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A serological survey of antibodies against bovine herpesvirus-1 in yak (*Poephagus grunniens*) in Arunachal Pradesh in India

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Summary

Serum samples were collected from 254 yak (*Poephagus grunniens*, presently *Bos grunniens*) in different yak tracts of India. These samples were then screened by virus neutralisation test (VNT) and avidin-biotin enzyme-linked immunosorbent assay (AB-ELISA) to study the seroprevalence of antibodies against bovine herpesvirus type 1 (BHV-1). The overall seroprevalence in yak was found to be 41% (105) by VNT and AB-ELISA. The sex of the animal, whether it was on a farm or free-ranging and the location of the different yak tracts did not seem to have any effect on seroprevalence. However, seroprevalence was found to increase with the age of the animals, being highest in yak older than three years of age (49%).

Yak generally share feeding, watering and grazing areas with other domestic and wild animals and this common ecological niche is thought to be a possible avenue of infection. This is the first time that the seroprevalence of antibodies against BHV-1 has been studied in yak in India.

Keywords

Avidin-biotin enzyme-linked immunosorbent assay – Bovine herpesvirus-1 – India – Infectious bovine rhinotracheitis – Infectious pustular vulvovaginitis – Seroprevalence – Virus neutralisation test – Yak.

Introduction

Infectious bovine rhinotracheitis or infectious pustular vulvovaginitis (IBR/IPV) is a highly infectious and economically important disease of domestic and wild bovines, caused by bovine herpesvirus type 1 (BHV-1) in the subfamily of *Alphaherpesvirinae* of the *Herpesviridae* family (4, 6). This virus is responsible for a wide range of clinical signs, including:

– rhinitis
– tracheitis
– laryngitis
– pustular vulvovaginitis
– conjunctivitis
– abortion
– reproductive failure
– decreases in milk production.

Thus, the disease may cripple livestock production, even though mortality is low in adult animals (4, 6). Most infections run a latent course following clinical recovery and, despite the development of neutralising antibody, the virus establishes its latency in pharyngeal, cervical, retropharyngeal or inguinal lymph nodes, the tonsils or trigeminal or sacral ganglia (9, 11). Stress, such as parturition or transport, can reactivate the latent infection so that intermittent shedding of the virus is quite common (6, 11). In this way, animals with latent infection often serve as a source of infection for other animals which come into contact with them, through respiratory, ocular and genital discharges (6, 11, 12). Therefore, identifying infected animals is a pre-requisite for eradicating this disease.

Serological tests, such as the virus neutralisation test (VNT) and enzyme-linked immunosorbent assay (ELISA), are usually employed to detect the acute, as well as convalescent, stage of infection. These tests can also detect the latent or carrier stage and have important implications for international trade as well as epidemiological studies (4, 12).

The yak (*Poephagus grunniens*) is a unique bovine species of great economic importance, confined to high hill- and snow-bound areas at 3,000 m to 5,000 m above mean sea level (msl) in the People's Democratic Republic of China, Mongolia, Bhutan, Nepal, Russia and India. This multi-purpose bovid is the only source of livelihood for the highlanders (1, 10). In India, the yak is considered a threatened species (1). Serological evidence of antibodies against BHV-1 has been demonstrated in cattle (2, 3, 4, 5) and buffalo (7). However, the epidemiological status of IBR is not known in yak, apart from a single report from China (10). The present paper seems to be the first comprehensive study to examine the status of IBR in yak in India.

Study population

From 2006 to 2008, sera were randomly collected from 254 animals from both an organised farm (at Nyukmadung) and free-ranging field animals from yak herds in six different areas (Chander, Mandla phudung, Nykamadung, Sella, Tawang and Jung). These areas are in the West Kameng (2,217 msl, from 91° 30' to 92° 40' East in longitude and 26° 54' to 28° 01' North in latitude) and Tawang (3,500 msl, at a latitude of 27° 48' and longitude of 97° 30') districts of Arunachal Pradesh, India (Fig. 1). These sera were de-complemented and subjected to avidin-biotin ELISA (AB-ELISA) and the VNT.

