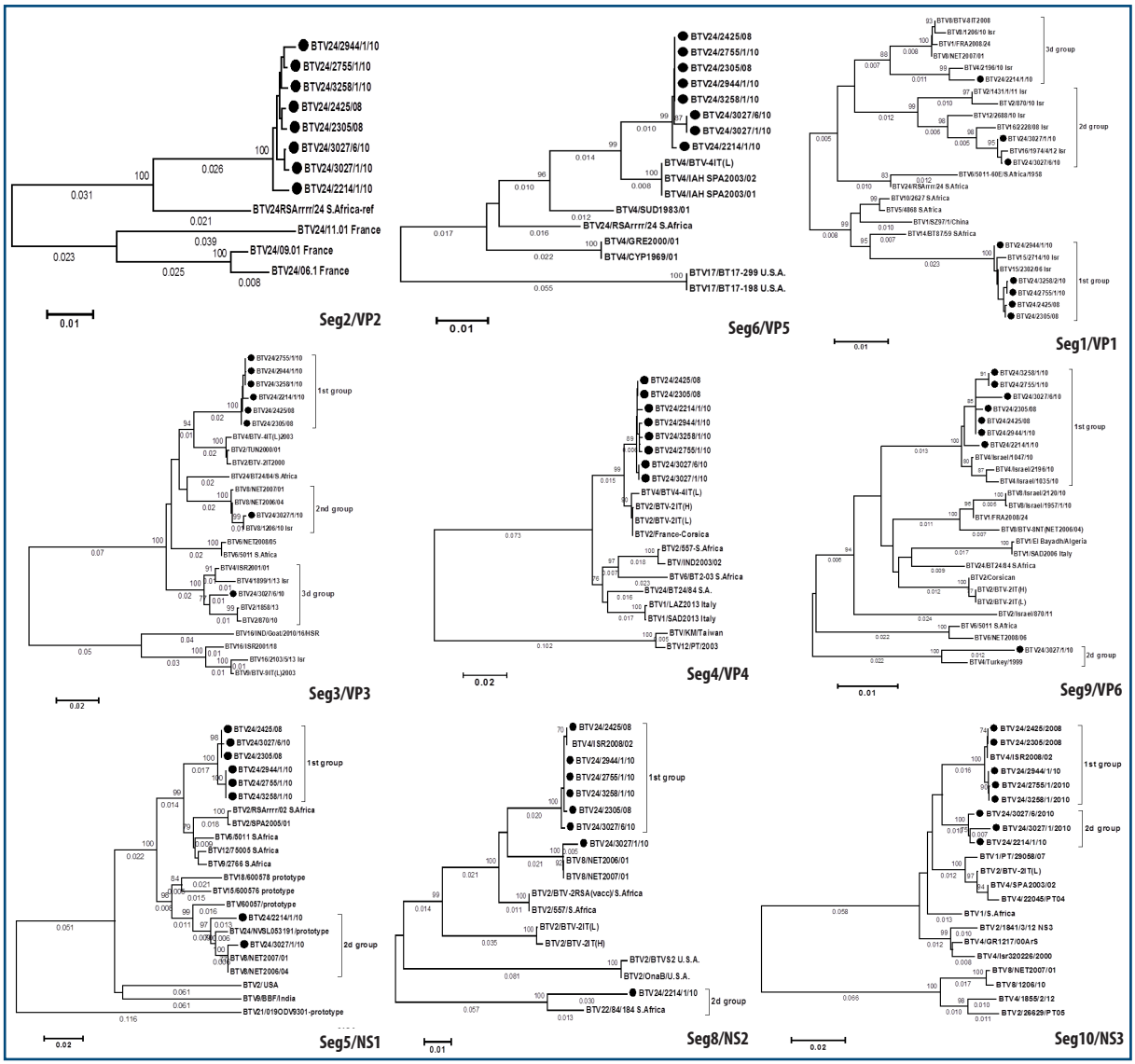

Figure 1. *Phylogenetic relationships of genes from 8 isolates of Bluetongue virus serotype 24 (BTV-24) in Israel, 2008-2010.* The phylogenetic trees, based on nucleotide sequences, were generated by neighbour-joining analysis with the Maximum Composite Likelihood model, by using MEGA 5.0 software (Tamura *et al.* 2011). The numbers below branches indicate neighbour-joining bootstrap values. The numbers above branches indicate branch lengths. The black circles indicate the studied Israeli BTV-24.

