

Serotypic identification of local Bluetongue virus isolates using Plaque Reduction Neutralization (PRN) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Techniques*

Volkan YILMAZ¹, Aykut OZKUL²

¹ Kafkas University, Faculty of Veterinary Medicine, Department of Virology, Kars; ² Ankara University, Faculty of Veterinary Medicine, Department of Virology, Ankara, Turkey.

Summary: Bluetongue virus (BTV) is a vector-borne disease of ruminants disseminated in the tropics and sub-tropics. It is also an important problem in the Middle East. The purpose of this study is serotypic identification of local Bluetongue viruses (BTV) isolated between 1998 and 2005. For this purpose, generic (g) and serotype specific (s) RT-PCR systems and the Plaque Reduction Neutralization Assay (PRNA) were used. Generic and serotype specific RT-PCR was performed on the segment 10 and segment 2 levels, respectively. Generic RT-PCR applications revealed specific DNA product (822 bp in length) in all 26 BTV field isolates. On the other hand, 19 BTV isolates were identified as serotype 9 and one isolate was found to be serotype 16, using the sRT-PCR technique. No DNA amplification was observed as a result of sRT-PCR for serotypes 2 and 4. Plaque reduction and neutralization assay (PRNA) was used for serologic identification of BTV isolates. Hyperimmune serums specific to three serotypes (BTV-4, BTV-9 and BTV-16) produced in rabbits, were used in PRNA. The test showed that 19 of the isolates were BTV-9, and the remaining two isolates were identified as BTV-4 and BTV-16.

Keywords: Bluetongue virus, serotyping identification, PRNA, RT-PCR

Yerel Mavidil virusu izolatlarının Plak Redüksiyon Nötralizasyon (PRN) ve Reverz Transkriptaz Polimeraz Zincir Reaksiyonu (RT-PZR) teknikleri ile serotipik identifikasyonu

Özet: Mavidil virusu dünyanın subtropik ve tropik bölgelerinde yaygın olan, ruminantların vektörle bulaşan hastalığıdır. Hastalık özellikle Ortadoğu için önem taşımaktadır. Bu çalışmada 1998-2005 yılları arasında izole edilen yerel BT viruslarının serotipik identifikasyonu amaçlanmıştır. Bu amaç için jenerik ve serotip spesifik RT-PZR sistemleri ile PRN testi kullanıldı. Jenerik RT-PZR ve spesifik RT-PZR sırasıyla segment 10 ve segment 2 düzeyinde gerçekleştirildi. Jenerik RT-PCR uygulamalarında 26 adet BTV izolatının tamamında spesifik DNA ürünü (822 bp. boyunda) elde edildi. Diğer taraftan sRT-PZR uygulaması sonunda ise izolatlardan 19 adedi serotip 9 ve 1 adedi serotip 16 olarak tanımlandı. Serotip 2 ve 4 için uygulanan spesifik RT-PZR sonunda herhangi bir DNA ürünü elde edilemedi. Plak redüksiyon ve nötralizasyon testi BTV izolatlarının serotipik identifikasyonu için kullanıldı. Tavşanlarda üretilen BTV-4, BTV-9 ve BTV-16'ya spesifik hiperimmün serumlar PRN testinde kullanıldı. PRN testi sonunda 19 izolat BTV-9, 1'er izolat BTV-4 ve BTV-16 olarak tanımlandı.

Anahtar Sözcükler: Mavidil virusu, serotipik identifikasyon, PRNT, RT-PZR.

Introduction

Bluetongue (BT) is an arthropod-transmitted disease of wild and domestic ruminants and is enzootic in many tropical and temperate regions, coincident with the distribution of competent *Culicoides* vector insects (13). It is caused by a virus classified within the prototype species *Bluetongue virus* (BTV) from the genus *Orbivirus*, the largest genus of viruses within the family Reoviridae, with double-stranded RNA genome which consists of ten segments (15). Twenty four serotypes have been identified so far (22).

Bluetongue has major implications for international trade in livestock (6), and is therefore found on the list of diseases which must be reported to the World Organization for Animal Health (18). BTV can infect most ruminant species, and although sheep frequently become clinically ill, goats and cattle usually remain asymptomatic (2, 12). It is believed that this reflects host-species-specific variations in the response of microvascular endothelial cells to BTV infection (3).

