Rapid detection of bluetongue virus in blood and organ samples using a capture enzyme-linked immunosorbent assay

O. Portanti, M. Luciani & G. F. Ronchi

Summary

An antigen capture ELISA for Bluetongue (BT) virus was developed using tissue culture supernatant to identify different BT virus (BTV) serotypes 1, 2, 4, 9 and 16, which have been incriminated in the current epidemic in the Mediterranean Basin. To obtain a positive result and after amplification in tissue culture, the minimum amount of infecting virus required was 100 $TCID_{50}$. Results from the antigen capture ELISA were compared with conventional methods for virus isolation and identification, such as virus amplification on embryonated chicken eggs (ECEs), followed by tissue culture and the direct immunofluorescence test. The sensitivity and specificity of the ELISA in infected tissue culture supernatant using homogenates of BTV-positive ovine and bovine organs and blood, without a previous step in ECEs, were 100%. The assay was also applied to homogenates of chicken embryo tissues, which had been infected with different BTV serotypes. This method enabled detection of the virus with 100% sensitivity and specificity rates, also using amplification in ECEs. Furthermore, among the various embryo tissues tested, liver was found to be the most suitable for use with ELISA. In experimentally infected ovine blood samples, the ELISA revealed the presence of the virus. Given the high sensitivity and specificity obtained with the BTV serotypes in this trial, the method should greatly facilitate BT diagnosis.

Introduction

Bluetongue (BT) virus belongs to the family *Reoviridae*, genus *Orbivirus*; 24 serotypes have been identified to date. The BT virus (BTV) is the aetiological agent of bluetongue disease in ruminants, a non-contagious disease that is transmitted to sheep and other domestic and wild ruminants by vectors belonging to the *Culicoides* genus (8, 12). Serotypes 1, 2, 4, 6, 9, 10, 13 and 16 are reported to prevail in the Mediterranean Basin (6, 22). Bluetongue has serious health and economic repercussions on livestock production of affected countries. Consequently, it is of uppermost importance to have rapid, sensitive and specific diagnostic methods so that effective health controls can be implemented and the spread of disease prevented. Diagnostic tests currently available for this purpose are: tissue culture isolation in mammals or insects, virus inoculation in laboratory animals and, more commonly, inoculation in embryonated chicken eggs (ECEs), followed by tissue culture testing in mammals or insects. Other techniques, such as the dot-blot, immunoelectron microscopy, immunoenzymatic tests and polymerase chain reaction (PCR) can be used to detect BTV in tissue cultures, arthropod vectors, organ and tissue samples, but they are of low sensitivity when applied to blood specimens, especially when sampled during the viraemic phase of infection

Istituto Zooprofilattico Sperimentale dell'Abruzzo & del Molise «G. Caporale» (IZSA&M), Teramo - Italy

Rapid detection of bluetongue virus in blood
and organ samples using a capture
enzyme-linked immunosorbent assay

O. Portanti, M. Luciani & G. F. Ronchi

(13, 19, 20, 23).

Immunoenzymatic assays are rapid, economical and easy to perform, and the sensitivity of the method can be enhanced by using serial amplification techniques of the virus possibly present in blood samples, organs, ECEs or tissue cultures (9, 10, 24). The aim of this study was to develop and validate an antigen capture ELISA (15, 16) for the detection of BTV in tissue cultures and ECEs after inoculation with the blood and organs of infected sheep or cattle.

Materials and methods

Monoclonal antibodies

Anti-BTV monoclonal Antibodies (MAbs) were produced by immunising Balb/c mice with a reference strain of BTV serotype 2 from the Onderstepoort Veterinary Institute (OVI) in South Africa. A quantity of 100 µg of viral proteins in Freund's complete adjuvant (FCA) was inoculated using the intraperitoneal route. Inoculation was repeated after 14 days with 100 µg of viral proteins in Freund's incomplete adjuvant (FIA). Two inoculations were then administered with 50 µg of viral proteins in FIA.

On day 55 a booster was administered with 100 µg of viral proteins. Splenocytes were used for cell fusion with mouse-myeloma Sp2/O-Ag-14 cell line. Hybridomas were cultured in Dulbecco's modified Eagle's medium with 20% foetal bovine serum, L-glutamine 2 mM, anphotericin-penicillin-streptomycin 100µg, gentamycin 50 mg/ml, nystatin 10 000 IU/ml and HAT 50µg. Hybridomas producing anti-BTV antibodies were cloned according to the limiting dilution method (2, 7, 14).

