Acta Phytopathologica et Entomologica Hungarica 36 (1–2), pp. 1–8 (2001)

Petri Dish-ELISA, a Simple and Economic Technique for Detecting Plant Viruses

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A large number of microtiter plates are needed for mass testing of planting material for viruses in seed certification and plant quarantine. In the case of poorly equipped laboratories, problems with availability of microtiter plates have economic implications for the broad acceptance of enzyme-linked immunosorbent assay (ELISA) for e.g. seed health testing in developing countries. In this experiment the potential of an alternative, cheaper technique was investigated. A conventional indirect ELISA procedure was followed for comparison between the polystyrene solid phases of plastic Petri dishes and microtiter plates for detection of three viruses belonging to the *Tobamovirus, Comovirus* and *Potyvirus* genera. A wax pen was used to divide the inner surface of a polystyrene Petri dish into many circles or squares. The hydrophobic boundaries thus provided were effective in separating 50 µl-drops throughout the ELISA procedure. In comparative assays of serial dilutions of the three viruses, the ELISA in Petri dishes resulted in similar or higher A_{405} values than the ELISA carried out in microtiter plates, suggesting a similar or better protein binding capacity of Petri dishes. The perspectives of this alternative method are briefly discussed.

Keywords: ELISA, microtiter plate, Petri dish, polystyrene.

Since its introduction in plant virology in the late 1970 (Voller et al., 1976; Clark and Adams, 1977) enzyme-linked immunosorbent assay (ELISA) has become the most widely used technique for various kinds of virus detection. Although ELISA puts fewer demands on equipment and facilities than the nucleic acid-based detection methods, performing conventional ELISA still meets some constraints in laboratories in the third world. In developing countries in the tropical and subtropical zones, where a great variety of virus-susceptible crop species are grown and the all-year virus vector pressure is high, management of virus diseases is particularly important. An essential part of this management is the availability and use of healthy planting material through seed certification and plant quarantine inspections, which are often not efficient due to a lack of laboratory equipment and materials. There is therefore a marked need for the development of simple, cheap, accurate and rapid testing methods suitable for large-scale routine use (Lange and Heide, 1986). Some simplified variants of enzyme-linked immunoassay (EIA) have been developed such as dot immunobinding assay (DIBA) using plain paper instead of expensive nitrocellulose membranes (Heide and Lange, 1988) and tissue blotting immunoassay (TBIA) (Lin et al., 1990 and others).

Several variants of ELISA have been developed, but they all, to our knowledge, have in common the need for microtiter plates and a spectrophotometric plate reader, often difficult to procure in the third world because of their high prices and the scarcity of foreign exchange for their import. The maintenance/repair of ELISA readers often also poses a difficulty.

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0238–1249/2001/$ 5.00

The experiments described here were undertaken to clarify the potential of a Petri dish-ELISA technique, designed by the authors, and to determine to what extent this technique fulfils the demands for sensitive and accurate routine detection of plant viruses.

Materials and Methods

Antigens and antisera

Dry leaf materials infected with tomato mosaic (ToMV, *Tobamovirus*), cowpea mosaic (CPMV, *Comovirus*) and blackeye cowpea mosaic (BlCMV, *Potyvirus*) were obtained from the virus culture collection of the Danish Government Institute of Seed Pathology (DGISP). The infected tissues, which have been stored over desiccant $CaCl_2$ were ground in the presence of phosphate buffered saline, 0.05M, pH7.2 (PBS) using 1 part of tissue + 9 parts of PBS (w/v). The extracts, considered to correspond closely to original extracts of fresh plant material as regards virus concentration, were centrifuged at 10,000 rpm for 10 min in a Heraus Biofuge-15 centrifuge to remove chloroplasts and plant debris. Series of dilutions were made by further diluting the supernatants from X100 to X25600 in PBS, thus with virus concentrations close to those which could be obtained from serial dilutions of fresh plant tissue. Extracts of healthy leaf material from the same species were prepared in the same manner for use as negative controls at corresponding serial dilutions. Polyclonal antisera of ToMV, CPMV and BlCMV were obtained from the antiserum bank of the DGISP. The antisera were diluted 1/1000 in serum buffer (PBS + 2% polyvinylpyrrolidone + 0.2% chicken egg albumin).

