Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to Influenza A and B and Parainfluenza Type 1 in Sera of Patients

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An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of antibodies to influenza A/Hong Kong/1/68, influenza A/Victoria/3/75, influenza B/Hong Kong/5/72, and parainfluenza type 1 viruses. Development and standardization of the method indicated that an acetone-ethyl alcohol mixture was a suitable fixative for the preparation of the solid-phase coupled antigen. The addition of sodium azide to the enzyme-conjugated solution and the concentrations of the enzyme-conjugate antiglobulin and test sera employed were all critical factors in the success of the ELISA procedure. The ELISA test was specific; there was no cross-reaction between influenza A and B or parainfluenza type 1 viruses. The concordance between ELISA and hemagglutination inhibition results suggested that both tests probably detected the same type of antibodies. The ELISA procedure was 8 to 64 times more sensitive than complement fixation and/or hemagglutination inhibition tests. Low levels of antibody in patients' sera were detected only by the ELISA test. During the course of the testing period false positive reactions were not encountered. The results of ELISA could be obtained within 3 h. The ELISA test required a very small amount of serum and, therefore, offered an opportunity to detect the presence of maternal antibodies to influenza viruses in blood collected from infants by heel prick.

Several studies have found the enzyme-linked immunoassay (ELISA) and radioimmunoassay to be of equal sensitivity (11, 20, 21). The quantitative determination of antigens or antibodies by ELISA was first described by Engvall and Perlmann (4, 5) and by Van Weemen and Schuurs (17). In ELISA, specific antibodies or antigens are quantitated by incubation of test fluids in tubes or microplates coated with antigen or specific antibody. The next step involves the addition of an enzyme-labeled reagent (anti-immunoglobulin or specific antibody) which, after reacting with the antigen-antibody complex already present on the solid phase, constitutes the basis for the final visualization of the total complex by means of a specific enzyme substrate. The optical density (OD) of the resulting soluble reaction product provides a measure of the amount of specific antibody or antigen in the test fluid.

During the past few years, ELISA has been developed for the detection of antibodies to a variety of viruses (1, 3, 6, 7, 10, 18). More recently, ELISA has been adapted for the detection of hepatitis A (11), hepatitis B surface antigen (8), the soluble antigen of Epstein-Barr virus (19), herpes simplex viruses (12) and the human reovirus-like agent (21).

In most cases, authors have used a modification of the previously reported procedure published by Ruitenberg et al. (13). In some cases, drastic changes have been made, with little or no explanation as to the reason. It is known that many factors can affect the outcome of the test. These factors must be strictly controlled if the test is to be applied in viral diagnostic laboratories.

The preparation of uniform antigen and/or antibody coupled to the solid phase, as well as the elimination of the nonspecific color are two main factors, among others, which need to be carefully standardized. This report describes standardization of these factors and the use of ELISA procedures for the detection of antibodies to influenza A/Victoria/3/75, influenza A/Hong Kong/1/68, influenza B/Hong Kong/5/72, and parainfluenza type 1 viruses in patients' sera.

MATERIALS AND METHODS

Antigen preparation. Seed virus preparations of influenza A/Victoria/3/75, influenza A/Hong Kong/1/68, influenza B/Hong Kong/5/72, and parainfluenza type 1 were kindly supplied by L. Spence (Department of Medical Microbiology, University of Toronto). Each virus was propagated in large (150 ml)

plastic tissue culture bottles containing a monolayer of African green monkey kidney cells. When the tissue became heavily infected (usually 3 to 5 days after inoculation), virus was released from the cells into the medium by three cycles of quick freezing and thawing. The total fluid was collected as the crude harvest of the virus. The preparation was then partially purified by low-speed centrifugation ($3,000 \times g$ for 20 min). The supernatant fluid was then collected and centrifuged again for 5 h at $100,000 \times g$ at 4°C in a Beckman L2-65B ultracentrifuge. The supernatant fluid was removed, and the pellet was then suspended in sterile phosphate-buffered saline solution (PBS) (pH 7.3) to 1/200 of the original volume.

Cell cultures. A 1-ml portion of African green monkey kidney cells suspension was planted in wells of the plastic Linbro tissue culture multiwell plates (24 flat-bottom wells per plate, Linbro Scientific Co. Inc.). The plates were sealed and incubated for 6 days at 37°C . The fluid was replaced with maintenance medium containing 2% fetal calf serum. The plates were then incubated for another 24 h, until they were infected with virus.

