

The Use of an Indirect Enzyme-linked Immunosorbent Assay to Detect Baculovirus in Larvae and Adults of *Oryctes rhinoceros* from Tonga

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SUMMARY

An indirect sandwich enzyme-linked immunosorbent assay (ELISA) was developed to detect the baculovirus of *Oryctes rhinoceros* in purified virus preparations and in extracts of *O. rhinoceros* larvae and adults collected in the field in Tonga. A 20 ng amount of purified virus was detected unequivocally but precise quantitative determinations of virus in insect extracts proved difficult because virus in solutions containing host protein gave lower absorbance values than a comparable purified virus standard. The implications for use of ELISA in ecological studies are discussed together with the choice of appropriate standards.

INTRODUCTION

The coconut palm rhinoceros beetle *Oryctes rhinoceros* was accidentally introduced into the Tongan islands in 1952 to 1961 (Catley, 1969). As part of a control programme, the baculovirus of *O. rhinoceros* (*Oryctes virus*) was deliberately released in 1970 to 1971 (Young 1974) and spread of the virus through the beetle population was monitored over the subsequent 2 years. Larvae and adult beetles were collected and incidence of virus was assessed by means of a bioassay technique (Zelazny, 1971) using healthy *O. rhinoceros* larvae; the method was tedious and time consuming. In a follow-up survey in 1978, the condition of coconut palms was scored and the beetle population was sampled as described by Young (1974). In this survey, however, the incidence of baculovirus was assessed by means of an indirect sandwich enzyme-linked immunosorbent assay (ELISA).

The direct ELISA has been used to detect insect viruses and to discriminate between them (Kelly *et al.* 1978*a, b*) but the assay has not as yet been used to detect virus in insects collected from natural populations. Kelly *et al.* (1978*b*) stated that 3 ng of *Heliothis armigera* baculovirus particles could be detected in a larval extract. The present paper describes an indirect ELISA method, its sensitivity and some of its problems.

METHODS

Oryctes rhinoceros larvae and adults. Larvae collected in Tonga were stored in plastic bags, with a maximum of five individuals per site comprising a single group and were frozen at -20°C before transfer to Auckland. Larvae were macerated in SET buffer (100 mM-NaCl, 1 mM-EDTA (sodium salt), 50 mM-tris-HCl, pH 8.0). The macerate was clarified at 4000 g for 5 min and virus was sedimented at 60000 g for 30 min and resuspended in SET buffer for analysis in the ELISA assay. A group of five larval sites which gave a negative response

in several ELISA tests was designated as virus free and the extracts were pooled and used as a diluent and as an antigen in some titrations. Individual samples were analysed on 30 to 60% (w/w) sucrose gradients in SET buffer at 90000 *g* for 3 h. Light-scattering bands containing virus were removed, pooled, diluted with SET buffer and sedimented at 60000 *g* for 30 min. The virus was resuspended in SET buffer, clarified at 4000 *g* for 5 min and aliquots of approx. 0.5 mg virus (A_{260} of 5) were stored at -90°C .

Protein determinations. Protein concentration was estimated by the method of Lowry *et al.* (1951); bovine serum albumin was used as a protein standard.

Preparation of antisera

Primary antibodies. Guinea-pigs were immunized by injecting 2 aliquots, each of approx. 0.5 mg virus mixed with Freund's complete adjuvant, intraperitoneally at a 7 day interval. The animals were exsanguinated by heart puncture 3 weeks later and a crude IgG fraction was obtained by precipitation with ammonium sulphate (40%, w/v).

Secondary antibodies. Rabbits were immunized intramuscularly with four samples each of approx. 0.5 mg of virus in SET buffer at 7 day intervals. The complement-fixing titre of the serum against 10 $\mu\text{g}/\text{ml}$ *O. rhinoceros* baculovirus was 1/1280. To produce pure unabsorbed IgG the serum was incubated on columns containing CNBr-activated Sepharose 4B (Pharmacia) linked with donkey anti-rabbit IgG. After thorough washing, the rabbit IgG was eluted with 0.2 M-glycine-HCl buffer, pH 2.2. The concentration of rabbit IgG was estimated spectrophotometrically, assuming $A_{280}^{0.1\%} = 1.4$. For use in the majority of tests, to ensure that the IgG preparations did not react with insect protein, the unfractionated serum was first absorbed with an extract of virus-free larvae. Then, 2.0 ml of serum were incubated with 0.5 ml of virus-free insect extract at 37°C for 3 h and centrifuged at 10000 *g* for 30 min before incubation on immunoabsorbent Sepharose columns.

