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Enzyme-labelled immunosorbent assay techniques in foot-and-mouth disease virus research

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SUMMARY

The indirect ELISA technique has been developed successfully to measure antibodies to foot-and-mouth disease virus (FMDV) in cattle sera. Preliminary studies using a standard serum assay show that reproducible results are obtained. The method should prove useful for the examination of antibody titres in sera from large numbers of cattle or other animals.

INTRODUCTION

Enzyme-labelled antibodies have been used in histopathology (Nakane & Pierce, 1966; Wicker & Avrameas, 1969) to detect virus antigens in tissue sections. Their use in quantitation procedures is relatively recent (Engvall & Perlmann, 1971, 1972; Van Weeman & Schuurs, 1971, 1972). Voller, Bidwell, Huldt & Engvall (1974), Voller, Bidwell & Bartlett (1975) and Voller & Bidwell (1976) applied a technique using microtitre plates to measure antibodies in various parasitic and viral diseases as well as to detect viruses (Voller, Bidwell & Bartlett, 1976). Wolters, Kuijpers, Kacaki & Schuurs (1976) applied an indirect ELISA technique to detect hepatitis B surface antigen, and Yolken *et al.* (1977) applied the technique for detection of a human reovirus-like agent of infantile gastroenteritis.

The principle of the indirect ELISA test has been explained fully by other authors (Voller *et al.* 1976). Briefly, it involves the adsorption of antigen to a solid phase, in this case polystyrene wells in haemagglutination plates. Sera containing specific antibodies to the antigen are added and allowed to react. Excess unreacted antibody is removed by suitable washing. An enzyme-conjugated antiserum to the species of animal in which the specific antibodies to the antigen were made, is then added. After washing to remove unreacted anti-antibody, a substrate solution is added which gives a colour reaction in time, depending on the amount of enzyme-conjugated antiserum attached to the wells.

The ELISA technique can also be applied to detect and compare viruses by adsorbing specific antibodies to the solid phase, and generally, can be used in ways analogous to radioimmunoassay techniques, showing similar sensitivities.

As a preliminary study of ELISA techniques as applied to FMD virus research, this paper describes the assay of bovine antibodies against FMD virus in cattle sera. Development of the assay involved examination of virus adsorption to solid phase, preparation of enzyme conjugated antisera, limitations of non-specific effects, and use of the assay in specific tests.

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The indirect ELISA technique described for other systems proved suitable for FMD virus.

MATERIALS AND METHODS

The preliminary experiments to investigate the application of the indirect ELISA techniques to FMD virus research were made in three stages.

Stage 1 involved the examination of virus adsorption to plastic haemagglutination plates, testing of conjugated antisera, and limited tests with positive antisera using routine methods outlined in the literature. The best conditions for the indirect ELISA for FMD virus were established.

Stage 2 involved the measurement of bovine antibodies from a collection of post-vaccination sera, using a dilution end-point method, where sera were diluted in a twofold series and added to wells containing adsorbed virus. The validity of the end-point results was checked by comparing them with serum neutralization test data already obtained on the sera.

Stage 3 involved the measurement of bovine antibodies using a limited number of antiserum dilutions, and comparing all results to a standard serum titration curve obtained for a known positive serum against virus. This was done to limit the number of assay points needed, for convenience where a large number of sera need to be assayed, and also to standardize the test so that comparison of results obtained at different times can be made.

Plates. Disposable polystyrene microhaemagglutination plates (Cooke M29 AR, Dynatech Laboratories, England) were used.

Virus diluent. Carbonate/bicarbonate buffer 0·05 M, pH 9·6 stored with 0·02 % sodium azide at 4 °C. This is termed 'carbonate buffer' in further descriptions.

PBS/Tween. Consisted of 8·0 g NaCl, 0·2 g KH_4PO_4, 2·9 g $Na_2HPO_4 . 12H_2O$, 0·2 g KCl, 0·5 ml Tween 20 in 1 l of distilled water; pH 7·4. This was stored at 4 °C.

Washing of plates. Was performed by flooding the wells with PBS-Tween, leaving for 3 min, and then emptying. This was repeated three times and the process is termed 'washing' in subsequent descriptions.

Enzyme. Alkaline phosphatase EC 3.1.3.1 type VII sigma from calf intestine. Specific activity of 1140 units/mg protein.

Diethanolamine buffer (10 %). Consists of 97 ml of diethanolamine, 800 ml water, 0·2 g of NaN_3, 100 mg $MgCl_2 . 6H_2O$; 1 M-HCl is added until the pH is 9·8. The total volume is made up to 1 l with distilled water. It was stored at 4 °C in the dark until used.

