

# Detection of bovine herpesvirus 1 sequences in yaks (*Bos grunniens*) with keratoconjunctivitis, using a highly sensitive nested polymerase chain reaction

S. Bandyopadhyay<sup>(1)\*</sup>, S. Das<sup>(1)</sup>, K.K. Baruah<sup>(1)</sup>, P. Chakravarty<sup>(1)</sup>, D. Chakrabarty<sup>(2)</sup>, T. Sarkar<sup>(2)</sup>, B. Pal<sup>(2)</sup>, S. De<sup>(3)</sup>, D. Pan<sup>(3)</sup>, A.K. Bera<sup>(3)</sup>, S. Bandyopadhyay<sup>(3)</sup> & D. Bhattacharya<sup>(3)</sup>

(1) National Research Centre on Yak, Dirang 790 101, Arunachal Pradesh, India

(2) Institute of Animal Health and Veterinary Biologicals, RRDL, 37 K B Sarani, Kolkata, India

(3) Indian Veterinary Research Institute, Eastern Regional Station, 37 Belgachia Road, Kolkata, India

\*Corresponding author: samiranvet@gmail.com

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## Summary

Thirty-seven yaks (*Bos grunniens*) with keratoconjunctivitis and 22 healthy yaks were used to investigate the role of bovine herpesvirus 1 (BoHV-1) in keratoconjunctivitis in yaks. Nucleic acid sequences of BoHV-1 glycoproteins B and E were detected in conjunctival swabs from all yaks with keratoconjunctivitis using a nested polymerase chain reaction (PCR). In 21 yaks, BoHV-1 sequences were detected along with *Moraxella bovis* (*M. bovis*) and *Neisseria* spp. The amplified BoHV-1 sequences were identical, and no nucleotide variation was observed when compared with a BoHV-1 reference strain using single-strand conformation polymorphism analysis of the amplified DNA sequences. Interestingly, BoHV-1 sequences could not be detected in samples from healthy yaks. However, conjunctival swabs from two healthy yaks (9.09%) yielded *M. bovis* and *Neisseria* spp. Samples from 35 yaks with keratoconjunctivitis showed positive reactions in an avidin biotin enzyme-linked immunosorbent assay for BoHV-1 antibodies; all the healthy yaks were seronegative. This is the first report of a possible association of BoHV-1 with keratoconjunctivitis in yaks.

## Keywords

Avidin biotin enzyme-linked immunosorbent antibody detection assay – Bovine herpesvirus 1 – Keratoconjunctivitis – *Moraxella bovis* – *Neisseria* spp. – Nested polymerase chain reaction – Single-strand conformation polymorphism – Yak.

## Introduction

The yak (*Bos grunniens*) is a unique bovine species of high economic importance in the hills and snowbound areas at 3,000 m to 5,000 m above msl (mean sea level) in China, Mongolia, Bhutan, Nepal, Russia and India (2, 13). The products and services provided by yaks include: meat and

milk; wool and leather for clothing, blankets, bags, implements, rugs and tents; bones for carving; transport for trade and agricultural production; and nutrient recycling. In addition, yaks serve as a financial asset and security for investment and family ceremonies (13). The yaks are reared under free-range conditions in the high hills where the air, water and pasture are free from any pollution, and their products (milk, meat, hair) are

considered organic. This multi-purpose bovid is the only source of livelihood for the rural tribal highlanders. Domesticated yaks (*B. grunniens*), a native of the Tibetan plateau, originated from wild yaks (*B. mutus przewalski*) (13). The yak population in India has decreased substantially over the years and the yak is now considered an endangered species (2).

Keratoconjunctivitis is the most common ocular problem encountered in Indian yaks (2, 3). It is also a serious concern in other parts of Asia (13). The clinical spectrum of the disease includes bilateral conjunctivitis, keratitis, conjunctival hyperaemia, chemosis, blepharospasm, photophobia and extreme restlessness (2). Corneal opacity and ulceration leading to partial or complete blindness are observed frequently. The disease has a high economic impact upon the local people that rear yaks, due to a sharp fall in milk yield, decrease in feed intake with loss of body weight, and decreased show value of the animals. Despite its overwhelming importance, little attention has been paid to the aetiological diagnosis of the disease. Recently, bacteria such as *Moraxella bovis* and *Neisseria* spp. have been detected in conjunctival swabs from yaks suffering from keratoconjunctivitis (3). However, there is no report of the involvement of bovine herpesvirus 1 (BoHV-1) in keratoconjunctivitis in yaks. The present report describes a case series of keratoconjunctivitis in yaks that was probably associated with BoHV-1.

