

## Isolation of bluetongue virus serotype-1 from aborted goat fetuses

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### Summary

Abortions and stillbirths were noticed in pregnant goats on a farm in the state of Gujarat, India. About 50% of the pregnant goats aborted or gave birth to dead kids. Bluetongue virus (BTV) antibody in the sera of affected goats was detected using a competitive enzyme-linked immunosorbent assay (ELISA). Viral antigen in the blood of these goats and in the aborted fetal spleens was detected using a sandwich ELISA. Two viruses (SKN-9, SKN-10) were isolated in cell culture from aborted fetal spleens and were confirmed as *Orbivirus* by demonstration of ten bands in RNA polyacrylamide gel electrophoresis and identified as BTV-1 by sequencing of the VP2 gene. Sequence analyses revealed that these isolates were very closely related to a BTV-1 (strain SKN-8) isolated from *Culicoides* vectors

captured on the same farm one month after the occurrence of abortion. Isolation of BTV-1 from fetuses is probably evidence of transplacental transmission of the wild-type strain because attenuated or laboratory-adapted BTV-1 strains have never been used in this region. This may have important implications in the epidemiology of bluetongue, considering the presence of many BTV serotypes in India.

### Keywords

Abortion – Bluetongue virus serotype-1 – Fetus – Goat – Transplacental transmission – VP2 gene – Wild type.

### Introduction

Bluetongue disease of sheep is caused by the bluetongue virus (BTV), an arbovirus of the genus *Orbivirus* in the family *Reoviridae*. The virus is transmitted by *Culicoides* biting midges, which are most abundant and active in hot and humid climates. Traditionally, the area where BTV and its vectors are present has been limited to latitudes between 40°N and 35°S; however, several species of *Culicoides* have been reported to be implicated in BTV transmission outside this geographic area (1). The host species susceptible to BTV infection include almost all domestic and wild ruminants. Sheep are the most common host and show the acute form of the disease, characterised by high fever, excessive salivation, congestion of the tongue, conjunctivitis, coronitis, and petechial haemorrhages and ulcers on the mucous membrane of the mouth. Cattle are considered natural reservoirs of the virus, because of the asymptomatic nature of the infection associated with a prolonged viraemia (2). Before the outbreaks of BTV-8 in northern and central Europe, bluetongue in bovines was defined as subclinical. However, in the 2006 epidemic, clinical signs (ulcers and necrosis of the muzzle, oral cavity, udder skin and teats; periocular dermatitis; oedema of the distal limbs; reproductive disorders) were described in a number of cases in cattle infected with BTV-8 (3, 4, 5). The strain is highly virulent, not only for sheep but also for cattle. Goats are also infected by BTV but the infection is asymptomatic or subclinical (6). The clinical signs in goats, if any, are a sharp drop in milk production and a high fever up

to 42°C (7); however, signs are less intense than usually seen in clinically diseased sheep and cattle (8).

The effects of BTV infection on reproduction are poorly understood. Abortion, stillbirth and fetal deformities have been attributed to BTV infection of sheep and cattle (9, 10, 11). Transplacental transmission of live attenuated strains has been described for BTV-2, -9 and -23 (12, 13) and studies clearly show that modification of field strains, for example BTV-10 and BTV-11, by growth in embryonated eggs or in cell culture can markedly increase their ability to cross the placenta and cause fetal infection (14, 15); nevertheless, it cannot be ruled out that other field strains could share the same properties. Before the 2006 incursion of BTV into Europe, the ability to cross the placenta and cause congenital fetal infection had been largely limited to attenuated BTV strains. In the European outbreak, the ability of BTV-8 (a field strain) to cross the placenta and infect the fetus was a major concern because of transplacental transmission and an increase in the numbers of abortions, stillbirths and fetal deformities in cattle (8, 16, 17, 18, 19). The evidence of transplacental transmission of this European strain is important because the vast majority of previous studies failed to detect transplacental transmission of wild strains of BTV (20). The BTV-8 strain represents the novel introduction of an exotic BTV strain/serotype into Europe, although details of its entry route remain unknown. Sequence analyses indicate that the strain is not derived from the BTV-8 vaccine strain that was used in the South African multivalent live attenuated vaccine (21).

