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# STANDARDIZATION OF THE DOT ENZYME-LINKED IMMUNOSORBENT ASSAY (DOT-ELISA) FOR EXPERIMENTAL PLAGUE

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*A dot enzyme linked immunosorbent assay (dot-ELISA) was previously developed to detect specific antibodies in rabbits sera immunized against F1A protein obtained from* Yersinia pestis. *This antigen was covalently linked onto the surface of dacron (polyethyleneterephthalate). Here, standard conditions are described for the optimization of this procedure: an amount of 20 ng of F1A protein was fixed onto dacron; anti-rabbit IgG peroxidase conjugate diluted 1:8,000 and 30% non-fat instant milk as blocking substance were used throughout the method. This procedure was compared with that employing nitrocellulose as solid-phase which showed to be more sensitive. However, the method based on dacron did not show false positive reactions against non-immunized rabbits sera at low antigen amount and diluted anti-IgG peroxidase conjugate.*

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

Plague, a bacterial acute disease caused by *Yersinia pestis*, occurs in men on three different clinical forms: bubonic, pneumonic, and septicemic (Coura et al., 1989). Wild rodents are natural reservoir of this bacillus and infect man and domestic animals by infected fleas as vector (Coura et al., 1989). The plague virulence is related to five factors (Brubraker, 1972): (1) VW antigens; (2) pesticin, fibrinolysin and coagulase; (3) capability to absorb iron; (4) capability to biosynthesize purine endogenously and (5) Fraction 1, a protein with antiphagocytic properties located in the capsular envelope of the bacillus *Y. pestis*. This latter fraction first isolated by Baker et al. (1947), is composed by two proteins: one bound to a small carbohydrate moiety (1A) and the other, a simple protein (1B).

The fraction 1A was used in this work since it is recognized as the principal antigen with immunization power and the only adequate for diagnosis purposes (Wake & Morgan, 1986; Coura et al., 1989).

A dot-immunoenzymatic assay was previously reported using a new solid phase (dacron) for plague diagnosis, and compared with a standard matrix-nitrocellulose (Montenegro et al., 1991). Dacron has been used as a support to immobilize enzymes (Weetall, 1970; Goldstein et al., 1977; Carvalho et al., 1986). In this work, the experimental conditions for the optimization of dot-ELISA using dacron was investigated.

## MATERIALS AND METHODS

Antigen F1A from *Y. pestis* avirulent strain A1122 was obtained according to Baker et al. (1952). Sera from immunized rabbits against *Y. pestis* F1A protein were prepared by standard procedures (Bahmanyar & Cavanaugh, 1976). Sera from non-immunized rabbits and 0.01 M phosphate buffered, pH 7.2, containing 0.8% w/v NaCl (PBS) were used as controls. Rabbits anti-IgG (prepared in goat) labeled with horseradish peroxidase and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were purchased from Serotec and Sigma, respectively. Tween 20 and bovine serum albumin were acquired from Inlab, Brazil. Dacron was produced by Rhodia do Brasil SA. Nitrocellulose filter (0.22 μm pore size) was obtained from Millipore Inc., USA. Whole and non-fat instant milk powder were acquired from

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Fleischmann & Royal Ltda., Brazil. All other reagents were of analytical grade obtained from Merck SA, Brazil.

Activation of dacron plates according to Oliveira et al. (1989) and modified by Montenegro et al. (1991).

Dot-ELISA was performed according to Pappas et al. (1984).

*Antigen titration* – F1A antigen, 4 mg/ml of protein according to Lowry et al. (1951), was diluted with PBS to concentration ranging from 80 to 0.078 µg/ml. Afterwards, 2.0 µl of each concentration were spotted onto the azide-dacron plates and nitrocellulose filters and kept at 37 °C for 30 min to dry. The antigen-dacron plates and antigen-nitrocellulose preparations were introduced into the wells of a Nunc multidish and stored at 4 °C overnight. For titration of the antigen, the conjugate was used at the dilution of 1:1,000.

*Blocking* – The following blocking substances were investigated: whole and non-fat instant milk powder (15% w/v), bovine serum albumin and goat serum (0.5, 1.0, 2.0 and 6.0% v/v) and gelatin (0.5, 1.0, 2.0 and 6.0% w/v). These solutions were prepared in 0.01 M phosphate buffer, pH 7.2, containing 0.8% w/v NaCl and 0.05% v/v Tween 20 (PBS-Tween 20). To each well, containing either the antigen-dacron plate or the antigen-nitrocellulose preparation, the blocking substance was added and kept overnight at 28 °C under gentle stirring. Then, the blocking solution was aspirated out.