## Materials and methods

### Study design, population and location

Samples for the study were collected over a period of more than three years (2006 to 2009). Thirty-seven yaks with keratoconjunctivitis, from both the organised farm of the National Research Centre on Yak (Nyukmadung, Arunachal Pradesh, India, 2,750 m above msl) and three different yak herds in the Nyukmadung Tom hills (3,000 m to 3,500 m above msl), were included in the study. The affected yaks showed unilateral or bilateral conjunctivitis, keratitis, profuse ocular discharge, photophobia and corneal opacity. Twenty-two apparently healthy yaks from both the farm and the Nyukmadung Tom hills were selected randomly and also included in the study.

### Collection of samples and bacteriological investigations

Bacterial cultures isolated from conjunctival swabs were maintained on nutrient agar/trypticase soy agar slants and were identified on the basis of their cultural, morphological and biochemical characteristics, as described previously (3). Blood was collected from each

animal via jugular vein puncture and the separated serum was de-complemented at 56°C for 30 min.

### Serological analysis

All the serum samples were subjected to avidin biotin enzyme-linked immunosorbent assay (Ab-ELISA) following a method described previously (4). The Ab-ELISA kit was obtained from the Project Directorate on Animal Disease Monitoring and Surveillance (PD\_ADMAS), Bangalore, India. Parallelism was assessed between serial dilutions of positive control sera and yak sera. A high degree of parallelism was observed in the percent positivity (PP) curve between serially diluted positive control sera and yak sera ( $p < 0.01$ ) using standard regression analysis. Plates coated with BoHV-1 antigen, and positive and negative control sera of bovine origin were supplied with the kit. Briefly, 100 µl of test and control sera at 1:100 dilutions in blocking buffer (containing 1% bovine gelatin and Tween 20 in phosphate buffered saline [PBS]) were added to respective wells in duplicate. The plates were incubated for 1 h at 37°C and washed subsequently. Thereafter, diluted biotinylated anti-bovine IgG (100 µl, 1:170 in blocking buffer) was added to each well and again incubated for 1 h at 37°C. After washing, 100 µl of diluted avidin-horseradish peroxidase conjugate (1:150 in blocking buffer) was added and the plates were incubated for 20 min at 37°C and then washed again. Finally, 100 µl of substrate chromogen mixture (100 µl of O-phenylene diamine dihydrochloride with 4 µl of 3% hydrogen peroxide) was added and the plates were incubated for 10 min at room temperature. The reaction was stopped by 50 µl of 1 M sulphuric acid. Absorbance was measured at 492 nm and the PP was calculated using the formula:

$$\text{PP (\%)} = \frac{\text{Average optical density [OD] of the sample} \times 100}{\text{Median OD of strong positive reference sample}}$$

A value greater than or equal to 40% was considered positive. The group with a PP >30% but <40% was interpreted as suspicious, and serum samples with a PP ≤30% were considered negative.

### DNA isolation and polymerase chain reaction

Samples of ocular discharge and swabs were suspended in sterile PBS (0.02 M, pH 7.2), heated at 95°C for 1 h and immediately cooled on ice prior to processing for nested PCR (n-PCR). Alternatively, DNA was extracted from ocular discharge using the phenol–chloroform–isoamyl alcohol method.

The glycoprotein B and E (gB and gE) genes of BoHV-1 were amplified using two sets of primers each (two external and two internal primers, listed in Table 1) (6).

**Table I****Oligonucleotide sequences used to amplify specific fragments from glycoproteins B and E of bovine herpesvirus 1, and polymerase chain reaction conditions**

After initial denaturation at 95°C for 5 min, *Taq* polymerase was added to each sample to increase the half-life of the enzyme

