Surveillance of bluetongue virus antibody in goats using a recombinant VP7-based indirect ELISA in the coastal saline area of West Bengal, India

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Summary

The authors describe the serological surveillance of bluetongue virus (BTV) group-specific antibody in goats of the coastal saline (Sunderban) area of West Bengal, India. A recombinant viral protein 7 (rVP7)-based indirect enzyme-linked immunosorbent assay (ELISA) was used to detect the antibody in sera. The bacterially expressed rVP7 was purified by affinity chromatography. The diagnostic performance of the assay was assessed by comparing it to the commercially available previously validated competitive ELISA. Using the control and 1 202 test sera, the cut-off value, sensitivity and specificity as well as other performance characteristics e.g. the Youden index, efficiency, positive and negative predictive value and prevalence were estimated. Field-collected goat sera (n = 1 202) were tested and a serological prevalence rate of 47% was observed in the study area.

Keywords

Bluetongue, Enzyme-linked immunosorbent assay, ELISA, Goat, India, Indirect enzyme-linked immunosorbent assay, Recombinant VP7, Serology, Surveillance, Virus.

Sorveglianza dell'anticorpo del virus della bluetongue nelle capre mediante ELISA indiretta basata sulla proteina VP7 ricombinante nell'area salina costale del Bengali occidentale, India

Riassunto

Gli autori descrivono la sorveglianza sierologica dell'anticorpo del virus della bluetongue (BTV) gruppo-specifico nelle capre dell'area salina costale (Sunderbun) del Bengali occidentale, India. Per rilevare la presenza dell'anticorpo nel siero è stata utilizzata un'analisi ELISA indiretta basata sulla proteina VP7 ricombinante (rVP7). La rVP7 espressa dai batteri è stata purificata mediante cromatografia di affinità. La rilevanza diagnostica dell'esame è stata valutata confrontando tale test con il test c-ELISA disponibile in commercio e già convalidato. Utilizzando i campioni di controllo e 1202 campioni in studio, sono stati calcolati il valore di cut-off, la sensibilità e la specificità nonché altre caratteristiche quali l'indice di Youden, l'efficienza, il valore predittivo positivo e negativo e la prevalenza. Dalle analisi dei campioni di siero di capra raccolti sul campo (n = *1202) è emersa una sieroprevalenza del 47% nell'area in studio, il che*

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indica la presenza di infezione del virus nelle capre di questa regione.

Parole chiave

Bluetongue, Capra, Dosaggio con immuno-assorbente legato all'enzima, Dosaggio con immunoassorbente legato all'enzima indiretto, ELISA, India, Sierologia, Sorveglianza, Virus, VP7 ricombinante.

Introduction

Bluetongue (BT) is an infectious, non-contagious, arthropod-borne viral disease that affects ruminants and is caused by the BT virus (BTV) which is the prototype species of the genus *Orbivirus*, family *Reoviridae* (17). The virus can infect most species of domestic and wild ruminants but clinical signs are usually most severe in domestic sheep and some species of deer, although in these species the clinical picture varies from subclinical infection to very severe and there can be death of some affected animals (8, 13, 14, 15). Cattle and goats usually have subclinical infections and, therefore, may serve as viral reservoirs for other species (29). However, some serotypes such as serotype 8, which has recently caused infection in northern Europe, exhibit greater virulence for cattle with marked clinical signs and even mortality (5, 30), as well as being a serious socio-economic concern (23, 24).

The acute form of BT in sheep is characterised by high fever, excessive salivation, and congestion of the tongue, petecheal haemorrhages and ulcers on the mucous membrane of the mouth. In addition, conjunctivitis, coronitis and reproductive disorders, leading to abortion and congenital abnormalities in newborn animals, have been reported (13). In goats, the most obvious clinical signs are a sharp drop in milk production and high fever (up to 42°C). Clinical signs in goats are not as clear as those observed in clinically diseased sheep and many infected animals will have subclinical infection. In some cases, goats may show oedema of the lips and head, nasal discharge and scabs on the nose and lips. Erythema of the skin of the udder and small subcutaneous haemorrhages can also be seen (4). Some BTV serotypes have been reported to cause weakness, pulmonary disease, abortion, foetuses with developmental abnormalities, kerato-conjunctivitis, anaemia and swollen joints (9).

