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# Comparative Neuropathology of Major Indian Bluetongue Virus Serotypes in a Neonatal BALB/c Mouse Model

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## Summary

Bluetongue virus (BTV) is neurotropic in nature, especially in ruminant fetuses and in-utero infection results in abortion and congenital brain malformations. The aim of the present study was to compare the neuropathogenicity of major Indian BTV serotypes 1, 2, 10, 16 and 23 by gross and histopathological lesions and virus distribution in experimentally infected neonatal BALB/c mice. Each BTV serotype (20 µl of inoculum containing $1 \times 10^5$ tissue culture infectious dose $[TCID]_{50}$/ml of virus) was inoculated intracerebrally into 3-day-old mice, while a control group was inoculated with mock-infected cell culture medium. Infection with BTV serotypes 1, 2 and 23 led to 65–70% mortality at 7–9 days post infection (dpi) and caused severe necrotizing encephalitis with neurodegenerative changes in neurons, swelling and proliferation of vascular endothelial cells in the cerebral cortex, cerebellum, midbrain and brainstem. In contrast, infection with BTV serotypes 10 and 16 led to 25–30% mortality at 9–11 dpi and caused mild neuropathological lesions. BTV antigen was detected by immunohistochemistry, direct fluorescence antibody technique and confocal microscopy in the cytoplasm of neuronal cells of the hippocampus, grey matter of the cerebral cortex and vascular endothelial cells in the midbrain and brainstem of BTV-1, -2, -10, -16 and -23 infected groups from 3 to 20 dpi. BTV nucleic acid was detected in the infected brain tissues from as early as 24 h up to 20 dpi by *VP7* gene segment-based one-step reverse transcriptase polymerase chain reaction. This study of the relative neurovirulence of BTV serotypes is likely to help design suitable vaccination and control strategies for the disease.



*Keywords:* BALB/c mice; bluetongue virus; histopathology; neuropathogenicity

## Introduction

Bluetongue (BT), a non-contagious, re-emerging arthropod-borne disease of sheep, cattle and other wild ruminants transmitted by biting midges of genus *Culicoides* (Mellor, 1990; Maclachlan and Guthrie, 2010), is caused by bluetongue virus (BTV), which belongs to the genus *Orbivirus* in the family Reoviridae (Pringle, 1999; Saminathan *et al.*, 2016). The double-stranded RNA (dsRNA) genome of BTV is formed by 10 segments encoding seven structural (VP1 to VP7) and four non-structural (NS1 to

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NS4) proteins (Ranjan *et al.*, 2015; Rao *et al.*, 2016). The BTV virion is an icosahedral particle assembled in a triple layered protein capsid. The outer capsid layer is formed by VP2 and VP5 proteins responsible for cell attachment and entry. The internal core is composed of VP3 (subcore) and VP7 (core surface layer) proteins (Ranjan *et al.*, 2015; Rao *et al.*, 2016). The core also contains three minor enzymatic proteins: VP1 (RNA-dependent RNA polymerase), VP4 (capping enzyme and transmethylase) and VP6 (RNA-dependent ATPase and helicase). The largest non-structural protein, NS1, forms tubules in the cytoplasm of BTV-infected cells. NS2 is a major component of the viral inclusion bodies, where morphogenesis and RNA replication take place. Two different isoforms, NS3 and NS3a, are glycoproteins involved in excretion of virus from infected cells against the innate immune response of the host cells. The recently discovered smallest BTV protein, NS4, has a role in replication of the virus in host cells (Ranjan *et al.*, 2015; Rao *et al.*, 2016).

At present, at least 27 distinct BTV serotypes have been recognized worldwide, which include the recently characterized putative BTV-27 serotype in goats from Corsica and France (Jenckel *et al.*, 2015). The serotype is predominantly determined by the VP2 segment, which is the most variable of BTV proteins and a main target of neutralizing antibodies (Huismans and Erasmus, 1981). BT is considerably variable in its clinical manifestations, which can range from asymptomatic infection to lethal haemorrhagic fever (MacLachlan *et al.*, 2009). The clinical outcome of BTV infection depends on the strain/topotype/serotype of BTV, passage history of the virus, virus dose, route of inoculation, age, species, breed, individual susceptibility and immune status of the infected host, nutritional status, stress and environmental factors such as solar irradiation and high temperature (MacLachlan *et al.*, 2009; Maclachlan and Guthrie, 2010). Great genetic and phenotypic variations called BTV topotypes have been observed, even within the same serotype, related to geographical locations (Gould and Pritchard, 1990). Variability in virulence exists among BTV strains due to frequent genome segment re-assortments adapting to new ecological zones (Shaw *et al.*, 2013).

Caporale *et al.* (2011) studied the molecular basis of BTV virulence in murine models. Genome segments encoding VP1, VP2 and NS2 revealed consistent differences between the virulent and attenuated strains of BTV. Multiple genome segments determined the virulence of BTV serotypes (Janowicz *et al.*, 2015).

The disease is enzootic in many tropical and subtropical regions and certain temperate regions. Since the first outbreak of BT in India was recorded during the 1960s, the country has been endemic for BT and there is evidence for circulation of at least 23 BTV serotypes (except for 19, 22, 25 and 26) evidenced by serology and/or virus isolation (Prasad *et al.*, 2009; Ranjan *et al.*, 2015; Ayanur et al., 2016; Rao *et al.*, 2016). Until now, 15 serotypes (BTV-1—4, -5, -6, -9, -10, -12, -16—18, -21, -23 and -24) have been isolated from animals in India (Ranjan *et al.*, 2015; Rao *et al.*, 2016). BTV-1, -2, -10, -16 and -23 are common and recently, an inactivated pentavalent vaccine comprised of these serotypes has been developed for use (Prasad *et al.*, 2009; Reddy *et al.*, 2010).

The BTV serotypes or strains are neuropathogenic in nature, occasionally causing fetal death and congenital cerebral malformations in newborn calves and lambs (Enright and Osburn, 1980; MacLachlan and Osburn, 1983; MacLachlan *et al.*, 1985; van der Sluijs *et al.*, 2013). Experimental in-utero infection of BTV in sheep and cattle induced fetal death and/or teratogenic effects, including severe encephalomalacia, hydranencephaly, porencephaly and retinal dysplasia varying with the gestational age of the fetus (Osburn *et al.*, 1971; Barnard and Pienaar, 1976; Luedke, 1985; Richardson *et al.*, 1985). Recently, in India, the *VP2* gene of the BTV-1 strain was isolated from the spleen of an aborted goat fetus by genome sequencing, indicating transplacental transmission of this naturally occurring strain. There has been no exposure to vaccine strains in this region (Chauhan *et al.*, 2014). Two strains of BTV serotype 11 (UC-2 and UC-8) were used to determine the difference in neuroinvasiveness in bovine fetuses and neonatal mice. It was found that UC-8 strains induced more severe necrotizing encephalitis in the cerebrum and cerebellum than the UC-2 strain (Waldvogel *et al.*, 1987, 1992a; Brewer and Osburn, 1998). It was also noticed that gene segment 5 of UC-8 was associated with neurovirulence in newborn mice (Carr *et al.*, 1995). Variations in virulence or pathogenesis among BTV serotypes have been reported experimentally in natural host sheep (Groocock *et al.*, 1982; Hamblin *et al.*, 1998; van der Sluijs *et al.*, 2013; Sánchez-Cordón *et al.*, 2013, 2015) and murine models of disease (Caporale *et al.*, 2011). The aim of the present study was to compare the neruropathogenicity of common Indian strains of BTV in BALB/c mice.

