Transplacental transmission of the Italian Bluetongue virus serotype 2 in sheep

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Bluetongue virus serotype 2, *Culicoides* cell line, Ewe, Fetus, Transplacental transmission.

Summary
In order to study the capability of a Bluetongue virus serotype 2 (BTV-2) field isolate to cross the placental barrier, 2 groups of 5 pregnant ewes were infected with a field BTV-2 Italian strain (Group A) or with the same strain passaged once in *Culicoides* cells (Kc) (Group B). Following infection, EDTA-blood and serum samples were collected weekly and tested for the presence of BTV RNA/infectious virus and anti-BTV-2 antibodies, respectively. At lambing, precolostral EDTA-blood and serum samples were collected from lambs and tested as before. The lambs were then sampled as scheduled for the dams. All sheep seroconverted on day 12 post-infection (pi) and remained seropositive throughout the sampling period (day 68 pi). BTV was isolated from day 7 pi to day 14 pi in animals of Group A and from day 5 pi to day 12 pi in animals of Group B. None of the 14 lambs born had pre-colostral antibodies. Three lambs born from two ewes of Group B were viraemic at birth and in one lamb infectious virus was isolated from blood up to 11 days of age. This study proved for the first time that a single passage of BTV-2 field strain in Kc cells is able to give to BTV the ability to cross the placenta barrier and infect foetal tissues.

Infezione transplacentare del ceppo italiano del sierotipo 2 del virus della Bluetongue nelle pecore

Parole chiave
Bluetongue virus sierotipo 2, Cellule di *Culicoides*, Feto, Pecora, Trasmissione transplacentare.

Riassunto
Per verificare se il ceppo italiano del sierotipo 2 del virus della Bluetongue (BTV-2) è in grado di attraversare la barriera placentare ed infettare i feti ovini, due gruppi di 5 pecore gravide sono stati infettati, rispettivamente, con il ceppo di BTV-2 di campo (Gruppo A) e con lo stesso ceppo passato una volta su cellule di *Culicoides* (Kc) (Gruppo B). Dopo l'infezione, a tutti gli animali sono stati effettuati settimanalmente prelievi di sangue in provette con e senza EDTA (siero) su cui rilevare, rispettivamente, la presenza di RNA virale e virus infettante, e anticorpi anti-BTV-2. Al parto, si è proceduto al prelievo di sangue e siero dagli agnelli prima dell'assunzione del colostro. I campioni prelevati sono stati analizzati come descritto per le pecore. In concomitanza con la madre, anche gli agnelli sono stati sottoposti a prelievo. Anticorpi anti-BTV-2 sono stati evidenziati in tutte le pecore a partire dal 12° giorno dall'infezione e per tutto il periodo di studio (68 giorni). Negli animali del gruppo A, BTV-2 è stato rilevato dal 7° al 12° giorno dall'infezione mentre in quelli del gruppo B dal 5° al 12° giorno. Dei 14 agnelli nati, nessuno presentava anticorpi pre-colostrali; tre agnelli nati da due pecore del gruppo B erano viremici alla nascita e virus infettante è stato isolato dal sangue fino all'11° giorno di vita. Il presente studio ha dimostrato per la prima volta che il ceppo italiano di BTV-2 è in grado di attraversare la barriera placentare di pecore dopo un solo passaggio su cellule Kc.

Introduction

Bluetongue (BT) is a viral non-contagious disease of wild and domestic ruminants caused by Bluetongue virus (BTV). Within the *Reoviridae* family, it represents the type species of the genus *Orbivirus* (Mertens and Diprose 2004). In the last few years next to the classical 24 serotypes, new serotypes have been described (Hofmann *et al.* 2008, Lefevre *et al.* 2008, Maan *et al.* 2011, Zientara *et al.* 2014, Schulz *et al.* 2016, Sun *et al.* 2016, Bumbarov *et al.* 2016, Savini *et al.* 2017, Marcacci *et al.* 2018, Lorusso *et al.* 2018). Sheep is the most susceptible species, but overt disease has also been reported in cattle, goats and wild ruminants (Elbers *et al.* 2008 a, b, Lefevre *et al.* 2008, Dal Pozzo *et al.* 2009, MacLachlan *et al.* 2009).

Even though BTV transmission mainly occurs through *Culicoides* biting midges, for some strains sporadic cases of oral (ingestion of infected placenta or colostrum) and vertical (transplacental) transmission have been reported (Menzies *et al.* 2008, Backx *et al.* 2009, Mayo *et al.* 2010, Van der Slujis *et al.* 2013, Batten *et al.* 2013, Rasmussen *et al.* 2013). According to the stage of pregnancy and strain involved, infection of fetuses with BTV can lead to embryonic death and return to service, abortion, congenital defects and stillbirth (Wouda *et al.* 2009, Santman-Berends *et al.* 2010, Saegermann *et al.* 2011). All these events might have serious economic repecussions. Vertical transmission of BTV in animals could also play an important role in the epidemiology of natural BTV infections (Maclachlan *et al.* 2009, Verwoerd and Erasmus 2004, Saegerman *et al.* 2011, Van der Sluijs *et al.* 2016). Some of these animals can be born infected, and may introduce the virus to new areas if the dam is transported, and allow BTV to overwinter (Van der Slujis *et al.* 2011, EFSA Scientific Opinion on bluetongue serotype 8 2011, Van der Sluijs *et al.* 2016).