BT is widely distributed across the tropical, subtropical and temperate regions of the world. In India, the first outbreaks of the disease in sheep and goat were reported in Maharastra in 1964 (25). It has now become clear that the disease is endemic and frequent outbreaks in small ruminants have been reported from many parts of the country (1, 12, 16). Serological surveillance for BTV antibodies in small ruminants has been conducted in the endemic states in the northern, central and southern areas of the country and a high degree of seropositivity (40%-80%) has been reported (19, 21, 28). In this study, the serological prevalence of disease in goats in the State of West Bengal was studied. The sheep population in this State is negligible. The climate of the coastal saline area of the State is hot and humid and there is a large population of biting midges. Several species of midges have been identified and confirmed to be present in the area (18, 26, 27). These insects feed on the domestic ruminants and, as a consequence, these hosts are subclinically infected and become seropositive. In the present study, a serological survey was conducted to detect BTV antibodies in goats using an indirect enzyme-linked immuno-sorbent assay (ELISA) with a recombinant viral protein 7 (rVP7) antigen (2, 20). The indirect ELISA was standardised against the previously described 'gold standard' competitive ELISA (c-ELISA).

Materials and methods

Positive and negative control sera and control sera for ELISA

The positive control serum was prepared in goats by hyperimmunisation of a goat with BTV-23. Pooled serum collected from kids born of seronegative goats was used as a negative control serum. Goat sera that tested positive and negative using the c-ELISA were considered as positive and negative control

sera, respectively. The control sera were used to determine the cut-off value.

Field serum samples from goats

Serum samples (n = 1 202) were collected from apparently healthy goats of the Black Bengal breed. Sampling was conducted in the winter months from different parts of the Sunderban area of the coastal saline zone of West Bengal. A total of 4 ml of blood was drawn from the jugular vein of goats in clot-activated evacuated tubes (Vacutainer, Becton Dickinson, New Jersey) and, after clotting, sera were separated by centrifugation and stored at –20°C.

Preparation of recombinant VP7 antigen

Escherichia coli (BL 21 strain) expressing histidine-tagged truncated VP7 protein of BTV-23 (20) was propagated in bulk for the preparation of recombinant antigen. The VP7 gene had been cloned in prokaryotic expression vector pET32a. Bacteria were propagated in 200 ml Luria Bertani (LB) media at 30°C under Isopropyl-β-D-thiogalactoside (IPTG) induction and harvested 8 h post induction. The culture was centrifuged at 6 000 × g for 10 min and the pellet was resuspended in 32 ml of guanidium lysis buffer, pH 7.8 (4 M guanidium hydrochloride, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 500 mM NaCl) by gentle rocking at room temperature for 15 min; the culture was then sonicated on ice using 9.9-second bursts for three cycles with a 9.9 sec pause between each burst. The lysate, thus prepared, was centrifuged at 10 000 × g for 15 min at 4°C and the supernatant loaded onto a column containing 4 ml nickel agarose (ProBond Resin, Invitrogen, Carlsbad, California) equilibrated with the binding buffer, pH 7.8 (6 M urea, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 500 mM NaCl). The column was then washed twice with six times the volume of the binding buffer; in addition, six subsequent washings were performed with imidazole washing buffer, pH 7.8 (20 mM imidazole, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 500 mM NaCl). The rVP7 was eluted with imidazole elution buffer, pH 7.8 (500 mM imidazole, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4 and 500 mM NaCl). The eluted fractions were then analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to check the purity of the recombinant protein and then stored at –80°C.

