Bluetongue virus serotype-1 in goats in the Pithoragarh area of Uttarakhand, India

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Summary
This short communication reports the results of a bluetongue sero-surveillance conducted in the Pithoragarh hills of Uttarakhand in India during the autumn of 2011. Unclotted blood and serum samples were collected from 51 goats for detection of bluetongue virus (BTV) antigen and antibodies. Of the 51 collected samples, 18 (35%) were positive to an indirect ELISA and 33 (64%) resulted positive to a BTV ELISA antigen. From a strong antigen-positive blood sample, a BTV was isolated (named as PTG-13) on cell culture and was subsequently confirmed as BTV-1 by RT-PCR and partial sequencing of genome segment-2. The goat serum samples were found to contain high titer of neutralising antibodies against BTV-23, nonetheless the virus could not be isolated. Interestingly, no neutralizing antibodies were detected against PTG-13 or other BTV-1 isolate, which suggests that sampling was probably done before the development of neutralizing antibodies against PTG-13 virus in the host. Isolation of BTV-1 (PTG-13) and presence of BTV-23 neutralizing antibodies in serum samples indicate that goats were probably infected with BTV-1 and 23 in different periods.

Virus della bluetongue sierotipo 1 in capre nell'area di Pithoragarh, Stato di Uttarakhand, India

Parole chiave
Bluetongue virus sierotipo-1, BTV-23, Capra, India.

Riassunto
Nella presente comunicazione sono riportati i risultati sulla presenza del virus della bluetongue (BTV) nell'area Pithoragarh, Stato di Uttarakhand, India, nell'autunno del 2011. Lo studio ha riguardato 51 campioni di sangue intero e siero di capra analizzati per rilevare la presenza di BTV e relativi anticorpi. Quando testati in laboratorio, 18 (35%) dei 51 campioni di siero sono risultati positivi all'ELISA indiretta mentre 33 (64%) campioni di sangue sono risultati positivi all'ELISA antigene per BTV. Dai campioni fortemente positivi è stato inoltre possibile far crescere il virus su tessuto colture (PTG-13). L'appartenenza del ceppo isolato al sierotipo 1 del BTV è stata confermata attraverso RT-PCR e sequenziamento parziale del genoma del segmento-2. Non è stato possibile invece isolare il sierotipo 23 nonostante i campioni di siero mostrassero titoli anticorpali elevati nel confronti di questo sierotipo. È possibile che il mancato rilevamento di anticorpi per PTG-13 o per altri ceppi di BTV-1 sia dovuto al fatto che il prelievo negli animali coinvolti nell'indagine sia stato effettuato prima che gli anticorpi per PTC-13 potessero svilupparsi. La presenza contemporanea di BTV-1 e di anticorpi per BTV-23 indica che gli animali testati sono stati infettati da BTV-1 e BTV-23 in momenti diversi.

Bluetongue is endemic in India. Outbreaks are frequent in sheep although domestic ruminants have high level of seropositivity throughout the country (6, 9, 23). Several serotypes of BTV have been isolated from different states of India, especially from the regions where sheep population is more abundant. Bluetongue virus serotype-1 was isolated from sheep and *Culicoides* vectors (11, 12, 20), and serotype-specific antibodies were detected against several serotypes including BTV-1 and 23 (7) in the North-Western part of the country (i.e. the states of Rajasthan and Gujarat). Seroprevalence and virus isolation results suggest that BTV-1 is the predominant serotype in the Northern and North-Western parts of India as recently reported (4, 8). In 1995, BTV-23 was isolated from sheep at foot-hills of Dehradun District of Uttarakhand State (18). The virus circulated in the region and 2 years later it caused an outbreak in sheep in a nearby place (Rishikesh). No other bluetongue outbreak, due to serotype-23 or any other serotype, has been subsequently reported in this region.

A nation-wide programme - All India Network Programme on Bluetongue - was then established in order to monitor and control the presence of bluetongue in domestic ruminants in the sub-Himalayan Indian plains and in the Deccan. Due to peculiarities of the terrain, very little BTV sero-surveillance studies have been conducted in the past in the hilly states of Northern India, especially in the state of Uttarakhand. The aim of this study was to assess the BTV situation in this region.