Bluetongue is endemic in India with frequent outbreaks in sheep, and BTV antibodies are widespread in domestic ruminants, including goats, throughout the country with a high level of seropositivity (22, 23, 24). Based on virus isolation and seroprevalence, the presence of 21 BTV serotypes has been reported in India (25). Strain BTV-serotype 1 (BTV-1) is mostly prevalent in the north-western regions and the virus has been isolated from sheep, goats and *Culicoides* (26, 27, 28). All the isolations that have been made in India were from vectors or diseased animals and there are no reports of BTV isolation from fetuses.

The occurrence of abortions and stillbirths in goats in Gujarat and the isolation of BTV-1 strains from aborted goat fetuses is reported here. These strains could be wild type because attenuated or laboratory-adapted BTV-1 strains or any other serotype have never been used as vaccines in Gujarat or anywhere else in India. The finding is important because transplacental transmission and fetal infection is mainly associated with attenuated BTV strains and not usually with wild-type strains.

## Materials and methods

### History of abortions and stillbirths in goats

Abortions and stillbirths occurred in goats on a livestock farm in Sardarkrushinagar, Gujarat, in July 2007. The farm housed sheep ( $n = 100$ ), goats ( $n = 140$ ), cattle ( $n = 350$ ) and buffalo ( $n = 200$ ) in separate enclosures. Among 25 pregnant goats (about three months of pregnancy), seven animals aborted and six others gave birth to dead kids (stillbirth). Abortion was observed only in goats, not in other species on the farm. Weight loss was noticed in many goats for a period of two to three months before the animals aborted. Apart from the abortions and stillbirths, no other bluetongue-related symptoms were noticed.

### Detection of antibodies in sera

After the observation of abortion, serosurveillance was conducted on the goats ( $n = 140$ ) to detect antibodies against BTV, peste des petits ruminants virus (PPRV) and *Brucella* spp. using commercially available antibody kits. The instructions provided by the manufacturers were followed. Competitive enzyme-linked immunosorbent assay (c-ELISA) kits were used to detect antibodies against BTV (bluetongue antibody c-ELISA test kit, Veterinary Diagnostic Technology Inc, Wheat Ridge, Colorado, USA) and PPRV (PPR c-ELISA kit, Indian Veterinary Research Institute, Mukteswar, India) (29). Antibody against *Brucella* spp. was determined in an indirect ELISA (AB-ELISA kit, ADMAS, Bangalore, India). Faecal smears from all the goats were examined with Ziehl-Neelsen staining

for the acid-fast paratuberculosis (Johne's disease) organism; the Johnin test was performed on samples from the goats that aborted or had stillborn kids.

### **Samples from fetuses and their dams**

Spleen homogenates from four aborted fetuses and unclotted blood from their dams were collected for serological and virological investigations. Tissues from other aborted fetuses or stillbirths were not collected as the goats are kept in outdoor pens and could have aborted or given birth to dead kids without being noticed.

### **Sandwich enzyme-linked immunosorbent assay for detection of bluetongue virus antigen**

The presence of BTV antigen was tested in EDTA-blood samples, fetal spleen tissues and cell culture supernatants (during virus isolation) using a polyclonal antibody-based group-specific sandwich ELISA (s-ELISA) (30). The assay plates were read at wavelength 492 nm on an ELISA reader and a value twice (or more) the mean optical density (OD) of the negative antigen control was considered as the positive/negative cut-off value (i.e. positive to negative (P/N) ratio  $\geq 2$ ). The assay specifically detects bluetongue viruses as a group with an analytical sensitivity of  $10^{2.4}$  TCID<sub>50</sub> per ml.