Primers		Reference	Product size (base pairs)	PCR conditions
External primers for gB	Forward: 5'-TACGACTCGTTCGCGCTCTC-3' Reverse: 5'-GGTACGTCTCCAAGCTGCC-3'	Fuchs <i>et al.</i> 1999 (6)	478	95°C for 30 s for denaturation, 60°C for 2 min for annealing and 72°C for 2 min for extension for 35 cycles
Internal primers for gB	Forward: 5'-TCTCGACCGGGGACATTATC-3' Reverse: 5'-GCCTCTTCGATCACGCAGTC-3'	Fuchs <i>et al.</i> 1999 (6)	385	95°C for 30 s for denaturation, 60°C for 2 min for annealing and 72°C for 1 min for extension for 10 cycles, followed by 95°C for 30 s for denaturation, 55°C for 2 min for annealing and 72°C for 1 min for extension for 25 cycles
External primers for gE	Forward: 5'-GCTTCGGTCGACACGGTCTT-3' Reverse: 5'-CTTTGTCGCCCGTTGAGTCG-3'	Fuchs <i>et al.</i> 1999 (6)	265	95°C for 30 s for denaturation, 55°C for 2 min for annealing and 72°C for 1 min for extension for 35 cycles
Internal primers for gE	Forward: 5'-CGCGCCCGTCTTTCTCCA-3' Reverse: 5'-CACGTCGGTGAAGCACTCG-3'	Fuchs <i>et al.</i> 1999 (6)	139	95°C for 30 s for denaturation, 60°C for 2 min for annealing and 72°C for 1 min for extension for 10 cycles, and 95°C for 30 s for denaturation, 53°C for 2 min for annealing and 72°C for 1 min for extension for 25 cycles

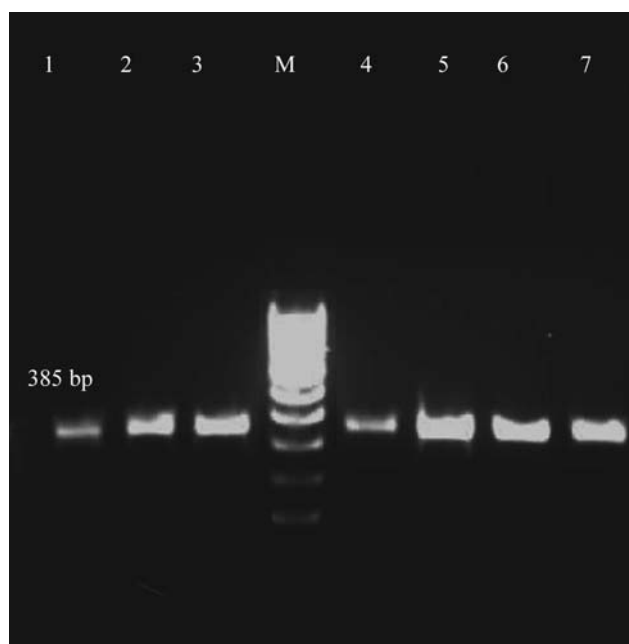
The PCR was conducted in 25 µl volumes containing 2.5 µl of 10X *Taq* polymerase reaction buffer, 1 U of *Taq* polymerase, 1 µl of 10 mM dNTP mix, 10-20 pmoles of each primer, 2.5 mM of magnesium chloride (Bangalore Genei Pvt Ltd, Bangalore, India) and 3 µl of template. Details of the PCR cycles and the conditions used for each of the primers are given in Table I. The amplified DNA products were analysed by electrophoresis in 2% agarose gels containing ethidium bromide (0.5 µg/ml). All samples were subjected to the n-PCR in triplicate, to avoid false-positive reactions. A BoHV-1.1 isolate and a no-template control were included in each PCR assay.

### Single-strand conformational polymorphism

Single-strand conformation polymorphism (SSCP) analysis was performed according to the method described by Xie *et al.* (15) with some modifications. In brief, 10 µl of each PCR product was denatured for 10 min at 94°C in 2X SSCP buffer (95% formamide, 0.025% xylene cyanole and 0.025% bromophenol blue) and chilled immediately on ice. The denatured samples were run in 10% acrylamide-bisacrylamide (29:1) non-denaturing gels at 60 volts for 12 h at 4°C. The pattern of bands was visualised using an ultraviolet transilluminator (DNR Bio-imaging system, Israel) after ethidium bromide staining.