## Materials and Methods

### *Virus*

BTV serotype 1 was isolated from a clinically affected cross-breed sheep during 1994 in Avikanagar in Rajasthan State, India (Prasad *et al.*, 1994). BTV-2 and

BTV-10 were isolated from clinically affected sheep during 2003 in the Tirunelveli District, Tamil Nadu State (Maan *et al.*, 2012) and Andhra Pradesh State (Bommineni *et al.*, 2008), India, respectively. BTV-16 was isolated from a clinically affected sheep during 2009 in Karnataka State, India (Ranjan *et al.*, 2016). BTV-23 was isolated from a clinically affected sheep during 1998 in Bangalore in Karnataka State, India (Tembhurne *et al.*, 2010). In this study, each BTV serotype is identified by the addition of a suffix in subscript indicating the country of origin and year of isolation, followed by the letter 'L' for 'low number of passages in culture'. We refer to the BTV serotypes as BTV-$1_{IND1994(L)}$, BTV-$2_{IND2003(L)}$, BTV-$10_{IND2003(L)}$, BTV-$16_{IND2009(L)}$ and BTV-$23_{IND1998(L)}$ strains. All of the BTV serotypes were maintained in baby hamster kidney-21 (BHK-21) cells and stored in freeze-dried form in the repository of the Virus Laboratory, Centre for Animal Disease Research and Diagnosis (CADRAD), ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh. The virus titre was determined as tissue culture infective dose ($TCID_{50}$) in BHK-21 cells by endpoint titration assay (Reed and Muench, 1938).

*Mice and Inoculation*

Pregnant inbred female BALB/c mice (6–8 weeks' old) in the later stage of gestation were procured from the Laboratory Animal Facility, CSIR-Central Drug Research Institute, Lucknow, India. The animals were housed in separate polypropylene cages in insect-proof accommodation of the experimental animal challenge house of the CADRAD, ICAR-IVRI, Izatnagar, India. The animals were provided with feed and water *ad libitum* and kept under controlled conditions (temperature 27 ± 2°C; relative humidity 30–55%) with 12/12 h light/dark cycle. All experiments were performed in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC).

*Experimental Design*

The litters of 3-day-old neonatal mice were divided into six groups and each consisted of 20 mice. Groups I, II, III, IV and V were inoculated intracerebrally (IC) with 20 µl of $1 \times 10^6$ $TCID_{50}$/ml of live Indian BTV serotypes 1, 2, 10, 16 and 23, respectively, and served as infected groups. Group VI mice were inoculated IC with 20 µl of cell culture medium to serve as a mock-infected control group. Two mice from each infected group were killed at 1, 3, 7, 9, 11, 15 and 20 days post infection (dpi) or earlier if they showed advanced clinical signs of encephalitis.

*Clinical Signs and Mortality Assessment*

The mice were observed for clinical signs, including ruffled fur, tremors, hunchback appearance, incoordination, paralysis and mortality until the end of the experiment. At specified time intervals, during necropsy examination, tissue samples from brain, spleen and lungs were collected into 10% neutral buffered formalin, RNAlater® (Applied Biosystems, Foster City, California, USA) and over ice (snap frozen in liquid nitrogen).

*Histopathology*

After fixation, tissue samples were processed routinely and embedded in paraffin wax. Sections (4–5 µm) were stained with haematoxylin and eosin (HE) (Luna, 1968).

The histopathological lesions were scored using a modified semiquantitative method (Maximova *et al.*, 2008, 2010): 0, no lesions; 1, minimal lesions; 2, mild lesions; 3, moderate lesions; and 4, severe lesions. This scale was assigned to BTV induced microscopical lesions of brain including: (1) cellular inflammatory infiltration of the brain parenchyma and meninges, (2) vascular endothelial swelling with microhaemorrhage and oedema, and (3) glial cell reaction with ischaemic neuronal degeneration and necrosis.

*Immunohistochemistry*

The avidin–biotin–peroxidase complex method of immunohistochemistry (ABC–IHC) was used to demonstrate BTV antigen in fixed brain tissue sections (anti-mouse Ig HRP detection kit, BD Biosciences, San Jose, California, USA) as described by Channappanavar *et al.* (2012).

*Immunofluorescence and Confocal Microscopical Studies*

Frozen tissues were sectioned (4–6 µm) on a cryostat microtome (Microm International GmbH, Walldorf, Germany) and sections were fixed to poly-L-lysine (Sigma–Aldrich, St. Louis, Missouri, USA) precoated glass slides and fixed immediately in chilled acetone for 10 min at −20°C. BTV-specific VP7 antigen was detected by the direct fluorescence antibody technique (d-FAT) using VP7-based group-specific fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (VMRD, Pullman, Washington, USA) as described by Umeshappa *et al.* (2011). The confocal fluorescence images were obtained using a confocal microscope (Olympus FV1000, Tokyo, Japan). Tissue morphology is indicated by differential interference contrast with scale bars on the images.

*One-step Reverse Transcriptase Polymerase Chain Reaction*

Viral RNA was extracted from the samples of brain, spleen and lung by using the RNeasy lipid tissue mini kit (Qiagen RNeasy kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The total RNA suspension was treated with RNase-free DNase to remove possible traces of genomic DNA. The one-step reverse transcriptase polymerase chain reaction (RT-PCR) was carried out by using the Quantitect-one step RT-PCR kit (Qiagen). The reaction occurred in a 25 µl volume with 2 µg RNA as template, 10 pmol of each forward and reverse primer and the rest of the components in the reaction, according to the manufacturer's instructions. The details of primers used to amplify VP7 segment of BTV is given in Table 1.

*Statistical Analysis*

Data from all the experimental groups were analyzed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA). Multiple comparisons between the groups were analyzed using two-way ANOVA followed by the Tukey–Kramer post-hoc test. Significance was assumed for $P \leq 0.05$. The Kaplan–Meier estimator method was used to draw survival curves and a log-rank test was used to compare survival rates between different BTV-infected groups.