The recombinant protein was concentrated and imidazole was removed by centrifugal ultrafiltration through a 10 000 molecular weight cut-off (MWCO) membrane (Millipore, Billerica, Massachusetts) and the quantity was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, Illinois). Approximately 12 µg of purified rVP7 per vial was lyophilised in the presence of a chemical stabiliser (60 mM trehalose dihydrate). The reactivity of freeze-dried rVP7 was evaluated with the standard positive control (PC) and negative control (NC) sera. Sufficient numbers of freeze-dried vials were preserved; these were used throughout the study.

Competitive ELISA

The c-ELISA was performed on goat sera using a commercial c-ELISA test kit (Bluetongue Antibody Test Kit, c-ELISA, Veterinary Medical Research & Development [VMRD] Inc. Pullman, Washington) in accordance with the procedures defined by the manufacturer. The assay is a monoclonal antibody-based test which detects BTV antibodies in ruminant sera. Briefly, 25 µl control sera (supplied with the kit) and test sera were added to the antigen-coated plate and incubated at room temperature for 15 min. Then antibody (monoclonal) peroxidase conjugate was added to each well and incubated for an additional 15 min at room temperature. The plate was then washed with the buffer (supplied) and 50 µl of substrate solution was added; the plates were held for 10 min at room temperature. The reaction was stopped by adding 50 µl of stop solution and reading was performed using an ELISA reader at 650 nm. Test sera that produced an optical density (OD) of less than 50% of the mean of negative controls were considered positive. Sera found positive and negative by this test were used as positive and negative control sera.

Procedure for the rVP7-based indirect ELISA

The indirect ELISA was performed using the procedure described by Richardson *et al.* (22). Freeze-dried rVP7 antigen was reconstituted and diluted in phosphate-buffered saline (PBS) (pH 7.5) to yield a concentration of 2.4 µg/ml. A 50 µl/well of diluted rVP7 (approximately 120 ng) was passively adsorbed onto ELISA plates (MaxiSorp, Nunc A/S, Rosklide) for 1 h at 37°C (or overnight at 4°C). Plates were then washed three times with PBS with 0.03% Tween 20 (PBST). The same washing procedure was followed after each incubation step. The unbound antigen was blocked by the addition of 100 µl of blocking buffer (3% skimmed milk powder, 2% gelatine in PBS) at 37°C for 1 h. After washing, 50 µl of sera (test and control sera diluted at 1:20 in blocking buffer) were added to the corresponding wells in duplicate. Following incubation at 37°C for 1 h, plates were washed and a volume of 50 µl of donkey anti-goat horseradish peroxidase (HRP) conjugate (Sigma, St Louis, Missouri) diluted 1:8 000 (in blocking buffer) was added. After incubation at 37°C for 1 h, plates were washed and 50 µl o-phenylenediamine dihydrochloride (OPD) (Sigma) substrate solution was added. Plates were incubated in the dark for 15 min at 37°C to develop the colour reaction; the reaction was terminated by adding 50 µl/well of 1M H_2SO_4. Optical densities (OD) were measured using a 492 nm reference filter in an ELISA reader.

Data analysis and evaluation of assay performance of rVP7-based indirect ELISA

ELISA results were expressed as the OD values generated and the cut-off value (positive/ negative threshold) for the indirect ELISA was determined by visual inspection of frequency distributions (10, 11). The positive and negative control sera (tested by c-ELISA) were assayed using the indirect ELISA and OD values were plotted as frequency distribution curves. The OD value corresponding to the point of intersection of the two curves was considered as the cut-off value.