In the beginning of 2009, subsequently to an outbreak of *peste des petits ruminants* (PPR) in the Pithoragarh area, some goat blood and serum samples were sent to our laboratory for detection of *peste des petits ruminants* virus (PPRV) antibodies. The sera resulted positive for PPRV antibodies. Serum and blood samples were also examined for BTV antibody/antigen and a number of samples were found positive. This prompted a further investigation on BTV seroprevalence on a fairly large number of samples from the area. In November 2011 serum and EDTA (Ethylenediaminetetraacetic acid) blood samples were collected from 51 goats of 3 villages around the town of Pithoragarh (altitude 1,635 m) in Uttarakhand State (16 from Swakot Village, 28 from Kalsin Village and 7 from Sintoli Village). They were brought to laboratory in ice and stored properly until further investigations were conducted. Incidentally, 1 month prior to sampling, an outbreak of PPR in goats was reported in these villages causing the death of 34 goats in the Swakot Village alone according to data received from the local veterinarians.

Presence of BTV antigen was tested in EDTA blood samples by a polyclonal antibody-based group-specific sandwich ELISA (s-ELISA) as described elsewhere (5). Out of 51 samples, 33 (64%) were positive, 11 of which showed high OD values (s-ELISA OD 1.00-1.25, negative control OD 0.22). The highest rate of the BTV antigen prevalence (87.5%) was noticed in Swakot Village, where a large number of goats died due to PPR a month before sampling. Lower prevalences were found in Kalsin (53.5%) and Sintoli Village (42.8). All the samples with the highest OD values originated from Swakot Village. Out of 51 serum samples, 18 (35%) were found positive for BTV antibody as tested by a group-specific indirect ELISA (9). In 12 animals both BTV antigen and antibodies were found.

Five samples were randomly chosen among the 11 blood samples with the highest OD values and were tested for virus isolation. The virus could successfully be isolated on BHK-21 cells from 1 sample (No. PTG-13) only, the isolate was named as PTG-13. At third passage, cytopathic effects (CPE) was clearly evident 24 hrs post infection (hpi). It was characterised by rounding and ballooning of cells (Figure 1). By 48 hpi, a degeneration was observed, aggregation and death of cells resulting into detachment from the surface of culture vessel. Aliquots of infected cell lysate of each passage were tested for BTV antigen by s-ELISA (5). Antigen was barely detectable at first passage. However, in subsequent steps more cells showed CPE and antigen quantity was also found to increase (Figure 1). The bluetongue antigen detection assay (s-ELISA) provides serological evidence that the virus isolate PTG-13 was a BTV. For further investigation, double stranded RNA (dsRNA) was extracted and purified from virus-infected cell culture (at third passage of virus)

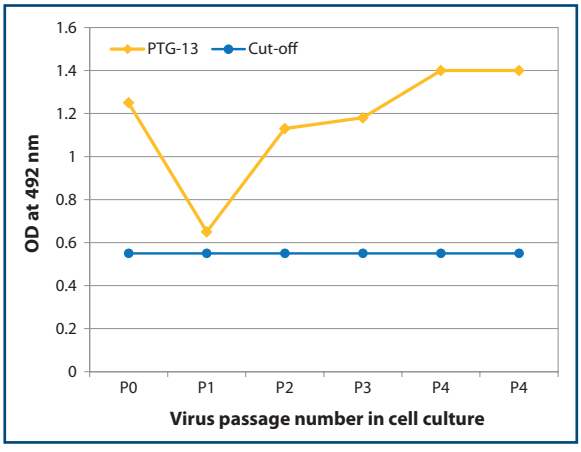

Figure 1. *Detection of BTV antigen by sandwich enzyme-linked immunosorbent assay in the infected cell lysates.* The cells were infected with PTG-13 virus and supernatants were collected after each passage for the assay. P0 indicates goat blood sample and P1-P5 indicates passage number of the virus isolate. Inset: Cytopathic effects (rounding and ballooning) produced by PTG-13 virus on BHK-21 cells at 24 hpi.

using Tri reagent (Sigma, St. Louis, MO, USA) according to the protocol described by Attoui *et al.* (1). The dsRNA was resolved by polyacrylamide gel electrophoresis (PAGE) and then stained with silver nitrate (10, 25). Ten bands, typical of BTV, or any Orbivirus (18) genome, were observed to have migrated according to their size (Figure 2a).

Polyacrylamide gel electrophoresis, silver staining and s-ELISA all confirmed PTG-13 as a BTV. The serotype of the virus was determined by RT-PCR coupled with sequencing of a fragment of genome segment-2 (seg-2). Considering the endemicity of BTV-1 in the Northern part of India, PCR was performed on PTG-13 virus with primers specific to seg-2 of the same serotype as per the procedure described in (4). A fragment of 828bp size (nucleotide position 50-878 bp) of seg-2 was amplified from PTG-13 virus and was visualised on agarose gel electrophoresis (Figure 2b). The PCR product was cloned and sequenced. BLAST (Basic Local Alignment Search Tool http://www.ncbi.nlm.nih.gov/) analysis of the sequence revealed 99% nucleotide identity (data not shown) with the seg-2 of recent BTV-1 isolates MKD-18 (GenBank accession No. HM014236), SKN-7 (JN558348) and SKN-8 (JN558349). PCR and partial sequencing of seg-2 confirmed PTG-13 as BTV-1.

