# Persistence of Bluetongue virus serotype 1 virulence in sheep blood refrigerated for 10 years

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**Keywords**
Blood,
Bluetongue,
Sheep,
Storage.

**Summary**
This paper reports that Bluetongue virus serotype 1 (BTV-1) infected blood collected during the 2006 Sardinia (Italy) epidemic from a ewe with clinical disease and stored at ~ 5 °C for 10 years, caused Bluetongue (BT)-like clinical disease and death when inoculated into a susceptible Sarda breed ram. Anatomo-histopathological examination and Real-Time Reverse Transcriptase PCR (Real-Time RT-PCR) confirmed the presence of BTV-1 in several tissues proving that the BTV-1 2006 isolate has maintained its infectivity and virulence.

## Persistenza della virulenza del sierotipo 1 del virus della Bluetongue nel sangue di pecora conservato a bassa temperatura per dieci anni

**Parole chiave**
Sangue,
Bluetongue virus,
Pecora,
Temperatura,
Conservazione.

**Riassunto**
In questo studio, sangue con virus Bluetongue sierotipo 1 (BTV-1), prelevato nel 2006 in Sardegna (Italia) da una pecora con una forma clinica di Bluetongue e conservato a ~ 5 °C per 10 anni è stato inoculato sperimentalmente in un ariete di razza Sarda. L'inoculo ha causato nell'ariete manifestazioni cliniche tipiche della Bluetongue con esito infausto. L'esame anatomo-istopatologico ha confermato la presenza di lesioni riferibili a Bluetongue, mentre la *Real-Time Reverse Transcriptase PCR* ha evidenziato la presenza di BTV-1 nel sangue e in molti altri tessuti. I nostri risultati dimostrano che l'isolato BTV-1 2006 ha mantenuto nel sangue per un periodo di 10 anni a ~ 5 °C sia l'infettività che una elevata virulenza.

Bluetongue virus (BTV) (*Orbivirus; Reoviridae*) is an arbovirus which causes Bluetongue (BT), a disease affecting wild and domestic ruminants with clinical signs being more prominent in sheep (MacLachlan *et al.* 2009). After inoculation into the skin of the susceptible host by the bite of some *Culicoides* species (Diptera; Ceratopogonidae), which act as vectors, BTV replicates in the regional draining lymph nodes from where it spreads to numerous organs, causing viraemia (Darpel *et al.* 2012). In the blood, BTV has been demonstrated to be strictly associated with mononuclear cells and, particularly, with erythrocytes (MacLachlan *et al.* 2009). Viraemia is considered long lasting, up to 63 days, in the ruminant host (MacLachlan 2004, Singer *et al.* 2001). Based on these characteristics, detection of BTV genome by Real-Time Reverse Transcriptase PCR (Real-Time RT-PCR) and/or *in vitro* isolation of the virus from blood are currently the preferred diagnostic tools for confirming infection in the absence of clinical signs.

Viability and infectivity of virus in whole clinical blood samples are affected by storage conditions, such as temperature and time (Wang *et al.* 2011), virus species might, however, play an important role.

Establishing the duration and persistence of viability and virulence of a specific virus in blood samples will be fundamental for understanding its ability to persist under various environmental conditions.

Expecially when field samples from potentially infected animals need to be transported to remote laboratories for confirmation. Virus stability should be undoubtedly taken into consideration in managing reference collections.

It has been demonstrated that BTV seems to be very stable in blood and tissue samples stored at 20˚C, 4˚C and -70˚C but not at -20˚C (Verwoerd and Erasmus 2004) and at high temperatures (Howell *et al.* 1967). Other chemical and physiological characteristics of BTV include stability in buffered lactose-peptone solutions at freeze-dried status (Verwoerd and Erasmus 2004) and instability below pH 6.5 as well as after removal of all extraneous protein (Owen 1964, Svehag *et al.* 1966). Moreover, BTV seems to be relatively resistant to lipid solvents, including ether and chloroform, but it is readily inactivated by disinfectants containing acids, alkali, sodium hypochloride and iodophors (Howell and Verwoerd 1971).

During the BTV serotype-1 (BTV-$1_{IT2006}$) epidemic in 2006 in Sardinia (Italy), samples of whole blood were routinely collected in ethylenediaminetetraacetic acid (EDTA) from sheep displaying clinical signs of BT. These samples were kept at 5 ± 3 °C and sent to the Istituto Zooprofilattico Sperimentale of Sardinia.