Segment 1 encoding internal protein VP1

Phylogenetic analysis of Seg1 sequences from the 8 Israeli isolates separated the viruses into 3 groups (Figure 1, Table III). The first group included 5 Israeli BTV-24 viruses: 2 isolated in 2008 and 3 in 2010. The VP-1 gene sequences from these isolates showed very high percentage identity (99.8%) with the VP-1 gene sequences from Israeli isolates of BTV-15, indicating that these segments were derived from a common ancestor.

The second group, consisting of 2 VP-1 gene sequences (BTV24/3027/6/2010 and BTV24/3027/1/2010), showed 93.5% identity with the VP-1 gene sequences in group 1, and the VP-1 gene in the second group showed 99.3-99.9% identity with Israeli BTV-16 VP-1 gene sequences (BTV16/2228/08 Isr and BTV16/1974/4/12 Isr).

The third group, consisting of 1 VP-1 gene sequence (BTV24/2214/1/2010), showed 93.2% identity with the group 1 VP-1 gene sequences, and was most closely related (99.2% identity) to a VP-1 gene sequence from an Israeli BTV-4 isolate (BTV4/2196/10).

The high percentage sequence identity (99.8%) between VP-1 gene from group 1 BTV-24 viruses and the Israeli BTV-15 strains indicated that this segment may have been reassorted or that these segments were derived from a common ancestor. Additionally, the low percentage identity among the 3 groups of VP-1 gene sequences indicates that the VP-1 gene in the viruses of groups 2 and 3 are likely to be

reassortants and that, possibly, they originate from the Israeli BTV-16 and BTV-4 field isolates.

Segment 3 encodes internal protein VP3

Phylogenetic analysis of Seg3 sequences from the 8 Israeli isolates separated the viruses into 3 groups (Figure 1, Table III). The first group included 6 of the Israeli BTV-24 strains: 2 isolated in 2008 and 4 in 2010. The percentage identity of the VP-3 gene sequences was 99.7%, indicating that these viruses were derived from a common ancestor. The VP-3 gene sequences from these isolates showed 95.7% identity with BTV2/TUN2000/01 (Table III).

The second group comprised only 1 VP-3 gene sequence (BTV24/3027/1/2010), which showed 99.4-99.9% identity with the Seg3 of the BTV-8 Israeli isolate (BTV8/1206/10 Isr) and the BTV-8 isolates from the Netherlands (BTV/NET2007/01 and BTV8/NET2006/04).

The third group comprised only 1 VP-3 gene sequence (BTV24/3027/6/2010), which showed 97.5% identity with VP3 gene sequences of BTV-2 (BTV2/1858/13 and BTV2/870/10) with BTV-4 (BTV4/ISR2001/01 and BTV4/1899/13 Isr). The percentage differences between the 1st and 2nd, the 1st and 3rd, and 2nd and 3rd groups of BTV-24 VP-3 gene sequences were 4.5%, 6.5%, and 6.4%, respectively. The low percentage identities among the three groups of VP-3 gene sequences indicates that the VP-3 genes in the viruses of groups 2 and 3 are likely to be reassortants, and that viruses from group 2 may have originated from the Northern European BTV-8.

Segment 4 encoding internal protein VP4

Phylogenetic comparative analysis of all BTV-24 Seg4 nucleotide sequences showed that the 8 Israeli isolates clustered into a single group (Figure 1, Table III). The percentage identity of Seg4 from all the Israeli isolates was > 99%, indicating that all the segments were descended from a common ancestor. This group clustered with 4 BTV-2 and BTV-4 strains isolated in Italy and Corsica [BTV-4IT(L) 2003, BTV-2IT (H and L) and BTV-2 Corsica] (Figure 1) and showed 98.8% identity with these viruses (Table III).