Tertiary antibodies. Goat IgG specific for rabbit IgG and linked with alkaline phosphatase was obtained from Miles Research Products, Miles Laboratories, Elkhart, Ind., U.S.A. and was used without further purification at a dilution of 1/1000.

Indirect ELISA technique. The assays were performed in polystyrene microtest plates (MTP. 1, Australian Disposable Products Pty, Ltd., Adelaide, South Australia). Coating, substrate, rinsing and sample buffers are described by Flegg & Clark (1979) and our indirect ELISA method is based on the procedures described by Clark & Adams (1977). Plates were coated with 100 μl primary antibody at 2 to 5 $\mu\text{g}/\text{ml}$ for 48 h at 37°C and reacted with 100 μl antigen diluted in sample buffer, for 6 h at 37°C . Then 100 μl of secondary antibody was added at 0.5 to 2 $\mu\text{g}/\text{ml}$ for 16 h at 37°C and followed by 100 μl of conjugated tertiary antibody for 4 h at 37°C . Finally 200 μl of substrate (*p*-nitrophenyl phosphate), at 0.5 mg/ml, was added and the plate was incubated at 37°C for at least 90 min. The reaction was stopped by adding 50 μl of 2 M-NaOH. The absorbance at 405 nm of the coloured reaction product was measured after diluting each sample to 0.8 ml. All assays were performed in duplicate.

RESULTS

Up to 5 mg of *Oryctes* virus were obtained from faecal samples from each infected adult beetle. The enveloped virus particles were relatively fragile and there was some loss of virus during gradient purification. Therefore, only one cycle of purification of virus was carried out and in the subsequent development of an ELISA method two factors had to be considered. First, that there might be antibodies to insect protein in the immune sera and second, that the immune sera might also react non-specifically with insect protein. Methods

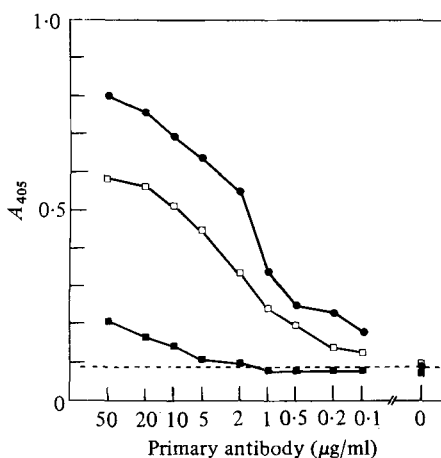


Fig. 1. Influence of the concentration of coating antibodies on detection of 1 µg/ml purified virus (●—●), virus plus insect extract (□—□) and insect extract (■—■). A 2 µg/ml amount of unabsorbed secondary antibody was used. ---, Background values of buffer control.

to identify and overcome these problems in the various stages of the modified ELISA technique were developed.

Titration of optimum concentration of primary antibody

The results of titrating a range of concentrations of primary antibody, from 50 to 0.1 µg/ml, are shown in Fig. 1. The antigen concentration was 1 µg of virus protein/ml and this was used either in buffer or diluted in a virus-free insect extract. A sample of the same virus-free insect extract was used as control. A 2 µg/ml amount of unabsorbed secondary antibody was used in all assays. At high concentrations of primary antibody, there was some reaction with insect extract and this was detected by the secondary antibody. The A_{405} values for the virus diluted in insect extract were consistently lower than the virus in buffer, but with both preparations there was little difference in A_{405} values in the range 50 to 5 µg/ml primary antibody. In some subsequent tests 20 µg/ml of primary antibody was used, although this was usually reduced to 2 to 5 µg/ml to ensure adequate detection of virus with minimal interference from insect extract.