Performance of the indirect ELISA was measured following the approaches described by Greiner and Gardner (7) to estimate the following:

- sensitivity (Se) = [TP/(TP+FN)] × 100
- specificity (Sp) = [TN/(TN+FP)] × 100
- Youden index (J) = [Se + Sp – 1]
- efficiency (Ef) = [(Se × p) + Sp x (1 – p)]
- positive predictive value (PPV) = [(p × Se)/{(P × Se) + (1 – p) (1-Sp)}] × 100
- negative predictive value (NPV) = {[(1 – p) × Sp]/ [(1 – p) × Se] + [p × (1 – Se)]} × –100
- apparent prevalence (Ap) = [(TP + FP)/ n] × 100
- true prevalence (p) = {[Ap+ (Sp – 1)]/[Se + (Sp–1)]} × 100

where:

- TP = true-positive sera
- FN = false-negative sera
- TN = true-negative sera
- FP = false-positive sera
- p = prevalence
- n = number of sera tested.

Results

Preparation of rVP7 antigen

The rVP7 antigen was obtained from recombinant bacterial culture which was purified by nickel affinity column chromatography. The purity of the recombinant protein was assessed by SDS-PAGE analysis of the column elutes. A single protein band with a molecular mass of about 36 kDa was obtained; this is similar to the expected histidine-tagged fusion VP7 protein (Fig. 1). There were no contaminating proteins in the preparation or, if there were, they did not affect the reactivity of recombinant protein in the indirect ELISA. The protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce, Rockford, Illinois) which determined that 5 µg of highly pure VP7 protein could be obtained from 1 ml of bacterial culture. The protein was dialysed to remove imidazole and lyophilised with trehalose.

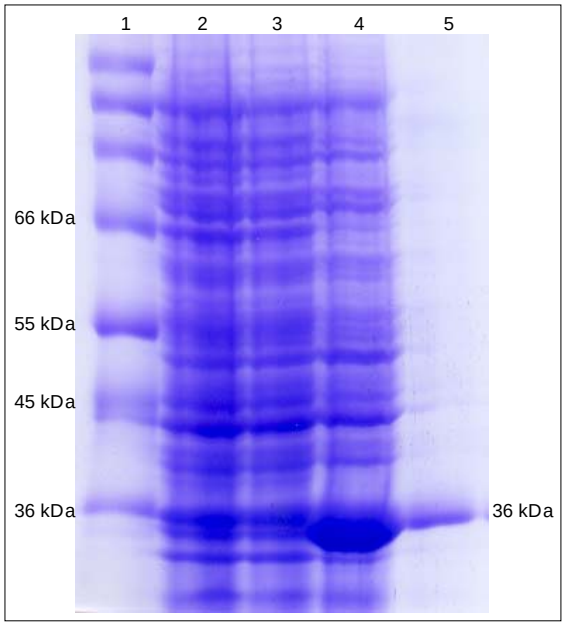

Figure 1
Purification of rVP7 from bacterial lysate by nickel agarose affinity chromatography
The bacterial lysate and column elute were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
The histidine-tagged rVP7 was obtained as a 36 kDa single polypeptide band (Lane 5) upon purification which is almost free of impurities
Lanes 2, 3 and 4 contain bacterial lysate transfected with empty pET32 vector, lysate harvested at 0 h post induction and lysate harvested at 8 h post induction, respectively
Lane 1 contains protein molecular weight marker (F Sigma # M4038)

Determination of cut-off value

The cut-off value was determined using the previously described frequency distribution method (10). Using the c-ELISA, goat sera ($n = 581$) were tested and the sera that were found positive and negative were considered as negative control sera ($n_1 = 322$) and positive control sera ($n_2 = 259$), respectively. These control sera were again assayed using the indirect ELISA and the frequencies of OD values of these two classes were plotted in frequency distribution curves. The intersection point (0.290) of two curves was considered as the cut-off OD value (Fig. 2).

Evaluation and standardisation of assay

Calculations of sensitivity, specificity and other parameters were conducted using a 2 × 2 table where the TP, FP, FN and TN were 259, 13, 8 and 322, respectively. High estimates of relative sensitivity (97%), relative specificity (96.12%) and other estimates of diagnostic accuracy (Youden index, efficiency, positive and negative predictive value) were derived from the data analysis (Table I).