Preparation of solid-phase coupled antigen. (i) Inoculation procedure. The monolayer cell cultures in 16 wells of each plate were inoculated with 200 median tissue culture infective dose of influenza A or B or parainfluenza type 1 virus. The remaining 8 wells were kept uninfected for use as negative controls.

(ii) Fixation procedure. When heavy infection was demonstrated in the monolayer cells by a cytopathic effect (CPE) or hemadsorption in representative cultures, a mixture of cold (-20°C) absolute ethyl alcohol and acetone (4:1) was added to each well and incubated for 30 min at room temperature. After the alcohol-acetone mixture was aspirated, the plates were air dried in a sterile cabinet. They were then stored at -70°C until used. In the present report, the fixed infected cells are referred to as solid-phase coupled antigen, whereas the fixed uninfected cells are referred to as solid-phase coupled no antigen. The latter were used as controls to detect nonspecific reactions.

(iii) BSA treatment. Bovine serum albumin (BSA) treatment was performed to eliminate nonspecific attachment to the solid support after the plates had been treated with the fixative. The wells containing solid-phase coupled antigen cells and coupled no antigen cells were brought to room temperature. A 1-ml portion of 1% BSA solution (1 g of BSA fraction V dissolved in 100 ml of PBS, pH 7.3) was added and incubated at room temperature for approximately 30 min. At the end of the incubation period, the solution was aspirated and the plates were air dried.

Serum diluent. Sera were diluted with 0.5% BSA solution containing 0.05% Tween 20.

Enzyme conjugate. Horseradish peroxidase-conjugated anti-human immunoglobulin G (IgG) or anti-rabbit IgG prepared in goat serum were supplied in liquid form by Miles Laboratories. This preparation was diluted 1:100 with 1% BSA solution containing 0.01% Tween 20 and 0.1% sodium azide.

Substrate solution. A 20-mg portion of *O*-dianisidine dihydrochloride (Sigma Chemical Co.) and 37.2 mg of ethylenediaminetetraacetic acid disodium salt were dissolved in 80 ml of acetate buffer (pH 3.8). A 50- μl portion of purified Triton X-100 and 450 μl of

30% H_2O_2 were added; then the final volume of the solution was adjusted to 100 ml with the acetate buffer.

Washing solution. All washing was done with 0.1 M PBS (pH 7.3) containing 0.01% Tween 20.

Negative and positive controls. The following three assays were performed. (i) The serum diluent was assayed as a negative control to determine the background nonspecific reactivity. (ii) Negative sera: preimmune rabbit sera, and human sera negative to influenza A and B and parainfluenza type 1 by complement fixation (CF) and by HAI were also assayed by ELISA. Only those sera which gave identical results when tested on solid-phase coupled antigen and on solid-phase coupled no antigen, were used as negative controls in the ELISA test. (iii) Positive control sera: immune rabbit and human sera positive to influenza A/Hong Kong/1/68 or influenza A/Victoria/3/75, influenza B/Hong Kong/5/72, and parainfluenza type 1 by CF and/or HAI were used as positive controls in ELISA test. Some of these sera were obtained from our reference service laboratory and some from L. Spence (Department of Medical Microbiology, University of Toronto).

Test procedure for ELISA. Usually, sera were tested at a starting dilution of 1:40 using BSA solution as diluent. A 0.2-ml portion of each serum dilution in BSA solution was inoculated into each of four wells, two containing solid-phase coupled antigen and two containing solid-phase coupled no antigen. This was done in triplicate for negative and positive control sera and in duplicate for BSA as a negative control. The plates were then covered and incubated at room temperature for 1 h, after which the wells were washed 10 times with the washing solution by an automatic uni-wash (purchased from Abbott Diagnostic Laboratories). A 0.2-ml amount of peroxidase-conjugated anti-human or anti-rabbit IgG prepared in goats was added to each well and incubated for an additional hour. The type of the conjugate used depended on whether the serum being tested was human or rabbit. The wells were then washed 15 times with the washing solution. Enzyme substrate solution (2 ml) was then added to each well. The enzyme reaction was allowed to proceed for 20 min at room temperature in the dark before it was stopped by adding 0.1 ml of 1 N NaOH. The substrate reaction time of 20 min was arbitrarily chosen for this standard procedure. A brownish-yellow color was developed as the result of the enzymatic reaction on the enzyme substrate. The intensity of the color was measured at 450 nm by a Brinkmann colorimeter 600/D with a stainless steel probe. The probe was immersed directly in the colored solutions in the wells, and the reading was taken within 15 min after the addition of the NaOH solution.

