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A solid-phase enzyme linked immunosorbent assay for the detection of African swine fever virus antigen and antibody

By R. C. WARDLEY, E. M. E. ABU ELZEIN,* J. R. CROWTHER and P. J. WILKINSON

The Animal Virus Research Institute, Pirbright, Surrey GU24 0NF, England

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SUMMARY

A solid-phase enzyme-linked immunosorbent assay was developed to measure both African swine fever virus (ASFV) antigen and antibody. Experiments showed it to be reproducible and able to detect limiting antigen concentrations of 50–500 HAD50/ml. The assay was more sensitive than those used at present to detect ASFV antibody and it is suggested that it might be of great diagnostic use in countries where African swine fever has recently appeared.

INTRODUCTION

Enzyme-linked immunosorbent assays (ELISA) have been described for many virus systems for the detection of both antigen and antibody (Voller & Bidwell, 1976; Voller, Bidwell & Bartlett, 1976). They involve the conjugation of an enzyme to specific antiserum which is then allowed to react with either antigen or antibody previously adsorbed to a solid phase. After a suitable reaction time, excess conjugated antibody is removed by washing and a substrate solution is added. This gives a colour reaction varying in intensity with the amount of conjugated antiserum which has attached to the reaction complex at the solid phase.

Provided the antibody to be conjugated is of a high specificity, the ELISA technique shows good sensitivity for both antigen and antibody detection. Conjugated antisera can be stored for long periods and the test read using a simple colorimeter. These characteristics make it an ideal test for both general laboratory and large-scale diagnostic work.

MATERIALS AND METHODS

Preparation of African swine fever virus antigen and antisera

These were prepared according to the methods described by Crowther, Wardley & Wilkinson (1979).

Conjugation of enzyme to gamma globulin

Alkaline phosphatase EC 3.1.3.1 type VII (Sigma Chemicals, U.K.) was conjugated with both the IgG fraction of the standard antiserum and the IgG

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fraction of a commercial goat anti-pig serum (Miles, U.K.). The method used was that described by Avrameas (1969) except that glutaraldehyde was used at a concentration of 0·05 % and the enzyme/IgG was incubated for 4 h instead of 2 h at room temperature. The working dilution was determined as described by Voller *et al.* (1976). Stock conjugated IgG was stored at 4 °C after the addition of ovalbumin (final concentration 5 %) and sodium azide (final concentration 0·02 %).

ELISA techniques

The following techniques were developed using a solid phase ELISA in microtitre plates. The conditions reported are optimal and were obtained by methods similar to those reported by Voller *et al.* (1976).

Antigen assay

A five-fold dilution series of stock virus was made in carbonate buffer (0·05 M carbonate/bicarbonate, pH 9·6, with 0·02 % sodium azide) and 100 μl amounts were added to microtitre plastic wells (disposable polystyrene haemagglutination plates M.129B – Dynatech Laboratories, U.K.) for 3 h at 37 °C or overnight at room temperature. Unattached virus was removed by flooding the wells with saline–Tween (0·9 M-NaCl, 0·05 % Tween 20 in distilled water), leaving for 3 min before emptying them and repeating the process a further two times. Each well then received 200 μl of a predetermined dilution of anti-ASFV IgG conjugate diluted in PBS–Tween (PBS plus 0·05 % Tween 20, pH 7·4, containing 3 % ovalbumin). Plates were incubated for 1 h at 33 °C and washed three times as described above before adding 200 μl of substrate solution (*p*-Nitrophenyl phosphate, 1 mg/ml – BDH Chemicals, U.K. – prepared immediately before use in carbonate buffer). Plates were left at room temperature for 30 min and the colour reaction stopped by the addition of 50 μl of 3 M-NaOH solution to each well. The colour intensity developed at each dilution was read spectrophotometrically at 405 nm. Controls included untreated wells and wells sensitized with Aujesky's Disease virus.

