Serological evidence of coronavirus infection in mithuns *(Bos frontalis)* from India

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Summary
Studies conducted on mithuns at the National Research Centre on Mithun, and mithuns found in free-ranging conditions in Nagaland, India, revealed that the overall prevalence of antibodies to coronavirus was 80% (95% confidence interval [CI] 77-83) when detected by a commercially available enzyme-linked immunosorbent assay. The highest seroprevalence (92%; 95% CI 89-95) was found in mithuns above three years of age, and the lowest (53%; 95% CI 36-70) in mithuns of 7 to 12 months old ($P = 0.00005$). The study also revealed a higher degree of positivity (++++) in mithuns above three years old than in those below one year. No statistically significant difference in seroprevalence was observed between male (80%; 95% CI 75-85) and female mithuns (80%; 95% CI 76-84). The seroprevalence was found to be higher (92%; 95% CI 89-95) in mithuns reared in semi-intensive systems compared with those kept under free-ranging conditions (56%; 95% CI 49-63; $P = 0.003$). This is probably the first serological evidence of coronavirus infection in mithuns. The possible role of adults in the epidemiology of coronavirus infection in mithuns is also discussed.

Keywords
Bos frontalis – Coronavirus – Mithun – Seroprevalence.

Introduction

The mithun *(Bos frontalis)* is a unique ruminant found in the hill regions of north-east India, Myanmar, Bhutan, Bangladesh, China and Malaysia. The Indian gaur *(Bos gaurus)*, also known as the 'Indian bison' and as the 'gayal', is the wild ancestor of the mithun. Chromosomally, the gaur and the mithun are identical (5). There are four distinct strains of mithun: Arunachal, Manipur, Mizoram and Nagaland. The mithun is a semi-wild animal, and its breeding selection is not controlled by humans. The body weight of adult mithuns varies from 400 kg to 600 kg. This animal is generally used for the production of beef of superior quality. The mithun produces milk with a fat percentage ranging from 11% to 13% (7); the milk-production potential of this animal has been recently

reviewed (1, 6). The mithun is used as a draught and pack animal due to its surefootedness on the steep hilly slopes. In tribal societies it is also used as a bridal gift and to barter in trade (10). The mithun prefers a moderate climate, dense forest and steep slopes, which are the general geographical features of this region. Mithuns generally are found at an altitude ranging from 300 to 3,000 metres above sea level (masl). Mithuns do not like warm temperatures and hot sun, and during the middle of the day they retire to the deep forest near small ponds, water springs or streams. Mithuns can be tamed by offering common salt. They are still kept in the forest under a free-grazing system, with no human attendance. The National Research Centre on Mithun has been established in Nagaland, India, to develop technologies and techniques for the scientific management of the mithun and to maximise their economic contribution. Workers at the

institute are attempting to domesticate this rare species of animal and maintain them under a semi-intensive system of management.

Bovine coronavirus can cause diarrhoea in dairy and beef calves ranging in age from one day to three months, but most of those affected are between one and two weeks of age (9). The virus is ubiquitous in cattle populations worldwide and the majority of adult cattle are seropositive (2). Bovine coronavirus has been isolated from wild ruminants with diarrhoea, and it has also been observed that wild animals may harbour coronavirus strains transmissible to cattle (13). Although mortality in mithun calves as a result of diarrhoea is frequently recorded at the institute farms (8), the viral causes of diarrhoea in this species have not been thoroughly investigated until now. Coronavirus may be one of the agents responsible for fatal diarrhoea in mithun calves, hence the relevance of the present study. There are several reports of coronavirus detection in other species of animal in India, but this is probably the first study of the prevalence of coronavirus antibodies in mithuns. The main objectives of the study were:

a) to determine the prevalence of coronavirus antibodies in serum samples from Indian mithuns

b) to analyse the association between seroprevalence and gender, age and system of management.

Materials and methods

Study location

This study was conducted on mithun farms of the National Research Centre on Mithun in Nagaland in India, and mithuns found in their natural habitat (free-ranging system) in the state of Nagaland. The state of Nagaland is located in the extreme north-east of the country and lies between latitude 25° 10' and 27° 4' N and longitude 93° 15' and 95° 15' E. The altitude varies from 194 to 3,826 masl. Average annual rainfall in the state is 2,000 mm. The summer mean temperature varies from 15°C to 30°C and the winter temperature ranges from 4°C to 25°C.

