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# Pathogenicity study in sheep using reverse-genetics-based reassortant bluetongue viruses

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ABSTRACT

Bluetongue (BT) disease, caused by the non-enveloped bluetongue virus (BTV) belonging to the *Reoviridae* family, is an economically important disease that affects a wide range of wild and domestic ruminants. Currently, 26 different serotypes of BTV are recognized in the world, of which BTV-8 has been found to exhibit one of the most virulent manifestations of BT disease in livestock. In recent years incursions of BTV-8 in Europe have resulted in significant morbidity and mortality not only in sheep but also in cattle. The molecular and genetic basis of BTV-8 pathogenesis is not known. To understand the genetic basis of BTV-8 pathogenicity, we generated reassortant viruses by replacing the 3 most variable genes, S2, S6 and S10 of a recent isolate of BTV-8, in different combinations into the backbone of an attenuated strain of BTV-1. The growth profiles of these reassortant viruses were then analyzed in two different ovine cell lines derived from different organs, kidney and thymus. Distinct patterns for each reassortant virus in these two cell lines were observed. To determine the pathogenicity of these reassortant viruses, groups of BTV-susceptible sheep were infected with each of these viruses. The data suggested that the clinical manifestations of these two different serotypes, BTV-1 and BTV-8, were slightly distinct and BTV-1, when comprising all 3 genome segments of BTV-8, behaved differently to BTV-1. Our results also suggested that the molecular basis of BT disease is highly complex.


## 1. Introduction

Bluetongue (BT), an insect-transmitted, non-contagious viral disease of domestic and wild ruminants is caused by bluetongue virus (BTV). The disease is characterized by inflammation of the mucous membranes, congestion, swelling, hemorrhages and is often accompanied with high mortality in sheep (Erasmus, 1975; Maclachlan et al., 2009). Although cattle and goats usually carry the virus for a certain period of time without showing any apparent clinical signs of disease, they are capable of transmitting the virus to other ruminants via biting *Culicoides* midges.

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BTV is present in a broad band of countries extending approximately between 40°N and 35°S (Mellor et al., 2000; Purse et al., 2005). Until 15 years ago, Europe was essentially BT-free apart from Cyprus; however, since 1998 at least one of the 26 serotypes of BTV has been active on the continent every year, mainly in the Mediterranean basin (Maclachlan and Guthrie, 2010; Mellor et al., 2008; Purse et al., 2005). In 2006 a highly pathogenic BTV-8 strain emerged for the first time in Northern Europe, spreading very rapidly and affecting thousands of herds. The same serotype re-emerged in 2007 and 2008, causing devastating disease not only in sheep but also in cattle with high morbidity and mortality (Elbers et al., 2008a, 2008b; Wilson and Mellor, 2009). Studies involving molecular epidemiology have also shown that the most severe disease in northern European sheep and cattle was caused by BTV-8 (Dal Pozzo et al., 2013; Martinelle et al., 2013; Purse et al., 2005). The phenotypic differences between BTV-8 compared with less virulent strains suggested that genetic background may be partly responsible. However, the mechanism of pathogenicity is still very poorly understood.

BTV is a member of the *Orbivirus* genus within the *Reoviridae* family. Like other members of the family, BTV has a genome of 10 segmented double-stranded RNA (segments S1–S10) that are enclosed within two capsids. While the inner core is made up of 5 highly conserved proteins (VP1, VP3, VP4, VP6 and VP7), the outer capsid consists of two variable proteins, VP2 (receptor-binding protein and serotype determinant) and VP5 (membrane penetration protein). In addition, BTV also encodes for 4 non-structural proteins (NS1–NS4), of which NS3 encoded by S10 is more variable than NS1 and NS2. NS3 is shown to be involved in virus trafficking and release from the infected host (Beaton et al., 2002; Celma and Roy, 2009). Recently it has been shown that NS3 is also involved in the regulation of the induction of interferon type 1 (Chauveau et al., 2013), suggesting a role in the innate immune response.

In this study, we designed reassortant viruses between BTV-8 and BTV-1 to establish the genetic basis of BTV pathogenicity. The rationale for designing reassortant viruses was based on the two most variable proteins of the outer capsid (VP2 and VP5) and the non-structural protein NS3, which is the most variable within BTV NS proteins. Reassortant viruses were generated using a reverse genetics (RG) system replacing these three RNA segments (S2, S6 and S10) of the low virulent strain, BTV-1, with that of highly virulent BTV-8, either singly or in combinations. The phenotypic characteristics of the disease caused by these reassortant viruses were analyzed by infection of sheep. Our results suggested that all three proteins together are involved in the disease outcome and that the molecular basis of BTV pathogenicity is highly complex.

## 2. Methods

### 2.1. Cells and viruses

BSR cells (BHK-21 subclone) were maintained in Dulbecco modified eagle medium (DMEM, Sigma–Aldrich) supplemented with 5% (v/v) fetal bovine serum (FBS, Invitrogen). PT and SFT-R cells (ovine-derived kidney and thymus cells respectively, Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut, Insel Riems, Germany) were maintained in minimum essential medium eagle (MEM, Sigma–Aldrich) supplemented with 10% (v/v) FBS.

BTV-1 (South African strain) and BTV-8 (Ardennes isolate) viral stocks were generated by infection of BSR cells and kept at 4 °C until use.

### 2.2. Recovery of reassortant BTV-1/BTV-8 viruses

Segments S2 (VP2), S6 (VP5) and S10 (NS3) (GenBank accession numbers: KJ872780–KJ872782) of BTV-8 were obtained using a sequence-independent cloning system as previously described (Boyce et al., 2008; Maan et al., 2007; Matsuo et al., 2011). Briefly, dsRNAs from purified core particles were ligated to a self-annealing primer before RT-PCR amplification using a specific primer. Each cDNA amplified from segments S2, S6 and S10 of BTV-8 was cloned into pUC19 and fully sequenced (Source Bioscience) before insertion of the T7 promoter at the 5′ end and insertion of a unique restriction enzyme site that generates the correct end of the segment at the 3′ end.

For synthesis of uncapped T7 transcripts for segments S2, S6 and S10, RiboMAX Large-Scale RNA Production System T7 (Promega) or TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific) kits were used according to manufacturer's instructions. Reassortant viruses between BTV-1 and BTV-8 were recovered from confluent monolayers of BSR cells after transfection with a full set of BTV T7 transcripts as described before (Boyce et al., 2008). Individual plaques were picked, amplified and virus stocks were kept at 4 °C.

### 2.3. Virus growth kinetics and characterization

The genomic dsRNA profile was analyzed for each reassortant. Monolayers of BSR cells were infected with reassortant or parental viruses and upon complete cytopathic effect, infected cells were harvested. The genomic dsRNA was purified with Tri reagent (Sigma) using standard methods. The parental origin of segments S2, S6 and S10 was determined by differential mobility in non-denaturing PAGE or by sequencing.

For virus growth study, monolayers of PT and SFT-R cells were synchronously infected at a multiplicity of infection (MOI) of 1 and samples were collected at 0, 12, 24 and 48 h (hours) post-infection (p.i.). Cells and supernatant were harvested, subjected to two freeze/thaw cycles and the total titer was determined by plaque assay in triplicate and expressed as plaque formation units per ml (PFU/ml) or by tissue culture infective dose 50 ($TCID_{50}$). The mean, standard deviation and the *p* values were also determined by Excel (Microsoft). Viral protein expression was determined by Western blot (WB) using specific antibodies against structural VP5, VP7 and non-structural NS3 proteins. As loading control, an antibody against β-actin (Sigma) was used. Each blotting experiment was