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Virus and Host Factors Affecting the Clinical Outcome of Bluetongue Virus Infection

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Running Title: Pathogenesis of Bluetongue

ABSTRACT

Bluetongue is a major infectious disease of ruminants caused by bluetongue virus (BTV), an arbovirus transmitted by *Culicoides*. Here, we assessed virus and host factors influencing the clinical outcome of BTV infection using a single experimental framework. We investigated how mammalian host species, breed, age, BTV serotypes, and strains within a serotype, affect the clinical course of bluetongue. Results obtained indicate that in small ruminants there is a marked difference in the susceptibility to clinical disease induced by BTV at the host species level, but less so at the breed level. No major differences in virulence were found between divergent serotypes (BTV-8 and BTV-2). However, we observed striking differences in virulence between closely related strains of the same serotype collected towards the beginning and the end of the European BTV-8 outbreak. As observed previously, differences in disease severity were also observed when animals were infected with either blood from a BTV-infected animal or from the same virus isolated in cell culture. Interestingly, with the exception of two silent mutations, full viral genome sequencing showed identical consensus sequences of the virus before and after cell culture isolation. However, deep sequencing analysis revealed a marked decrease in the genetic diversity of the viral population after passaging in mammalian cells. In contrast, passaging in *Culicoides* cells increased the overall number of low frequency variants compared to virus never passaged in cell culture. Thus, *Culicoides* might be a source of new viral variants and viral population diversity can be another factor influencing BTV virulence.

48 **IMPORTANCE**

49 Bluetongue is one of the major infectious diseases of ruminants. It is caused by an arbovirus known as
50 Bluetongue virus (BTV). The clinical outcome of BTV infection is extremely variable. We show that
51 there are clear links between the severity of bluetongue and the mammalian host species infected,
52 while at the breed level differences were less evident. No differences were observed in the virulence
53 of two different BTV serotypes (BTV-8 and BTV-2). In contrast, we show that the European BTV-8 strain
54 isolated at the beginning of the bluetongue outbreak in 2006 was more virulent than a strain isolated
55 towards the end of the outbreak. In addition, we show that there is a link between the variability of
56 the BTV population as a whole and virulence and our data also suggest that *Culicoides* cells might
57 function as an "incubator" of viral variants.

58

INTRODUCTION

Bluetongue is one of the major infectious diseases of ruminants and is caused by bluetongue virus (BTV), a virus transmitted from infected to uninfected hosts by *Culicodes* biting midges (1). BTV is the type species of the genus *Orbivirus* within the virus family *Reoviridae* and possesses a genome consisting of 10 segments of double-stranded RNA (dsRNA) encoding for 7 structural and 4 non-structural proteins (1-3). The icosahedral particle is organized as a triple layer of capsid shells (4, 5). The outer capsid is formed by VP2 and VP5, while the inner layer is composed of two major proteins, VP3 (sub core) and VP7 (core), encasing the 10 genomic segments of linear dsRNA and three minor enzymatic proteins, VP1 (RNA-dependent RNA polymerase), VP4 (RNA capping enzyme) and VP6 (RNA-dependent ATPase and helicase) (2, 4, 5). In addition, BTV expresses four non-structural proteins (NS1, NS2, NS3 and NS4) involved in virus replication, morphogenesis and in counteracting the innate immune system of the host (3, 6, 7).

There are at least 26 BTV serotypes (BTV-1 to BTV-26) circulating worldwide. Serotypes are determined primarily by differences in the outer capsid protein VP2, which induces neutralizing antibodies in infected animals (8-13).

Bluetongue is enzootic in areas where the mammalian reservoirs, the virus and the insect vector have the opportunity to coexist in climatic conditions conducive to BTV replication and transmission. As a result, historically BTV was present exclusively in tropical and subtropical areas of the world where suitable conditions exist. However, in the last 10-20 years the global distribution of bluetongue, similarly to some of the other vector borne diseases, has expanded dramatically, potentially due to a variety of factors including an increased global travel and commerce, deforestation and climate change (14-17).

An interesting aspect of bluetongue is the extreme variability of the clinical outcome as a result of BTV infection. In many cases, BTV induces only mild or inapparent clinical infections, while in others it can

kill the infected host. Symptoms of Bluetongue have been mainly attributed to the damage of small blood vessels increasing vascular permeability and resulting in hyperemia, congestion, vascular thrombosis, localized / diffused edema, hemorrhages and erosion of the mucous membranes. The main clinical signs of affected animals include fever, depression, respiratory distress and anorexia (18-21).

This variability of clinical outcomes induced by BTV has been attributed to a variety of factors such as species, breed, age and the immune status of the mammalian host, as well as the serotype/strain of the virus (21-24). In general, sheep, yak, llamas, and alpacas have been described as the most sensitive species to BTV–induced disease. Cattle and other wild ruminants have a certain degree of resistance to disease, although they are fully susceptible to infection. Cattle show longer periods of viremia and are, therefore, considered reservoirs of infection (21, 25-31). Goats are also susceptible to BTV infection but do not appear to be very susceptible to disease, although contrasting reports appear in the literature and the heterogeneous experimental conditions used in different studies make it difficult to compare the available data (19, 24, 32-36).

The immunologic status of infected animals understandably has a major influence on the susceptibility to infection and explains why outbreaks of bluetongue typically occur when susceptible animal species are introduced into endemic areas, or when virulent strains of BTV reach previously unexposed ruminant populations (21). Animals infected with a specific BTV serotype produce long lasting neutralizing antibodies with limited cross protection against heterologous serotypes (37). Environmental factors, such as the exposure to solar radiation or high temperatures, can also exacerbate the disease symptoms (38, 39).

While infection of sheep in the tropics and subtropics is common, clinical disease in indigenous breeds is rarely observed. The North European breeds of sheep have been described to be very susceptible to BTV-induced disease as opposed to African or South-East Asian breeds (19, 22, 40-47). Within the

same sheep breed, or even within the same flock, there may be considerable differences in the severity of the disease occurrence in individual animals (21, 23).