Titration of optimum concentration of secondary antibody

Three antigen preparations were also used to titrate secondary antibody, in the form of pure IgG. (Fig. 2). There was marked absorption and detection of insect extract especially at higher concentrations of unabsorbed secondary antibody (Fig. 2a). Virus in buffer was detected adequately at secondary antibody concentrations of 5 to 0.5 µg/ml and again, the presence of insect extract resulted in a marked depression in A_{405} . Virus was readily detected at a secondary concentration of 0.5 µg/ml with minimal interference from insect extract; this concentration was therefore selected for subsequent tests. The results shown in Fig. 2(b) indicate that absorption with insect extract considerably reduced the reaction of secondary antibody with insect extract relative to the response of *Oryctes* virus. Although the presence of insect extract masked virus detection slightly, this effect was less than with unabsorbed antibody. A concentration of 0.5 µg/ml was selected for further titrations since this concentration detected virus readily, reaction with insect protein being only slightly greater than 'background'.

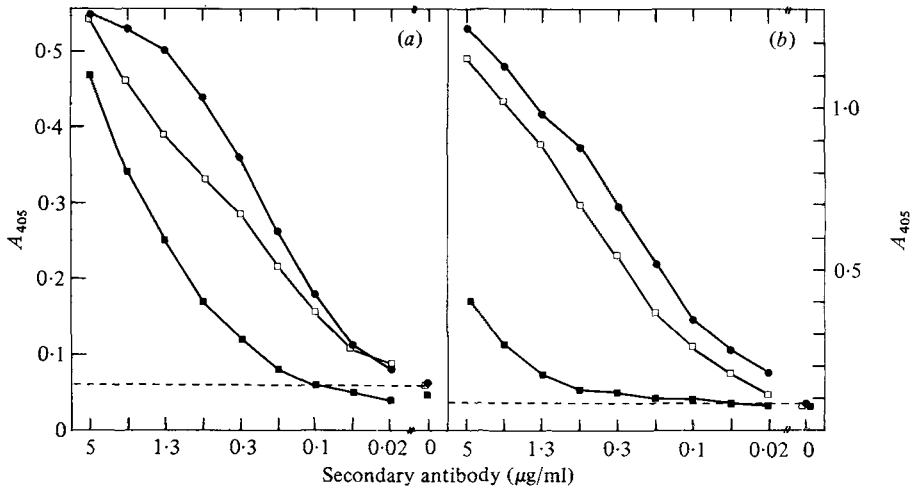


Fig. 2. Absorbance values obtained in ELISA with various concentrations of (a) unabsorbed secondary antibody and (b) absorbed secondary antibody, and 1 $\mu\text{g/ml}$ of purified virus (●—●), virus plus insect extract (□—□) and insect extract (■—■). Coating antibody concentrations were (a) 20 $\mu\text{g/ml}$ and (b) 5 $\mu\text{g/ml}$.