## Results

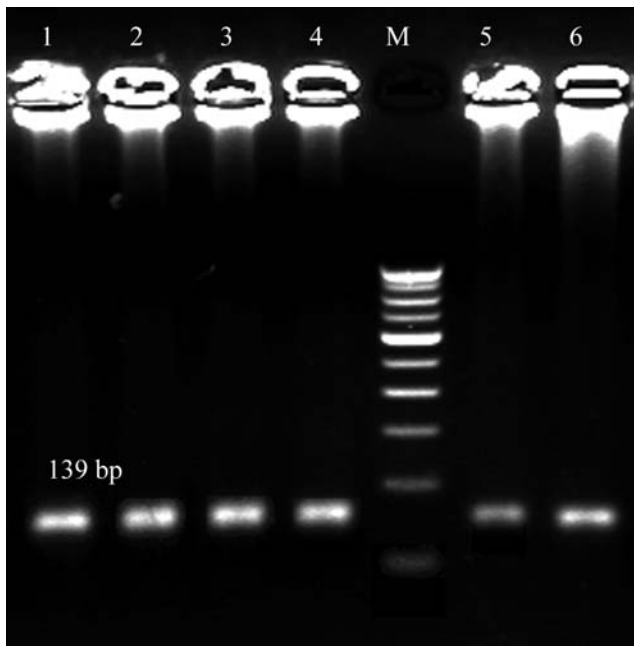
Amplified products specific for BoHV-1 were detected in conjunctival swabs from all 37 yaks with keratoconjunctivitis using gB (Fig. 1) and gE (Fig. 2)



**Fig. 1**  
**Representative profile of amplification of glycoprotein B (385 base pairs [bp]) from bovine herpesvirus 1 using nested polymerase chain reaction from samples of ocular secretion or conjunctival swabs from yaks with keratoconjunctivitis**

Lane 1: positive control. Lanes 2 to 7: samples collected from yaks with keratoconjunctivitis. M: 100 bp ladder

specific n-PCR. However, only 22 samples were positive using the external sets of primers specific for gB and gE. Fifteen samples became positive only in the nested reaction using the internal sets of primers. In 21 samples, viral DNA was detected simultaneously with *M. bovis* and



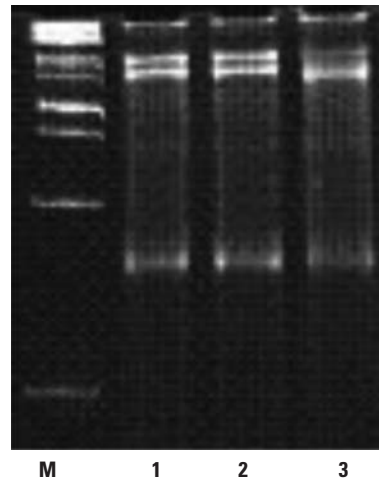
**Fig. 2**  
**Representative profile of amplification of glycoprotein E (139 base pairs [bp]) from bovine herpesvirus 1 using nested polymerase chain reaction from samples of ocular secretion or conjunctival swabs from yaks with keratoconjunctivitis**  
 Lane 1: positive control. Lanes 2 to 6: samples collected from yaks with keratoconjunctivitis. M: 100 bp ladder

*Neisseria* spp. Specific viral PCR products could not be detected in the conjunctival swabs from the healthy yaks. However, *M. bovis* and *Neisseria* spp. were both recovered from the conjunctival swabs of two healthy yaks. All the amplified sequences of gB (Fig. 3) and gE (Fig. 4) were found to be identical, and when they were compared by SSCP with the amplified segments of a known positive BoHV-1 control no nucleotide variation was observed.

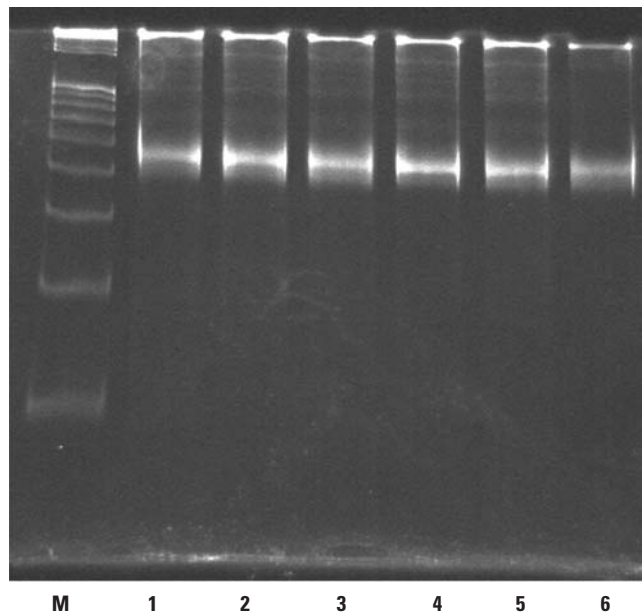
Antibodies reactive with BoHV-1 were detected in 35 yaks with keratoconjunctivitis. In all these yaks, the PP values exceeded 40%. Samples from 11 yaks were highly reactive, with PP values of more than 60%. Two yaks (PCR positive) with keratoconjunctivitis were categorised as suspect on the Ab-ELISA, with a PP value <40% but >30%. Of the 22 healthy yaks, seven could be categorised as suspect (PP value <40% but >30%) and 15 were seronegative, with PP values <30%.

