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Quantitation of serological cross-reactivity between two geographical isolates of *Oryctes* baculovirus by a modified ELISA

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Summary

An assay was developed for quantitation of the antigenic relationship between viruses, by modification of the indirect ELISA. The principle of this method is to estimate the epitopes not shared between the related viruses, after titration of the antibodies specific to the common epitopes as in a blocking ELISA. In practice, varying concentrations of purified virus are preincubated with a fixed dilution of heterologous or homologous antiserum and the unbound antibodies present in the mixture are back titrated with virus particles bound to microtitre plates. The antigenic relationship is described in terms of differentiation index (DI) and total antigenic reactivity (TAR). This method has been used to quantitate cross-reactivity between two geographically different isolates of *Oryctes* baculovirus.

Modified ELISA; Antigenic cross-reactivity; *Oryctes* baculovirus isolate; Total antigenic reactivity

Introduction

Variations of enzyme linked immunosorbent assays (ELISA) are increasingly being used for the study of antigenic relationship between viruses (Barbara et al., 1978; Kelly et al., 1978; Koenig, 1978; Clark and Bar-Joseph, 1984). Indirect ELISA (Jaegle and Van Regenmortel, 1985) and its variations such as double an-

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tibody sandwich ELISA (Van Regenmortel and Burckard, 1980), ELISA using $F(ab')_2$ fragments of virus specific immunoglobulins as the capture antibodies (Barbara and Clark, 1982) and protein A sandwich ELISA (Edwards and Cooper, 1985; Hughes and Thomas, 1988) have been used to quantitate serological cross reactions among viruses. We now describe another modification of indirect ELISA. The modified procedure has been used to discriminate between two geographical isolates of *Oryctes* baculovirus. *Oryctes* baculovirus is a viral pathogen of *Oryctes rhinoceros*, an insect pest of coconut and oil palm and has been successfully used as a biocide to control the pest (Bedford, 1980). A Philippines isolate of *Oryctes* baculovirus (PV 505) has been previously characterised (Crawford and Sheehan, 1985; Crawford et al., 1985). The viral genome is approximately 128 Kbp and 27 protein bands have been visualised after electrophoresis. The Indian isolate of *Oryctes* baculovirus is a large bacilliform enveloped virus with a genome (DNA) size of approximately 126 Kbp (Mohan and Gopinathan, unpublished). Analysis of the total viral proteins revealed 43 species ranging from 9.9–217 kDa. An antigenic comparison between the two virus isolates is described.

Materials and Methods

Viruses and antisera

The Indian isolate (KI) of *Oryctes* baculovirus was obtained from the wild population of rhinoceros beetles, *Oryctes rhinoceros*, in Kerala State, India. The virus isolate PV 505 was obtained from Dr. Crawford, DSIR, Auckland, New Zealand. Both KI and PV 505 were purified from the excreta of infected *O. rhinoceros* beetles (Payne et al., 1977) by a series of sucrose gradient centrifugations (Payne, 1974). The virus preparations were further purified by immunoaffinity chromatography. For the latter purpose antisera to total viral proteins from both isolates were raised in rabbits using standard immunisation procedures. The sera from different bleedings were pooled and inactivated, and the titres were determined. Virus specific antibodies were coupled to CNBr-activated Sepharose 4B (Pharmacia) and used for affinity chromatography. The adsorbed virus particles were eluted at 0.23–0.25 M NaCl in 50 mM Tris-HCl, pH 8.0, and the peak fractions (based on $A_{260/280nm}$) were pooled and centrifuged at 100000 × *g* for 1 h to pellet the virus.

ELISA

Two different ELISA procedures were adopted. Polystyrene microtiter plates (Nunc, Denmark) were used throughout.

Indirect ELISA. The procedure described by Clark and Barbara (1987) was followed except for the use of whole antibody molecules instead of $F(ab')_2$ fragments. Briefly, the wells were coated overnight with 100 µl of 2.5 µg/ml of KI and PV505 and reacted with doubling dilutions of anti-KI antiserum (1/200 to 1/204800). In the reverse titration 5 µg/ml PV 505 and KI were used for coating the wells and reacted with doubling dilutions of anti-PV505 antiserum (1/200 to 1/204800). Af-

finity purified goat-antirabbit IgG conjugated to alkaline phosphatase at 1/3000 dilution was used for detection. The working concentrations of viruses and antisera were determined by prior chequerboard titration.