ELISA procedures

An indirect-ELISA procedure, similar to that described by Hobbs et al. (1987) for the DAC (direct antigen coating) method, was followed when comparing the polystyrene solid phases of plastic Petri dishes and microtiter plates (Nunc, Maxisorp™ F=96, Denmark). The comparative experiments were performed simultaneously. Standard polystyrene Petri dishes of 9 cm diam. (Kerial Plast, Denmark) were prepared for ELISA by dividing the inner surface into many circles or squares with a hydrophobic substance, either by use of a DAKO™ pen used for immuno histochemical tests or, even more efficiently, by a wax-based crayon (of the kind used for children's colouring books). A paper template of 45 squares (1x1 cm) placed under the Petri dish was used for convenient dividing and for numbering the positions of the circles/squares. Thus in each Petri dish up to 45 samples could be processed. An orientation point was marked both on the plate edge and the template. Any liquid loaded remained drop-shaped inside the area of the square or circle due to the hydrophobic action of the wax, which made a barrier around the drop *(Fig. 1).*

Throughout the Petri dish procedure, 50 µl of each of the reactants were loaded carefully into the centre of each circle or square using a precision pipette. Eight replicates were made for each dilution. After loading the antigen dilutions, the Petri dish was incubated under a lid for 1 h at 37 ºC. Then the Petri dish was unloaded in one swift movement

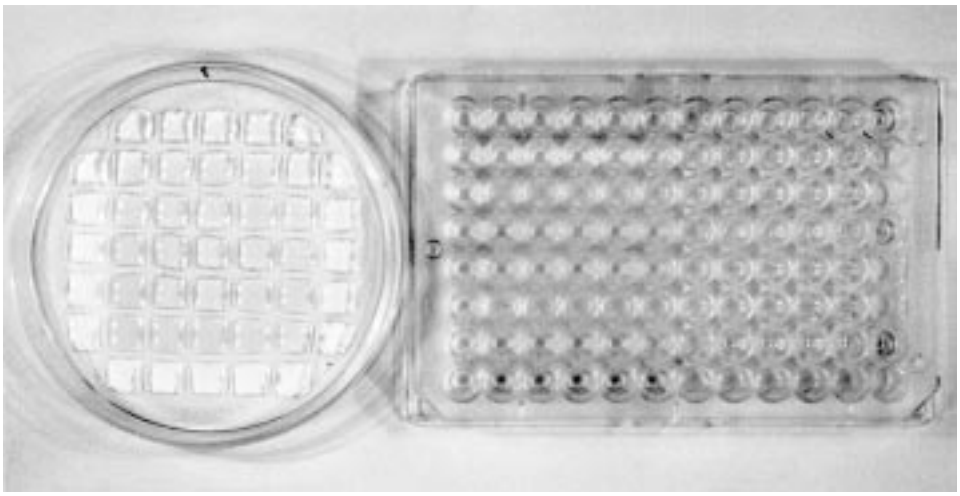

Fig. 1. ELISA carried out in polystyrene Petri dish and microtiter plate. As shown, reagents remain drop-shaped inside the squares drawn by a hydrophobic wax pen

and rinsed with PBST (PBS + 0.02% KCl + 0.05% tween 20) followed by covering the inner surface with three changes of PBST. Washing was done by slowly rotating the buffer inside the plate for 3 min between each change. After the wash the Petri dish was gently blotted by touching the inner surface of the plate with a piece of filter paper without causing any damage to the hydrophobic wax lines. The two next reactants, i.e. dilutions of crude specific antisera and swine anti-rabbit serum enzyme conjugate, were loaded into the circles/squares and allowed to incubate under a lid, each for 1 h at 37 ºC. Washing as described above followed both incubations. Finally, each square or circle was loaded with substrate solution (approx. 0.4 mg/ml of p-nitro phenyl phosphate in substrate buffer). In the experiments comparing Petri dish-ELISA with microtiter plate-ELISA, each sap dilution was loaded and processed in four replicates.

A parallel procedure in microtiter plates was carried out, using 100 µl of each reactant and three replicates per dilution, but otherwise according to Hobbs et al. (1987) and with the same concentrations, incubation times and temperatures as used for Petri dishes.