Confirmatory test. The specificity of each of the sera positive for antibodies to influenza A or B or parainfluenza type 1 virus was confirmed by blocking the antibodies with homotypic antigen by the following procedure. A 100- μl amount of the serum was pipetted into each of two conical centrifuge tubes. To the first tube, 100 μl of the partially purified, concentrated homotypic virus were added. To the second tube, a 100- μl amount of serum diluent was added. Both tubes were incubated for 18 h at 4°C , then centrifuged at $3,000 g$ for 15 min. A sample of the supernatant fluid was then diluted with the serum

diluent to a final dilution of 1:40. Positive and negative control sera were processed in the same way as the test sera. The sera treated with the virus and the control sera were assayed by ELISA for residual antibodies. The ODs of the color developed for each serum when treated and not treated with the virus were compared. If the OD of the specimen was reduced by 60% or more after it had been treated with the homotypic virus, the specimen was considered positive.

CF. The CF procedure used was the microtiter technique described by Sever (15). Usually, sera were tested at a starting dilution of 1:8.

HAI. The HAI procedure used was that described by Hierholzer et al. (9). Sera were tested at a starting dilution of 1:10.

Hemadsorption method. The rate of infection was tested by the hemadsorption method described by Shelokov et al. (16).

RESULTS

Results of ELISA were interpreted on the following bases. (i) The 0.5% BSA solution (serum diluent), when used as inoculum, should yield negligible OD values. The OD values obtained reflected the nonspecific color developed as the result of nonspecific attachment of the conjugate to the solid phase. Since BSA solution was used as a diluent for the negative and positive sera, this step was necessary to check whether there was any exogenous factor in the BSA solution which might produce nonspecific color in the test.

(ii) The negative and positive sera, when tested on solid-phase coupled no antigen, should also yield negligible OD values. The intensity of the color developed, reflected the amount of the nonspecific antibodies (IgG molecules) attached to the solid phase.

(iii) The negative and positive sera when tested on solid-phase coupled antigen should show a significant difference in the OD values. The difference in these values between the positive and negative sera reflected the amount of antibodies in the positive specimen to which we refer as ELISA specific activity for antibody. This value can be also expressed as an ELISA ratio by the following equation:

ELISA ratio

$$= \frac{\text{OD of the unknown specimen} - \text{OD of BSA}}{\text{OD of the negative control} - \text{OD of BSA}}$$

If the ratio was 2 or more, the specimen was considered to be positive for the antibody in question. If the ratio was less than 2, the specimen was considered to be negative. The end point titer of a positive serum was defined as the highest dilution which gave a value of 2 for the ELISA ratio.

Determination of a suitable fixative. The success of the ELISA technique for detection of viral antibody depends mainly on the preparation of a solid-phase coupled viral antigen which retains immunological reactivity. Four different fixatives were compared for preparation of solid-phase coupled antigen. These were methyl alcohol, ethyl alcohol, a mixture of 1 part of acetone plus 4 parts of ethyl alcohol, and 2% formaldehyde in PBS (pH 7.3). The first three fixatives were used at -20°C , whereas the fourth was used at room temperature. The fixation time was 30 min in each case. The rest of the procedure was carried out as described above.

The results recorded in Table 1 were an average of four determinations. Values obtained for the ELISA specific activity for antibody were similar when methyl alcohol, ethyl alcohol, or a mixture of acetone and ethyl alcohol (1:4) were used as fixatives. However, when 2% formaldehyde was used as a fixative, the value of the ELISA specific activity for antibody was considerably reduced, and the ELISA ratio was less than 50% of that obtained when the other fixatives were used. Although ethyl alcohol, methyl alcohol, or the mixture of ethyl alcohol and acetone gave similar results, the latter was chosen for the standard procedure. When the cells were fixed with the mixture of ethyl alcohol and acetone, they were firmly attached to the solid support and were not affected by the extensive washings involved in the various steps of the procedure. The plates were stored at -70°C for several months without any appreciable loss in antigenicity.

