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EVALUATION OF THREE SEROLOGICAL TESTS FOR THE DETECTION OF HUMAN PLAGUE IN NORTHEAST BRAZIL

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The passive haemagglutination (PHA) test, enzyme-linked immunosorbent assay (ELISA) and the dot enzyme-immunosorbent assay (DOT-ELISA) were used to detect the levels of IgG antibodies against the Fraction 1 (F1) antigen of Yersinia pestis *in sera of plague-infected patients from Northeast Brazil. Twenty three selected PHA-positive sera of subjects with bacteriological confirmation of plague were also positive in the DOT-ELISA but only 19 were detected by the conventional ELISA technique. Another group of 186 serum samples from subjects diagnosed as plague-infected by clinical and epidemiological parameters, but PHA-negative, were screened with DOT-ELISA and 11 gave positive results. The specificity of the assays on the serological detection of plague was confirmed in inhibition tests using purified F1 antigen. These results suggest that DOT-ELISA can be an useful, simple and more sensitive alternative for the serodiagnosis of plague in Northeast Brazil.*

Key words: *Yersinia pestis* – fraction-1 antigen – passive haemagglutination test – ELISA – DOT-ELISA

The passive haemagglutination test (PHA) has been the recommended choice for the serodiagnosis of *Yersinia pestis,* the causative agent of bubonic plague, over the last 30 years (Baltazard et al., 1956; WHO, 1970; Bahmanyar & Cavanaugh, 1976). The PHA proved to be an useful technique for laboratories localized in developing countries based on its simplicity, low cost, fast results and rather good sensitivity. Application of other alternative tests to detect plague antibodies in humans, as the enzyme-linked immunosorbent assay (ELISA) and the solid-phase radioimmunoassay (SPRIA), had been reported but their uses are still restricted to well equipped research laboratories (Cavanaugh et al., 1979; Hudson et al., 1980; Williams et al., 1982, 1986). In general, these techniques show some advantages over the PHA as significant higher sensitivities and the possibility to test individual classes of immunoglobulins (Hudson et al., 1980; Williams et al., 1982). However, the elevated costs represented by these new diagnostic methods prevent their widespread use in under developed countries.

Recently, a dot enzyme-linked immunosorbent assay (DOT-ELISA) was described and applied to the diagnosis of different pathogenic organisms (Pappas et al., 1983, 1984a, 1984b; Santos et al., 1987). The method proved to be quite reliable and sensitive when compared to conventional ELISA test and represented only a fraction of the costs usually spent with ELISA or SPRIA (Pappas et al., 1984a, b; Santos et al., 1987).

In Brazil, human plague is still detected in the Northeast region and outbreaks are registered every few years with considerable mobidity and mortality rates (Almeida et al., 1985, 1989). The serological detection of plague is based solely on the PHA test which proved to be satisfactory as an accessory test for diagnosis of plague (Almeida et al., 1981, 1987). However, the high incidence of patients with clear symptons of plague but without seroconversion casts doubts on the sensitivity of the PHA (Almeida et al., 1981, 1989). Attempts to use other serological tests as immunofluorescence and bacterial agglutination did

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not improve the sensitivity of the serological detection of plague (Almeida et al., 1987; Brito et al., 1989).

In order to evaluate the performance of other serologic tests on the diagnosis of human plague, we have compared the PHA test with ELISA and DOT-ELISA using sera from patients collected at different plague foci in Northeast Brazil during the last 10 years. Two groups of sera from the serum collection of the plague section at the Centro de Pesquisas Aggeu Magalhães were specifically selected based on their previous reactions with the PHA test and clinical and epidemiological characteristics. The present results suggest that the DOT-ELISA is more sensitive than PHA and ELISA assays for the serodiagnosis of human plague.

MATERIALS AND METHODS

Sources of sera – Human sera were obtained from patients living at different plague foci in Northeast Brazil during the last 10 years. The normal field procedure involved collection of sera from suspected plague-infected persons based on clinical symptoms and epidemiological evidences as part of a government program (SUCAM) to surveil and control the bubonic plague. A total of 209 human sera, kept frozen at the plague section of the Centro de Pesquisas Aggeu Magalhães, were specifically chosen for this study. The samples were divided in two groups based on their reactivity in the PHA test. The first group was represented by 23 samples of PHA-positive sera which were further confirmed in inhibition tests and isolation of plague bacillus. The second group of sera were chosen from a larger collection of PHA-negative samples without bacteriologic confirmation but with strong clinical and epidemiological evidences as presence of lymphadenitis, fever, and records of plague in the same premise at the time of the initial symptoms.

