

Solid-phase radioimmunoassay techniques for the detection of African swine fever antigen and antibody

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(Received 19 December 1978)

SUMMARY

A solid phase radioimmunoassay (RIA) has been successfully developed to measure both African swine fever virus (ASFV) antigen and antibody. Studies show that the assay is reproducible and will detect limiting antigen concentrations equivalent to 50–500 HAD₅₀/ml. Both direct and indirect antibody RIA have been developed and have proved to be approximately 100 times more sensitive than the complement fixation test at present available and 1000 times more sensitive than the immuno-electro-osmophoresis test for the detection of ASFV antibody.

INTRODUCTION

African swine fever (ASF) is a highly contagious disease of swine caused by an icosahedral deoxyribovirus (Almeida, Waterson & Plowright, 1967). The original African isolates were rapidly fatal for European pigs (Montgomery, 1921), although passage both in the laboratory and in the natural state has produced chronic forms of the disease. A number of different systems for virus assay have been developed (Pan *et al.* 1974; Malmquist & Hay, 1960), some of which require adaptation of the virus to continuous swine cell lines and others present difficulties because of the problems associated with the use of primary pig tissue. Antibody detection has also presented many difficulties. The complement fixation (CF) test, despite the high pro-complementary activity of pig serum, and electrophoretic tests have continued to be the most satisfactory means of measuring antibody titres (Pan *et al.* 1974).

In the present report we evaluate different methods for the radioimmunoassay (RIA) of both ASF virus and ASF antibody and describe the optimum conditions for such tests.

MATERIALS AND METHODS

Preparation of antigen

A Ugandan isolate of ASFV (Hess *et al.* 1965) which had previously been passed 60 times in pig kidney cells was inoculated at a multiplicity of 10 onto pig kidney monolayers. After adsorption for 1 h at 37 °C the monolayers were washed three

times in phosphate buffered saline (PBS) and maintained in Eagle's medium supplemented with antibiotics. Maximum cytopathic effect occurred in 2–3 days and the cells were then freeze-thawed three times and centrifuged at 2000 *g* for 10 min. Small samples of the supernatant were stored at -70°C . Uninfected monolayers treated in the same way were used as controls.

Preparation of antisera

Antisera (prepared by Mr A. Greig of this Institute at the East African Veterinary Research Organization, Muguga, Kenya) to African isolates of ASFV were made by intramuscular inoculation of 50 kg pigs with 10^7 HAD50 of a tissue culture adapted avirulent ASFV Uganda isolate. Ten days later pigs received 10^4 HAD50 of a virulent Tengani isolate with subsequent injections of virulent Kirawira, Hinde and Queen Elizabeth isolates each at further 10-day intervals. Serum was collected 10 days later, heat inactivated (56°C , 30 min) and stored at -20°C . Serum was used either as untreated standard antiserum or as an IgG fraction prepared using DEAE cellulose (Fahey & Terry, 1973). The IgG fraction of both the standard antiserum and a commercial goat anti-pig IgG (Miles Laboratories, England) were labelled with $\text{Na }^{125}\text{I}$ (Radiochemical Centre, Amersham, England) by the method described in Williams & Chase (1967).

RIA techniques

The following tests were developed using solid phase RIA on flexible microtitre plates (soft polyvinyl micro ELISA plates – Dynatech Laboratories, U.K.). The conditions reported are optimal and were obtained by methods similar to those described by Rosenthal, Hayashi & Watkins (1973).

Antigen assay. Five-fold dilution series of stock virus, diluted in phosphate buffered saline (PBS), were added in $50\ \mu\text{l}$ amounts to microtitre plate wells. Antigen was dried in a stream of warm air (37°C) and then the plates were washed four times in PBS by flooding and emptying the wells (Rosenthal *et al.* 1973).