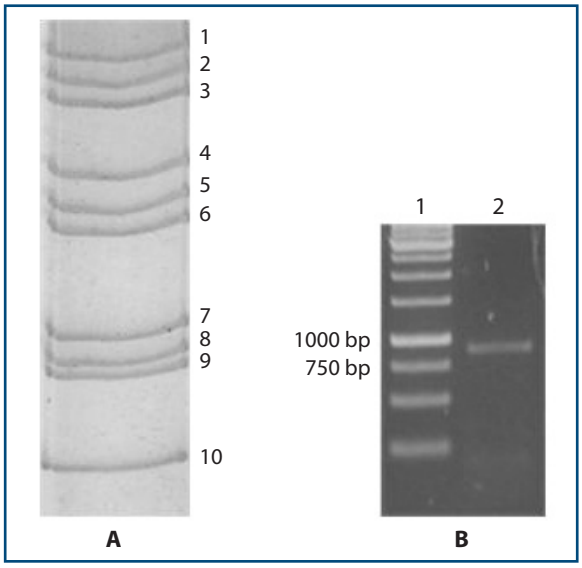

Figure 2. *Polyacrylamide gel electrophoresis and polymerase chain reaction.*

(A) BHK cells (in 75 cm^2 flask) were infected with the PTG-13 virus, and viral genomic dsRNA was extracted and resolved in 10% polyacrylamide gel (5h, 120 V) and silver-stained.

Ten dsRNA segments, typical of BTV (or any Orbivirus) genome, were observed (segments are numbered).

(B) Agarose gel electrophoresis of PCR product.

Amplification of segment-2 of PTG-13 virus was done by RT-PCR using BTV-1 VP2 (segment-2) gene-specific primers. An 886-bp (nucleotide position 7-893 of segment-2) product was amplified (lane-2) using primers described by Biswas *et al.* 2010 (4). Lane-1 contains 1Kb ladder DNA molecular weight marker.

Serum neutralisation test (SNT) was done by microneutralisation assay as described in (15) to detect neutralising antibodies against BTV-1 and 23 in goat sera. Ten serum samples from those goats which showed high OD values in the BTV ELISA antigen test were used against 200 $TCID_{50}$ of each of the following viruses: BTV-1 [isolates PTG-13 and MKD-18 (4)] and BTV-23 Dehradun isolate (17). Out of 10, 6 samples were from Swakot village (PTG-1, 5, 10, 11, 12 and 14) including the sample number PTG-13, which yielded the isolate BTV-1 PTG-13, and 4 samples were from Kalsin Village (PTG-17, 18, 24 and 31). It was interesting to note that no neutralising antibodies were detected in any of the above goat sera (including the sample No. PTG-13) against PTG-13 and MKD-18. However, high level of neutralising antibodies against BTV-23 Dehradun isolate was detected in all serum samples, including the sample No. PTG-13, at 1:128 dilution and in 6 samples at 1:256 dilution.

In this study, a BTV-1 (PTG-13) has been isolated on cell culture from an asymptomatic goat. No neutralising antibodies against the PTG-13 virus or MKD-18 isolate of BTV-1 were detected in goat sera. Furthermore, high level of neutralising antibodies was detected against BTV-23. Based on the previous reports of virus isolation (3, 4, 8, 12, 13, 17, 20, 22), it could be considered that prevalence of BTV-1 and BTV-23 is higher in the North-Western states and foot hills of Uttarakhand (Figure 3) and so, we have attempted to search neutralizing antibodies in the goat sera against these 2 serotypes. However, antibodies against other BTV serotypes could have been present in the goats involved in this study; similarly viraemia caused by other serotypes could not be excluded. The presence of BTV-1 viraemic animal suggests that BTV-1 was circulating at the time of sampling. The infection in goats with 2 serotypes, in fact, could have occurred in distinct periods. Failure to isolate BTV-23 may be due to absence of the virus in the animals at the time of sampling. The appearance of BTV-23 neutralising antibodies in the blood circulation coincided with the decline in virus titre. In general, the peak of bluetongue viraemia occurs in the first 2 weeks of infection, before the appearance of neutralising antibodies, and then the virus titre drops rapidly to a low level when infection persists for a month or more (14, 26).