Anti-F1 antigen antibodies were raised in rabbits immunized with 5 mg of purified *Y. pestis* F1 protein emulsified in 1 ml of Freund's incomplete adjuvant and inoculated intravenously (ear vein). After three weeks, an additional 1 mg of F1 antigen without adjuvant was injected and the animals were bled ten days later.

Antibodies against *Y. pestis* A1122 were raised in rabbits immunized with one s. c. injection of 1.5×10^6 cells followed by four i. v. injections of 1.5 to 4.5 x 10^6 bacteria at one week intervals. Blood samples were removed one week after the last injection. *Y. pseudotuberculosis* and *Y. enterocolitica* antisera were supplied by the Centers for Disease Control (Plague Branch, Fort Collins, U. S. A.). Antisera against *Salmonella, E. coli* (Instituto Oswaldo Cruz, Rio de janeiro, Brazil) and *Shigella* (Inlab, São Paulo, Brazil) were purchased for this work.

The passive haemagglutination test (PHA) – The PHA test routinely performed for the serodiagnosis of plague followed the procedure described by World Health Organization (WHO, 1970) using formaldehyde-fixed and F1 sensitized sheep red blood cells.

The enzyme-linked immunosorbent assay (ELISA) – The ELISA test was carried out as described by Voller et al. (1976). Each well of a Limbro flat botton 96-well microtitre plate (Flow Laboratories, cat. no. 76-003-05, Virginia, U. S. A.) was sensitized with 5 µg of the F1 antigen of *Y. pestis* in 100 µl of coating buffer at pH 9.6. Plates were incubated at 37 °C for 1 h followed by three washings with phosphate buffered saline with 0.05% Tween 20 (PBST). Unspecific binding was blocked with 0.5% bovine serum albumine (BSA) in PBST for 30 min at room temperature followed by three washings with PBST. Test sera were diluted in PBST and 100 µl was applied to each well and incubated at 37 °C during 1 h. After three washings with PBST, 100 µl of previously PBST-diluted horseradish peroxidase-conjugate immunoglobulins (1:2,000 or 1:6,000 dilutions for anti-rabbit and anti-human IgG conjugates, respectively) (Dakkopatts, Norway) were added to the wells and incubated for 1 h at 37 °C. Unbound conjugates were removed by three washings with PBST and the color reaction was carried out with 100 µl per well of freshly prepared 0.03% 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (Sigma, St. Louis, U. S. A.) in developer buffer (0.1 M Na_2HPO_4, 0,05 M citric acid) containing 0,005% H_2O_2. After incubation at room temperature in the dark for one hour the plates were read in a BIO-RAD EIA-spectrophotometer model 2550 at 405 nm.

The DOT-enzyme-linked immunosorbent assay (DOT-ELISA) – The DOT-ELISA was carried out essentially as described by Santos et

al. (1987). Forty nanograms of purified F1 antigen were spoted on 0.5 cm x 0.5 cm nitrocellulose paper squares (Sheicher and Schuell, Dassel, Germany). The paper squares were allowed to dry at room temperature for approximately 30 min, transfered to a 24 well Limbro flat botton cell culture plate (Flow Laboratories, cat. no. 76-033-05, Virginia, U. S. A.) followed by 1 h incubation with 0.5 ml of PBST containing 0.5% of BSA at room temperature. The samples were washed twice with PBST with 5 min interval and, then incubated with 0.5 ml PBST-diluted sera for 1 h at room temperature. After five washings with PBST (5 min intervals) the samples were incubated with horseradish peroxidase goat anti-human IgG conjugate or swine anti-rabbit IgG conjugate for 1 h at room temperature using the same dilutions employed for the ELISA assays. Finally, after three additional washings with PBST, the samples were briefly rinsed with 0.1 M Tris-HCl pH 7.5 and subjected to the developing reaction with 0.5 ml of 0.06% 4-chloronaphtol (Sigma, St. Louis, U. S. A.) in 0.1 M Tris-HCl pH 7.5 containing 0.005% H_2O_2. Positive reactions were usually visible after 15 min at room temperature. Washing with tap water was used to stop the reactions.

