

THE USE OF DACRON PLATES FOR DOT ENZYME-LINKED IMMUNOSORBENT ASSAY (DOT-ELISA)

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Dacron (polyethyleneterephthalate) is proposed as a matrix for dot-ELISA procedures, as an alternative to nitrocellulose. Plates of dacron were partially hydrazinolyzed and hydrazide groups introduced were converted to azide groups. The derivative dacron-antigen was covalently linked on to the plates through these azide groups. The derivative dacron-antigen was exhaustively washed according to CROOK and antigen was still fixed onto the plates. Protein F1A purified from Yersinia pestis was used as a model. Titration of sera from immunized and non immunized rabbits against this protein was carried out by employing the dot-ELISA method. No significant difference was observed using dacron-antigen and nitrocellulose-antigen preparations. However, both procedures showed to have a significant better performance in comparison with the passive hemagglutination method. The specificity and reproductibility of the dot-ELISA assay using both preparations showed a similar behaviour. Nitrocellulose preparation was stable at 4 °C, 28 °C and -20 °C for 90 days, whereas the dacron-antigen derivative was stable only when stored at 4 °C. Dacron-antigen derivative could be re-used when the spot developing was proceeded using 4-chloro-1-naphthol as substrate.

Key words: dacron – dot-ELISA – *Yersinia pestis*

Hawkes et al. (1982) proposed an immunoassay using nitrocellulose as a support in which the antigen was applied as a spot and called it "dot-immunobinding". Afterwards, Pappas et al. (1983) described a similar method to diagnose visceral leishmaniasis using the second antibody marked with an enzyme and named it "dot-ELISA". This procedure has been widely used because it does not need either sophisticated equipment or specialized personnel. Nitrocellulose is the most widely used matrix due to its ease of handling and the quality of the colour developed (Renner, 1988) although it is very expensive. Other supports have been proposed, such as, diazobenzyloxymethyl (Renart et al., 1979), cellulose acetate membrane (Schaltmann & Pongs, 1980), nylon (Hendry & Herrmann, 1980), diazophenylthioether (Reiser & Wardale, 1981) and filter paper (Rapak & Szewczuk, 1986; Fletcher, 1987).

Dacron (polyethyleneterephthalate) has been used to immobilize enzymes yielding active derivatives (Weetall, 1970; Goldstein, 1977; Carvalho Jr et al., 1986, 1987; Oliveira et al., 1989). Here, a "dot-ELISA" using dacron (plates) as a solid phase is proposed, to which antigen obtained from *Yersinia pestis* was covalently linked. Results were compared with the dot-ELISA on nitrocellulose filters and with the passive hemagglutination assay (PHA).

MATERIALS AND METHODS

Antigen F1A from *Yersinia pestis* avirulent strain A1122 was obtained according to Baker et al. (1952). Sera from rabbits immunized against *Yersinia pestis* F1A antigen protein were prepared following the standard procedure (Baltazard et al., 1956). Sera from non-immunized rabbits and phosphate buffered saline (0.01 M phosphate buffer, pH 7.2, containing 0.8% w/v NaCl – PBS) were used as controls. Rabbit antisera against *Shigella flexneri* polyvalent and *Alkalescens-Dispar* (Inlab, Brazil), *Escherichia coli* invasive polyvalent, *Escherichia*

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coli enteropatogenic classic A group and *Salmonella* polyvalent (Oswaldo Cruz Institute, Brazil), *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Center Disease Control, U.S.A.) were obtained to test cross reactivity. Anti-rabbit IgG labelled with horseradish peroxidase was purchased from Serotec, Oxford, England. 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 4-chloro-1-naphtol were from Sigma, Chem. Co., St Louis, MO. Tween-20 was acquired from Inlab. Dacron was produced by Rhodia do Brazil SA. Nitrocellulose filter (0.22 μ m pore size) was from Millipore, Inc., Bedford, MA. All other reagents of analytical grade were obtained from Merck S.A., Brazil.

Activation of dacron plates (based on Oliveira et al., 1989) – Dacron plates measuring approximately 5 x 5 x 2 mm were incubated with a mixture composed of hydrazine hydrate (10 ml) and methanol (100 ml) at 40 °C for 8 h with continuous stirring. The hydrazide-dacron derivative obtained was washed twice with methanol 100% (50 ml) and methanol 90% v/v (50 ml). The hydrazide groups were converted to azide groups by incubating the treated plates (up to 20) in 5% w/v NaNO₂ (2 ml) in 0.6N HCl (16 ml) at 28 °C (room temperature) for 25 min with stirring. Azide-dacron plates were then washed twice with deionized water, twice with 1 M NaCl and twice with deionized water, dried and used immediately (azide groups are unstable).