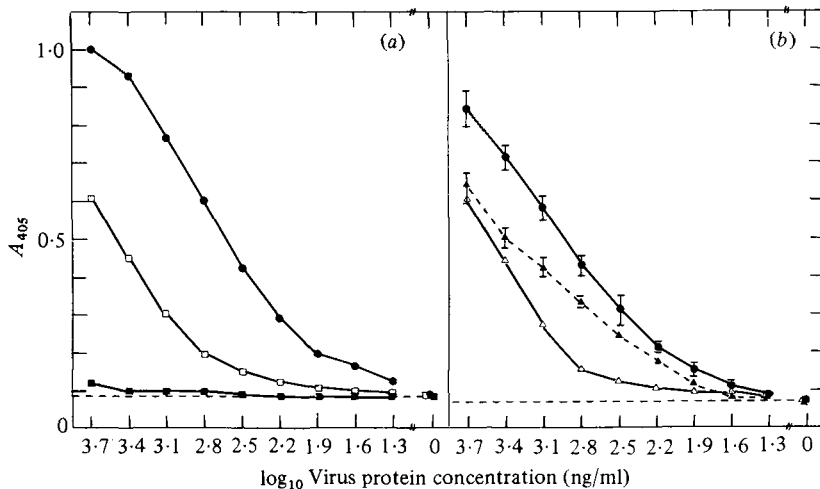


Fig. 3. (a) Effect of various concentrations of antigen on the A_{405} obtained with 10 $\mu\text{g/ml}$ coating antibody and 0.5 $\mu\text{g/ml}$ of absorbed secondary antibody in ELISA, using purified virus (●—●), virus plus insect extract (□—□) and insect extract (■—■). (b) Effect of various concentrations of antigen on the A_{405} obtained with 0.5 $\mu\text{g/ml}$ absorbed secondary antibody in ELISA, using 5 $\mu\text{g/ml}$ coating antibody. ●—●, Purified virus diluted in buffer; ▲—▲, virus diluted in constant insect protein; Δ—Δ, virus plus protein diluted in buffer; ---, background values of buffer control. Vertical bars represent the standard error of the mean.

Titration of Oryctes virus

Using primary antibody at 20 $\mu\text{g/ml}$ and absorbed secondary antibody at 0.5 $\mu\text{g/ml}$, titrations were made of purified virus in buffer, a series of dilutions in buffer of a virus preparation plus insect extract and a dilution series of an equivalent concentration of insect extract as a control. Detection of insect extract was minimal, even at high protein concentrations (Fig. 3a) and virus in buffer was detected unequivocally with A_{405} values above 'background' down to 20 ng virus protein/ml, the lowest dilution tested. When an equivalent

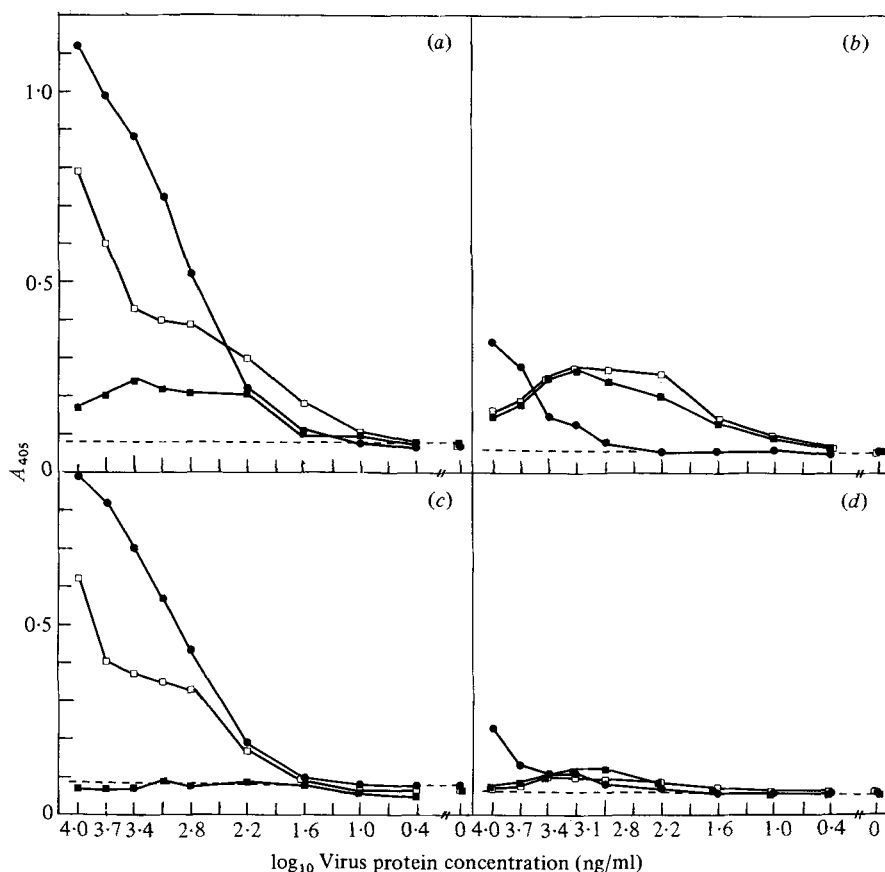


Fig. 4. Absorbance values obtained in ELISA with various concentrations of antigens using (b, d) 5 $\mu\text{g/ml}$ pre-immune primary antibody, (a, c) 5 $\mu\text{g/ml}$ immune primary antibody, (a, b) 0.5 $\mu\text{g/ml}$ unabsorbed secondary antibody and (c, d) 0.5 $\mu\text{g/ml}$ absorbed secondary antibody. ●—●, Purified virus; □—□, virus plus insect extract; ■—■, insect extract.