## Results

*Clinical Signs and Mortality*

Three-day-old mice inoculated IC with BTV serotypes showed mild clinical signs of lack of growth and immobility, followed by neurological tremors that started between 4 and 6 dpi. Mice infected with BTV serotypes 1, 2 and 23 showed 65–70% mortality and maximum mortality was observed at 7–9 dpi. In contrast, mice infected with BTV serotypes 10 and 16 showed mortality of 25–30% and maximum mortality was observed at 9–11 dpi. All control mice inoculated with cell culture fluid without virus survived until the end of the experiment. Survival plots for the groups of mice inoculated with different BTV serotypes are shown in Fig. 1a.

*Gross and Histopathological Lesions*

Gross lesions in the brain of BTV infected mice were vascular congestion of the cerebral meninges and focal haemorrhages. Microscopical examination of the cerebral meninges showed focal areas of

**Table 1**
**Oligonucleotide primers used for amplification of BTV genome**

| *Gene* | *Primer sequence (5′-3′)* | *Product size (base pairs)* | *Annealing temperature (°C)* | *Reference* |
|---|---|---|---|---|
| BT-VP7/F-839 | GAG ATC ACA CAT GGC ATG G | 243 | 54 | Umeshappa *et al.* (2010) |
| BT-VP7/R-1081 | CGT GCA AAG TGG ACT ACA C | | | |

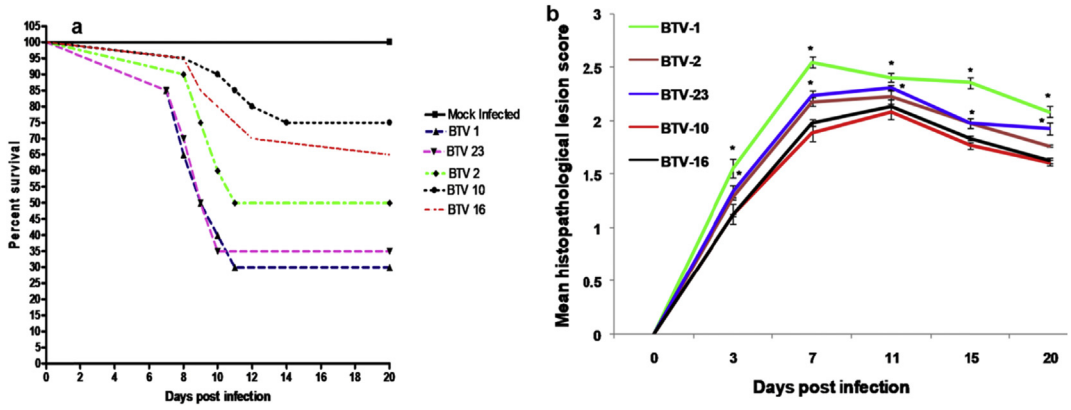


Fig. 1. Percent survival plots (a) and mean histopathological lesion score (b) in mice inoculated with different BTV serotypes ($n = 20$ mice/group). Litters of 3-day-old neonatal BALB/c mice were monitored for survival after IC injection with 20 µl of $1 \times 10^5$ $TCID_{50}$/ml of five live Indian BTV serotypes. Significant (*) difference ($P < 0.05$) was observed between the groups at each time point using the log-rank test.

lymphocytic infiltration (Fig. 2a) and congestion and swelling of endothelial cells lining the meningeal capillaries of mice infected with BTV serotypes 1, 2 and 23 at 3–5 dpi. Perivascular oedema, increased perineuronal space, marked swelling and proliferation of vascular endothelial cells, resulting in reduced intraluminal space of cerebral capillaries, were noticed at 5–7 dpi in mice inoculated with BTV-1, -2 and -23 (Figs. 2b and c). The neurons showed degenerative changes with swollen cell bodies at 7 dpi. Areas of haemorrhage in the subventricular zones of the forebrain, due to leakage from cerebral microcapillaries, were noticed at 7–9 dpi in mice inoculated with BTV-1, -2 and -23. The neurons also showed morphological

