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A Quantitative Enzyme-linked Immunosorbent Assay for Bovine Herpesvirus Type 1 (BHV-1) Antibody

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Abstract. A quantitative enzyme-linked immunosorbent assay (ELISA) was developed to detect and measure antibody to bovine herpesvirus type 1 (BHV-1) in cattle sera. The optical density produced from a single dilution of test serum was compared with a standard curve and the results were read and printed out from a computer interfaced to a multichannel ELISA reader. The printed results were expressed in ELISA units. The ELISA results obtained on 370 cattle sera were compared with those of the serum neutralisation test (SNT). An agreement of 90·5% was obtained when reciprocal SNT titres equal to or greater than 4 and IgG ELISA units equal to or greater than 50 were taken as indicative of a specific reaction. Of the 370 sera, 35 gave discrepant results of which 21 were SNT positive/IgG ELISA negative and 14 were SNT negative/IgG ELISA positive. When the SNT positive sera negative in the IgG ELISA were tested in an IgM ELISA, 19 were found to be positive. Thus, when the IgG and IgM ELISA results were combined the overall agreement between the ELISA and SNT increased to 95·7%. The IgG ELISA had a sensitivity of 82·4% and specificity of 94·4% relative to the SNT, whereas the combined IgG and IgM ELISA results gave a sensitivity and specificity of 98·3% and 94·4% respectively. There was a good positive correlation between the two tests (r = 0·86).

Introduction

Bovine herpesvirus type 1 (BHV-1), also known as infectious bovine rhinotracheitis-infectious pustular vulvovaginitis (IBR-IPV) virus is associated with a wide variety of clinical entities including respiratory infection, conjunctivitis, vulvovaginitis, abortion and less commonly encephalitis and generalized systemic infection.[18] It is one of the most important agents of bovine respiratory disease, either directly or as a predisposing factor for bacterial infection, most commonly *Pasteurella haemolytica*.[32]

Several procedures have been used for the detection of BHV-1 antibody in cattle sera including indirect fluorescence[1] indirect hemagglutination[23] reverse passive hemagglutination[16] and, complement fixation. Although serum neutralization (SNT) is accepted as the standard test and is commonly performed for the serological diagnosis of BHV-1 infections[3,7,21,26] it is slow and requires expensive cell culture facilities. The advent of the enzyme-linked immunosorbent assay (ELISA) has enabled more rapid and sensitive quantitative detection of IgG[5,6,9,14,20,29] and IgM antibodies.[15]

Several workers[2,4,10,17,25] have demonstrated that ELISAs employing a single dilution of serum can be standardized and correlate well with other serological assays.

Since it is necessary to detect latent carriers in BHV-1 control schemes and to demonstrate freedom from antibodies for animal certification in international trade, it is now imperative to have an international standard test with an agreed system of result expression. However, the currently used standard SNT is not well defined and the ELISA still suffers a major drawback of lack of uniformity in result expression.[13,19]

This paper describes work to provide a simple adaptable quantitative means of result expression and also to provide guidelines for international standardisation of testing for BHV-1 antibodies.

Materials and methods

Bovine serum

Attempts were made to obtain standard positive and negative international reference sera against BHV-1 but none could be traced.

The standard positive serum used in this study was kindly supplied by Dr A. G. Rae at the Moredun Research Institute. This serum had been obtained

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from an IBR-free calf hyperimmunized with gradient-purified live virus (strain 6660)[27] emulsified in Freund's complete adjuvant followed three weeks later by the same virus in Freund's incomplete adjuvant. Three weeks later the same virus without adjuvant was given intravenously. The virus used for these injections had infectivity titres of 8·1 or 8·9 $\log_{10}$ $TCID_{50}$/ml. The calf was bled 10 days after the last injection. The standard negative serum was a preinoculation serum from the same calf.

A total of 370 serum samples submitted to the Moredun Research Institute for analysis from different parts of Scotland were used in the study. One hundred and twenty six samples were paired sera collected from 63 animals at the time of respiratory disease (acute phase serum) and three to four weeks later (convalescent phase sera). The remaining sera were collected from 244 different animals which were bled once. All sera were heat inactivated at 56°C for 30 min and stored in small aliquots at −20°C before use. The sera were tested in the SNT and IgG ELISA by independent operators before the results of each test were compared.

Cell cultures and virus

A semicontinuous cell line of embryonic bovine trachea (EBTr) cells was used for virus growth as previously described.[28]

The '6660' field strain of IBR virus was used after four passages including three plaque purifications, for the preparation of both the SNT and ELISA antigens according to the methods of Hebert *et al.* (1985).[18] Control ELISA antigen was prepared in the same way from uninfected EBTr cells.

