ANTIGEN-CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY USING MONOCLONAL ANTIBODY FOR DETECTION OF BLUETONGUE VIRUS ANTIGEN

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ABSTRAK

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Deteksi antigen virus *bluetongue* dengan menggunakan antibodi monoklonal yang spesifik terhadap virus *bluetongue* pada uji antigen secara *antigen-capture enzyme-linked immunosorbent assay* (*antigen-capture* ELISA) telah diterapkan. Uji ini spesifik untuk virus *bluetongue* dan tidak dapat mendeteksi virus yang berhubungan erat dengan *bluetongue* seperti virus *epizootic haemorrhagic disease of deer* (EHD). Teknik *antigen-capture* ELISA merupakan metode alternatif pengganti uji agar gel imunodifusi dan imuno-dot blotting untuk mendeteksi antigen bluetongue pada jaringan yang terinfeksi, biakan jaringan Vero, *Aedes albopictus* dan BHK-21.

Kata kunci: ELISA, antibodi monoklonal, deteksi antigen, BTV

ABSTRACT

SENDOW, I. 1997. Antigen-capture enzyme-linked immunosorbent assay using monoclonal antibody for detection of bluetongue virus antigen. *Jurnal Ilmu Ternak dan Veteriner* 2 (4): 258-262.

An antigen-capture enzyme-linked immunosorbent assay (ELISA) using a specific anti-bluetongue virus group was applied to detect bluetongue viral antigen. The test was specific for bluetongue viruses and did not detect the closely related epizootic haemorrhagic disease of deer viruses (EHDV) and other orbiviruses. It was easy to perform and could be established in laboratories which have simple facilities. The antigen-capture ELISA technique is an alternative method to agar gel immunodiffusion and immuno-dot blotting tests to detect bluetongue antigen in infected tissues, Vero cells, *Aedes albopictus* cells and BHK-21 cell cultures.

Keywords: ELISA, monoclonal antibody, antigen detection, BTV

INTRODUCTION

Bluetongue virus (BTV) is an insect-borne virus which infects cattle, sheep, goats and wild ruminants (PARSONSON and SNOWDON, 1985; SENDOW *et al.*, 1991). The agent belongs to the Orbivirus genus in the Reoviridae family (GORMAN *et al.*, 1983). The isolation and identification of BTV as the causative agent of Bluetongue disease relies on isolation with cell culture (JOCHIM, 1985; LUEDKE *et al.*, 1985), embryonated chicken eggs (FOSTER and LUEDKE, 1968) or susceptible sheep (GARD *et al.*, 1988) from blood of infected animals or from insect vectors. The presence of virus is indicated by observation of cythopathic effect (CPE), clinical disease of sheep or dead embryos respectively. Identification of the virus isolate is achieved by examination of infected cell cultures using the fluorescence antibody technique (CYBINSKI and ZAKREWENZKY, 1983), the agar gel immunodiffusion test (DELLA-PORTA *et al.*, 1985) or the immuno-dot blotting test (AFSHAR *et al.*, 1987). These procedures can be labour intensive, time consuming, and expensive. The immuno-dot blotting test, although newer technology than the other two tests, cannot detect BTV antigen in tissue suspensions or in certain cell cultures such as *Aedes albopictus* cells due to the high background (SENDOW *et al.*, 1992). Since these methods are technically demanding and time consuming, an alternative method was investigated to detect and identify BTV group antigen in diagnostic specimens including infected cell cultures and tissues by a monoclonal antibody-based antigen-capture ELISA. The performance of this assay has been evaluated by testing samples originating from embyonated chicken eggs (ECE) infected with blood from sheep with experimental BTV infections.

MATERIALS AND METHODS

Samples

Sheep blood

Nine sheep were infected with BTV serotypes 1 and 21 (SENDOW *et al.*, 1993), and four sheep were infected with blood containing no BTV as control. Heparinized blood was collected every day for 25 days. These blood

samples were tested by antigen-capture ELISA and also inoculated into ECE.

