

Research Article



Bluetongue Virus in Morocco from 2004-2012

DRIF KAMAR^{1*}, ELHARRAK MEHDI², LOUTFI CHAFIQA², FASSI FIIHRI OUAFAA¹

¹Microbiology Immunology and Contagious Disease, Agronomy and Veterinary Institute Hassan II; ²Department of Virology, BIOPHARMA, Rabat, Morocco.

Abstract | Bluetongue (BT) is an infectious, non-contagious disease of domestic and wild ruminants caused by Bluetongue Virus (BTV). The disease is quite prevalent in Morocco where serotypes BTV-1 and BTV-4 were found circulating since 2006. Despite use of attenuated bivalent vaccine, outbreaks are not uncommon each year along with lack of knowledge in geographical evolution of each serotype. Here, in this retrospective study from 2004-2012, we have determined current status of BTV prevalence and its serotypes together with techniques used for virus isolation, its antigen and antibody detection. Overall the prevalence of BTV was found to be 77.61% (728/938) and 76.94% (307/339), respectively. Although, we noticed some variation in year-wise distribution of each serotype particularly BTV-1 where BTV-4 was more prevalent than BTV-1 (45.77% vs. 40.59%). The study provides a baseline data upon circulating serotypes of BTV in Morocco over the years that will certainly help devising its control strategies in future.

Keywords | Bluetongue, Ruminants, Morocco, Vaccination, Prevalence

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***Correspondence** | Drif Kamar, Agronomic and Veterinarian Institute Hassan II, MIMC Department, IAV, Rabat, Morocco; **Email:** drif.kamar@gmail.com

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INTRODUCTION

Bluetongue virus (BTV) is an arthropod-borne emerging pathogen of wild and domestic ruminants that is closely related to Epizootic hemorrhagic disease virus (EHDV). The virus is transmitted by *Culicoides* with potential to spread rapidly to far off countries (Gollapalli et al., 2012; Maan et al., 2012a; Maan et al., 2012b; Maan et al., 2012c; Rao et al., 2012). The causative agent, Bluetongue virus, is the prototype of genus *Orbivirus* within the family *Reoviridae* (Mertens et al., 2005). With segmented genome (Seg-1 to Seg-10) of linear double-stranded RNA (dsRNA) (Mertens et al., 2009), BTV includes 26 distinct serotypes (Mertens et al., 2005; Chagnat et al., 2009; Maan et al., 2011). The disease may occur as acute or sub-clinical form characterized by a febrile response, inflammation and congestion leading to facial oedema and hemorrhages and ulceration of the mucous membranes.

Since 2004, the disease is endemic in Morocco with two different serotypes; BTV-4 in 2004 and BTV-1 in 2006. Though from 2005, necessary prophylactic campaigns using bivalent attenuated vaccine were started, outbreaks even in the vaccinated farms were not uncommon. This

vaccine failure may be attributed to the nature of farming systems adapted in Morocco that varies between intensive and extensive farming and allows disease spread through vector, the midges. This retrospective study provides an insight into the geographic spread and evolution of BTV serotypes in Morocco since its first epidemic in 2004.

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of 1337 BTV suspected samples consisting of sera (339) and blood (938) were collected from different outbreaks since 2004. During the first BT epidemics, sera were received from outbreaks and subsequently processed ELISA, however since 2007, both sera and blood added with anti-coagulant (EDTA) were received from outbreaks. All of samples were collected from sick sheep showing clinical signs suspected of BT (Table 1).

VIRUS ISOLATION

Blood samples collected from 2004 to 2009 were inoculated into embryonated chicken eggs (ECE) via yolk sac route. The eggs were incubated for 5 days at 35°C and examined daily. Embryos that died between 36 hrs. to 5 days

Table 1: Geographic provenance and number of samples collected from 2004 to 2012

Origin	2004	2006	2007	2008	2009	2010	2011	2012
Souss massa draa			28		21	49	30	6
Gharb-Chrarda-Beni hssen	9		19			32	20	12
Chaouia-ouardigha	1		7	22		10		
Marrakech-Tensift-Al haouz		1	6	7	66	46	4	10
Region de l'oriental		102	1		23	1	6	2
Grand Casablanca			2	1			1	11
Rabat-Sale-Zemmour-Zaer	24		25	9	3	11	6	2
Doukkala-Abda				2				
Tadla-Azilal		1	10		9	7	5	
Meknes-Tafilalet	9	1	53	25	66	133	4	6
Fes-Boulmane	3	6	15	29	69	46		5
Taza-Al hoceima-Taounate	4		1	6	5			
Tanger-Tetouan	9		25		1	10		
Unknown area	8	8	30	19	34	25	16	5
Totaux	67	119	222	120	297	350	92	70

post infection (PI), showing hemorrhagic lesions, were tested by RT-PCR. From 2009 – 2012, virus isolation was performed on cell culture, VERO and BHK cell lines.