### **Isolation and identification of bluetongue virus**

BHK-21 cells were grown in tissue culture flasks (25 cm<sup>2</sup>) in Glasgow modified minimum essential medium (GMEM, Sigma), supplemented with 10% fetal bovine serum. About 0.5 ml of a 10% suspension of aborted fetal spleen was homogenised and lysed with sonic vibrations and clarified by centrifugation. The supernatant was filtered through a 0.2 µm membrane and the filtrates added to a pre-formed layer of BHK-21 cells. Viruses were adsorbed to the cells at 37°C for 1 h and the cell layer was washed three times with PBS. The cells were covered with culture medium containing 2% fetal bovine serum (US Origin FBS, Invitrogen, catalogue no. 16140-071, certified negative for bluetongue virus antibodies) and incubated at 37°C. Infected cells

were observed for cytopathic effects. Virus was harvested at four days post-infection and about 300 µl inoculum was passaged for the next cycle. In total, five blind passages were done. Samples of the fifth passage were tested for BTV or antigen and the nucleic acid.

The isolated virus was identified in the s-ELISA and a diagnostic reverse-transcription polymerase chain reaction (RT-PCR) (31) for the detection of antigen and nucleic acid, respectively. Infected cells from the fifth passage were frozen and thawed and the total RNA was extracted following a standard method. The RNA was used to amplify the NS1 gene of BTV following the method described by Dangler *et al.* (31).

### **Extraction and purification of bluetongue virus double-stranded RNA**

Double-stranded RNA (dsRNA) was extracted and purified, using the method adapted from Clarke and McCrae (32) and Squire *et al.* (33). Briefly, BTV-infected cell cultures were scraped from the surface of 75 cm<sup>2</sup> flasks and total RNA was extracted using phenol-chloroform (3:2). Single-stranded cellular RNA was removed by precipitation with lithium chloride and the dsRNA was dissolved in Tris-EDTA buffer. The preparation was stored in absolute alcohol at -70°C or used for polyacrylamide gel electrophoresis.

### **Polyacrylamide gel electrophoresis of double-stranded RNA and silver staining**

These procedures were adapted from the methods described by Squire *et al.* (33), Herring *et al.* (34) and Promega Protocols (35). The dsRNA was electrophoresed in a 10% discontinuous SDS-free polyacrylamide gel at 140 V for 5 h. Gels were then fixed with acetic acid and stained with silver nitrate.

### **Sequencing of full-length segment-2 of bluetongue virus isolates and sequence analysis**

Full-length amplification of BTV segment-2 was achieved by generating overlapping PCR products using three pairs of primers

(27). The PCR amplicons were cloned and the plasmids containing the expected insert were sequenced using the BigDye Terminator<sup>®</sup> v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, CA, USA) on an automated sequencer (Applied Biosystems 3130 Genetic Analyzer), following the manufacturer's instructions. Nucleotide sequences were aligned using the CLUSTAL W algorithm (36) implemented in the MegAlign of the DNASTAR program package (DNASTAR Inc, Madison, USA). Phylogenetic comparison was made using MEGA4 software (37). The evolutionary history was inferred using the neighbour-joining method (38). Evolutionary distances were computed using the Kimura-2 parameter method (39) and are in the units of the number of base substitutions per site. The bootstrap consensus tree inferred from 10,000 replicates (40) was taken to represent the evolutionary history of the taxa analysed.

## Results

### Serosurveillance of the goats

Overall, 10% (14/140) of goat serum samples tested positive for BTV antibody. Twelve of 25 pregnant goats and six of seven goats that aborted tested positive for BTV antibody. No antibody against PPRV was detected in any of the goats. Antibody against *Brucella* spp. was detected in two goats but in none of the pregnant goats. Acid-fast organisms were demonstrated with Ziehl-Neelsen staining in 12 faecal smears. The goats that aborted or had stillborn kids were negative in the Johnin test and no acid-fast organisms were observed in their faecal smears. No apparent weight loss was noticed in these goats.

### Serological tests on the tissue samples

All the collected blood samples and fetal spleen tissues tested positive for BTV antigen in the s-ELISA (Fig. 1). One blood sample (GB04) tested strongly positive in comparison with the positive control of the assay. Sera tested positive for BTV antibody in agar gel immunodiffusion (Table I).