Iodinated anti-ASFV IgG appropriately diluted in PBS, containing a final concentration of 3% bovine serum albumin (BSA), pH 7.4, was then added in $50\ \mu\text{l}$ amounts to each well. Plates were incubated at 37°C for 2 h while being shaken at 60 rev./min using a rotary shaker (R100 Rotatest, Luckham Ltd, U.K.). The iodinated antibody was then aspirated from each well and the plates were washed ten times in running tap water. The amount of radioactivity associated with each well was determined by cutting up the plate and putting the cups into plastic tubes which were counted in a gamma counter (MR1032, Kontron, U.K.). Controls included untreated wells and wells sensitized with Aujeszky's Disease virus.

Antibody assays. Antibody RIA was performed using an inhibition test or an indirect technique. (a) The inhibition test involved drying $50\ \mu\text{l}$ amounts of standard antibody IgG, appropriately diluted in PBS, onto wells. Plates were then washed four times in PBS before the addition of $50\ \mu\text{l}$ of a dilution of standard antigen in PBS to each well (approximately 10^6 HAD50/well). Plates were incubated for 2 h at 37°C with shaking and washed a further four times with PBS

before the addition of 50 μ l amounts of a dilution series of test and standard antisera in PBS/1% BSA. After a further similar incubation period and washing, 125 I labelled anti-ASFV serum diluted in PBS/3% BSA was added to each well and again incubated, after which the 125 I antibody associated with each well was determined as described in the assay for antigen detection. Controls were normal pig serum and the standard antiserum. The 100% inhibition of iodinated antibody attachment to the plate was measured in wells receiving a 1/5 dilution of standard antiserum, whereas the 0% inhibition was measured in wells without competing antiserum. The % inhibition at any serum dilution was then calculated from the formula below:

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

(b) The indirect test for antibody involved drying 50 μ l amounts of standard antigen diluted 1/5 in PBS/1% BSA (approximately 10^6 HAD50/well) onto plates. After washing four times in PBS, 50 μ l amounts of dilutions of test or control pig serum in PBS/1% BSA were added to each well. Plates were rotated for 2 h at 37 °C and then washed four times in PBS before the addition of 50 μ l amounts of an optimum dilution of iodinated anti-species IgG in PBS/3% BSA. Plates were then handled in a similar manner to that described above. Controls included pre-inoculation sera, normal pig sera and standard antiserum. Results were expressed as test counts at dilution A minus normal pig serum counts at dilution A.

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 RIA techniques and the CF and IEOP tests.

RESULTS

Detection of antigen

The use of untreated tissue culture virus and iodinated anti-ASFV IgG produced a reproducible and sensitive assay. Figure 1 shows the data from two assays performed with standard antigen. Results indicate that, with the IgG used, the limiting concentration measurable was equivalent to 50–500 HAD50/well. The relation between infectivity and RIA antigen measurement was linear from this detection limit to approximately 10^8 HAD50/ml under these conditions.

This method of antigen detection was chosen after experiments with a number of alternative conditions and systems. These included an indirect assay and also attempts to increase the specificity of the procedure by producing higher con-