The first report of transplacental transmission of BTV dates back to 1955, when Shultz and DeLay found that vaccination of pregnant ewes with an *in ovo*-attenuated BTV vaccine caused the birth of weak and malformed lambs. Since then, studies on mother-to-foetus transmission of BTV in ruminants were conducted under various experimental settings using either cell- or *in ovo*-adapted strains. The interest on this issue peaked after the north-European BTV-8 outbreak, that was responsible for abortions and teratogenesis in cattle (Barnard and Pienaar 1976, Gibbs *et al.* 1979, Stott *et al.* 1982, Richardson *et al.* 1985, Parsonson *et al.* 1987, MacLachlan *et al.* 2000, De Clerq *et al.* 2008 a, b, Menzies *et al.* 2008, Darpel *et al.* 2009, Van der Slujis *et al.* 2011, Van der Slujis *et al.* 2013). It was the first time that a field isolate was able to cross the placental barrier. Before BTV-8 incursion, vertical transmission was proven only for strains adapted on cells or passaged into embryonated chicken eggs (ECE). Even if field isolates are capable of causing abortion in pregnant animals, they have never been detected in foetal tissues (Van der Slujiis *et al.* 2011). It is for this reason that BTV field isolates have been supposed not able to cross the placental barrier and that abortion to likely be the result of the disease in the dam. However, no experimental trials have ever been designed and performed to support or refute this supposition. From what just said, it can also be deduced that passages in an artificial culture system -like ECE or cell culture may favour phenotypic changes like changes in virulence, tissue tropism and ability to cross the placenta. It has been demonstrated that one passage on embryonated chicken eggs (ECE) and two passages on mammalian cells, were capable of establishing an infection in ovine foetuses (Van der Slujiis *et al.* 2011). Recently, it was also demonstrated that two passages, one on Kc cells (a cell line derived from *C. sonorensis*), and one on CPT-Tert cells (ovine choroid plexus cells) were enough to give to the BTV-2 Italian strain the capability of crossing the placental barrier and infect ovine fetuses (Rasmussen *et al.* 2013).

Concerning BTV vertical transmission, much has therefore been achieved since 1955 but much has still to be done. Improving our knowledge on the mechanism(s) behind the vertical transmission of BTV is of paramount importance in order to better understand the biology and epidemiology of the virus. In this trial, the capability to cross the ewe placental barrier of two BTV-2 strains, one never passaged into cell lines and one passaged once into Kc cells, has been evaluated.

Materials and methods

Ethics

All procedures on animals were accomplished according to the Italian Decreto Legislativo 4 March 2014, no. 26 on the protection of animals used for scientific purposes, and were approved by the Italian Ministry of Health.

Animals

Twenty-nine healthy Bergamasca ewes were housed in the facilities of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' (IZSAM). The animals were uniquely identified by ear tag numbers. The ewes were treated with deltamethrin (Butox Pour on, MSD Animal Health, Milan, Italy), dewormed and confirmed negative for the presence of BTV RNA by real time RT-PCR and anti-BTV antibodies by competitive ELISA (cELISA) and virus neutralisation (VN).

The oestrus was synchronized by means of intravaginal sponges soaked with flugestone acetate (Crono-gest sponge, MSD Animal Health, Milan, Italy) kept *in situ* for 15 days, followed by the administration of 400 IU of equine chorionic gonadotropin (Crono-Gest PMSG, MSD Animal Health, Milan, Italy) at removal. Two days later, four rams were introduced in the flock for three weeks.

Ten ewes were subsequently diagnosed pregnant by ultrasound examination and enrolled in the study.

Two weeks before inoculation, ewes were randomly divided into 2 groups and housed in insect-proof barns. BTV-free status of the animals was rechecked as described above.

Automatic temporized dispenser of pyrethroids operated during the entire experiment and ewes were treated monthly with deltamethrin. One blacklight trap operated between the first door and the entrance of the barns and further two traps operated within the barns.

Challenge virus

The BTV-2 used for the challenge derived from the spleen of a sheep naturally infected during the 2001 BTV outbreak in Sardinia, Italy (internal reference number 80306/2001). It is worth to note that the outbreak occurred before the beginning of the first BTV vaccination campaign which started in 2002 and that the BTV-2 strain used in this trial is genetically distinct from the BTV-2 vaccine strain (Elia *et al.* 2008, Savini, unpublished results).

