Latex Agglutination Tests for Measurement of Antiplague Antibodies

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A latex agglutination test was evaluated as a method for detection and titration of antiplague antibodies. Slide and microtiter techniques using polystyrene latex particles coated with specific fraction-I antigen of *Yersinia pestis* were found to be comparable in specificity and sensitivity to serological tests commonly used in laboratory practice. The latex agglutination titers correlated well with those measured by the World Health Organization standard method of indirect hemagglutination, although there was a tendency for the former to be a little lower than the latter. With further study, the latex agglutination test may have application in the seroinvestigation of plague infection in rodents.

The latex agglutination (LA) test, using antigen-coated particles, was first developed by Singer and Plotz in dealing with the rheumatoid factor (6, 7). Later, the slide LA test for rheumatoid arthritis (5) and the microtiter LA test (4) for histoplasmosis were described. The techniques are straightforward and have been applied to serological investigations with various kinds of bacterial and other antigens. However, no report of using this technique for plague has been published so far. This paper describes experiments in which we successfully prepared a plague LA antigen and used it for measurement of antiplague antibodies. The data were compared with those obtained by the World Health Organization (WHO) standard method of indirect hemagglutination (IHA) (2, 8, 9) to confirm the specificity and sensitivity of the test.

MATERIALS AND METHODS

Plague bacilli. Three strains of *Yersinia pestis*, Yreka, A1122, and MII40, were used. The first two strains have "envelope," i.e., fraction-I (FR-I), antigen, which is missing in strain MII40. The organisms, cultivated by the method previously described (8), were suspended in physiological saline and killed by heating at 56°C for 60 min.

Sera. Adult mice, rats, and rabbits procured from a local farm were subcutaneously inoculated with the killed bacilli. At appropriate times thereafter, the blood was obtained by cardiac puncture, and serum was separated and stored at -20°C until ready for use. Sera from wild rodents captured in the harbor areas of Kobe and Sakaide during the period 1975 to 1976 were also tested. Just before the experiment, the

serum was inactivated by heating at 56°C for 30 min. When needed, the samples were diluted with glycine-buffered saline (consisting of 7.507 g of aminoacetic acid, 2.0 g of sodium azide, and 5.844 g of NaCl in 2 liters of distilled water, adjusted to pH 8.2 by 0.1 N NaOH) supplemented with 0.25% bovine serum albumin.

FR-I-coated polystyrene latex particles. The FR-I antigen was extracted from strain A1122 bacilli by the method of Baker et al. (1). Polystyrene latex particles (Bacto latex, 0.81 µm, Difco; or SDL-59, 0.9 µm, Takeda Chemical Industry, Japan) were suspended in the glycine-buffered saline, to which the FR-I antigen was added at a concentration of 50 µg/ml. After being kept at room temperature for 30 min, bovine serum albumin was added to a final concentration of 0.5%, and the mixture was kept at 5°C for a further 12 h for completion of coating. The antigen-coated particles were washed by centrifugation in the bovine serum albumin-containing, glycine-buffered saline three times, each at 4,000 rpm for 10 min, and were finally suspended in the same buffered saline. This LA antigen proved stable at 4°C for at least 12 months. Just before use, the antigen was again washed in the bovine serum albumin-containing, glycine-buffered saline.

LA tests. (i) Slide-LA method. One drop (0.05 ml) of serum and 1 drop (0.05 ml) of antigen were put on a slide and mixed thoroughly with a glass rod. After incubation at 37°C for 1 h in a humid chamber, the reaction was read under a light microscope (×100). The second reading was done after reincubation at 37°C for an additional hour. A control test was set using noncoated latex. The positive reaction appeared as agglutination of particles (see Fig. 1).

(ii) Micro-LA method. Serial dilutions of serum, 0.025 ml each, were made in a U-type microtiter plate (Cook Engineering Co.). To each well, 0.025 ml of LA was added. After thorough mixing, the plates were sealed with a plastic tape and incubated at 37°C. The reaction was read at 2 h (preliminary) and at 16 to 18

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h (final) with the aid of a mirror. A control test was set using noncoated latex. The positive reaction appeared as an irregular ring formation of agglutinated particles, whereas a smooth-contoured sedimentation of particles was regarded as the negative reaction (Fig. 2).

Other serological tests. A microplate modification of the IHA test (2) was performed as described previously (8, 9). The specificity of the reaction was confirmed by carrying out in parallel the nonspecific hemagglutination (3), FR-I antibody inhibition (9), and 2-mercaptoethanol (ME)-acetone-treated IHA (8) tests.

