

Status of sheep sera to bluetongue, peste des petits ruminants and sheep pox in a few northern states of India

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Summary

Bluetongue (BT), peste des petits ruminants (PPR) and sheep pox are the most economically important viral diseases of sheep in India. Serum samples obtained from sheep in five northern states of the country were screened for antibody against these agents to explore the extent of spread of these infections. A total of 516 serum samples were screened for the presence of antibodies against BT and PPR viruses. Of these, 155 samples were also tested for antibodies against sheep pox virus. BT antibodies were found in 293 (56.8%) animals, PPR virus antibodies in 215 (41.7%) and sheep pox virus antibodies in 106 (68.3%). Of the serum samples tested, 25.2% were positive for antibodies against all three viruses. These findings clearly demonstrated not only the enzootic nature of disease, but also the co-existence of antibodies to more than one of these viruses which would indicate that concurrent infections were common. Therefore, control measures should focus in combating all three diseases simultaneously by exploring the possibility of a trivalent vaccine or the use of multiple genes expressing vectored vaccine.

Keywords

Bluetongue, India, Peste des petits ruminants, Sheep, Sheep pox, Seropositivity.

Situazione dei sieri ovini per febbre catarrale degli ovini, peste dei piccoli ruminanti e vaiolo ovino in alcuni stati del nord dell'India

Riassunto

La febbre catarrale degli ovini (bluetongue: BT), la peste dei piccoli ruminanti (PPR) e il vaiolo ovino sono le malattie virali delle pecore più importanti dal punto di vista economico in India. Campioni di sangue provenienti da pecore di cinque stati del nord del Paese sono stati esaminati per anticorpi verso tali agenti allo scopo di valutare la diffusione di queste patologie. Sono stati esaminati 516 campioni di sangue per la ricerca di anticorpi nei confronti di virus BT e PPR. Di questi, 155 campioni sono stati anche testati per anticorpi contro il vaiolo ovino. Anticorpi BT sono stati trovati in 293 animali (56,8%), anticorpi per PPR in 215 (42,7%) e anticorpi contro il vaiolo ovino in 106 animali (68,3%). Tra i campioni testati, il 25,2% è risultato positivo per anticorpi nei confronti di tutti e tre i virus. Questi dati dimostrano chiaramente non solo la natura enzootica della malattia, ma anche la coesistenza di anticorpi verso più di un virus, cosa che sta a significare che le infezioni concorrenti sono comuni. Per tali ragioni le misure di controllo dovrebbero focalizzarsi sulla lotta alle tre patologie esplorando la possibilità di un vaccino trivalente o con l'uso di vaccini in grado di esprimere geni multipli.

Parole chiave

Bluetongue, Febbre catarrale degli ovini, India, Pecora, Peste dei piccoli ruminanti, Sieropositività, Vaiolo ovino.

Introduction

Sheep play a major role in the rural agrarian economy of India. They provide animal protein to the ever-growing human population at a comparatively low cost and are important in areas where crop and dairy farming are uneconomical. Sheep also play a significant role in the livelihood of a large proportion of small and marginal farmers and landless labourers (1). India ranks fifth in terms of sheep population in the world (34). Of the 6.5% total gross domestic product contribution from the livestock sector, the share contributed by sheep and sheep by-products is highly significant (20). However, optimum productivity is hampered by various infectious diseases, among which bluetongue (BT), peste des petits ruminants (PPR) and sheep pox are economically the most significant.

These three viruses belong to different viral families, namely: sheep pox virus (family *Poxviridae*, genus *Capripoxvirus*), PPR virus (PPRV, family *Paramyxoviridae*, genus *Morbillovirus*) and BT virus (BTV, family *Reoviridae*, genus *Orbivirus*). All three viruses can have a major impact on sheep productivity and profitability but they also affect other livestock species, causing varying degrees of direct and indirect losses. Sheep are the primary host for sheep poxviruses. The occurrence of BT in sheep is associated with morbidity and mortality rates of 80% and 30%, respectively (22), whereas both morbidity and mortality due to PPR can reach 100% in severe outbreaks, but mortality may be less than 50% in milder outbreaks. In a fully susceptible flock, the morbidity rate of sheep pox may be 75% (22) and mortality may be 50%. However, in some cases, the sheep pox mortality rate may be 100%, depending on the virulence of the virus, age and immune status of the host and climatic factors (22, 28). In India, PPR alone incurs losses of up to 1 800 million Indian rupees (approximately US\$43 million)

annually (3) and the situation is almost the same for BT (17) and sheep pox (10).

