*Veterinaria Italiana*, **45** (3), 443-448

# A sandwich ELISA for the detection of bluetongue virus in cell culture using antiserum against the recombinant VP7 protein

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**Summary**

A polyclonal antibody-based sandwich enzyme-linked immunosorbent assay (s-ELISA) was developed for the detection of bluetongue virus (BTV). The test used antiserum against BTV and antiserum against the bluetongue (BT) core protein. The antiserum against the virus was used as a capture antibody and the antiserum against the protein was used for detection. In this study, antiserum to recombinant viral protein 7 (rVP7) was used as a detection antibody in place of anti-core antiserum. The assay was used to detect the BT serotypes found in India, namely: 1, 2, 9, 15, 18 and 23. The modified sandwich assay was able to detect BTV serotypes in cell culture supernatants. The use of anti-rVP7 antiserum as the detection antibody avoids the tedious and expensive purification of BTV core particles.

**Keywords**

Antiserum, Anti-core antiserum, Anti-rVP7 antiserum, Bluetongue, ELISA, Enzyme-linked immunosorbent assay, India, Sandwich ELISA, Virus.

## ELISA sandwich per il rilevamento del virus della bluetongue in colture cellulari mediante antisiero contro la proteina VP7 ricombinante

**Riassunto**

*È stato messo a punto un dosaggio ELISA sandwich basato su un anticorpo policlonale per il rilevamento del virus della bluetongue (BTV). Nel test sono stati utilizzati l'antisiero contro il BTV e l'antisiero contro la proteina core della bluetongue, rispettivamente come anticorpo di cattura e rilevamento. In questo studio l'antisiero verso la proteina virale 7 ricombinante (rVP7) è servito da anticorpo di rilevamento in sostituzione dell'antisiero anticore. Il dosaggio ha consentito di individuare i sierotipi della BT in India: 1, 2, 9, 15, 18 e 23. L'ELISA sandwich con tecnica modificata ha permesso di rilevare i sierotipi del BTV nei surnatanti della coltura cellulare. L'impiego dell'antisiero anti-rVP7 come anticorpo di rilevamento ha consentito di evitare la purificazione, laboriosa e costosa delle particelle core del BTV.*

**Parole chiave**

Antisiero, Antisiero anti-core, Antisiero anti-rVP7, Bluetongue, Dosaggio con immunoassorbente legato all'enzima, ELISA, ELISA sandwich, India, Virus.

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## Introduction

Bluetongue virus (BTV) is an arbovirus that belongs to the genus *Orbivirus* within the Family *Reoviridae* (9). The virus can infect most ruminant species. However, clinical signs of bluetongue (BT) disease occur mostly in sheep, some species of deer and, occasionally, in cattle. BTV has a 10-segment genome and each segment encodes for a viral protein (VP), seven of which are structural and three are non-structural. The outer capsid proteins, VP2 and VP5, are the serotype determinants and are responsible for generation of serotype-specific neutralising antibody (8). The major core protein, VP7, determines serogroup specificity (14). Isolation and identification of BTV and identification of BT antibody are essential steps in the laboratory confirmation of BTV infection.

Traditionally, laboratory confirmation of BTV is performed by intravenous egg inoculation followed by passages in mammalian cells (17). This virus isolation procedure is tedious and may take up to five weeks to complete. Consequently, alternative methods for BTV isolation or BT nucleic acid identification have been sought. These methods include dot-blot, immuno-electron microscopy, reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (5, 10, 11, 15). The real-time RT-PCR is the method used most commonly for the direct detection of BT antigen. These *in vitro* techniques, singly or in various combinations, have been applied to the identification of BTV antigen in cell cultures, eggs, insect vectors and ruminants. Enzyme-linked immunosorbent assay (ELISA) techniques, especially antigen-capture or sandwich ELISA (s-ELISA), for the detection of virus antigen, have a number of advantages, including being economical, specific and rapid. Additionally, large numbers of clinical or laboratory samples can be screened in a short time by these assays; this makes them very useful when conducting epidemiological studies.