Modified indirect ELISA. The indirect ELISA was modified by introducing preincubation of virus with heterologous/homologous antisera to block antibodies against common epitopes between the two viruses, thus enabling the detection and quantitation of epitopes specific to the virus bound to ELISA plate. In this modification, varying concentrations of KI were preincubated with a fixed dilution of homologous (antiKI) or heterologous (anti-PV505) antisera. The excess of unbound antibodies present, if any, were titrated against a fixed concentration of KI or PV505, bound to microtitre wells. The first two rows of wells were coated with 100 μl of KI (2.5 μg/ml) in standard phosphate buffered saline (PBS), pH 7.8. This served as 'capture' antigen. The last well in each row contained PBS alone as control. The wells were washed thrice with PBS (Tween-20 was excluded to prevent any damage to viral envelope) and each well was blocked with 150 μl of 2% bovine serum albumin (BSA) in PBS, at room temperature (RT, 23°–25°C) for 2 h.

KI and PV505 were serially diluted in PBS in two separate sets of Eppendorf tubes, to contain 10, 7.5, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 μg/ml of free antigen. Equal volumes of 1/2000 diluted anti-KI antiserum in PBS+0.5% BSA were added, mixed well and incubated at RT for 3 h. The antiserum used was always homologous to the 'capture' antigen employed. The working concentrations of 'capture' and 'free' antigens and dilution of antiserum used in the assay were determined beforehand by chequerboard titration. The samples (100 μl each) from KI + antiKI sets were added to the first row of wells and 100 μl samples of PV505 + antiKI set were added to the second row of wells in the microtitre plate. The plates were incubated at RT for 2 h and washed thrice with PBS. Subsequently 100 μl of 1/3000 diluted goat-antirabbit IgG coupled to alkaline phosphatase (Pel Freez Biologicals, U.S.A.) in PBS + 0.5% BSA was added and incubated at RT for 1 h. This was followed by washing thrice with PBS and addition of 100 μl of p-ni-

TABLE 1

Schematic representation of the modified ELISA

1.	Coat two rows of wells in a microtitre plate with purified KI (as 'capture' antigen) for overnight at 4°C.
2.	Make serial two fold dilutions of: (a) KI ('free' antigen) and (b) PV505 ('free' antigen) separately in two sets of tubes. Add diluted antiKI antiserum to both sets of tubes and incubate for 2 h at RT.
3.	Add 2 (a) to the first row of wells and 2 (b) to the second row of wells.
4.	Incubate for 3 h at RT.
5.	Wash, add diluted goat-antirabbit IgG-HRP (or alkaline phosphatase) conjugate to both rows of wells and incubate.
6.	Add *o*-phenylene diamine (or *p*-nitrophenyl phosphate) in appropriate buffers to the wells, develop colour and read absorbance 450/405nm.
7.	In parallel, coat the third and fourth row of wells with purified PV 505 and perform the assay as above, using KI and PV 505 as free antigens and antiPV505 antiserum.

trophenyl phosphate (Sigma, 0.1% dissolved in 9.7% v/v ethanolamine, pH 9.8, in PBS) to each well and incubated for 1/2 h at RT. The reaction was stopped with 50 µl of 3 N NaOH and A_{405nm} was read in an ELISA reader (Biotech Instruments, U.S.A.).

The assay was also repeated using goat-antirabbit IgG conjugated to horse radish peroxidase (HRP). In this detection system, *o*-phenylenediamine (0.04% in 0.05 M sodium citrate buffer, pH 5.0, containing 0.05% of 30% H_2O_2) was used as substrate. The enzyme reaction was carried out for 1/2 h, stopped with 50 µl 4 N H_2SO_4 and A_{450nm} was recorded. As controls, preimmune serum, no 'capture' antigen and no 'free' antigen were included in each row (Table 1).