To increase the volume of the inoculum and evaluate the vitality of the strain, two sheep were injected. Briefly, approximately one gram of the spleen was homogenized and diluted 1:10 in phosphate buffered saline (PBS) added with antibiotics and antimycotics. The homogenate was centrifuged at 5,000 rpm for 10 minutes and the supernatant inoculated subcutaneously to a BTV-free sheep (internal id number 84372). A BTV-free ram (internal id number 84359) was used as second animal. It was further inoculated subcutaneously with 30 ml of PCR-positive blood collected from the first sheep. Blood samples were collected daily from the ram by jugular venepuncture, and BTV RNA-positive samples were stored at - 80 °C (virus stock BTV-2_{blood}).

BTV-2_{blood} was also propagated in Kc cells for 10 days, and the infected tissue culture was harvested, titrated and stored at - 80 °C until use (virus stock BTV-2_{Kc}; titer 5.97 $\log_{10}$ $TCID_{50}$/mL) .

Experimental design

The 10 pregnant ewes were divided into 2 groups of 5 animals each, namely Group A and Group B. At approximately 120 days of gestation, animals of Group A were inoculated subcutaneously with 10 mL of BTV-2_{blood} while the 5 ewes of Group B with 2 mL of BTV-2_{Kc}. Each group was located in the same insect-proof stable but in different pens.

Two not-pregnant, virologically and serologically negative sheep were added to the infected groups (one per group) and used to monitor eventual BTV circulation.

Rectal temperatures and clinical signs of all animals were monitored daily for 21 days. A rectal temperature ≥ 40 °C was considered fever.

EDTA-blood and serum samples were collected from all animals three times a week and once a week for 68 days, respectively. Blood was tested for the presence of BTV RNA by real time RT-PCR and for the presence of infectious virus by virus isolation (VI); serum was tested against BTV antibodies by cELISA and by VN assay.

After lambing, precolostral EDTA-blood and serum from lambs as well as colostrum from ewes were collected, and tested serologically and virologically as previously described. Subsequently, lambs were sampled as scheduled for the ewes.

Whenever possible, placentas were collected and screened for the presence of viral RNA and infectious virus. All samples were kept at 4 °C until analysis (for a maximum of 2 days).

Necropsy and tissue collections

In case of death, animals underwent post-mortem examination and gross lesions were recorded.

Tissue samples from brain, heart, spleen, lungs, ileus, kidney and lymph nodes (submandibular, mesenteric, mediastinic, precrural and popliteal) were removed and splitted into two aliquots: one was fixed in 10% buffered formaldehyde for histology and immunohistochemistry (IHC) and one homogenised in PBS (1:10). Once clarified by centrifugation at 5,000 rpm for 15 minutes, homogenates were stored at 4 °C until analysis (for maximum of 2 days).

Histology and immunohistochemistry

Tissue samples were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin wax. Subsequently, tissue blocks were sectioned at a thickness of 5 µm, stained with haematoxylin and eosin (HE) and examined microscopically.

Sections of collected tissues were also processed for IHC: briefly, tissue sections were heat-treated for antigen retrieval (121 °C x 8 min in 0.01 M citrate buffer, pH 6.0) and incubated overnight with an

anti-BTV VP7 monoclonal antibody (ab22070, Abcam, UK) diluted 1:1000. Immune reactions were revealed using a polymer-based peroxidase technique (Envision Plus kit, Denmark). Positive and negative controls were included in all IHC reactions.

Entomological surveillance

Insects were collected once a week and placed in ethanol 70%. *Culicoides* midges were identified according to complex, species (when possible), sex and age (Goffredo and Meiswinkel 2004). Insects were pooled according to date of collection, trap number, species and age, and pools tested for the presence of BTV RNA.

Laboratory tests

Serum and colostrum were tested against BTV antibodies by a cELISA (Lelli *et al.* 2003) and VN test, as described in the OIE Terrestrial Manual (OIE 2018).

The detection of BTV RNA in EDTA-blood samples, organ homogenates and *Culicoides* pools was performed by using the one step real time RT-PCR targeting the segment 10 of BTV genome, as described in the OIE Terrestrial Manual (OIE 2018).

Virus titration and isolation were attempted on EDTA-blood samples and organ homogenates as described by Savini and colleagues (Savini *et al.* 2009).

$TCID_{50}$ was calculated by using the method of Reed and Muench (Reed and Muench 1938).

Statistical analysis

For statistical purposes, antibody titer was expressed as the reciprocal of the highest serum dilution (N) able to inhibit at least 75% of the virus CPE. Data were plotted as Log_2 of the mean values obtained.

The results of real-time RT-PCR are defined by the number of the cycle threshold (Ct) at which the specific amplicon was revealed.