COMPETITIVE ENZYME LINKED IMMUNOSORBENT ASSAY (cELISA)

Serum samples were tested for antibodies against BTV using a commercial competitive enzyme linked immunosorbent assay (cELISA) kit (IDVET Innovative Diagnostics ID Screen Bluetongue Competition ID-Vet, Montpellier, France) as per manufacturer’s instructions.

VIRUS NEUTRALIZATION TEST (VNT)

To classify the BTV positive sera according to their serotype, a virus neutralization test (VNT) was carried out (OIE terrestrial manual., 2014). Samples were tested for the presence of neutralizing antibodies. Serial dilutions (1:10) of heat-inactivated sera were incubated for 1 h at 37°C with an equal volume (50 ml) of BTV (100 TCID₅₀ with potential to produce pathological change in 50% of cell cultures inoculated). Approximately 10⁴ BHK-21 cells in 100 mL of medium were added per well and, after incubation for 5 days at 37°C with 5% CO₂, the microplate was read using an inverted microscope. Two replicates of each serum were tested with BTV-1 and BTV-4 available in Biopharma Lab, Rabat, Morocco. Neutralizing antibody titers were calculated according to Spearman– Karber’s formula and expressed as the last dilution of serum that neutralizes 100% of cytopathic effect (CPE) Serum samples with a virus neutralization titer inferior to 1.3 log₁₀ were considered positive to that particular serotype, BTV-1 or BTV-4.

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION PCR RT-PCR

Blood samples were collected from 938 local and/or cross-

bred animals. Samples were centrifuged and blood was kept at -20°C until assayed for the presence of BTV.

Viral RNA was extracted from blood using commercially available High Pure RNA Isolation Kit (Roche, Germany) following the instructions provided with kit. Superscript III Platinum One-Step RT-PCR system (Invitrogen, USA) was used to detect antigen using group specific primers BTV-S5-F1 5’-GGCAACYACCAAACATGGA-3’ and BTV-S5-R1 5’- AAAGTYCTCGTGGCATTWGC-3’.

Reverse transcription and amplification were performed by using a 7300 Real Time PCR System (Applied Biosystems). To provide specific information regarding isolate serotype, the primers for specific serotypes were used to amplify the segment 2, allowed to identify the serotype of each tested sample.

Table 2: Different type of samples used in this study

Year	Nombre of samples	Sera samples	Blood samples	Samples isolated in egg and cell culture
2004	67	67		36
2006	119	119		13
2007	222	97	125	13
2008	120	12	108	8
2009	297	24	273	11
2010	350	40	310	9
2011	92	7	85	5
2012	70	33	37	15

RESULTS

Based upon RT-PCR, overall prevalence of BTV was found to be 77.61% (728/938) where BTV-4 was more

prevalent than for RT PCR we use only blood BTV-1 (45.77% vs. 40.59%). Year wise, interestingly, BTV-1 was detected in all samples (100%) collected during the year 2007 with further decrease in 2011 (46.15%) to complete absence in 2012. Contrary to BTV-1, BTV-4 remains relatively constant throughout except 2007 (Table 3).

Table 3: Prevalence of BTV-1 and BTV-4 using a RT-PCR

Année	BT Positif	BTV1	BTV2
2007	67,2	100	
2008	14,81	43,75	56,25
2009	53,84	52,38	47,61
2010	78,06	31,40	68,59
2011	76,47	46,15	53,84
2012	78,37		100

Using ELISA, anti-BTV antibodies were detected in 307 sera with an overall seroprevalence of 76.94% (307/339) in different area of Morocco. VNT showed higher prevalence of antibodies against BTV-1 than BTV-4 (75.89% vs 24.10%). The prevalence was significantly higher during 2004–2007 (72.31%) than during 2008–2012 (27.68%) (Table 4).