BTV is transmitted by midges of the genus *Culicoides*. The sheep pox virus is spread by aerosol, direct contact with affected hosts and possibly by *Stomoxys calcitrans*, whereas PPRV spreads primarily by aerosol and fomites due to direct contact with contaminated material and diseased hosts. In India, a total of 21 BTV serotypes have been reported, based on sero-surveillance or virus isolation (19). However, no such sero-diversity is observed in respect of sheep pox virus and PPRV. The virulence of these viruses may vary depending, at least partly, upon the strain of virus.

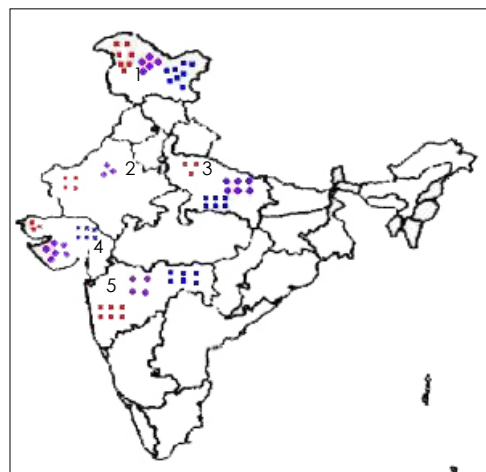
The present study aimed to explore the proportion of animals with antibody against each of the viruses individually and to determine if these viruses occur simultaneously in serum samples collected from five northern states of India. Standard tests/kits were used to screen for antibodies against each of the viruses. The study also aimed to identify the geographic distribution of antibodies to each of the viruses and to establish how many of the animals had antibody against more than one of the viruses.

Materials and methods

Field serum samples

Sheep serum samples ($n=516$) of unknown antibody status were collected from different geographic locations of the states selected from state- and privately owned sheep farms and from small flock owners and individual farmers. The samples were collected from the five northern states of the country, namely: Uttar Pradesh, Jammu and Kashmir, Maharashtra, Rajasthan and Gujarat (Fig. 1). The serum samples were collected from most of the animals with clinical signs of PPR and sheep pox. The clinical signs of PPR included fever, anorexia, necrotic stomatitis with gingivitis, diarrhoea and bronchopneumonia, whilst those for sheep pox were fever, anorexia, increased respiratory rate, oedema of the eyelids, nasal discharge and cutaneous lesions on the less hairy parts of the body. Samples were collected from different areas in

each state by field veterinarians and were despatched to state veterinary biological institutes. The biological institutes sent the samples to our laboratory for further confirmation. The serum samples were kept frozen (-20°C). They originated from different Indian sheep breeds and were only collected from animals over 12 months of age. All 516 serum samples were tested for antibodies against BTV and PPRV. All the animals tested had clinical signs that were suggestive of PPRV infection and had no history of PPR vaccination. Vaccination is not practised against BT as there is no vaccine currently available in the country for field use. Only 155 samples were tested for antibody against sheep pox virus and these were from animals suspected of being infected with sheep pox virus. There was no history of sheep pox vaccination in these flocks during the previous 10 years. Only 155 of the 516 samples collected were tested for antibody against sheep pox virus as the sheep pox vaccination status of the remainder of the animals was unknown.



- Bluetongue
- Peste des petits ruminants
- Sheep pox

- 1 Jammu and Kashmir
- 2 Rajasthan
- 3 Uttar Pradesh
- 4 Gujarat
- 5 Maharashtra

Each colour represents approximately 10% (fractions are rounded off to the nearest integer) proportion positivity of sera to a respective disease as indicated in a different colour

Figure 1
Proportion of bluetongue, peste des petits ruminants and sheep pox virus antibodies in sheep in a few northern states of India

Detection of antibodies against bluetongue virus

BTV-specific antibodies were detected using the agar gel immunodiffusion (AGID) test, indirect enzyme-linked immunosorbent assay (i-ELISA) and the competitive ELISA (c-ELISA).