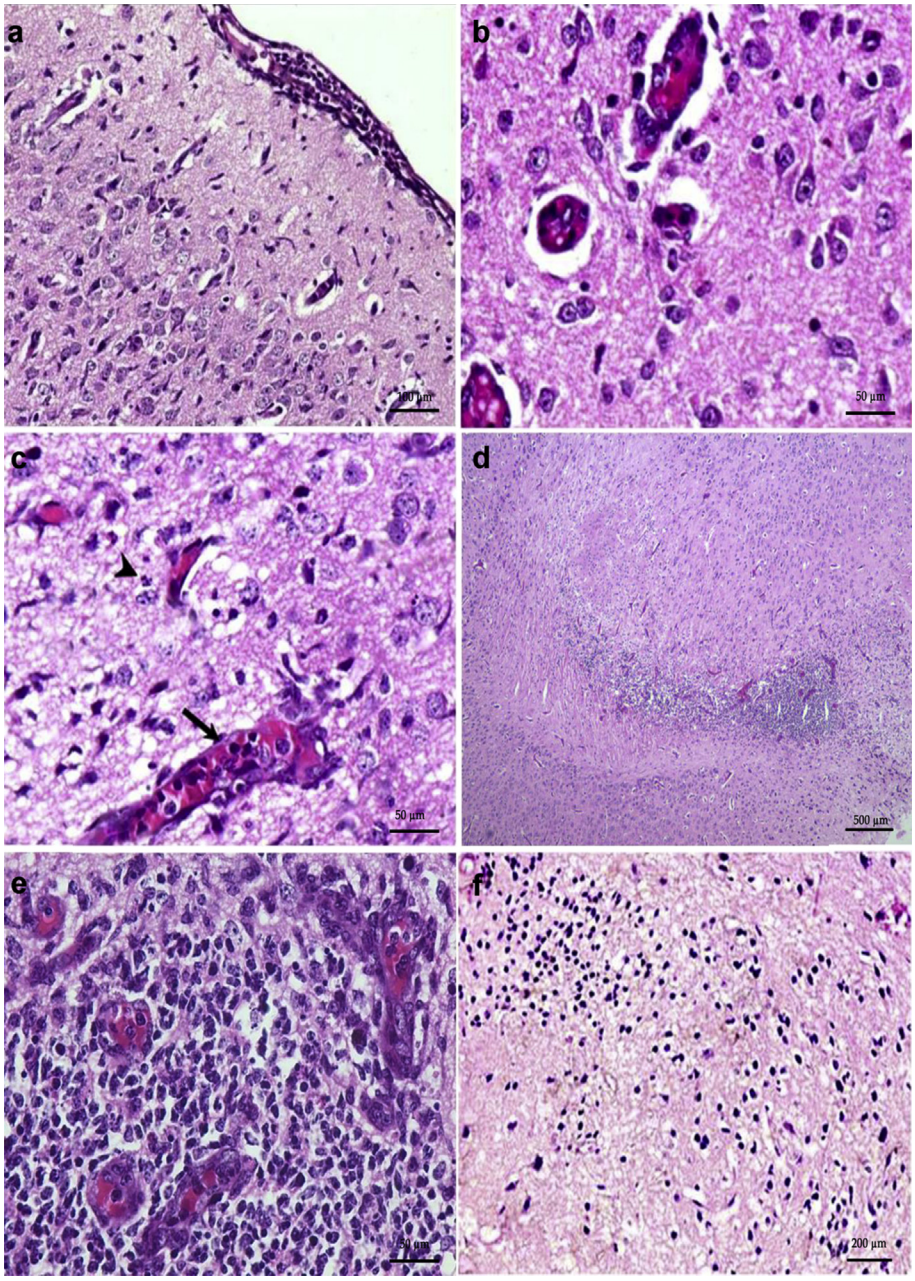


Fig. 2. Histopathological lesions in different groups. (a) Cerebral meninges showing infiltration of mononuclear cells at 3 dpi in a BTV-23-inoculated mouse. HE. Bar, 100 µm. (b) Perivascular oedema and swelling of endothelial cells leading to reduced intraluminal space of capillaries at 5 dpi in a BTV-1 inoculated mouse. HE. Bar, 50 µm. (c) Vascular endothelial cell proliferation and swelling (arrow) leading to reduced intraluminal space of capillaries and neurons showing apoptosis (arrowhead) at 7 dpi in a BTV-2 inoculated mouse. HE. Bar, 50 µm. (d) Focal areas of necrosis with infiltration of mononuclear cells in the forebrain at 9 dpi in a BTV-1 inoculated mouse. HE. Bar, 500 µm. (e) Necrotic areas with infiltration of mononuclear cells at 11 dpi in a BTV-23 inoculated mouse. HE. Bar, 50 µm. (f) Increased number of glial cells at 15 dpi in a BTV-2 inoculated mouse. HE. Bar, 200 µm.

features of apoptosis-like cell shrinkage, condensed cytoplasm and pyknosis with dark eosinophilic cytoplasm at 9–11 dpi in mice inoculated with BTV-1, -2 and -23 (Fig. 2c). Bilaterally symmetrical non-suppurative necrotizing encephalitis in the forebrain, brainstem and cerebral cortex was noticed at 9–11 dpi (Figs. 2d and e). Reactive gliosis was characterized by multifocal infiltration of glial cells into inflammatory areas at 11–15 dpi (Fig. 2f). In contrast, sections of brain from mice infected with BTV serotypes 10 and 16 revealed mild infiltration of lymphocytes into the meninges at 3–7 dpi and mild swelling of endothelial cells in capillaries at 9–11 dpi. No gross or histopathological lesions were detected in the spleen or lung. The control mice showed no BTV-associated histopathological lesions.

*Semi-quantitative Histopathological Lesion Grading*

The BTV induced histopathological lesion scores were significantly ($P \leq 0.05$) higher in mice infected with BTV-1, -2 and -23 serotypes than those receiving BTV-10 and -16 serotypes (Fig. 1b). The magnitude of the histopathological lesions gradually increased after inoculation and peaked at 7 dpi, persisting up to 11 dpi and followed by a decrease in intensity of lesions after 15 dpi in mice inoculated with BTV-1, -2 and -23. Groups inoculated with BTV-10 and -16 serotypes revealed significantly ($P \leq 0.05$) lesser magnitude of lesions at 3–7 dpi, which peaked at 11 dpi; however, sharply decreased levels were noticed at 15 dpi (Fig. 1b).

*Distribution of Bluetongue Virus Antigen*

BTV antigen was detected by IHC in sections of brain from mice infected with BTV-1, -2, -10, -16 and -23 serotypes from 3 to 15 dpi. BTV antigen was localized to the cytoplasm of neuronal cells of the hippocampus, the grey matter of cerebral cortex and vascular endothelial cells in the midbrain and brainstem (Figs. 3a and b). BTV antigen was demonstrated by d-FAT in the brain of mice infected with BTV-1, -2, -10, -16 and -23 serotypes from 3 to 20 dpi. BTV antigen was detected in the cytoplasm of neuronal cells, mainly in the cerebral cortex and subventricular areas (Figs. 4a and e). BTV antigen was localised to the neurons as seen by confocal microscopy at 7 and 11 dpi (Figs. 5a and d). In contrast, no positive labelling was detected in the spleen and lungs of BTV-infected mice and brain from control mice inoculated with cell culture medium containing no virus.

*Molecular Detection of Bluetongue Virus*

BTV nucleic acid was detected by *VP7* gene segment-based one-step RT-PCR in infected brain as early as 24 h and remained positive up to 20 dpi (Fig. 6).

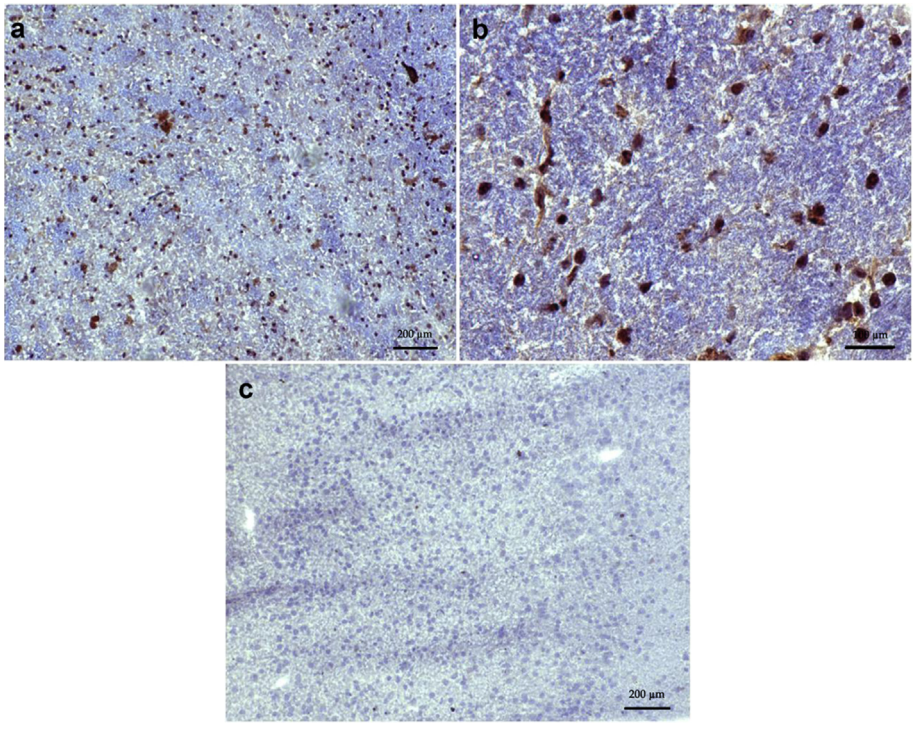


Fig. 3. Immunohistochemical detection of BTV-1 (a) and BTV-23 (b) antigen in sections of neonatal mouse brain at 7 dpi. IHC. Bars, 200 µm (a), 100 µm (b). (c) Negative control showing no positive immunoreactions for BTV antigen. IHC. Bar, 200 µm.