In the same ELISA plate the third and fourth rows were coated with 5 µg/ml PV505 as 'capture' antigen. Anti-PV505 antiserum (sodium sulfate fraction, diluted 12 800×) was incubated with either PV505 or KI (as 'free' antigen) and the assays were carried out as in the previous case.

Results

The steps of the modified ELISA are schematically summarised in Table 1. In this method the antibodies were preincubated with varying concentrations of either homologous or heterologous virus before incubating with the microtitre plate-bound virus which was always homologous to the antiserum used. Fig. 1A depicts the titration curves of the modified ELISA using HRP-antibody conjugate. As expected, there was an overall reduction in absorbance for the homologous antigen-antibody systems viz. KI+antiKI antiserum (with KI as 'capture' antigen) and PV 505 + anti PV 505 (PV 505 as 'capture' antigen). A notable feature (Fig. 1A) is the conspicuous lag in the homologous antigen-antibody system at higher 'free' virus concentrations. This reflects the near total neutralisation of antibodies by the 'free' virus. For instance, when varying concentrations of PV 505 as 'free' antigen were titrated against antiPV 505, a lag phase until ln 7.6 was observed indicating the total binding of antibodies to 'free' antigen or absence of unbound antibodies for colour development. In the KI+anti PV505 system, antibodies against epitopes on PV 505 not common with KI are available for binding to the 'capture' antigen and hence the absence of a lag period. Applying a principle similar to that of serological differentiation index (SDI) used for the quantitation of antigenic relationship among viruses (Jaegle and Van Regenmortel, 1985), the concentration of 'free' virus accounting for a particular reference absorbance value (0.79 in Fig. 1A) for all the curves, was computed. It can be further seen in Fig. 1A that a concentration of ln 5.8 (=330 ng virus/ml) of KI as 'free' antigen was able to produce a blocking effect on the colour development to the same extent as ln 7.6 (= 1998 ng/ml) of PV505. The differentiation index (DI, defined as the difference in ln virus concentrations accounting for the same reference absorbance value) was 1.8. In the reverse assay with PV 505 as 'capture' antigen and antiPV505 antibodies, the DI was only 0.75. This indicated the lack of reciprocal cross reactivity to the same degree between KI and PV 505. Such a phenomenon was reported for other