Characterisation

Clones were checked for MAb production using indirect ELISA (17). The same test was used to monitor cross-reactions with African horse sickness (AHS) virus and epizootic haemorrhagic disease

(EHD) virus of deer (1, 12, 26). The MAb isotype was determined using the ImmunoPure® monoclonal antibody isotyping kit I (Pierce, Rockford). MAbs were screened using immuno-Western blotting (11, 25). Electrophoresis of BTV-2- reference strain viral proteins was run at 20 mA on Mini-Protean 3 electrophoresis cell and 12% Tris-HCl polyacrylamide gel (Bio-Rad Laboratories, California). Transfer to nitrocellulose membrane 0.45 µm, was run at 35 mA for 55 min by Mini Trans-blot® Electrophoretic transfer cell (Bio-Rad Laboratories, California). Nitrocellulose membrane was saturated with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 3% skimmed milk for 1 h at 37°C. Purified and peroxidase-conjugated MAbs (18) were diluted in TBS-T and incubated overnight at 4°C. After washing with TBS-T, nitrocellulose strips were dipped in the Opti-4-Chloro-1-Naphthol substrate kit (Bio-Rad Laboratories, California) until immune complexes were detected.

Capture polyclonal antibodies

A hyperimmune rabbit serum was produced by immunising a New Zealand rabbit with a BTV-2 reference strain. A quantity of 200 µg of viral proteins in FCA was inoculated six times by intradermal injection over a period of 50 days. The immunoglobulin G (IgG) fraction was purified from sera by protein A affinity chromatography performed on a chromatographic system and recombinant protein A prepacked column (HiTrap rProtein A FF, 5 ml, Amersham Biosciences), binding buffer Na-phosphate 0.1 M, pH 7.0, eluting buffer glycine-HCl 0.1 M, pH 3.0 and neutralising buffer Tris-HCl 1 M, pH 9.0. The IgG fraction was used as capture-antibody for the BTV antigen capture ELISA. The same purification method was applied in IgG-isotype MAbs.

Bluetongue virus

Standardisation of BTV antigen capture ELISA was

O. Portanti, M. Luciani & G. F. Ronchi

Rapid detection of bluetongue virus in blood
and organ samples using a capture
enzyme-linked immunosorbent assay

performed using the following virus serotypes: BTV-1 reference strain, BTV-2 reference strain, BTV-2 attenuated strain, BTV-4 reference strain, BTV-9 reference strain, BTV-9 attenuated strain and the BTV-16 reference strain (kindly provided by the OVI), and Italian field strains of BTV-2 and BTV-9. To validate the method, samples were obtained from experimentally infected animals. The following samples were tested: three ovine brains, seven ovine spleens, one bovine brain, five bovine spleens and fourteen ovine blood samples collected during the viraemic phase of disease. Samples from uninfected animals were also collected, as follows: four ovine spleens, one bovine spleen and six ovine blood samples.

Virus identification
Virus serogroup and serotype of ovine and bovine organs experimentally infected were confirmed by immunofluorescence and virus neutralisation tests (VN) (5, 21)

Sample preparation from organs and blood
Brain and spleen samples were homogenised with sterile quartz powder phosphate-buffered saline (PBS) containing nystatin 5 000 IU/ml, streptomycin 10 mg/ml, gentamycin 250 µg/ml and penicillin 10 000 IU/ml (PBS-AB). The homogenate was sonicated on ice 3x15 sec and centrifuged at 1 250 g for 20 min at 4°C. Supernatants were stored at 4°C. Organs from chicken embryos were processed using the same procedure. Blood samples in ethylene-diaminetetra-acetic acid (EDTA) were centrifuged at 230 g for 20 min at 4°C. Pellets were suspended in PBS-AB and centrifuged, then suspended in lactose-peptone and sonicated.

Virus isolation in cell culture
Vero cells cultures were infected with different BTV serotypes at different titres (10^2–10^3-10^4 TCID$_{50}$) and with samples from organs and blood samples. When either the characteristic cytopathic effect (CPE) was

fully developed or at most within six days, supernatants were harvested and centrifuged at 1 000 g for 10 min at 4 °C. Supernatants were titrated after centrifugation and tested using the BTV antigen capture ELISA. Virus titres were expressed as TCID$_{50}$/50 µl. Supernatants from cell cultures without CPE were also collected and tested. Negative controls were performed using uninfected cell cultures.