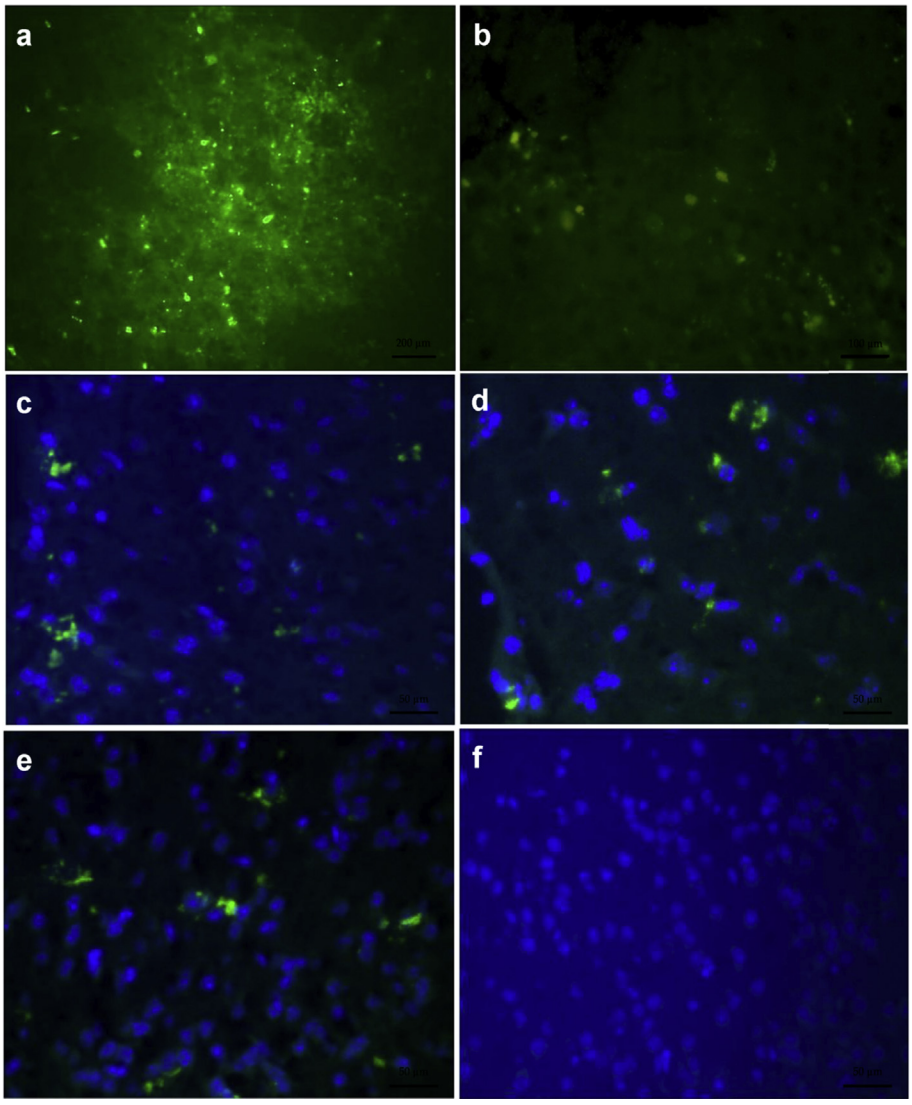


Fig. 4. Bluetongue virus VP7 antigen detection by direct fluorescent antibody technique (d-FAT) in neonatal mice brain at 7 dpi from different groups. (a, b) Brain showing strong and diffuse positive bright apple green fluorescence signals for BTV-1 (a) and weak signals for BTV-23 (b) antigen. Bars, 200 µm (a), 100 µm (b). (c–e) Brain showing positive signals for BTV-1 (c), BTV-23 (d) and BTV-10 (e) antigen with blue coloured nucleus (DAPI). Bars, 50 µm. (d) Negative control showing no positive immunoreactions for BTV antigen. Bar, 50 µm.

However, spleen and lung were negative for BTV RNA at all times. The results of BTV methods in brain tissue are summarized in Table 2.

## Discussion

In the present study, significant differences were found in the nature of neuropathogenic lesions caused by different serotypes of Indian BTV. BTV-infected neonatal mice developed non-suppurative necrotizing encephalitis of the cerebral cortex, midbrain and brainstem. Three serotypes, BTV-1, -2 and -23, were found to be more neuropathogenic in mice, while serotypes BTV-10 and BTV-16 were relatively non-pathogenic. Variations in pathogenesis or virulence among BTV serotypes have been reported in sheep and murine models of disease (Groocock *et al.*, 1982; Hamblin *et al.*, 1998; Caporale *et al.*, 2011; van der Sluijs *et al.*, 2013; Sánchez-Cordón *et al.*, 2013, 2015). Australian BTV serotype 20 was compared with American BTV serotype 17 in sheep and very mild clinical signs and viraemia were detected at 5 dpi in animals infected with BTV-20. In contrast, animals infected with BTV-17 showed pyrexia, severe hyperaemia of the oral mucosa and nasolabial area and viraemia was detected from 3 to 20 dpi (Groocock *et al.*, 1982). Caporale *et al.* (2011) compared the in-vivo pathogenic potential of BTV