Dotting (based on Santos et al., 1988) – F1A antigen (4 mg/ml of protein, according to Lowry et al., 1951) was diluted to 10.0 μ g/ml with PBS. A small drop of this diluted antigen solution (2 μ l) was spotted with a Hamilton syringe onto the dried azide-dacron plate and onto nitrocellulose filter and kept at 37 °C for 30 min to dry. The antigen-dacron plate and antigen-nitrocellulose filter were introduced into the well of a Nunc multidish and stored at 4 °C overnight. All materials were prepared in duplicate and handled with forceps.

Blocking – To each well was added 30% w/v non-fat milk (Fleischmann and Royal Ltda., Brazil) solution (0.6 ml) prepared in PBS, pH 7.2, containing 0.05% (v/v) Tween-20 (PBS-Tween) and kept during 15 min with gentle stirring. The blocking solution was aspirated out and the plates were washed three times with PBS-Tween with shaking, during 1 min

each; the last wash was preceded by a 10 min incubation without stirring.

Primary incubation – The serum samples and controls were serially diluted in PBS-Tween and introduced into the wells containing the blocked antigen-dacron plates or antigen-nitrocellulose filters. The plates and filters were shaken for 1 min at room temperature, incubated for 30 min and the washing procedure was carried out as described above.

Secondary incubation – Anti-rabbit IgG peroxidase conjugate (IgG-HRP) diluted 1:8,000 in PBS, pH 7.2, was pipetted (0.6 ml) into each well, the plate shaken for 1 min and incubated for 30 min. Again, the plates and filters were washed five times as described before.

Development of the spot – Antigen-dacron plates and antigen-nitrocellulose filters were incubated for 10 min with shaking at room temperature, with a solution containing 0.25 mg/ml 3,3'-diaminobenzidine and 0.005% (v/v) H₂O₂ prepared in 0.1 M citrate-phosphate buffer, pH 5.0. After 10 min the substrate was removed and distilled water was added. The positive reactions were assessed by development of brown spots.

Passive Hemagglutination Assay (PHA) – The PHA was carried out according to procedures described by the WHO Expert Committee on Plague (1970).

Re-use of dacron – Dot-ELISA was developed on the antigen dacron derivative by using 4-chloro-1-naphtol (3.0 mg/ml in methanol 99.5% v/v) and 0.012% v/v H₂O₂ prepared in 0.1 M Tris/HCl, pH 7.5 and an incubation times of 20 min at 28 °C. A blue spot developed in the positive reaction whereas no spot was present in the negative reaction. Positive and negative plates were incubated in 0.1 M glycine/HCl buffer, pH 2.2, with stirring at 28 °C for 1 h to remove the antibodies. Afterwards, the same plates were used for another dot-ELISA employing the same procedure but changing antigen dacron plates positions, namely, the previous positive antigen dacron plate was used for the control test and vice versa.

Crook washing (Crook et al., 1970) – Antigen-dacron plates were washed according to Crook et al. (1970) with stirring at 28 °C for 10 min in each step.

Temporal and thermal stabilities of the antigen – Antigen-dacron plates and antigen-nitrocellulose filters were stored at 28 °C, 4 °C and –20 °C for a time ranging from 7 to 90 days. Then, dot-ELISA was carried out according to the methodology described using positive sera in PBS-Tween and anti IgG-HRP conjugate in PBS, dilutions of 1:100 and 1,000, respectively.

Statistical analysis – The titers obtained from PHA and dot-ELISAs using dacron and nitrocellulose were statistically analysed according to the variance test. The probability level (p) considered significant was 0.05.

RESULTS

The titration (reciprocal titers) of sera obtained from eleven rabbits immunized against *Yersinia pestis* (F1A) by using PHA and dot-ELISA on nitrocellulose and dacron is shown in Table. All the PHA titers were positive ($\geq 1:16$) according to Butler & Hudson (1977). The rabbit number three presented equal reciprocal titers in all procedures whereas the number four showed a higher value for PHA compared with the immunoenzymatic assays.

TABLE

Titration of sera from rabbits immunized against *Yersinia pestis* by using passive hemagglutination and dot-ELISA on nitrocellulose and dacron (reciprocal titers)

Rabbit	Passive hemagglutination	Nitrocellulose	Dacron
1	2,048	32,768	32,768
2	4,096	8,192	16,384
3	2,048	2,048	2,048
4	2,048	1,024	1,024
5	128	1,024	1,024
6	64	2,048	256
7	128	16,384	16,384
8	512	16,384	16,384
9	128	2,048	8,192
10	16	2,048	16,384
11	64	1,024	16,384

Sera from ten non immunized rabbits did not show positive reactions when using PHA or dot-ELISA with dacron as solid phase. However, four sera showed false positive results at a dilution of 1:128 when using nitrocellulose as

matrix. No dot-ELISA positive reaction was observed using the PBS control.