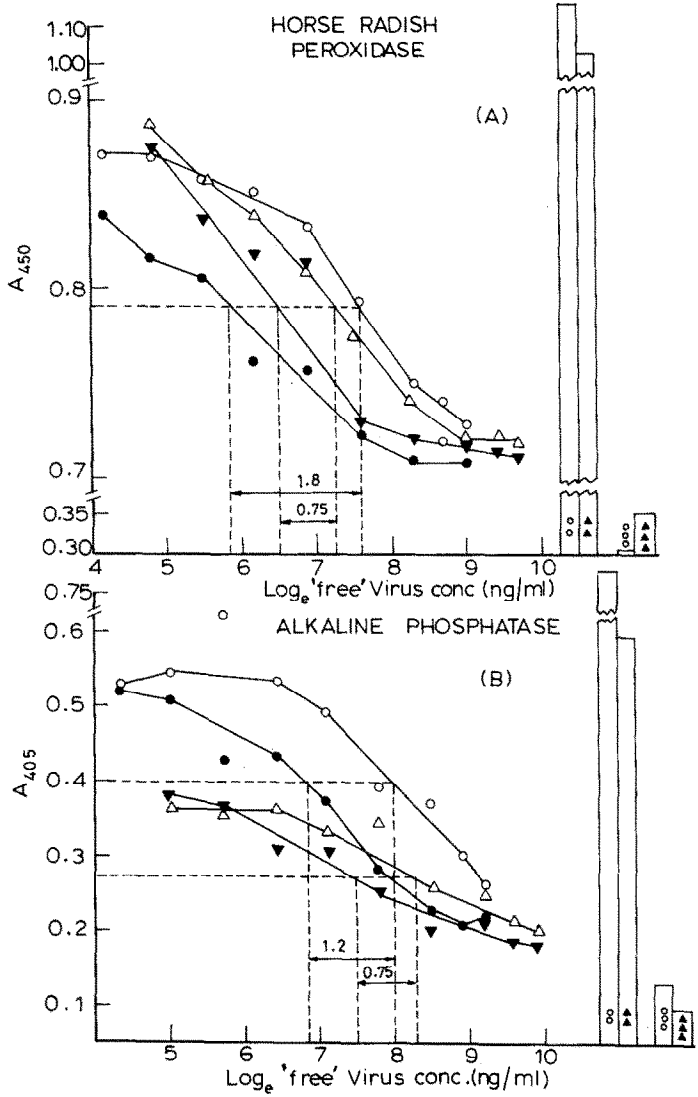

Fig. 1. Antigenic relatedness between the *Oryctes* baculovirus isolates using modified ELISA. Experimental details are given in text. (○) PV505 titrated against antiKI antibodies; (●) KI titrated against antiKI antibodies; 'capture' antigen was 2.5 μg/ml KI in both; (⊗) positive control; (△) KI titrated against antiPV505 antibodies; (▼) PV505 titrated against antiPV505 antibodies; 'capture' antigen was 5 μg/ml PV505 in both; (▲) positive control, (○▲)-negative controls. The enzyme detection systems used were HRP (in Fig. 1A) and alkaline phosphatase (in Fig. 1B).

virus systems also (Barbara and Clark, 1982). The slopes of the curves in Fig. 1A were transformed into regression lines using the least squares method (Clark and Barbara, 1987) in order to obtain an accurate value of DI and the DI values were computed from these (Fig. 2A and Table 2).

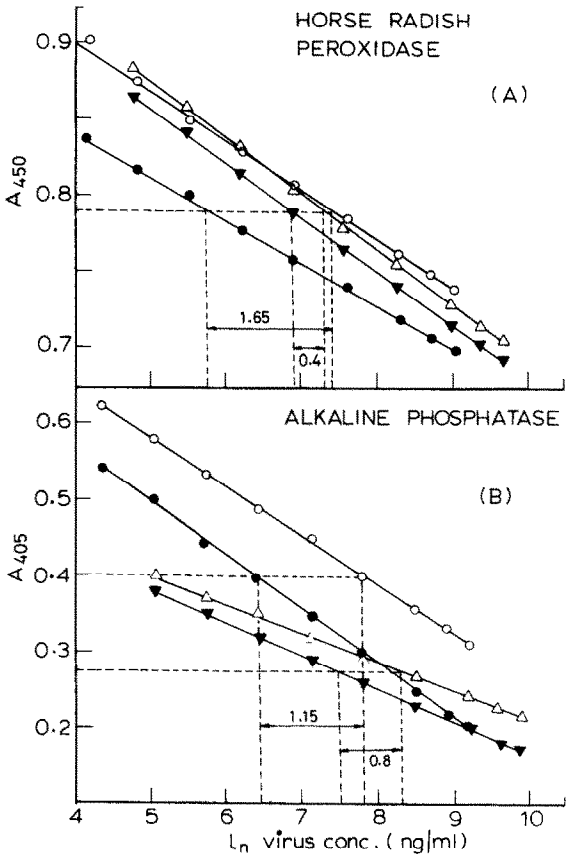

Fig. 2. Regression analysis. (A) Regression lines for curves in Fig. 1A; (B) regression lines for curves in Fig. 1B.

The nature of the curves obtained in the assays performed with alkaline phosphatase antirabbit IgG conjugate system (Fig. 1B) was similar to that obtained with HRP system, including the lack of reciprocal cross reactivity between the virus isolates. Transformation of values in Fig. 1B into regression lines is shown in Fig. 2B and the DI values obtained in this system are shown in Table 2.

Different batches of KI and PV 505, independently purified, yielded consistent DI values in this modified ELISA method. The ELISA test was always carried out with freshly purified preparations. Long storage or freeze-thawing of virus stocks gave inconsistent DI values mainly due to the breakdown of viral proteins (as inferred from the SDS-PAGE pattern of stored viruses).