The selected blocking substance was further tested at different concentrations and incubation times ranging from 1.0 to 30%, w/v. and from 1 min to 15 hr, respectively. Immunized and non-immunized rabbit sera were used at dilution of 1:100 in PBS-Tween 20 and anti-rabbit IgG-HRP at dilution of 1:1,000.

*Washing* – The plates were washed three times with PBS-Tween 20, with shaking for 1 min. In the last washing the plates and filters were incubated for 10 min before the aspiration.

*Primary incubation* – Sera from the rabbits immunized against the F1A antigen of *Y pestis* and sera from the non-immunized rabbits were serially diluted in PBS-Tween 20 from 1:8 to 1:65,536 and introduced into the wells containing the blocked antigen – dacron plates or antigen – nitrocellulose preparations. These materials were shaken for 1 min and incubated for 30 min at 28 °C. The washing procedures were carried out as above for three times.

*Titration of the anti-rabbit IgG peroxidase conjugate (IgG-HRP)* – IgG-HRP was diluted in PBS at dilutions ranging from 1:1,000 to 1:256,000. In this experiment the antigen was used at an appropriate concentration previously established. Again the plates were washed three times as described above.

*Development of the spot* according to Montenegro et al. (1991).

## RESULTS

Dot-ELISA in dacron plates, for the detection of the anti-*Y. pestis* antibody in rabbit sera showed that the minimum concentration of F1A antigen to provide the best performance was equal 10 µg/ml of protein (Fig. 1). Under this concentration a decrease of titers was observed in both matrices (dacron and nitrocellulose). Nevertheless, under 0.312 µg/ml of F1A protein the antigen-dacron derivatives showed lower titers than antigen nitrocellulose preparations. It is worthwhile to notice that at concentration 0.078 µg/ml the titer for the antigen-dacron derivative was equal to the observed for the control (non-immunized rabbits) whereas titer of 1:512 was found for the antigen-nitrocellulose preparation. Negative reactions were observed for the antigen-dacron derivative when non-immunized rabbit sera were used even at concentration as high as 80 µg/ml of antigen F1A protein. However, false positive reactions were observed for the antigen-nitrocellulose preparations at protein concentrations from 80 to 10µg/ml F1A protein yielding titer equal to 1:128.

No false positive reaction was detected for both matrices when PBS was used as control.

According to these results one can notice that antigen-nitrocellulose preparations were capable to detect lower amounts of antibody (higher sensitivity) compared with the antigen-dacron derivatives.

The dilution of IgG-HRP conjugate equal to 1:8,000 showed the best performance using both matrices (Fig. 2). However, higher dilutions yielded lower results for the dacron derivative (negative results at dilution of 1:64,000) than the nitrocellulose preparation (positive results

