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Original Article

Tetanus Antibody Assay Combining In-House ELISA and Particle Agglutination Test and Its Serosurvey Application in a Province in Turkey

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SUMMARY: In order to determine a practically useful quantitative assay method for tetanus antibody in a large-scale seroepidemiological study, a method combining an in-house ELISA with a particle agglutination test (KPA) was evaluated in comparison with the in vivo mouse neutralization test. Serum samples with mouse neutralization antibody titers 0.01 IU/ml (the minimum protective level) or below showed considerable overestimation of antitoxin titers up to 1.0 IU/ml when studied by in-house ELISA alone. On the other hand, the KPA values were highly correlated with the mouse test, even in cases of titers equal to 0.01 IU/ml or below. The combination of these two procedures, in which in-house ELISA values of 1.0 IU/ml or below were replaced by KPA values, provided a high correlation in antibody titers with the mouse test (r = 0.968). We applied this combined method to a tetanus seroepidemiological survey in a province in Turkey. The survey included 347 subjects from the healthy population, and the quantitative analyses showed high antibody levels in children and young adults and significantly low levels among adults aged 40 or over. A characteristic distribution of antibody titers in each age group was also demonstrated.

INTRODUCTION

In 1997, Refik Saydam National Hygiene Center (RSHC) and the General Directorate of Primary Health Care of the Ministry of Health of Turkey, with the cooperation of the Japan International Cooperation Agency, implemented a project for infectious disease control in Turkey. Seroepidemiological surveillance on vaccine-preventable diseases including tetanus, diphtheria, pertussis, polio, measles, and hepatitis B, was conducted in 2000 - 2001 (1,2). Three provinces with geographically distinct locations and different socio-economic levels were selected for the survey; one each from the regions of the Black Sea (Samsun), the Mediterranean (Antalya), and the South-East Anatolia (Diyarbakir). This was a cross-sectional study involving both urban and rural health centers. Serum samples from randomly selected healthy subjects were analysed to determine the current states of immunity to vaccine-preventable diseases. The project also aimed to establish laboratory methods for routine use in seroepidemiological surveillance in RSHC.

For infectious disease surveillance in a population, seroepidemiological study on antibody prevalence is essential, and it requires a method technically and economically suitable to test a large number of samples. Also, the method should preferably be applicable to various antigens in a laboratory. ELISA is a suitable method for this purpose; it is cost effective and can evaluate antibodies against various antigens using the same procedure.

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According to a generally adopted classification, a tetanus antitoxin level of 0.01 IU/ml is considered to be the minimum protective level though it affords incomplete protection; 0.1 IU/ml is considered protective; and 1.0 IU/ml or above offers long-term protection (3-5). The reliable titration of such protective levels is necessary to predict the immunity against tetanus in a given population. ELISA has been widely used for tetanus seroepidemiology. However, human sera with low antibody titers are reportedly often overestimated when titrated by ELISA (3,6). To overcome this problem, the tetanus antibody assays with ELISA in the early studies were supplemented by a neutralizing antibody assay in mice for low-titer sera (7,8). Subsequent modifications of the ELISA with additional procedures such as a competitive assay, a toxin binding inhibition assay, and a double-antigen procedure were then described (9-12).

The purpose of the present study was to determine a practically useful method for a quantitative assay of tetanus antibodies in a large number of serum samples, covering even the minimum protective level or lower. We evaluated a combined assay method in which the antibody titers equal to or less than 1.0 IU/ml with in-house ELISA were replaced by those of a particle agglutination test (KPA). We also describe here the result of the application of this combined method to a seroepidemiological survey conducted in a province as a part of a project for infectious disease control in Turkey. Our study showed that this combined assay method made it possible to provide accurate assessment of age-specific tetanus antibody prevalence in the areas under study.