Serotypes/strains of BTV with different degrees of virulence have been described in the literature. For example, the North European BTV-8 strains that spread since 2006 in Northern Europe is considered highly virulent as it induced high levels of mortality in naïve sheep and in some cases also caused severe clinical disease in cattle (48-51). On the other hand, it is interesting to note that no clinical cases of disease were observed even in sheep when BTV-8 reached Northern Italy and Sardinia a few years later (G. Savini, personal communication). Other serotypes related to vaccine strains (BTV-6, BTV-11, BTV-14) have entered Europe briefly, in general showing very little pathogenicity in the field (52-54).

Bluetongue is experimentally reproducible and several studies have addressed, directly or indirectly, the variability of the clinical outcome resulting from BTV infection, although at times with contradictory results (55, 56). The heterogeneous experimental conditions used in different studies make it difficult at times to compare the available data. For example, many of the BTV strains used in experimental studies have been passaged more or less extensively in cell culture and this can potentially lead to attenuation of virulence (57, 58). In addition, some reports in the literature stress that experimental infection using BTV strains isolated in mammalian cell cultures from lethal cases of bluetongue most often results only in the induction of mild clinical signs of the disease (39, 59, 60). Thus, some investigators have used blood from viremic animals as an inoculum and this appeared to be a very effective way to induce severe clinical signs in the infected animals (20, 61). However, the induction of severe clinical signs of bluetongue have also been reported using BTV passaged in cell culture (62) or virus isolated in embryonated eggs (32, 40).

Here, we used a single experimental framework and standardised conditions in order to systematically assess virus and host factors influencing the clinical outcome of BTV infection. We evaluated

differences in susceptibility to BTV-induced disease in goats, and sheep of different breeds. In addition, we studied differences in the virulence of two divergent BTV serotypes (BTV-2 and BTV-8), as well as the virulence of different BTV-8 strains isolated at the beginning and end of the North European outbreak of 2006-2008. Finally, we evaluated whether genetic bottlenecks (63) exist that can influence BTV adaptation in *Culicoides* and mammalian cells and also how these influence virulence.

MATERIAL AND METHODS

Cells. Mammalian cells were grown at 37°C in a humidified atmosphere supplemented with 5% CO2. BHK-21, BSR (a clone of BHK-21 cells), and African green monkey VERO cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). CPT-Tert cells are sheep choroid plexus cells immortalized with the simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (hTERT) and were grown at 37°C in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% FBS (64). KC cells (65) were derived from *Culicoides sonorensis* larvae and grown at 28°C in Schneider's insect medium supplemented with 10% FBS.

Virus strains and titrations. BTV-$8_{NET2006}$ (Pirbright reference collection number NET2006/04) was originally isolated from a naturally infected sheep during the 2006 outbreak in Northern Europe and has been previously described (3). BTV-$8_{NET2007(blood)}$ was derived from the spleen of a sheep infected with blood derived from a naturally infected cow in the Netherland during the 2007 BTV-8 outbreak as already described (66). Further viruses were isolated *in vitro* from BTV-$8_{NET2007(blood)}$ after (i) 1 passage in KC cells (BTV-$8_{NET2007(1KC)}$), (ii) 1 passage in KC and 1 passage in BHK_{21} cells (BTV-$8_{NET2007(1KC/1BHK)}$) and (iii) 1 passage in KC and 2 passages in BHK_{21} cells (BTV-$8_{NET2007(1KC/2BHK)}$).

BTV-2_{IT2000} and BTV-8_{IT2008} were derived from naturally occurring outbreaks of bluetongue in sheep in Italy and were isolated in 2000 and 2008 respectively. All viruses used in this study were isolated in KC cells and subsequently passaged twice in BHK-21 cells before use in experimental infections. Virus stocks were prepared by infecting BHK-21 cells at a multiplicity of infection (MOI) of 0.01 and collecting the supernatant when obvious cytopathic effect (CPE) was observed. Supernatants were clarified by centrifugation at 500 g for 5 min and the resulting virus suspensions aliquoted and stored at 4°C. Virus titers were determined by standard plaque assays (67). In order to compare the growth of the various BTVs strains used in this study, CPT-Tert cells were infected at a multiplicity of infection (MOI) of 0.01 and supernatants collected at 8, 24, 48, 72 and 96h post infection (p.i.). Samples from

each time point were subsequently titrated by endpoint dilution analysis in BSR cells, and titers were expressed as 50% tissue culture infective doses ($TCID_{50}$). Each assay was repeated at least twice using two different virus stocks.

BTV genome sequencing. The complete genome sequences were derived from the following strains: BTV-8_{IT2008}, BTV-$8_{NET2007(blood)}$, BTV-$8_{NET2007(1KC)}$, BTV-$8_{NET2007(1KC/1BHK)}$ and BTV-$8_{NET2007(1KC/2BHK)}$. dsRNA was extracted from the spleen or infected cells as previously described (57). Full length genome segments were amplified from dsRNA using SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) using primers complementary to the 5' or 3' end termini of the viral genome segments. The genome of BTV-8_{IT2008} was sequenced using the Sanger method. For the other viruses, equimolar, purified PCR products of the 10 genomic segments of each virus were pooled, sheared by focused sonication (Covaris), followed by size selection using Ampure XP magnetic beads. Illumina MiSeq libraries were generated using the KAPA real-time Library preparation kit (KAPA), further quantified using qRT-PCR (KAPA) and sequenced using an Illumina MiSeq with a 300 cycle cartridge as suggested by the manufacturers. Analysis of genetic diversity was carried out using CLC Genomic Workbench Version 6.0.1 (CLC bio). After quality assessment and the removal of sequencing artefacts, reads were mapped using BTV-$8_{NET2006}$ as a reference sequence and the consensus sequences extracted. Reads with a similarity fraction below 70% were omitted in the final assembly. Single nucleotide polymorphisms were identified using the quality based variant detection function within CLC Genomics Workbench Version 6.0.1. Total sample reads were mapped to the consensus sequence of each segment and variants were called using as parameters nucleotides with total coverage of over 100 reads and central quality score of Q20 or higher. Average quality score per nucleotide was above Q35.8 in all samples. The mean depth of coverage per variant in each viral genome was between 8154 and 12461. Presence of both forward and reverse read were required to call a variant while the frequency threshold was arbitrarily set at 0.1%.