The serological difference between the two viruses was confirmed by the conventional indirect ELISA in which fixed concentrations of KI/PV 505 were titrated against serial dilutions of homologous and heterologous antisera. SDI as described by Jaegle and Van Regenmortel (1985) was computed from the regression lines (Fig. 3B) for the titration curves in Fig. 3A (see Table 3).

TABLE 2

Quantitation of cross-reactivity by modified ELISA

Enzyme-antibody conjugate (Detection system)	'Capture' antigen	'Free' antigen	Antiserum	ln Virus concentration (Fig. 1A,B)	Regression lines (Fig. 2A,B)	Differentiation index (DI)	Total antigenic reactivity (TAR) (ng)	TAR ratios KI:PV505
Goat-antirabbit IgG-horse radish peroxidase	KI	KI	antiKI	5.8	5.75		314[b]	
						1.65[a]		1:5.21
	KI	PV 505	antiKI	7.6	7.40		1636	
	PV 505	KI	antiPV505	7.25	7.3		1480	
						0.4		1.49:1
	PV 505	PV 505	antiPV505	6.50	6.9		992	
Goat-antirabbit IgG-alkaline phosphatase	KI	KI	antiKI	6.8	6.45		633	
						1.35		1:3.86
	KI	PV 505	antiKI	8.0	7.8		2441	
	PV 505	KI	antiPV505	8.25	8.3		4024	
						0.8		2.23:1
	PV 505	PV 505	antiPV505	7.5	7.5		1808	

[a]Values based on regression lines.
[b]Based on virus protein content.

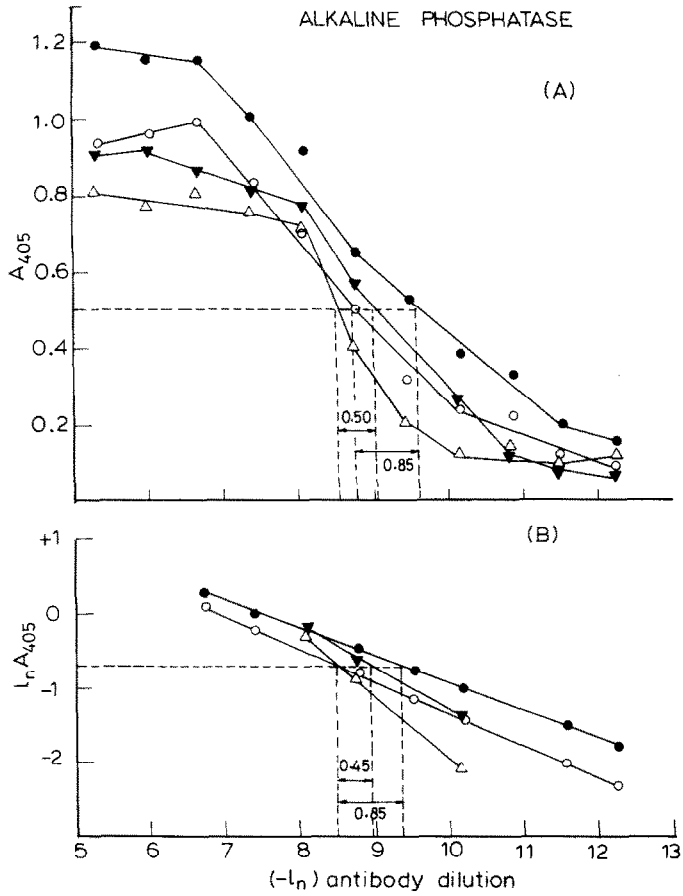

Fig. 3. Computation of serological differentiation index (SDI). (A) SDI calculation for the titration of viruses with homologous and heterologous antisera. (●) KI titrated against antiKI antibodies; (○) PV505 against antiKI; (△) KI against antiPV505; (▼) PV505 against antiPV505. (B) Double ln transformation of values in Fig. 3A into regression lines.