## Discussion

The presence of BoHV-1 related DNA exclusively in the conjunctival swabs obtained from yaks with keratoconjunctivitis suggests that BoHV-1 may play an important role in keratoconjunctivitis in yaks. The specificity of the PCR system applied (6) was determined



**Fig. 3**  
**Single-strand conformation polymorphism analysis of amplified glycoprotein B sequences of positive and test samples**  
 M: 1 kilobase ladder. Lane 1: positive virus control (BoHV-1.1). Lanes 2 and 3: test samples



**Fig. 4**  
**Single-strand conformation polymorphism analysis of amplified glycoprotein E sequences of positive and test samples**  
 M: 1 kilobase ladder. Lane 1: positive virus control (BoHV-1.1). Lanes 2 to 6: test samples

by analysis of the DNA of BoHV-1 and the related alphaherpesviruses BoHV-5, N569, and caprine herpesvirus 1 (CpHV-1). Using CpHV-1 as a template, no amplification was found in either PCR with the primer pairs used here. With the primer pair gB-1-gB-2 and BoHV-5 DNA as the template, a fragment of the same size as that obtained with BoHV-1 DNA was amplified, whereas PCR with primers gE-1-gE-2 was negative. The PCRs were



not validated for recently detected ruminant alphaherpesviruses such as bubaline herpesvirus and cervid herpesviruses 1 and 2 (CvHV-1, CvHV-2) which show a high degree of sequence homology to BoHV-1, especially for the gB gene (11).

Taken together, the results of the PCR investigations strongly implicate a role of BoHV-1 as a causative agent of keratoconjunctivitis in yak. On the other hand, a role of other ruminant alphaherpesviruses cannot be ruled out without sequence determination of the amplified PCR products.

However, in the SSCP analysis no nucleotide variations could be detected in the amplified genes (gB and gE) in comparison with those of the known positive control (BoHV-1). The SSCP is a highly sensitive technique for the detection of micro-lesions in DNA, such as single-base substitutions, small deletions, small insertions or microinversions. These results provide strong evidence that these yaks were actually infected with BoHV-1. In our previous study (4), keratoconjunctivitis was detected in 29 of 105 seropositive yaks.

In the present study, pathogens such as *M. bovis* and *Neisseria* spp. were also detected, in combination with BoHV-1, in 21 of 37 yaks with keratoconjunctivitis. The clinical severity was more prominent in these yaks.



**Fig. 5**  
**Infectious bovine keratoconjunctivitis (pink eye) in a yak**  
The animal was seropositive for bovine herpesvirus 1 (BoHV-1). Apart from BoHV-1, *Moraxella bovis* and *Neisseria* were also detected in the conjunctival swab of the animal. Here, corneal opacity appears as the principal sign

Corneal opacity, which spread centrifugally, was the principal manifestation, together with copious ocular discharge and severely hyperaemic bulbar and scleral conjunctiva (Fig. 5). In cattle, BoHV-1 and *M. bovis* co-infection has been reported to cause a severe form of keratoconjunctivitis (8). Multi-drug resistant (MDR) *M. bovis* and *Neisseria* spp. have been recovered from yaks and mithun (*Bos frontalis*) with keratoconjunctivitis in a previous study (8). However, in the present study, *M. bovis* and *Neisseria* spp. could not be isolated from 16 yaks with keratoconjunctivitis (43.24%). In these animals, the corneal opacity was reduced or absent (Fig. 6) and the lesions were confined to the corneo-scleral junction. Outbreaks of conjunctivitis are not uncommon in cattle following BoHV-1 infection, even without the simultaneous involvement of *M. bovis* (8). In none of the healthy yaks were BoHV-1 related sequences detected. Interestingly, the conjunctival swabs from two apparently healthy yaks from the Nyukmadung Tom hills yielded *M. bovis* and *Neisseria* spp. It was confirmed by the farmers that keratoconjunctivitis had occurred in these yaks previously. Infected animals have been reported to be carriers of both bacteria for a period exceeding one year following clinical recovery (8, 14). The possible role of BoHV-1 as a causative agent of keratoconjunctivitis in yak requires further validation by controlled infection of seronegative animals with BoHV-1 and with BoHV-1 in combination with *M. bovis* and *Neisseria* spp.