Other methods – Purification of the F1 antigen from *Y. pestis* strain A1122 grown at 37 °C was carried out as described by Baker et al. (1952). Inhibition tests were performed as described above except by the incorporation of 100 μg/ml purified F1 antigen in the buffer solutions used to dilute the primary antibodies.

RESULTS

Specificity and lack of cross reactivity of sera raised against bacteria closely related to *Y. pestis* were evaluated with the PHA, ELISA, and DOT-ELISA tests using rabbit polyclonal antibodies against *Y. pestis* A1122, *Y. pseudotuberculosis, Y. enterocolitica, Salmonella sp., Shigella sp.*, and *E. coli* (commensal and invasive). As can be seen on Table I all serologic tests proved to be specific for *Y. pestis* and no cross reactivity was detected when the purified F1 protein was used as antigen. Sera from healthy humans and patients with Chagas' disease and schistosomiasis were also negative in the three tests (data not shown).

The sensitivity of the tests were compared with 23 bacteriologic positive human sera collected during the period between 1982 to 1986. Table II shows that all sera were positive for PHA as well as for IgG DOT-ELISA whereas the anti-F1 IgG ELISA could detect only 19

TABLE I

Reaction of rabbit hyperimmune sera against gram-negative bacteria closely related to *Yersinia pestis* in PHA, ELISA and DOT-ELISA using the purified F1 protein as antigen

Tested Sera	Serological assay used[a] PHA	ELISA	DOT-ELISA
F1[b]	1,024	1,024	4,096
Y. pestis A1122	4,096	2,048	8,192
Y. pseudotuberculosis	–	–	–
Y. enterocolitica	–	–	–
Salmonella sp.	–	–	–
Shigella sp.	–	–	–
E. coli (non pathogenic)	–	–	–
E. coli (enteroinvasive)	–	–	–
Control[c]	–	–	–

–: titres lower than 1/16.
a: reciprocal antibody titres.
b: rabbit immunized with purified *Y. pestis* F1 antigen.
c: sera from healthy non-infected rabbits.

TABLE II

Serological analysis of human sera from plague-infected patients with bacteriologic confirmation from Northeast Brazil

Patient number	Year of blood collection	Serologic assay used[a] PHA	ELISA	DOT-ELISA
1	1980	128	64	256
2	1982	16	64	512
3	1982	512	64	512
4	1982	512	64	16,384
5	1982	128	64	1,024
6	1982	256	64	1,024
7	1982	16	16	128
8	1985	512	4,096	65,536
9	1986	64	32	512
10	1986	32	2,048	8,192
11	1986	64	256	8,192
12	1986	64	128	512
13	1986	1,024	32	1,024
14	1986	128	64	516
15	1986	64	16	516
16	1986	128	64	516
17	1986	32	16	128
18	1986	128	16	1,024
19	1986	64	–	256
20	1986	128	64	256
21	1986	16	–	32
22	1986	16	–	256
23	1986	64	–	32

–: Titres lower than 1/16.
a: Reciprocal antibody titres.

samples (82.6%). In all but two sera, the titres obtained with IgG DOT-ELISA were clearly higher than the values recorded with PHA. On the other hand, the titres detected by ELISA were similar or even smaller than those obtained with PHA (Table II). Specificity of the results was further demonstrated in inhibition tests which decreased the titres by at least four times (data not shown).

In order to evaluate the presence of false PHA-negative results in 186 sera samples collected from plague-infected subjects, based on clinical and epidemiological evidences but PHA-negative, were analyzed by IgG ELISA and IgG DOT-ELISA. Eleven samples (5.9% of the sera examined) derived from a plague outbreak bursted in 1986 at the Paraíba State gave positive results by the IgG DOT-ELISA only (Table III). These samples were submitted to inhibition tests using purified F1 antigen and all titres were reduced to background level further demonstrating their specificity (data not shown).