Differences between the mean neutralizing antibody titers and Ct per sampling day, among the different groups, were analyzed using the non-parametric Wilcoxon-Mann-Whitney test for independent groups. A probability of $P < 0.05$ was set as significance level.

Results

During the entire experimental period, only 1 parous midge belonging to the Obsoletus complex was collected from the blacklight trap placed between the first door and the entrance of the barns. It was negative when tested for the presence of BTV RNA.

Clinical signs

Most animals had fever from day 6 to day 11 post-infection (pi).

On day 14 pi, ewe no. 4 (Group A) died before parturition. Clinical signs initially included nasal discharge, salivation and conjunctivitis, and later mild coronitis, severe facial oedema and finally dyspnoea. Between days 20 and 32 pi, the remaining 9 ewes gave birth to 14 lambs: six were born from Group A and 8 from Group B. Lamb no. 12 from ewe no. 1 (Group A) was born dead.

Apart from this event, no abortions were reported during the trial, however 6 lambs, 1 from Group A and 5 from Group B died within the first 10 days of age. The neonatal mortality rate was 43%.

On day 37 pi, an ewe of Group B (no. 10) was euthanized due to severe neurological signs (inability to stand, hyperextension of front and hind limbs and opisthotonus) unresponsive to antibiotic therapy and supportive care.

Gross pathology, histology and immunohistochemistry of ewes

Two ewes, one per group, died during the study.

At necropsy, the carcass of ewe no. 4 (Group A) showed a generalized yellowish gelatinous subcutaneous oedema, particularly of the head, with sparse subcutaneous petechiae and hemorrhagic suffusions; petechiae and suffusions were also evident on oesophageal mucosa and adventitia, spleen and pleura.

Other gross lesions included hydropericardium and haemorrhages at the base of the pulmonary artery, lymphadenomegaly, and pulmonary oedema with presence of foamy fluid in trachea.

Histologically, perivasal oedema and congestion were detected in the brain. Heart was affected by an interstitial myocarditis with necrosis (Figure 1a) and spleen by an inflammatory process characterized by lymphocytic karyorrhexis (Figure 1b). Lungs showed a catharral alveolitis with congestion and secondary contamination by bacteria.

All organs tested PCR-positive, but virus isolation was inconclusive.

The size of the foetus was normal for its gestational age. Macroscopic lesions included renal hyperaemia and splenic petechiae. All sampled organs tested PCR-negative.

At necropsy, the carcass of the ewe (no. 10) of group B had erosions of the oral mucosa, diffuse

cutaneous haematomas, haemorrhagic mediastinic and head lymph nodes and consolidated pulmonary areas. *Clostridium* spp. were isolated from spleen, liver, brain, lungs and kidney, and *Escherichia coli* and *Pasteurella multocida* from lungs. Only brain and lymph nodes tested positive for the presence of BTV RNA and stained positive for VP7.

Histologically, a severe and diffuse pneumonia and an interstitial myocarditis with fibrosis and degeneration were observed. Lymph nodes were hyperplastic, and retropharyngeal lymph nodes were positively stained for VP7 (Figure 1c).