Table 1. Effect of insect extract on the A_{405} values produced in ELISA for *Oryctes baculovirus*

Antigen	Antigen concentration (μg virus protein/ml)							
	5	2.5	1.25	0.63	0.31	0.16	0.08	0
(a) Purified virus in buffer	0.54	0.38	0.28	0.20	0.14	0.11	0.09	0.05
(b) (a) with insect extract wash before antigen	0.57	0.43	0.30	0.21	0.15	0.11	0.09	0.07
(c) (a) with insect extract wash after antigen	0.39	0.26	0.19	0.12	0.11	0.10	0.08	0.07
(d) Purified virus in insect extract	0.35	0.20	0.12	0.10	0.09	0.09	0.08	0.08

concentration of virus in insect extract was diluted in buffer, titration showed that the A_{405} was substantially lower than the comparable series of dilutions of virus alone in buffer (Fig. 3a), the minimum detection level of virus being reduced to about 60 ng/ml.

The diagnosis of virus in relatively crude extracts of *Oryctes* larvae and adults could possibly be affected by the presence of varying levels of insect extract so this phenomenon was examined further. Three parallel dilution series of virus were made: one in buffer, one in a constant, high concentration of insect extract and the third, like that illustrated in Fig. 3(a), was a dilution series in buffer of virus plus insect extract (Fig. 3b). The detection level in the purified virus dilution series is about 30 ng/ml but both curves for virus in insect

Table 2. *Oryctes virus infection in samples of larvae from different breeding sites diagnosed by indirect sandwich ELISA*

Virus concentration per site (μg)	No. of sites	% of total sites per category
5-20	7	3.4
21-100	2	1.0
101-2000	5	2.4
> 2000	1	0.5
No virus	192	92.7
Total sites	207	

Table 3. *Oryctes virus infection in samples of adult beetles diagnosed by indirect sandwich ELISA*

Virus concentration per beetle (μg)	No. of beetles	% of total
5-20	23	24.7
21-100	5	5.4
101-2000	25	26.9
> 2000	25	26.9
No virus	15	16.1
Total	93	

extract and the virus detection levels are lower than that for virus in buffer. Interestingly, the curve for the dilution series of virus plus insect extract is substantially lower than that for virus diluted in constant, high concentrations of insect extract. Fivefold replications were used in this experiment to determine reproducibility of the assay between plates. The standard errors were so low (Fig. 3*b*) that relatively small differences between treatments, even at low antigen concentrations, can be compared with confidence.

The preliminary titrations showed that there were substantial interactions between insect extract and primary and secondary antibody, and also that the presence of insect extract depressed the A_{405} produced by virus and reduced the detection level. To investigate the mechanisms behind these interactions, three dilution series of insect extract, virus and virus plus insect extract were titrated using immune and pre-immune guinea-pig primary antibody and, in each case, using either unabsorbed or absorbed secondary antibody. The results are shown in Fig. 4(*a* to *d*). The responses obtained with pre-immune primary sera are of particular interest. At high concentrations, especially above 500 ng/ml, virus adheres non-specifically to pre-immune primary antibody and this is detected by either absorbed or unabsorbed secondary antibody. Insect extract, with or without virus, adheres to pre-immune primary antibody with a broad optimum at 50 ng to 2.5 $\mu\text{g}/\text{ml}$. This is clearly detected by unabsorbed secondary antibody and only slightly by absorbed secondary antibody. With immune primary antibody, insect extract alone is detected only by unabsorbed secondary antibody. With either absorbed or unabsorbed secondary antibody, insect extract interferes with the detection of virus.

Purified virus in buffer and virus diluted in insect extract were then titrated with absorbed secondary antibody and compared with two titrations of purified virus in buffer in which the plates were washed with a constant level of insect extract before and after adding antigen (Table 1). In the latter treatment a marked depression in A_{405} was obtained, similar to that when purified virus and insect extract were added to the plates together.