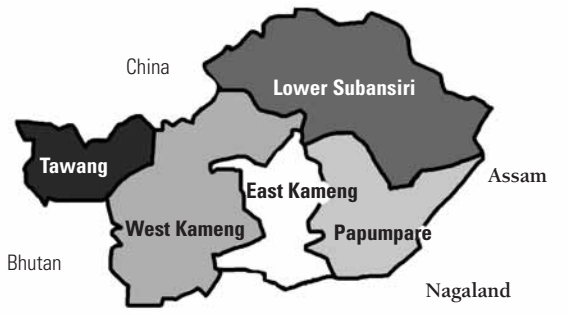

Fig. 1
Map of eastern Arunachal Pradesh showing the location of the Tawang and West Kameng districts
West Kameng and Tawang fall within the eastern Himalayan provinces of India and have a long international border with Bhutan in the South-west and China in the North
Source: All India Coordinated Research Project on the 'Improvement of feed resources and nutrient utilization in raising animal production', National Research Centre on Yak, 2005 to 2006

Serological testing

Avidin-biotin enzyme-linked immunosorbent assay

The AB-ELISA kit was obtained from the Project Directorate on Animal Disease Monitoring and Surveillance, Bangalore, India. Plates coated with BHV-1 antigen, and positive and negative control sera of bovine origin, were supplied with the kit. Parallelism was assessed between the serial dilutions of the positive control sera and yak sera. A higher degree of parallelism was observed in the percentage positivity curve between serially diluted positive control sera and yak sera ($p < 0.01$), using the standard regression test. Therefore, it was concluded that yak immunoglobulin G (IgG) is highly similar to bovine IgG, if not identical.

Briefly, 100 µl of the test and control sera (1:100 dilutions in blocking buffer, containing 1% bovine gelatin and Tween 20 in phosphate buffered saline) was added to the respective wells, in duplicate. The plates were then incubated for 1 h at 37°C and subsequently washed. Thereafter, diluted (100 µl, 1:170 in blocking buffer) biotinylated anti-bovine IgG 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 incubated for 20 min at 37°C and washed again. Finally, 100 µl of substrate chromagen complex (100 µl of O-phenylene diamine dihydrochloride with 4 µl of 3% H_2O_2) was added and the plates were incubated for 10 min at room temperature. The reaction was stopped by 50 µl of 1 M H_2SO_4. Absorbance was taken at 492 nm and percentage positivity (PP) was calculated using the formula below, where OD stands for optical density:

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

A value greater than or equal to 40 was considered positive.

Virus neutralisation test

This test was performed using the method recommended by the World Organisation for Animal Health (12). Briefly, two-fold dilution of yak sera was mixed with an equal volume of BHV-1 containing a 50% tissue culture infective dose of $10^{2+0.5}$, in 96-well, flat-bottomed, cell-culture grade microtitre plates, in duplicate. For each undiluted test serum, one extra well was kept to check the toxicity of the serum on the cells, if any. The plates were incubated at 37°C with 5% CO_2 for 24 h. At the end of incubation, 100 µl of cell suspension was added to each well (trypsinised Madin-Darby bovine kidney cells in Eagle's

minimum essential medium, 3×10^4 cells per well). All the plates were again incubated at 37°C with 5% CO_2, for 3 to 5 days. The plates were microscopically examined each day for cytopathic effects (CPEs) and the test was validated by checking positive, negative and cell controls.

The positive control in two-fold dilution gave absolute neutralisation of the virus with no CPE. The negative control gave no neutralisation when used undiluted. In the cell control, only the cell culture medium was added, without any virus, and the monolayer remained intact during the test.

Statistical analysis

The chi-squared (χ^2) test was used to compare seroprevalence in relation to the sex, age, type (farm or free-range) and location (different yak tracts) of the animal. The confidence interval (CI) was determined by Fisher's exact test. All analysis was performed in Graph Pad Prism software, version 4.0.

Results

Seroprevalence of infectious bovine rhinotracheitis

Of 254 animals tested, 105 were found to be positive by VNT and AB–ELISA (prevalence 41%: 95% CI = 36, 48).