Agar gel immunodiffusion test

BTV serotype 23 soluble antigen was prepared from infected baby hamster kidney 21 (BHK-21) cells (16). The standard method was followed using seven well patterns on plates of 1% agarose in 0.85% NaCl (14). Antigen was added to the centre well, unknown sera samples were added to every other well and positive control sera from sheep infected with BTV serotype 23 were added to the remaining three wells. In addition, BTV-negative sheep sera and non-infected BHK-21 cell culture antigen were used as controls.

Indirect enzyme-linked immunosorbent assay

BTV-23 was propagated and purified as previously described (16, 18). The i-ELISA for the detection of antibodies to BTV was performed as explained by Afshar *et al.* (2) with modifications (6). In summary, flat-bottomed 96-well, polystyrene microtitration plates were coated with 50 µl/well of a 1:100 dilution of BTV-23 purified virus antigen in phosphate buffered solution (PBS) (0.01M, pH 7.2). The plates were incubated at 37°C for 1 h under constant orbital shaking. The wells were washed three times with washing buffer (0.002M PBS + 0.05% Tween 20) and 100 µl of blocking buffer (3% gelatin + 3% skimmed milk powder in PBS) was added to each well. After incubation and washing of the plates, 50 µl of a 1/20 dilution of serum in blocking buffer were added to each well and the plates were incubated for 1 h. The anti-sheep immunoglobulin G (IgG)-horse radish peroxidase (HRPO) conjugate, 50 µl/well (1:10 000 in blocking buffer) was added and the plates were incubated for 1 h at 37°C. Substrate solution of O-phenylenediamine (OPD) 0.4 mg/ml + 4 µl of 3% H₂O₂ was added to each well and the plates were observed for 15 min during the development of colour. The

reaction was stopped with 1M H₂SO₄. The absorbance values were measured at a wavelength of 492 nm.

Test validation

The antigen and serum dilutions that gave the greatest difference in absorbance at 492 nm (75% of the plateau) between positive and negative (P/N) in a chequerboard titration were selected. Test sera also included weak-positive and negative serum samples. Before testing the field samples of unknown status, the i-ELISA was validated by detection of antibody in 142 known positive and 138 negative serum samples. The negative cut-off was established as twice the mean optical density (OD) values of the known negative population (13); this value was 0.180. The controls with the following OD values were also included in the test:

- positive (0.250±0.03)
- negative (0.089±0.01)
- blank (0.068±0.009)
- conjugate (0.07±0.007).

Competitive enzyme-linked immunosorbent assay

The same 516 serum samples were also screened using a commercially available c-ELISA kit in accordance with the instructions of manufacturer (Veterinary Diagnostic Technology, Inc., Wheat Ridge, Colorado).

Detection of antibodies against the peste des petits ruminants virus

The same 516 field serum samples were screened for antibodies against the PPRV using the c-ELISA kit that had been developed by our laboratory and is currently employed for the sero-surveillance of PPRV throughout India. The test has been standardised and calibrated based on the guidelines of the World Organisation for Animal Health (*Office International des Épizooties*: OIE) (30, 31).

Detection of antibodies against the sheep pox virus

The sheep pox virus antibodies were detected by using the counter immunoelectrophoresis (CIE) and i-ELISA described below.

Counterimmunoelectrophoresis

The serum samples were screened for antibodies against sheep pox virus using the CIE test in accordance with the standard procedure (29). In brief, the positive antigen sample was prepared from a 10% suspension of scab material collected from sheep experimentally infected with the Srinagar strain of sheep pox virus that had been propagated in sheep by needle passage. Electrophoresis was performed on 1% agarose in 50 mM Tris-Borate buffer (pH 8.3) at 20 volts/cm for 45 min, on glass microscopic slides. The gel was dried and stained with Coomassie brilliant blue to observe the precipitin bands.

Indirect enzyme-linked immunosorbent assay

Indirect ELISA for the detection of antibodies against sheep pox virus was performed as described by Hosamani *et al.* (11, 12) with modifications (5). Briefly, sheep pox virus antigen was prepared by sonicating infected Vero cells and then 50 µl/well of a 1/50 dilution of the supernatant, containing viral proteins in phosphate buffer saline, was added to each well of a flat-bottomed 96-well plate and incubated at 37°C for 1 h. A blocking solution (3% skimmed milk powder + 1% gelatin in PBS) was added to the wells containing antigen and incubated for 1 h at 37°C. A total of 50 µl of a 1/100 dilution of serum were added to each of the two wells and the plates were incubated for 1 h at 37°C. A sheep anti-IgG-HRP conjugate, 1:10 000, 50 µl/well, was added and incubated at 37°C for 1 h. The plates were washed three times between each step with PBS containing 0.05% Tween 20. The subsequent steps were as described above.