Virus isolation in embryonated chicken eggs
Ten- to thirteen-day-old embryonated chicken eggs were intravenously inoculated (3, 4) with 100 TCID$_{50}$ of either the BTV-2 reference strain, Italian field strains of BTV-2, BTV-4 reference strain, BTV-9 reference strain or the BTV-16 reference strain. After seven days, brain, heart and liver samples were collected from embryos. Uninfected tissues were used as negative controls. Supernatants were tested with the BTV antigen capture ELISA.

BTV antigen capture ELISA
ELISA 96-well- microplates were coated with anti-BTV polyclonal antibodies at a final concentration of 20 µg/ml in carbonate/bicarbonate buffer (50 mM, pH 9.6). A volume of 100 ml was added to each well. Plates were kept at room temperature (RT) (17-25°C) overnight and then washed with PBS containing 0.05% Tween 20. A 50 ml volume of each sample was dispensed into the wells and incubated by gently shaking for 1 h at 37°C. After washing, 50 ml of the selected MAb (6C5F4D7), conjugated with horse-radish peroxidase (anti-VP7-BTV), were dispensed in all wells. Plates were stored at 37°C. After further washing, 100 µl of chromogen substrate (3.3´, 5.5´- tetramethylbenzidine liquid substrate system for ELISA) were added. Plates were kept for 30 min at RT. The colorimetric reaction was then stopped with 50 µl of sulphuric acid 0.5 N and the optical density (OD) measured at 450 nm with a microplate reader. The positive controls (infected tissue culture supernatant or liver, heart and brain homogenates from infected

Rapid detection of bluetongue virus in blood
and organ samples using a capture
enzyme-linked immunosorbent assay

O. Portanti, M. Luciani & G. F. Ronchi

Table I
Supernatants bluetongue virus-infected cell cultures tested with the antigen capture ELISA.

Dilution of cell colture supernatant	P:N[1] ratio of culture supernatant of BTV-infected Vero cells								
	BTV serotypes								
	Reference BTV-1	Reference BTV-2	Italian BTV-2	Attenuated BTV-2	Reference BTV-4	Reference BTV-9	Italian BTV-9	Attenuated BTV-9	Reference BTV-16
Undiluted	5.8	5.9	5.6	5.9	5.9	5.5	5.8	5.6	5.9
1:2	5.2	5.3	5.0	5.7	5.2	5.0	5.2	4.9	5.9
1:4	4.5	4.8	4.3	5.3	4.6	4.9	5.0	4.4	5.9
1:8	3.6	4.0	3.1	4.7	4.0	4.6	4.6	3.9	5.8
1:16	2.4	2.8	2.1	3.6	3.3	4.1	4.1	3.1	5.3
1:32	<2	<2	<2	2.6	2.5	3.3	3.3	2.2	4.8
1:64	<2	<2	<2	<2	<2	2.4	2.4	<2	4.1
1:128	<2	<2	<2	<2	<2	<2	<2	<2	3.4
Virus titre[2]	3.4	3.1	3.6	3.9	3.7	4.7	4.3	4.4	5.2

[1] P:N ratio: positive sample ≥ 2; negative sample <2
[2] Log_{10} $\mathrm{TCID}_{50}/50$ µl

ECE) and negative controls (uninfected tissue culture supernatant or liver, heart and brain homogenates from uninfected ECE) were included in the test. Samples were then classified as positive when the OD was equal to or greater than twice the OD of the negative controls (P:N$\geq$ 2).
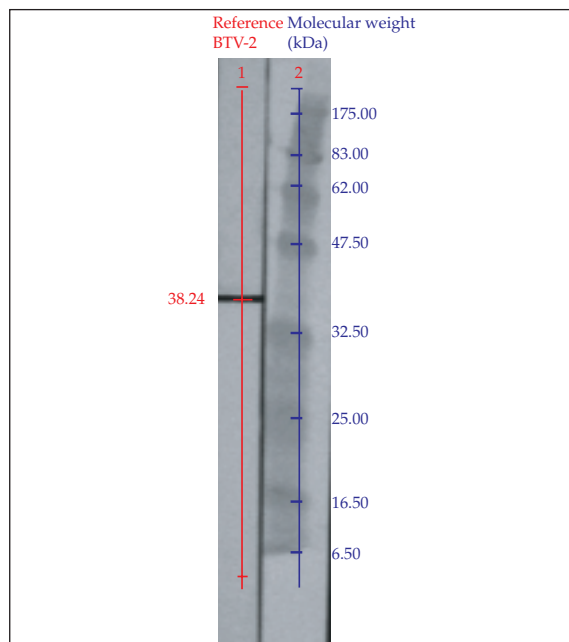