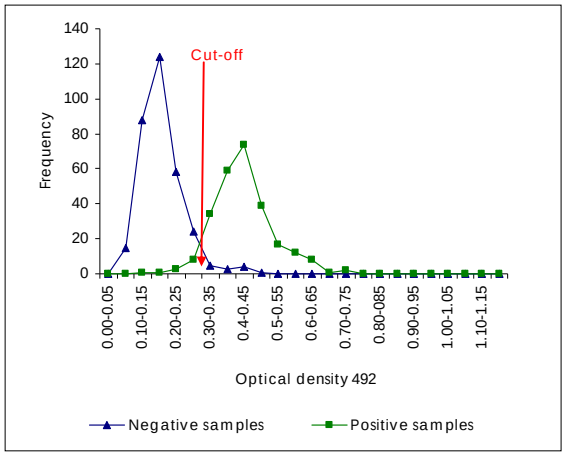

Figure 2
Determination of cut-off value of the rVP7-based indirect enzyme-linked immunosorbent assay
The cut-off value was determined by frequency distribution of negative control ($n_1 = 322$) and positive control ($n_2 = 259$) serum samples on the basis of their mean optical density (OD_{492}) value derived from the assay
The intersection point of two curves (arrow head) was considered as the cut-off value of 0.290

Table I
Performance of rVP7-based indirect enzyme-linked immunosorbent assay

Measure	Value
Cut-off (optical density)*	0.290
Sensitivity	97.00%
Specificity	96.12 %
Youden index (J)	0.93
Efficiency	96.56%
Positive predictive value	96.19%
Negative predictive value	96.94%

* determined by the frequency distribution method (10)

Prevalence of bluetongue virus antibody

The field-collected goat serum samples ($n = 1\,202$) from the coastal saline area of West Bengal were tested using the indirect ELISA. Based on the cut-off OD value of 0.290, as determined by the frequency distribution

method, the prevalence of BTV antibodies in goats was 47.25%.

Discussion

Bluetongue is endemic in India and a high degree of seroprevalence in small ruminants has been reported by several workers, particularly in the states of Rajasthan, Himachal Pradesh, Gujarat, Maharashtra, Karnataka, Tamil Nadu and Andhra Pradesh, where the sheep populations are high. In the eastern areas of India (Bihar, Orissa, West Bengal), sheep populations are much lower; goats are the principal small ruminants reared in these areas and population are high. In the study area of West Bengal, the sheep population is less than one tenth that of goats. The climate of this area is hot and humid, which is very suitable for the propagation of *Culicoides* species.

The BTV c-ELISA is listed by the World Organisation for Animal Health (*Office International des Épizooties*: OIE) (31) as a prescribed test for the detection of BT antibody. BTV c-ELISA kits are available from three licensed manufacturers in the United States; in this study, the kits used as controls were from one of the following companies:

- VMRD, Pullman, Washington
- Veterinary Diagnostic Technology Inc., Wheat Ridge, Colorado
- DiagXotics, Wilton, Connecticut.

These kits are very expensive and the cost can be very high if they are used for screening large numbers of sera in a vaccination or serological surveillance campaign. Consequently, a cost-effective recombinant VP7-based indirect ELISA was developed (2, 20) and used in this study to detect BTV group-specific antibody in goats.

Conclusions

The indirect ELISA is a simple and rapid method for the detection of antibodies, but a purified well-characterised antigen is needed (6). The production and purification of VP7 of BTV, as described in this paper, is a simple method to manufacture large quantities of a quality purified immunodiagnostic reagent. In addition, the protein was stabilised by freeze-drying with trehalose, a potent stabiliser.