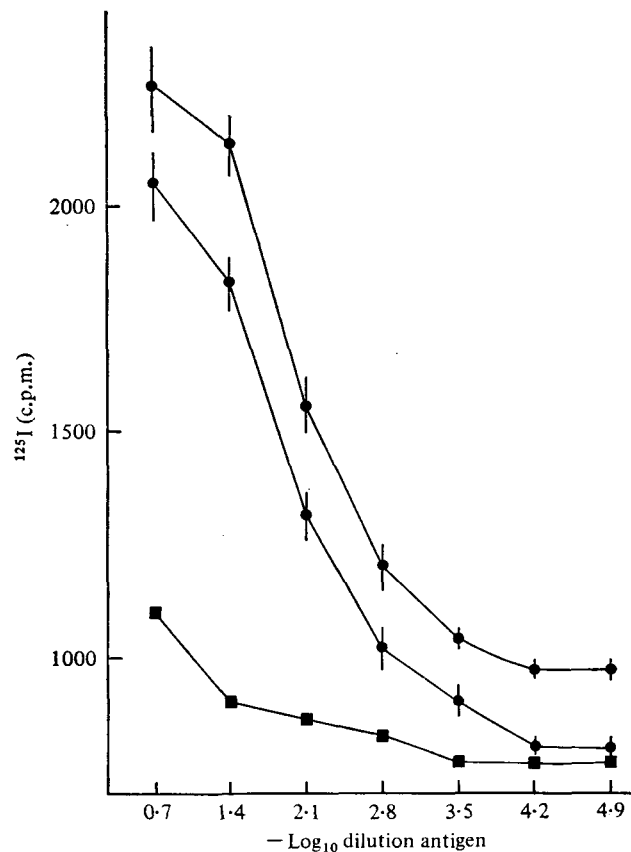


Fig. 1. Quantification of African swine fever virus using solid phase radio-immunoassay. Graph shows two assays of infected tissue culture, ●-●. Bars represent $2 \times$ SD from mean of quadruplicate determinations. Control antigen, ■-■ (Aujeszky's Disease virus).

centrations of specific anti-ASFV antibody for ^{125}I labelling by immunoadsorption techniques as described by Gilman & Docherty (1977). Both these methods failed to increase the sensitivity of the assay. Further experiments examining different incubation times, the concentration of BSA used, the application of antigen to plates without drying and the substitution of BSA by non-ionic detergents all failed to improve the test significantly.

Antibody inhibition test

The results of a set of experiments to measure anti-ASFV antibody using the RIA antibody inhibition technique are shown in Fig. 2. The conditions under which the test was performed were also investigated during the development of this standard assay. A crucial point was the sensitization of the plates with anti-ASFV IgG before the addition of antigen. Theoretically, this step could be omitted since ASFV has been shown to attach directly to the plates. However, results are then erratic and irreproducible. Once the plates had been sensitized with anti-ASFV IgG, the concentration of antigen used did not appear to be critical. This

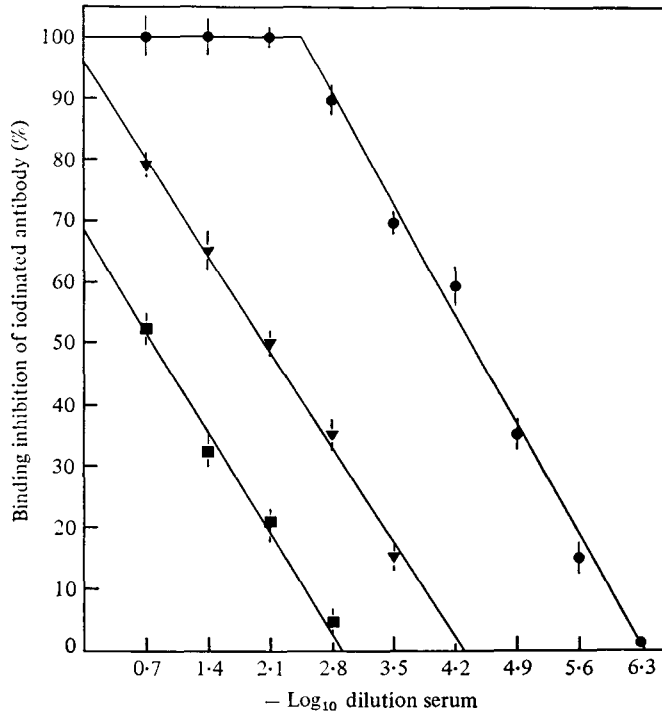


Fig. 2. Antibody inhibition assay showing the results for three different antisera. Each point represents the mean percentage inhibition from four determinations. The bars represent $2 \times$ SD from the mean.

was probably because the antiserum on the solid phase dictated adsorption from the medium. Further experiments examining incubation times, different BSA concentrations and the use of non-ionic detergents did not significantly alter the sensitivity of the test.