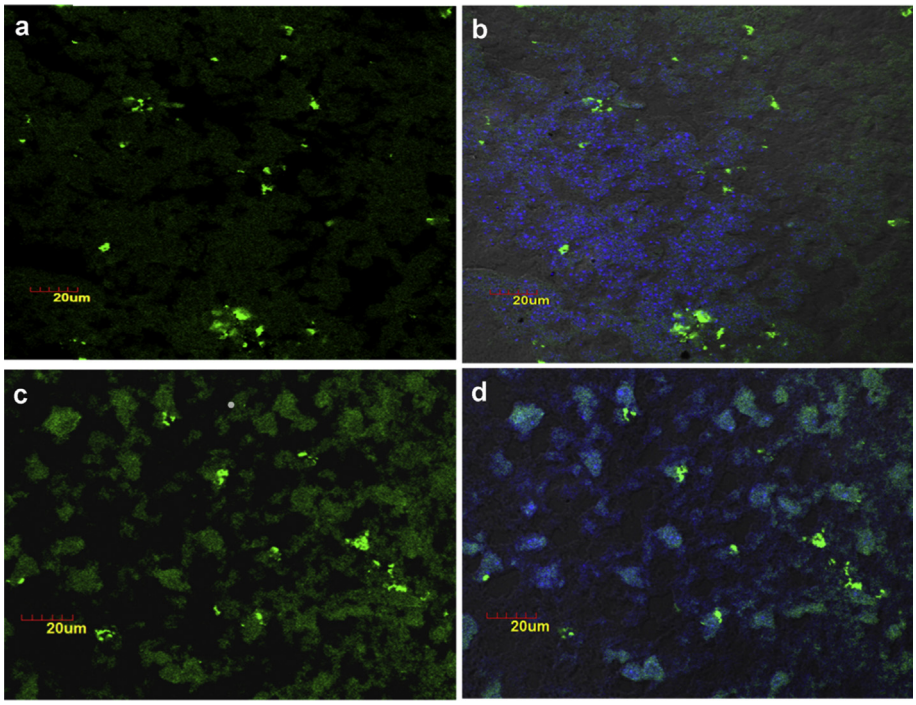


Fig. 5. Bluetongue virus VP7 antigen localization in brain tissue by confocal microscopy at 7 dpi in BTV-1 (a,b) and BTV-23 (c,d) inoculated mice. (a, c) FITC; (b,d) merged DIC images.

serotypes BTV-2, BTV-4 and BTV-9 isolated from naturally occurring outbreaks of BT in sheep in the Republic of South Africa and Italy. Significant differences were observed in mortality and histopathological lesions among Italian BTV serotypes, even when all were low-passaged strains. Further, BTV-1-infected sheep showed a longer clinical course, more severe clinical signs and gross lesions and increased serum levels of pro-inflammatory cytokines than those infected with BTV-8 (Sánchez-Cordón *et al*., 2013, 2015). European isolates of BTV-1 have more transplacental transmission potential when compared with isolates of BTV-8 (van der Sluijs *et al*., 2013). Moreover, BTV-1 induced severe central nervous system defects such as hydranencephaly and porencephaly, while these lesions were not observed in the BTV-8-infected fetuses. The differences in neuropathogenic lesions were also observed with different strains of BTV-11 (Waldvogel *et al*., 1986; Brewer and Osburn, 1998) in experimentally-infected neonatal mice. Similar lesions were reported in the brain of fetal calves and lambs infected experimentally with BTV-10 and -13 serotypes (Osburn *et al*., 1971; McLachlan and Osburn, 1983; MacLachlan *et al*., 1985; Waldvogel *et al*., 1992b). Immunofluorescence studies could detect BTV VP7 antigen in neuronal cells of the cerebral cortex and subventricular zones as early as 3 dpi. The distinct spread of green fluorescence signals was detected in the cytoplasm of neuronal cells of mice infected with BTV by confocal microscopy. IHC in infected brain showed positive labelling in the cytoplasm of neuronal cells and vascular endothelium of microcapillaries. The immunohistochemical and immunofluorescence demonstration of viral antigen gave the indication that BTV serotypes showed neuroinvasive and replicative potential in neurons and had the ability to

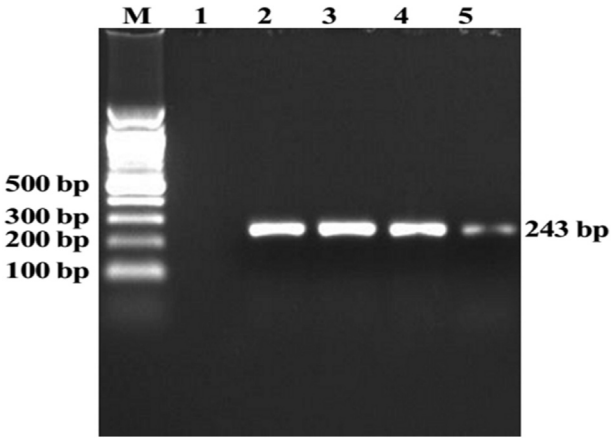


Fig. 6. Bluetongue virus nucleic acid detection from neonatal mice brain by one-step RT-PCR at 7 dpi. Lane M, 100 base pair (bp) DNA ladder; Lane 1, no template control (NTC); Lane 2, BTV-1; Lane 3, BTV-23; Lane 4, BTV-2; Lane 5, BTV-10.

**Table 2**
**BTV detection in brain at different time intervals**

| *Days post infection* | *Detection method* | | |
|---|---|---|---|
| | *Direct fluorescence antibody technique* | *Immunohistochemistry* | *One-step RT-PCR* |
| 0 | − | − | − |
| 1 | − | − | + |
| 3 | + | + | + |
| 7 | + + + | + + | + |
| 11 | + + + | + + + | + |
| 15 | + + | + | + |
| 20 | + | − | + |

−, no signal; +, weak signal; + +, moderate signal; + + +, strong signal.

damage vascular endothelial cells of brain. These results were in concurrence with the results of Narayan and Johnson (1972), Caporale *et al.* (2011) and Channappanavar *et al.* (2012).

BTV nucleic acid was detected by one-step RT-PCR in the brain of infected mice as early as 1 dpi until the end of the experimental period; however, no viral RNA was detected in the spleen and lung. This indicates selective replication and restriction of virus within the target brain tissue of neonatal mice. RT-PCR can be used as an additional tool to study BTV neuropathogenesis in neonatal mice due to its sensitivity and specificity. The differences in severity of lesions caused by different BTV serotypes depend on virus factors such as BTV serotypes, strain/topotype, passage history of the virus, virus dose, inoculation route and host factors such as age, species, breed, individual susceptibility, immune status of the infected host, nutritional status, stress and environmental factors such as solar irradiation and high temperature (MacLachlan *et al.*, 2009; Maclachlan and Guthrie, 2010). The genetic heterogeneity between different BTV serotypes is mainly attributable to variable expression of diseases in susceptible hosts (Carr *et al.*, 1994; De Mattos *et al.*, 1994; Bonneau *et al.*, 2002; Caporale *et al.*, 2011; Janowicz *et al.*, 2015). It remains crucial to elucidate exactly which viral factors are involved in causing the variable virulence. Caporale *et al.* (2011) studied the molecular basis of virulence of various BTV serotypes in murine models of experimental infection and reported that genome segments encoding VP1, VP2 and NS2 showed consistent differences between the virulent and attenuated serotypes/strains of BTV (Caporale *et al.*, 2011). Recently, it has been reported that multiple genome segments determine the virulence of the BTV-8 serotype, including VP2 and NS3, which are the primary determinants of virulence, but VP1, VP5, VP4, VP6 and VP7 also contribute to virulence (Janowicz *et al.*, 2015). Furthermore, it has been reported that differences in the pathogenesis or virulence between BTV serotypes is not due to greater virus replication or an early intense antibody response, suggesting that viral load and antibody levels do not influence the pathogenicity of BTV serotypes (Sánchez-Cordón *et al.*, 2013). In contrast, a direct link exists between the pathogenicity of BTV serotypes, the severity of vascular lesions and serum concentrations of acute phase proteins (APPs) (Sánchez-Cordón *et al.*, 2013). The changes from necrotizing lesions to non-suppurative meningoencephalitis from 1 to 10 dpi revealed considerable ability of mice of that age to respond immunologically to BTV infection (Gowland, 1965; Richards and Cordy, 1967). This is the first study of the comparative neuropathogenicity of major Indian BTV serotypes, but further work is needed to determine the genetic basis involved in these differences.