RESULTS

Optimal test conditions. Preliminary experiments indicated that the glycine-buffered saline was highly suitable for dilution of the antigen and serum. Other conditions that proved to be optimal were: (i) pH 8.2 for reaction medium; (ii) 50- μ g/ml final concentration of FR-I antigen (see Table 1); and (iii) 0.25% final concentration for antigen-coated latex particles. The time of incubation and reaction reading, 1 and 2 h, as described in Materials and Methods, was determined after repeated preliminary tests; incubation at 37°C for more than 2 h usually brought about nonspecific reactions. Heat-inactivated sera gave clearer results than fresh sera, which showed nonspecific reactions.

Parallel titration of antiplague antibodies by LA and IHA methods. Sera from mice and rats immunized with plague bacilli were titrated for antiplague antibodies by the slide-LA, micro-LA, and IHA methods in parallel. The data obtained are summarized in Tables 2 (for mouse sera) and 3 (for rat sera). Similar tests were performed with immune rat sera collected at intervals after inoculation of bacilli (see Table 4).

The micro-LA titers correlated well with those obtained by the IHA method, although the former tended to be a little lower than the latter. The slide-LA reaction was also shown to parallel both the micro-LA and IHA reactions; the slide-LA became positive 7 days after inoculation of

FIG. 1. Anti plague slide-LA tests. (A) Positive reaction produced by mixing immune rabbit serum and FR-I-coated latex particles is shown. In (B), in which the immune serum and control noncoated particles were mixed, no agglutination occurs. The immune serum was obtained from an adult rabbit injected subcutaneously three times with heat-killed strain A1122 bacilli; 14 days after the third injection, the serum was taken and inactivated by heating at 56°C for 30 min.

FIG. 2. Anti plague micro-LA tests. Each well contains immune mouse serum diluted (twofold, serially from 1 [1:4] to 6) and latex particles (antigen coated in A; noncoated control in B). Wells 1 to 4 of (A) show the positive reaction.

TABLE 1. *Parallel micro-LA and slide-LA tests using various concentrations of FR-I antigen and antiserum*

Concn of FR-I antigen ($\mu\text{g/ml}$)	Dilution of antiserum ^a						
	8	16	32	64	128	256	512
2,500	++ ^b (+++) ^c	— (+++)	— (++)	— (—)	— (—)	— (—)	— (—)
1,000	+++ (+++)	+++ (+++)	++ (++)	+ (+)	— (+)	— (—)	— (—)
500	+++ (+++)	+++ (+++)	+++ (+++)	+ (+)	— (—)	— (—)	— (—)
250	+++ (+++)	+++ (+++)	++ (++)	++ (+)	+ (—)	— (—)	— (—)
125	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)	+ (+)	— (—)	— (—)
50	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)	— (++)	— (—)
25	+ (+++)	+ (+++)	++ (+++)	+++ (+++)	+++ (++)	— (—)	— (—)
0	— (—)	— (—)	— (—)	— (—)	— (—)	— (—)	— (—)

^a Rabbits procured from a local farm were inoculated subcutaneously with heat-killed strain A1122 organisms three times (3.0×10^9 , 1.2×10^9 , 1.2×10^9).

^b Micro-LA titer.

^c Slide-LA titer is given in parentheses.

TABLE 2. *Parallel slide-LA, micro-LA, and IHA tests for detection of antiplague antibodies in laboratory mouse sera*

No. of mice	Test	No. of samples showing titer of:										No. of sam- ples show- ing titer of 1:8 or more (%)	No. of sam- ples show- ing positive slide-LA (%)
		<4	4	8	16	32	64	128	256	512	1,024		
138 ^a	Micro-LA	0	11	18	38	34	29	8	0	0	0	127 (92)	138 (100)
	IHA	0	0	1	10	14	53	32	26	2	0	138 (100)	
18 ^b	Micro-LA	18	0	0	0	0	0	0	0	0	0	0	0
	IHA	18	0	0	0	0	0	0	0	0	0	0	
54 ^c	Micro-LA	54	0	0	0	0	0	0	0	0	0	0	0
	IHA	54	0	0	0	0	0	0	0	0	0	0	

^a Each mouse was given a single subcutaneous injection of heat-killed *Y. pestis*, strain Yreka (1.4×10^8 organisms), and 14 days thereafter the serum was taken.

^b Each mouse was given a single subcutaneous injection of heat-killed *Y. pestis*, strain MII40 (5.4×10^8 organisms), and 14 days thereafter the serum was taken.

^c Nonimmunized control mice.

bacilli when the IHA titers were 32 or more. It is noteworthy that the sera from mice inoculated with strain MII40 bacilli gave a completely negative reaction; this reflects the fact that this strain lacks envelope, or FR-I, antigen. The findings reported in our previous paper (8) were confirmed in Table 4: (i) antiplague titers of serum after treatment with acetone or ME-ace-

tone paralleled those obtained by the WHO standard method; and (ii) the antibodies detected early after the inoculation of bacilli were ME sensitive, whereas those found in later stages were ME resistant.