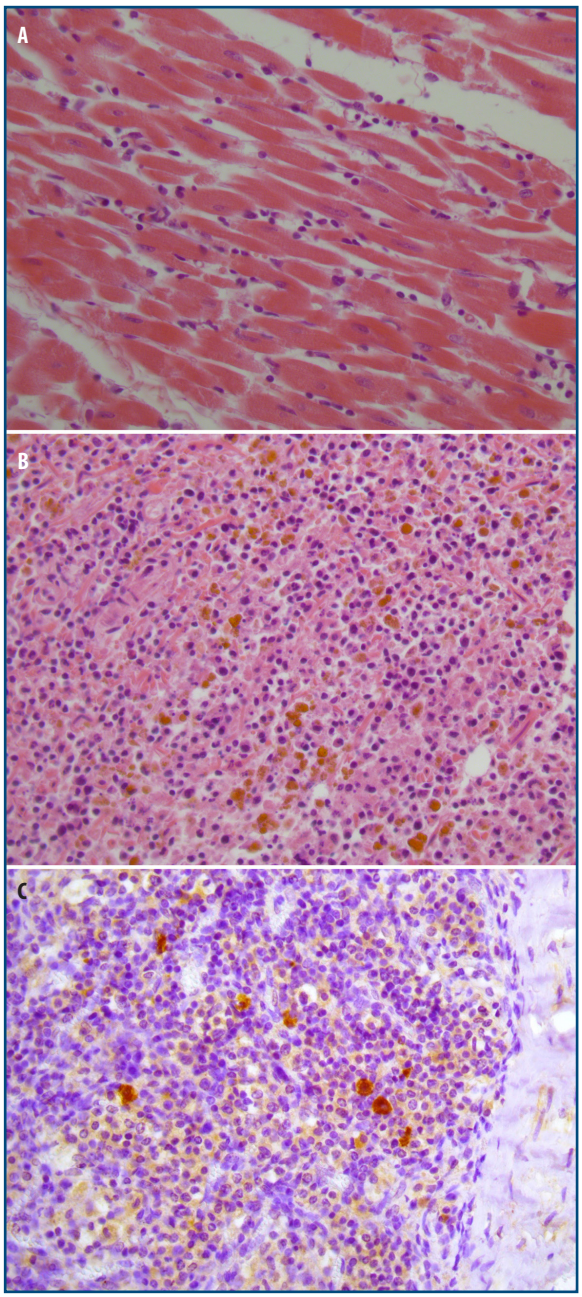

Figure 1. *Examples of tissue sections from dead ewes.* All sections have been stained with haematoxylin and eosin and are shown at a magnification 40X. **A.** Heart of a sheep of Group A (infected with BTV-2_{blood}) showing phlogosis and necrosis. Immunohistochemistry (IHC) was negative. **B.** Spleen of the same sheep of a), showing karyorrhexis of lymphocytes. **C.** Retropharyngeal lymph node of a sheep of Group B (infected with BTV-2_{Kc}), showing macrophages and lymphocytes positive for BTV-VP7 by IHC (brown spots).

Gross pathology, histology and immunohistochemistry of lambs

A total of 6 lambs born from 5 ewes died (1 lamb born from a Group A ewe and 5 lambs born from Group B ewes). Gross lesions were not indicative of BTV infection.

Lambs born from ewes of group A

Lamb no. 32 was born dead. All tested organs were negative to BTV RT-PCR.

Lambs born from ewes of group B

The sampled tissues collected from those lambs

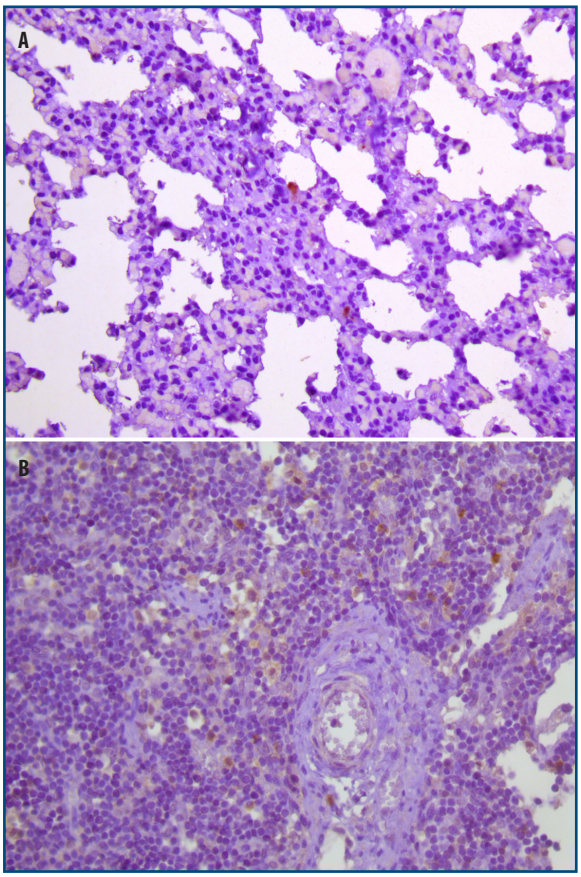

Figure 2. *Examples of tissue sections from dead lambs.* All sections have been stained with haematoxylin and eosin and are shown at a magnification 40X. **A.** Histological section of the lung of lamb no. 3, born from a sheep of Group B (infected with BTV-2_{Kc}). Interstitial macrophages positive for BTV-VP7 by immunohistochemistry (IHC) are shown (brown spots). **B.** Retropharyngeal lymph node of the same lamb of a) showing macrophages and lymphocytes positive for BTV-VP7 by IHC (brown spots).

which were viraemic at birth were BTV RT-PCR positive.

Lamb no. 23 was viraemic from birth till death which occurred ten days later. Gross lesions included pulmonary atelectasis and enteritis with ascites. *E. coli* was isolated from liver, ileus and ascitic fluid.

Except for the heart, all sampled organs were BTV RT-PCR positive. No relevant histological lesions were found. VP7 was detected in spleen, liver and retropharyngeal and meseraic lymph nodes (Figure 2, a-b).

Lamb no. 24 died 5 days after birth. It was viraemic from birth till death.

Gross lesions included pulmonary hyperaemia, splenic petechiae, enlargement of mesenteric and mediastinic lymph nodes, sub-epicardial suffusion, hyperaemia of intestinal mucosa, renal congestion, mild ectasia of meningeal blood vessels. All sampled organs were positive to BTV RT-PCR.