Conjugates and substrate

Horseradish-peroxidase (HRPO)-conjugated heavy chain specific rabbit– anti-bovine IgG_1, IgG_2 and IgM were obtained (Nordic Immunological Laboratories Ltd). Equal volumes (50 μl) of heavy chain specific anti-bovine IgG_1 and anti-bovine IgG_2 were mixed[22] and used at an optimal predetermined dilution for testing all sera. Only selected sera were tested for IgM antibodies with the anti-bovine IgM conjugate.

The substrate was 40 mg orthophenylene diamine (OPD) (Sigma) per 100 ml of citrate phosphate buffer pH 5 (0·1 M citric acid, 0·2 M Na_2HPO_4) containing 10 μl of 30% hydrogen peroxide (H_2O_2).

ELISA procedure

The infected cell lysate was used as the BHV-1 antigen for the ELISA. Flat-bottomed 96-well polyvinyl plates (Cooke M129A Dynatech Laboratories, Billinghurst, Sussex) were coated overnight at 4°C with 100 μl/well of BHV-1 antigen at an appropriate predetermined dilution in 50 mM carbonate-bicarbonate buffer pH 9·6. The wells in column one were not coated and were used as a blank in each plate. During the assay, standard positive and negative test sera and conjugate were diluted in 0·5 M sodium chloride buffered to pH 7·2 with 0·1 M phosphate, 0·05% Tween 20, 1 mM EDTA and 0·5% ovalbumin. A standard positive serum was made up in 8 four-fold dilutions between 1/16 and 1/262144 for the standard curve. 50 μl volumes of each dilution in duplicate were transferred into the wells of column two and three, and 50 μl volumes of the negative standard serum into column four of each plate. Each test sample was tested in duplicate (in row) at a single dilution of 1/50 and the plates incubated for 2 h at room temperature in a humid box. After washing, 50 μl of the appropriately diluted HRPO-conjugated rabbit-anti-bovine IgG_1 + IgG_2 or IgM conjugate was added to wells of column two to twelve, and incubated for 1 h at room temperature. After the final washing step, 100 μl of OPD was added to each well. The reaction was stopped after 40 min of incubation at 37°C in the dark by addition of 25 μl of 2·5 M sulphuric acid. The optical density (OD) was read at 492 nm wavelength (against substrate/acid blank) in a multichannel recorder (Titertek Multiscan Flow Laboratories) interfaced to a BBC computer and Epson FX-80 printer (Acorn Computer Ltd, Cambridge, U.K.) using a Titertek Multiskan interface (Flow Laboratories) and a 3187 serial interface type 312B (Flow Laboratories).

The computer programme was designed to fit data from the standard curve to a second polynominal equation.[8] Optical density versus reciprocal of dilution of standard were used in the equation: $y = a + b(\log x) + c(\log x)^2$ where y = optical density, x = reciprocal of dilution of the standard positive serum, a = value of y intercept, b = slope of the line and c = constant. The titre of the standard serum was taken as being equal to the number of units of antibody contained therein. The level of antibody in the test sera was derived directly from the equation and expressed as ELISA units.

Serum neutralization test

Virus neutralizing antibody assay was carried out using the modified serum neutralization test described by Bitsch (1978) in a microtitre plate system using approximately 100 $TCID_{50}$ of the '6660' strain IBR virus.

Table 1. Comparison of IgG_1 + IgG_2 ELISA results and SNT titres of sera which were negative in both tests or positive in both tests

	SNT Titre	No. of sera	OD_{492} Mean ± SD	ELISA units Median	ELISA units Range*
Negative Sera ELISA Units < 50 (OD_{492} < 0·1) Total – 237	<2	223	0·052 ± 0·023	29	0–48
	2	7	0·056 ± 0·027	34	23–40
	3	7	0·052 ± 0·012	40	28–46
Positive (OD_{492} > 0·1) Total 98	4	3	0·142 ± 0·014	54	52–63
	6	3	0·123 ± 0·016	78	52–78
	11	9	0·147 ± 0·026	78	53–101
	16	3	0·192 ± 0·035	77	58–117
	22	8	0·180 ± 0·042	115	67–256
	32	4	0·181 ± 0·070	165	52–355
	45	10	0·204 ± 0·075	143	61–448
	64	4	0·259 ± 0·123	204	63–831
	90	12	0·250 ± 0·071	207	66–1941
	128	13	0·307 ± 0·105	330	106–2676
	180	6	0·358 ± 0·062	804	413–1252
	256	6	0·341 ± 0·101	463	415–2511
	360	3	0·403 ± 0·074	1313	881–2350
	512	2	0·437 ± 0·107	—	898–2954
	720	3	0·515 ± 0·039	2655	1328–3479
	1024	4	0·461 ± 0·097	3711	904–4966
	1440	5	0·524 ± 0·091	3344	2466–9708