The neonatal mouse is apparently a good laboratory animal model to study the neuropathogenesis of virulent and avirulent BTV serotypes/strains. The distinction between virulent and non-virulent BTV serotypes/strains and identification of viruses that cause neuropathogenesis represents critical information for informing policy makers with regards to the design of suitable vaccines and for conducting cost−risk analysis prior to the implementation of control measures and control strategies to reduce the economic losses due to reproductive failures in pregnant sheep and cattle.

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## Conflict of Interest Statement

The authors declare that they have no conflicts of interest with respect to their authorship or publication of this article.

## References


Ayanur A, Singh KP, Cherian S, Singh V, Nandi S *et al.* (2016) Sero-epidemiology and molecular detection of bluetongue virus in Indian ruminants. *Veterinaria Italiana*, **52**, 305–311.

Barnard BJ, Pienaar JG (1976) Bluetongue virus as a cause of hydranencephaly in cattle. *Onderstepoort Journal of Veterinary Research*, **43**, 155–158.

Bommineni YR, Reddy YN, Rao PP, Sudheer D, Susmitha B *et al.* (2008) Isolation of bluetongue virus serotypes 2, 9 and 15 from sheep in India. *The Veterinary Record*, **163**, 545–546.

Bonneau KR, Topol JB, Gerry AC, Mullens BA, Velten RK *et al.* (2002) Variation in the NS3/NS3A gene of bluetongue viruses contained in *Culicoides sonorensis* collected from a single site in southern California. *Virus Research*, **84**, 59–65.

Brewer AW, Osburn BI (1998) Sequential distribution of neurovirulent and avirulent strains of bluetongue virus in neonatal mice by RT-PCR. *Archives of Virology*, **143**, 145–155.

Caporale M, Wash R, Pini A, Savini G, Franchi P *et al.* (2011) Determinants of bluetongue virus virulence in murine models of disease. *Journal of Virology*, **85**, 11479–11489.

Carr MA, Brewer AW, Osburn BI (1995) Growth of a neuroinvasive strain of bluetongue virus in suckling mice. *Archives of Virology*, **140**, 915–925.

Carr MA, de Mattos CC, de Mattos CA, Osburn BI (1994) Association of bluetongue virus gene segment 5 with neuroinvasiveness. *Journal of Virology*, **68**, 1255–1257.

Chauhan HC, Biswas SK, Chand K, Rehman W, Das B *et al.* (2014) Isolation of bluetongue virus serotype 1 from aborted goat fetuses. *Revue Scientifique et Technique de l'OIE*, **33**, 803–812.

Channappanavar R, Singh KP, Singh R, Umeshappaa CS, Ingale SL *et al.* (2012) Enhanced pro-inflammatory cytokine activity during experimental bluetongue virus-1 infection in Indian native sheep. *Veterinary Immunology and Immunopathology*, **145**, 485–492.

De Mattos CC, de Mattos CA, Osburn BI, MacLachlan NJ (1994) Evolution of the L2 gene of strains of bluetongue virus serotype 10 isolated in California. *Virology*, **201**, 173–177.

Enright FM, Osburn BI (1980) Ontogeny of host responses in ovine fetuses infected with bluetongue virus. *American Journal of Veterinary Research*, **41**, 224–229.

Gould AR, Pritchard LI (1990) Relationships amongst bluetongue viruses revealed by comparisons of capsid and outer coat protein nucleotide sequences. *Virus Research*, **17**, 31–52.

Gowland G (1965) Induction of transplantation tolerance in adult animals. *British Medical Bulletin*, **21**, 123–128.

Groocock CM, Parsonson IM, Campbell CH (1982) Bluetongue virus serotypes 20 and 17 infections in sheep: comparison of clinical and serological responses. *Veterinary Microbiology*, **7**, 189–196.

Hamblin C, Salt JS, Graham SD, Hopwood K, Wade-Evans AM (1998) Bluetongue virus serotypes 1 and 3 infection in Poll Dorset sheep. *Australian Veterinary Journal*, **76**, 622–629.

Huismans H, Erasmus BJ (1981) Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort Journal of Veterinary Research*, **48**, 51–58.

Janowicz A, Caporale M, Shaw A, Gulletta S, Di Gialleonardo L *et al.* (2015) Multiple genome segments determine virulence of bluetongue virus serotype 8. *Journal of Virology*, **89**, 5238–5249.

Jenckel M, Bréard E, Schulz C, Sailleau C, Viarouge C *et al.* (2015) Complete coding genome sequence of putative novel bluetongue virus serotype 27. *Genome Announcement*, **3** e00016-15.

Luedke AJ (1985) Effect of bluetongue virus on reproduction in sheep and cattle. *Progress in Clinical & Biological Research*, **178**, 71–78.

Luna LG (1968) *Manual of Histologic Staining Methods*, 3rd Edit., Armed Forces Institute of Pathology, McGraw-Hill, New York, pp 25–32.

Maan NS, Maan S, Guimera M, Pullinger G, Singh KP *et al.* (2012) Complete genome sequence of an isolate of bluetongue virus serotype 2, demonstrating circulation of a western topotype in southern India. *Journal of Virology*, **86**, 5404–5405.

MacLachlan NJ, Osburn BI (1983) Bluetongue virus-induced hydranencephaly in cattle. *Veterinary Pathology*, **20**, 563–573.

MacLachlan NJ, Osburn BI, Ghalib HW, Stot JL (1985) Bluetongue virus-induced encephalopathy in fetal cattle. *Veterinary Pathology*, **22**, 415–417.

MacLachlan NJ, Drew CP, Darpel KE, Worwa G (2009) The pathology and pathogenesis of bluetongue. *Journal of Comparative Pathology*, **141**, 1–16.