BTV was observed in the 19th century in Africa, but was only described in 1902 by Spreull. One of the

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* This study is summarized by the first author from his PhD Thesis with same title.

first reports out of Africa was from Cyprus in 1943. The first outbreak seen in Turkey was in Hatay in 1944-1947. This outbreak was controlled with strict measures. The second appearance was in the Aegean region in 1977, later on the Marmara and Mediterranean regions were affected as well. The virus was identified as BTV Type-4 (25, 27). The disease has been detected serologically and virologically in many studies so far and type 4, 9 and 16 have been identified in Turkey.

BTB outbreaks have occurred annually in the Mediterranean Basin since 1998, which result in significant economic losses (14). During the last decade five different serotypes of BTB have been detected circulating in the Mediterranean basin (BTB-1, 2, 4, 9, 16). BTB-15 has also been detected in Israel, but does not appear to have spread to adjacent countries. The distinct strains of BTB-1 and 4 have also been detected in the western Mediterranean region (1).

The plaque reduction neutralization test has long been recognized as one of the most accurate methods of detecting antibodies. The commonly used method of constant serum-varying virus combined with a quantal assay has been so severely criticized elsewhere as to make discussion of it unnecessary. While the plaque

reduction test is extremely accurate and quantitative, its limitations due to antigenic variation among isolates are as yet unknown. It appears to be a simple and very useful procedure for working with BTB (23).

The study described here examines the identification of BTB serotypes that were circulating in western parts of Turkey between 1998 and 2005, using RT-PCR methods and Plaque Reduction Neutralization assay (PRNA).

Materials and methods

Viruses and plaque assay: A total of 26 BTB isolates were used in the study. All of the viruses were isolated in the virology laboratory of the Central Veterinary Research and Control Institute - Ankara from blood and organ samples obtained from sick and/or dead animals and transferred to the laboratories of the Virology Department at the Ankara University School of Veterinary Medicine. The samples received had originated from 7 provinces, two of which were in southern Turkey, while the rest were from western Turkey (Table 1). A total of 26 BTB isolates were inoculated onto the Vero AFFSA cells and 21 of them reached satisfying infectivity titer as calculated by native plaque titration assay.

Table 1. BTB isolates, their geographical distribution and further details.

Tablo 1. BTB izolatlarının coğrafik dağılımları ve özellikleri

Isolate Code	Province	County	Year	Species	Cell/passage number
TR-1	MUGLA	DALAMAN	1999	SHEEP	VERO/5
TR-2	IZMIR	MENEMEN	1998	SHEEP	VERO/2
TR-3	AYDIN	BOZDOGAN	1998	SHEEP	VERO/3
TR-4	IZMIR	MENEMEN	1999	SHEEP	VERO/2
TR-5	MUGLA	DALAMAN	1998	SHEEP	VERO/6
TR-6	IZMIR	BUCA	1998	SHEEP	VERO/3
TR-7	CANAKKALE	YENICE	1999	SHEEP	VERO/2
TR-8	ANTALYA	MANAVGAT	1999	SHEEP	VERO/2
TR-9	IZMIR	TIRE	1999	SHEEP	VERO/4
TR-10	AYDIN	SOKE	1998	SHEEP	VERO/3
TR-11	ANTALYA	MANAVGAT	1999	SHEEP	VERO/2
TR-12	AYDIN	CINE	1998	SHEEP	VERO/3
TR-13	AYDIN	CENTER	2001	SHEEP	VERO
TR-14	AYDIN	CINE	2001	SHEEP	VERO
TR-15	AYDIN	CINE	2001	SHEEP	VERO
TR-16	AYDIN	GERMENCİK	2000	SHEEP	VERO
TR-17	AYDIN	CINE	2001	SHEEP	VERO
TR-18	AYDIN	BOZDOĞAN	2005	SHEEP	VERO/3
TR-19	DENİZLİ	ACIPAYAM	1998	SHEEP	VERO/1
TR-20	AYDIN	CINE	1999	SHEEP	VERO/1
TR-21	ANTALYA	SERİK	1999	SHEEP	VERO/1
TR-22	MUGLA	MILAS	1999	SHEEP	VERO/1
TR-23	İCEL	CENTER	1999	SHEEP	VERO/1
TR-24	IZMIR	BUCA	1999	SHEEP	VERO/1
TR-25	AYDIN	BOZDOGAN	1999	SHEEP	VERO/1
TR-26	ANTALYA	SERİK	1999	SHEEP	VERO/1