Table 4: Prevalence of BTV1 and BTV4

Year	Sera	BT1	BT4
2004	67		44,77
2006	119	96,63	
2007	97	79,38	
2008	12	16,66	
2009	24	68,42	31,57
2010	40	62,06	37,93
2011	7	60	40
2012	33	16,66	83,33

BTVs from 2004 to 2009 were isolated in eggs while, from 2009 to 2012, they were isolated in cell culture. The isolated strains were tested through RT-PCR to constitute a strains collection for Biopharma lab (Table 6).

DISCUSSION

The Moroccan livestock includes approximately 2.8 million cattle, 17.5 million sheep, 5 million goats and 190.0 camels. Both weather conditions (www.agriculture.gov.ma) and the geographic location of Morocco are the most important factors involved in the emergence of the BT in The Mediterranean Basin; a potential source of BTV for Europe and North Africa (Mellor and Wittmann, 2002). Additionally, the international trade of animals has also a role toward introduction of new viruses across different geographies.

In Morocco, Bluetongue was reported in 50s where serological investigation showed presence of BTV-10. Since then, this area was free until 2004 when BTV-4 appeared in North West of Atlantic coast, and spread to other regions within a few months. In 2006 and 2007, BT re-appeared with serotype 1 that caused severe epidemics in North Africa region. In 2008, numbers of outbreaks were reduced with disease only in cattle sentinels. BT is currently endemic in Morocco and both serotype 4 and 1 have been reported after 2009. This study allowed us to map the distribution of BTV-1 and 4 in order to understand the geographic distribution of each serotype. The outcomes of this study revealed that both serotypes 1 and 4 were geographically present in a separate area but each year the distance between them become shorter. Furthermore, both serotypes (1 and 4) were reported in the same provinces last years. The resurgence of serotype 4 in 2009 has caused increased number of outbreaks with more pronounced virulence signs than those in 2004. Full genome sequencing has revealed that this BTV-4 was a reassortant virus containing fragments of BTV-1 genome (unpublished communication). Although first epidemics of BTV-1 have affected many regions in Morocco, serological and molecular results of this study confirm dominance of BTV-4 mainly after 2009. BTV-1 seems limited in a few areas while BTV-4 was found spreading largely in Morocco (Figure 1). This new epidemiological situation can be attributed to antigenic diversity within BTV serotypes. A co-circulation of both serotypes (BTV1, BTV4) has been confirmed in many areas in Morocco where BTV-4 assigned outbreaks could probably be another re-assortment between BTV-4 and BTV-1, explaining why there is a reduction of BTV-1 assigned outbreaks during the last years.

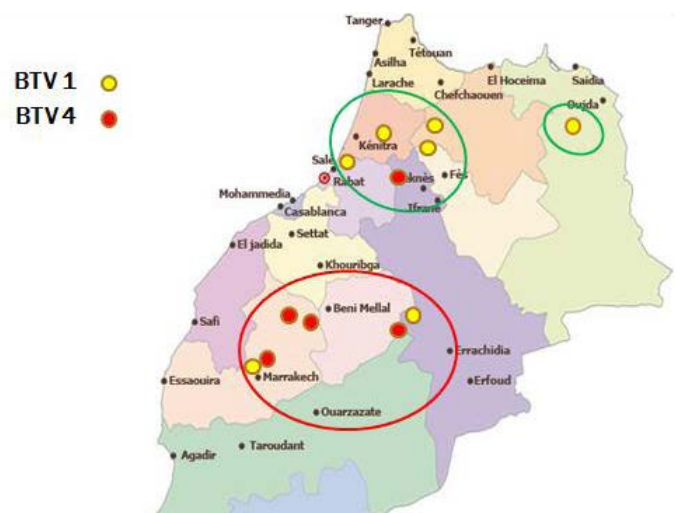


Figure 1: Geographic evolution of BTV-1 and BTV-4 in 2009

Though many vaccination campaigns were carried out since 2005, both BTV-1 and BTV-4 spread significantly in 2010–2011 (Figure 2 and Figure 3).