Embryonated chicken eggs (ECE)

Each of 100 µl infected and control sheep blood sample was diluted ten fold with sterile phosphate buffered saline (PBS) and inoculated intravenously into 11 day old ECE and then incubated at 33.5° C for 5 days. Embryos were candled every day and observed for death. Embryos dying at the first day post inoculation were discarded while embryos dying on the second to fifth day post inoculation were harvested and homogenized in 10% PBS, then centrifuged at 750 g for 10 minutes. Supernatants were stored at 4° C prior to testing by antigen-capture ELISA or passage in cell culture.

Cell culture

a. *Aedes albopictus* cell culture

Each of 100 µl of supernatant of infected ECE was inoculated into *Aedes albopictus* (C6/36) cell cultures and then incubated at room temperature for 5 days. Cythopathic effect (CPE) was not observed in this type of cell culture, hence passaging in susceptible marker cells such as BHK-21 or Vero cells is usually nesessary. The reminder of C6/36 infected cells were stored at 4° C for testing by antigen-capture ELISA.

b. BHK-21 and Vero cell culture

Monolayer culture of BHK-21 and Vero cells inoculated with C6/36 cells infected BTV were blind passaged three times to detect viral isolates. Infected BHK-21 and Vero cells were incubated at 37° C for 5 days and observed for CPE. Samples containing virus produced CPE in the infected cells. When CPE observed in 80% of the cell monolayer, cultures were harvested and stored at 4° C prior to testing. Reference Epizootic Haemmorhagic Disease of deer (EHDV) serotype 5 (CSIRO 157) was also inoculated into BHK-21 and Vero cells. CPE was also observed and used for testing.

Insect suspension

Insect collected in the field were identified to species, and pooled. Each pool of species of *Culicoides* contained up to 250 insects. Each pool of insect were ground in 1 ml of PBS for testing by antigen-capture ELISA.

Antigen-capture Enzyme linked immuno-assay (Antigen-capture ELISA)

The method was a modified from MECHAM *et al.* (1990). Ninety six well microtitre soft plates (Disposable) were coated with 100 µl of 1:100 dilution or optimal dilution of monoclonal antibody BTV group 20E9/B7/G2 (LUNT *et al.*, 1988) in phosphate buffered saline (PBS) and incubated at room temperature with mechanical shaking for 2 hours, or overnight at 4° C. Plates were then washed three times in PBS-Tween 20 (5% Tween 20 in PBS) and dried on tissue paper. A volume of 50 µl of each test sample (insect suspension, infected sheep blood, chicken embryonated egg, C6/36, Vero and BHK-21 cell culture) was added directly into wells in duplicate and incubated at room temperature with shaking for 45 minutes before washing in PBS-T three times. Polyclonal sheep anti-BTV sera was added in 50 µl volumes into all wells and then incubated again for 45 minutes at room temperature in shaker before washing in PBS-T three times. This was followed by reaction with 50 µl of rabbit anti sheep conjugated peroxidase (Biorad) diluted at optimal dilution or 1:1000 in PBS containing 5% casein, and incubated at room temperature with shaking for 45 minutes before washing in PBST four times. Substrates such as TMB (KPL prod.) or ABTS were added in 100 µl volumes to all wells and incubated for 10 minutes before adding 100 µl of the stopper HCl 1 M if TMB substrate was used. No stopper was needed if ABTS was used. The plates were read using an ELISA reader with 405 nm filter if ABTS was used, and 650 nm if TMB substrate was used. Positive reaction could be seen visually, yellow with TMB substrate or green with ABTS. Negative samples produced no colour. An optical density of two times higher than the negative of OD controls was judged as a positive reaction.

RESULTS

Bluetongue virus antigen was not detected in infected samples of sheep blood, ECE tissue suspension and *Aedes albopictus* (C6/36) cell lines by the immuno-dot blotting test (SENDOW *et al.*, 1992). However, BTV antigen was easily demonstrated by antigen-capture ELISA test in those samples, except samples of blood (Table 1).