Effect of Tween-20 on the ELISA test. In the original procedure described by Engvall and Perlmann (4), a solution of PBS and 0.05% Tween 20 was used as a washing fluid. Our pilot experiments showed that PBS containing 0.01% Tween 20 was as efficient as 0.05% Tween 20, but superior to PBS alone. Addition of Tween 20 to the serum diluent and/or to the conjugate diluent had a slight effect in reducing the nonspecific background color. Therefore, Tween 20

TABLE 1. *Effect of different fixatives on the detection of antibodies to influenza A/Victoria/3/75 by ELISA*

Fixative	ELISA sp act ^a	ELISA ratio ^b
Methyl alcohol	0.58	11
Ethyl alcohol	0.70	11
Acetone and ethyl alcohol (1:4)	0.70	12
2% Formaldehyde	0.30	5

^a OD positive - OD negative.

^b OD of positive - OD of BSA/OD of negative - OD of BSA.

was incorporated in the washing fluid, serum diluent, and conjugate diluent in concentrations of 0.01%, 0.05%, and 0.01%, respectively.

Effect of NaN_3 on the ELISA test. Sodium azide has been used as an inhibitor for peroxidase enzymatic activity (14). Therefore, no sodium azide was added to the washing fluid. The addition of NaN_3 to the serum diluent had no effect on the ELISA end results. But when NaN_3 was added to the conjugate diluent, the nonspecific background reactivity was reduced by approximately 60%. A total of 53 negative sera from children were assayed in duplicate at a dilution of 1:40 by the ELISA method. The test was carried out twice, with and without sodium azide in the conjugate diluents. The OD values ranged from 0.12 to 0.32 with an average mean value of 0.17 ± 0.05 when the sera were assayed without the addition of NaN_3 to the conjugate diluent. On the other hand, when 0.1% of NaN_3 was added, the results obtained ranged from 0.05 to 0.15 with an average mean value of 0.1 ± 0.02 . All values in both cases were within three standard deviations from the average mean value.

Figure 1 illustrates the effect of different concentrations of NaN_3 on the ELISA test. The nonspecific color as well as the absolute values of the OD for the sera positive for antibodies to influenza A/Victoria/3/75 were decreased by increasing the NaN_3 concentrations in the conjugate diluent. ELISA specific activity and ELISA ratios were slightly higher when 0.1% sodium azide was added to the conjugate.

Determination of the optimal conjugate dilution for ELISA. The use of an optimal dilution of enzyme-conjugated anti-IgG is essential to obtain maximum specific activity and to minimize nonspecific binding of the conjugate which may yield false-positive results. Therefore, the optimal dilution of conjugate was determined by titration before use. Positive and negative control sera were assayed at two different dilutions (1:40 and 1:160), using serial dilutions of the conjugate. The ELISA specific activity for antibody at each dilution of the conjugate was determined. Results in Fig. 2 illustrate that a 1:100 dilution of the conjugate was the optimal dilution which produced the maximum ELISA specific activity. This dilution was selected for routine use.

Antigen-antibody reaction time. The effect of incubation times on the antigen-antibody reactions by ELISA at room temperature (22°C) was investigated to determine the length of time required for maximum binding. Sera positive and negative for antibodies to influenza A/Victoria/3/75 were assayed at different dilutions for two incubation times (1 and 18 h). Figure 3 illustrates that the ELISA specific activities for antibodies were higher when the antigen-antibody was incubated for 18 h. The end point titer of the serum doubled when the incubation period was increased from 1 to 18 h. A 1-h incubation time was chosen for the present standard procedure to obtain the results of the test within 3 h. Similar results were obtained

FIG. 1. Effect of different concentrations of sodium azide in the conjugate diluent on the optical density of negative and positive sera for antibodies to influenza A/Victoria/3/75. Reaction time for substrate was 20 min.

FIG. 2. Effect of the concentration of peroxidase-conjugated goat antibody to human IgG on the specific activity in ELISA for the detection of antibodies to influenza A/Victoria/3/75. Reaction time for the substrate was 20 min.

when sera positive for antibodies to influenza A/Hong Kong/1/68 or influenza B/Hong Kong/5/72 or parainfluenza type 1 were assayed.

Assessment of a negative cut-off value for ELISA. It is known that ELISA is more sensitive than CF and/or HAI (10); therefore, specimens which are negative by CF or HAI for antibodies to influenza A or B or parainfluenza type 1 are not necessarily negative by ELISA.

