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DEVELOPMENT AND USEFULNESS OF THE GELATIN-PARTICLE-AGGLUTINATION TEST FOR TITRATION OF ANTIBODIES AGAINST DIPHTHERIA, PERTUSSIS AND TETANUS TOXINS

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SUMMARY: The gelatin-particle-agglutination (PA) test for titrating antibodies against diphtheria, pertussis and tetanus toxins was developed and used for assaying 65 sera from healthy children to assess the antitoxin acquisition in relation to the administration of adsorbed diphtheria-purified pertussis-

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tetanus (DPT) combined vaccine. The antitoxin titers obtained by the PA test and the conventional methods were correlated well; the correlation coefficient of the diphtheria antitoxin titers between the PA test and the cell culture method was 0.908, that of the tetanus antitoxin titers between the PA test and the passive hemagglutination test 0.968, and that of anti-pertussis toxin titers between the PA test and polystyrene-ball ELISA 0.885. The PA test was shown to be useful in both developed and developing countries, since it is simple to perform, sensitive and specific, and the three antitoxins can be titrated by the same procedure.

INTRODUCTION

For implementation of vaccination against diphtheria, pertussis and tetanus, seroepidemiological survey is the most reliable method to assess the prevalence of the diseases and the effects of vaccination and to find the individuals who should be vaccinated. The serological tests, however, are often hampered because of the requirement for expensive equipment or for complicated procedures which often need the training of workers. Moreover, a distinct assay method should be used for each disease. Since trivalent diphtheria-pertussis-tetanus (DPT) vaccine was being used worldwide, it will be practically of great benefit, if these three antibodies can be assayed by the same procedure.

In this report, we describe development of the gelatin-particle-agglutination (PA) test for determination of the three antibodies. The gelatin particles used as a carrier of the antigens were the same as those used for HTLV-1 and HIV antibody assay (1,2). The test has routinely been applied for screening at blood banks and other institutions. The test is simple and rapid to perform and does not require any expensive equipment.

We applied the test we developed to the surveillance of Japanese healthy children for the antibodies, and the antibody titers against the three antigens were compared in relation to the vaccination history. The test proved to be not only simple to perform but also sensitive and specific. Therefore, it is suitable to perform in developing countries, where WHO has reinforced the Expanded Program of Immunization (EPI).

MATERIALS AND METHODS

Preparation of PA reagents: The gelatin particles used were artificial spherical ones made of gelatin and gum arabic (3). The gelatin particles were activated with 2.5 µg of tannic acid/ml for 10 min at 37 C, washed, mixed with an equal volume of each antigen, and incubated for 60 min at 37 C. The particle suspensions were then lyophilized and stored at 4 C until used.

Antigens used for coating gelatin particles: Tetanus and diphtheria toxoids purified for vaccine production were used. The original tetanus toxin contained 0.594 mg protein/ml and 250 Lf/ml, and diphtheria toxin 2.438 mg protein/ml and 1,100 Lf/ml. Pertussis toxin (PT), a preparation for acellular pertussis vaccine, was purified from a culture of Tohama strain by salting out with ammonium sulfate, zonal centrifugation, and collecting separately from the filamentous hemagglutinin (FHA) (4). It contained 1.05 mg protein/ml and 58,000 ELISA units/ml.

Reference antibodies: Reference materials (lyophilized) used were the national standard tetanus antitoxin (immune horse serum) lot 3, with a titer of 50 international unit (IU)/ampoule, the national standard diphtheria antitoxin (immune horse serum) lot 9, 1,060 IU/ampoule, and anti-PT-anti-FHA human immunoglobulin lot JN1H-10, containing 250 anti-PT ELISA unit (EU)/ampoule, and 400 anti-FHA EU/ampoule. Before use, each was dissolved in saline and it was diluted to a desired concentration.

Sample sera: The samples were 65 sera collected from healthy children in Shizuoka Prefecture in 1990 for the purpose of pertussis seroepidemiology under the project of the National Epidemiological Surveillance of Vaccine Preventable Diseases (5,6). The donor's ages and vaccination histories are shown in Table II.

PA test for antibody titration: The procedures were the same as described for the HTLV-I antibody assay (1). Twofold serial dilutions (25 µl) of a sample serum were made on a U-bottomed 96-well microplate and each dilution was mixed with an equal volume of a sensitized particle suspension. The mixtures were allowed to stand for 2 hr at room temperature and read for agglutination with the naked eye. To the first well of the serum dilutions, added were nonsensitized gelatin particles instead of sensitized ones to serve as a control for nonspecific reaction. The highest dilution showing positive agglutination was determined and the PA titer was expressed as twice the highest net serum dilution factor.