Lamb no. 27 was weak at birth and died at the age of 2 days. Gross lesions included mild enlargement of meseraic and mediastinic lymph nodes, hyperaemia of intestinal mucosa and mild ectasia of meningeal vessels. All sampled organs were negative to BTV RT-PCR and virus isolation.

Twin lambs no. 33 and no. 34 died at the age of 2 days. Gross lesions included enteritis with ascites, sub-epicardic petechiae, a wide superficial haemorrhage of the right ventricle. *E. coli* was isolated from liver, gut and ascitic fluid. Organs collected from both animals were negative to the RT-PCR for BTV.

Serological and virological results in ewes

The animals added to each group for monitoring BTV circulation, remained negative to BTV virological and serological tests throughout the entire experimental period.

Two animals, one of Group A and one of Group B, first showed ELISA BTV antibodies on day 5 pi. From day 12 pi till the end of the trial, all animals were positive to the BTV c-ELISA. Analogously, neutralizing antibodies were detected in all animals starting from day 12 pi till the end of the sampling period (on day 68 pi) (Figure 3).

The highest antibody titer was detected on day 26 pi in Group A (mean 1:360) and on day 19 pi in Group B (1:704) (Figure 3).

On day 12 pi, the mean antibody titer of Group B was significantly higher than in Group A ($p < 0.01$) (Figure 3).

BTV RNA was detected in all infected ewes starting from day 3 pi in Group B and day 5 pi in Group A. Then, viral RNA concentration increased further, peaking on days 7-10, and remaining steady throughout the sampling period.

The mean CT value observed in the animals of Group A on day 5 pi was significantly higher than that observed in Group B. Subsequently, the Ct value trend of both groups was almost completely comparable (Figure 4).

All pregnant ewes had at least one-day of infectious viraemia (from day 7 pi in Group A and from

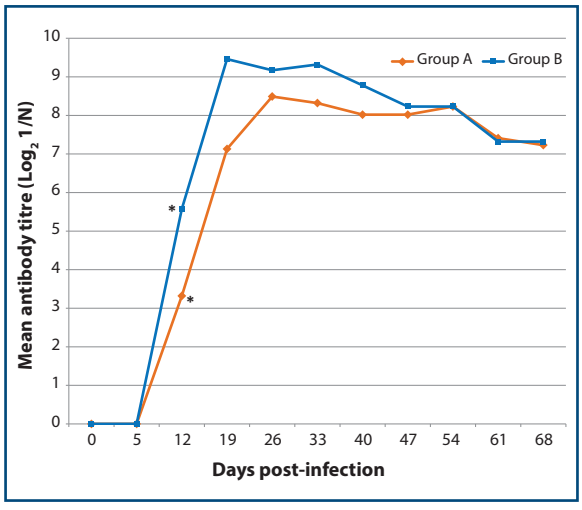

Figure 3. *Mean neutralizing antibody titer in ewes per sampling day.* Group A: ewes infected with BTV-2_{blood}; Group B: pregnant ewes infected with BTV-2_{Kc}. Antibody titer is expressed as the reciprocal of the highest serum dilution (N) able to inhibit at least 75% of the virus cytopathic effect (CPE). Data are plotted as Log_2 of the mean values obtained per ewe per sampling day.
Group A: ewes infected with BTV-2_{blood}; Group B: ewes infected with BTV-2_{Kc}. * = $p < 0.01$.

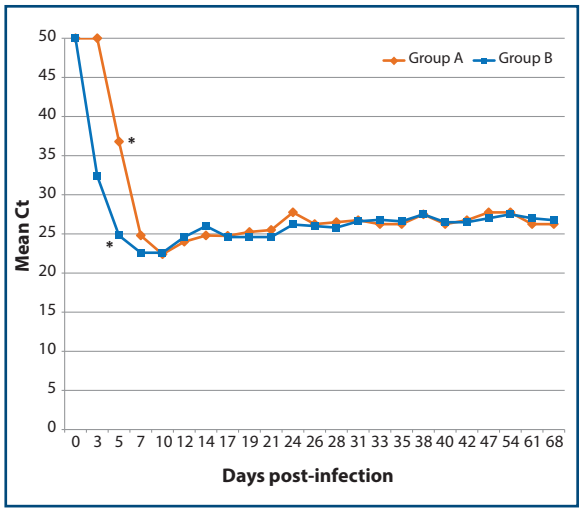

Figure 4. *Mean Cycle threshold (Ct) values in ewes per sampling day.* Group A: ewes infected with BTV-2_{blood}; Group B: pregnant ewes infected with BTV-2_{Kc}. * = $p < 0.05$.

day 5 pi in Group B). In two animals of Group A, viraemia lasted till day 14 pi, while in Group B viraemia was not longer than 12 days (3 ewes). In all infected animals BTV titers never were higher than 2.3 $\log_{10}$ $TCID_{50}$/mL.