Segment 9 encoding internal protein VP6 and nonstructural protein NS4

Phylogenetic analysis of Seg9 sequences from the 8 Israeli isolates separated these viruses into 2 groups (Figure 1, Table III). The first group comprised 7 of the Israeli BTV-24 viruses with > 99% identity among their Seg9 segments, indicating that these segments were derived from a common ancestor. They showed 99.6% identity with BTV4/Israel/1047/10 virus isolate (Table III). The second group comprised 1 Israeli (BTV24/3027/1/10) and 1 BTV-4 Turkish (BTV-4/Turkey/1999) isolate (Figure 1), whose identity did not exceed 98.3%. The 2 groups of viruses shared 93.6% identity, which indicated that the VP6 gene of BTV24/3027/1/10 isolate from group 2 was likely to be a reassortant.

Segment 5 encoding nonstructural protein NS1

Phylogenetic analysis of Seg5 sequences from the 8 Israeli isolates clustered the viruses into 2 groups (Figure 1, Table III). The first group included 6 of the Israeli BTV-24 viruses, which shared more than 99% identity, indicating that these 6 Seg5 originated from a common ancestor, possibly from South Africa (Figure 1).

Two other Israeli BTV-24 viruses (BTV24/2214/1/2010 and BTV24/3027/1/2010), with 97.2% identity, belonged to the second group. The NS1 gene sequence from 1 of these viruses (BTV24/3027/1/2010) showed a 99.4% identity with the NS1 gene from the Northern European strains of BTV-8 isolated in the Netherlands (BTV8/NET2007/01 and BTV8/NET206/04), whereas BTV24/2214/1/14 showed 97.9% identity with BTV24/NVSL053191/ prototype isolate (Table III). The low percentage identity of 92.5% between the 2 groups of viruses indicates that the NS1 genes of the second group were likely to be reassortants, possibly derived from the Northern European strain of BTV-8.

Segment 8 encoding nonstructural protein NS2

Phylogenetic analysis of Seg8 sequences from the 8 Israeli isolates clustered the viruses into 3 groups (Figure 1, Table III). The first group comprised 6 Israeli BTV-24 and one BTV-4 (BTV4/ISR2008/02) viruses that shared 99.8% similarity; the second comprised 1 Israeli virus (BTV24/3027/1/10) and BTV-8 strains isolated in the Netherlands, which shared 99.4% identity.

The third group comprised 1 Israeli isolate (BTV24/2214/1/10) and 1 South African BTV-22 isolate (BTV22/84/184 S.Africa), which shared about 95.7% similarity. The percentage identity between the BTV-24 viruses from first and second groups was about 95%, whereas the percentage identity between BTV-24 viruses from the first and the third groups was about 84.6-85.2% (Figure 1). The low homology among the NS2 genes from the 3 groups of Israeli viruses makes it highly likely that the viruses of groups 2 and 3 are reassortants.

Segment 10 encoding nonstructural protein NS3

Phylogenetic analysis of Seg10 sequences from the Israeli isolates clustered the viruses into 2 groups

(Figure 1, Table III), which shared about 96.5% identity. The first group comprised of 5 Israeli BTV-24 and 1 BTV-4 (BTV4/ISR2008/02) viruses with 99.6-99.7% identity, indicating that these 5 NS3 segments originated from a common ancestor. The Seg10 from the second group of Israeli BTV-24 isolates clustered with BTV-1, BTV-2, and BTV-4 isolates from the Mediterranean area [BTV1/PT/29058/07, BTV2/BTV-2IT(L), BTV4/22045/PT04, BTV4/SPA2003/02], with 97.5-96% similarity. Separation of the Seg10 sequences of Israeli BTV-24 isolates into 2 groups indicated possible reassortment of Seg10 sequences from the second group.