Antibody assays

The inhibition test. The IgG fraction of the standard antiserum was diluted in carbonate buffer to 10 μg/ml and added in 200 μl amounts to microtitre plate wells (disposable polyvinyl micro-ELISA plates – Dynatech Laboratories, U.K.). After incubation for 3 h at 37 °C or overnight at room temperature, the plates were washed three times and a 1/5 dilution of stock virus (approximately 10^6 HAD50/ml) in carbonate buffer was added in 200 μl amounts to each well. The plates were incubated for 1 h at 37 °C and washed as described, before the addition of five-fold dilutions of the 200 μl amounts of test serum in PBS-Tween. After a further incubation period of 1 h at 37 °C, plates were again washed and the routine described above for the addition of conjugated anti-ASFV IgG and substrate followed.

Controls were normal pig serum and the standard positive antiserum. The 100 % inhibition of conjugated antibody attachment to the plate was measured in wells receiving a 1/5 dilution of standard antiserum, whereas 0 % inhibition was

measured in wells without competing antiserum. The % inhibition at any serum dilution was then calculated from the formula below:

$$\%\ \text{inhibition of test serum} = \frac{0\%\ \text{inhibition reading} - \text{test antibody reading}}{0\%\ \text{inhibition reading} - 100\%\ \text{inhibition reading}} \times 100.$$

The indirect test. The method used for this assay of antibodies against ASFV is as described by Abu Elzein & Crowther (1978) for foot-and-mouth disease virus. Haemagglutination plates were sensitized with ASFV (approximately 10^6 HAD50/well) and washed before the addition of dilutions of test serum in PBS-Tween. After incubation and washing, enzyme-labelled anti-species IgG suitably diluted in PBS-Tween 3 % ovalbumin was added to the plates, which were then incubated for a further 2 h and washed before the addition of substrate. Controls included pre-inoculation sera, normal pig sera and standard positive antiserum. Results are expressed as:

Test sera readings at a given dilution
− normal pig serum readings at the same dilution

Viral infectivity assays

Viral infectivity was assayed by haemadsorption in pig bone marrow cultures (Malmquist & Hay, 1960) using ten tubes per dilution.

Complement fixation (CF) and immune electro osmophoresis (IEOP) assays

CF tests were performed with periodate-treated sera (Akao *et al.* 1962) and IEOP using the assay of Pan, DeBoer & Hess (1972). ASFV antibody titres from ten pigs which had either died or recovered from infection with different ASFV isolates were compared using the ELISA techniques and the CF and IEOP tests.

RESULTS

Detection of antigen

Figure 1 shows the data from three assays performed with the standard antigen. The results indicate that the limiting antigen concentration detected was equivalent to 50–500 HAD50/well and the relation between infectivity and the ELISA measurement was similar to that described using the radio immuno assay technique (Crowther *et al.* 1979). Experiments using different buffers, incubation times, the effect of drying antigen to plates and an indirect antigen assay all failed to improve the sensitivity of the test significantly.

Antibody inhibition test

The results of experiments to measure ASFV antibody in recovered pigs, using the ELISA antibody inhibition technique, are shown in Figure 2. The conditions under which this test was performed were also investigated during the development of this assay and those described were found to be optimum. The results show that, at high concentrations, the serum from some animals completely inhibits the binding of conjugated antibody (Fig. 2B and E). The titres of each serum can be read at the 50 % inhibition level and, provided a standard antiserum is included