Test validation

Using a chequerboard titration, the sheep pox antigen (Srinagar strain) and serum dilutions that revealed maximum difference in absorbance (75% of the plateau) at 492 nm between positive and negative (P/N) were selected to test the serum samples. Other controls included weak sheep pox virus and positive and negative sera. The optimised i-ELISA was validated before testing the field

samples of unknown status by testing 132 pre-vaccinated samples with the virus neutralisation test (VNT) ($n=132$) and 132 post-vaccinated ($n=132$) serum samples. The negative cut-off was determined by plotting the frequency distribution of pre-vaccinated sheep pox virus antibody-free and vaccinated sheep pox-positive sheep against OD₄₉₂ values. The point of intersection of the frequency distribution of vaccinated and pre-vaccinated sheep was taken as the negative cut-off (13). After visually inspecting the frequency distribution of vaccinated and pre-vaccinated animals, the negative cut-off was 0.2.

Proportion of infected animals

The proportion of animals with antibodies against two or three of the viruses was determined using the more sensitive ELISA techniques.

Statistical analysis

The c-ELISA results for BT ($n=516$) and PPR ($n=516$) and i-ELISA results for sheep pox ($n=155$) were used to calculate the beta distribution. The 95% confidence intervals (CI) and the prevalence level in sheep for antibody against each virus was calculated through Beta ($s+1, n-s+1$) distribution using Excel®, where 's' is the total number of positives and 'n' is the total number of samples tested. The probability distribution of the percentage of positive animals shows not only the most probable value of antibody presence, but also the level of uncertainty due to sample size.

Results

Antibodies to BTV, PPRV and sheep pox virus in Indian sheep using different serological tests and their significant differences are shown in Table I. Antibodies against BTV were found in 56.8%, 53.5% and 37.9% of animals, respectively, when tested with the i-ELISA, c-ELISA and AGID. Antibodies against PPRV were detected in 41.7% of animals using the c-ELISA. The following were the results of the tests for antibodies against sheep pox virus:

- 68.3% i-ELISA-positive
- 27.7% CIE-positive for sheep pox using the CIE test.

The results indicate that the ELISA formats are more sensitive than AGID and CIE. Consequently, all the following summaries of antibody results, as well as Table II and Figures 1 and 2, are based on the BTV and PRV c-ELISA and sheep pox i-ELISA. The c-ELISA formats used in this study for BT and PPR antibody detection were monoclonal antibody-(MAb) based. The c-ELISA for BT uses a MAb raised against BTV group-specific (VP7) antigen; the PPR MAb is against the haemagglutinin (H) protein. A summary of the BTV, PPRV and sheep pox virus antibody test results from sheep in the northern states of India are shown in Figure 2. The distribution of antibodies against BT, PPR and sheep pox viruses are shown in Table II and Figure 1 according to geographic origin. It is clear from

Table I
Antibodies against bluetongue, peste des petits ruminants and sheep pox viruses
in Indian sheep using different serological methods

Disease	Number tested	Number positive	Proportion positive (%)	Confidence interval (95%)
Bluetongue				
AGID	516	196	37.9*	(33.90, 42.25)
i-ELISA	516	293	56.8*	(52.47, 60.99)
c-ELISA	516	276	53.5*	(49.17, 57.75)
Peste des petits ruminants				
c-ELISA	516	215	41.7	(37.49, 45.97)
Sheep pox				
CIE	155	43	27.7*	(21.30, 35.28)
i-ELISA	155	106	68.3*	(60.68, 75.19)

* significantly different ($p \leq 0.05$)
AGID agar gel immunodiffusion
i-ELISA indirect enzyme-linked immunosorbent assay
c-ELISA competitive enzyme-linked immunosorbent assay
CIE counter-immunoelectrophoresis

Table II
Bluetongue, peste des petits ruminants and sheep pox virus antibodies in Indian sheep according to geographic origin