MacLachlan NJ, Guthrie AJ (2010) Re-emergence of bluetongue, African horse sickness, and other orbivirus diseases. *Veterinary Research*, **41**, 35.

Maximova OA, Brian RM, Alexander GP (2010) High-throughput automated image analysis of neuroinflammation and neurodegeneration enables quantitative assessment of virus neurovirulence. *Vaccine*, **28**, 8315–8326.

Maximova OA, Ward JM, Asher DM, St Claire M, Finneyfrock BW *et al.* (2008) Comparative neuropathogenesis and neurovirulence of attenuated flaviviruses in nonhuman primates. *Journal of Virology*, **82**, 5255–5268.

Mellor PS (1990) The replication of bluetongue virus in *Culicoides* vectors. *Current Topics in Microbiology and Immunology*, **162**, 143–158.

Narayan O, Johnson RT (1972) Effects of viral infection on nervous system development. I. Pathogenesis of bluetongue virus infection in mice. *American Journal Of Pathology*, **68**, 1–14.

Osburn BI, Silverstein AM, Prendergast RA, Johnson RT, Parshall CJ (1971) Experimental viral-induced congenital encephalopathies. I. Pathology of hydranencephaly and porencephaly caused by bluetongue vaccine virus. *Laboratory Investigation*, **25**, 197–205.

Prasad G, Garg AK, Minakshi Kakker NK, Srivastava RN (1994) Isolation of bluetongue virus from sheep in Rajasthan State, India. *Revue Scientifique et Technique de l'OIE*, **13**, 935–938.

Prasad G, Sreenivasulu D, Singh KP, Mertens PPC, Maan S (2009) Bluetongue in the Indian subcontinent. In: *Bluetongue*, P Mellor, M Baylis, P Mertens, Eds., Elsevier, Amsterdam, pp. 167–189.

Pringle CR (1999) Virus taxonomy: the universal system of virus taxonomy updated to include new proposals ratified by international committee on taxonomy of viruses during 1998. *Archives of Virology*, **144**, 422–429.

Ranjan K, Minakshi P, Prasad G (2015) Bluetongue: Indian perspective. *Acta Virologica*, **59**, 317–337.

Ranjan K, Prasad M, Lambe U, Guray M, Prasad G (2016) Molecular typing of bluetongue virus 16 from Karnataka State of India. *Journal of Microbiology and Related Research*, **2**, 43–49.

Rao PP, Hegde NR, Reddy YN, Krishnajyothi Y, Reddy YV *et al.* (2016) Epidemiology of bluetongue in India. *Transboundary and Emerging Diseases*, **63**, 151–164.

Reddy YKM, Manohar BM, Pandey AB, Reddy YN, Prasad G *et al.* (2010) Development and evaluation of inactivated pentavalent adjuvanted vaccine for bluetongue. *Indian Veterinary Journal*, **87**, 434–436.

Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. *American Journal of Hygiene*, **27**, 493–497.

Richards WPC, Cordy DR (1967) Bluetongue virus infection: pathologic responses of nervous systems in sheep and mice. *Science*, **156**, 530–531.

Richardson C, Taylor WP, Terlecki S, Gibbs EP (1985) Observations on transplacental infection with bluetongue virus in sheep. *American Journal of Veterinary Research*, **46**, 1912–1922.

Saminathan M, Rana R, Ramakrishnan MA, Karthik K, Malik YS *et al.* (2016) Prevalence, diagnosis, management and control of important diseases of ruminants with special reference to Indian scenario. *Journal of Experimental Biology and Agricultural Sciences*, **4**, 338–367.

Sánchez-Cordón PJ, Pérez de Diego AC, Gómez-Villamandos JC, Sánchez-Vizcaíno JM, Pleguezuelos FJ *et al.* (2015) Comparative analysis of cellular immune responses and cytokine levels in sheep experimentally infected with bluetongue virus serotype 1 and 8. *Veterinary Microbiology*, **177**, 95–105.

Sánchez-Cordón PJ, Pleguezuelos FJ, Pérez de Diego AC, Gómez-Villamandos JC, Sánchez-Vizcaíno JM *et al.* (2013) Comparative study of clinical courses, gross lesions, acute phase response and coagulation disorders in sheep inoculated with bluetongue virus serotype 1 and 8. *Veterinary Microbiology*, **166**, 184–194.

Shaw AE, Ratinier M, Nunes SF, Nomikou K, Caporale M *et al.* (2013) Re-assortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment. *Journal of Virology*, **87**, 543–557.

Tembhurne PA, Mondal B, Pathak KB, Biswas SK, Sanyal A *et al.* (2010) Segment-2 sequence analysis and cross-neutralization studies on some Indian bluetongue viruses suggest isolates are VP2-variants of serotype 23. *Archives of Virology*, **155**, 89–95.

Umeshappa CS, Singh KP, Pandey AB, Singh RP, Nanjundappa RH (2010) Cell mediated immune response and cross-protective efficacy of binary ethylenimine-inactivated bluetongue virus serotype-1 vaccine in sheep. *Vaccine*, **28**, 2522–2531.

Umeshappa CS, Singh KP, Rudragouda C, Sharma K, Nanjundappa RH *et al.* (2011) A comparison of intradermal and intravenous inoculation of bluetongue virus serotype 23 in sheep for clinico-pathology, and viral and immune responses. *Veterinary Immunology and Immunopathology*, **141**, 230–238.

van der Sluijs MT, Schroer-Joosten DP, Fid-Fourkour A, Vrijenhoek MP, Debyser I *et al.* (2013) Transplacental transmission of bluetongue virus serotype 1 and serotype 8 in sheep: virological and pathological findings. *PLoS One*, **8**, e81429.

Waldvogel AS, Anderson CA, Higgins RJ, Osburn BI (1987) Neurovirulence of the UC-2 and UC-8 strains of bluetongue virus serotype 11 in newborn mice. *Veterinary Pathology*, **24**, 404–410.

Waldvogel AS, Anderson GA, Phillips DL, Osburn BI (1992a) Association of virulent and avirulent strains of bluetongue virus serotype 11 with premature births of late-term bovine fetuses. *Journal of Comparative Pathology*, **106**, 333–340.

Waldvogel AS, Anderson CA, Phillips DL, Osburn BI (1992b) Infection of bovine fetuses at 120 days gestation with virulent and avirulent strains of bluetongue virus serotype 11. *Comparative Immunology, Microbiology and Infectious Diseases*, **15**, 53–63.

Waldvogel AS, Stott JL, Squire KR, Osburn BI (1986) Strain-dependent virulence characteristics of bluetongue virus serotype 11. *Journal of General Virology*, **67**, 765–769.

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