Table 2. Generic and serotype specific primers used in the study.
Tablo 2. Çalışmada kullanılan jenerik ve serotip spesifik primerler

Primer Code	Location (nt)	Nucleotide sequences (5'-3')	Product size (bp)	Specificity
BTV-Gen/10/F	1-22	GTAAAAAAGTGTCGCTGCCATG	822	Generic
BTV-Gen/10/R	798-822	GTAAGTGTGTAGCGCCGCATACCCT		
BTV-2/2/852-F	852-874	GAG/TT/CTG/ATGTCGGATATTA/GTCACG	1804	Type-2
BTV-2/2/2656-R	2656-2638	TCGTGCGACACTTCG/AATAG		
BTV-4/2/200-PF	200-221	GCTTAACTATAAAACCAACGAGG	2324	Type -4
BTV-4/2/ 2524R	2524-2505	AACTTGGACGTCACAACAGG		
BTV-9/2/1473-F	1473-1491	GGC/GAATATATTTCTA/GATGG	1128	Type -9
BTV-9/2/2601-R	2601-2584	GGAAAAGTATTGGCAAT		
BTV-16/2/2010F	2010-2029	GAACGTTGTATGATGTCTCG	762	Type -16
BTV16/2/2772R	2772-2752	GCCGAAGGTGCGATCTGGCCG		

Table 3. Group of rabbits and inoculation procedure.
Tablo 3. Tavşan grupları ve inokulasyon yöntemi.

Group no	Rabbit number	BTV Serotype	Immunization Procedure
I	2	BTV-4	Concerning serotype, 4 inoc. (id) with 15 days apart
II	2	BTV-9	Concerning serotype, 4 inoc. (id) with 15 days apart
III	2	BTV-16	Concerning serotype, 4 inoc. (id) with 15 days apart
IV	2	Control	DMEM, 4 inoc. (id) with 15 days apart

RNA extraction, generic and serotype specific RT-PCRs: Viral RNA was extracted from 200 µl of cell-culture supernatant from BTV infected Vero cells using the "Viral RNA extraction kit" (Roche, Germany) as described in the manual. The follow-up reverse transcription (RT) was performed using the "RevertAid kit" protocol based on random hexamer priming (Fermentas, Lithuania) according to the manufacturer's instructions. For Seg-10 amplification, the generic RT-PCR primers designed previously (17) were used (Table 2). The PCR was initiated by a single denaturation step at 96 °C for 10 min, followed by 40 cycles of 1 min at 58 °C, 1 min at 72 °C and 1 min at 94 °C. The mixture was then incubated, as a single step, for 1 min at 50 °C and 10 min at 72 °C for final extension. Five microliters of each PCR product was analyzed in 1% agarose (Prona, Spain) gels containing ethidium bromide (Sigma, USA). On the other hand, serotype specific RT-PCR was carried out on segment 2 with primer sets, two of which had been previously designed (16) while the remaining two (BTV-9 and BTV-16) were designed within this study (Table 2). The serotype specific RT-PCR was performed in conditions similar to that described for generic RT-PCR.

Hyperimmune Serum Production: In order to obtain the type-specific hyperimmune serums which are the main component of the PRNA test, rabbits were inoculated with three serotypes of BTV (BTV-4, BTV-9 and BTV-16). The rabbits were divided into 3 inoculation groups, each consisting of two rabbits, and two of the rabbits were kept separate to serve as a control

group. Groups 1 to 3 were inoculated with BTV-4, BTV-9 and BTV-16, respectively, while group 4 was injected with Dulbecco's modified Eagle's medium (DMEM) to keep them as negative control animals. The injections were made intradermally (id) every fifteen days at multiple region on the back after shaving the skin (Table 3). After the fourth injection, the animals were premedicated with xylazine HCl (Rompun, Bayer) and bled by cardiac puncture. Heat inactivated serum samples were tested with the microneutralization test (5) in order to not only estimate serotype specific antibody titer, but also to see possible cross-reactions between BTV serotypes.

Plaque reduction neutralization test: PRNA was performed as previously described (19). Briefly, each BTV isolate with 200 pfu in 200 µL was mixed with an equal volume of undiluted hyperimmune serums raised in rabbits and incubated at 37°C for 1 h., subsequently the mixtures were inoculated onto Vero monolayers grown in 24-well plates. After 1 hour of incubation to provide cellular adsorption of free virus particles, the cells were overlaid with 3.2% carboxymethyl cellulose in 2x DMEM for the plaque assay. Plaques were scored on day 6 post-inoculation after fixing cells with a 10% formaldehyde solution and subsequent staining with 0.37% crystal violet. The viruses were serotypically identified based on their counted plaque numbers. Briefly, a seventy percent inhibition in plaque numbers by any anti-serotype hyperimmune serum was used as the identification criteria for the BTV isolates.