Survey of antiplague antibodies in sera from wild rodents captured within harbour areas. Sera from wild rodents (including *Rattus*

TABLE 3. *Parallel slide-LA, micro-LA, and IHA tests for detection of antiplague antibodies in laboratory rat sera*

No. of rats	Test	No. of samples showing titer of:									No. of samples showing titer of 1:8 or more (%)	No. of samples showing positive slide-LA (%)
		<4	4	8	16	32	64	128	256	512		
49 ^a	Micro-LA	0	11	16	17	4	1	0	0	0	38 (77.5)	49 (100)
	IHA	0	1	7	7	16	14	3	1	0	48 (97.9)	
8 ^b	Micro-LA	8	0	0	0	0	0	0	0	0	0	0
	IHA	8	0	0	0	0	0	0	0	0	0	
24 ^c	Micro-LA	25	0	0	0	0	0	0	0	0	0	0
	IHA	25	0	0	0	0	0	0	0	0	0	

^a Each rat was given a single subcutaneous injection of heat-killed *Y. pestis*, strain Yreka (10^9 organisms), and 7 to 21 days thereafter the serum was taken.

^b Each rat was injected subcutaneously with heat-killed *Y. pestis*, strain MII40, two times (10^9 organisms each time), and 7 days after the second injection the serum was taken.

^c Nonimmunized control rats.

TABLE 4. *Parallel titration of antiplague antibodies by slide-LA, micro-LA, and IHA methods using immune rat sera*

Rat no. ^a	Interval after injection (days)	Antibody titer measured by:						
		Slide-LA	Micro-LA	WHO standard method ^b			IHA	
				IHA	NSHA	FRIAI	Acetone treatment	ME-acetone treatment
1	7	+ (-) ^c	16 (<4) ^d	64	<4	<4	64	16
	14	+ (-)	32 (<4)	32	<4	<4	32	16
	21	+ (-)	8 (<4)	32	<4	<4	8	8
2	7	+ (-)	16 (<4)	64	<4	<4	64	<4
	14	+ (-)	16 (<4)	64	<4	<4	64	32
	21	+ (-)	8 (<4)	32	<4	<4	32	16
3	7	+ (-)	32 (<4)	64	<4	<4	64	<4
	14	+ (-)	32 (<4)	128	<4	<4	128	128
	21	+ (-)	64 (<4)	128	<4	<4	128	64
4	7	+ (-)	16 (<4)	32	<4	<4	32	<4
	14	+ (-)	16 (<4)	32	<4	<4	32	16
	21	+ (-)	8 (<4)	32	<4	<4	8	8
5	7	+ (-)	16 (<4)	32	<4	<4	32	<4
	14	+ (-)	16 (<4)	64	<4	<4	64	32
	21	+ (-)	4 (<4)	64	<4	<4	64	64

^a Animals, procured from a local farm, were given a single subcutaneous injection of heat-killed *Y. pestis*, strain Yreka (10^9 organisms).

^b NSHA, Nonspecific hemagglutination; FRIAI, FR-I antibody inhibition.

^c Control reaction with noncoated particles is given in parentheses.

^d Titer of control reaction with noncoated particles is given in parentheses.

rattus and *R. norvegicus*) captured in the harbour areas of Kobe and Sakaide during the period 1975 to 1976 were titrated for antiplague antibodies in parallel by the slide-LA, micro-LA, and WHO-IHA methods, as well as by our methods involving acetone or ME-acetone treatment (8). Of 400 samples, all were negative except one, which showed positive reactions by slide-LA (+++), micro-LA (1:8), IHA (1:64), acetone-treated IHA (1:32), and ME-acetone-treated IHA (1:8). The isolation of plague bacilli from tissues of this positive animal was unsuccessful.

DISCUSSION

Although the IHA test (2, 9) has been widely used for serological diagnosis of plague, the necessity of developing a more convenient method(s) for the same purpose is obvious. In view of the usefulness of the LA technique, we investigated the slide-LA and micro-LA methods by using latex particles coated with FR-I antigen. Antisera were obtained from laboratory mice and rats experimentally immunized by inoculation of plague bacilli. The titers obtained

by the micro-LA method showed good correlation with those obtained by the IHA method, although the former titers tended to be a little lower than the latter. The slide-LA reaction became positive 7 days after inoculation of plague bacilli when the titers reached 1:32 or more. The same tests were performed with sera collected from wild rodents captured within harbor areas. Although all but one sample showed negative reactions, the overall results indicated that the antiplague LA methods had a good specificity compared with the standard methods. The data, in addition, again confirmed our previous finding (8) that treatment of test serum with acetone or ME-acetone is useful for analysis and interpretation of antibody titers. The LA tests described herein can be used as convenient methods for detection and titration of antiplague antibodies and are particularly applicable to serosurveillance of plague when dealing with a large number of serum samples.

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