### **Bluetongue virus isolation and identification**

Four spleen tissue samples were blind-passaged in BHK-21 cells. Up to the third passage any cytopathic effect was difficult to detect under the microscope, but at the fourth passage and above an effect was clearly evident. Viruses were isolated from aborted fetus spleen samples of goat no. 04 and goat no. 25 and designated as SKN-9/India/2007 and SKN-10/India/2007, respectively (referred to as SKN-9 and SKN-10). The identity of the viruses was confirmed as BTV in s-ELISA and RT-PCR. Samples of both isolates tested strongly positive for BTV antigen at the fifth passage (Fig. 1). A diagnostic RT-PCR was used to amplify the BTV NS1 gene (31) and products of expected size – (273 bp) were amplified from both the isolates and were cloned and sequenced. Nucleotide sequences were analysed to verify the identity of the PCR products. Matching the sequences with those available in the public database confirmed that they were of the BTV NS1 gene (data not shown).

### **Polyacrylamide gel electrophoresis of the bluetongue virus genome and serotype identification**

The genome segment migration patterns (electropherotypes) of the BTV isolates were similar and ten dsRNA segments were resolved according to their size (Fig. 2). Considering the endemicity of BTV-1 in the state of Gujarat and the recent isolation of BTV-1 from *Culicoides* (28), the SKN-9 and SKN-10 isolates were considered likely to be BTV-1. Accordingly, a set of primers for BTV-1 (primer set P3/P4 on VP2/segment-2) (27) was used to amplify a fragment of genome segment-2 (830 bp, nucleotide position 1186–2015). The correct-size products were amplified and were cloned and sequenced. BLAST analysis ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) of the sequences revealed 99% nucleotide identity (data not shown) with segment-2 of Indian BTV-1 isolate MKD-18 (GenBank accession no. HM014236). By using PCR and sequencing, not by using epidemiology, it was demonstrated that isolates SKN-9 and SKN-10 belong to serotype BTV-1.

### Sequencing of the full-length VP2 gene and phylogenetic analyses

Sequences of the overlapping PCR products were assembled to obtain the full-length VP2 gene of SKN-9 and SKN-10, and these have been submitted to GenBank under accession numbers JX101694 and JX101695, respectively. The gene coding for protein VP2 is 2886 bp in length in both the isolates and it has 961 amino acids. BLAST analysis of these sequences confirmed that they are cognate with VP2 gene sequences previously published for other BTV-1 isolates/strains (GenBank accession no. HM014236; NJ558348 [SKN-7] and NJ558349 [SKN-8]) (27, 28). The nucleotide sequences of gene VP2 of SKN-9 and SKN-10 were compared with the recently isolated Indian BTV-1 isolates SKN-7, SKN-8 and MKD-18, and more than 99% nucleotide identity was observed among them. Phylogenetically, Indian isolates form a single monophyletic group closest to the Australian isolates (Fig. 3).

### Discussion

Serological and virological investigations in the present study demonstrated the presence of BTV in affected goats and aborted fetuses, suggesting that BTV infection may be one of the causes of abortion and stillbirth in goats. Viral antigen was detected in the blood of four goats and their aborted fetuses by s-ELISA, indicating that the virus was transmitted transplacentally. The virus was isolated in cell culture of the spleens of aborted goat fetuses and the identity of the virus was confirmed in s-ELISA and sequencing of the PCR-amplified viral gene. Overall, 48% (12/25) of the pregnant goats tested positive for BTV antibody and more than 50% (13/25) of them aborted or gave birth to dead kids. Among seven goats that aborted, six were positive for BTV antibody. Brucellosis, a common cause of abortion in goats, was ruled out since only 0.014% (2/140) of goats at the farm tested positive for antibody to *Brucella* spp. and all the pregnant goats tested negative. Paratuberculosis organisms were detected in some of the goats but not in the pregnant ones; furthermore, paratuberculosis is not known to cause abortion.