In the indirect ELISA procedure the total antibodies are available to the detection system. In contrast, in the modified ELISA described the level of remaining antibodies (obtained after adsorption with 'free' antigen) only are available for detection. Hence if the midpoints of the titration curves (Fig. 1A and B) are 2-fold or more than the basal values of negative controls (pre-immune serum and no 'capture' antigen controls) the data can be considered significant.

TABLE 3

Quantitative measurement of cross-reactivity between KI and PV505 by indirect ELISA

	(-ln value)	SDI[a]
KI titrated against antiKI antiserum	9.35	
		0.85
PV505 titrated against antiKI antiserum	8.50	
PV 505 titrated against anti PV505 antiserum	8.95	
		0.45
KI titrated against antiPV505 antiserum	8.50	

[a]See Fig. 3B.

Discussion

In the indirect ELISA described by Jaegle and Van Regenmortel (1985) for the estimation of antigenic cross reactivity, the viruses are antigenically discriminated based on the titre of antibodies required to elicit the same absorbance value. The viruses being compared are titrated separately. The modified ELISA described by us involves blocking of antibodies against epitopes common to both viruses by 'free' antigen and thus become unavailable to the 'capture' antigen bound to the plate. Hence, only the antibodies for which there are no epitopes on 'free' antigen to bind, or cross reacting antibodies with greater affinity for epitopes on 'capture' antigen would finally be responsible for the formation of the colour product of the detection system. The intensity of the colour, therefore, serves as a measure of the heterogeneity between the 'free' and 'capture' antigens under standardised concentrations of all the three reactants (done using chequerboard titration). When the 'capture' and 'free' antigens refer to the same virus, the concentration of antibodies binding to 'capture' antigens is a function of the concentration of 'free' antigen, because the higher the 'free' antigen concentration, the lower would be the concentration of antibodies available to the 'capture' antigen. At high ratios of free:capture antigens (e.g. 3:1, 4:1 used in the experiment) the 'free' antigens would bind almost all the available antibodies during preincubation leaving nothing for the 'capture' antigens. In practice, however, a basal absorbance (Fig. 1A, B) in the regions of high 'free' antigen concentration was obtained. This is due to dissociation of a small proportion of antibodies from 'free' antigen and binding to 'capture' antigens. Conversely at low concentrations of 'free' antigen, a high absorbance results.

In assays where the 'free' and 'capture' antigens were different viruses, antibodies having no specific epitopes on 'free' antigen should bind to the 'capture' antigen independent of the concentration of 'free' antigens. Hence even at high ratios of 'free':'capture' antigen, the colour formed is due to antibodies binding to epitopes specific to 'capture' antigens. In addition, crossreacting antibodies with greater affinity towards 'capture' antigen would also contribute to the colour in-

tensity. The modified ELISA takes into account the heterogeneity between viruses not only due to virus-specific epitopes but also the interplay of the relative affinities of cross-reacting epitopes since the viruses being compared are in the same reaction mixture.

This modified procedure has been applied to the quantitation of serological cross-reactivity between two different geographical isolates of *Oryctes* baculovirus.

Differentiation index (analogous to SDI) has been used to measure the overall antigenic heterogeneity between KI and PV 505. Also, in the modified ELISA established by us, it was possible to equate the viruses being compared (in ng protein) in terms of total reactive antigens when titrated against the homologous or heterologous antiserum. For instance, in Table 2, 1 ng of KI is equivalent to 5.21 ng of PV 505 in total antigenic reactivity (TAR) when titrated against antiKI antiserum, whereas 1.49 ng of KI is equivalent to 1 ng of PV 505 against antiPV 505 antiserum. Hence the difference in TAR expressed as a ratio is a more direct measure of the heterogeneity in the total reactivity of antigens between the viruses. The consistently low DI obtained between KI and PV 505 (0.4 and 0.8 in Table 2) with PV 505 as 'capture' antigen and antiPV 505 antiserum, as against the values 1.65 and 1.35 obtained when KI was used as 'capture' antigen and antiKI antiserum, leads us to infer that epitopes on KI are stronger in cross-reactivity and hence are able to bind a larger proportion of antibodies in antiPV 505 serum compared to the binding affinity of PV 505 towards antibodies in antiKI serum. Lack of such reciprocal cross-reactivity to the same degree has been reported for other viruses also (Barbara and Clark, 1982). SDI between KI and PV 505 (Table 3) calculated as per indirect ELISA procedure (Jaegle and Van Regenmortel, 1985) also substantiated the antigenic heterogeneity between KI and PV 505. This method also supported the skewed reciprocal crossreactivity.