Animals and management practices

Because of the difficulty of obtaining large numbers of animals (mithuns are generally found in deep forests and their habitat is not easily accessible), only 160 mithuns (semi-intensive = 106; free-ranging = 54) were used for the study. These 160 animals comprised 54 males and 106 females. The animals were kept on two farms using a semi-intensive system and one using the free-ranging system of management. The number of animals chosen in each farm and their age groups are shown in Table I. The

Table I
Distribution and age groups of animals in different management systems

Type of management system	Number of animals	Age group	Number of animals
Semi-intensive system			
Farm 1	55	2-12 months	18
		1-3 years	16
		> 3 years	21
Farm 2	51	2-12 months	15
		1-3 years	8
		> 3 years	28
Free-ranging system	54	2-12 months	9
		1-3 years	16
		> 3 years	29

criteria for inclusion of a particular age group of animals in the study was the same for all farms and was based on the availability of animals within that particular age group. Depending upon the availability, animals were categorised into four age groups: 2 to 6 months, 7 to 12 months, 1 to 3 years and more than 3 years. Only animals whose age group was known were included in the study. The animals in the present study were individually identified by metal ear tags bearing unique identification numbers.

The animals kept under the semi-intensive system of management were allowed to graze in the forested areas of the farms specially developed for mithuns. During the night the mithun were tethered in their respective sheds. In addition to sufficient green fodder (which mainly consisted of forest leaves, young plants and grasses), their daily food rations normally included a concentrate mixture given according to the body weight of the animal. Though free-range mithuns are found in deep forests, they are periodically (at monthly intervals) brought to the open fields by their owners to be offered salt, which is a common practice of mithun rearers. Mithun farmers keep full records of their stock, including birth records. The sera collected from these animals were separated, labelled and stored at −20°C until testing.

Serological examination

Sera were analysed for antibodies to coronavirus using a commercially available competitive enzyme-linked immunosorbent assay test (Coronavirus ELISA serum testing kit, Cypress Diagnostics, Belgium) using the manufacturer's recommended procedure. The sensitivity and specificity of the ELISA, obtained using sera from mithun, were 95% and 98%, respectively. Field serum samples from cattle were also collected and tested simultaneously with mithun sera for comparison purposes.

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Out of 72 cattle tested for the presence of antibodies to coronavirus, 86% (62/72) were found to be positive. The test was performed as described below.

Diluted serum samples (1:20 in the supplied dilution buffer) were added to the wells of the 96-well microplate supplied with the kit (which had been previously sensitised with a bovine coronavirus-specific polyclonal antibody) at 100 µl per well (two wells per sample). This protocol was repeated for the reference positive and negative sera. The conjugate (peroxidase-coupled bovine coronavirus-specific monoclonal antibody; diluted 1:20 in the dilution buffer) was added at 100 µl per well, and then the plate was incubated at room temperature for 1 h. After incubation, the plate was washed three times with diluted washing solution. One hundred microlitres of an indicator mixture comprising the chromogen (tetramethylbenzidine) and substrate (hydrogen peroxide) was added to each well, and then the plate was incubated at room temperature for 10 min in the dark. To measure the optical density (OD) the stop solution was added at 50 µl per well and readings were obtained using an ELISA reader with a 450 nm filter. The mean optical density was calculated for the positive and negative sera and for each sample. The percentage inhibition (% inh) for each tested sample and for the positive reference serum was calculated by applying the following formulae:

% inh sample = [(OD negative – OD sample)/ OD negative] × 100

% inh positive = [(OD negative – OD positive)/ OD negative] × 100

The test was not validated unless the following two conditions were fulfilled:

OD negative – OD positive > 0.7

% inh positive > 50%

The positivity of each sample was determined as in Table II.

Table II
Relationship between calculated values of inhibition percentage and the positivity of serum samples

Calculated Value	Positivity
% inh < 20	0
20 < = % inh < 40	+
40 < = % inh < 60	++
60 < = % inh < 80	+++
80 < = % inh	++++

Data analysis

Data were analysed using standard methods (12). Chi-squared (χ^2) and Fisher's exact tests were used to compare seroprevalence values with respect to age, gender and management system. Analyses were performed using Statistical Package for the Social Sciences (SPSS) 10.0 software for Windows taking a probability (P) value < 0.05 as statistically significant. Confidence limits for the proportions were established by the exact binomial test with 95% confidence intervals (CI).