Disease	Number tested	Number positive	Proportion positive (%)	Confidence interval (95%)
Bluetongue^(a)				
Maharashtra	195	116	59.5 ^(a)	(52.47, 66.13)
Jammu and Kashmir	135	98	72.6	(64.50, 79.41)
Gujarat	14	3	21.4 ^(b)	(07.79, 48.09)
Rajasthan	158	56	35.4	(28.41, 43.18)
Uttar Pradesh	14	3	21.4	(07.79, 48.09)
Peste des petits ruminants^(a)				
Maharashtra	195	84	43.1	(36.32, 50.10)
Jammu and Kashmir	135	64	47.4	(39.16, 55.80)
Gujarat	14	9	64.3	(38.38, 83.66)
Rajasthan	158	49	31.0	(24.32, 38.62)
Uttar Pradesh	14	9	64.3	(38.38, 83.66)
Sheep pox^(c)				
Maharashtra	65	37	56.9 ^(b)	(44.79, 68.26)
Jammu and Kashmir	67	55	82.1 ^(b)	(71.20, 89.41)
Gujarat	14	9	64.3	(38.38, 83.66)
Uttar Pradesh	9	5	55.6	(26.24, 81.29)

- a) competitive ELISA
- b) significantly different ($p \leq 0.05$)
- c) indirect ELISA

Table II that the proportion of BT seropositivity is significantly different for Maharashtra and Gujarat ($p \leq 0.05$) states. For sheep pox, significant differences were observed between the states of Maharashtra and Jammu and Kashmir ($p \leq 0.05$). No such significant geographic differences ($p \leq 0.05$) were recorded for PPR.

The proportion of sheep with antibodies for both BTV and PPRV was 25.6% (132/516) (the 95% CI would be 22.01% to 29.52%), whereas 47.8% (74/155, 95% CI: 40.02% to 55.58%) and 37.4% (58/155, 95% CI: 30.19% to 45.27%) of the animals were harbouring antibodies against BTV and sheep pox virus, and PPRV and sheep pox virus, respectively. The spatial pattern of the two infections in Indian sheep is shown in the Table III. It is clear from the results that the proportion of sheep that gave positive results for both BT and PPR are significantly different between Rajasthan and Maharashtra and Jammu and Kashmir ($p \leq 0.05$). The magnitude of combined seropositivity of the serum samples to BTV, PPRV and sheep pox virus was 25.2% (39/155, 95% CI: 18.99% to 32.55%). The proportions of positivity against all the three infections in Maharashtra and Uttar Pradesh were 30.8%

(20/65, 95% CI: .20.89% to 42.85%) and nil, respectively. Multiple infection in sheep of Jammu and Kashmir and Gujarat were, 23.9% (16/67, 95% CI: 15.29% to 35.38%) and 21.4% (3/14, 95% CI: 7.79% to 48.09%), respectively.

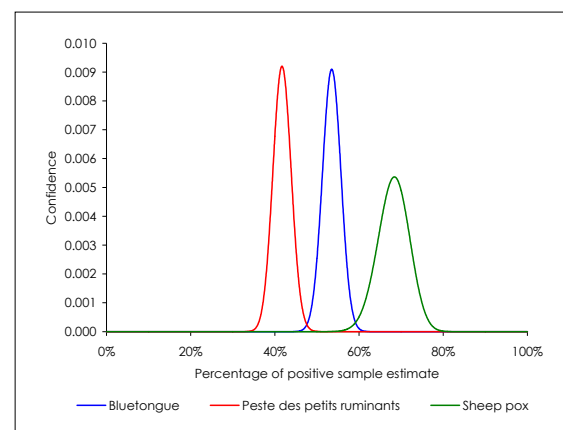


Figure 2
Confidence of the percentage of sheep with bluetongue, peste des petits ruminants and sheep pox antibodies in a few northern states of India

Discussion

Sheep play an important role in the agricultural economy of many countries. Despite their comparative innate natural

Table III
 Multiple viral antibodies in Indian sheep according to geographic origin

Diseases	Number tested	Number positive	Proportion positive (%)	Confidence interval (95%)
Bluetongue ^(a) and peste des petits ruminants ^(a)				
Maharashtra	195	56	28.7 ^(b)	(22.83, 35.44)
Jammu and Kashmir	135	51	37.8 ^(b)	(30.04, 46.21)
Gujarat	14	3	21.4	(07.79, 48.09)
Rajasthan	158	19	12.0 ^(b)	(07.86, 18.03)
Uttar Pradesh	14	3	21.4	(07.79, 48.09)
Peste des petits ruminants ^(a) and sheep pox ^(c)				
Maharashtra	65	26	40.0	(28.95, 52.19)
Jammu and Kashmir	67	24	35.8	(25.39, 47.83)
Gujarat	14	6	42.9	(21.27, 67.71)
Uttar Pradesh	9	2	22.2	(06.67, 55.61)
Bluetongue ^(a) and sheep pox ^(c)				
Maharashtra	65	33	50.8	(38.88, 62.57)
Jammu and Kashmir	67	38	56.7	(44.77, 67.92)
Gujarat	14	3	21.4	(07.79, 48.09)
Uttar Pradesh	9	0	0.0	(0.0, 25.89)