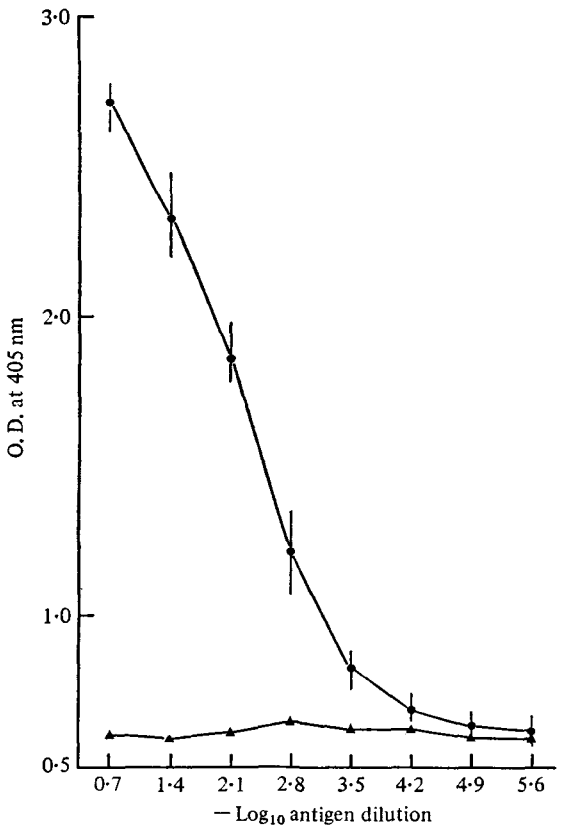

Fig. 1. Quantification of African swine fever virus using micro-ELISA. Graph shows the mean results from three separate titrations of ASFV antigen in dilutions of infected tissue culture. Each point represents the mean of six determinations (●——●). The bars represent 2 × SD from the mean of each point. Control antigen (Aujesky's Disease virus) was also titrated in the same system (▲——▲).

in each assay, test titres can be expressed in terms of this standard, thus enabling a direct comparison of assays performed at different times to be made. Sera from normal pigs and pigs which had recovered from Aujesky's Disease all gave negative results.

Indirect antibody test

Assays of three ASFV antibody positive sera are shown in Fig. 3. High concentrations of sera inhibit the attachment of the conjugated antibody to the solid phase and it is not until the antisera have been diluted to $> 10^{-1.25}$ that a maximum optical density reading is obtained. Despite this inhibition, the attachment of conjugated antibody at low dilutions of antisera is well in excess of that seen with normal pig serum controls, thus making the test useful for screening sera in epidemiological surveys.

Comparison of ELISA techniques and the CF and IEOP tests

Ten sera from pigs which had received different isolates of ASFV were tested for antibody using both ELISA techniques and the CF and IEOP tests. The

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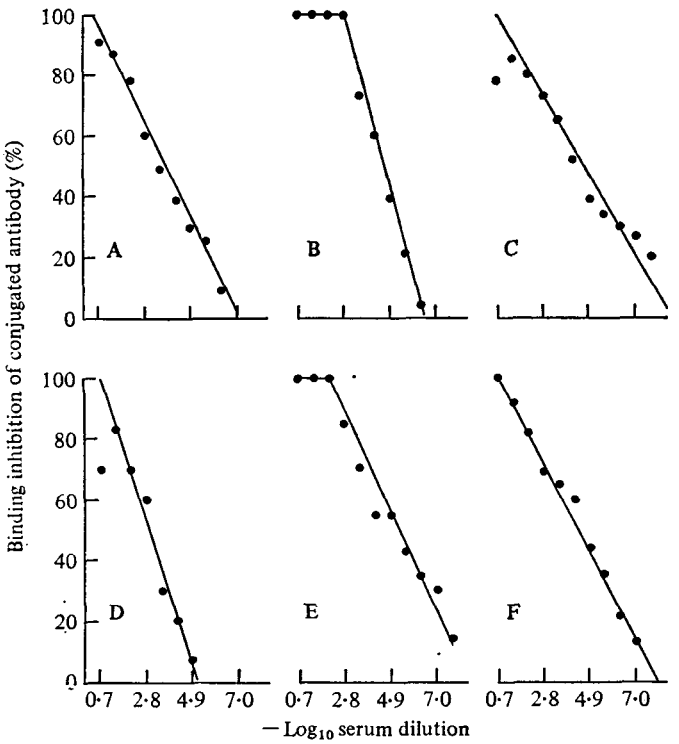

Fig. 2. Antibody inhibition assay showing the results from six different antisera. Each point represents the mean % inhibition from four determinations.