Except for two animals (one from Group A and one from Group B), BTV was also detected by RT-PCR in all the collected placentas.

Serological and virological results in lambs

At birth, none of the lambs had antibodies against BTV. Subsequently, all lambs seroconverted due to passive transfer of colostral maternal antibodies.

Lambs born from ewes of Group A

All lambs born from ewes of Group A were negative to BTV RT-PCR for the entire length of the trial.

Lambs born from ewes of Group B

Three lambs (no. 22, no. 23 and no. 24) born from 2 ewes infected with BTV-2_{Kc} were viraemic at birth.

Twin lambs no. 22 and no. 23 were born on day 22 pi and lamb no. 24 on day 23 pi. At the time of birth, the respective ewes resulted positive to BTV RT-PCR but negative to virus isolation.

Lamb no. 22 was BTV RT-PCR positive till 90 days of age (day 112 pi). Infectious BTV was isolated from EDTA blood samples up to 11 days of age (day 33 pi), 21 days after the latest BTV isolation from its mother. The titer of neutralising antibodies detected in the colostrum was 1:160 whereas the lamb had a titer of 1:80 up to 60 days of age (last serum sampling day).

Lambs no. 23 and no. 24 were BTV RT-PCR positive till death (10 and 5 days of age, respectively). Infectious virus was isolated from lamb no. 23 untill 6 days of age (day 28 pi; 16 days after latest BTV isolation from its mother) and from lamb no. 24 untill 3 days of age (day 26 pi; 14 days after BTV isolation from its mother).

Discussion

In this study all infected ewes seroconverted and developed viraemia and RNA-aemia after BTV-2 infection. As in other experimental infections (Caporale *et al.* 2014), ewes infected with the BTV strain grown *in vitro,* became viraemic earlier than those infected with the wild strain never passaged into tissue culture.

Both types of inoculum (BTV-2_{blood} or BTV-2_{Kc}) were also capable of causing death, and comparable severe clinical signs. In other similar studies, the clinical signs observed in sheep when infected with blood originating from a naturally BTV-infected animal, were more severe than those observed in animals infected with cell-adapted viruses (MacLachlan *et al.* 2008, Eschbaumer *et al.* 2010, Caporale *et al.* 2014). In this experiment, BTV-2_{Kc} was passaged only once in *Culicoides* cells while in all mentioned studies, the number of cell passages of the inoculum was three as a minimum, and included at least one passage on mammal cells. The exiguous number of passages to which the inoculum has been subjected or, alternatively, the type of cell lines (insect) used in this trial might therefore be at the origin of this scarce attenuation of the BTV-2. However, based on these results, it is hard to say whether it was because the strain was passaged only once on tissue culture (TC), because it was passed on *Culicoides* derived cells only or because it was never passaged on mammal cells.

On the other hands, the fact that both inocula were able to reproduce viraemia and clinical signs was fundamental in order to study the occurrence of vertical transmission of BTV-2 strains in ewes. This trial, if on the one hand demonstrated that one passage into Kc cell, although not capable of reducing the virulence of BTV-2, was able to confer to the Italian BTV-2 strain the capacity of crossing the placental barrier of ewes, on the other hand, confirmed that, for BTV-2 field isolates, crossing the placenta is an exceptional event.

The BTV-2 strain used in this study was responsible for the most severe BTV outbreak occurring in Italy in 2000-2001, causing more the 500,000 clinical cases and deaths. Although many abortions were reported, BTV-2 was never detected in foetal tissues (Savini *et al.* 2014). In the same way, with the only exception of BTV-8 European strain, wild-type BTV strains have never been found in ruminant foetal organs (Elbers *et al.* 2008, Dal Pozzo *et al.* 2009, Méroc *et al.* 2009, EFSA Scientific Opinion on Bluetongue serotype 8).

Transplacental infection has been, instead, frequently reported for BTV strains with behind a lab story implicating several passages in an artificial culture system (MacLachlan *et al.* 2000, Shimshony *et al.* 1980, Savini *et al.* 2014). Rasmussen and colleagues (Rasmussen *et al.* 2013) in a recent study, injected ewes with the same strain used in this trial and proved that the Italian BTV-2 strain was able to cross the placenta after being passaged once in Kc cells and once in CPT-Tert cells.