Enzyme substrate. A solution of *p*-nitrophenyl phosphate (1 mg/ml) (BDH Chemicals, England) in 10 % diethanolamine buffer pH 9·8 was prepared immediately before use

Goat antibovine IgG antiserum. This was obtained from Miles Laboratories Ltd., U.K. The gamma-globulin fraction from the serum was prepared according to the method described by Fahey & Terry (1973) using DEAE-cellulose column chromatography. Gamma globulin from a non-immune bovine serum was also prepared for standardization of the conjugate. Total protein concentration was adjusted to 5 mg/ml after spectrophotometric examination (Wood, Thompson &

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Goldstein, 1965). Samples were stored at −20 °C in siliconized glass vials (Repelcote-treated; Hopkin and Williams, England).

Conjugation of enzyme with antibovine gamma globulin

Alkaline phosphatase was conjugated with goat anti-gamma globulin according to the method described by Avrameas (1969). The working dilution was determined by examination of the reaction between a non-immune bovine gamma globulin adsorbed onto wells and enzyme-labelled antibody dilutions, as described by Voller *et al.* (1976). A dilution of 1/400 was found to be satisfactory. Stock conjugate was stored at 4 °C after the addition of ovalbumin to a final concentration of 5%.

Virus

Type O_1BFS 1860 FMD virus was grown and purified as described by Brown & Cartwright (1963), using 1% SDS in place of deoxycholate. Radioactive ^{35}S-methionine-labelled virus was grown and purified as described by Harris & Brown (1977). Virus was dispensed in 0·5 ml volumes in siliconized glass vials, and stored at −70 °C. Before use, samples were dialysed against PBS, pH 7·2, to free the virus from sucrose which was found to interfere with the ELISA test.

Test sera

Samples of bovine sera were obtained from the Vaccine Research Department, AVRI, Pirbright, and were from cattle 21 days after vaccination with different doses of O_1BFS 1860 vaccine. Serum against Rinderpest virus was obtained from the Department of General Virology, AVRI, Pirbright.

Examination of the adsorbance of purified O_1BFS 1860 to plates

Purified ^{35}S-methionine-labelled virus was diluted to different concentrations in carbonate buffer or PBS, pH 7·2. The radioactivity was determined in each sample by pipetting 20 μl onto a glass fibre disk, air drying, and placing into 5 ml of scintillation fluid (0·1 g POPOP, 4·0 g PPO in 1 l of toluene). The radioactivity was determined using a liquid scintillation counter (Unilux II: Nuclear Chicago). Samples (0·2 ml) of each virus dilution were pipetted into wells which were then incubated at different temperatures for various times (Fig. 1). The well contents were then examined for radioactivity. Each well was then washed three times with PBS-Tween. At each stage the washings were examined for total radioactivity. Finally, the wells were dried and the radioactivity associated with the well measured. Results indicating the percentage of total input radioactive virus counts remaining adsorbed to wells are shown in Fig. 1.

Effect of buffers on the integrity of the virus

Purified radioactive virus was diluted in carbonate buffer or PBS, pH 7·2. Samples (0·2 ml) were incubated overnight (O/N) at room temperature (RT). The integrity of the virus was examined by centrifugation on linear 15–45% sucrose

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gradients. After fractionation of the gradients the radioactivity was determined as above. Any change in sedimentation pattern from untreated virus was noted.

Methods of assay

Serum dilution end-point method

The sera were diluted from 1/25 in twofold dilutions in PBS-Tween. Triplicate samples of each dilution (0·2 ml) were then added to sensitized wells (2 μg/ml virus, incubated O/N at RT). Similar dilutions of anti-rinderpest and anti-A_{24} bovine sera were also added to sensitized wells. Plates were then incubated at 37 °C for 2 h after which they were washed. Enzyme-labelled anti-bovine gamma-globulin diluted 1/400 in PBS-Tween, containing 3 % ovalbumin, was then added (0·2 ml/well) and the plates were incubated for 2 h at 37 °C. The plates were then washed and each well received 0·2 ml of the substrate solution. The plates were incubated at RT for 45 min, and the reaction was then stopped by the addition of 50 μl of 3 M-NaOH solution. The test was read by eye, the highest dilution of the serum in the last well showing detectable colour was determined as the end-point. Results for repeated tests using this method are shown in Table 1.

Examination of sera using standard serum dilution curves

In order to standardize the test to take into account different conjugate preparations, different concentrations of sensitizing virus and possible differences in laboratory methods, and also to limit the number of dilutions of sera necessary in the test, a method using a standard serum was developed. A known titre positive serum was designated as standard and all results comparing the bovine sera are related to this.