An s-ELISA for the detection of BTV (or antigen) has recently been developed in our laboratory. The assay uses anti-BTV antiserum as the capture antibody and anti-core antiserum as the detection (tracing) antibody (2). This assay is sensitive and has a threshold for virus detection of 2.4 $\log_{10}$ $TCID_{50}$. This sensitivity is comparable to the antigen detection assay that has been described by Thevasagayam *et al.* (16). However, production of the antisera and especially the anti-core antiserum, is difficult as preparation and purification of BTV core particles is very tedious and time-consuming. Substitution of anti-core antiserum (the detection antibody in the s-ELISA) with a monoclonal antibody or monospecific polyclonal VP7 antibody is a simpler method for the purification of BTV core particles. The BTV VP7 protein has recently been expressed in our laboratory (12) and the recombinant protein has been used to develop a group-specific anti-recombinant viral protein (rVP7) antiserum that can be used in an indirect ELISA for detection of BTV antibodies in sera (1). In this study, we investigated the potential of the anti-rVP7 antiserum as a detection antibody in s-ELISA.

## Materials and methods

### Virus propagation and antigen preparation

Six serotypes of BTV, BTV serotypes 1, 2, 9, 15, 18 and 23, were each grown in baby hamster kidney-21 (BHK-21) cells in 75 $cm^2$ flasks in order to prepare antigen for s-ELISA. When complete cytopathic effect (CPE) of the cultures was observed, cells were harvested, disrupted by sonic vibration and centrifuged at 3 000 × g for 15 min. The titre of the viruses isolated was measured by the method of Reed and Muench (13) and ranged between $10^{5.8}$ and $10^{6.7}$ $TCID_{50}$/ml. The supernatant (about 20 ml) was used as antigen in the ELISA. The negative control was the uninfected cell culture lysate which was prepared in the same way.

The BTV VP7 was expressed as a histidine-tagged protein in bacteria (*Escherichia coli*) cloned in a pET32a vector (12) and purified by nickel affinity column chromatography. The purified rVP7 antigen was freeze-dried and used for the preparation of antiserum in

guinea-pigs. The BTV-23 (6) virion and core particles were purified by the method described by Mertens *et al*. (7) and were used for the preparation of antiserum in rabbits and guinea-pigs, respectively.

### Antiserum production

Two rabbits and four guinea-pigs were immunised with the purified BTV 23 virions (whole virus) and core particles, respectively. A quantity of 20 µg purified virion and 10 µg core particle were dissolved in 200 µl of 0.1 M Tris-HCl, pH 8.0 and emulsified with equal volumes of Freunds complete adjuvant. Each rabbit and guinea-pig received 20 µg purified virion and 10 µg core particle, respectively, subcutaneously at several sites. Inoculation was repeated after 21 days with Freunds incomplete adjuvant using the same amount of antigen. Sera were collected 7 days after the final inoculation, pooled species-wise and stored lyophilised after measuring the antibody titre. Similarly, anti-rVP7 antiserum was prepared in four guinea-pigs, pooled and stored lyophilised.

### Sandwich ELISA procedure

This assay was performed as described by Chand *et al.* (2). Briefly, ELISA plates (Maxisorp Nunc A/S, Rosklide) were coated with the capture antibody (rabbit anti-BTV antiserum) in carbonate bicarbonate buffer, pH 9.6 (60 mM sodium carbonate, 40 mM sodium bicarbonate) at 37°C for 1 h. The plates were washed three times with washing buffer (phosphate buffer solution [PBS] containing 0.05% Tween-20) and blocked by adding blocking buffer (4% skimmed milk powder and 2% gelatin in PBS). The antigen was added after incubation and washing. The plates were incubated at 37°C for 1 h, washed and then detection antibody (guinea-pig anti-core antiserum or anti-rVP7 antiserum) was added to all wells. After incubation and washing, anti-guinea-pig horseradish peroxidase (HRP) conjugated antibody (Dako, Glostrup) was added at a dilution of 1:2 000. After a final washing step, a freshly prepared substrate/ chromogen mixture (hydrogen peroxide/ orthophenylene diamine) was added to all wells. The colour was allowed to develop for 10 min and the reaction was stopped by adding 50 µl of 1 M $H_2SO_4$. The plates were read at 492 nm wavelength on an ELISA reader. A value twice (or more) the mean optical density (OD) value of the negative antigen control was considered as the positive/negative cut-off value (i.e. positive to negative [P/N] ratio ≥2).