- a) competitive ELISA
- b) significantly different ($p \leq 0.05$)
- c) indirect ELISA

resistance to various infectious diseases, there are viruses that can cause a drastic reduction in productivity. This poses an adverse impact on the agricultural economies of some countries. BT, PPR and sheep pox are three diseases that can have a major impact on sheep productivity and cause tangible and intangible losses. Given their potential to spread rapidly across international borders inflicting serious socio-economic repercussions, these diseases are all notifiable to the OIE.

Although there is some similarity in the clinical signs of BT, PPR and sheep pox, each disease is caused by a significantly different virus. In the present study, results clearly show that the infections are endemic in many of the northern states of India, namely: Uttar Pradesh, Jammu and Kashmir, Gujarat, Rajasthan and Maharashtra; the percentage of positive samples does vary between states.

BT was reported 128 years ago from South Africa, when European breeds of sheep were introduced. Globally, 24 BTV serotypes have been reported (36). In India, a total of 21 serotypes have been identified; 11 of the virus serotypes have been isolated and the diagnosis of the remaining 10 types was based on serology (32). The occurrence of BT disease involves complex interaction of factors, in particular host, agent, environment (referred to

as a 'triad') and the presence of the appropriate vectors. In India, there have been isolated reports of the use of experimental monovalent, bivalent and trivalent inactivated BT vaccines (24, 27, 32); however, a vaccine is not yet available commercially and vaccination is not practised. There were more seropositive animals in this study using the c-ELISA than in previous studies in which only the AGID test was used (4, 7, 9, 21, 25, 32). This demonstrated that more seropositive animals are detected if the more sensitive c-ELISA is used. Other factors that can affect seroprevalence of BT are husbandry practices (7) and climatic factors that may favour the activity of *Culicoides* vectors and the basic ecology of the virus (32). BT antibody has also been found in goats, cattle and buffalo in India (32). Goats and cattle usually suffer from subclinical infections of BTV (26), but may serve as amplifying hosts that contribute to the spread of infection in sheep. In addition, complex ecological circumstances, such as global warming, may enable the vectors to survive in niches where they previously could not. The changing pattern of land use, with cattle replacing sheep, may be another contributing factor, since some of the insect vectors that spread BTV feed more readily on cattle than on sheep (33). More importantly, India possesses more

infectious strains of BTV than any other country and clinical cases of BT have been reported in much of the country (15).

The results of this study confirm that there is apparently widespread natural infection with PPR (41.7%); this incidence is only slightly lower than that of BT. This high rate of infection in sheep could possibly be due to low case fatality rate of PPR. Compared to goats, sheep are kept for many years for wool and meat. Many sheep are infected and most recover; they develop antibody that lasts for the life of the animal, resulting in a higher proportion of animals with antibodies to PPR virus. Consequently, the seroprevalence of PPR may not be related to the susceptibility or resistance to PPRV as previously reported (30). Nevertheless, the present results are in agreement with those reported previously for the southern states of India (31). In Turkey, the occurrence of PPRV infection did not vary substantially in relation to the geographic locations of sheep and goats tested (23) or to the sheep population of the area. A similar seroprevalence was also recorded in earlier reports (30).

The principal objective of our investigation was to explore the simultaneous presence of antibodies to BTV, PPRV and sheep pox virus. Accordingly, a relatively high number of serum samples had antibodies to BTV and sheep pox virus, BTV and PPRV, PPRV and sheep pox virus, as well as to all three viruses. Based on these observations, it could be

inferred that the three infections are widespread in these states. Therefore, control measures should be designed to reduce the occurrence of these infections by vaccination, preferably by employing combined vaccines or by using a vectored vaccine containing multiple antigens of these viruses. A number of combined vaccines and recombinant vectored vaccines have been tested with success in small ruminants (8, 13, 35).

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