After 30 min incubation at room temperature the substrate was examined visually for a yellow colour development indicating positive reactions, and after a further 30 min the substrate reaction was stopped both in Petri dishes and microtiter plates by adding 5 µl of a 3 M NaCl solution to each square/circle and each well. Then the reactions in microtiter plate wells were read spectrophotometrically by an ELISA reader (Titertek Multiscan™) at 405 nm wavelength. To read the absorbance values of Petri dish reactions by ELISA reader, samples from all the 8 replicates of each dilution were transferred into wells of a clean microtiter plate by a precision pipette. Three sets of Petri dish replicates per dilution were combined in this way making three wells of 100 µl. All sets of Petri dish replicates were combined in this way, and finally a comparison between the absorbance values from the microtiter plate ELISA and the Petri dish-ELISA for detecting the three viruses was made.

Results

The data presented are reading averages of replicates from two experiments, performed in both Petri dish and microtiter plate. Absorbance values representing ELISA reactions of serially diluted ToMV, CPMV, and BlCMV-infected samples and healthy controls are presented in *Table 1*, and for convenient comparison also graphically in *Fig. 2*. Antigen-antibody reactions for all dilutions of infected samples were positive in the polystyrene Petri dishes as well as in the microtiter plates processed in parallel. Even at the highest dilution (1:25600) of infected tissue in buffer the viruses could be detected in both systems, still with absorbance values 7–16 times higher than in the corresponding dilution of healthy material. Dilutions ranging to the limit of detection and beyond were not included in these experiments, the purpose of which was to compare the sensitivity of two detection systems.

Table 1 (A)

Comparison between ELISA in polystyrene Petri dish and microtiter plate for detection of tomato mosaic virus (average absorbance values)

Antigen Dilution	ToMV *Tobamovirus*			
	Petri dish		Microtiter plate	
	H*	I	H	I
	**			
1/100	0.086 *0.002*	2.010 *0.004*	0.061 *0.003*	1.134 *0.005*
1/200	0.075 *0.002*	1.965 *0.004*	0.051 *0.001*	0.795 *0.006*
1/400	0.074 *0.003*	1.838 *0.005*	0.049 *0.001*	0.777 *0.003*
1/800	0.071 *0.001*	1.767 *0.004*	0.047 *0.001*	0.722 *0.001*
1/1600	0.071 *0.001*	1.662 *0.003*	0.046 *0.002*	0.618 *0.006*
1/3200	0.068 *0.002*	1.311 *0.004*	0.045 *0.001*	0.599 *0.008*
1/6400	0.065 *0.003*	1.113 *0.005*	0.045 *0.001*	0.542 *0.003*
1/12800	0.051 *0.001*	0.829 *0.006*	0.039 *0.001*	0.530 *0.003*
1/25600	0.040 *0.001*	0.665 *0.002*	0.011 *0.001*	0.425 *0.004*

* H = healthy, I = infected,

** values are the standard deviation within each mean of absorbance readings.

The data presented show that the A_{405} values of infected samples for the 3 viruses at the dilutions 1:200–1:6400 were often one and a half and sometimes two times higher for ELISA performed on plastic Petri dishes compared with those of microtiter plates. Readings tended to be more similar at higher dilutions (1:12800 and 1:25600) for infected samples with ToMV and CPMV *(Table 1* and *Fig. 2).*

The A_{405} values for healthy samples were proportionally low in both systems. However, the ratios of corresponding A_{405} values for infected samples/A_{405} values for healthy samples, indicating the specificity and sensitivity of the assays, were higher for the Petri dish (PD) system than for the microtiter plate (MP) system. E.g. for BlCMV the

Table 1 (B)

Comparison between ELISA in polystyrene Petri dish and microtiter plate for detection of cowpea mosaic virus (average absorbance values)

Antigen Dilution	CPMV *Comovirus*							
	Petri dish				Microtiter plate			
	H*		I		H		I	
1/100	0.064	*0.001***	2.181	*0.005*	0.082	*0.001*	1.638	*0.005*
1/200	0.058	*0.002*	1.846	*0.007*	0.072	*0.003*	1.452	*0.005*
1/400	0.057	*0.001*	1.604	*0.005*	0.054	*0.003*	1.423	*0.004*
1/800	0.052	*0.001*	1.446	*0.007*	0.053	*0.003*	0.963	*0.006*
1/1600	0.051	*0.001*	1.323	*0.004*	0.050	*0.002*	0.884	*0.005*
1/3200	0.050	*0.001*	1.121	*0.002*	0.049	*0.002*	0.660	*0.004*
1/6400	0.049	*0.001*	0.880	*0.003*	0.044	*0.003*	0.618	*0.006*
1/12800	0.049	*0.001*	0.619	*0.008*	0.043	*0.001*	0.529	*0.001*
1/25600	0.049	*0.001*	0.567	*0.008*	0.043	*0.001*	0.417	*0.001*