The ELISA procedure was followed as above; however, in each test a standard curve was obtained relating to the optical density (OD), at 405 nm to the colour developing against a twofold dilution series of the standard positive serum. Test sera were diluted 1/25, 1/50 and 1/100, the OD after the test was read and related to the standard curve. Values for the test sera were only read in the linear region of the standard curve. The method was examined using selected bovine sera. The intratest variation was determined using two sera from the group and titrating them nine times in the same test.

The test was repeated at a different time to determine the inter-test variation between the titres of the test sera relative to the standard curve.

Neutralization tests

The neutralizing antibody titres of the sera were determined in microtitre plates using the method described by Golding, Hedger & Talbot (1976).

RESULTS

Figure 1 shows the percentage of total virus added to the wells remaining after the washing procedures. The best conditions for virus adsorption involved the use of carbonate buffer and incubation O/N at RT. Sensitization of the wells with

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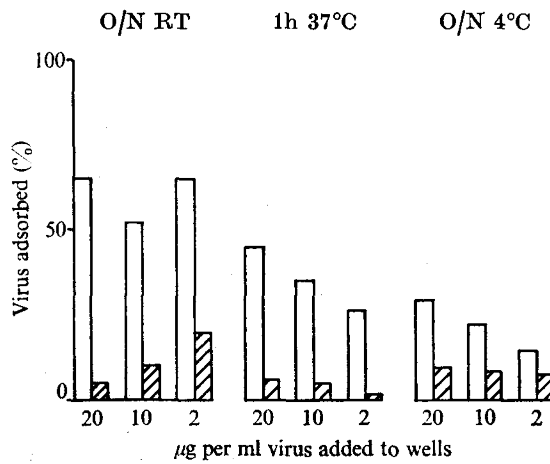

Fig. 1. Examination of virus adsorption. Diluent: □, Carbonate buffer; ▨, PBS.

Table 1. *Comparison of end-point ELISA and serum-neutralization results*

Vaccine dilution	Serum number*	Log_{10} 1/titre ELISA test	Log_{10} 1/titre Neutralization test
1/50	2541	2·6	2·00
	2542	< 1·4	< 0·75
	2543	2·0	1·50
	2545	2·3	1·40
	2546	< 1·4	0·90
	2547	1·7	1·95
	2548	< 1·4	< 0·75
1/10	2549	2·0	1·50
	2550	1·7	1·65
	2551	1·4	1·80
	2552	2·0	2·10
	2553	1·7	1·95
	2554	2·0	2·55
	2555	2·6	2·40
	2556	2·6	2·10
1/2	2557	1·7	1·50
	2558	2·0	2·55
	2559	2·3	2·55
	2560	2·0	1·80
	2561	2·6	2·55
	2563	2·6	2·10
	2564	2·6	2·40
	Anti-rinderpest	< 1·4	ND
	Anti-A_{24} FMDV	< 1·4	ND
	Non-sensitized wells	< 1·4	ND

ND, not done. * Sera diluted 1/50.

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Table 2. *Titration of bovine sera against standard serum using indirect ELISA test*

Sera at 1/50	Mean OD 405 nm (n = 3) 1	2	Standard deviation of mean 1	2	Dilution of standard serum at test OD 1	2	Relative titre of test serum as % standard 1	2
2542*	0·36	0·37	0·020	0·015	—	—	—	—
2545	0·71	0·75	0·015	0·020	4570	4360	1·09	1·14
2546*	0·38	0·37	0·020	0·030	—	—	—	—
2548*	0·37	0·39	0·010	0·015	—	—	—	—
2552	0·71	0·73	0·020	0·015	4570	4470	1·09	1·11
2555	0·98	0·99	0·015	0·015	3160	3020	1·58	1·66
2556	1·00	1·01	0·010	0·005	3020	2950	1·66	1·69
2558	0·81	0·80	0·005	0·020	3890	3980	1·29	1·26
2559	0·81	0·81	0·020	0·015	3890	3890	1·29	1·29
2560	0·77	0·78	0·020	0·005	4270	4170	1·17	1·20
Anti-rinderpest*	0·36	0·36	0·015	0·015	—	—	—	—
Anti-A_{24}	0·29	0·31	0·020	0·030	—	—	—	—

n, Number of times serum tested.
1 and 2 refer to separate experiments.
The means of experiments do not differ significantly (99 % confidence limits).
* Sera tested at 1/25 dilution.