Bluetongue virus antibodies are widespread in ruminants in the state of Gujarat (22), and in buffalo the presence of neutralising antibodies against as many as 11 BTV serotypes has been reported (41). A study in Gujarat also found that the prevalence of BTV antibody was much higher in ruminants (cattle, buffalo and goats) that aborted than in the clinically healthy animals (42). This suggests that BTV infection was one of the causes of abortion, although this was not confirmed by virus isolation from aborted fetuses or antigen detection in the animals. In the present study, two isolates (SKN-9, SKN-10) of BTV-1 were isolated from the spleen of aborted goat fetuses, which is evidence of transplacental transmission of the wild-type strain. The SKN-9 and SKN-10 isolates are neither attenuated nor laboratory-modified, because at present there is no bluetongue vaccination (inactivated or live) programme in Gujarat or in the rest of India, although some inactivated vaccines (43, 44) are under experimental trial.

In July 2007, abortion and stillbirth occurred in goats on a livestock farm at Sardarkrushinagar, Gujarat. Blood samples from some dams and spleens from their aborted fetuses were collected for serological and virological investigations, which led to the isolation of two BTV isolates (SKN-9 and SKN-10) from fetal spleens. During August, *Culicoides* were captured at the same farm and virus (BTV-1, isolate SKN-8) was isolated from the insects (28). Nucleotide sequencing of the complete VP2 gene of these three BTV-1 isolates showed that they were very strongly related (99.1% to 99.4% nucleotide identity among SKN-8, SKN-9 and SKN-10), suggesting that they are probably identical. Isolate SKN-8 has probably been transmitted from *Culicoides* to goats, crossed the placenta and eventually infected the fetus (28). However, it is essential to study the antigenic properties and sequence the entire genome of these three isolates to prove that they are identical.

In the past, BTV transplacental transmission was mainly associated with laboratory-adapted strains, in particular with live attenuated vaccine strains. However, the 2006 European outbreaks have provided ample evidence for transplacental transmission of a wild-type BTV

strain (serotype-8), leading to abortion and fetal abnormalities in cattle and sheep (19, 45). Nonetheless, the mechanism by which BTV-8 adapted to cross the placenta and infect the fetus remains unknown. Other wild-type BTV strains have also been shown to be vertically transmitted and to cause abortion in cattle and sheep in natural infection (11, 46). The present study provides evidence of transplacental transmission of a field strain of BTV (serotype-1) in goats. Other field strains from other serotypes may also be vertically transmitted and infect the ruminant fetus as many BTV serotypes are known to be circulating in India.

## Conclusions

Abortions and stillbirths occurred in a flock of goats and BTV antigen was detected in the blood of dams and the corresponding fetal spleens. The cause of the abortions and stillbirths may be attributed to BTV infection of the pregnant animals. Two viruses (BTV-1) were isolated from the fetal spleens and found to be very closely related to a recent BTV-1 strain isolated from *Culicoides*. This is an indication of transplacental transmission of a wild-type BTV strain and is not the consequence of any vaccination programme.

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**Table I**  
**Examination of goat blood and spleen of aborted fetuses for**  
**bluetongue virus antigen and virus isolation**

Samples	AGID <sup>(a)</sup>	s-ELISA <sup>(b)</sup>	Virus isolation in BHK-21 cells
Blood, goat no. 02, aborted <sup>(c)</sup>	+	+	n/a
Blood, goat no. 04, aborted <sup>(c)</sup>	+	+	n/a
Blood, goat no. 25, aborted <sup>(c)</sup>	+	+	n/a
Blood, goat no. 52, aborted <sup>(c)</sup>	+	+	n/a
Spleen of aborted fetus, goat no. 02		+	Failed
Spleen of aborted fetus, goat no. 04		+	Isolated
Spleen of aborted fetus, goat no. 25		+	Isolated
Spleen of aborted fetus, goat no. 52		+	Failed