Nucleotide sequence accession numbers. Sequences of BTV-2_{IT2000} and BTV-8_{IT2008} have been deposited in GenBank and were assigned accession numbers KM053268 to KM053277 (BTV-2_{IT2000}) and KM053258 to KM053267 (BTV-8_{IT2008}). The raw data used for deep-sequencing analyses are available upon request.

Experimental infections in mice. Transgenic mice deficient in type I interferon (IFN) receptor (129sv IFNAR$^{-/-}$; B&K Universal Ltd.) were maintained at biosafety level 3. For each experiment, groups of adult mice matched for sex and age (n=5 per group), were infected intraperitoneally with 300 PFU of virus or mock-infected as indicated in the Results section. Mice were examined for clinical symptoms daily until the experiment was concluded at 14 days post infection.

***In vivo* pathogenicity studies.** Animal experiments were carried out at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale" (Teramo, Italy) in accordance with local and national approved protocols regulating animal experimental use (protocol n. 10933/2011 and 7440/2012). Studies were conducted using a total of 65 sheep and 10 goats held in an insect-proof isolation unit with veterinary care. All animals were confirmed to lack antibodies towards BTV using a BTV blocking ELISA as previously described (68). The absence of BTV-specific antibodies was confirmed for each animal using a BTV-specific qRT-PCR in blood samples (see below). For this study, all animals were infected intradermally with a total of $2X10^6$ PFU (in 5 ml) of the specific BTV strains indicated below by multiple inoculations in the inner leg and in the pre-scapular areas. Negative controls were inoculated with 5ml of mock-infected cell-supernatant. Groups (n=5 animals per each group) of domestic goats, 8 months old Dorset, and two years old Sardinian, Dorset and Italian mixed breed sheep were infected with BTV-$8_{NET2006}$. Two additional groups of Sardinian sheep were inoculated with BTV-8_{IT2008} or BTV-2_{IT2000}. Two additional groups of Sardinian sheep (n=5 per group) were inoculated with either 5ml of infected blood (BTV-$8_{NET2007(blood)}$) or with the same virus after passage in KC and BHK_{21} cells (BTV-$8_{NET2007(1KC-2BHK)}$). All viruses used in this study have the same passage history (1 passage in KC cells and

two passages in BHK_{21} cells) unless indicated otherwise. 5 goats and 25 sheep (5 adult Dorset, 5 young Dorset, 5 Italian mixed breed and 10 Sardinian sheep) were used as negative controls and were inoculated with uninfected cell culture media. Blood samples were collected (with EDTA) from all infected animals, daily for 15 days post-infection and thereafter at day 17, 19, 21 and 28 p.i. when the experiment was concluded. The blood samples were analyzed for the presence of viremia by qRT-PCR (see below). Serum samples were collected from each animal on the day of the inoculation (day 0) and then at day 7, 14, 21 and 28 p.i. Sera were tested by virus neutralization assay for the presence of BTV-specific antibodies. Body temperature and clinical signs were recorded daily, beginning a week before inoculation, until day 15 p.i. and subsequently at day 17, 19, 21 and 28 pi. Fever was defined as rectal temperature above 40°C. Clinical signs were scored using a clinical reaction index (CRI) with minor modifications as already described (66) (Table S1).

Virus neutralization assays. The presence of neutralizing antibodies in infected sheep and goats, against the BTV strains used was assessed by neutralization assays testing serial 2-fold dilutions of sera as already described (69). Briefly, serum dilutions (1:10 to 1:1,280) and a fixed amount of virus (100 $TCID_{50}$) were incubated for 1h at 37°C in 96 well plates, whereupon 100 µl suspension of VERO cells (3×10^5/ml) was added to each well in minimum essentials medium (MEM). Plates were incubated for 6–7 days at 37°C, 5% CO_2 after which monolayers were then scored for cytopathic effect (CPE). The titer of neutralizing antibodies in each serum sample was determined by endpoint dilution assays (70). Values reported for each sample are the $\log_{10}$ of the 50% endpoint (proportionate distance, PD) of 4 replicates performed using VERO cells.

qRT-PCR. Viremia in experimentally infected animals was assessed by qRT-PCR as already described (57, 69). Briefly, blood samples (500 µl) were pre-treated with 1 ml cold distilled water on ice for 10 min and then centrifuged at 4°C for 10 min at 13000 *g*. Armored RNA (Asuragen, USA) was added to

each sample before RNA extraction and used as an internal control to verify RNA extraction efficiency. Total RNA was extracted from the resulting cellular pellet, using the High Pure Nucleic Acid extraction kit (Roche, Nutley, New Jersey), in accordance with the manufacturer's instructions. The quality of the samples was further assessed by amplifying the sheep β-actin gene as previously described (71). For each sample, 250 ng of RNA was used in a one-step qRT-PCR reaction employing primers/probes for segment 5 (encoding NS1) of BTV and the armored control RNA. Samples were analysed using a 7900HT fast real-time PCR system and the sequence detection system software SDS, version 2.3 (Applied Biosystems). BTV genome copy numbers expressed as $\log_{10}$/µg of total RNA were derived using a standard curve generated from the amplification of *in vitro* transcribed synthetic BTV segment 5 RNA using the mMESSAGE mMACHINE T7 Ultra Kit (Ambion), according to the manufacturer's instructions. Signal levels with CT values ≥ 40 were considered negative.

Statistical analysis. Statistical analysis was carried out using the software Prism (GraphPad). Significance of differences in body temperature between groups of infected animals was estimated by calculating the total area under the curve (AUC) of body temperatures between days 3 to 11 p.i. for each animal. Significant differences between groups were calculated using an unpaired t test or ANOVA as appropriate. The AUC relative to the levels of BTV RNA in the blood was calculated for each animal from day 1 p.i. to the end of the experiment and groups were compared using an unpaired t test or ANOVA as appropriate. In addition, significant differences in the peak levels of viremia were also compared using an unpaired t test or ANOVA as appropriate.