Table 3. *Repeat serum titration using indirect ELISA test*

Serum number	Mean OD at 405 nm (n = 9)	Standard deviation	Standard error of mean
2555	0·99	0·010	0·006
2556	1·00	0·010	0·006

n, Number of times sera tested.

2 μg/ml of virus was found to be satisfactory for the detection of bovine antibodies; approximately 0·25 μg remained associated with each well. A maximum of 0·4 μg per well virus in PBS remained even with high (20 μg/ml) amounts of sensitizing virus.

Virus integrity appeared unaffected by carbonate buffer. No breakdown into 12S components was observed after examination of the sucrose density gradients. The effect of carbonate buffer on the immunological reactivity of the virus was not studied extensively, but wells sensitized with virus in PBS gave the same results for the standard serum titrations, indicating that similar antigen/antibody reactions were being examined. The addition of 3 % ovalbumin to the conjugate was essential to minimize non-specific effects.

Results using the dilution end-point method are shown in Table 1, and are compared with the neutralization antibody titres. Exactly the same end-points were obtained in three experiments in the ELISA test using the same conjugate. All sera found negative (< 0·75) by the SN tests were found negative (< 0·50 OD) using the standard curve or (< 1·4) using the end-point method in the ELISA test.

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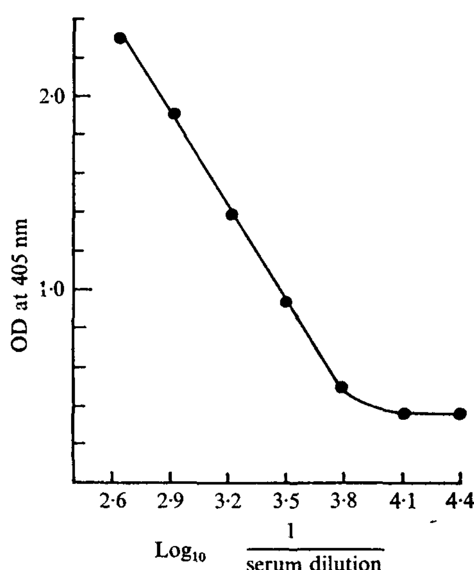

Fig. 2. Standard serum titration curve.

Tables 2 and 3 show the results from two experiments for selected bovine sera using the standard serum procedure. Figure 2 shows a typical standard curve with a linear relationship for OD against serum concentration from 0·5 to 2·4 OD. The use of bovine anti-rinderpest and anti type A24 FMD virus serum, or the use of non-sensitized wells, all gave negative results, i.e. OD values below 0·5 under the conditions used in the test, indicating the specificity of the test for type O FMD virus antibodies. Sera were termed negative when values less than 0·5 OD were obtained at a 1/25 dilution. Examination of the OD of positive sera at different dilutions showed that the dilution was related to the OD as found in the standard serum curve, i.e. parallel lines to the standard curve could be constructed. This indicated that a similar virus/antibody reaction was being measured in the test and standard sera.

DISCUSSION

Preliminary results indicate that the indirect ELISA technique can be successfully used to quantify antibodies to FMD virus from cattle sera.

FMD virus O_1BFS 1860 was successfully adsorbed to the surface of microtitre plates. A concentration of 2 μg/ml was sufficient to give reproducible results, although it was possible to adsorb up to 2·5 μg of virus to each well using carbonate buffer. The buffer system used did not affect the physical integrity of the virus, and did not appear to affect the detection of bovine antibody in this test.

Use of the standard serum dilution curve gave reproducible results for the antibody titres from test to test using a single dilution of serum, which has obvious advantages over dilution end-point methods. The relation between the ELISA technique results and the neutralizing antibody titres is complicated since the ELISA measures total antibody to O_1BFS whereas the neutralization test measures

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only neutralizing antibody. Scatter diagrams relating the ELISA and SN results indicated a low degree of correlation ($r = 0{\cdot}6930$) between the two tests. However, the test to test variation between individual serum titres for the neutralization test has been found to be high in this laboratory, giving unsatisfactory results, whereas the ELISA technique appears to give a low inter-test variation when a standard serum is used. The use of the ELISA test to obtain consistent results for the amount of total bovine antibody, as well as the examination of the amounts of IgM, IgG and IgA antibody using specific antibovine conjugated antisera, is now being examined. Antibodies from a large number of vaccinated cattle are being determined and a full statistical evaluation of the use of the ELISA technique using standard serum curves of known immunological activity, will be made. Parameters such as the use of crude pelleted virus preparations and the use of inactivated virus to sensitize the plates, are also being examined.

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