## Results

In the s-ELISA, rabbit anti-BTV-23 antiserum was used as the capture antibody and guinea-pig anti-core antiserum or anti-rVP7 antiserum was used as detection antibody. Depending upon the type of detection antibody used, the assay was designated as s-ELISA I (anti-core antiserum as detection antibody) or s-ELISA II (anti-rVP7 antiserum as detection antibody). The optimum dilutions of capture and detection antibody were selected using a chequerboard titration against fixed dilution/ concentration of BTV-23 cell culture antigen. The best and the most satisfactory result was obtained at a dilution of 1:2 000 for capture antibody and 1:400 for detection antibody, while the dilution of the positive antigen and negative antigen controls was 1:5. At these dilutions, the signal-to-noise ratio (P/N ratio) was estimated to be >6. These dilutions and concentrations were maintained and followed throughout the study.

Both the assays detected BTV serotypes 1, 2, 9, 15, 18 and 23 in the cell culture supernatants. The average P/N ($OD_{492}$) values for the positive and negative antigen controls generated in the s-ELISA II were slightly lower than those of the s-ELISA I (Fig. 1).

The limit of detection (i.e. sensitivity) of the assays was determined using cell culture supernatant of BHK-21 cultures infected with BTV-23 ($10^{6.7}$ $TCID_{50}$/ml) or BTV-2 ($10^{6.5}$ $TCID_{50}$/ml). These viruses were diluted 10-fold and 50 µl of each dilution was tested by the assays. The detection limit of the s-ELISA I for BTV-23 and BTV-2 was an antigen dilution of $10^{-3}$ which was equivalent to $10^{2.4}$ and $10^{2.6}$ $TCID_{50}$/ml, respectively, for the s-ELISA I. For the s-ELISA II, the limit was estimated to be $10^{3.1}$ and $10^{3.2}$ $TCID_{50}$/ml, (Fig. 2).

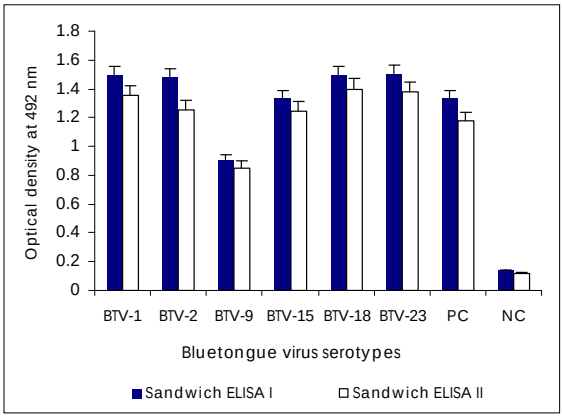
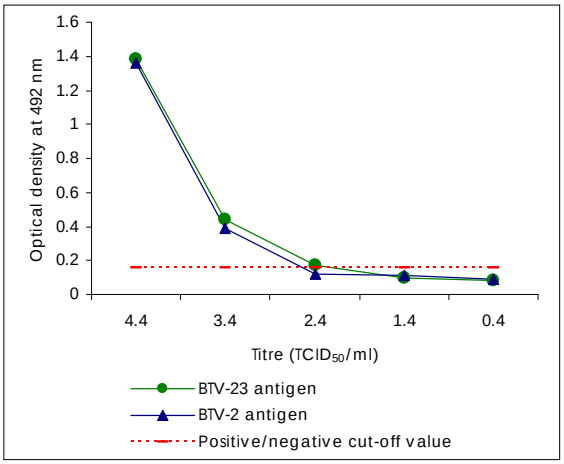



Figure 1
Detection of bluetongue virus serotypes using two sandwich ELISA formats
The dilution of test antigens was 1:2
The dilutions of capture and detection antibody were 1:2 000 and 1:400, respectively
Positive control (PC) and negative control (NC) antigens were used at a dilution of 1:5
The overall optical density values were greater in sandwich ELISA I than in sandwich ELISA II
Results are mean values ± standard deviation of three different experiments