A representative aliquot of these samples that tested positive to BTV-1 by nested RT-PCR (Shad *et al.* 1997)

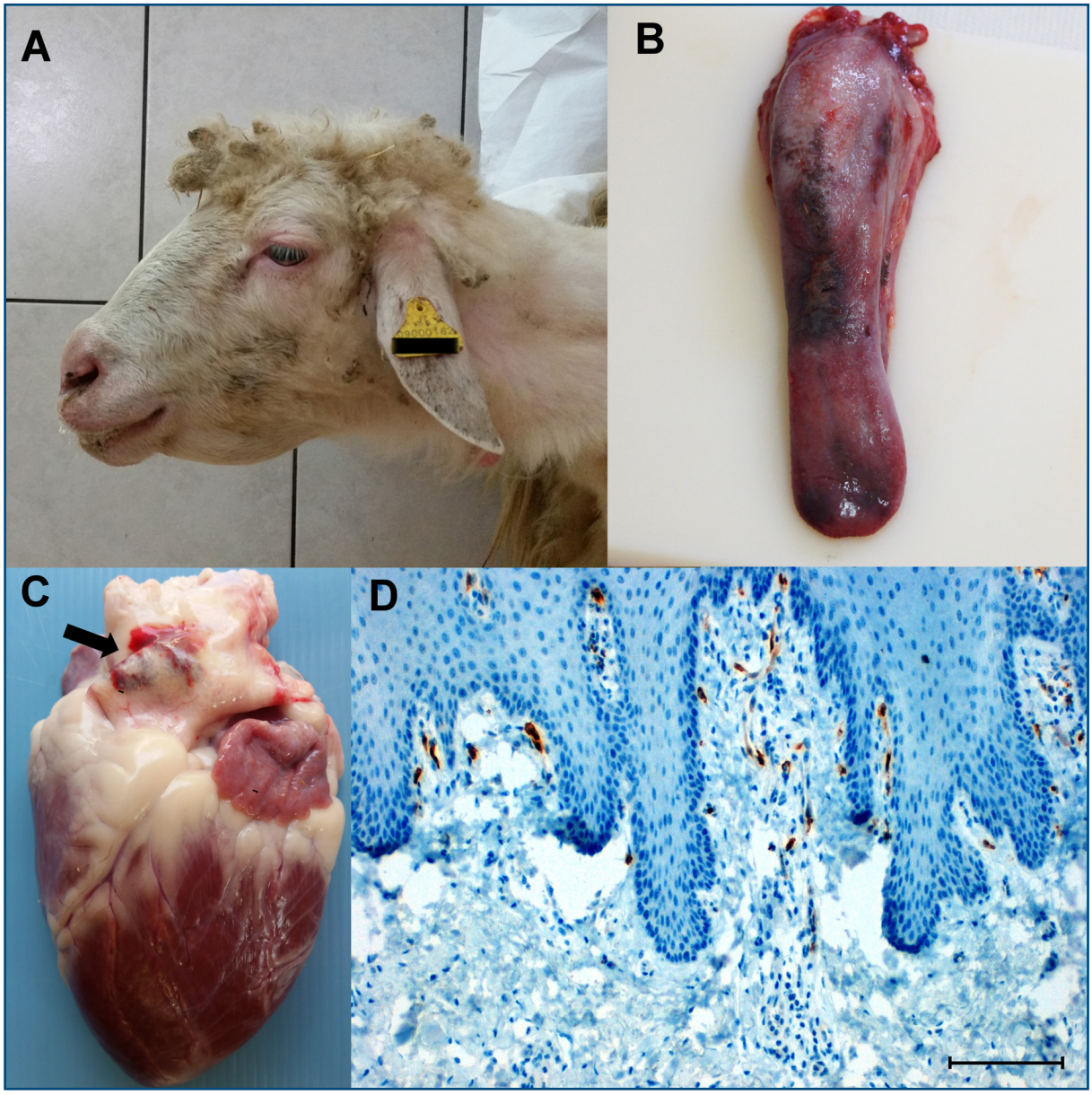


**Figure 1.** *BTV- serotype 1 infected ram.* Subcutaneous oedema, hyperemia of periorbital areas and nostrils (**A**); hemorrhagic and necrotic erosions in the dorsal surface of the tongue (**B**); sub-intimal hemorrhage in the pulmonary artery (**C**); Immunoreactivity for BTV- NS2 protein of the endothelial cells of the small capillaries in the *lamina propria* of the tongue, diaminobenzidine chromogen and Mayer hematoxylin counterstain. Bar = 100 µm.

were stored at 5 ± 3 °C to be used as positive controls in future diagnostic activities. Some of samples were stored under these conditions for 10 years. In accordance with the Quality Control Systems at our laboratory, thermometry instrumentation for monitoring the temperature was installed inside the refrigerator.

In 2016, the limited experimental data available on this issue and a need to reactivate this virus for use as infectious inoculum in a larger experimental study, prompted us to determine the stability and viability of the BTV-$1_{IT2006}$ isolate.