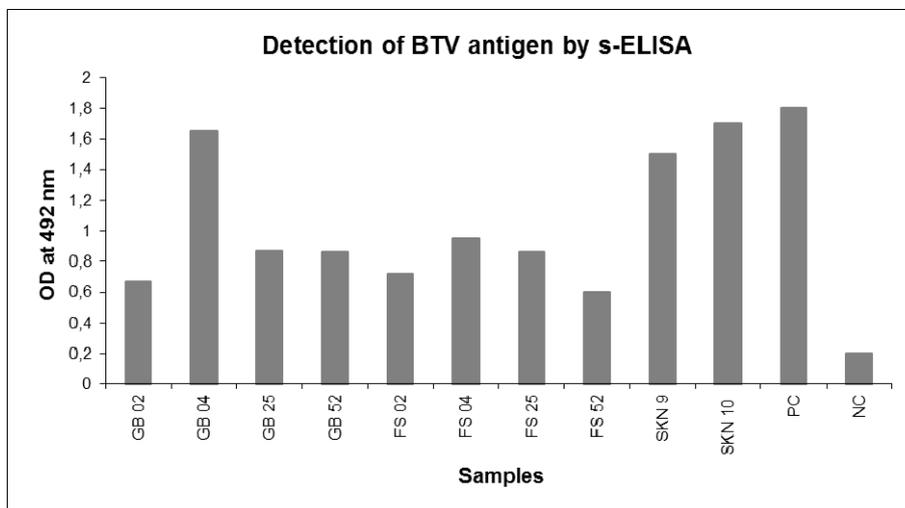
a) AGID: bluetongue-agar gel immunodiffusion test kit (Veterinary Diagnostic Technology, Inc, Wheat Ridge, Colorado, USA)

b) s-ELISA: polyclonal antibody-based sandwich enzyme-linked immunosorbent assay for the detection of bluetongue virus group-specific antigen (30)

c) Serum samples also tested positive for bluetongue virus antibody in a competitive ELISA (Bluetongue antibody c-ELISA test kit, Veterinary Diagnostic Technology, Inc, Wheat Ridge, Colorado, USA)

BHK-21: baby hamster kidney-21

n/a: not attempted



BHK-21: baby hamster kidney-21

FS: fetal spleen

GB: goat blood

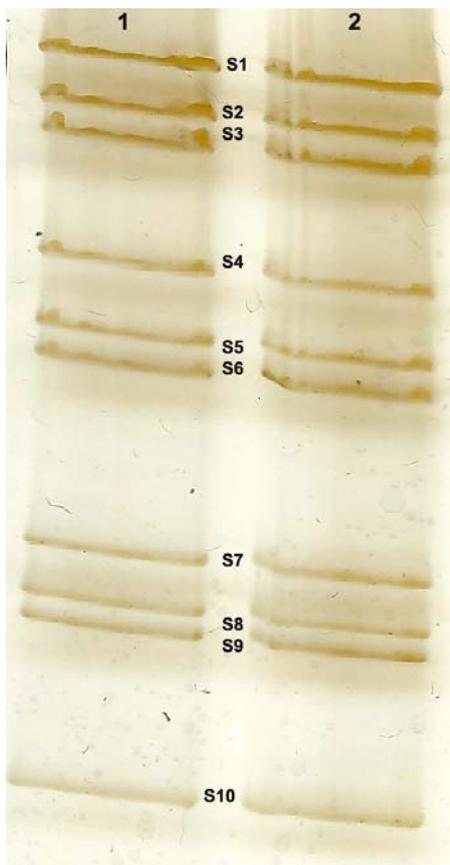
NC: negative control

PC: positive control

**Fig. 1**

**Sandwich enzyme-linked immunosorbent assay to detect bluetongue virus antigen in goat blood, fetal spleen and infected cell culture supernatants**

Bluetongue virus was isolated from aborted fetal spleens (FS04, FS25) by culture on BHK-21 cells. The isolated viruses (SKN-9, SKN-10) showed high optical density values at passage five



**Fig. 2**

**Polyacrylamide gel electrophoresis of the double-stranded RNA genome of bluetongue virus isolated from aborted fetal spleen**

Viral RNA was extracted from infected cell culture, resolved in 10% polyacrylamide gel and silver stained. Segments S1–S10 were visible for isolates SKN-9 (lane 1) and SKN-10 (lane 2). The two isolates showed similar genome migration profiles