To assess the feasibility of using ELISA for the detection of antibodies to influenza viruses in routine diagnosis, negative samples must be assayed to determine the negative cut-off value.

Infection with influenza is widespread among general populations; therefore, one can assume that a majority of the population acquires antibodies to one or more types of influenza viruses. Therefore, the specimens used for this experiment were sera collected from children 6 to 24 months of age. These sera were negative for influenza A and B and parainfluenza type 1 by CF and HAI. The sera were then assayed at a 1:40 dilution by ELISA for antibodies to influenza A/Hong Kong/1/68. The specimens were tested simultaneously for antibodies and for non-specific reactions using solid-phase coupled antigen and solid-phase coupled no antigen, respectively.

Figure 4 illustrates that the OD values ob-

tained for the 53 sera tested in duplicate on solid-phase coupled antigen, ranged from 0.05 to 0.15 with a mean value of 0.10 ± 0.02 . Similar

FIG. 3. Titrations of human control serum positive to influenza A/Victoria/3/75 at 1- and 18-h incubation periods. Arrows indicate the end point titers. ELISA ratio = OD of test serum - OD of BSA/OD of negative control - OD of BSA.

FIG. 4. Normal distribution histogram of the OD values of children's sera negative for antibodies to influenza A/Hong Kong/1/68. Sera were tested in duplicate.

values were obtained when the same sera were tested on solid-phase coupled no antigen (values ranged from 0.03 to 0.16 with a mean value of 0.10 ± 0.04).

The similarity of results obtained in testing these sera against the solid-phase coupled antigen and against solid-phase coupled no antigen, confirmed that these sera did not contain antibodies to influenza A/Hong Kong/1/68. Therefore, these OD values were considered to be true negative values. All values obtained were within three standard deviations from the negative mean.

Samples which gave ELISA ratios greater than 2 were always positive for HAI or CF antibodies to influenza A/Hong Kong/1/68. Therefore, two times the negative control mean (equivalent to approximately seven standard deviations) was chosen to be the cutoff value for distinguishing negative from positive values.

These analyses offer an opportunity to standardize the test not only within any laboratory but also between several different laboratories.

Standard curves for dose response. The dose response test was performed in duplicate in serial twofold dilutions on four different positive reference human sera. Each serum was positive for antibodies to either influenza A/Victoria/3/75, influenza A/Hong Kong/1/68, influenza B/Hong Kong/5/72, or parainfluenza type

1 virus. Each serum was tested by ELISA against its homotypic antigen. A sigmoid shaped curve was obtained in all cases (Fig. 5). The levelling of the curve at high antibody concentrations evidently reflected saturation of the available antigen sites on the solid support. The levelling at low antibody concentrations was probably due to nonspecific binding of the conjugated anti-IgG to the solid-phase coupled antigen. Several other sera were titrated by the same method, and similar types of curves were obtained.

Specificity of ELISA. Serial twofold dilutions of an immune rabbit serum positive for antibodies to influenza A/Victoria/3/75 were assayed by ELISA and HAI against homotypic antigen (influenza A/Victoria/3/75) as well as against heterotypic antigen (influenza A/Hong Kong/1/68). Figure 6 illustrates that the heterotypic antibody "titers" were 1:20 and <1:80 by HAI and ELISA, respectively, and that the homotypic antibody titers were $\leq 1:160$ to 1:1,000 by HAI and ELISA, respectively.

Comparison of ELISA, HAI, and CF for the detection of antibodies to influenza A and B and parainfluenza in human reference sera and in clinical specimens. Reference human sera and 11 paired clinical specimens (acute and convalescent) were tested for antibodies to influenza A and B and parainflu-

FIG. 5. Titrations of four human antisera to (1) influenza A/Hong Kong/1/68, HAI titer 1:80; (2) influenza A/Victoria/3/75, HAI titer 1:40; (3) influenza B/Hong Kong/5/72, HAI titer 1:40; (4) parainfluenza type 1 CF titer 1:16. These sera were tested against their homotypic viruses.

FIG. 6. Titration of immune rabbit serum (IRS) to influenza A/Victoria/3/75 against (1) influenza A/Victoria/3/75 virus (A/VIC), ■...■; and against (2) influenza A/Hong Kong/1/68 virus (A/HK), X----X.

enza type 1 by the ELISA, HAI, and CF methods.