MATERIALS AND METHODS

Mouse neutralization test: Tetanus test toxin (4.4×10^6

LD_{50}/ampoule) supplied by the National Institute of Infectious Diseases, Tokyo, was used for the in vivo assay. The test was performed by the lethal end point method using Swiss mice of either sex, weighing 17-20 g, randomly bred in our own establishment. Sera were titrated at the L+/1000 dose of tetanus toxin. The range of serum dilutions to be tested depended on the tetanus antitoxin content of the sera, which had been previously determined by in-house ELISA. Three different dilutions of serum were incubated with tetanus toxin for 1 h at room temperature, and 0.4 ml of the mixtures was then injected subcutaneously into each of two mice. The mice were observed for the following 5 days, and the symptoms and time of death were recorded. A control series of mice, injected with tetanus toxin mixed with defined amounts of the International Standard for Tetanus Antitoxin (1,400 IU/ampoule, National Institute for Biological Standards and Control [NIBSC], Potters Bar, UK) were included in every experiment, and titers of the samples were calculated according to these results. The amount of tetanus antitoxin in each serum was expressed in International Units per ml (IU/ml). The cut-off value used for the test in our laboratory was 0.01 IU/ml.

KPA: KPA was performed using a kit manufactured by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. The kit was made of polypeptide artificial carrier particle (porous spheres of poly γ-methyl-L-glutamate) stained with Reactive Blue solution, sensitized with highly purified tetanus toxoid using tannic acid, and provided in lyophilized form (13,14). The test was performed according to the manufacturer's instructions. For brief, a volume of 25 μl of twofold serial dilutions of test sera was mixed with a 25 μl suspension of the coated particle in the wells of a U-bottomed microplate. The plate was held for 2 h at room temperature. Non-agglutinated particles settled to form a clear spot with a smooth circumference in the center of the well that was judged to be negative. Agglutinated particle formed a large rough ring that was judged to be positive. The reference serum of the kit was assayed in parallel in each test, and the result was used to determine the minimum detectable level. The antibody titer of the test serum was calculated by multiplying the end point dilution of positive agglutination and the minimum detectable titer of the reference serum. According to the manufacturer, the cut-off value of the test is 0.01 IU/ml.

In-house IgG ELISA (In-house ELISA): An in-house ELISA for anti-tetanus toxoid IgG antibody was conducted using 96-well flat-bottom plates (Greiner, no. 655001, Frickenhausen, Germany) with a purified tetanus toxoid (Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan). The plates were coated with tetanus toxoid (100 μl at 2 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6) overnight in a refrigerator in a humid atmosphere. Then 125 μl of blocking buffer (PBS containing 0.5% BSA) was distributed into each well and incubated for 1 h at 37°C in an Incubator/shaker (Labsystem, Helsinki, Finland). After every step, plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T). Eight twofold serial dilutions of test sera and the reference antiserum were prepared with PBS containing 0.5% BSA and 0.05% Tween 80, and then added to the plates. After being incubated for 1 h at 22°C, Fc-specific alkaline phosphatase-conjugated goat anti-human IgG (Seikagaku Kogyo, Tokyo, Japan) diluted in PBS-T was distributed to each well, and plates were held for 1 h at 22°C. Finally, p-nitrophenyl phosphate (Wako, Tokyo, Japam) in diethanolamine buffer (1 mg/ml, pH 9.6) was added to each well. After 1 h at 22°C, the reaction was stopped by 3M NaOH solution, and the plates were read at $A_{405/630}$ by an ELISA reader (Labsystem).

The International Antitoxin Unit was determined using the reference antitoxin (Anti-tetanus immunoglobulin, human, lyophilized, 120 IU/ml, NIBSC), using a parallel line assay (15). A standard curve was drawn for each plate, and the curve drawn by the optical densities of test serum dilutions, which was linear and parallel to the standard curve, was interpolated. Statistical significance in the test was set at a probability of $P = 0.05$. The cut-off value of the test was determined to be 0.01 IU/ml.

Study population and serum samples: This study was part of the national seroepidemiological surveillance of vaccine-preventable diseases, conducted by the Ministry of Health of Turkey in three selected provinces in 2000-2001 (1). For the surveys, study subjects were selected randomly from each age group by health centers in the given areas, and questionnaires and blood samples were collected after obtaining informed consent to participate (2).