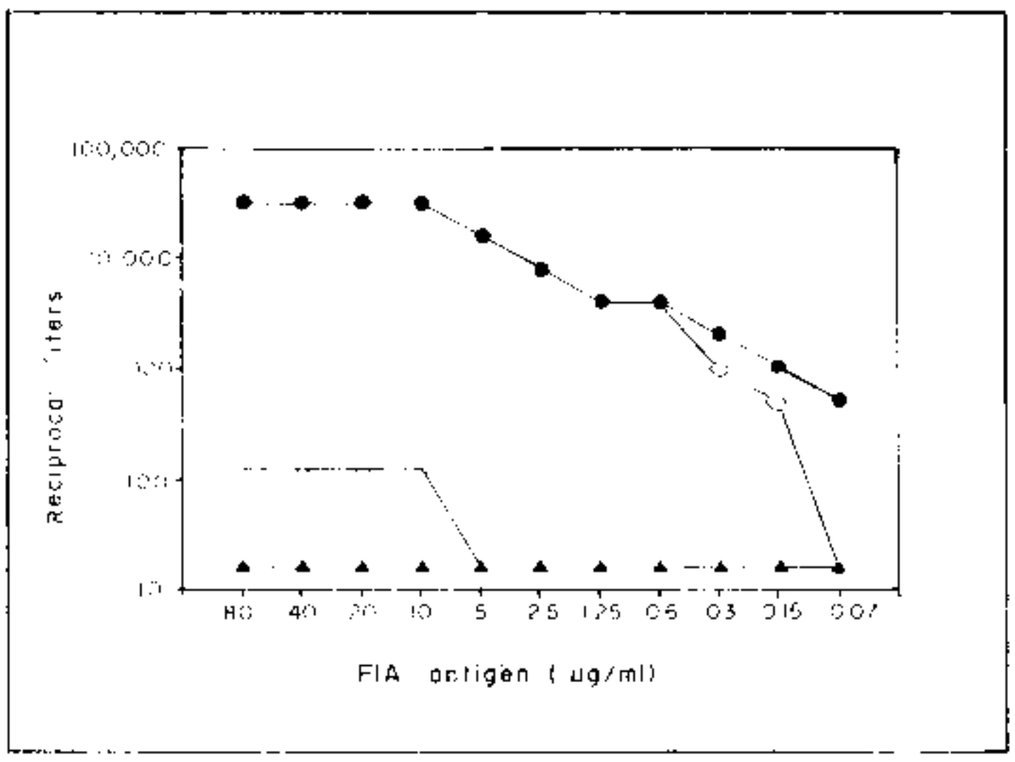


Fig. 1: determination of optimal antigen concentration for dot-ELISA. Decreasing concentrations of F1A antigen from *Yersinia pestis* were dotted onto dacron plates and nitrocellulose filters, incubated with immunized and non-immunized rabbit sera and titers determined visually. Antigen-dacron – O; Antigen-nitrocellulose – ●; antigen-dacron control – ▲; antigen-nitrocellulose control – Δ.

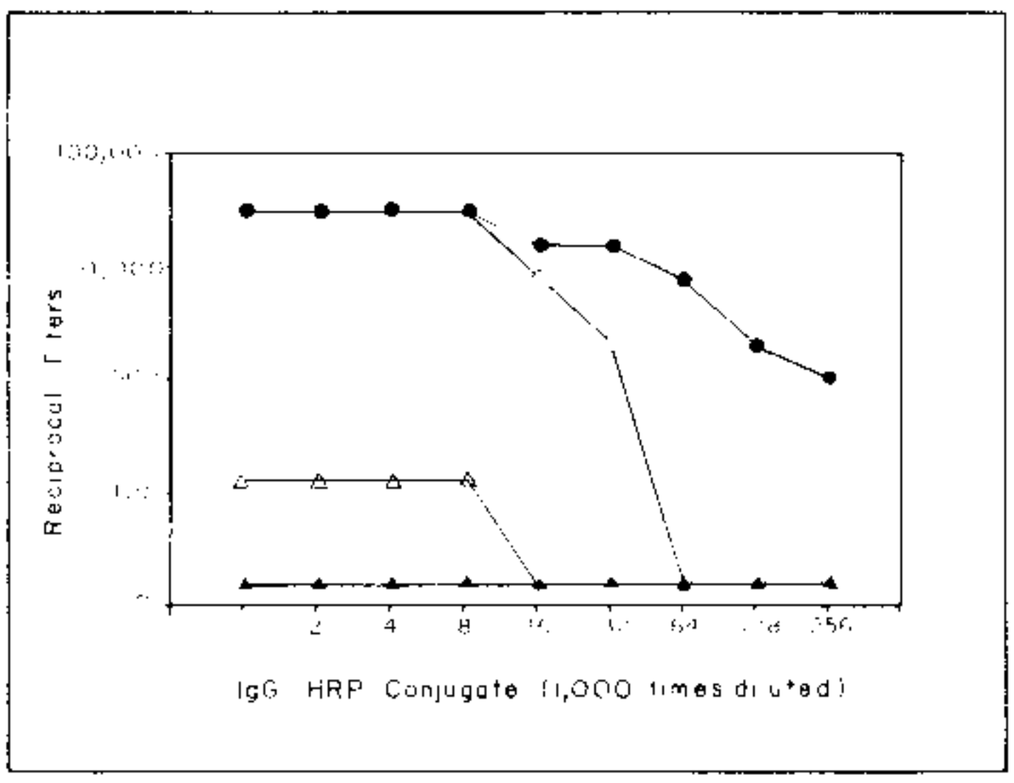


Fig. 2: determination of optimal conjugated dilution for dot-ELISA. Increasing dilution of anti-IgG-peroxidase conjugate was added to wells containing antigen-dacron derivative and antigen-nitrocellulose preparation, incubated with immunized and non-immunized rabbit sera and determined visually. Antigen-dacron – O; Antigen-nitrocellulose - ●; antigen-dacron control - ▲; antigen-nitrocellulose control – Δ.

until dilution of 1:256,000). No false positive reaction was observed for titration of the non-immunized rabbits sera using the antigen-dacron derivative whereas false positive reaction was detected by using the antigen-nitrocellulose preparation, presenting a titer of 1:128 until IgG-HRP dilution of 1:8,000. No false positive reactions were observed when PBS was used as control in both matrices.