It was possible to isolate the PTG-13 virus from goat blood because sampling was done probably within the first 2 weeks of infection (when the viraemia was at the peak) before appearance of neutralising antibody against PTG-13 virus. This also explains the absence of neutralising antibody in the goat serum samples against the PTG-13 virus. Isolation of BTV from blood is more successful when sample is collected at peak viraemia. However, in the later phase of infection virus isolation is possible

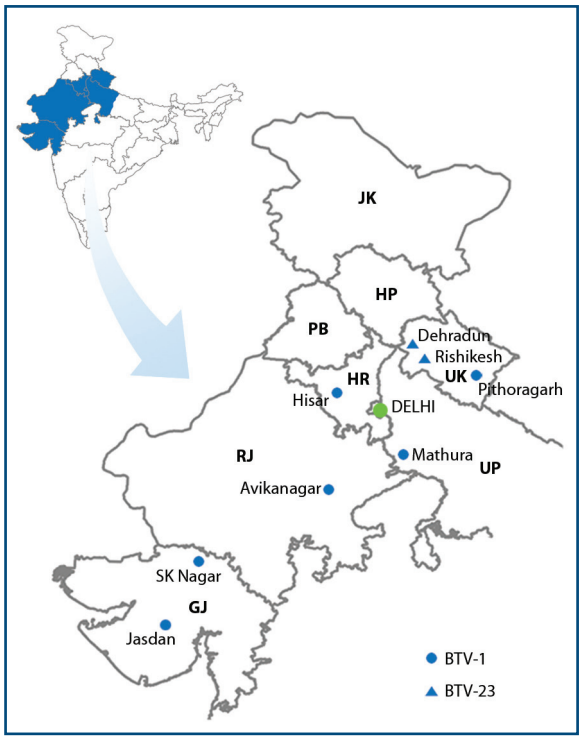

Figure 3. *Map showing the North-Western states of India where strains of BTV-1 and 23 have been isolated.* Bluetongue virus serotype-1 was isolated from Hisar (12), Avikanagar (20), Mathura (isolate MKD-18, 4), Jasdan (isolate SKN-7, 8), SK Nagar (Sardarkrushinagar, isolate SKN-8, 8) and Pithoragarh (isolate PTG-13, this study). Bluetongue virus serotype-23 was isolated from Dehradun (17) and Rishikesh (22).
JK: Jammu & Kashmir; HP: Himachal Pradesh; PB: Punjab; UK: Uttarakhand; HR: Haryana; RJ: Rajasthan; UP: Uttar Pradesh; GJ: Gujarat.

even in the presence of neutralising antibodies; because bluetongue virus is strongly cell-associated during viraemia, and there is strong evidence that this cellular association protects circulating virus from elimination by neutralising antibodies during prolonged infection (2). Due to this fact, we have selected viraemic animals for SNT (Serum neutralization test) with the expectation that both virus (BTV) and its neutralizing antibody would be present together in blood. In this case, the detection of both virus and its neutralising antibodies in the same animal would have been a strong evidence of infection. However, we could not detect BTV-1 antibodies in the viraemic animals but the chance to find BTV-1 antibodies would have been higher in non-viraemic animals.

It is worth mentioning that highest rate of BTV antigen prevalence (87.5%) and the highest OD values in the BTV ELISA antigen were observed in the goats of Swakot Village where a heavy mortality was reported due to PPR. It seems that there is a correlation between PPR infection and surge of BTV antigen (or virus) in small ruminants if the latter virus is already present in the host. In several occasions, BTV has been isolated from sheep or goats naturally infected with the PPRV (4, 18) and in such cases of mixed infection, the PPR symptoms become prominent (19). The surge of BTV antigen or virus may be due to flare up of virus that was already present inthe goats (in this study) because of stress or immunosuppressive conditions. The role of PPRV for suppressing the immune system of the goats should not be ignored in this case. A few cases of mixed viral infections in sheep and goats involving PPRV and orf virus, goat pox virus or BTV have been reported in India and other countries (16, 17, 24). Morbilliviruses, in general, have been shown to cause suppression of the host immune system (27, 28). Recent studies show that experimental PPRV infection in goats can lead to marked suppression of host immune response accompanied by severe leucopaenia (21).

BTV-23 was isolated more than 15 year ago from Dehradun and Rishikesh, and at present, seems to have established its presence at Pithoragarh (a place at hills about 220 Km North-East to Dehradun) as evidenced in this study. Bluetongue virus serotype-1, which normally circulates in the North Indian valleys, has now invaded the hills. Finally, the isolation of BTV-1 (PTG-13) from goat blood, the presence of high titre neutralising antibodies in goat sera against BTV-23 and the absence of neutralising antibody against PTG-13 virus suggest that goats were naturally infected with BTV-1 and 23 in 2 moments in time.

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