For the comparative study between the tests, 62 serum samples from Samsun Province with different antibody levels by ELISA were used. More sera with low ELISA titers were selected and titrated by the mouse neutralization test and KPA, respectively. The sera studied to determine the applicability of the method to seroepidemiology were collected in 2001 from Antalya Province from subjects including a healthy population ranging in age from 6 months to 91 years old

Table 1. Number of subjects and vaccination history, Antalya, 2001

Age group	Total	Sex		Area		Tetanus vaccination dose				
		Male	Female	Rural HC	Urban HC	Non	1-2	3-4	5≤	unknown
0-1	28	13	15	14	14	0	2	26	0	0
2-3	31	14	17	17	14	1	0	29	0	1
4-5	26	11	15	12	14	0	0	25	0	1
6-7	27	10	17	12	15	1	0	16	10	0
8-9	32	18	14	17	15	0	2	2	27	1
10-19	33	14	19	18	15	0	6	6	18	3
20-29	41	16	25	19	22	0	12	14	6	9
30-39	40	16	24	18	22	2	10	10	12	6
40-49	42	20	22	24	18	2	14	8	3	15
50-	47	25	22	23	24	3	12	4	1	27
Total	347	157	190	174	173	9	58	140	77	63

HC: health centers.

(Table 1). The group of 347 subjects consisted of males and females, from rural and urban areas, with no statistically significant difference between groups ($P > 0.05$).

Statistical analysis: Statistical analysis was carried out using the chi-squeare test and F test. A titer of less than 0.01 IU/ml was set to 0.005 IU/ml for calculating the geometric mean titer (GMT).

RESULTS

Comparison of antibody titers measured by KPA or in-house ELISA with those by in vivo neutralization test in mouse: Sixty-two sera were used for the comparison. The in vivo neutralization test in mouse was taken as the standard test. Correlations of the values obtained by the KPA and the in-house ELISA against the mouse test are shown in Figs. 1a and 1b, respectively.

In the mouse test, 37 of the 62 sera provided antibody titers equal to or above 0.01 IU/ml, and 25 sera were below this level. The antibody values determined by KPA correlated well with those by the mouse test; 37 sera were >0.01 IU/ml and 25 were below this level (Fig. 1a). On the other hand, in the in-house ELISA, 23 of 25 serum samples that were below 0.01 IU/ml by the mouse test showed values between 0.1 IU/ml and 1.0 IU/ml (Fig. 1b). Thus, within this low-titer range, the in-house ELISA values resulted in a mixture of specific and non-specific reactions in which it was impossible to differentiate the presence of protective antibody from non-specific reactivity. The results indicated that the in-house ELISA was most useful to estimate samples above 1.0 IU/ml. Under this level, however, the method is not acceptable, and the serum titers should be determined by either the mouse neutralization test or KPA.

Combination of tetanus antibody titers of in-house ELISA above 1.0 IU/ml and KPA equal to or below 1.0 IU/ml: Figure 1c shows the correlation analysis of the combined assay method in which the tetanus antibody titers >1.0 IU/ml (17 of 62 serum samples) were based on the results of the in-house ELISA and those 1.0 IU/ml or below (45 of 62 serum samples) were based on the KPA. In order to evaluate the combination of the two methods statistically, the correlation coefficients and the regression lines against the mouse test were calculated separately for the titers from the two methods of the combined line, excluding double-negative titers in both tests (<0.01 IU/ml, $n = 24$) in order to avoid adverse affects resulting from their significant number.

In Fig. 1c, the correlation between titers of the mouse test and a range >1.0 IU/ml of the combined line (titers based on the in-house ELISA, $n = 17$) was r = 0.903, and the regression line equation was Y = 1.1509 X − 0.1569. The correlation between titers of the mouse test and a range of <1.0 IU/ml of the combined line excluding the double-negatives in both tests (titers based on the KPA, $n = 21$) was r = 0.921, and the regression line equation was Y = 0.9399 X − 0.1607. No statistically significant difference was found between the correlation coefficients of the two component tests of the combined line, which were calculated separately (r = 0.903 and r = 0.921, $P > 0.05$), nor between their intercepts (–0.1569 and –0.1607, $P > 0.05$). The correlation between the titers of the mouse test and the combined line of the two tests excluding the double-negatives in both tests was r = 0.968 ($n = 38$), and the regression line equation was Y = 1.0429 X − 0.0922.