Table 4. Comparative results of BTV isolates with gRT-PCR and sRT-PCR/PRNA
 Tablo 4. BTV izolatlarının jenerik RT-PZR ile serotip spesifik RT-PZR/PRNT'nin karşılaştırmalı sonuçları

ISOLATE NO	gRT-PCR	sRT-PCR/ PRNA			
		BTV-2	BTV-4	BTV-9	BTV-16
TR-1	+	-/	-/-	+/+	-/-
TR-2	+	-/	-/-	+/+	-/-
TR-3	+	-/	-/-	+/+	-/-
TR-4	+	-/	-/-	+/+	-/-
TR-5	+	-/	-/-	+/+	-/-
TR-6	+	-/	-/-	+/+	-/-
TR-7	+	-/	-/-	+/+	-/-
TR-8	+	-/	-/-	+/+	-/-
TR-9	+	-/	-/-	+/+	-/-
TR-10	+	-/	-/-	+/+	-/-
TR-11	+	-/	-/-	+/+	-/-
TR-12	+	-/	-/-	+/+	-/-
TR-13	+	ND	ND	ND	ND
TR-14	+	ND	ND	ND	ND
TR-15	+	ND	ND	ND	ND
TR-16	+	ND	ND	ND	ND
TR-17	+	ND	ND	ND	ND
TR-18	+	-/	-/-	-/-	+/+
TR-19	+	-/	-/-	+/+	-/-
TR-20	+	-/	-/-	+/+	-/-
TR-21	+	-/	-/-	+/+	-/-
TR-22	+	-/	-/-	+/+	-/-
TR-23	+	-/	-/+	-/-	-/-
TR-24	+	-/	-/-	+/+	-/-
TR-25	+	-/	-/-	+/+	-/-
TR-26	+	-/	-/-	+/+	-/-

ND: not determined

Results

Identification using generic and serotype-specific RT-PCR: RT-PCR using a generic primer set revealed specific DNA product (822 bp in length) in all of 26 BTV field isolates, while serotype specific RT-PCR identified 20 BT viruses, of which 19 were BTV-9 and the other one was BTV-16. On the other hand, one of the isolates (TR-23) was not able to be identified by serotype specific RT-PCR. There was no specific DNA amplification as a result of sRT-PCR for serotypes 2 and 4. Serotype distribution of local BTV isolates is given in Table 4.

Antibody production serotyping with PRNA: Immunization protocols for each rabbit group were performed successfully. VN assay revealed that all of the hyperimmune serums produced had 50% neutralizing antibody titer at their 1:10 dilutions for all respective BTV serotypes. Based on this observation, PRNA was carried out using undiluted hyperimmune serums. Three replicates of the PRNA test revealed that nineteen of the BTV isolates were identified as serotype 9 while the remaining two isolates were identified as serotypes 4 and 16, respectively.

Discussion and Conclusion

This study was carried out to identify local Bluetongue viruses (BTV) isolated between 1998 and 2005. For this purpose, generic (g) and serotype specific (s) RT-PCR were used as molecular diagnostic systems and Plaque Reduction Neutralization Assay (PRNA) as a conventional diagnostic system. Generic RT-PCR was performed on segment 10 using primers designed previously (17). On the other hand, sRT-PCR was carried on segment 2 with primer sets, two of which had been designed previously (16) while the remaining two (BTV-9 and BTV-16) were designed within this study.

Seg-10, the smallest genome segment, encodes NS3 and NS3A proteins (21). NS3 is a membrane protein that plays a role in the egress of the virus from infected cells (10). Other important features of NS3 include the two transmembrane domains which allow it to act as a viroporin, and extracellular portions known to form a loop that are thought to be important in terms of recognition by cells (9). Almost no amino acid substitutions were observed in the extra-cellular and glycosylation sites of the NS3 proteins of isolated BT viruses. Recent studies (8) indicate that NS3 protein

depends on the host cell and is not a particularly suitable target to detect BTV nucleic acid in infected mammalian cells. On the contrary, in our study, it was possible to detect specific DNA product (822 bp in length) in all of the BTV field isolates using generic RT-PCR. This was derived from all of the targeted NS3 sequences.