Indirect antibody tests

Data from experiments are shown in Fig. 3. The curve shapes are typical of those produced by this assay, although in theory high concentrations of positive antisera might be expected to produce a constant maximum number of counts. The exact reasons why high concentrations of antibody give this 'prozone-like' effect remain to be elucidated. The bell-shaped form of these curves presents some difficulties in the reading of serum titres. Because of the inhibition seen at high antibody concentrations, titres can only be estimated beyond the peak where the slopes are parallel. A full series of antibody dilutions was tested so as to determine this region. The inclusion of a standard antiserum control allows titres obtained from different tests to be compared.

Comparison of RIA with CF and IEOP tests

The results shown in Table 1 for RIA of antibody are compared with CF and IEOP titres of the same ten sera. The RIA techniques proved most sensitive since

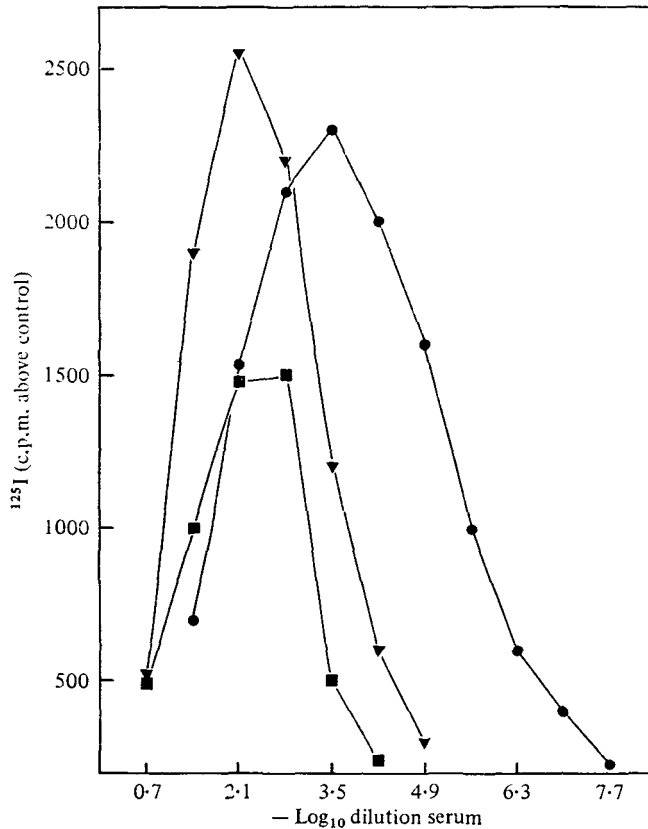


Fig. 3. Indirect RIA for quantification of antibody to ASF virus. The results for three different antisera are shown. The graph relates the amount of iodinated anti species antiserum attaching to wells at different serum dilutions. Points represent means from four determinations, background control values of normal pig serum.

antibody was detected at higher dilutions of test serum. In general, RIA titres were found to be 10–100 times higher using the antibody inhibition test and 100–1000 times higher with the indirect antibody RIA.

DISCUSSION

Radioimmunoassay techniques have proved useful in many fields of biological research. The assays are technically simple and rapid to perform and employ small amounts of reagents. Moreover, once tests have been standardized, automated gamma counting and computational facilities allow antigen and antibody amounts to be calculated and recorded. We have shown here that these techniques can be applied to ASF to provide rapid sensitive assays for both antigen and antibody. Present methods for the detection of ASFV rely mainly upon assays in primary pig tissues. Although other methods such as immunofluorescence (Heuschele, Coggins & Stone, 1966) and electrophoresis (Pan *et al.* 1974) can be used, they are of little value when quantification of virus is necessary. By its