* H = healthy, I = infected,

** values are the standard deviation within each mean of absorbance readings.

Table 1 (C)

Comparison between ELISA in polystyrene Petri dish and microtiter plate for detection of blackeye cowpea mosaic virus (average absorbance values)

Antigen Dilution	BICMV *Potyvirus*							
	Petri dish				Microtiter plate			
	H*		I		H		I	
1/100	0.064	*0.005***	2.327	*0.008*	0.082	*0.001*	1.684	*0.005*
1/200	0.058	*0.002*	2.179	*0.006*	0.072	*0.001*	1.272	*0.003*
1/400	0.057	*0.002*	2.107	*0.004*	0.054	*0.001*	1.159	*0.002*
1/800	0.052	*0.001*	1.943	*0.004*	0.053	*0.001*	1.009	*0.002*
1/1600	0.051	*0.001*	1.759	*0.006*	0.050	*0.002*	0.993	*0.004*
1/3200	0.050	*0.001*	1.552	*0.009*	0.049	*0.002*	0.725	*0.001*
1/6400	0.049	*0.001*	1.149	*0.003*	0.044	*0.001*	0.560	*0.002*
1/12800	0.049	*0.001*	1.086	*0.007*	0.043	*0.001*	0.509	*0.002*
1/25600	0.049	*0.001*	0.599	*0.005*	0.043	*0.001*	0.293	*0.001*

* H = healthy, I = infected,

** values are the standard deviation within each mean of absorbance readings.

average ratio was 15 in the MP, but 30 in the PD, and for CPMV 17 and 24, respectively. The ratios of the corresponding infected/healthy values in *Table 1* varied from 38 (2.179/0.058 for BlCMV in PD at dil. 200) to 7 (0.293/0.043 for BlCMV in MP at dil. 25600). The results demonstrated improved detectability in the PD system regardless of virion types that in this case represented three virus genera. The visual evaluation of the reactions in the PD system was at least as easy as of those in the MP system *(Fig. 1).*