The BTV-$1_{IT2006}$ load in the aforementioned field blood samples was evaluated by Real-Time RT-PCR (OIE Manual 2014) using an AB 7900HT Fast System (Thermo Fisher Scientific). Quantifications were expressed as $TCID_{50}$/ml equivalents by using a standard curve generated from the amplification of three replicates of RNAs, isolated from several (n = 5) 10-fold dilutions of known viral infectious titer in VERO cells ($10^{4.43}$ $TCID_{50}$/ml BTV-1). Standard curve efficiency (Vaerman *et al.* 2004) was 99.9% with $R^2 = 0.99$ ($P < 0.0001$). RNA level in blood pool resulted $10^{3.55}$ $TCID_{50}$/ml equivalents. May-Grunwald-Giemsa staining smears from these BTV-$1_{IT2006}$ infected EDTA blood samples showed that the erythrocytes were still intact with no significant lysis.

After quantification, 10 ml of this blood was mixed with a 5 ml solution of 1,000,000 U.I. of penicillin and 300,000 U.I. of streptomycin, and injected intravenously into a susceptible ram free from antibodies against BTV. The ram was housed in an insect-secure facility. Clinical examination, and blood sampling were carried out daily, to evaluate the viraemia and antibody response against BTV.

Five days post infection (p.i.), the inoculated ram displayed severe conjunctivitis, nasal discharge, hyperaemia of periorbital areas, anorexia, lameness, prostration, and subcutaneous oedema in the inter-mandibular space (Figure 1A). Areas of hemorrhagic-necrotic erosions were observed on the dorsal mucosal surface of the tongue, which exhibited an evident bluish staining (Figure 1B). Progressive worsening of clinical status was observed until death 10 days p.i. Body temperature and viraemia monitoring are shown in Figure 2A-B. Interestingly, the seroneutralization assay was not able to detect neutralizing antibodies in the collected serum samples, thus demonstrating a high virulence of this BTV-1 isolate.

At necroscopy, sub-intimal hemorrhages in the pulmonary artery (Figure 1C), foci of necrosis on the papillary muscles of the left ventricle and multifocal hemorrhages in the endocardium were observed. The digestive system, particularly the rumen, showed vascular congestion, hemorrhages and

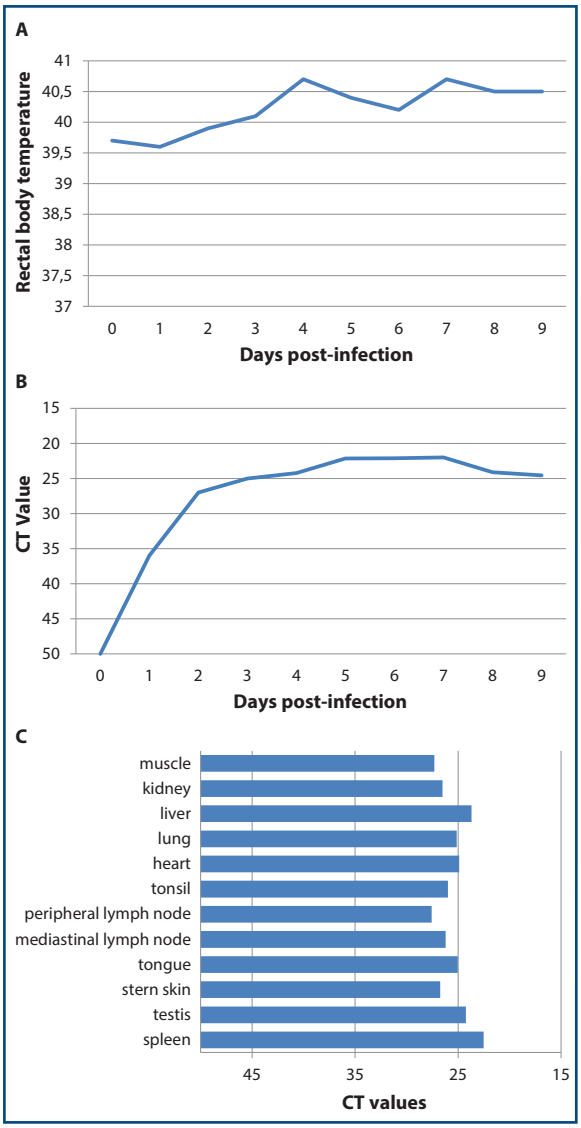


**Figure 2.** *Trend of rectal body temperature (**A**), Real-Time RT-PCR Threshold cycle (CT) values for BTV-serotype 1 (BTV-1) in blood (**B**) and others tissues (**C**) of a ram intravenously inoculated with BTV- 1 naturally infected sheep blood stored at 5 ± 3°C for 10 years.*

erosions in the mucosa. Peripheral lymph nodes were enlarged, edematous and hemorrhagic. These gross lesions were mirrored by microscopical changes characterized by severe perivasculitis/vasculatis of the small vessels with endothelial hypertrophy, oedema, vessel congestion and hemorrhages in the derma of the skin as well as in the *lamina propria* of the digestive mucosae. By immunohistochemical staining, BTV NS2 protein was detected in the endothelial cells of the small capillaries of the tongue, under the epithelium-*lamina propria* junction (Figure 1D). Real-Time RT-PCR detected BTV RNA in several organs (Figure 2C).