Table 1. The detection of BTV antigen from different specimens using antigen-capture ELISA

Specimen	Ag - C - ELISA	Identification
Blood	0/408	ND*
ECE	54/408	BTV serotypes 1, 21
Aa cells	44/303	BTV serotypes 1, 21
BHK-21	44/303	BTV serotypes 1, 21
cells	12/62	BTV serotypes 1, 21
Vero cells	2/83	BTV serotypes 1, 21
Insect susp.		
Total	166/1,567	

*ND = Not done
ECE = Embryonated chicken eggs

BHK-21 and Vero cell cultures inoculated with EHDV serotype 5 (CSIRO 157) were also tested using

antigen-capture ELISA. The result indicated that EHDV antigen was not detected using this monoclonal antibody.

Insect suspensions were also used in this test. The result indicated that antigen-capture ELISA test detected the BTV antigen in such suspensions. However, not all samples of insect can be assessed by antigen-capture ELISA directly.

In monitoring experimental infection with BTV, antigen-capture ELISA test proved sensitive. Table 2 and Table 3 indicate a corelation between embryo death with haemorrhages with positive results in the antigen-capture ELISA. Embryos inoculated with blood from sheep at 15 days post BTV infection and dying 2 dpi, without producing haemorrhages, gave no reaction in the antigen-capture ELISA. However, the antigen-capture ELISA detected virus from all sheep infected with BTV at 7 to 12 days post infection.

DISCUSSION

Diagnosis of BTV relies on detection of seroconversion, virus isolation or both from infected animals. Blood samples are commonly used for isolation and identification of BTV. However, this process depends on inoculation of ECE and cell cultures, followed by serological identification of the virus. These procedures are sensitive and specific, but take several weeks to complete (SENDOW *et al.*, 1993). The antigen-capture ELISA test described above will overcome this problem, as the test can be completed in maximum of 7 days.

In the immuno-dot blotting test, antigen was not detected from samples from *Aedes albopictus* cell cultures or tissue suspensions due to the high background (SENDOW *et al.*, 1992). In antigen-capture ELISA test, this background problem was eliminated and gave a more efficient and inexpensive assay.

No blood samples collected from infected sheep gave positive reactions in this test, even blood samples that produced dead embryos with haemorrhages or dwarfed embryos when inoculated into ECE. Inability of the antigen-capture ELISA test to detect BTV directly from blood samples of infected sheep probably was a reflexion of the low titre of virus, with the peak viraemia in infected sheep are below the threshold titer

Tabel 2. The detection of BTV antigen in chicken embryonated egg inoculated with sheep blood infected experimentally with BTV serotype 21

Day of inoculation	Animal No.													
	0941		948		863		041		939		947		944	
	E	C	E	C	E	C	E	C	E	C	E	C	E	C
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	-	+H	+	-	-	-	-	+H	+	-	-	-	-
5	-	-	+H	+	+H	+	-	-	+H	+	-	-	-	-
6	+	-	+H	+	+H	+	+H	+	+H	+	-	-	-	-
7	+H	+	+H	+	+H	+	+H	+	+H	+	-	-	-	-
8	+H	+	+H	+	+H	+	+H	+	+H	+	-	-	-	-
9	+H	+	+H	+	+H	+	+H	+	+H	+	-	-	-	-
10	+H	+	+H	+	+H	+	+H	+	+H	+	-	-	-	-
11	+H	+	+H	+	+H	+	+H	+	+H	+	-	-	-	-
12	+H	+	+H	+	+H	+	+H	+	+H	+	-	-	-	-
13	+H	+	-	-	+H	+	+	±	+H	+	-	-	-	-
14	+H	+	-	-	+	±	-	-	+H	+	-	-	-	-
15	+	-	-	-	+	-	+	-	+	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* E = dead embryo
H = Haemorrhage
C = AG - C - ELISA

Tabel 3. The detection of BTV antigen in chicken embryonated egg inoculated with sheep blood infected experimentally with BTV serotype 1