RESULTS

Replication kinetics *in vitro* and virulence in mice of BTV-2_{IT2000}, BTV-$8_{NET2006}$ and BTV-8_{IT2008}. In order to investigate virus and host factors affecting the clinical outcome of BTV infection we initially focused on three different strains of bluetongue: a BTV-2 strain isolated from Italy in 2000 (BTV-2_{IT2000}), a BTV-8 strain isolated from the Netherlands in 2006 (BTV-$8_{NET2006}$) and a BTV-8 strain isolated in Italy in 2008 (BTV-8_{IT2008}).

Firstly, we assessed the ability of all viruses to replicate in sheep CPT-Tert cells. No major differences were observed in the replication kinetics of the viruses regardless of the serotype and strain used in the assay (Fig. 1A). We next assessed the virulence of each strain in $IFNAR^{-/-}$ mice as these mice succumb to wild type BTV infection (57, 72). Mice were inoculated intraperitoneally with 300 PFU of the BTV strains above. All of the mice inoculated with the various BTV strains showed clinical signs around 3 days p.i. characterized by ocular discharge, apathy and lethargy. All BTV infected mice died between 6 and 8 days post-infection while no signs of disease were observed in the control mock-infected mice (Fig. 1B).

Influence of species, breed and age of the mammalian host on the clinical outcome of BTV infection. Several studies investigating the factors that affect the clinical outcome to BTV infection have already been published (1, 20, 21, 73). Here, we aimed to assess the variables affecting the pathogenesis of bluetongue in a single experimental framework. First, we assessed the outcome to BTV infection in 2 year-old goats and sheep of three different breeds (the Northern European Dorset Poll, the Italian Sardinian sheep and a mixed breed from Central Italy). An additional group of Dorset poll sheep, 8 months old in age, were also used in the study. We deliberately used viruses isolated in KC cells and subsequently passaged twice in BHK-21 for all the experimental infections carried out in this study. This strategy allowed us to use viruses minimally passaged *in vitro* and with the same history in cell culture.

Sheep infected with BTV-$8_{NET2006}$ developed classic clinical signs of bluetongue including fever (defined here as body temperature > 40°C), which started 4-5 days p.i., depression, anorexia, respiratory distress, increase in salivation, facial edema and hyperaemia of nasal and buccal mucosa (Fig. 2 and Supplementary Fig. S1, showing data for each individual animal). Overall, no major differences in clinical signs were observed between the three sheep breeds used in this study nor between 8 month old and 2 year old Dorset poll sheep. In addition, no significant differences ($p > 0.05$) were observed in the levels of fever or the cumulative number of days with fever between all the sheep groups. However, one sheep in the mixed breed infected group had to be euthanized because of onset of severe clinical signs. Consequently, the general and total clinical score of the infected mixed breed group was higher than the other groups (Fig. 2A). In all the infected groups, BTV RNA in the blood peaked at about 5 days p.i. then slowly decreased, although it remained detectable up to 4 weeks p.i. at which point the experiment was concluded (Fig. 2C and Supplementary Fig. S1). Neutralizing antibodies were detected at day 7 p.i., peaked by 14 days p.i. and then remained essentially constant for the duration of the experiment (Fig. 2D).

On the other hand, goats after BTV-$8_{NET2006}$ infection showed no clinical signs nor fever throughout the duration of the experiment (28 days) (Fig. 2A-B). Differences in the body temperature between day 3 and 10 post-infection were statistically significant between goats and each of the groups of sheep used above ($p< 0.0001$). The onset of viremia in goats was delayed, compared to infected sheep, peaking at 10 days post-infection. Average levels of BTV RNA in the blood were at least 10 fold higher in goats, compared to infected sheep, between day 9 and 16 p.i. but overall the differences observed were not statistically significant due to individual variations (ANOVA $p= 0.45$) (Fig. 2C and Supplementary Fig. S1). All mock-infected sheep and goat controls used in this study showed no clinical signs, and

298 remained negative for the presence of both viral RNA in the blood and neutralizing antibodies towards
299 BTV (Fig. S2).

300 **Influence of BTV strain and serotype on the clinical outcome of BTV infection.** We also assessed the
301 pathogenicity of different BTV serotypes, as well as different virus strains within a single serotype. The
302 severity of disease observed in sheep inoculated with either BTV-2_{IT2000} or BTV-$8_{NET2006}$ was largely
303 equivalent, with both viruses inducing typical clinical signs observed in bluetongue (Fig. 3A). In
304 contrast, animals infected with BTV-8_{IT2008} showed only a mild transitory fever but no other clinical
305 signs (Figs. 3B and Supplementary Fig. S3 showing data for each individual animal). Excluding the
306 temporary pyrexia displayed by some animals at day 1 p.i., BTV-$8_{NET2006}$ and BTV-2_{IT2000} induced
307 cumulatively 17 and 18 days of fever in their respective groups of infected sheep. In contrast, BTV-
308 8_{IT2008} induced only 8 cumulative days of fever. Overall, we also observed that on average sheep
309 infected with BTV-$8_{NET2006}$ or BTV-2_{IT2000} displayed higher levels of fever compared to sheep infected
310 with BTV-8_{IT2008}, although differences were not statistically significant (ANOVA p= 0.17). BTV-8_{IT2008},
311 BTV-$8_{NET2006}$ and BTV-2_{IT2000} all induced similar levels of viremia (ANOVA p= 0.54) and neutralizing
312 antibodies in infected sheep (Fig. 3C-D).

313 We next sequenced the complete genomes of BTV-$8_{NET2006}$ and BTV-8_{IT2008} in order to determine the
314 genetic basis for the different phenotypes of these two viruses. We detected a total of 24 nucleotide
315 mutations between BTV-$8_{NET2006}$ and BTV-8_{IT2008}, including 16 silent mutations and 8 non-synonymous
316 mutations leading to differences in the viral VP1, VP2, VP4, NS1, NS2 and VP6 proteins (Fig. 4).