Passive hemagglutination (PHA) test for tetanus antitoxin (7): A 2.5% (V/V) suspension of tanned, formalinized sheep red blood cells (FSRC) was sensitized with an equal volume of purified tetanus toxoid (15 Lf/ml) for 30 min at 37 C, followed by washing and resuspending in 2.5% (V/V) in saline. Serum samples were each diluted 1:5 with saline, inactivated for 30 min at 56 C, and absorbed with 0.05 ml of 20% (V/V) unsensitized FSRC per ml of a serum dilution for 60

min at 37 C, and centrifuged. The supernatant served for assay. Three lines of 25% increasing serial dilutions of each serum sample were made on a microplate and the average value was taken as HAU.

Cell culture method (CCM) for diphtheria antitoxin: The micro-cell-culture-color-change method with VERO cells (8) was used.

PS-ball ELISA for anti-pertussis toxin: A commercially available ELISA Kit for anti-pertussis toxin assay (Takeda Chemical Industries Ltd., Osaka) with antigen-coated polystyrene balls as the solid phase (the PS-ball ELISA method) (5,9,10) was used according to the manufacturer's manual.

DPT vaccine for immunization: An adsorbed diphtheria-purified pertussis-tetanus combined vaccine (4) commercially available was used.

RESULTS

Preparation of PA Reagents

The gelatin particles were sensitized by mixing with each antigen as described in Materials and Methods. The optimal conditions for pH of the buffer and antigen concentration for sensitization with each antigen were investigated with their reference antisera and three kinds of gelatin particles with different electrophoretic mobilities.

The conditions tested for tetanus and diphtheria toxoids were the pH range from 5.0 to 8.0 in 0.15 M PBS and the antigen concentrations ranging from 5 to 300 µg/ml. Those for pertussis toxin were the pH range of 6.0-8.5 and the antigen concentrations of 5-80 µg/ml. The optimal conditions obtained preparing the PA reagents and the results of the PA test with the reference antiserum are shown in the upper part of Table I.

Based on the results, the sensitivity and the theoretical value were calculated: PA at 1:16 corresponded to 0.08 HAU for tetanus antitoxin, 0.016 IU for diphtheria antitoxin and 1.6 EU for anti-pertussis toxin.

Antitoxin Titration of Sera from Healthy Children

According to the results shown in Table I, the gelatin particles coated separately with each of the three antigens were prepared, with which the sera from healthy children were assayed (Table II). Comparisons of the results with those of the conventional assays are shown in Fig. 1. With all three antigens, high correlations were demonstrated: the correlation coefficients calculated

Table I. Preparation of the PA reagents and the results of the PA test

	Tetanus	Diphtheria	Pertussis
Optimal pH for sensitization	pH 6.0	pH 6.0	pH 8.0
Antigen concentration	150 µg/ml	150 µg/ml	10 µg/ml
Reference antibody			
Conventional method	10 HAU/ml	1 IU/ml	25 EU/ml
PA titer	1:2,048	1:1,024	1:256
PA sensitivity at PA 1:16	0.08 HAU/ml	0.016 IU/ml	1.6 EU/ml
PA sensitivity on human sera tested in this experiment			
at PA 1:16	0.10 HAU/ml	0.042 IU/ml	1.2 EU/ml

among those with positive results in at least either of the two methods were 0.968 between PHA and PA for tetanus antitoxin, 0.908 between CCM and PA for diphtheria antitoxin and 0.885 between EU and PA for anti-pertussis toxin.

From the data shown in Fig. 1, regression analyses were made and the sensitivities of the PA tests were calculated. The corresponding antibody titers obtained by the conventional assays at PA 1:16 were 0.10 HAU/ml for tetanus, 0.042 IU/ml for diphtheria and 1.2 EU/ml for pertussis.

Concerning the tetanus antitoxin assay, the sensitivity was nearly that expected from the data of the reference antitoxin as shown to the bottom of Table I; the PA titer at 1:16 corresponded to 0.10 HAU/ml as compared with 0.08 HAU/ml in the reference antitoxin test. As shown in Fig. 1, when PA at 1:16 and higher was taken as tetanus antitoxin positive, 36 HAU-positive samples (over 0.02 HAU/ml) were all positive, and when PA at 1:32 and higher was taken as positive, only one (No. 72, 0.119 HAU/ml) was in the negative category.

Concerning the diphtheria antitoxin assay, the PA titer at 1:16 corresponded to 0.04 IU/ml of the CCM titer, 1/2.6 less sensitive than expected from the data of reference antitoxin shown in Table I. As a result, when PA 1:16 and higher were considered as positive, two of 11 sera possessing CCM titers of 0.02-0.08 IU/ml were in the negative category, and seven of these 11 sera became negative when PA 1:32 and higher were taken as positive.