The influence of sex, age, location and type of animal on seroprevalence

Although the seroprevalence of IBR was slightly higher among female yak (42%: 95% CI = 35, 51) than among males (40%: 95% CI = 31, 50), the difference was not statistically significant (Table I).

The seroprevalence of IBR was highest in yak that were more than three years of age (49%: 95% CI = 40, 59), followed by yak between one and three years (45%: 95% CI = 36, 57). Yak below one year (23%: 95% CI = 14, 36) came last. Statistical analysis showed a significant difference ($p < 0.05$) among the different age categories of yak (Table I).

The seroprevalence of IBR was higher in animals from Sella (44%: 95% CI = 31, 64), Chander (44%: 95% CI = 30, 65) and Nyukmadung (43%: 95% CI = 34, 54). However, the difference was not statistically significant (Table I).

Similarly, the seroprevalence was higher among farm yak (44%: CI = 34, 57) than among free-ranging yak (40%: CI = 24, 35). Again, the difference was not statistically significant (Table I).

Discussion

The overall seroprevalence of IBR in yak was found to be 41%, using VNT and AB-ELISA. Previously, antibodies

Table I
Risk factors for seroprevalence of antibodies against bovine herpesvirus-1 in yak in India

Risk factors	Total number of animals tested	Seropositive		χ^2	Degree of freedom*	p-value
		percentage	95% CI			
Sex						
Male	101	40%	31, 50	0.20	1	0.64
Female	153	42%	35, 51			
Age						
6 m to 1 year	61	23%	14, 36	11.4	2	0.003
1-3 years	86	45%	36, 57			
> 3 years	107	49%	40, 59			
Yak tract (location)						
Nyukmadung	98	43%	34, 54	0.69	5	0.98
Sella	36	44%	31, 64			
Jung	27	37%	23, 61			
Chander	32	44%	30, 65			
Tawang	29	41%	27, 64			
Mandla phudung	32	37%	24, 59			
Type of animal						
Farm yak	72	44%	34, 57	0.39	1	0.52
Free-ranging yak	182	40%	24, 35			

CI: confidence interval
χ^2 : chi-squared value
*degree of freedom: number of values in the final calculation that are free to vary

against BHV-1 had been detected in 36% of the yak tested in China (10). Specific antibodies against BHV-1 have also been detected in cattle (29%) and buffalo (8%) in India, during recent years (5, 7). In a separate study, serological evidence of BHV-1-specific antibodies was found in 45% of breeding bulls (3). Yak are generally reared under the transhumance system of migration and, during winter or a lean season, they come down to lower altitudes in search of feed. As a result of their close contact with other domestic or wild animals, they may acquire the infection (1). Moreover, yak live in herds, providing an easy avenue for the transmission of pathogens (1).

The seroprevalence of IBR was slightly higher in yak cows (42%) than among bulls (40%). Natural servicing is usually practised in yak herds, and repeated use of an infected bull could disseminate the infection and thus contribute to increased seropositivity among yak cows. Nowadays, farmers from comparatively lower altitudes prefer yak hybrids. The indiscriminate use of semen from infected bulls to inseminate yak cows may also be implicated in this higher seroprevalence among female yak, since semen is an ideal vehicle for BHV-1 infection (5). The virus establishes latency in the sacral ganglia in male animals after sexual transmission (12). Recrudescence, reactivation and shedding of the virus were reported in carrier bulls at the time of mating or during periods of prolonged stress (5, 6, 12).

It was observed that the seroprevalence of IBR was highest among yak aged more than three years (49%), followed by yak aged between one and three years (45%), and yak aged less than one year (23%). In cattle (2, 4), the seroprevalence of IBR was found to increase with age. This is probably due to greater exposure to infected or carrier animals as the animal ages.

In regard to location, the seroprevalence of IBR was found to be higher in Sella (44%), Chander (44%) and Nyukmadung (43%). However, the difference was not statistically significant. The prevalence of antibodies against BHV-1 was higher among farm animals (44%) than in free-ranging yak (40%). However, again, the difference was not statistically significant. A higher seroprevalence among farm animals may be due to the closer proximity of these animals.