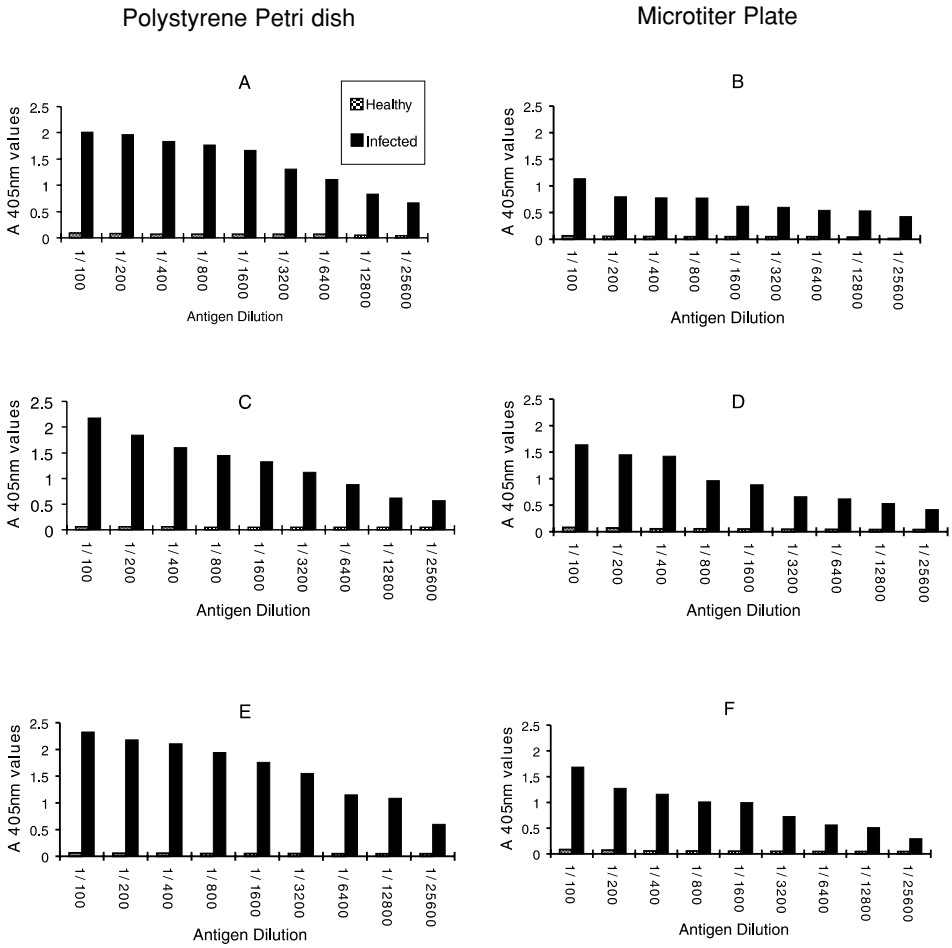

Fig. 2. Comparison between ELISA carried out in polystyrene Petri dish and microtiter plate for detection of 3 viruses belonging to different genera. A–B for ToMV (tobamovirus), C–D for CPMV (comovirus) and E–F for BICMV (potyvirus)

Discussion

A comparison of the absorption values of the two methods suggests a similar or even better protein-binding ability of the polystyrene of the Petri dishes used here than that of the microtiter plates. This was reproduced in a number of comparative tests, made before and after those reported here, and which included also viruses like cucumber mosaic cucumovirus and tomato ringspot nepovirus (data not shown). The performance of different batches or other brands of Petri dishes was not investigated.

An obvious risk one could expect from the Petri dish method is an unintended mixing of reactants between the drops. However, this occurred surprisingly rarely during the several tests made prior to, as well as in, the actual experiments. Provided that the wax boundaries were made accurately and any drops of remaining buffer after wash were removed before adding the subsequent reactant, none of the reactive drops merged. Also, no mixing took place during normal handling, observing that heavy shaking or shocks were avoided when moving the Petri dishes e.g. to and from the incubator.

The colour intensity was easier to rate visually in the drops on the Petri dish bottom than in the wells of the microtiter plate, where disturbing effects of reflection in the walls of the wells occurred. For both systems a light box or a white paper was used as background. To ensure correct conclusions without a spectrophotometric reader it is, however, particularly for the Petri dish method recommendable, prior to each virus/host/test reagent combination in the routine mass-testing to be undertaken, to adjust the test set-up in such a way that clear-cut positive and negative reactions will be obtained. This means choosing the optimum dilutions of extract, antiserum and conjugate, and, if composite samples are tested, not least adjusting the maximum number of plants or seeds to be represented in each sample, allowing the clear-cut detection of one infected plant or seed, even if it has a low virus content, among X healthy ones.

The use of other enzyme/substrate systems such as penicillinase/penicillin using bromothymol blue as the colour indicator (Sudarshana and Reddy, 1989; Abraham and Albrechtsen, 2001, in press) which could improve the visual rating in Petri dish-ELISA because of the clear change from blue to yellow in the presence of virus, should be investigated. Also the performance of other ELISA procedures in the Petri dish assay, such as double-antibody-sandwich (DAS) or triple-antibody-sandwich (TAS) ELISAs should be evaluated.

The main advantage of the Petri dish-ELISA method is the reduced costs. An ELISA reader is not needed, only 50 µl or less is required of each reactant compared to 100 or 200 µl normally used in microtiter plates, and if three Petri dishes replace one microtiter plate, the cost of solid support will be reduced 5- to 8-fold. Compared with the use of microtiter plates Petri dish-ELISA requires, however, some preparative work and more accuracy in the application of the reactants and thereby a slight increase in time/labour, which should be included when calculating the over-all cost.

An alternative way to reduce costs could be the re-use of microtiter plates as suggested by e.g. Manandhar et al. (1996). In this work cleaning microtiter plates by a detergent following a prescribed procedure allowed the safe use of each plate up to four times. It will, however, require the procurement of microtiter plates.

The results indicate that ELISA can be performed as sensitively and specifically in a polystyrene Petri dish as in a microtiter plate. Virions belonging to three virus genera were equally well detected, suggesting that the detectability of different virion types in polystyrene Petri dishes is comparable to that of microtiter plates. The simplicity and the reduced costs may make the method applicable for large-scale testing in poorly equipped laboratories.

Acknowledgement

We are indebted to Drs S. B. Mathur and C. N. Mortensen for their comments and support for the idea of the research work. Thanks to Mrs Lise Hasle for her excellent technical assistance and to Mr M. Ragab for photographing.

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