ELISA diagnoses of field collected Oryctes

On each ELISA plate a fivefold dilution series of each of nine extracts of insects from individual sites was compared to a standard fivefold dilution series of virus plus added virus-free insect extract. The standard dilution series was repeated once on each plate. When the A_{405} values for two or more dilutions from each site were clearly above 'background', that site was considered to be virus infected. The virus concentration at each infected site was estimated by comparison of dilutions of insect extract and of virus standards which gave equivalent A_{405} values, and multiplication by the appropriate dilution factor. The results of diagnoses on 207 larval breeding sites are presented in Table 2. Most of the 16 positive sites contained less than 500 ng of virus. By contrast (Table 3) 84 % of adult beetles sampled were virus infected, the majority producing at least 100 μ g virus. Table 3 is a summation of diagnoses made on faecal samples collected from each beetle over 2 months and finally on extracts from the cadavers. Some beetles excreted several mg of virus during this period.

DISCUSSION

The indirect sandwich ELISA was considered to be rather more convenient than the direct sandwich system as commercially available enzyme-linked anti-rabbit IgG can be used thus avoiding the need to conjugate several immune sera with enzyme. Specific and non-specific interactions between primary antibody and insect extract (Fig. 1, 4) were not considered important provided that the secondary antibody was absorbed with insect extract; detection of insect extract was then almost completely eliminated (Fig. 2, 3*a*, 4). The specificity of the secondary antibody was therefore a critical factor in the unequivocal detection of virus, though the limit of detection was similar using either absorbed or unabsorbed serum (Fig. 4*a*, *c*). Absorbed secondary antibody at a concentration of 0.5 μ g/ml gave adequate detection of virus since reactions with insect extract were little different from 'background'.

The indirect sandwich ELISA was an efficient means of titrating as little as 20 ng/ml of purified virus. However, when virus and insect extract were diluted together there was a depression of A_{405} and a reduction in the detection limit of virus. This was not a result of interaction between secondary antibody and insect material since absorbed secondary antibody no longer reacted serologically with insect extract (Fig. 4). More likely, insect extract impeded the combination of virus particles with secondary antibody and possibly also with primary antibody (Table 1), thus reducing the sensitivity of the assay. This effect renders the precise quantification of virus levels in crude insect extracts impossible.

Similar results have been obtained by Kelly *et al.* (1978*b*) with assay of iridescent virus type 22 in extracts of *Galleria mellonella* larvae. In contrast these authors found little effect of host protein on detection of a baculovirus in *Heliothis armigera* larvae. Some plant extracts mask detection of plant viruses by ELISA (Clark & Adams, 1977; Flegg & Clark, 1979). Crawford *et al.* (1978) showed that although high levels of extraneous material did not alter the detection level of a direct sandwich RIA for baculovirus polyhedrin, the contaminants reduced binding of labelled antibody by up to 30 %.

Thus the ELISA technique may be effective when assaying purified virus but may, at best, be only semi-quantitative when determining the level of virus in crude host extracts using purified virus as a standard. Our results suggest that quantification may be improved by using as a standard a dilution series of virus in an appropriate host extract.

The data for the single sampling (Table 2) probably underestimate the effects of virus upon the larval population, because incipient infections, below the level of detection of the

assay, were probably present. Nevertheless, 7.3 % of the larval breeding sites were shown to contain virus-infected larvae. By contrast, 84 % of the sample of adults collected in Tonga were shown to be infected with virus and some were so heavily infected that they excreted milligram quantities of virus each week over a period of 1 to 8 weeks (E. C. Young & J. F. Longworth, unpublished data). The amounts of virus in the adults (Table 3) are probably an overestimate since they were made on serial faecal samples which contain very low levels of insect protein by comparison with extracts of whole insects; the virus plus insect extract standard may not have given the best estimate of virus concentration in the adults. However, the diagnoses of presence or absence of virus are accurate.

The results of this survey, and the diagnoses of virus infection by means of the ELISA method are consistent with previous surveys and bioassays for virus (Young 1974; Zelazny, 1976). Virus-infected adults are long-lived and mobile and, though infertile, they continue to mate and attempt to lay eggs. Thus they transmit virus to their partners when mating and to larvae by defecation in the breeding sites which they visit. The ELISA technique is a convenient, specific and sensitive diagnostic tool with which to approach studies on the ecology of insect viruses.

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