The results indicated that the replacement of in-house ELISA values equal to 1.0 IU/ml or below with KPA values could be applicable for tetanus seroepidemiology in the whole titer range including levels as low or lower than that of minimum protection.

Application of the combined method to analysis of tetanus antibody prevalence in Antalya: The method combining the in-house ELISA and the KPA was applied for the quantitative assay of community-based seroepidemiology against tetanus in Antalya. All of the 347 serum samples were titrated by in-house ELISA and the 138 samples with titers 1.0 IU/ml or below were reassessed by KPA.

The distribution of tetanus antibody titers in each age group is illustrated in Fig. 2, which clearly shows a characteristic

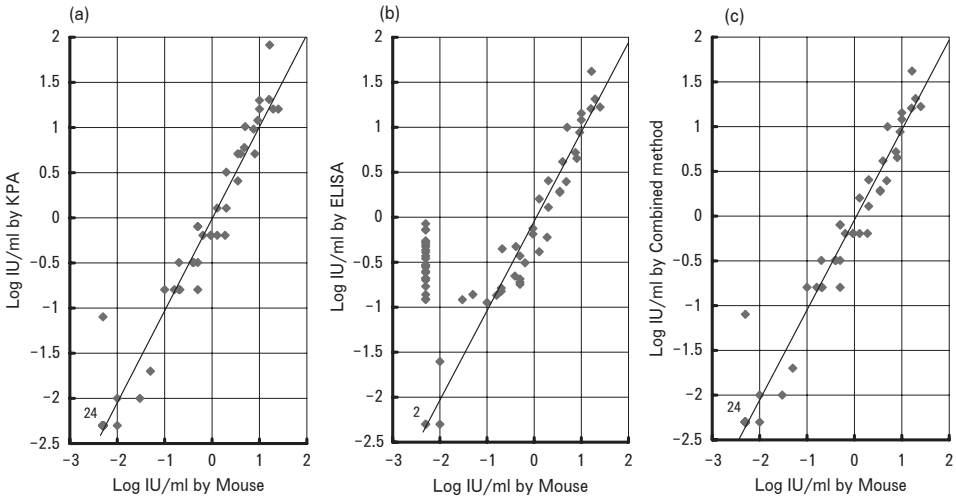

Fig.1. Correlation of tetanus antibody titer measurements using four methods. The in vivo neutralization test in mouse, along with in vitro KPA, in-house ELISA, or the combined method of in-house ELISA with KPA, were used for measurements ($n = 62$). A titer of less than 0.01 IU/ml was set to 0.005 IU/ml in the figures. The number at the lower left in each figure indicates the number of multiple marks for the double-negatives (<0.01 IU/ml) – that is, on both tests.
(a) Correlation of antibody titers in mice and KPA. r = 0.901, excluding double-negatives (<0.01 IU/ml) in both tests. (b) Correlation of antibody titers in mouse and in-house ELISA. r =0.903 in the range of >1.0 IU/ml. (c) Correlation of antibody titers in mouse and the method combining in-house ELISA (>1.0 IU/ml) with KPA(<1.0 IU/ml). r = 0.968 excluding double-negatives (<0.01 IU/ml) in both tests.