317 **Effect of cell culture adaptation on BTV virulence.** Published reports suggest that, in some cases,
318 infection of target species using blood directly from a naturally BTV infected animal induces more
319 severe clinical signs than tissue culture adapted virus (20, 61). In the context of the experimental
320 framework used in this study, we inoculated two groups of Sardinian sheep with either blood from a
321 BTV - infected animal (BTV-$8_{NET2007(blood)}$), or the same virus isolated in cell culture after a single

passage in KC cells and two passages in BHK_{21} (BTV-$8_{NET2007(1KC-2BHK)}$). As assessed by qRT-PCR, the infected blood contained approximately 100 fold less viral RNA than the inoculum of BTV-$8_{NET2007(1KC-2BHK)}$ (data not shown). Sheep infected with BTV-$8_{NET2007(blood)}$ displayed a higher clinical score and reached statistically significant higher levels of fever (p=0.01) compared to sheep inoculated with BTV-$8_{NET2007(1KC-2BHK)}$ (Figs. 5A-B and Supplementary Fig. S4). Sheep infected with BTV-$8_{NET2007(blood)}$ displayed 27 cumulative days of fever as opposed to 16 shown by sheep infected with BTV-$8_{NET2007(1KC-2BHK)}$. In addition, the levels of viral RNA in the blood were also consistently and considerably higher (10 to 1000 fold; p=0.018) in sheep infected with BTV-$8_{NET2007(blood)}$ compared to those found in BTV-$8_{NET2007(1KC-2BHK)}$ infected sheep (Figs. 5C and Supplementary Fig. S4). Interestingly, viremia was delayed by 2 days in BTV-$8_{NET2007(blood)}$ infected animals. In addition, we did not find neutralizing antibodies at 7 days post-infection in any of the sheep infected with BTV-$8_{NET2007(blood)}$ (Fig. 5D). In contrast, all sheep infected with BTV-$8_{NET2007(1KC-2BHK)}$ had BTV neutralizing antibodies by day 7 p.i. No differences in the levels of neutralizing antibodies were found at later time points between sheep infected with BTV-$8_{NET2007(blood)}$ and BTV-$8_{NET2007(1KC-2BHK).}$ Thus, as proposed in other studies (20, 61), infection of sheep with BTV collected directly from infected animals and never passaged in tissue culture induced more severe clinical signs than the homologous virus passaged even minimally in tissue culture.

BTV population diversity influences virulence. Next, we aimed to link the phenotypic differences observed above between sheep inoculated with BTV-$8_{NET2007(blood)}$ or BTV-$8_{NET2007(1KC-2BHK)}$ to genetic changes that might occur in the virus following cell culture adaptation. We analysed the genomes of BTV-$8_{NET2007(blood)}$ and BTV-$8_{NET2007(1KC-2BHK)}$ by deep sequencing, using the same stocks utilised in the experimental infections described above. We also analysed the intermediate viruses BTV-$8_{NET2007(1KC)}$ and BTV-$8_{NET2007(1KC-1BHK)}$. Furthermore, in order to test the reproducibility of the results obtained, we repeated in parallel the adaptation in KC and BHK_{21} cells of BTV-$8_{NET2007(blood)}$ in an independent set of

experiments. Altogether, we analysed the full genome of 7 viral samples: BTV-$8_{NET2007(blood)}$, and two independent isolates of BTV-$8_{NET2007(1KC)}$, BTV-$8_{NET2007(1KC-1BHK)}$ and BTV-$8_{NET2007(1KC-2BHK)}$.

We found that the consensus sequences of BTV-$8_{NET2007(blood)}$ and BTV-$8_{NET2007(1KC-2BHK)}$ were identical with the exception of two silent mutations in segments 1 (nt 2756) and segment 4 (nt 1431) (Fig. 6). Both point mutations were selected after the initial passage in KC cells and in both independent experiments.

RNA viruses, due to their high mutation rates, do not exist as a single genotype but as a complex of variants (also referred to as quasispecies), each possessing unique random mutations (74, 75). Consequently, we analysed BTV-$8_{NET2007(blood)}$ and the effect on its population diversity after passaging *in vitro* in KC and BHK_{21} cells.

In Fig. 7 we have plotted the degree of variability at each nucleotide position of each genomic segment before and after passaging in cell culture. A nucleotide is plotted, and is referred to as a "variant", if it represents at least 0.1% of the viral population. In general, the number of variants was higher in the virus before cell passaging, or after one passage in KC cells, compared to what observed even after a single passage in BHK_{21} cells. Interestingly, for 9 of the 10 segments in the first set of experiments, and for 8 of the 10 segments in the second set of experiments, the number of variable nucleotides was higher in the virus passaged once in KC cells as compared to the virus from blood before passage in cell-culture. There was a larger number of variants with a frequency between 0.1 and 0.29% in BTV-$8_{NET2007(1KC)}$, while the number of variants with a frequency > 0.4% was several fold higher in BTV-$8_{NET2007(blood)}$ (Fig. 8). The two silent mutations selected in the consensus sequence of BTV-$8_{NET2007(1KC-2BHK)}$ were already present as high prevalence variants in BTV-$8_{NET2007(blood)}$ (14.9% for nt 2756 of segment 1 and 10.4% for nt 1431 of segment 4) (dots circled in red in Fig. 7). On the other hand, other variants present with a frequency of about 10% in segment 3 and segment 6 were not selected after

passage *in vitro*. Essentially, the same results were obtained in the two independent sets of experiments.

DISCUSSION

Most infections of susceptible hosts by pathogenic viruses result in clinical manifestations that can vary greatly in their severity. For some viruses, such as avian influenza virus for example, low and highly virulent strains are distinguishable by clear genotypic differences (76). Nevertheless, in some circumstances, even infection of susceptible hosts with highly pathogenic viruses can result in mild or unapparent clinical symptoms.

Bluetongue is a disease characterised by a highly variable clinical spectrum (21-24). Understanding the basis for this variability is complicated by the fact that BTV exists in nature as many diverse strains representing different serotypes, topotypes and reassortant viruses often co-circulating in the same geographical area. In addition, BTV can infect a variety of ruminant species, each with different genetic and immunological backgrounds. Furthermore, BTV is transmitted by different species of *Culicoides* in diverse ecological contexts. There have been several studies concerning naturally occurring bluetongue or experimentally induced disease, clearly indicating that factors related to both the mammalian host and the virus can influence the outcome of BTV infection (55). However, it is not always straightforward to compare data from different studies. Thus, the weight given to different host or virus factors in determining the clinical outcome to BTV infection can differ in heterogeneous ecological or experimental settings.