TABLE III

Serological analysis of plague-infected patients diagnosed by clinical and epidemiological parameters but negative for PHA

Origin	Number of sera	Serologic positive cases (%) PHA	ELISA	DOT-ELISA
Paraiba	103	–	–	11 (10.6)
Ceara	43	–	–	–
Rio G. Norte	17	–	–	–
Bahia	16	–	–	–
Pernambuco	2	–	–	–
Control[a]	5	–	–	–
Total	186	– (0)	– (0)	11 (5.9)

–: titres lower than 1:16.
a: healthy non-infected adults.

DISCUSSION

The serological detection of plague by the PHA was described more than 30 years ago and represents one of the most simple and fast techniques available for third world countries where *Y. pestis* can still be found (WHO, 1970; Bahmanyar & Cavanaugh, 1976). In Brazil, the PHA has been used routinely by our group for the serological detection of plague in the last ten years (Almeida et al., 1981, 1987, 1989). Since its implementation as a large scale screening test the PHA proved to be an invaluable tool in surveillance programs but it still has a secondary role on the diagnosis of the disease in humans where clinical and epidemiological evidences are the major parameters used (Almeida et al., 1981, 1987, 1989).

The results obtained in the present work demonstrated that even though all three tests employed were specific for *Y. pestis* the DOT-ELISA proved to be more sensitive than the ELISA and PHA tests. Moreover, eleven PHA-negative serum samples (5.9% of the samples tested) from patients diagnosed as plague infected gave positive results only with the IgG DOT-ELISA, corroborating the better performance of this assay. It should be noted that all experiments using ELISA and DOT-ELISA were based on the detection of circulating IgG antibody levels whereas the PHA is able to detect all humoral immunoglobulins. Taking into account that many samples were collected during the initial stages of infection, when circulating IgM is higher then the IgG blood levels, we can assume that values obtained with ELISA or DOT-ELISA could be even higher if conjugate antibodies against total immunglobulins were used.

The fact that the great majority of the sera collected from plague-suspected patients continued to be non-responsive, both in the IgG ELISA as well as in the IgG DOT-ELISA, can be attributed to several factors as: lack of response of the patients immune system to the F1 antigen or presence of strains not producing the F1 antigen; samples obtained in a too early stage of infection for a serological response; clinical symptoms associated with non plague infections; and low sensitivities of the serological tests. The first two possibilities are improbable since all *Y. pestis* strains so far isolated in Northeast Brazil were able to produce the F1 antigen. Low anti-F1 antibody levels could explain negative serological results in samples collected a few days after the first symptons but since no paired samples were available this possibility could not be proved. Fever and lymphadenitis associated with other diseases as mononucleosis could be also attributed to some negative serological results but in all cases the epidemiological evidences were very strong and reports of *Y. pestis* isolation in the same premise at the same time of symptoms onset were available for most of the PHA-negative samples analysed (unpublished observation).

The high incidence of serological negative results in samples of plague suspected patients were reported by several authors (Cavanaugh et al., 1979; Almeida et al., 1981; Williams et al., 1986). Williams and colleagues (1982) reported a considerable improvement of the diagnosis of plague by means of ELISA to detect the antigenemia during acute phase. However, even with this approach more than 50% of serum samples of plague-suspected patients collected during a plague outbreak in Namibia were negative including seven cases in which the plague bacillus could be isolated (Williams et al., 1986). Similar problems were observed in Northeast Brazil since the PHA test was introduced as a routine assay for the serological survey of plague in humans, rodents and carnivorous (Butler et al., 1980; Almeida et al., 1981, 1987, 1989). Although the number of positive cases detected by DOT-ELISA among PHA-negative sera are still low in percentage (5.9%) it represents a significant improvement on the serological detection of plague. These results also suggest that the lack of seroconversion in suspected plague-infected patients could be attributed to the low sensitivities of the assays used.

The considerable higher sensitivity, easy and fast procedure and possibility to discriminate specific immunoglobulins classes make the DOT-ELISA a potential diagnostic tool of plague. The stability of the F1 antigen on nitrocellulose sheets for rather long periods, even without refrigeration (unpublished observation), adds additional optimism on the use of the DOT-ELISA as a better choice for routine diagnosis of plague in Brazil and other third world countries. Additional attempts to further improve the sensitivity and simplicity of the DOT-ELISA for the detection of human plague is under current investigation.

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