Results

The overall prevalence of antibodies to coronavirus in the studied mithuns was found to be 80% (95% CI 77-83). The difference in prevalence of antibodies to coronavirus among different age groups of mithun was found to be statistically significant (P = 0.00005). It was observed that the seroprevalence increased with an increase in age of the animals, with the exception of the 7-12 months age group (Table III). The highest seroprevalence was observed in mithuns more than 3 years old (92%, 95% CI 89-95), with the lowest seroprevalence recorded in mithuns aged between 7 and 12 months (53%, 95% CI 36-70). The proportion of animals that showed the highest degree of positivity (++++) also increased with increasing age; the

Table III
Seroprevalence of coronavirus antibodies by age for 160 mithuns in India

Age group	Number of animals tested	Number of positive animals	Prevalence (%)	95% CI	Number of animals with ++++ positivity
2-6 months	10	7	70	42-98	1
7-12 months	32	17	53	36-70	3
1-3 years	40	32	80	68-92	8
> 3 years	78	72	92	89-95	50
Total	160	128	80	77-83	62

χ^2 = 22.45
degree of freedom = 3
P = 0.00005

highest percentage (69%) of ++++ positivity was recorded in mithuns above 3 years of age (Table III). No statistically significant difference in seroprevalence (P = 0.90) was observed between male and female mithuns (Table IV).

The association of management system with seroprevalence was found to be statistically significant (P = 0.003) (Table V). A higher seroprevalence (92%, 95% CI 89-95) was observed in mithuns reared in the semi-intensive system, in comparison to mithuns kept under the free-range system of management (prevalence = 56%, 95% CI 49-63).

Discussion

The serological results obtained in this study indicate that mithuns in India are exposed to coronavirus infection. Although there are no comparable studies on coronavirus infection in mithun, it is possible to compare the results with findings in other bovine species, such as cattle. The present study revealed that mithuns of all age groups are susceptible to coronavirus infection. This finding is in agreement with the findings of Durham *et al.* (3), who observed that cattle of all age groups are susceptible to coronavirus infection.

An increase in seroprevalence was evident in older mithuns. Although such an association has been observed in related bovine species, such as cattle (9), the present findings could not be verified due to the lack of substantiating evidence in mithuns. The increase in the prevalence of antibodies to coronavirus with age could be due to the fact that, as animals grow older, they are more likely to be exposed to the virus as they are more likely to come into contact with other animals that have recovered from the disease but remain as carriers. Likewise, the highest degree of positivity (++++) was observed in adult mithuns (above 3 years of age); this could be due to repeated exposure to infection or a long lasting immune reaction. Though various authors (4, 11) have observed severe diarrhoea in adult cows due to coronavirus infection, the role of coronavirus as an aetiological agent of diarrhoea in adult mithuns is yet to be explored; this would require a detailed study involving a large sample of the mithun population.

Gender, in contrast to age, had no influence on the prevalence of antibodies to coronavirus in mithuns. This indicates that, under the same management system, both male and female mithuns have an equal chance of acquiring coronavirus infection. The higher prevalence of antibodies to coronavirus in mithuns kept in the semi-intensive system in comparison to mithuns found in free-range conditions could be due to the difference in management practices, such as stocking density.

As intensive mithun farming is gradually being adopted by the mithun rearers in the north-eastern hill region of the country, the status of this animal in relation to different diseases will have a strong influence on strategies for disease investigation programmes because of the increased chance of intermingling of this species with other ruminants. The role of coronavirus as an aetiological agent in mithun calf mortality is another important area that needs to be thoroughly investigated. The sample size of the present study was based on the availability of animals; efforts to collect large numbers of samples from animals in

Table IV

Seroprevalence of coronavirus antibodies by gender for 160 mithuns in India

Gender	Number of animals tested	Number of positive animals	Prevalence (%)	95% CI
Male	54	43	80	75-85
Female	106	85	80	76-84
Total	160	128	80	77-83

χ^2 = 0.01
degree of freedom = 1
P = 0.90

Table V

Seroprevalence of coronavirus antibodies by management system for 160 mithuns in India

Management system	Number of animals tested	Number of positive animals	Prevalence (%)	95% CI
Semi-intensive (n = 2)	106	98	92	89-95
Free-ranging (n = 1)	54	30	56	49-63
Total	160	128	80	77-83

χ^2 = 28.17
degree of freedom = 1
P = 0.003

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the wild situation did not succeed due to the inaccessibility of their natural habitat. Despite this shortcoming, the authors feel that the study does provide an insight into the prevalence of antibodies to coronavirus in this rare species of animal.