In this study, we dissected both host and virus factors that can affect the clinical outcome of BTV infection. The use of a uniform experimental framework has allowed us to rigorously interrogate both experimental questions addressed in past studies (55), as well as explore hitherto unanswered questions. First of all, as suggested previously (24, 32-36), we confirmed that while both sheep and goats are fully susceptible to BTV (in this case BTV-8) infection, the former are more susceptible than goats and more likely to develop clinical disease. The levels of viremia in BTV-infected goats were not different (if anything higher) compared to those observed in infected sheep. These data confirm that

BTV is able to replicate to high levels in goat tissues but cellular damage, either induced by the virus or the host immune responses, does not likely occur. We do not know if goats would be more susceptible to disease if we had used higher infectious doses. We have used $2X10^6$ PFU of BTV in our experimental infections and this is likely far more infectious virus than is transmitted in nature by infected midges. In addition, studies in sheep using as little as $10^{1.4}$ $TCID_{50}$ were able to induce infection in this animal species (66). In two previous studies, also using BTV-8 isolates from the Netherlands, some of the experimentally infected goats developed mild clinical signs, fever and viremia (34, 36). However, in both studies, goats were infected intravenously (34, 36) and in one of them animals were infected at day 62 of gestation (36). Another study used BTV-4 which was isolated in embryonated chicken eggs and passaged seven times in BHK_{21}. Only 1 of 11 goats (of two different breeds) infected with this virus showed transient pyrexia, but at the same time 10 of 12 inoculated sheep did not show fever or signs of disease either (32). Thus, this study confirmed that the mammalian host species is certainly one of the main factors that determine the clinical outcome to BTV infection.

We did not find major differences in the susceptibility of sheep breeds from the Mediterranean area (Sardinian and Italian mixed breed) and Northern European breeds (Dorset poll) to bluetongue, despite their distinct geographical, historical and breeding backgrounds (47). Thus, variations in the susceptibility to bluetongue of different sheep breeds might not be as pronounced as originally thought. It is also important to stress that bluetongue itself was first discovered in European breeds imported into South Africa (77). Those breeds showed a higher susceptibility to bluetongue than local animals, although the influence of herd immunity on the latter could have also played a role. It is therefore difficult to weigh the influences of the host's genetic background, previous BTV exposure or the insect vector on the susceptibility to the disease in that particular context.

We have also analysed the influence of divergent viral serotypes, and closely related but distinct strains within the same serotype, on the clinical outcome of bluetongue. BTV-$8_{NET2006}$ is considered to

421 be a highly pathogenic virus (both in terms of morbidity and mortality) and the cause of one of the
422 largest outbreaks of bluetongue in history (48-51). However, in our experimental setting, we did not
423 find any difference in virulence between BTV-$8_{NET2006}$ and another serotype such as BTV-2_{IT2000} which
424 was isolated in Italy in the year 2000 from a naturally occurring case of bluetongue in sheep. Another
425 study, comparing the virulence of BTV-1 isolated from Algeria and a 2006 isolate of BTV-8 from
426 Belgium concluded that the former was more virulent than the latter (78). Although in that particular
427 study, the cell culture passage history was not described and viruses were inoculated sub-cutaneously,
428 it appears that the overall data suggest that in itself BTV-$8_{NET2006}$ is not necessarily more virulent than
429 other BTV serotypes, such as BTV-2 or BTV-1, that have been circulating in Europe in the last decade. It
430 is likely that other factors, such as the rapid spread of the infection to an extremely large number of
431 fully susceptible and naïve hosts (never previously exposed even to heterologous BTV serotypes)
432 contributed to the number of severe cases of disease observed during the Northern European
433 outbreak caused by this strain of BTV.
434 The BTV-$8_{NET2006}$ strain was isolated from samples collected at the beginning of the European outbreak
435 of this virus. Since the original cases identified in 2006 in central Europe, BTV-8 moved in subsequent
436 years towards several surrounding geographical areas (including southwards). Interestingly, in
437 Northern Italy and in Sardinia, BTV-8 (termed in this study BTV-8_{IT2008}) was only detected at the
438 serological level in a few animals but it was not associated with clinical disease (G. Savini, personal
439 communication). We showed conclusively in our study that BTV-8_{IT2008} was less virulent than BTV-
440 $8_{NET2006}$. BTV-8_{IT2008} accumulated several non-synonymous mutations in structural and non-structural
441 proteins (including VP1, VP2, NS1 and NS2) already implicated in attenuation of tissue culture adapted
442 BTV-2, BTV-4 and BTV-9 (57). Thus, this study formally proves the appearance of less virulent strains
443 during a BTV outbreak. The comparative smaller number of severe cases of bluetongue in endemic
444 areas might depend upon several factors, including the levels of herd immunity, the decrease in

445 virulence of circulating BTV strains, and possibly, on the long term selection of genetically resistant
446 individual animals.
447 Finally, we further investigated the observation that experimental infection of sheep with blood
448 collected from naturally occurring cases of bluetongue appears to induces, in general, more severe
449 clinical cases compared to the disease induced in sheep infected with viruses isolated in tissue culture
450 or embryonated eggs (20, 61). Indeed, we have confirmed in our experimental framework that sheep
451 inoculated with BTV-$8_{NET2007(blood)}$ displayed a more severe disease and higher levels of viremia than
452 those infected with the virus isolated in cell culture (BTV-$8_{NET2007(1KC/2BHK)}$). It is unlikely that factors
453 present in the infected blood could be the cause of more severe clinical signs in sheep. Importantly,
454 the highest levels of fever and the most severe clinical signs in sheep infected with BTV-$8_{NET2007(blood)}$
455 were observed between Day 6 and 11 p.i. when the levels of BTV in the blood where at their highest.
456 Virus passaging in tissue culture can lead to adaptive changes in the viral genotype that could in turn
457 affect viral virulence. However, we found only 2 synonymous mutations between the consensus
458 sequence of BTV-$8_{NET2007(blood)}$ and the cell culture isolated virus BTV-$8_{NET2007(1KC-2BHK).}$ Both mutations
459 were present in approximately 10% of the variants of BTV-$8_{NET2007(blood)}$ and interestingly they were
460 both selected in two independent experiments. It is possible that these silent mutations in some way
461 affect viral virulence. In addition, the sequencing methods used did not cover the non-coding regions
462 of each segment and therefore we may have also missed other important mutations. However,
463 overall there appears to be very little (or no variation at all) at the consensus sequence level (at least
464 for BTV-8) of viruses isolated from blood or minimally passaged in cell culture. RNA viruses have the
465 highest error rates (10^{-4}-10^{-6} per nucleotide site per genome replication) of any microorganism due to
466 their RNA-dependent RNA polymerase lacking proofreading activity during RNA synthesis (79, 80). As
467 such, RNA viruses exist as a population of variants, genetically closely related but distinct from their
468 consensus sequence. It is rational to argue that the opportunity to quickly adapt and generate diverse