Table I displays the results obtained by using several substances to block non specific binding sites on the antigen dacron and nitrocellulose preparations. Whole and non-fat instant milk presented the best results according to this Table. However, the non-fat instant milk showed a slight better background than whole milk. Thus the farther milk was chosen as the blocking substance and it was investigated for concentration and incubation time conditions (Table II).

TABLE I

Blockage of unspecific binding sites of the antigen-dacron and antigen-nitrocellulose preparations by several substances. F1A antigen from *Yersina pestis* (20 μg) was covalently fixed on dacron and spotted on nitrocellulose. Sera from immunized rabbits (dilution of 1/100) and anti-rabbit IgG-HRP (dilution of 1/1,000) were used throughout dot-ELISA according to Pappas et al. (1984). The blocking substances (0.6 ml) prepared in PBS-Tween 20 were added to the wells containing the antigen-solid phase preparations and incubated for 15 h at 28 °C

| Concentration (%) | Whole milk | Non-fat milk | Bovine serum albumin | Goat serum | Gelatine |
|---|---|---|---|---|---|
| | antigen-dacron/antigen-nitrocellulose | | | | |
| 15 | ++/++ | ++/++ | nd | nd | nd |
| 6 | nd | nd | ++/+ | ++/++ | –/– |
| 2 | nd | nd | ++/– | ++/++ | +/– |
| 1 | nd | nd | +/– | +/+ | +/– |
| 0.5 | nd | nd | +/– | +/– | –/– |

++ good; + regular; – negative and nd = not determined.

TABLE II

Blockage of unspecific binding sites of the antigen-dacron and antigen-nitrocellulose preparations by non-fat instant milk at different concentration and incubation time. Antigen concentration, sera and IgG-HRP dilutions were as described in Table I

| Incubation time | Concentration (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antigen-dacron | | | | | | Antigen-nitrocellulose | | | | | |
| | 30 | 20 | 15 | 10 | 5 | 1 | 30 | 20 | 15 | 10 | 5 | 1 |
| 15 hr | ++ | ++ | ++ | ++ | + | + | ++ | ++ | ++ | ++ | ++ | ++ |
| 2 hr | ++ | ++ | ++ | ++ | + | +/– | ++ | ++ | ++ | ++ | ++ | ++ |
| 1 hr | ++ | ++ | ++ | ++ | +/– | – | ++ | ++ | ++ | ++ | ++ | ++ |
| 30 min | ++ | ++ | ++ | ++ | – | nd | ++ | ++ | ++ | ++ | ++ | ++ |
| 15 min | ++ | ++ | ++ | ++ | nd | nd | ++ | ++ | ++ | ++ | ++ | + |
| 10 min | ++ | ++ | ++ | ++ | nd | nd | ++ | ++ | ++ | ++ | + | + |
| 5 min | ++ | ++ | ++ | + | nd | nd | ++ | ++ | ++ | ++ | + | + |
| 1 min | ++ | ++ | ++ | +/– | nd | nd | ++ | ++ | ++ | ++ | +/– | +/– |

++ good; + regular; +/– weak; – negative and nd = not determined.

Fig. 3 illustrates the spots developed according to the procedure described for the dot-ELISA using dacron as a matrix comparing with those using nitrocellulose.