Table 1. *Comparison of ELISA techniques with the CF and IEOP test for the measurement of antibody*

	Antibody inhibition test		Indirect antibody test			
Sera	$-\log_{10}$ 50% inhibition titre	S.E.*	$-\log_{10}$ of titres read at 0·3 OD†	S.E.	$-\log_{10}$ CF test titre	$-\log_{10}$ IEOP test titre
1	4·2	4·1	4·5	4·8	3·9	0·7
2	5·0	5·6	4·0	5·7	3·4	0·7
3	7·0	3·9	4·9	3·2	3·7	0·7
4	4·9	4·7	3·5	6·7	3·1	0·7
5	3·0	6·2	2·8	4·1	2·7	Negative
6	5·7	4·2	4·2	5·7	3·0	0·7
7	4·9	3·7	2·0	8·2	ND‡	Negative
8	7·0	8·9	3·0	7·1	3·0	0·7
9	Negative	—	1·4	3·6	Negative	0·7
10	Negative	—	1·4	4·1	Negative	Negative

* S.E. Standard error of the mean (%).

† Titres read in the parallel part of the curve in the region after peak OD have been observed.

‡ ND, Not done.

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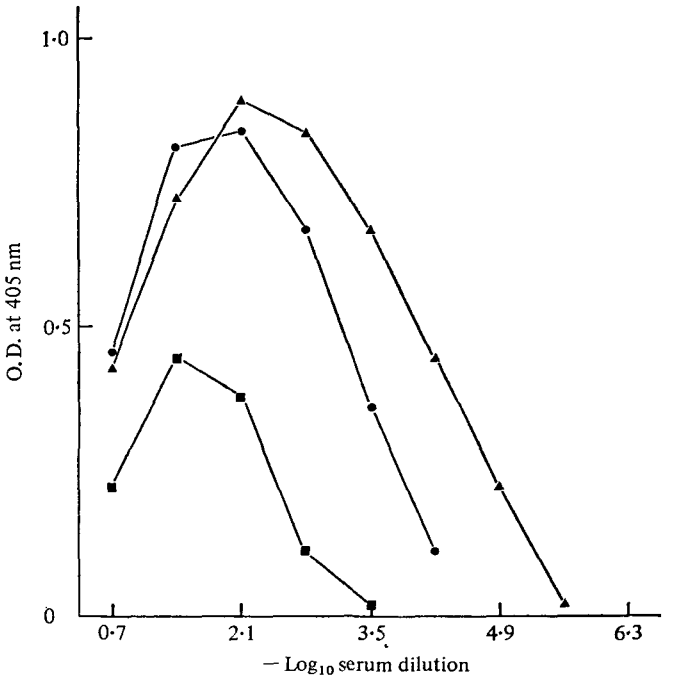

Fig. 3. Indirect ELISA for quantification of antibody of ASFV. The results for three different antisera are shown. The graph relates the amount of enzyme-linked antispecies antiserum attaching to wells with different test serum dilutions. Points represent means from four determinations above background control values obtained using a non-immune pig serum.

results are shown in Table 1 and show that, in general, the ELISA is at least ten times more sensitive in detecting ASFV antibody than either of the other two tests.

DISCUSSION

Preliminary results indicate that the ELISA technique can be successfully applied to the measurement of both ASFV antigen and antibody.

Antigen can be attached to the microtitre plate wells and the buffer system used does not appear to affect viral antigenicity. The direct assay for antigen gives reproducible results and, by always including a standard antigen of known titre, test samples could be calculated from a standard curve, giving an assay which has advantages over the infectivity test.

Both the assays for antibody appear more sensitive than the CF or IEOP tests, although the titres obtained with the IEOP test are lower than those described in other laboratories (Pan *et al.* 1972). This discrepancy in IEOP test sensitivity probably relates to the quality of the soluble antigen preparation used in the test. Normal tissue culture virus can be used in both ELISA techniques, which should facilitate the standardization of the test and the comparison of results from different laboratories. Recent work in this laboratory (Crowther *et al.* 1979)

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comparing a radio immuno assay technique for ASFV antigen and antibody detection shows it to be of similar sensitivity to the ELISA.

Although the assays described here are useful research tools which will advance our knowledge of ASFV, they could also be adapted for use in field laboratories for both antigen and antibody detection. Recently ASF has spread to Malta, Sardinia and South America. In such cases diagnostic tests have to be rapidly learnt and the ELISA technique lends itself to such a situation. As the conjugated antibody is stable over a long period of time, this could be produced and tested elsewhere, so that field laboratories would need only a minimum of equipment to allow them to carry out diagnosis.

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