viral populations is critical for the survival of RNA viruses (74) in the face of selective pressures, including the innate and adaptive antiviral responses of the host. For example, polioviruses mutants with a high fidelity polymerase (and thus low population diversity) display an attenuated phenotype in mice, despite possessing identical consensus sequences to the virulent wild type viruses (81-83).

We found that BTV-$8_{NET2007(blood)}$ contained the largest number of high frequency variants. However, when BTV-$8_{NET2007(blood)}$ was passaged in insect KC cells the resulting viral population (BTV-$8_{NET2007(1KC)}$) showed the overall highest number of variants, even higher (~ 60%) than those in the blood before tissue culture isolation. A severe genetic bottleneck was observed after viral passaging in mammalian BHK_{21} cells with the resulting viruses (BTV-$8_{NET2007(1KC/1BHK)}$ and BTV-$8_{NET2007(1KC/2BHK)}$) showing the smallest degree of variability.

These data suggest that BTV virulence is not only affected by changes in the viral proteins selected at the consensus level, but also by the genetic variability of the population as a whole. This hypothesis is also supported by previous observations made in a limited number of genes before the advent of deep-sequencing (84, 85). In a study that analysed segment 2 of a virulent strain of BTV-1, Gould and Eaton (84) showed that the consensus sequence did not change after a single passage in tissue culture that resulted in viral attenuation. In addition, Bonneau and colleagues (85) showed that the number of variants observed in segment 2 and 10 of plaque purified BTV-10 increased during transmission of the virus between ruminants and insect vectors, but without changes to the consensus sequence.

Thus, "flat" populations containing a relatively small number of variants appear to be less virulent than more variable populations.

In addition, our data also suggest that *Culicoides* cells might function as a natural source of new BTV variants. BTV is an arbovirus and as such must adapt rapidly to replicate in hosts as different as a warm blooded mammal and insects. An increased variability of replication in *Culicoides* cells might allow BTV to adapt faster to different selective pressures present in the invertebrate and vertebrate hosts. These

493 data also reinforce the notion that it is critical to avoid the use of modified live vaccines that induce
494 even transient viremia in vaccinated animals. The transmission of vaccine strains in the Culicoides
495 population might then lead to the emergence of "new" strains with the potential to revert to their
496 original phenotype.
497 Our study has not taken into consideration factors related to the invertebrate host (e.g. species, sites
498 and number of "infectious" bites) that could affect BTV pathogenesis. The insect host certainly plays a
499 role in modulating the interaction between virus and the mammalian host as some studies are
500 beginning to suggest (86). It is possible that transmission of BTV by different species of *Culicoides*, in
501 different geographical areas, could influence the pathogenesis of bluetongue in different ways. This is
502 an exceedingly important area of research that will need to be addressed in the coming years.
503

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510

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Figure Legends

Figure 1. *In vitro* replication kinetics and pathogenicity in mice of the BTV strains used in this study. **A**. Replication kinetics of BTV-2_{IT2000}, BTV-$8_{NET2006}$ and BTV-8_{IT2008} in sheep CPT-Tert cells. Cells were infected at MOI 0.05, and supernatants were collected 8, 24, 48, 72 and 96 h post infection. Supernatants were then titrated in BSR cells by limiting dilution assays. Experiments were repeated independently three times and data are represented as averages of the experiments. Error bars indicate standard errors. **B**. Survival plots of 129sv $IFNAR^{-/-}$ mice inoculated intraperitoneally with 300 PFU of BTV-$_{2IT2000}$, BTV-$8_{NET2006}$ and BTV-8_{IT2008}. Mice were observed for 2 weeks post inoculation for the presence of clinical signs of systemic disease. All the viruses used in this study killed all the infected mice between day 6 and 8 post-inoculation. None of the five mock-infected mice showed any clinical symptoms (not shown in the figure) and survived throughout the observation period.

Figure 2. **Experimental infection of goats and different sheep breeds with BTV-$8_{NET2006}$**. **A**. Graphs showing clinical signs recorded in BTV infected goats and various sheep breeds including Sardinian, mixed breed and Dorset poll (n= 5 per each group). Animal were all of approximately 2 years of age with the exception of an additional group of 8 months old Dorset poll that are indicated as "Dorset (young)". Animals were scored daily after infection using a clinical index score (shown in Table S1) taking into account general symptoms, respiratory signs, fever, need for veterinary intervention or death. General symptoms included are depression, anorexia, facial and feet lesions. Each group of 5 animals was infected with the same dose of BTV-$8_{NET2006}$ intradermally. Scores shown for respiratory symptoms, general symptoms and fever represent the average values collected for each group (± standard error) during the duration of the entire experiment (28 days). Total scores are instead the cumulative values for each symptom within a group collected throughout the observation period. **B.** Body temperature (average per group; values per each individual animal are shown in Fig. S1) of

771 animals infected with BTV-$8_{NET2006}$. Physiological temperature in sheep ranges normally between 38.3
772 and 39.9°C (black broken lines). Fever in this study was recorded when rectal temperature was above
773 40°C. In experimentally infected animals fever appeared between day 5 and 6 post-infection. **C.** BTV
774 RNA in blood samples of experimentally infected sheep and goats. Viral RNA was detected by qRT-PCR
775 and values are expressed as log_{10} copy number per µg of total RNA. Note that goats reached the
776 highest level of BTV RNA in the blood. **D**. Neutralizing antibodies towards BTV in experimentally
777 infected animals. Sera were collected at the times indicated following experimental infection (time 0)
778 and subjected to neutralization assays as indicated in Materials and Methods. Values shown are
779 averages +/- standard deviations and represent the log_{10} of the 50% endpoint (proportionate distance,
780 PD). Mock infected goats and sheep (data shown in Figure S2) did not show any clinical sign of
781 bluetongue, maintained a physiological temperature throughout the experiment and did not have any
782 detectable BTV RNA or neutralizing antibodies.