Table 2 illustrates that the end point titers obtained by ELISA were 8 to 64 times higher than those obtained by either CF or HAI tests. No cross reaction could be detected between influenza A and/or B and/or parainfluenza type 1 when the ELISA method was used.

In the first five clinical cases, positive serological reactions were demonstrated for antibodies to influenza A by the three methods. The increase in the antibody titers between acute- and convalescent-phase sera was much greater by ELISA than by CF or HAI tests. There was no increase in the level of antibodies to influenza B or to parainfluenza type 1 in any of these five patients by any method used.

In the last six cases, significant increases in antibodies to influenza B/Hong Kong/5/72 were demonstrated by the three methods. No increase in the level of antibodies to influenza A or to parainfluenza type 1 was seen by any of the three methods.

These results indicate that ELISA results concur with CF or HAI results. The high sensitivity and specificity of ELISA qualify this technique to be used in laboratory diagnosis. The present ELISA method has been shown to be a reliable one, and very little day-to-day variation in the results has been observed. The maximum change in the end point titer obtained with any serum tested at different times was twofold.

Comparison between the ELISA technique and HAI for the detection of antibodies to influenza A in unknown sera. A total

of 43 sera of unknown antibody content were tested by ELISA and HAI methods for the presence of antibodies to influenza A. The contrast in color intensities of positive and negative sera was obvious to the naked eye. Therefore, results were read visually as well as colorimetrically. Complete agreement was found between both methods of reading. As seen in Table 3, there was 75% agreement between the two tests. Eleven sera were positive by ELISA but negative by HAI, and these were confirmed to be positive for antibody to influenza A/Victoria/3/75 by the blocking test described above. A total of 20 sera were positive by ELISA and positive by HAI: 12 were negative for ELISA and negative for HAI. The results indicate that the low levels of antibody which cannot be detected by HAI are easily detected by ELISA methods.

DISCUSSION

The reliability of any immunological assay depends on the strict standardization of all reagents and procedures used. Without such standardization, comparison of results from assay to assay would be difficult, if not impossible.

The present investigation illustrates that after controlling the different factors affecting the test, the ELISA technique can become a useful addition to the currently available methods (CF and HAI) for detection of antibodies to influenza A and B and parainfluenza type 1 viruses in patients' sera.

A major factor which plays a role in the success of any solid-phase immunoassay for detection of antibody is the preparation of a uniform solid-phase coupled antigen. Most previous investigators have followed the method described previously by Engvall and Perlmann (4) or Ruitenberg et al. (13), in which the solid-phase coupled antigen is prepared by physical adsorption of the antigen to the plastic support. This method has recently been criticized by Chessum and Denmark (2) who have indicated that the antigen attaches unevenly to the solid support. This could create serious problems in using the method for laboratory diagnosis, especially when acute- and convalescent-phase specimens are tested. Difference in titers could be due to unevenly coated surfaces rather than to an actual difference in the amount of the specific antibodies in the sera. Pilot experiments done in our laboratory have confirmed their observations; unless antigens used for coating the solid surface were partially purified and concentrated, the results were not reproducible. Preparation of concentrated, partially purified antigen is a cumbersome and time-consuming procedure.

To circumvent this problem, the solid-phase

TABLE 2. CF, HAI, and ELISA titers of antibodies to influenza A and B and parainfluenza type 1 in human reference sera and clinical specimens

Patient specimens	Reciprocal of antibody titers							
	Influenza A/Victoria/3/75			Influenza B/Hong Kong/5/72			Parainfluenza type 1	
	CF	HAI	ELISA	CF	HAI	ELISA	CF	ELISA
Reference human sera								
Anti-influenza A	128	320	≥5,120	<16 ^a	<10	40	<16 ^a	80
Anti-influenza B	8	10	<40	512	320	>1,600	8	<40
Anti-parainfluenza type 1	8		40	<8		<40	16	160
Clinical specimen								
I Acute	8	<10	10	8	<10	<40	<2	<40
Convalescent	64	80	2,560	8	<10	<40	2	<40
II Acute	64	160	2,560	<8	<10	<40	<4 ^a	<40
Convalescent	512	320	>10,240	<8	10	80	<4 ^a	<40
III Acute	8	<10	10	8	20	80	<2 ^a	<40
Convalescent	64	80	640	8	20	160	<2 ^a	<40
IV Acute	<8	<10	40	<8	<10	<40	<2 ^a	<40
Convalescent	128	80	2,560	<8	<10	<40	2	<40
V Acute	<8	<10	40	<8	<10	<40	2	<40
Convalescent	128	40	2,560	<8	<10	<40	<4 ^a	<40
VI Acute	<8	<10	<40	<8	<10	<40	<8	<40
Convalescent	8	<10	<40	32	40	640	<8	<40
VII Acute	<8	40	<40	<8	10	<40	<8	<40
Convalescent	<8	40	40	64	80	1,280	<8	<40
VIII Acute	8	80	80	<8	10	40	8	<40
Convalescent	8	80	80	32	40	640	8	<40
IX Acute	<8	<10	<40	<8	10	<40	<8	<40
Convalescent	<8	<10	<40	64	80	640	<8	<40
X Acute	<8	<10	<40	<8	<10	<40	<8	<40
Convalescent	<8	<10	<40	32	40	650	<8	<40
XI Acute	<8	10	40	<8	<10	40	<8	<40
Convalescent	<8	10	40	32	40	320	<8	<40