*Note: Range is the lowest and highest ELISA unit value recorded for each SNT titre

Statistical analysis

Regression analysis, Chi-square test, paired *t*-test, and the non-parametric rank correlation by the Spearman method were carried out on the ELISA and SNT results for the 98 Scottish sera positive in both tests to determine the relationship, relative sensitivity and specificity of the two tests.

Results

ELISA IgG and SNT results

Comparison of the IgG ELISA and SNT results showed that 98 sera were positive (ELISA units > 50; SNT > 4) and 237 sera were negative (ELISA units < 50; SNT < 4). The results comparing ELISA units and SNT titres are given in Table 1. For any given SNT

Table 2. Comparison of IgG_1 + IgG_2 ELISA and SNT results

ELISA results	SNT results Positive (>4)	SNT results Negative (<4)	Total	% agreement (a + d/grand total)
Positive (>50)	98*	14†	112	90·5%
Negative (<50)	21‡	237§	258	
Total	119	251	370	

Relative sensitivity and specificity of the tests: ELISA compared to SNT a/a + c = 98/119 = 82·4%; d/d + b = 237/251 = 94·4%. SNT compared to ELISA a/a + b = 98/112 = 87·5%; d/d + c = 237/258 = 91·9%.

* number of samples positive in both tests; † number of samples positive in ELISA but negative in SNT; ‡ number of samples positive in SNT but negative in ELISA; § number of samples negative in both tests.

Table 3. Agreement between detection of BHV-1 antibody by (IgG_1 + IgG_2) and IgM ELISA and SNT

	SNT results Positive (>4)	SNT results Negative (<4)	Total	% agreement (a + d/grand total)
ELISA results Positive (>50)	117*	14†	131	95·7%
Negative (<50)	2‡	237§	239	
Total	119	251	370	

Relative sensitivity and specificity of the tests: ELISA compared to SNT a/a + c = 117/119 = 98·3%; d/d + b = 237/251 = 94·4%. SNT compared to ELISA a/a + b = 117/131 = 89·3%; d/d + c = 237/239 = 99·2%.

* number of samples positive in both tests; † number of samples positive in ELISA but negative in SNT; ‡ number of samples positive in SNT but negative in ELISA; § number of samples negative in both tests.

titre ELISA units varied considerably but the greater the SNT titre, the greater the median ELISA units demonstrated and there was a clear quantitative relationship between the results of the two tests.

This left 35 sera with repeatable discrepant results, virtually all of which gave low positive results in one test or the other. The final categorization of the 370 sera tested in the IgG_1 + IgG_2 ELISA compared with the results previously obtained in the SNT are summarised in Table 2. The results show that 21 sera were SNT positive/IgG ELISA negative, and 14 were SNT negative/IgG ELISA positive. The overall agreement between the two tests was 90·5%.

Because it was considered likely that the positive result in the SNT was due to the presence of IgM, the 21 sera which were IgG_1 + IgG_2 ELISA negative/SNT positive were tested for the presence of IgM by ELISA. All but 2 were positive by IgM ELISA. Combining the IgM ELISA results with the IgG_1 + IgG_2 ELISA results increased the sensitivity and overall agreement between the ELISA and SNT (Table 3).

Of the paired serum samples tested it was found that 3 pairs had shown significant seroconversion in

Table 4. Paired serum samples which showed significant sero-conversion in the SNT and ELISA

Sample Number	Optical density (OD) at 492 nm	ELISA units	Reciprocal SNT titre
K51/3	0·03	<50	<4
K51/4	0·19	166	90
K287/1	0·02	<50	<4
K287/2	0·26	382	128
K351/1	0·12	61	16
K351/2	0·29	484	128