BTV was reported for the first time in Turkey in 1944. The presence of four BTV serotypes (e.g. BTV-2, BTV-4, BTV-9, BTV-16) has been reported in Turkey since that time (4, 11, 14, 26). In this study, 19 BTV isolates were identified as serotype 9 and one isolate was determined to be serotype 16 based on sRT-PCR. DNA amplification was not observed as a result of sRT-PCR for serotypes 2 and 4. Based on these results, it is concluded that BT infection in Turkey was caused dominantly by BTV-9 between 1995 and 2005. These assessments thoroughly conform with BTV epidemics which have been circulating in neighboring countries to the west in the same period. By considering the geographical locations of BTV isolates and the date of occurrence, we assume that BTV-2 does not exist in Turkey, although BTV-9 was isolated predominantly in western Anatolia and a few isolates of BTV-4 and 16 were detected in southern and southwestern Anatolia.

In this study, a total of 26 BTV isolates were inoculated onto the Vero AFFSA cells and 21 of them reached satisfying infectivity titer as calculated by native plaque titration assay. However, Vero AFFSA cell culture adaptation of 5 BTV isolates (TR13-17) was failed and no titer was observed by native plaque titration assay performed in case. This situation was thought to be due to Vero AFFSA cell-virus incompatibility or adaptation problem. Consequently, these isolates were not subjected to serotypic identifications by sRT-PCR and PRNA. It is pointed out in this study that following isolation and adaptation to cell culture must be fulfill before diagnostic differentiation.

The isolate TR-23 was identified as serotype 4 by PRNA, but not by RT-PCR. This is most probably caused by incompatibility between primer and target gene sequences as designated primers belonged to Mediterranean Basin isolates, and so BTV-4 primers did not match the target cDNA of isolate TR-23. This observation was found in concordance to several studies in which serotypic discrimination of BTV-4 European isolates and vaccine strains has been performed (16, 28).

Hiperimmunization of rabbits with concentrated viruses for up to 8 weeks did not result in excessive neutralizing of the antibody titer against the serotypes. However, neutralization antibodies can be obtained by immunizations against active infection after inoculations in sheep with similar concentrations (20). When the results obtained after hyperimmunization were evaluated, it was concluded that the antibody titer obtained from

rabbits was low and that it might be possible to obtain a higher antibody titer from animals other than rabbits.

Although PRNA is a labor intensive and time-consuming technique that requires a high level of skill, it has long been recognized as one of the most accurate methods of detecting BTV antibodies (23). The commonly used method of constant serum-varying virus combined with a quantal assay has been so severely criticized elsewhere as to make discussion of it unnecessary (23). While the plaque reduction test is extremely accurate and quantitative, its limitations due to antigenic variation among isolates are as yet unknown. It appears to be a simple and very useful procedure for work with BTV. In this study, hyperimmune serums specific to three serotypes (BTV-4, BTV-9 and BTV-16) produced in rabbits were used in PRNA. The test showed that 19 of the isolates were BTV-9, and the remaining two isolates were identified as BTV-4 and BTV-16. Similarly, PRNA is regarded as the 'gold standard' method for identification of some arboviruses (e.g. Bunya-, Flavi- and Togavirus) (7, 19, 24).

In conclusion, in view of BT epidemic's seasonality and that fact that it is rapidly spread via insects, BTV must be identified by accurate and rapid methods without viral isolation in samples from infected animals. Field screening of BTV infections in endemic countries, and the diagnostic value of RT-PCR applications must be enhanced. Therefore, real-time RT-PCR that is extremely accurate and advanced in-vitro amplification techniques must be developed and utilized for rapid, yet accurate identification of BTV serotypes. On the other hand, as RT-PCR performance is directly influenced by the laboratory or user profile and is an expensive technique, PRNA can be used for serotypic identification of BTV isolates in diagnostic laboratories that do not have molecular diagnostic systems based on similar performance in identifying BTV serotypes found in this study.

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Adress for correspondence

Dr. Volkan Yılmaz

Kafkas Üniversitesi Veteriner Fakültesi

Viroloji Anabilim Dalı, Paşaçayır, Kars,

Tel: +90 474 2426807/1107, Faks: +90 474 2426847

E-mail: volkankara1980@hotmail.com