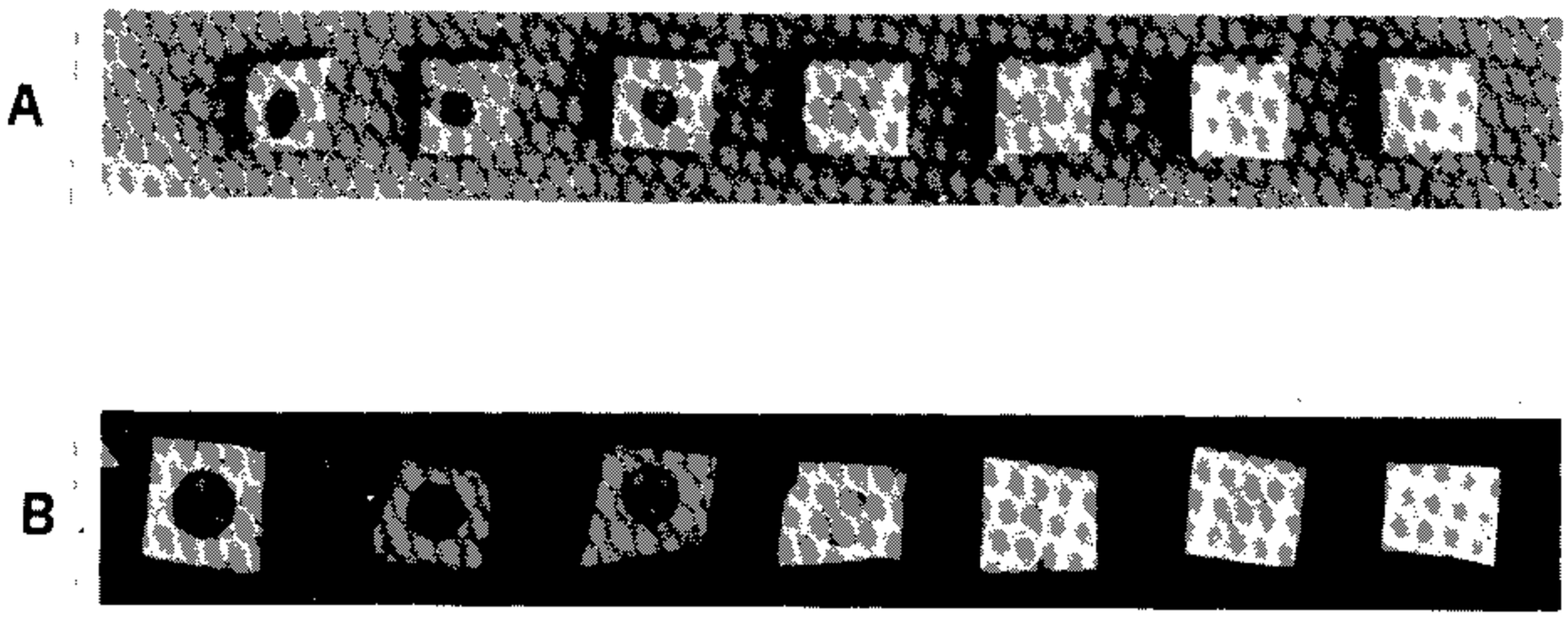


Fig. 3: spots developed by using dot-ELISA procedure based on dacron and nitrocellulose matrices. The spots were developed according to Montenegro et al. (1991). Sera and IgG-HRP dilutions were as described in Table I. Antigen concentrations were 160, 80, 40, 20, 10, 5 and 2.5 ng for dacron (A) and nitrocellulose (B) preparations.

## DISCUSSION

For the standardization of the dot-ELISA to detect *Y. pestis* antibodies in rabbits sera it is essential for the reproducibility and sensitivity of the immunoenzymatic assay to establish the best conditions of antigen concentration, IgG-HRP conjugate dilution, blocking substance, and its incubation time.

Blocking substances are used in ELISA to block undesired free sites on the matrix to protein fixation. Thus, non specific protein bindings are avoided during the antigen-antibody complex formation. The choice of this blocking substance is very important to the sensitivity and reproducibility of the assay. It depends on several features, such as, support, antigen and antibody. Mohammad & Esen (1989) only suggested the use of washing solution containing non ionic detergents to overcome this problem. In this work, it has been observed that in addition to the non ionic detergent (Tween 20) a blocking substance was necessary. Instant whole and non-fat milk showed to be the best blocking substances. This observation is in accordance to previous reports from Vogt et al. (1987). Gelatin and BSA were not efficient as they are not so heterogeneous substances as milk casein (Kenna et al., 1985). Although non-fat instant milk showed good performance at minimal concentration and incubation time, the reproducibility was not satisfactory for the antigen-dacron plate. Then, the optimal parameters for both conditions using this preparation were established to be 30%, w/v, and 15 min, respectively. Similar conditions were kept when using the antigen-nitrocellulose filter, for comparison reasons. Perhaps, antigen-dacron plate required higher concentrations of instant non-fat milk and incubation time than antigen-nitrocellulose filter because of its larger surface area.

The immunoenzymatic assay presented in this paper showed that more sensitivity was observed when the antigen-nitrocellulose preparation was used as the solid-phase in the dot-ELISA procedure. This can be attributed to the porous nature of nitrocellulose which allows absorption of larger amounts of protein whereas the protein links only onto the surface of the dacron. On the other hand, a false positive reaction was described for the antigen-nitrocellulose preparation when non-immunized rabbit sera was used for the test. Probably, the blocking substance was not enough to prevent nonspecific interactions between the antigen F1A from *Y. pestis* and antibodies present in the non-immunized sera. Again, the antigen linked to the surface of the dacron allowed better conditions of blocking substance interaction. Finally, the explanation for the positive reaction for the antigen-nitrocellulose preparation using diluted IgG-HRP conjugated (1:256,000) can also be due to the higher amount of protein (F1A protein) absorbed by this matrix whereas the antigen-dacron derivative showed negative reaction at IgG-HRP dilution of 1:64,000.

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