Western blots of total proteins of PV 505 and KI when developed with homologous and heterologous antisera showed differences, albeit small, in the mobilities of protein bands (Mohan and Gopinathan, unpublished data). When restriction endonucleases were used to discriminate the genomes of KI and PV505, there were no differences in the restriction patterns with respect to 7 enzymes and barely detectable differences were seen with 3 enzymes (data not shown). The genome size is large (approx. 126 Kbp); there are many restriction fragments and it was difficult, therefore, to detect these minor differences. DNA reassociation kinetics in solution between homologous and heterologous DNA fragments also did not reveal any difference, because of the limited sensitivity of this method (Mohan and Gopinathan, unpublished data). For these reasons, the present method can be considered as a superior alternative.

Acknowledgement

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References

Barbara, D.J. and Clark, M.F. (1982) A simple indirect ELISA using $F(ab')_2$ fragments of immunoglobulin. J. Gen. Virol. 58, 315–322.

Barbara, D.J., Clark, M.F., Thresh, J.M. and Casper, R. (1978) Rapid detection and serotyping of prunus necrotic ring spot virus in perennial crops by enzyme-linked immunosorbent assay. Ann. Appl. Biol. 90, 395–399.

Bedford, G.O. (1980) Biology, ecology and control of palm rhinoceros beetles. Ann. Rev. Entomol. 25, 309–339.

Clark, M.F. and Barbara, D.J. (1987) A method for the quantitative analysis of ELISA data. J. Virol. Methods 15, 213–222.

Clark, M.F. and Bar-Joseph, M. (1984) Enzyme immunosorbent assays in plant virology. In: K. Maramorosch and H. Koprowski (Eds.), Methods in Virology, Vol. VII, pp. 51–85. Academic Press, London.

Crawford, A.M., Ashbridge, K., Sheehan, C. and Faulkner, P. (1985) A physical map of the *Oryctes* baculovirus genome. J. Gen. Virol. 66, 2649–2658.

Crawford, A.M. and Sheehan, C. (1985) Replication of *Oryctes* baculovirus in cell culture: viral morphogenesis, infectivity and protein synthesis. J. Gen. Virol. 66, 529–539.

Edwards, M.L. and Cooper, J.I. (1985) Plant virus detection using a new form of indirect ELISA. J. Virol. Methods 11, 309–319.

Hughes, J.d.'A. and Thomas, B.J. (1988) The use of protein A-sandwich ELISA as a means for quantifying serological relationships between members of the tobamovirus group. Ann. Appl. Biol. 112, 117–126.

Jaegle, M. and Van Regenmortel, M.H.V. (1985) Use of ELISA for measuring the extent of serological cross-reactivity between plant viruses. J. Virol. Methods 11, 189–198.

Kelly, D.C., Edwards, M.L. and Robertson, J.S. (1978) The use of enzyme-linked immunosorbent assay to detect and discriminate between, small iridescent viruses. Ann. Appl. Biol. 90, 369–374.

Koenig, R. (1978) ELISA in the study of homologous and heterologous reactions of plant viruses. J. Gen. Virol. 40, 309–318.

Payne, C.C. (1974) The isolation and characterization of a virus from *Oryctes rhinoceros*. J. Gen. Virol. 25, 105–116.

Payne, C.C., Compson, D. and De Looze, S.M. (1977) Properties of the nucleocapsids of a virus isolated from *Oryctes rhinoceros*. Virology 77, 269–280.

Van Regenmortel, M.H.V. and Burckard, J. (1980) Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assays (ELISA). Virology 106, 327–334.