Reference BTV-2   Molecular weight (kDa)

Figure 1
Immuno-Western blotting: anti-VP7-BTV compared to reference BTV-2.

Results

Monoclonal antibodies
MAb 6C5F4D7 was selected from the 44 hybridomas obtained. The MAb isotype was IgG_{2a} light-chain-K. The MAb selected by immuno-Western blotting showed a protein band of about 38 kDa corresponding to BTV-VP7 and therefore named anti-VP7-BTV (Fig. 1). No cross reactivity was seen when AHS and EHD viruses were tested by indirect ELISA.

Capture-ELISA-BTV sensitivity and specificity
The tissue culture supernatants, in which the different BTV serotypes used for method standardisation were contained, were titred in Vero cells and examined by BTV antigen capture ELISA at the same time. The BTV antigen capture ELISA detected the virus up to 100 TCID_{50} for all serotypes used. All BTV serotypes were detected in undiluted infected tissue culture supernatants. The diluted supernatants of different BTV serotypes were considered positive with a positive/negative ratio of ≥ 2 in BTV antigen capture ELISA (Table I). The infected organs and blood samples showed positive results in BTV antigen capture ELISA.

O. Portanti, M. Luciani & G. F. Ronchi

Rapid detection of bluetongue virus in blood
and organ samples using a capture
enzyme-linked immunosorbent assay

Table II
Comparison of sensitivity and specificity of bluetongue antigen capture ELISA with bluetongue virus isolation in cell culture.

Sample	Bluetongue antigen capture		Cell culture		CPE[1]	VN[2]
	Positive	Negative	Positive	Negative		
Ovine spleen	2		2		4+	BTV-9
Ovine spleen	3		3		3+	BTV-2
Ovine spleen	1			1	-	BTV-9
Ovine spleen	1			1	-	BTV-2
Ovine brain	2		2		4+	BTV-9
Ovine brain	1			1	-	BTV-9
Bovine spleen	3		3		3+	BTV-9
Bovine spleen	1		1		4+	BTV-2
Bovine spleen	1		1		3+	BTV-9
Bovine brain	1			1	-	BTV-2
Ovine spleen		4		4	-	-
Bovine spleen		1		1	-	-
Ovine blood	12		1		2+	BTV-2, BTV-9
Ovine blood	2			2	-	BTV-2, BTV-9
Ovine blood		6		6	-	-

[1] Cytopathic effect
[2] Virus neutralisation test

Four organs and two blood infected samples did not show a CPE on tissue culture but tested positive in the BTV antigen capture ELISA (Table II).

Comparison of results of the BTV antigen capture in different chicken embryo tissues

In brain, heart and liver homogenates supernatants from ECEs infected with different BTV serotypes, a positive/negative ratio of >2 was observed in the BTV antigen capture ELISA. The liver homogenates usually showed positive/negative ratios that were greater than in brain and heart homogenates (Table III).

Discussion

Capture-ELISA-BTV can be considered an alternative method to conventional techniques for virus isolation and identification based on viral amplification on ECEs followed by different steps in tissue cultures and virus detection by immunofluorescence. Capture-ELISA-BTV is a reliable detection method for BTV screening of infected blood and organ samples of ovine and bovine animals in which just one single amplification step is performed in tissue cultures.

Sensitivity and specificity of the BTV antigen capture ELISA using tissue cultures for amplification was 100%. No false-positive or false-negative-samples were recorded during the study. Tissue cultures infected with BTV which did not show CPE after six days of incubation were all positive to the BTV antigen capture ELISA.