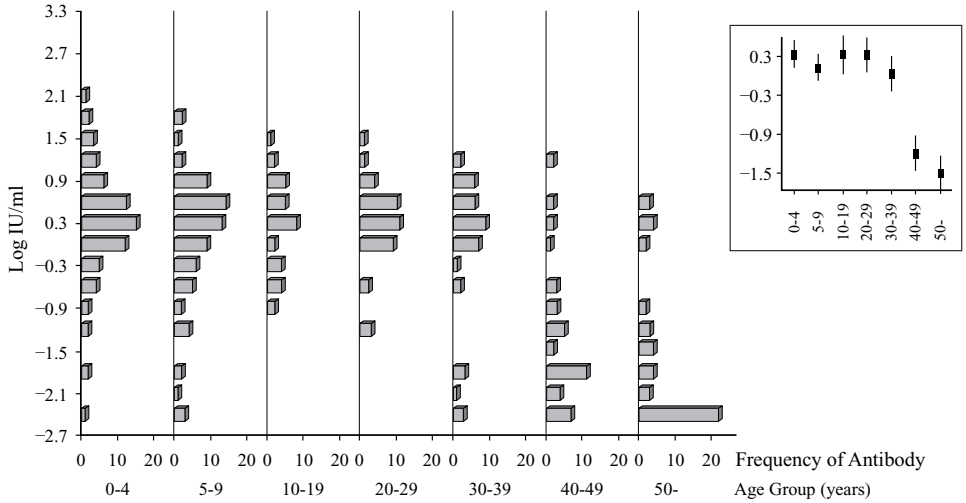

Fig. 2. Tetanus antibody prevalence in each age group in Antalya Province, Turkey, 2001. Tetanus antibody titers were determined by the combined method of in-house ELISA and KPA (n = 347). Inset shows geometric mean titers (GMTs) and confidence interval of antibody titers in each age group. A titer of less than 0.01 IU/ml was set to 0.005 IU/ml for calculating GMT. The x-axis of the inset indicates age groups in years, and the y-axis indicates Log_{10} IU/ml.

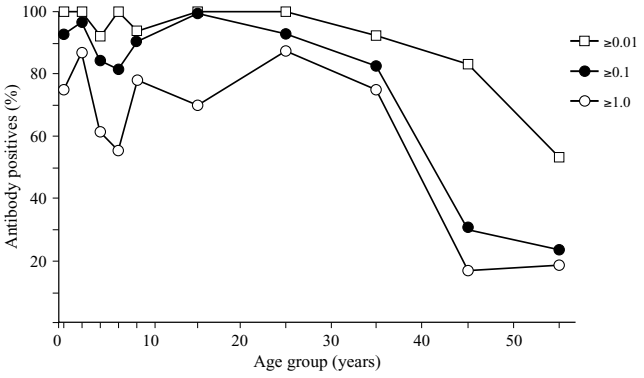

Fig. 3. Age group distribution at three different tetanus antibody levels, Antalya Province, Turkey, 2001. Tetanus antibody titers were determined by the combined assay method of in-house ELISA and KPA. n = 347.

distribution of tetanus antibody in each age group. The great majority of the children and young adults had antibody titers above the minimum protective level, and GMTs for the age groups up to those in their 30s were more than 1.10 IU/ml (log_{10} value 0.04, the inset of Fig. 2). In contrast, in groups of subjects in their 40s and those over 50, the antibody titers were scattered, and a significant number of persons lacked the minimum protective level of antibody. Seven of 42 subjects in the 40s age group (16.7%; 95% confidence interval [CI] 31.4 - 7.0%) and 22 of 47 subjects ≥50 years (46.8%; CI 61.9 - 32.1%) were revealed to have antibodies under the minimum protective level of 0.01 IU/ml. The remarkably low GMTs in these older groups compared with levels in other age groups are also demonstrated in the inset of Fig. 2 ($P < 0.01$); 0.065 IU/ml (log_{10} value - 1.19, CI 0.118 - 0.035 IU/ml) for subjects in their 40s, and 0.032 IU/ml (log_{10} value -1.494, CI 0.057 - 0.018 IU/ml) for those ≥50 years.

In order to demonstrate tetanus antibody prevalence in Antalya, three different antibody levels were used (Fig. 3). The antibody-positive rates were 89.6 % (CI 92.6 - 85.9%) for ≥0.01 IU/ml, 74.1% (CI 78.6 - 69.1%) for ≥0.1 IU/ml, and 60.2% (CI 65.4 - 54.9%) for 1.0 IU/ml in total subjects. The antibody prevalence above 0.1 IU/ml, which is considered to be a fully protective level against tetanus, was more than 82% in the groups of children and adults up to those in their 30s, but was markedly reduced to 31.0% (CI 47.1 - 17.6%) in subjects in their 40s, and 23.4% (CI 38.0 - 12.3%) in ≥50 years old ($P < 0.01$). In addition, at the higher antibody levels of 0.1 IU/ml and 1.0 IU/ml shown in Fig. 3, a certain reduction was seen in groups of those 4 - 5 and 6 - 7 years of age compared with those in the younger age group, but the rates increased again at 8 - 9 years of age. The reduction in antibody levels was statistically significant ($P < 0.05$).