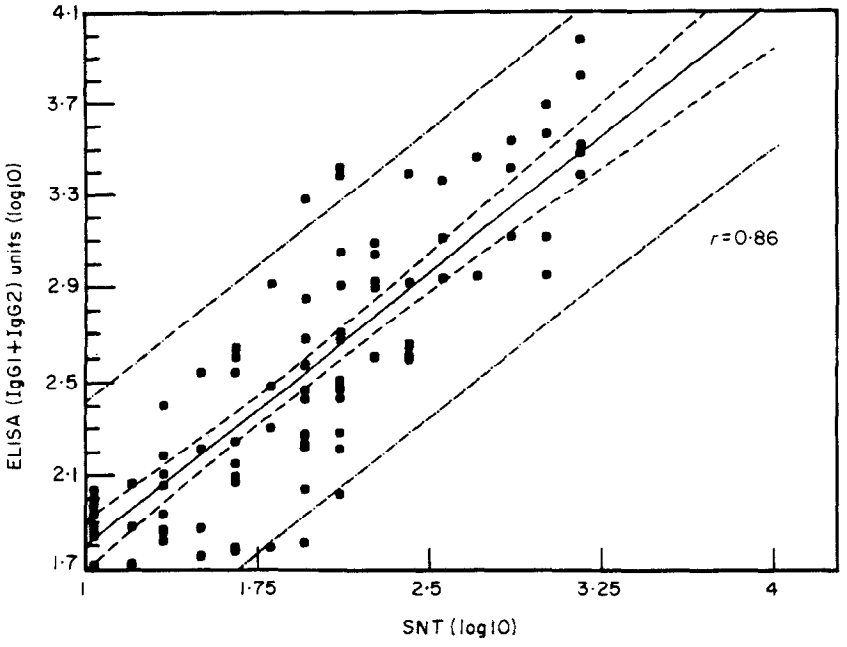

Figure 1. Regression curve for comparison of ELISA assay to SNT. ——— fitted regression line from the equation: $y = a + bx$ – – – – 95% confidence limits; – · – · – · – 95% prediction limits.

the SNT test. All three also showed significant seroconversion in the IgG_1 + IgG_2 ELISA (Table 4).

Statistical comparison of ELISA and SNT results

The regression analysis and correlation coefficient of the results from the IgG_1 and IgG_2 ELISA and SNT on positive sera are shown in Fig. 1. There was a very good positive correlation coefficient ($r = 0{\cdot}86$, at $P \ll 0{\cdot}001$) between the ELISA and SNT (for the 98 serum samples with ELISA units >50) when data were subjected to simple linear regression analysis and the non-parametric rank correlation by the Spearman method.

Discussion

The purpose of this study was to develop a simple rapid ELISA capable of providing results which can be evaluated and compared between laboratories for the testing of sera in IBR control schemes and for the screening of animals for international trade prior to export and after importation.

Although the SNT is the accepted test for screening animals for international trade, it is recognized to suffer from interference by non-antibody neutralizing factors in some sera.[11,12] Other disadvantages include slowness and requirement for expensive cell culture facilities, and so far there is no single established test protocol. Many laboratories now use ELISA routinely for diagnosis of BHV-1 infections. However, the major drawbacks hindering the recommendation of ELISA for adoption as an international standard test are the great variation in the ELISA techniques and the difficulties likely to be experienced with standardization between laboratories, together with the lack of any agreed system of expression of results.[13]

The system of standardization adopted in the ELISA developed in the present study was the inclusion of a series of eight appropriate standard positive reference serum dilutions, and eight replicates of a standard negative reference serum on each plate to compensate for interplate variations associated with variable incubation times, temperature etc. As previously pointed out,[24] the quantitation of the results by comparing the activity of the serum samples to that of a standard reference serum in each test plate gives an opportunity to standardize the test not only within one laboratory but also between several laboratories.

We have clearly shown a good positive correlation between the SNT and the ELISA ($r = 0{\cdot}86$). Both tests were able to detect animals exposed to BHV-1 with a high degree of accuracy and agreement when cut-off values were fixed i.e. by excluding sera with low SNT titre (<1:4) and low ELISA titre (<50 units) in the comparison of the two tests. As widely acknowledged by others[30,31] at low discrimination dilutions of some sera, the ELISA may be positive when there is no discernible activity in the SNT. It is also known

that such low SNT titres of <1:2 or 1:4 are difficult to assess in an SNT.[29]

Therefore, to formulate guidelines for standardized ELISA for BHV-1 serology particular considerations should be given to the provision and availability of an international control reference serum or preferably a battery of sera such as the ones which exist for coronavirus (WHO reference serum), Brucellosis or Toxoplasmosis together with a standard antigen, labelled antiglobulin sera, and specification of a standard ELISA protocol with a single internationally agreed system for the expression of results which is readily comparable between laboratories.

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