	PHA (0.02 HAU/ml)		
	-	+	Total
PA (1:16) -	29	0	29
+	0	36	36
Total	29	36	65

	CCM (0.02 IU/ml)		
	-	+	Total
PA (1:16) -	27	2	29
+	0	36	36
Total	27	38	65

	ELISA (10 EU)		
	-	+	Total
PA (1:64) -	41	0	41
+	2	22	24
Total	43	22	65

Fig. 1. Correlation in antibody titers between PA and the conventional methods. Conventional methods for antibody titration were passive hemagglutination (PHA) for tetanus antitoxin, micro-cell-culture-color-change method with VERO cells (CCM) for diphtheria antitoxin, and ELISA with antigen-coated polystyrene balls (PS-ball ELISA method) for anti-pertussis toxin. "r" indicates correlation coefficient and "b" regression coefficient.

To increase sensitivity, another lot of the PA reagent sensitized with the antigens at a higher concentration (300 µg/ml) was prepared. With this lot, five of the seven samples with low PA titers were assayed; four (No. 56, 57, 70 and 71) showed elevated titers by one dilution and another one (No.99) unchanged (see Table II). The difference between the hyperimmune reference antibody and individual human sera can be explained for by the difference in the avidity of the antibodies.

Concerning pertussis toxin antibody assay, the PA titers at 1:16 (corresponding to 1.2 EU/ml) were close to the lowest detectable level of ELISA (1.0 EU/ml) (10), and all samples with >10 EU/ml, the level suggested as anti-pertussis toxin positive by others (11), showed the PA titers of >1:64. When the samples lower than 10 EU/ml were considered, however, some inconsistency was noted between PA and ELISA.

Correlation of the Titers of the Three Antibodies with the Vaccination History

1) The tetanus antitoxin and vaccination history. All 35 children who had been vaccinated more than once showed the tetanus antitoxin in both PA and PHA. One (No. 71 in Table II) of 30 children who had not reportedly been vaccinated showed some tetanus antitoxin in both PA (1:32) and PHA (0.19 HAU/ml, data not shown). Since the case had also some diphtheria antitoxin of the minimum detectable titer, it is most likely that the case had received a vaccine at least once.

The results showed that detection of the antitoxin should indicate a history of tetanus vaccination including DPT vaccine, because the tetanus antibody can not be acquired naturally (12).

2) Relationship among the three antitoxins. Of the 65 children, from whom samples were obtained, most that had reportedly received DPT vaccination contained antibodies against diphtheria, pertussis and tetanus toxins. Of the 65 samples tested, five gave inconsistent results with the tetanus antitoxin; one (No. 15) in diphtheria and the other four in pertussis (No. 57 was excluded from the diphtheria antitoxin-negative category since it was positive by the improved reagent). No. 15 in Table II had antibody only to diphtheria on a minimal detectable level. The sample showed also a minimum titer by the conventional CCM test (0.02 IU/ml, data not shown) and thus it might be a specific antibody to diphtheria or some related antigens.

The other three had both tetanus and diphtheria antitoxins but lacked detectable anti-pertussis toxin antibody (No. 57, 71, 72). Their negative results

Table II. DPT vaccination history and antibody titers against diphtheria, pertussis and tetanus toxins determined by the PA test