Summary of phylogenetic analyses

Phylogenetic analysis of the segments encoding the outer coat proteins (Seg2 and Seg6) and the VP4 internal protein (Seg4) revealed that all 8 Israeli BTV-24 isolates were descended from 1 common ancestor. All tested Israeli strains were clearly distributed into 4 variants by phylogenetic characterization of Seg1, Seg3, Seg5, Seg8, Seg9, and Seg10. Five of the viruses isolated between 2008 and 2010 shared a high level of identity among all 9 segments, and made up variant 1. A second variant (BTV24/3027/6/10), isolated in 2010, showed significant variation from variant 1 in 3 of its gene segments (VP1, VP3 and NS3 genes).

A third variant (BTV24/3027/1/10) showed significant variation from variant 1 in 6 of its gene segments (VP1, VP3, VP6 and NS1, NS2 and NS3 genes). Interestingly, VP3, NS1, and NS2 genes of the third variant of the Israeli BTV-24 isolate could have originated from the European BTV-8. A fourth variant (BTV24/2214/1/10) showed significant variation from variant 1 in 4 of its gene segments (VP1, NS1, NS2 and NS3 genes). The marked differences among the 4 BTV-24 variants, in sequence identity among 6 out of 9 of the gene segments, indicate the occurrence of a high level of genetic reassortment.

Discussion

A wide range of serotypes of BTV has been circulating in Israel for many years (Brenner *et al.* 2010, Brenner *et al.* 2011, Bumbarov *et al.* 2012, Shimshony 2004), and BTV-24 strains have been frequently detected, along with other serotypes, from 2008 to 2010. The co-circulation of many different serotypes of BTV in Israel makes it an ideal location to investigate the degree and levels of reassortment in the field. The present study set out to investigate whether natural reassortment of BTV viruses had occurred in the field and whether there were links between disease manifestations and the genetic properties of these viruses. Five of the 8 Israeli BTV-24 strains showed similar clustering to the 9 segments that were sequenced, indicating that these viruses were likely to have been derived from a common BTV-24 ancestor. In the examined farms, classical clinical signs were observed (Table I). At the same time, 3 of the BTV-24 isolates (BTV24/3027/1/2010, BTV24/3027/6/2010, and BTV24/2214/1/2010) from 2 sheep flocks showed diverse clustering of various internal genes (Table I), and nervous-like symptoms were observed in these flocks.

The subdivision into phylogenetic groups and the low homology between viruses from different groups (Seg1, Seg3, Seg5, Seg8, Seg9, and Seg10) indicate that a high frequency of natural reassortment of these genes is occurring under field conditions. Data from the present investigation suggest that nervous-like symptoms, like those that were observed in sheep (in farms in southern and northern Israel) affected by BTV-24, may be associated with the unique reassortments in some internal genes (Table I). It was observed that the genes encoding NS3 (Seg10) were found to be very similar among all 3 viruses within the second phylogenetic group (Figure 1).

Interestingly, nervous-like symptoms were observed in the 2 sheep flocks that were affected by BTV-24 genetic variants 2, 3, and 4, but not in those affected by genetic variant 1. It is therefore possible that these nervous-like clinical signs could be associated with the genetic characteristics of the viruses, possibly with the NS3 gene.

However, it is also possible that the nervous-like clinical signs observed in these sheep flocks were being caused by an alternative undiagnosed infectious agent, and that BTV was also circulating at the same time.

The limited number of observations in the present study cannot support far-reaching conclusions about the relationship between the genetic characteristics of the internal genes (genotypes), on the one hand, and the clinical disease (phenotype). The incomplete genetic data regarding both Israeli and non-Israeli BTV viruses do not enable us to evaluate the similarity between viruses that may have undergone reassortment with the Israeli BTV-24 isolates and viruses from other BT serotypes, and to deduce from which viruses those segments could be obtained. More detailed investigations are required in order to consider these possible relationships.

Nonetheless, we can conclude that genetic reassortment of various internal genes is freely occurring among circulating BTV-24 strains in the field in Israel. The high levels of genetic reassortment observed within the circulating BTV-24 strains in Israel may be causing changes in the viral phenotype,

which may contribute to the diverse range of clinical manifestations that are currently being observed in Israeli cattle, sheep, and goats (Brenner *et al.* 2010, Brenner *et al.* 2011, Bumbarov *et al.* 2012).

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