The selection of an optimal threshold (cut-off) and the determination of the diagnostic sensitivity, specificity and other measures of test accuracy are critical. In this study, we used the previously validated c-ELISA (VMRD kit) to classify a limited number of field-collected goat sera into positive and negative groups, which were used as control sera. These control sera were used to determine the cut-off value for the rVP7-based indirect ELISA, using the frequency distribution method described by Jacobson (10). The determination of the cut-off value by inspection of frequency distributions has the advantage of being simple and flexible and requires no statistical calculations or assumptions regarding the normality of two distributions. The determination of performances of a test depends upon the estimates of diagnostic accuracy (PPV, NPV and Ef), which depends on disease prevalence (3). A high efficiency estimate (96.56%) was obtained, indicating a high probability of the test to correctly classify sera. The Youden index (0.93) also indicated a high probability of correct classification in the present study.

BTV antibody was present in a large percentage of goats in the study area (47% prevalence), which indicates there has been virus infection in goats in this region and consequently this is a threat to the native breed of sheep that are found there. These results indicate that other domestic ruminants, e.g. cattle and buffalo, are probably also infected with BTV. There is a need for an extensive antibody and virus survey of BTV in the animals of this region.

Our results demonstrate that the indirect ELISA, using the recombinant VP7, has the potential to be used in serological surveillance for BTV antibody. To determine the exact prevalence, a larger number of samples should to be tested.

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References

1. Aruni A.W., Kathiresan D., Rani R.U., Subbaraj R., Reddy Y.K.M., Saravanabava K., Purushothaman V. & Koteeswaran A. 2006. Outbreak of bluetongue in southern districts of Tamilnadu. *Indian Vet J*, **83**, 679.
2. Biswas S.K. 2005. Evaluation of diagnostic efficiency of recombinant truncated VP7 protein of bluetongue virus serotype 23. MVSc thesis submitted to Deemed University, Indian Veterinary Research Institute, Izatnagar, 10-46.
3. Brenner H. & Gefeller O. 1997. Variation of sensitivity, specificity, likelihood ratios and predictive values with disease prevalence. *Stat Med*, **16**, 981-991.
4. Dercksen D., Groot Nibbelink N., Paauwe R., Backx A., van Rijn P. & Vellema P. 2007. First outbreak of bluetongue in goats in The Netherlands. *Tijdschr Diergeneeskd*, **132**, 786-790.
5. Elbers A.R.W., Backx A., Ekker H.M., van der Spek A.N. & van Rijn P.A. 2008. Performance of clinical signs to detect bluetongue virus serotype 8 outbreaks in cattle and sheep during the 2006 epidemic in The Netherlands. *Vet Microbiol*, **129**, 156-162.
6. Gravell M., Dorsett P.H., Gutenson O. & Ley A.C. 1977. Detection of antibody to rubella virus by enzyme-linked immunosorbent assay. *J Infect Dis*, **136** (Suppl.), S300-S303.
7. Greiner M. & Gardner I.A. 2000. Application of diagnostic tests in veterinary epidemiological studies. *Prev Vet Med*, **45**, 43-59.
8. Howerth E.W., Greene C.E. & Prestwood A.K. 1988. Experimental induced bluetongue virus infection in the white-tailed deer: coagulation, clinical pathologic and gross pathologic changes. *Am J Vet Res*, **49**, 1906-1913.
9. Inverso M., Lukas G.N. & Weidenbach S.J. 1980. Caprine bluetongue virus isolation. *Am J Vet Res*, **41**, 277-278.
10. Jacobson R.H. 1998. Validation of serological assays for diagnosis of infectious diseases. *Rev Sci Tech*, **17**, 469-485.
11. Jacobson R.H. 2004. Principles of validation of diagnostic assays for infectious diseases. *In* Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), Fifth Ed. Office International des Épizooties, Paris, 21-29.
12. Jain N.C., Sharma R. & Prasad G. 1986. Isolation of bluetongue virus from sheep in India. *Vet Rec*, **119**, 17-18.
13. MacLachlan N.J. 1994. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp Immunol Microbiol Infect Dis*, **17**, 197-217.
14. MacLachlan N.J. 2004. Bluetongue: pathogenesis and duration of viraemia. *Vet Ital*, **40**, 462-467.
15. Mahrt C.R. & Osburn B.I. 1986. Experimental bluetongue virus infection of sheep; effect of previous vaccination: clinical and immunologic studies. *Am J Vet Res*, **47**, 1191-1197.
16. Mehrotra M.L., Shukla D.C. & Khanna P.N. 1996. Studies on bluetongue disease in India – isolation & serotyping of fields isolates. *Indian J Comp Microbiol Immunol Infect Dis*, **17**, 8-13.
17. Mertens P.P.C., Maan S., Samuel A. & Attoui H. 2004. *Oribivirus, Reoviridae*. *In* Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses (ICTV) (C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Deseelberger & L.A. Ball, eds). Elsevier/Academic Press, London, 466-483.
18. Mondal A.K. & Nandi N.C., 1989. Fauna of Sundarban mangrove ecosystem, West Bengal, India. *Fauna Conserv*, **3**, 1-97.