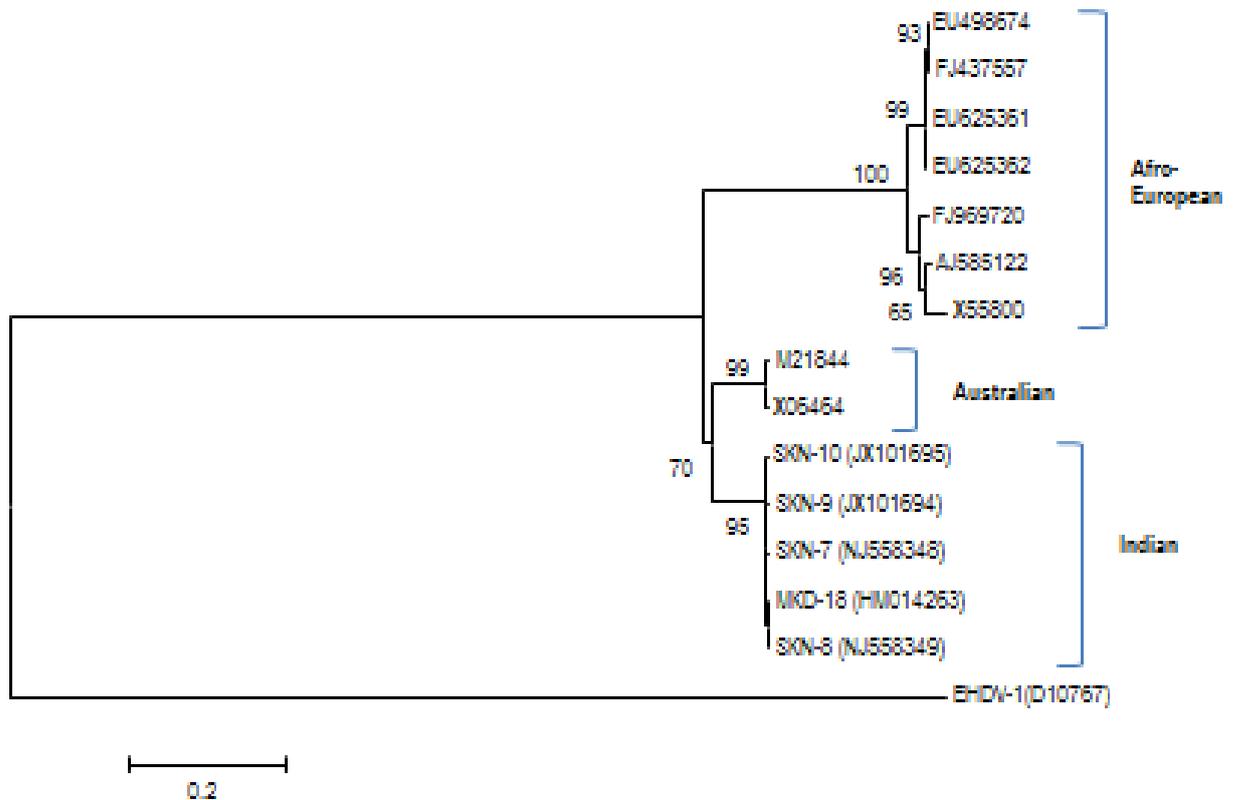


Fig. 3.

**Fig. 3**  
**Neighbour-joining tree of VP2 nucleotide sequences depicting phylogenetic relationships among bluetongue virus serotype-1 isolates from India and other countries**

The bluetongue virus-1 (BTV-1) isolates used in the phylogenetic analyses are from Portugal (EU498674), France (FJ437557), Algeria (EU625361), Morocco (EU625362), South Africa (FJ969720, AJ585122/South Africa reference strain RSAr1, X55800 South Africa vaccine strain) and Australia (X06464, M21844/isolate CS156). Epizootic haemorrhagic disease virus (EHDV-D10767) was used as an out group

Bootstrap support values deduced from 10,000 resamples are presented at the nodes. The GenBank accession numbers of the sequences of Indian isolates are given in parentheses. Scale indicates 0.2 nucleotide substitutions per site