The virus amplification level in tissue cultures was sufficient to detect the presence of the virus in all samples tested with the BTV antigen capture ELISA. Since the MAb used in the assay is specific for the BTV serotypes, BTV antigen capture ELISA did not reveal related orbiviruses, such as AHS virus and EHD virus. The procedure is not only easy to perform, but is less costly and more rapid when compared with the methods widely used in virology laboratories. To perform the BTV antigen capture ELISA, only seven days were required (BTV amplification on tissue-cultures: six days; and BTV antigen capture ELISA: one day). The method is suitable for bluetongue diagnosis to control the spread of infection and can be considered an important tool for surveillance and eradication programmes. Virus amplification in ECEs proved to be an alternative method in comparison

Rapid detection of bluetongue virus in blood
and organ samples using a capture
enzyme-linked immunosorbent assay

O. Portanti, M. Luciani & G. F. Ronchi

to the use of tissue cultures for the viral identification by BTV antigen capture ELISA. Liver homogenates from chicken embryos showed higher values of P/N-ratios than embryo heart and brain homogenates. This result, together with the fact that the liver can be homogenised with ease, suggested its use as a target organ for BTV antigen capture ELISA. The application of BTV antigen capture ELISA to other BTV serotypes not investigated in this study could

require amplification in embryonated eggs rather than in tissue cultures, especially when wild BTV strains, which do not adapt to tissue cultures, are used. This could be of interest in future investigations. Other detection techniques for antigens and nucleic acids such as immunoblotting, PCR and nucleic acid probes involve higher costs and are less suitable than BTV antigen capture ELISA for the screening of large numbers of samples.

Table III
Comparison of bluetongue antigen capture ELISA and isolation in embryonated chicken eggs.

ECE[1] tissue	BTV[2]	Bluetongue antigen capture ELISA[3]	Infectivity[4]
Brain	Italian BTV-2	3.2	2.8
Heart	Italian BTV-2	4.2	3.7
Liver	Italian BTV-2	7.1	5.2
Brain	Reference BTV-2	2.3	2.3
Heart	Reference BTV-2	4.1	3.4
Fegato	Reference BTV-2	4.4	3.9
Cervello	Reference BTV-4	3.8	3.0
Heart	Reference BTV-4	4.0	3.2
Liver	Reference BTV-4	4.6	4.0
Brain	Reference BTV-9	3.0	3.1
Heart	Reference BTV-9	5.0	4.7
Liver	Reference BTV-9	6.1	5.8
Brain	Reference BTV-16	3.1	2.4
Heart	Reference BTV-16	3.8	3.3
Liver	Reference BTV-16	4.9	5.0

[1] Embryonated chicken egg
[2] Infected BTV serotype in embryonated chicken eggs
[3] P:N ratio: positive sample ≥ 2 ; negative sample < 2
[4] Log_{10} $TCID_{50}$/50 μl.

Acknowledgements

References

1. Appleton J. A. & Letchworth G.T., 1983. Monoclonal antibody analysis of serotype-restricted and unrestricted bluetongue viral antigen determinants. *Virology,* **124**, 286-299.

2. Campbell A.M., 1987. Monoclonal antibody technology. In Laboratory Techniques in biochemistry and molecular biology 13, Edited by Burdon RH and van Knippernberg P. H. Published by Elsevier Science BV Po Box 211 1000 AE Amsterdam The Netherlands.

3. Foster N. M. & Luedke A. J. 1968. The direct assay for bluetongue virus by intravascular inoculation of embryonating chicken eggs. *Am J Vet Res*, **29**, 749-753.

4. Foster N.M., Luedke A.J. & Metcalfe H.E. 1972. Bluetongue in sheep and cattle: efficacy of embryonating chicken eggs in viral isolation. *Am J Vet Res*, **33**, 77-81.

5. Gard G.P. & Kirkland P.D. 1993. Bluetongue virology and serology. *In*: Corner, L.A., Bagust, T., J., (eds). Australian Standard Diagnostic Techniques for Animal Diseases. CSIRO for the standing Committee on Agriculture and Resource Management, East Melbourne, Australia.

6. Gibbs E. P. J. & Greneir E.C. 1994. The epidemiology

O. Portanti, M. Luciani & G. F. Ronchi

Rapid detection of bluetongue virus in blood
and organ samples using a capture
enzyme-linked immunosorbent assay

of bluetongue. *Comp Immun Microbiol Infect Dis,* **17**, 207-220.

7. Goding J.W. 1993. Monoclonal antibodies: principles and practice. Production and application of monoclonal antibodies in cell. Biology, biochemistry and immunology. Third Edition. Academic Press Limited London.