Conventional methods of long-term preservation of viruses usually entail setting up a vial of virus suspension, prepared by specific protocols, and storage at ultra-temperatures (-70°C),

cryopreservation in liquid nitrogen and freeze-dried techniques (Gould 1999).

We demonstrate that BTV collected from an infected sheep does maintain infectivity and virulence in blood sample with EDTA for 10 years at 5 ± 3 °C. This suggests that there are alternative and easily accessible options for the long-term storage and transport of BTV. Our study focused on the use of natural host bioassay as a measure of BTV stability in blood samples. In this regard, the clinical, pathological and virological aspects observed correspond with those commonly reported in natural or experimental cases with highly pathogenic BTV, which causes acute fatal disease (MacLachlan *et al.* 2008), thus excluding the possibility that other unknown infectious agents could have been preserved in the blood.

Among the mammalian viruses, smallpox is believed to be capable of surviving for very long periods of time (Wolff and Croon 1968, Gould 1999).

However, no other characterized examples of animal virus long-term survival with a decade long perspective, in tissue samples or excreta kept at natural circumstances, have been reported.

*In vitro* and *in vivo* experimental studies have shown the tropism of BTV for a number of cell types, including monocytes/macrophages, lymphocytes, dendritic cells and microvascular endothelium (MacLachlan *et al.* 2009). In the viremic phase of the infection, BTV is engulfed into the cell membrane invagination of bovine erythrocytes, remaining infectious despite the presence of neutralizing antibodies (Brewer and MacLachlan 1992, Parsonson and McColl 1995).

We speculate that this intimate association between BTV and the erythrocyte membrane not only facilitates a prolonged viraemia and consequently the infection of biting vectors, but that it also functions as a mechanism protecting the viability of the virus, even outside of the host organism. However, our infected blood samples were stored in EDTA Vacutainer®, and it cannot be ruled out that this anticoagulant played a role in preserving virus infectivity. Indeed, EDTA enhanced preservation of structural components of the blood cells (Banfi *et al.* 2007), thereby protecting the viral capsid in the red blood membrane. Interestingly, May-Grunwald-Giemsa staining showed that erythrocyte membranes in the BTV-$1_{IT2006}$ blood were intact. Nevertheless, EDTA seems to have conflicting effect on virus infectivity. On the one hand, it extended Food and Mouth Disease virus (FMDV) activity for up to seven weeks (Crick *et al.* 1966), while on the other hand, viral load of Mason-Pfizer monkey virus (M-PMV), a D-type retrovirus causing immunodeficiency syndrome and tumors in old world monkeys, significantly decreased after seven days in $K_3$EDTA blood (Qin *et al.* 2014). In addition, EDTA reduced Dengue virus (DENV) liberation from BHK-21 cells in $Ca^{++}$-free culture, at contrary, no effect was observed on Chikungunya virus (Matsumura and Yamashita 1978).

It can be mention that other Orbiviruses, e.g. African horse sickness virus (AHSV), are strictly associated with erythrocytes during viraemia (MacLachlan and Guthrie 2010) and can maintain their stability and infectivity for one year in washed erythrocytes held at ~ 5 °C (House *et al.* 1990). Similarly, DENV, also a RNA arbovirus in the family *Flaviviridae*, maintains its replication ability when stored in buffer containing platelets and erythrocytes (Sutherland *et al.* 2016). BTV is an arbovirus, hence, resistance in the environment does not seem to be of epidemiological importance. However, *Culicoides* midges are poikilothermic and it is known that environmental temperature can influence replication and virulence of BTV inside the insect (Mullens *et al.* 1995, Paweska *et al.* 2002).

Although we did not determine if there was a reduction in the virus titre over the 10-year storage and only one sample was tested, we believe that this finding offers the opportunity to conduct retrospective studies on stored tissues and to have an easy, functional and practical way of storing BTV infected samples. Finally, our result stimulates interest for gaining a better understanding of the biological mechanism by which viruses preserve infectivity for extended periods in tissues and fluids collected from infected hosts.

## Ethics statement

The protocol of the slaughtering procedures, involving the ram investigated herein, was officially approved by the Service for Animal Welfare of the IZS, according to the guidelines n. I 09 044. The experiment was carried out in agreement with the prescriptions of Italian National Law (Art. 31 Decr. Leg. 4 march 2014 n. 2; Permission: N. 1248/2015-PR)

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