19. Naresh A. & Prasad G. 1995. Relative superiority of c-ELISA for detection of bluetongue virus antibodies. *Indian J Exp Biol*, **33**, 880-882.
20. Pathak K.B., Biswas S.K., Tembhurne P.A., Hosamani M., Bhanuprakash V., Prasad G., Singh R.K., Rasool T.J. & Mondal B. 2008. Prokaryotic expression of truncated VP7 of bluetongue virus (BTV) and reactivity of the purified recombinant protein with all BTV type-specific sera. *J Virol Methods*, **152**, 6-12.
21. Ravishankar C., Krishnan Nair G., Mini M. & Javaprakasan V. 2005. Seroprevalence of bluetongue virus antibodies in sheep and goats in Kerala State, India. *Rev Sci Tech*, **24**, 953-958.
22. Richardson M.D., Turner A., Warnock D.W. & Llewellyn P.A. 1983. Computer-assisted rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of aspergellosis. *J Immunol Methods*, **56**, 201-207.
23. Saegerman C., Hubaux M., Urbain B., Lengelé L. & Berkvens D. 2007. Regulatory aspects concerning temporary authorisation of animal vaccination in case of an emergency situation: example of bluetongue in Europe. *Rev Sci Tech*, **26**, 395-414.
24. Saegerman C., Berkvens D. & Mellor P. 2008. Bluetongue epidemiology in the European Union. *Emerg Infect Dis*, **14**, 539-544.
25. Sapre S.N. 1964. An outbreak of bluetongue in goats and sheep in Maharastra State. *Indian Vet Rev*, **15**, 69.
26. Sinha S. & Dasgupta S.K. 1991. *Monohelea unica* Sinha and Dasgupta. A new biting midge (Ceratopogonidae) from West Bengal, India. *J Bengal Natl Hist Soc*, NS, 26-28.
27. Sinha S., Majumdar A. & Chaudhuri P.K. 2005. New species of predaceous midges of the genus *Alluaudomyia* Kieffer, 1913 (Insecta, Diptera, Ceratopogonidae) from the coastal regions of West Bengal, India. *Zoosystema*, **27**, 115-122.
28. Sreenivasulu D., Subba Rao M.V., Reddy Y.N. & Gard G.P. 2004. Overview of bluetongue disease, viruses, vectors, surveillance and unique features: the Indian sub-continent and adjacent regions. *Vet Ital*, **40**, 73-77.
29. Taylor W.P. 1986. The epidemiology of bluetongue. *Rev Sci Tech*, **5**, 51-56.
30. Thiry E., Saegerman C., Guyot H., Kirten P., Losson B., Rollin F., Bodmer M., Czaplicki, G., Toussaint J.-F., Clercq K.D., Dochy J.-M., Dufey J., Gilleman J.L. & Messeman K. 2006. Clinical cases of bovine bluetongue in Belgium. Bluetongue in northern Europe. *Vet Rec*, **159**, 327.
31. World Organisation for Animal Health (Office International des Épizooties: OIE) 2004. Bluetongue. *In* Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). Fifth Ed. OIE, Paris, 195-210.