Surprisingly, high levels of specific antibodies against BoHV-1 were detectable in 35 out of 37 yaks with keratoconjunctivitis only. The Ab-ELISA is employed widely for detection of BoHV-1 antibodies in



**Fig. 6**  
**Conjunctivitis in yaks seropositive for bovine herpesvirus 1**  
Inflammation and oedema are prominent in the conjunctiva at the corneo-scleral junction, with no or little involvement of the cornea. Only bovine herpesvirus 1 was detected using nested polymerase chain reaction (both gB and gE specific) from the conjunctival swab of the animal

epidemiological studies and eradication programmes. However, an incubation period of one to three weeks is generally required to develop serologically detectable titres in the ELISA (6, 8). This was probably the reason for lower levels of BoHV-1 antibodies in two affected animals (categorised as suspicious) in which viral DNA could be detected using the n-PCR. The study proved the usefulness of the n-PCR for the detection of BoHV-1 in clinical samples. The test has been found to be more sensitive than virus isolation (9) and serological tests, at least in the early stage of infection (6) in cattle. In experimentally infected calves, BoHV-1 DNA was detected in peripheral blood mononuclear cells using n-PCR at eight months post infection, but no virus was isolated (12). Similar findings were reported by Takiuchi and co-workers, who detected BoHV-1 in association with abortion in cattle herds in Brazil (10). The interpretation of serological analysis is very difficult, owing to the antigenic similarity of ruminant alphaherpesviruses that are related to BoHV-1. Indeed, these viruses cross-react in ELISA and seroneutralisation tests. The indirect ELISA tests available currently are unable to discriminate between these related alphaherpesviruses (11).

In yaks, keratoconjunctivitis is found mainly in the summer and autumn. This is probably due to the abundance of flies (*Musca domestica*) and the intensity of sunlight during these seasons. Flies and intense ultraviolet irradiation are important predisposing factors for keratoconjunctivitis, in addition to the presence of microorganisms (1). Profuse hair growth around the eyes is also an important contributing factor because of constant irritation (2).

The widespread presence of BoHV-1 or a related alphaherpesvirus in yaks can be attributed to the way yak husbandry is practised in India. Yaks are generally reared under a transhumance system of migration and during the lean season they come down to a lower altitude in search of feed. During this period, they share common grazing and sources of water with other domestic and wild animals such as hill cattle and mithun (2). Moreover, yaks are kept in herds of 50 to 200 animals. Thus, increased contact

between animals provides an easy avenue for the transmission of pathogens (4). Higher rainfall in the hills may also be a predisposing factor for the relatively high rate of infection (5). People from lower altitudes often prefer yak hybrids instead of pure yaks, and the indiscriminate use of infected or carrier cattle bulls and infected frozen semen for insemination of yak cows could be a route of transmission of the pathogen to yaks. However, the likelihood of inducing an ocular condition by the venereal transmission of virus is very remote. Yaks have been found to be deficient in trace minerals (7), especially in copper and zinc. These minerals are known to play an important role in fine-tuning the immunological status of animals, in the absence of which animals become more prone to infection with BoHV-1.

## Conclusions

In conclusion, the predominant presence of BoHV-1 DNA sequences in the yaks with keratoconjunctivitis indicates that BoHV-1 or a closely related ruminant alphaherpesvirus may play a vital role in keratoconjunctivitis in yaks. Rather than virus isolation, which is a time-consuming procedure, n-PCR can be applied in the field to detect viral DNA in clinical samples. In the early phase of infection or in immunocompromised animals, n-PCR can be used instead of the commonly employed serological tools.