## Discussion

The most important aspect of an s-ELISA is the production of antiserum against purified antigens. The quality of capture and detection antibodies relies upon the purity of antigens. Moreover, the purity of the antigens also results in a better signal-to-noise ratio and sensitivity of the assay. The methods employed in this study for preparation of BTV virions and core particles guaranteed that the antigens used in the test were pure (7). The preparation and purification of BTV core particles, however, is cumbersome and tedious. The BTV core is composed of five structural proteins (VP1, VP3, VP4, VP6 and VP7); VP7, which contains the group-specific antigenic determinants (3), is the major protein and comprises approximately 36% of the core particle (4). Most of the antibodies in the anti-core antiserum are directed against the VP7 protein. With this knowledge, it was decided to use anti-rVP7 antiserum, instead of anti-core antiserum, as a detection antibody in the s-ELISA for the detection of BTV antigen (2). Purification of rVP7 from bacterial culture is easy and an unlimited amount of pure antigen can be obtained with much less labour. Purification of an equivalent quantity of BTV core particles is much more labour-intensive and is also very expensive.

a) Sensitivity of sandwich ELISA-I




b) Sensitivity of sandwich ELISA-II

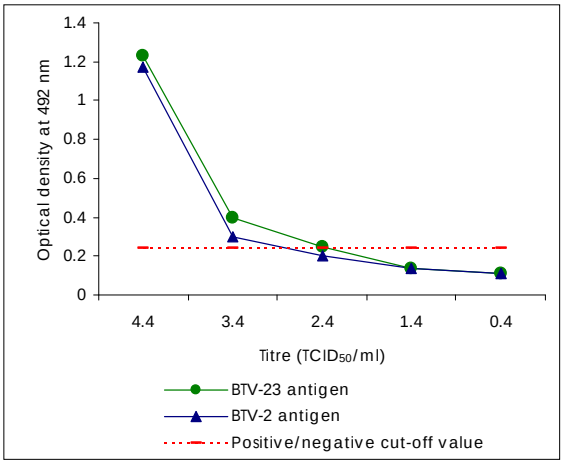



Figure 2
Comparative sensitivity of sandwich ELISA I and II
Viruses with known titres were serially diluted 10-fold and assayed with two systems
(a) The detection limit of sandwich ELISA I was $10^{2.4}$ and $10^{2.6}$ $TCID_{50}$/ml for bluetongue virus (BTV) serotype 23 and BTV-2, respectively
(b) The detection limit of sandwich ELISA II was $10^{3.1}$ and $10^{3.2}$ $TCID_{50}$/ml, respectively

Two formats, namely, s-ELISA I (rabbit anti-BTV antiserum as capture antibody and guinea-pig anti-core antiserum as detection antibody) and s-ELISA II (rabbit anti-BTV antiserum as capture antibody and guinea-pig anti-rVP7 antiserum as detection antibody) were compared for the detection of different BTV serotypes in infected cell culture by

testing the supernatants from each. Both the s-ELISA systems detected all the BTV serotypes used in the study; however, the P/N ($OD_{492}$) values were lower in s-ELISA II (Fig. 1). This was due to a larger number of epitopes in the core particle compared to the VP7 protein alone. For the same reasons, the sensitivity (limit of detection) of the s-ELISA II was less (about 10-fold) than that of the s-ELISA I (Fig. 2). With this measure of sensitivity established for s-ELISA II, it could then be used to detect cell culture-grown BT viruses. It was hoped that the sensitivity for the detection of the wild viruses in clinical blood samples would be the same; however, this study of clinical samples had not yet been conducted.

## Conclusions

In conclusion, anti-rVP7 antiserum can be used as a detection antibody in the s-ELISA. The use of this reagent allows the elimination of the tedious and expensive methods for purification of BTV core particles. The potential of this anti-rVP7 antiserum (s-ELSA II) should now be investigated for the detection of viruses in blood and tissue samples from animals experimentally and naturally infected with BTV.

## Acknowledgments

The authors would like to thank the Director of the Indian Veterinary Research Institute (IVRI) and the Coordinator of All-India Network Project on Bluetongue (AINP-BT) for providing facilities. Karam Chand and Baneswar Sing received a fellowship from the IVRI.

## Grant support

This work was supported by the AINP-BT of the Indian Council of Agricultural Research (ICAR).

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