8. Gorman B.M. 1990. The bluetongue virus. *Curr Top Microbiol Imm*, **162**, 1-19.

9. Hawkes R.A., Kirkland P.D., Sanders D.A., Zhang F., Li Z., Davis R.J. & Zhang N. 2000. Laboratory and field studies of antigen capture ELISA for bluetongue virus. *J Virol Methods*, **85**, 137-149.

10. Hossein M., Hawkes R.A., Kirkland P.D. & Dixon R. 1998. Rapid screening of embryonated chicken eggs for bluetongue virus infection with an antigen capture ELISA. *J Virol Methods,* **75**, 39-46.

11. Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T. *Nature* (London), **227**, 680-685.

12. Lelli R., Portanti O., Langella V., Luciani M., Di Emidio B. & Conte A.M. 2003. Produzione di un kit ELISA competitiva per la diagnosi sierologia della bluetongue. *Vet Ital*, **47**, 5-13.

13. Malavasi F. & A. Bargellesi-Sevesi 1992. Anticorpi monoclonali tecniche di base Ph. D. 02. In: I manuali delle scuole, Scuola superiore di oncologia e scienze biomediche. Dipartimento di Genetica, Biologia e Chimica Medica Università degli Studi di Torino, Istituto di Chimica Biologica Università degli Studi di Genova.

14. McColl K.A. & Gould A.R. 1991. Detection and characterisation of bluetongue virus using the polymerase chain reaction. *Virus Res*, **21**, 19-34.

15. Mechan J.O. 1993. Detection of bluetongue virus from blood of infected sheep by use of an antigen-capture enzyme-linked immunosorbent assay after amplification of the virus in cell culture. *Am J Vet Res,* **54**, 370-372.

16. Mechan J.O., Dean V.C., Wigington J.G. & Nunamaker R.A. 1990. Detection of bluetongue virus in *Culicoides* varipennis by antigen capture ELISA assay. *J Med Entomol*, **27**, 602-606.

17. Morgan-Capner P.H., Pullen, J.R.,. Pattison, D.E,. Bidwell, A., Bartlett B. & Voller A.1979. A comparison of three tests for rubella antibody screening. *J Clin Pathol*, **32** (6), 542-545.

18. Nakane P.K. & Kawaoi A. 1974. Peroxidase-labeled antibody. A new method of conjugation. *J Histochem Cytochem*, Dec, **22** (12), 1084-91.

19. Nunamaker R.A., Mecham J.O., Holbrook F.R & Lockwood J.A. 1997b. Applications of dot-blot, ELISA, and immunoelectron microscopy to field detection of bluetongue virus in *Culicoides varipennis* sonorensis: an ecological perspective. *J Med Entomol,* **3**, 24-28.

20. Nunamaker R.A., Mecham J.O., Wigington J.G. & Ellis J.A. 1997a. Bluetongue virus in laboratory-reared *Culicoides* varipennis sonorensis: applications of dot-blot, ELISA, and immunoelectron microscopy. *J Med Entomol,* **34**, 18-23.

21. OIE, 2004, Manual of standards for Diagnostic tests and Vaccines. 5 ed, chapter 2.1.9 Bluetongue: 195-210

22. Pini A. 1976. A study on the pathogenesis of Bluetongue: replication of the virus in the organs of infected sheep *Onderstepoort J Vet Res*, **43**, 159-164.

23. Shad G., Wilson W.C., Mecham J.O. & Evermann J.F. 1997. Bluetongue virus detection: a safer reverse trascriptase polymerase chain reaction for prediction of viremia in sheep. *J Vet Diagn Invest*, **9**, 118-124.

24. Stanislawek W. L., Lunt R.A., Blacksell S.D., Newberry K.M., Hooper P.T. & White J.R. 1996. Detection by ELISA of bluetongue antigen directly in the blood of experimentally infected sheep. *Vet Microbiol*, **52**, 1-12.

25. Towbin H., Staehelin T. & Gordon S. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose: procedure and some application. *Proc Natl Acad Sci USA,* **76**, 4350-4354.

26. Zhou E.M. & Afshar A. 1999. Characterisation of monoclonal antibody to epizootic hemorrhagic disease virus of deer (EHDV) and bluetongue virus by immunisation of mice with EHDV recombinant VP7 antigen. *ResVet Sci,* **66**, 247-252.