The statistical analysis showed a significant difference (p < 0.05) between PHA and the dot-ELISAs. However, no significant difference was observed between dot-ELISAs using dacron or nitrocellulose as solid phases.

No cross-reaction was detected with sera from rabbits immunized against *Shigella flexneri* polyvalent, *Escherichia coli* invasive polyvalent, *Escherichia coli* enteropathogenic classic A group, *Alkalescens-Dispar*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* using dacron. Only a faint reaction on the nitrocellulose matrix was observed with sera from rabbits immunized against *Salmonella* polyvalent at a dilution of 1:8.

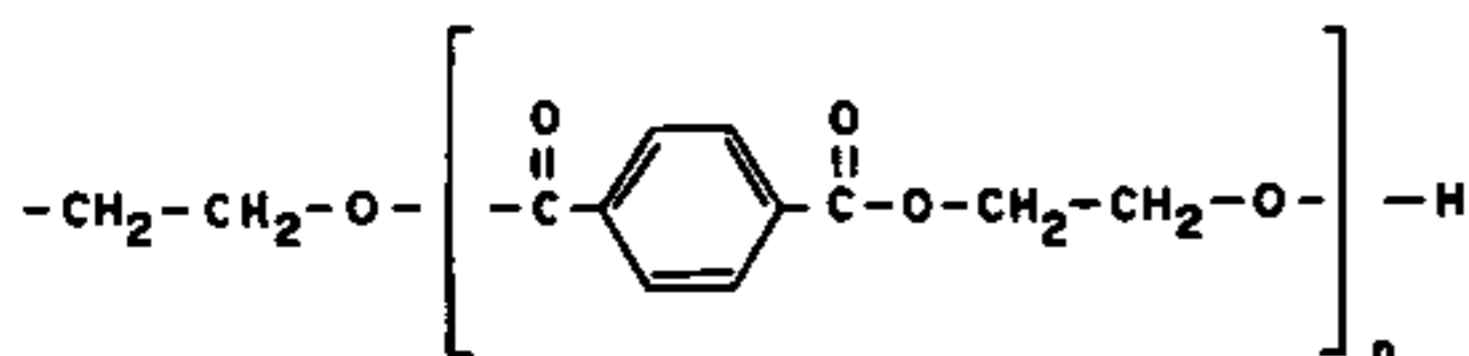
The re-use of the antigen-dacron plates in the dot-ELISA showed to be efficient only when 4-chloro-1-naphtol was used as the developer. The blue spots were removed from the positive reaction plate after glicine/HCl treatment. On the other hand, the spot developed by using 3-3'-diaminobenzidine could not be removed using several treatments (ethanol 99.5%, v/v; methanol 99.5%, v/v; petroleum ether 50%, v/v; and sodium hyperchloride 2.5%, v/v). When dot-ELISA was performed employing the positive plate washed with glicine/HCl buffer as control, the reaction became negative. On the contrary, the control plate also washed as above showed a positive reaction when for the primary incubation immune serum was used.

The antigen-dacron derivative, even after being washed as recommended by Crook et al. (1970) still showed a positive reaction in the dot-ELISA.

Dot-ELISA carried out on antigen-dacron plates stored at 4 °C for 90 days still showed the same spots as those recently prepared. However, antigen-dacron plates stored at 28 °C and –20 °C were only efficient for 15 and 60 days, respectively. The nitrocellulose was efficient in all the three temperature for 90 days.

DISCUSSION

Dacron is a polyester with the following structure:



with n assuming values of approximately 15,000.

This polyester was chemically treated so that azide groups were introduced into the molecule and the antigens (proteins) would be covalently bound on the support (Oliveira et al., 1989).

In the Table can be observed that PHA, with exception of rabbits 3 and 4, yield lower results than those obtained in the dot-ELISAs, using either nitrocellulose (Almeida & Ferreira, 1989) or dacron.

The specificity of dot-ELISA proceeded on both matrices can be considered as similar although there was a discrete reaction against *Salmonella* polyvalent using nitrocellulose as the solid phase. Probably, due to the porous configuration of this support, the blocking substance was not efficient enough since a false positive reaction was also observed for the non immunized rabbit sera.

The positive reaction of the dot-ELISA performed on the antigen-dacron derivative after being washed according to Crook et al. (1970) strongly suggest that covalent linkage is established between the antigen and the dacron matrix. This washing (acid, basic and high ionic strength treatments) removes proteins fixed non covalently on supports.

Furthermore, a covalent linkage between antigen and dacron is also suggested by the ability for re-use of the derivative after glycine/HCl treatment.

The results show that the use of dacron as a matrix instead of nitrocellulose presents the following advantages: a) it is easily available; b) the antigen is covalently linked to it, so that it can be re-used and c) no false positive reaction was detected; d) sensitivity was similar for both matrices.

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