783 **Figure 3. Virulence of BTV-2_{IT2000}, BTV-$8_{NET2006}$ and BTV-8_{IT2008}.** Clinical scores (**A**), rectal temperature
784 (**B**), viremia (**C**) and neutralizing antibodies (**D**) of Sardinian sheep (n= 5 per group) infected with either
785 BTV-2_{IT2000}, BTV-$8_{NET2006}$ or BTV-8_{IT2008}. Description of graphs in each panel have been described in the
786 legend of Fig. 2. Note that experimental infections of sheep (Dorset poll, Dorset poll "young", Sardinian
787 or mixed breed) and goats with BTV-$8_{NET2006}$ and Sardinian breed sheep with BTV-2_{IT2000}, or BTV-8_{IT2008}
788 were carried out at the same time but are shown separately in Figures. 2 and 3 to facilitate the
789 narrative. Consequently, the same sets of data for the Sardinian sheep infected with BTV-$8_{NET2006}$ are
790 shown both in Figures 2 and 3. Fever and viremia data for each individual animal are shown in Figure
791 S3. Note that sheep infected with BTV-8_{IT2008} display very mild clinical signs, only a transitory fever and
792 lower levels of viremia compared to sheep infected with BTV-2_{IT2000} and BTV-$8_{NET2006}$.

793 **Figure 4. Genetic differences between BTV-$8_{NET2006}$ and BTV-8_{IT2008}.** Schematic representation of the
794 10 genomic segments of BTV-$8_{NET2006}$ and BTV-8_{IT2008}. Mutations in BTV-8_{IT2008} compared to BTV-

795 $8_{NET2006}$ are indicated with red dots. Non-synonymous mutations are highlighted with black asterisks,
796 and the position of the mutated amino acid residue. Note that the length of the schematic genome
797 segments and the relative position of synonymous and non-synonymous mutations in the cartoon are
798 indicative only.

799 **Figure 5. Experimental infection of Sardinian sheep with BTV-$8_{NET2007(blood)}$ and BTV-$8_{NET2007(1KC-2BHK)}$.**
800 Clinical scores (**A**), rectal temperature (**B**), viremia (**C**) and neutralizing antibodies (**D**) of Sardinian
801 sheep (n= 5 per group) infected with either BTV-$8_{NET2007(blood)}$ or BTV-$8_{NET2007(1KC-2BHK)}$. Graphs in each
802 panel have already been described in the legends of Figure 2. Fever and viremia data for each
803 individual sheep are shown in Figure S4. Note that sheep infected with BTV-$8_{NET2007(blood)}$ displayed
804 more severe clinical signs, higher levels of fever and viremia compared to sheep infected with BTV-
805 $8_{NET2007(1KC-2BHK)}$.

806 **Fig 6. *In vitro* adaptation of BTV-$8_{NET2007(blood)}$.** The effects of adaptation *in* vitro of BTV-$8_{NET2007(blood)}$
807 was assessed by comparing the genomic sequences of BTV-$8_{NET2007(blood)}$ with the sequences of viruses
808 isolated *in* vitro after passaging in *Culicoides* KC cells (1 passage) and two further passages in BHK_{21}
809 cells. Schematic representation of the experiment is shown at the top of the figure. Two independent
810 experiments (represented with blue or red arrows) were carried out and sequences of the viral
811 genome were obtained after each passage *in vitro*. The cartoon shows the schematic representation
812 of individual genomic segments of BTV. Mutations found in the consensus sequences of the cell
813 culture passaged viruses are shown as red or blue dots indicating the two independent experiments.
814 Only two synonymous mutations were selected in Seg-1 and Seg-4 immediately after passage in KC
815 cells in both independent experiments and were conserved after further passaging in BHK_{21} cells.

816 **Fig 7. Viral population diversity of BTV-$8_{NET2007(blood)}$ before and after isolation in cell culture.** Changes
817 in nucleotide diversity of BTV-$8_{NET2007(blood)}$ amplified directly from the spleen of an infected sheep
818 were compared with sequences of the same virus after isolation in KC and BHK_{21} cells. Differences

were assessed by deep sequencing as described in Materials and Methods. Total reads of individual genome segments were mapped to consensus sequences and single nucleotide polymorphisms (SNPs) were assigned above the arbitrary 0.1% frequency threshold. On the graph each dot represents the percentage of nucleotides difference (y-axis) from the consensus sequence of each nucleotide composing the individual genomic segments of the virus (x-axis). The total number of variable nucleotides (> 0.1%) for each sample is shown in the right corner of each plot. Dots circled in red in Seg-1 and Seg-4 of BTV-$8_{NET2007(blood)}$ are those nucleotides that have been selected in the majority of the viral populations after passage *in vitro.*

Fig 8. Frequency distribution of variable nucleotide in BTV-$8_{NET2007(blood)}$, BTV-$8_{NET2007(1KC)}$, BTV-$8_{NET2007(1KC-1BHK)}$ and BTV-$8_{NET2007(1KC-2BHK)}$. Histograms showing for each virus the number of nucleotides with % variation falling within defined borders ("bins"). Panels A-B and C-D represent data of two independent experiments. Note that panels B and D have a different scale in the y-axis compared to panels A and C as the frequency of variants present in more than 0.4% of the total population was significantly lower compared to variants presented in panels A and C.