This study further reduced the number of cell passages of BTV-2 and proved that one passage into Kc cells is still sufficient to grant the virus the competence to infect fetuses and lead to the birth of viraemic lambs. To the best of our

knowledge, this is the first time that such a finding is reported. The effects induced on the neonatal development by BTV when crossing the placental barrier, largely depend on the period of gestation and stage of maturation of the foetus. The infection may result in prenatal death with abortion or stillbirth, malformation such as hydranencephaly and blindness, abnormal behavior like tremor and 'dummy syndrome', or normal term neonates (Oberst 1993). In this trial, ewes were infected in the last month of pregnancy, normal terms lambs were then expected. Of the 5 ewes infected with BTV-2_{Kc}, two gave birth to 3 viraemic lambs. Although not unanimously accepted, the birth of viraemic offsprings can play an important epidemiological role. In Northern Ireland, viraemic calves from imported PCR-negative dams were blamed as the cause of the BTV introduction in the country (Menzies *et al.* 2008). It has been hypothesized that the virus was 'ferried' undetected in foetal tissues (a 'Trojan horse' mechanism). Because of that, specific control measures are adopted when pregnant animals have to be moved from BTV-8 infected to BTV-8 free areas.

Vertical transmission has also been implicated as one of the possible strategies behind the overwintering process (Takamatsu *et al.* 2003, White *et al.* 2005, Wilson *et al.* 2008). It has been thought that BTV can overwinter in foetal tissues to reemerge months later in viraemic offspring. In such a way, the infectious viraemic period can be extended beyond that of the dam (Gibbs *et al.* 1979, Richardson *et al.* 1985, De Clerq *et al.* 2008, van der Sluijs *et al.* 2011, Savini *et al.* 2012). In this trial, infectious virus was isolated in 3 lambs born from ewes infected with BTV-2_{Kc} at 3, 6 and 11 days of age (day 33 pi), which means 14, 16 and 21 days, respectively, after the last VI positivity detected in the mother blood samples. In lamb no. 22, the only BTV positive lamb which survived in this study, BTV RNA was instead detected until 90 days of age (day 112 pi). It is well known that an animal positive to RT-PCR but negative to virus isolation is not infectious to vectors (MacLachlan *et al.* 1994, Bonneau *et al.* 2002), in this trial, the infectious viraemic period was then extended for a maximum of 21 days, a period probably not long enough to allow virus overwintering, even in temperate regions. Of the two BTV-2 strains used in this experiment, the BTV-2_{Kc} was the only one capable to cross the placenta of ewes. Since the BTV RNA concentrations and the antibody response detected in the blood samples of Group A and Group B animals were similar, this ability could not be ascribed to different levels of viraemic titers triggered by the BTV-2 infections. In other words, this new acquired capacity of the BTV-2_{Kc} did not depend on the host response.

It is known that virus populations are not made of a single member with a defined nucleic acid sequence. Rather, they are dynamic distributions of nonidentical but related members called a quasispecies. The consequence of a quasispecies is that most viral infections are initiated not by a single virion, but a population of particles. The progeny produced after this infection results from selective forces that operate inside the infected host (Eigen 1993). As other RNA viruses, BTV also exists as a quasispecies (Domingo and Holland 1997, Bonneau *et al.* 2001). It is likely that in the BTV population virions capable of crossing the placenta barrier and virions which don't have this capacity coexist, with the latter more represented. In this study, Kc cell lines have probably acted as a molecular sieve, by selecting minor viral variants with phenotypic changes which, among other characteristics, include the ability to cross the placenta. Although, theoretically, phenotypic changes accumulate only over several subcultivations (Kirkland and Hawkes 2004, Pedersen *et al.* 2009, Caporale *et al.* 2014), in this study an unique passage into Kc cells was sufficient to confer to the BTV-2 wild strain the ability to cross the placenta of ewes. This finding supports Caporale and colleague hypothesis (Caporale *et al.* 2014) that Kc cells might function as a natural source of new BTV variants, because an increased variability of replication in *Culicoides* cells allows BTV to adapt faster to different selective pressures.

At birth, none of the lambs had BTV antibodies, and seroconversion occurred only after the ingestion of colostrum. It is known that ovine foetuses are able to mount an immune response against BTV at 95-100 days of gestation, approximately 10 days after they become infected (Silverstein *et al.* 1963, Osburn *et al.* 1971, Enright and Osburn 1980, Miyasaka and Morris 1988, Tizard 1996, Hoffmann *et al.* 2013). In this trial, ewes were infected at about 120 days of gestation and viraemic lambs were born 22-23 days post-infection. According to theory of Van der Slujis and colleagues (Van der Slujis 2011), in this trial BTV-2_{Kc} probably infected foetal tissues not earlier than days 12-13 pi, so lambs did not have enough time to mount an humoral immune response detectable at birth.

Conclusions

Although, under many aspects, BTV is a very well-characterized virus, the mechanisms behind transplacental transmission are not clearly defined yet.

This study proved that a single passage of BTV-2 field strain in Kc cells is able to give to BTV the ability to cross the placenta barrier and infect foetal tissues. It adds another piece to the intricate puzzle of

BTV transplacental transmission and improves our understanding of this phenomenon.

Further research, however, is needed to disclose the viral genes/proteins and the mechanisms involved in this event in order to design live vaccines not capable of crossing the placenta and infecting foetuses.

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