Day of inoculation	Animal No.											
	937		935		726		BP7		934		940	
	E	C	E	C	E	C	E	C	E	C	E	C
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	+	-	+	-	-	-	-	-
4	-	±	-	-	+	+	+	+	-	-	-	-
5	+H	+	-	-	+	+	+H	+	-	-	-	-
6	+H	+	+H	+	T	NT	+H	+	-	-	-	-
7	+H	+	+H	+	+H	+	+H	+	-	-	-	-
8	+H	+	+H	+	T	NT	+H	+	-	-	-	-
9	+H	+	+H	+	+H	H	+H	+	-	-	-	-
10	+H	+	+H	+	+H	+	+H	+	-	-	-	-
11	+H	+	+H	+	+H	+	+H	+	-	-	-	-
12	+H	+	+H	+	+	+	+H	+	-	-	-	-
13	+H	+	+H	+	+	-	+	-	-	-	-	-
14	+H	+	+H	+	-	-	-	-	-	-	-	-
15	+H	+	+H	+	-	-	+	-	-	-	-	-
16	+H	+	+H	+	-	-	-	-	-	-	-	-
17	+	-	+	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-

E = presence of dead embryonated chicken egg
C = antigen - capture ELISA
T = toxic
NT = not tested

(MECHAM, 1993). To enhance the sensitivity of the antigen-capture ELISA, blood samples were inoculated into cell culture or ECE prior to detection. LUNT *et al.* (1988) demonstrated that monoclonal antibody (Mab) to Bluetongue virus group 20E9/B7/G2 was specific for BTV viruses and can distinguish between BTV and other related virus including Epizootic Hemorhhagic Disease of deer (EHD) virus. In the current work, EHD virus serotype 5 (CSIRO 157) in cell culture was used as a comparison and the results indicated that EHDV did not react with the BTV Mab.

In the field, BTV and EHDV may exist together in domestic and wild ruminant populations (FOSTER *et al.*, 1980). Because these viruses are related and can cause similar diseases in ruminants, it is important to distinguish between them. The antigen-capture ELISA using specific Mab against BTV was serogroup-specific for representative of 22 BTV serotypes (LUNT *et al.*, 1988) and did not react with EHDV.

Not all samples from insect suspensions will react in antigen-capture ELISA directly. After passaged in ECE, BTV antigen can be detected. This result showed that low titre of virus cannot be detected. Using tissue culture infected fluid, when CPE was observed, the antigen-capture ELISA still gave a result.

The antigen-capture ELISA test results indicated that BTV antigen cannot be detected in blood sample, but can be detected in infected ECE and cell culture. At present isolation of BTV should follow those steps; ie ECE and passaging in the cell culture due to its sensitivity (GOLDSMIT *et al.*, 1975). Using the previous method, detection of BTV antigen should be attempted in cell culture suspensions such as in BHK-21 or Vero cells, but not in ECE by immuno-dot blotting test. This antigen-capture ELISA test could detect BTV antigen in ECE before passaging to the cell culture, so if the results showed no antigen, passaging in the cell culture need not be conducted. This will reduce time, and use of reagents.

Clear interpretation was shown in this test, and it can be read visually. This test has advantages to be applied in the small laboratory in Indonesia with limited facilities such as no ELISA reader. In this case, if trace results are observed, the test should be repeated from the second and third passaged in ECE.

Apart from these advantages of antigen-capture ELISA, the disadvantage of the test is that serotyping of

BTV cannot be attempted. Serum neutralization, plaque inhibition and plaque reduction tests still would be needed to confirm the BTV serotypes.

Unlike the fluorescence antibody technique (FAT) or AGID tests, this assay is very easy to perform and allows the screening of several hundred samples in a day. This procedure should be particularly useful in discriminating between samples infected with BTV and EHD viruses, which often occurred sympatrycally (FOSTER *et al.*, 1980). It is currently being applied to field-collected specimens.

The used of antigen-capture ELISA test for detection and identification of BTV group viruses from infected animals should be a value to diagnosticians and epidemiologist because its sensitivity, specificity, rapidity, practicality and relative economy.

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