Code No.	Vaccination history**				Age	PA titer		
	I-1	I-2	I-3	II		Tetanus	Diphtheria	Pertussis
1	-	-	-	-	1	< 16	< 16	< 16
2	-	-	-	-	1	* < 16	* < 16	< 16
3	-	-	-	-	< 1	< 16	< 16	< 16
4	-	-	-	-	1	< 16	< 16	< 16
5	-	-	-	-	1	< 16	< 16	< 16
6	-	-	-	-	1	< 16	< 16	< 16
7	-	-	-	-	1	< 16	< 16	< 16
8	-	-	-	-	1	< 16	< 16	< 16
9	-	-	-	-	< 1	< 16	< 16	< 16
10	-	-	-	-	< 1	< 16	< 16	< 16
11	-	-	-	-	1	< 16	< 16	< 16
13	-	-	-	-	1	< 16	< 16	< 16
14	-	-	-	-	1	< 16	< 16	< 16
15	-	-	-	-	< 1	< 16	16	< 16
16	-	-	-	-	< 1	< 16	< 16	< 16
17	-	-	-	-	< 1	< 16	< 16	< 16
18	-	-	-	-	1	< 16	< 16	< 16
20	-	-	-	-	< 1	< 16	< 16	< 16
22	+	-	-	-	2	32	64	64
23	+	+	+	-	3	*128	*512	*64
26	-	-	-	-	2	< 16	< 16	< 16
28	+	+	+	-	3	128	64	128
29	-	-	-	-	2	< 16	< 16	< 16
31	+	+	+	+	3	*2048	*2048	512
33	+	+	+	+	3	>2048	1024	128
34	-	-	-	-	3	< 16	< 16	< 16
35	+	+	+	-	3	128	256	< 16
39	-	-	-	-	2	< 16	< 16	< 16
41	+	+	-	-	4	128	1024	256
43	+	+	+	+	4	2048	256	128
44	-	-	-	-	5	< 16	< 16	< 16
47	+	+	+	+	4	*1024	*2048	512
48	+	+	+	+	4	1024	512	128
49	+	+	+	+	6	512	256	32
51	+	+	+	+	4	>2048	2048	128
52	+	+	+	-	6	512	1024	256
55	+	+	+	+	4	512	512	1024
56	+	+	+	-	6	32	16#	16
57	+	+	+	+	6	32	< 16#	< 16
58	+	+	+	+	5	512	64	128
59	+	+	+	-	4	64	128	32
61	+	+	+	+	7	*1024	*512	32
62	+	+	+	+	8	* 256	*128	64
64	+	+	+	+	7	256	1024	128
69	+	+	+	+	7	256	64	128
70	+	+	+	+	9	64	16#	16
71	-	-	-	-	7	32	16#	< 16
72	+	+	+	-	7	16	64	< 16
73	+	+	+	+	9	1024	128	64
74	+	+	+	+	9	128	128	64
75	+	+	+	+	9	256	64	32
76	+	+	+	+	8	256	512	64
77	+	+	+	+	9	*64	*32	*16
81	-	-	-	-	< 1	< 16	< 16	< 16
84	-	-	-	-	1	< 16	< 16	< 16
85	-	-	-	-	< 1	< 16	< 16	< 16
88	-	-	-	-	3	< 16	< 16	< 16
89	-	-	-	-	2	< 16	< 16	< 16
91	+	+	+	+	6	1024	1024	512
92	+	+	+	+	4	512	1024	256
95	+	+	+	+	9	128	32	128
96	+	+	+	+	9	1024	256	512
98	+	+	+	-	8	64	32	256
99	+	+	-	+	8	*32	*16	*16
100	-	-	-	-	1	< 16	< 16	32

* PA titer after absorption with unsensitized particles.

PA titer by the improved reagent was 1:32.

** I-1, I-2 and I-3 indicate the first, second and third primary vaccination, respectively, and II indicates a booster vaccination.

were consistent with those of the pertussis ELISA test, indicating that the pertussis toxin antibody was not acquired or below the detectable level; it is notable that their antitoxin titers against diphtheria and tetanus were low in parallel. No. 100 serum showed a low level of antibody to pertussis toxin but none of diphtheria or tetanus antitoxins.

DISCUSSION

This study had two main purposes: one was to examine the sensitivity and specificity of the newly developed PA test in comparison with the conventional methods and the other its acceptance and usefulness for practical use in developed and developing countries.

For the conventional method for tetanus antitoxin assay, we used PHA, which had not been accepted as an alternative of the legal assay method, *in vivo* neutralization in mice, although the correlation between PHA and mice neutralization has been established (7). Therefore, the final evaluation of the tetanus PA test should be made by the neutralization test in mice. In spite of such a drawback, the tetanus PA test was well comparable in the sensitivity and specificity with PHA assay.

The lower sensitivity of the diphtheria PA test with the first lot of the reagents was improved by coating the gelatin particles with the antigen of an increased concentration.

For the anti-pertussis toxin titer, 10 EU/ml was set as a cut-off value for the positive antibody level in the ELISA test (10). In our results, all samples with >10 EU had PA titers $\geq 1:64$, and even two with lower titers than 10 EU/ml (1.25 and 5) were 1:64 in PA. Thus, for confirmation of the recent infection or diagnosis purpose of the onset of the diseases, the PA test is sensitive enough. When sera with titers lower than 10 EU/ml were considered, however, some showed PA titers inconsistent with the ELISA titers. Therefore, further comparative studies are needed to use the PA test to detect antibody of lower levels; for example, to detect transplacentally transmitted maternal antibodies in infants or to assess the response to a primary vaccination.

It is well known that no tetanus antitoxin may be acquired naturally, and detection of the antitoxin should be considered to have been acquired by a tetanus toxoid vaccination. Our comparative studies among DPT antibodies by the PA

test confirmed that the antitoxin to tetanus is a reliable indicator of tetanus or DPT vaccination. The results are most useful for implementation of EPI when the vaccination rate is unknown or needs to be confirmed. For this purpose, the same procedures for serological assay for antibodies against diphtheria, pertussis and tetanus toxins by the PA test will be of great benefit at the practical sites.

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