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

Sera	Antibody inhibition assay			Indirect antibody assay			-log ₁₀ IEOP test titre	
	-log ₁₀ 50% inhibition titre	S.E.*	-log ₁₀ 0% inhibition titre	S.E.	-log ₁₀ titres† read at 1000 c.p.m.	-log ₁₀ end point titre		S.E.
1	3.3	4.9	4.9	4.9	5.8	7.5	5.2	0.7
2	3.2	5.1	4.2	4.8	5.2	6.5	8.1	0.7
3	3.4	5.9	4.9	5.1	5.2	6.5	4.9	0.7
4	3.4	4.3	5.3	4.2	5.6	6.8	5.7	0.7
5	1.2	5.6	3.4	3.2	4.0	5.5	7.1	0.7
6	3.3	3.6	4.5	4.2	5.6	6.7	4.3	0.7
7	1.2	11.4	3.8	8.1	3.1	4.5	7.9	Negative
8	1.6	6.7	3.6	7.1	3.0	4.4	8.1	0.7
9	Negative	—	Negative	—	3.4	4.8	4.8	Negative
10	0.1	7.6	2.3	4.1	3.0	4.5	5.7	Negative

* Standard error of the mean (%).

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

nature, the infectivity assay will always be the most sensitive test available, although by the time tissue cultures are prepared, inoculated and read, the assay takes between 7 and 10 days. The RIA for antigen described here can be performed in 5 h, allows many samples to be tested at one time and, since many replicates of each sample can be assayed, allows greater confidence to be placed on titres obtained.

As ASFV isolates cross-react extensively in serological tests (Hess, 1971), it is possible to use antisera to any isolate for the detection of other isolates. It is probable, however, that as yet undetermined antigenic differences do exist between the isolates and thus we have chosen to use serum prepared against many isolates in an effort to maximize the sensitivity of the assay. The use of available antisera gives detection limits equivalent to 50–500 HAD₅₀.

A standard antigen preparation whose infectivity has been accurately titrated in primary pig tissue can be included in the antigen assay, so that test samples can then be calculated using the standard RIA curve. This assumes that the RIA is measuring infectivity or that the two parameters bear a direct relationship. It is probable that the RIA is, in fact, measuring both infective virus and soluble antigen but in our experiments there is a proportional relationship which allows infectivity values to be deduced from an RIA count.

Neutralizing antibodies against ASFV have never been unequivocally demonstrated (Hess, 1971) and the determination of ASFV antibodies has relied largely on gel diffusion and CF tests. IEOP is used as a screening test for large numbers of sera (Pan *et al.* 1974). Because of the procomplementary activity of pig sera, CF tests are of little use either in routine diagnosis or for sera with low titres.

RIA is more sensitive than either IEOP or CF tests (Table 1) and, unlike these, RIA does not need specially prepared antigen. In both IEOP and CF tests, antigen preparations will influence the serum titre obtained. In our tests the IEOP values appear about ten-fold lower than those recorded in other laboratories (Pan *et al.* 1974). Even so, the RIA test would still be between 10 and 1000 times more sensitive in antibody detection.

The indirect antibody RIA presents some difficulties in the calculation of serum titres. When screening large numbers of samples, however, this problem does not arise as sera will be scored as either positive or negative for ASFV antibody. In such a test, samples could be assayed as three different dilutions in an effort to overcome the inhibitory effect seen at high serum concentrations and regarded as positive if the counts recorded at any dilution were above those for a normal pig serum. The simplicity of the indirect assay makes it more attractive for such large-scale surveys, whereas the antibody inhibition test might be better applied to research. The results of the inhibition assay show that the slopes obtained using different competing antisera are not always the same. This probably indicates differences in avidity of homologous and competing (heterologous?) antibodies for the specific antigens used on the solid phase. Direct comparisons of serum titres using this technique can be made only when the slopes are the same.

The authors wish to thank Mr S. Williams for enthusiastic technical assistance.

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