Table 5: The provenance of viruses isolated in Morocco (2004-2012)

BTV outbreaks	2004	2006	2007	2008	2009	2010	2011	2012
Souss Massa			2		2	2	1	2
Marrakech		4			2	2		2
Chaouia ouardigha casa				3				2
Rabat-Salé	6		3					
Gharb	9		3			2	2	2
Tanger-Tetouan	9		3					
Oriental		5			3		2	3
Meknes	6		2	3	2	2		2
Fes		4		2	2	1		2
Taza-Alhocima	6							
Total	36	13	13	8	11	9	5	15

Table 6: PCR serotyping of BTV isolated in cell culture and eggs

Year	virus isolation in cell culture	virus isolation in eggs	BTV-1	BTV-4
2004	-	36		36
2006		13	13	
2007		13	13	
2008		8	6	2
2009		11	1	4
2010	9		4	5
2011	5			5
2012	15			15

eral times. Thus, a Moroccan study confirm the role of cattle in maintaining both serotypes 1 and 4, using VN assay (Kamar et al., 2014). In addition to this, circulation of BTV-8 in cattle was also proved by the same study which indicated a silent circulation of this serotype without showing any clinical signs or subsequent economic impact. Moreover in 2010, another full genome sequencing of samples from sheep showed another BTV-4 reassortant that contains segments from serotypes 1, 4 and 8 (unpublished communication). This further explains an increase in prevalence of BTV-4 compared to other serotypes.



Figure 2: Geographic evolution of BTV-1 and BTV-4 in 2010

In 2012, except one outbreak of serotype 1 reported in July in the eastern region of the country, virus detected from all reported cases was typed as BTV-4 (Figure 4).

The persistence of BTV in Moroccan livestock remains unclear and the presence of reservoirs were involved sev-



Figure 3: Geographic evolution of BTV-1 and BTV-4 in 2011

Another study showed that domestic dogs seroconvert when infected with BTV. During a BT outbreak in Morocco caused by the same strain of BTV-1 that was used to infect the dogs in an experimental study, a high percentage of the domestic dogs sampled were found to be seropositive for BTV-1 that spread through the ruminant population in the region (Oura et al., 2013). This showed that dogs are capable of being infected with BTV-1, however there lacks data to determine whether dogs mounted

a viraemia in response to BTV-1 infection. Another study showed persistence of viral RNA in blood of dogs, which may be an evidence for a low level replication or could be indicative of persistence of viral inoculum (Oura et al., 2011). Likewise, Batten et al. (2011) revealed isolation of BTV from blood of infected camels indicating that they may be capable of transmitting virus to local *Culicoides* midge population. This suggests that BTV is not depending only to sheep and cattle but canine and camels are also involved in outbreaks at Moroccan region.

script. CL carried out the virus isolation, participated in the serological and molecular studies and helped to draft the manuscript. OFF participated in the design of the study and performed the results analysis. ME participated in the design of the study and coordination. All authors read and approved the final manuscript.

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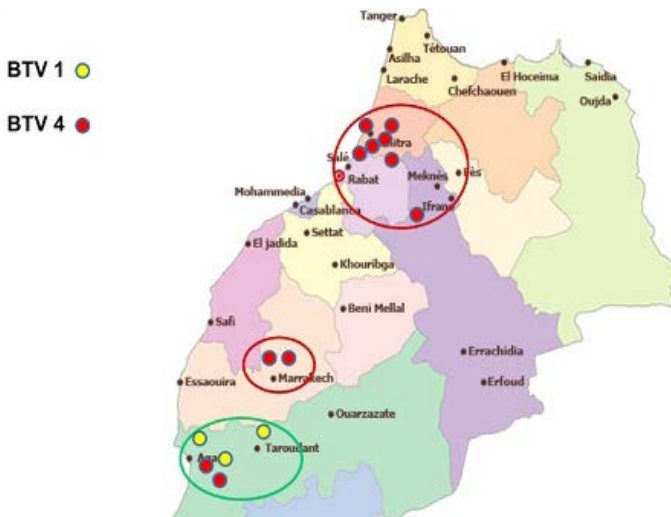


Figure 4: Geographic evolution of BTV-1 and BTV-4 in 2012

The BT outbreaks spread north-west to western Morocco and the evolution of BTV depend upon many factors especially BTV antigenicity, reservoir of viruses and probably silent circulation of other serotypes. Addition to that, vector *C. imicola* continues to be most prevalent vector mainly with global warming and wind systems reported last years in the Mediterranean region.

CONCLUSION

In our study, the dominance of BTV-4 was confirmed mainly after 2009 as compared to BTV-1. Distribution of serotypes circulating in Morocco discloses presence of a new BTV-4 which resists all conditions and adapt easily to changes experienced in Morocco. To that end, investigations based upon full genome sequencing should be carried out to clarify the genetic transformation of BTV serotypes isolated in Morocco.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

KD carried out the molecular characterization studies, the serological studies, virus isolation and drafted the manu-

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