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## Détection de séquences de l'herpèsvirus bovin 1 chez des yaks (*Bos grunniens*) atteints de kératoconjonctivite au moyen d'une technique d'amplification en chaîne par polymérase nichée de haute sensibilité

S. Bandyopadhyay, S. Das, K.K. Baruah, P. Chakravarty, D. Chakrabarty, T. Sarkar, B. Pal, S. De, D. Pan, A.K. Bera, S. Bandyopadhyay & D. Bhattacharya

### Résumé

Trente-sept yaks (*Bos grunniens*) atteints de kératoconjonctivite et 22 yaks en bonne santé ont été soumis à des tests visant à déterminer le rôle joué par l'herpèsvirus bovin de type 1 (BoHV-1) dans l'apparition de cas de kératoconjonctivite chez le yak. La technique d'amplification en chaîne par polymérase (PCR) nichée a détecté des séquences nucléiques des glycoprotéines B et E du BoHV-1 dans tous les écouvillons conjonctivaux prélevés sur des yaks atteints de kératoconjonctivite. Dans 21 cas, *Moraxella bovis* (*M. bovis*) et *Neisseria* spp. ont été détectés en même temps que les séquences du BoHV-1. Les séquences amplifiées du BoHV-1 étaient identiques et aucune variation nucléotidique n'a été constatée par rapport à une souche de référence du BoHV-1 lors de l'analyse de polymorphisme de conformation simple-brin (SSCP) des séquences amplifiées d'ADN. Aucun produit d'amplification de séquences du BoHV-1 n'a été obtenu à partir des échantillons provenant de yaks en bonne santé. En revanche, *M. bovis* et *Neisseria* spp. ont été retrouvés dans les écouvillons conjonctivaux de deux yaks en bonne santé (9,09 %). Les échantillons issus de 35 yaks atteints de kératoconjonctivite ont donné des résultats positifs à l'épreuve immuno-enzymatique à l'avidine-biotine pour la détection d'anticorps dirigés contre le BoHV-1. Il s'agit du premier rapport décrivant une probable association de l'infection par le virus BoHV-1 et de la kératoconjonctivite chez les yaks.

### Mots-clés

Amplification en chaîne par polymérase nichée – Analyse de polymorphisme de conformation simple-brin – Épreuve immuno-enzymatique à l'avidine-biotine pour la détection d'anticorps – Herpèsvirus bovin de type 1 – Kératoconjonctivite – *Moraxella bovis* – *Neisseria* spp. – Yak.

## Detección de secuencias del herpesvirus bovino 1 en yaks (*Bos grunniens*) afectados de queratoconjuntivitis, utilizando una reacción en cadena de la polimerasa anidada de gran sensibilidad

S. Bandyopadhyay, S. Das, K.K. Baruah, P. Chakravarty, D. Chakrabarty, T. Sarkar, B. Pal, S. De, D. Pan, A.K. Bera, S. Bandyopadhyay & D. Bhattacharya

### Resumen

Los autores describen un estudio encaminado a dilucidar el papel que cumple el herpesvirus bovino 1 (HVBo-1) en la queratoconjuntivitis del yak. Para ello se analizaron treinta y siete yaks (*Bos grunniens*) con queratoconjuntivitis

y 22 animales sanos. Tras aplicar a exudados nasales una reacción en cadena de la polimerasa (PCR) anidada, se detectaron secuencias de ácidos nucleicos de las glicoproteínas B y E del HVBo-1 en todas las muestras procedentes de yaks enfermos. En 21 casos, esas secuencias se acompañaban de la presencia de *Moraxella bovis* (*M. bovis*) y *Neisseria* spp. Las secuencias del HVBo-1 eran idénticas, sin que el análisis de polimorfismos de la conformación unicitenaria de las secuencias de ADN amplificadas revelara variación alguna en los nucleótidos con respecto a los de una cepa de referencia del virus. Las PCR de muestras de yaks sanos no depararon ningún elemento procedente del HVBo-1. Sin embargo, en los exudados nasales de dos yaks sanos (9,09%) se observó la presencia de *M. bovis* y *Neisseria* spp. Las muestras de los 35 animales con queratoconjuntivitis dieron resultado positivo a un ensayo inmunoenzimático (ELISA) con avidina/biotina de detección de anticuerpos contra el HVBo-1. Todos los yaks sanos resultaron seronegativos. Es la primera vez que se describe una posible asociación del HVBo-1 con la queratoconjuntivitis en los yaks.

#### Palabras clave

Ensayo inmunoenzimático con avidina/biotina para detección de anticuerpos – Herpesvirus bovino 1 – *Moraxella bovis* – *Neisseria* spp. – Polimorfismos de la conformación unicitenaria – Queratoconjuntivitis – Reacción en cadena de la polimerasa anidada – Yak.



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