A summary of the vaccination histories of the subjects collected by questionnaires are shown in Table 1. High vaccination rates of more than 90% for ≥3 doses are shown among children in groups under 10 years of age, but in groups of subjects in their 40s or over 50, only 16 of 89 subjects had ≥3 doses. Moreover, many of these older subjects had an unknown history of tetanus vaccination. In addition, a large percentage of children had, by 8 - 9 and 10 - 19 years of age, received a 5th injection of tetanus vaccine, in contrast to 3 - 4 doses received by those in the younger groups. It was observed that antibody prevalence pattern was correlated with vaccination histories throughout all subject groups.

DISCUSSION

Although ELISA is widely used for tetanus seroepidemiology, the overestimation that occurs in a significant number of low-titer sera poses problems (3,6,16). Our study confirmed that the sera with antibody titers less than 0.01 IU/ml by the mouse neutralization test gave a considerable titer variation up to 1.0 IU/ml with in-house ELISA, as described previously (9). As a result, the in-house ELISA failed to discriminate between in vivo neutralizing antibody and non-specific reactivity at low antibody levels. KPA was easy to perform, and the results were well correlated with the in vivo mouse neutralization method for all antibody examined in a serological survey. However, this method was more expensive and not cost effective for assay of a large number of samples. On the other hand, the combination of these two methods was acceptable for tetanus seroepidemiology; it can obtain reliable antibody titers quantitatively at a comparatively lower

cost. In this study, this method was applied for community-based tetanus seroepidemiology, which was done for the first time in Turkey, in contrast to the studies on limited age groups previously reported from this country (17-19).

It is generally accepted that the antibody against tetanus toxin cannot be obtained naturally and that the antitoxin detected in human sera is exclusively induced by vaccination (3). Immunization against tetanus in Turkey started in 1937 and was accelerated in 1985 with the National Vaccination Campaign. At present, based on the National Routine Immunization Program of the Ministry of Health, children receive their primary vaccination against tetanus with 3 doses of diphtheria-pertussis-tetanus vaccine (DPT) in the 2nd, 3rd, and 4th months after birth, and the 4th dose at 16 - 24 months. Subsequently, 2 booster doses with adult form diphtheria-tetanus vaccine (dT) are offered at primary school and another dose with tetanus toxoid (TT) is offered during high school. In addition, women receive two TT doses in their first pregnancy. Men in the army are also given additional TT. According to the data from the General Directorate of Primary Health Care, Antalya was one of the provinces with the highest immunization coverage in Turkey; the reported vaccination coverage in Antalya for the 3rd dose of DPT during the past 5 years was approximately 90% on average, against approximately 80% on average throughout the country (20).

Our study demonstrated that children and younger adults possess a high antibody level of immunity against tetanus, whereas a large proportion of the older population lacked even the minimum protective level. The evidence of low-titer antibodies in a significant percentage of these older age groups, as shown in Fig. 2 and also at the 0.01 IU/ml level in Fig. 3, may suggest the waning of the antibody during the years after immunization. Further, it is considered very likely that older groups had received insufficient vaccination during childhood, given that the national vaccination program for children was not fully in place before 1985. Also, the vaccination rates of these groups in our study were significantly low, and many subjects in these groups did not know their vaccination history.

The high rate of minimum antibody level among children with a certain reduction in antibody titers in those from 4 to 7 years of age indicated that after the four primary immunizations by 24 months old, the antibody titers decreased until the booster in the primary school period, which once more elevated the children's antibody levels. This result provides evidence for the effectiveness of booster injections during the primary school period, confirming that the national vaccination schedule implemented in Turkey is adequately providing tetanus immunity among the young population.

Our results showed that a significant portion of the general population possessed the tetanus antibody in a range not measurable by in-house ELISA, and these subjects had to be reassessed by KPA. We successfully conducted a large-scale, quantitative seroepidemiological study covering as low as the minimum protective level by applying the combined method described above. However, further improvement of in-house ELISA for tetanus seroepidemiology remains to be considered.

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