The relative sensitivity and specificity of the AB-ELISA were found to be 100% for detecting BHV-1-specific antibodies in yak. This indicates that the AB-ELISA, which is a simpler test than the VNT, can effectively be used in future.

This study indicates that antibodies against BHV-1 are widespread among the yak of these regions in India. Clinical features indicative of BHV-1 infection were also detected in these yak, for example:

– reproductive disorders, including abortion and infertility

– keratoconjunctivitis

– respiratory illness (1).

However, the subfamily of *Alphaherpesvirinae* of the *Herpesviridae* family encompasses a cluster of six viruses that are antigenically similar to BHV-1 (8). Thus, the possibility that these yak were infected by a different, antigenically related virus, rather than BHV-1 itself, cannot be excluded.

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Enquête sérologique sur la présence d'anticorps dirigés contre l'herpèsvirus bovin de type 1 chez le yak (*Poephagus grunniens*) dans l'Arunachal Pradesh, Inde

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Résumé

Des échantillons de sérum ont été prélevés sur 254 yaks (*Poephagus grunniens*, actuellement *Bos grunniens*) provenant de différents sites où cette espèce est présente en Inde. La neutralisation virale et l'épreuve immuno-enzymatique à l'avidine-biotine (ELISA A-B) ont été utilisées pour déterminer la prévalence sérologique des anticorps dirigés contre l'herpèsvirus bovin de type 1 (BHV-1). Les résultats des deux épreuves ont révélé une prévalence sérologique globale chez les yaks de 41 % (105). Le sexe des animaux, leur état sauvage ou domestique et leur site d'origine ne jouent apparemment aucun rôle sur la prévalence sérologique enregistrée. En revanche, l'âge semble être un facteur déterminant, la prévalence augmentant avec l'âge des animaux et atteignant un pic chez les yaks âgés de plus de trois ans (49 %).

Les yaks partagent avec d'autres espèces domestiques et sauvages les sites d'abreuvement et les pâturages qu'ils fréquentent ; cette niche écologique commune constitue une route probable d'infection. Il s'agit de la première étude visant à déterminer la prévalence sérologique des anticorps dirigés contre le virus BHV-1 chez le yak en Inde.

Mots-clés

Épreuve de neutralisation virale – Épreuve immuno-enzymatique à l'avidine-biotine – Herpèsvirus bovin de type 1 – Inde – Prévalence sérologique – Rhinotrachéite infectieuse bovine – Yak.

Encuesta serológica sobre los anticuerpos contra el herpesvirus bovino 1 presentes en yaks (*Poephagus grunniens*) de Arunachal Pradesh, India

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Resumen

En este estudio para establecer la seroprevalencia de los anticuerpos contra el herpesvirus bovino 1 (HVB-1) se tomaron muestras de 254 yaks (*Poephagus grunniens*, actualmente *Bos grunniens*) de distintas regiones de India y se procedió a una detección sistemática mediante la prueba de neutralización del virus y el ensayo inmunoenzimático con avidina/biotina (ELISA-AB). Ambas pruebas mostraron una prevalencia total del 41% (105) en los yaks. Aparentemente, la seroprevalencia no varía con el sexo, la cría en libertad o en explotaciones, ni las regiones de origen de los animales. Por el contrario, se

encontró que aumentaba con la edad de los especímenes y era superior en los yaks mayores de tres años de edad (49%).
Los yaks suelen compartir las zonas donde se alimentan, beben y pastorean con otros animales domésticos y salvajes. Se supone que el origen de la infección puede encontrarse en esos nichos ecológicos comunes. Se trata del primer estudio sobre la prevalencia de los anticuerpos contra el HVB-1 en yaks en India.

Palabras clave
Ensayo inmunoenzimático con avidina/biotina – Herpesvirus bovino 1 – India – Prueba de neutralización viral – Rinotraqueítis infecciosa bovina – Seroprevalencia – Yak.

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