As mithun live in small herds of twenty to forty animals, and frequently come into contact with other livestock species while grazing and browsing, there is the potential for disease to be transmitted from one species to another. Surveillance of coronavirus infection in all domestic and sylvatic animals in mithun-inhabited areas is thus an important exercise if one wishes to know the exact status of this disease in mithun. More detailed studies, involving larger populations of mithuns and other livestock, should be undertaken to reveal the role of mithun in transmitting coronavirus infection to other livestock species, and vice versa. Such a study would also increase our understanding of the epidemiology of coronavirus infection in the mithun.

Conclusion

The present study revealed that mithuns in India are exposed to coronavirus infection. Under the prevailing agro-climatic conditions of India, the highest prevalence of antibodies to coronavirus was found in adult mithuns (above 3 years of age) in comparison to the younger age group. A significant influence of management system on coronavirus seroprevalence was observed in mithuns kept under a semi-intensive system of management.

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Confirmation sérologique de l'infection à coronavirus chez le gayal *(Bos frontalis)* en Inde

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Résumé
Une étude sérologique réalisée sur des gayals du Centre national de recherche sur le gayal ainsi que sur des gayals vivant en liberté à Nagaland, Inde, a montré que la prévalence globale des anticorps dirigés contre les coronavirus, telle que révélée par dosage immunoenzymatique à l'aide d'une trousse commerciale, était de 80 % (avec un intervalle de confiance [IC] de 95 % compris entre 77 et 83). La prévalence se trouvait être la plus élevée (92 % ; IC 95 % 89-95) chez les gayals âgés de plus de trois ans, et la plus faible (53 % ; IC 95 % 36-70) chez ceux âgés de 7 à 12 mois ((P = 0,00005). L'étude a également révélé que la positivité était d'un ordre de grandeur plus élevé (++++) chez les gayals âgés de plus de trois ans que chez ceux de moins d'un an. Aucune différence significative au plan statistique n'a été constatée en fonction du sexe des animaux, la prévalence étant, chez les mâles, de 80 % avec un IC 95 % de 75-85 et, chez les femelles, également de 80 % avec un IC 95 % de 76-84. La prévalence se trouvait être plus élevée (92 % ; IC 95% 89-95) chez les gayals élevés dans des systèmes semi intensifs que chez ceux vivant en liberté (56 % ; IC 95 % 49-63 ; P = 0,003). Il s'agit probablement de la première confirmation sérologique d'une infection à coronavirus chez le gayal. Les auteurs examinent également le rôle des adultes dans l'épidémiologique de l'infection à coronavirus chez le gayal.

Mots-clés
Bos frontalis – Coronavirus – Gayal – Prévalence sérologique.

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Prueba serológica de infección por coronavirus en ganado gayal *(Bos frontalis)* de India

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Resumen
Los autores exponen estudios realizados en el Centro Nacional de Investigación sobre Ganado Gayal, y en ejemplares de ese ganado criados al aire libre en Nagaland, India, que mostraron que la prevalencia general de los anticuerpos contra coronavirus alcanzaba un 80% (intervalo de confianza del 95% [IC 95%]: 77-83) cuando se los detectaba con un ensayo inmunoenzimático disponible en el comercio. La mayor seroprevalencia (92%; IC 95%: 89-95) se encontró en animales de más de tres años de edad, y la más baja (53%; IC 95%: 36-70) en ejemplares de 7 a 12 meses de edad (P = 0,00005). El estudio también mostró una mayor positividad (++++) en el ganado gayal de más de tres años de edad que en los animales menores de un año. No se observó diferencia estadística significativa alguna entre machos (80%; IC 95%: 75-85) y hembras (80%; IC 95%: 76-84). La seroprevalencia era mayor (92%; IC 95%: 89-95) en los animales criados en sistemas semi-intensivos que en los ejemplares camperos (56%; IC 95%: 49-63; P = 0,003). Se trata, probablemente, de la primera prueba serológica de infección por coronavirus en ganado gayal. En este artículo también se examina el posible papel de los animales adultos en la epidemiología de la infección por coronavirus en ganado gayal.

Palabras clave
Bos frontalis – Coronavirus – Gayal – Seroprevalencia.
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