^a Anticomplementary at lower dilutions.

coupled antigen was prepared by fixing infected monolayer cell culture to the support surface. This method would appear to be superior to coating the plastic plates with the antigen by physical adsorption since the results obtained by the present method were consistent and reproducible. The shelf life of the solid-phase coupled antigen is several months at -70°C . Also, the present technique is highly versatile and can be adapted to detect the presence of influenza viruses in patients' specimens (F. R. Bishai, ELISA in diagnostic virology. Proceedings of the International Symposium on Enzyme-Labeled Immunoassay of Hormones and Drugs, Ulm (Doneau), West Germany, 10-11 July, 1978, in press).

Another important factor in all enzyme-labeled assays is nonspecific reactivity. This poses difficulties both in identifying weakly positive reactions and in interpreting high negative values. In ELISA, the addition of Tween-20 to PBS for washings reduces partially the background reactivity but does not eliminate it completely.

Our results have shown that the addition of 0.1% NaN_3 to the conjugate diluent considerably reduces the background reactions. The reasons for the effect of NaN_3 on the background reactions are not yet known. The ODs for 106 determinations of negative control sera diluted 1:40 varied between 0.05 and 0.15, with a mean value of 0.1 ± 0.02 . The present method has also permitted the examination of undiluted serum if necessary. The great reduction in the background color permits reading results visually if a colorimeter is not available.

The ELISA procedure described was 8 to 64 times more sensitive than either CF or HAI tests for the detection of antibody titers to influenza A or B or parainfluenza type 1 viruses.

Approximately 25% of the 43 unknown sera studied were positive for antibodies to influenza A/Victoria/3/75 by ELISA but were negative by HAI test. These sera were confirmed to be positive when ELISA blocking tests were performed. The sera which were negative by HAI but positive by ELISA thus appear to contain a

low level of antibodies which are below the detectability level of the HAI test.

The high sensitivity of this test permits the examination of very small volumes of serum specimens. Therefore, we are able to detect antibodies to influenza A and B and/or parainfluenza type 1 viruses in heel-prick blood from infants. The droplet of blood is collected on filter paper, then it is eluted in 1% BSA solution and tested by our ELISA method. This method offers a new simple method for the detection of maternal viral antibodies in newborn children.

No pretreatment of sera is required for ELISA, whereas sera tested by the HAI procedure must be treated by receptor-destroying enzyme to remove nonspecific inhibitors of hemagglutination. The CF test requires standardization of the antigen, hemolysin, erythrocytes, and complement daily and overnight incubation, but the ELISA method can be completed within 3 h. The test is a useful technique for epidemiological surveys as well as laboratory diagnosis.

ELISA was specific in that there was no cross reaction between antibodies to influenza A or B or parainfluenza type 1 viruses. Also when ELISA was used, the cross-reactivity of antibodies to two related strains of influenza A (influenza A/Victoria/3/75 and influenza A/Hong Kong/1/68) was minimal; the degree of the cross-reactivity between two related strains was slightly less or similar to that seen by the HAI test. It is therefore probable that the type of antibodies which are detected by ELISA are similar to those detected by the HAI method.

During the course of the testing period we did not encounter any false positive reactions. The 75% agreement of the present technique with the HAI test and the demonstration of seroconversion in several patients by the present ELISA are evidence that this method is as specific as the HAI test.

The advantage of using ELISA to detect antibodies to influenza viruses also applies to other diseases with different etiologic agents.

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