

Influenza A and B virus IgG and IgM serology by enzyme immunoassays

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SUMMARY

Enzyme immunoassays (EIA) for IgG and IgM antibodies against influenza A and B virus are described. One hundred and seven subjects with a clinical diagnosis of acute respiratory infection (influenza, bronchitis or pneumonia) were selected for this study during two epidemics of influenza A which occurred in Finland in 1983 and 1985. Paired sera and nasopharyngeal secretions were obtained from all subjects. The sera were tested for influenza A and B antibodies by IgG and IgM EIAs and by complement fixation tests. The nasopharyngeal secretions were tested by an indirect EIA for influenza A and B antigens.

The IgG EIA was found to be better than complement fixation for the diagnosis of influenza A infections: only 22% of the significant increases detected by this test were also positive by complement fixation. The additional contribution of the IgM EIA to the number of positives was minimal.

It was also found that testing a single 1/1000 dilution of serum for influenza A and 1/100 dilution for influenza B in the IgG EIA gave as many positives as the conventional method of testing several dilutions.

INTRODUCTION

Complement fixation and hemagglutination inhibition are the conventional techniques used widely to detect antibody to influenza viruses in diagnostic virology, although the complement-fixation test is relatively insensitive. Both techniques are difficult to automate and are also liable to non-specific interference.

For several years solid-phase enzyme immunoassay techniques have been applied to influenza serology (Leinikki & Pässilä, 1976; Bishai & Galli, 1978; Lambre & Kasturi, 1979; Masihi & Lange, 1980). The advantages of enzyme immunoassays (EIAs) over the conventional techniques include greater sensitivity in detecting antibody and convenience (Hammond, Smith & Noble, 1980; Madore, Reichman & Dolin, 1983; Julkunen, Pyhälä & Hovi, 1985).

Several procedures have been published for processing raw absorbance data from EIAs to express the amount of antibody in the specimens (de Savigny & Voller, 1980). We have evaluated single- and multiple-dilution versions of an IgG EIA for detecting antibodies to influenza A and B viruses and then compared IgG and IgM EIAs and complement fixation serology with the detection of influenza

A viral antigens from nasopharyngeal secretions in the diagnosis of influenza A infections.

MATERIALS AND METHODS

Subjects

Influenza A and B antibody-negative serum specimens used in the preliminary optimization of the enzyme immunoassays were obtained from infants whose entire lifetime was confined to a period devoid of influenza epidemics, and whose maternal antibodies had declined to undetectable levels.

Paired sera with significant (fourfold or greater) increases in complement-fixing antibodies against influenza A (17 subjects) and B (29 subjects) were selected from sera stored in the laboratory.

One hundred and seven individuals (57 males and 50 females) with acute respiratory infection, clinical influenza, bronchitis or pneumonia were included in the study. Paired serum specimens, collected with 1–3 weeks' interval between them, and one or several specimens of nasopharyngeal secretions had been submitted to the Department of Virology from all these subjects during one of the two influenza A epidemics in Finland in 1983 and 1985. The mean age of the subjects at the time of diagnosis was 25·8 years (range 1–98, with 58% of subjects being less than 10 years old).

Influenza A and B virus antigen preparation for enzyme immunoassays

Strains of influenza A and B viruses (A/Fin/34/80 (H3N2) and B/USSR/100/83) isolated and adapted to grow in hens' eggs were inoculated into the allantoic cavity of 10-day-old embryonated hens' eggs. After incubation at 36 °C for 70 h the eggs were chilled at 4 °C, the allantoic fluid was collected and clarified by centrifugation at 2000 *g* for 15 min. Viral antigen was pelleted from the clarified supernatant by ultracentrifugation at 100 000 *g* for 1 h. Excess allantoic fluid proteins were removed from the pellets by two successive washes with phosphate buffered saline (PBS, pH 7·4). Thereafter they were finally resuspended in PBS by sonication and stored at –20 °C until use. A negative control antigen was prepared from uninfected allantoic fluid in the same way.

Enzyme-immunoassay for influenza A and B IgG and IgM antibodies

Polystyrene microtitre wells (Microstrip, Eflab, Finland) were coated with various concentrations of influenza A and B antigens (see below) by incubating 100 μ l of the antigen in PBS in each well overnight at room temperature. Thereafter the wells were washed once with PBS, sealed and stored at 4 °C until use. Paired sera with previously detected fourfold or greater increase in complement-fixing antibodies to influenza A and B virus were used in the preliminary optimization of the enzyme immunoassay (EIA).

Amounts of influenza A and B antigens varying between 0·25 and 2 μ g per well (measured as total protein in the antigen preparations) were used in an antigen titration. The absorbance values obtained with a strong positive serum were maximal when antigen amounts of 1 μ g (influenza A) and 0·5 μ g (influenza B) per well were used for coating, therefore these amounts were used subsequently.

Serum specimens were diluted in PBS containing 5% normal porcine serum

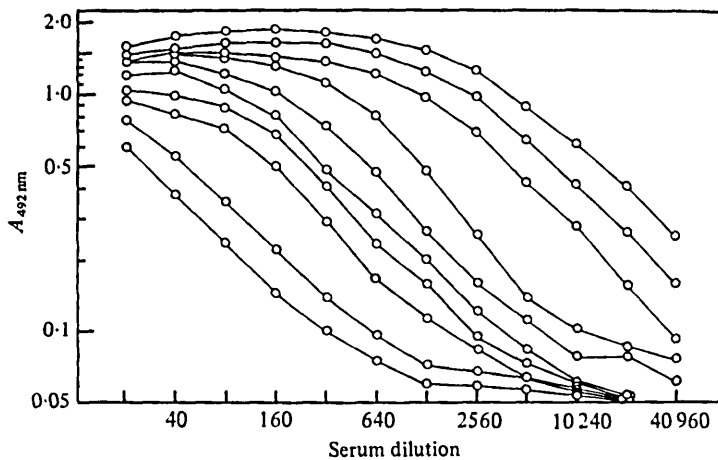


Fig. 1. A representative array of titration curves of the influenza A enzyme immunoassay. All pairs of sera used in the preliminary optimization of the assay had significant increases in complement-fixing antibodies.

(influenza A) or 0.5% bovine serum albumin (influenza B) and 0.1% Tween-20 (dilution buffer), and 100 μ l of the dilutions were incubated in antigen-coated microtitre wells at 37 °C for 2 h. Thereafter the wells were washed three times with PBS containing 0.1% Tween-20 (wash buffer), incubated at 37 °C for 1.5 hours with 100 μ l of horseradish peroxidase-labelled porcine antibodies against human IgG or IgM (Orion Diagnostica, Espoo, Finland) in dilution buffer. After the second incubation the wells were washed twice with wash buffer, once with plain PBS, and 100 μ l of substrate solution was added (0.3% *ortho*-phenylene-diamine and 0.02% hydrogen peroxide in citrate buffer, pH 5.5, prepared immediately before use). The wells were incubated at room temperature protected from direct light until satisfactory colour development. The enzyme reaction was stopped by adding 150 μ l of 1 M hydrochloric acid and the absorbances were read vertically at 492 nm using an automatic eight-channel spectrophotometer (Titertek Multiskan, Labsystems, Finland) connected to a Hewlett-Packard 87 microcomputer for convenient processing of the original absorbance data.

The pairs of sera with significant increases in complement fixing antibodies against influenza A or B were tested in the EIA in twofold dilutions from 1/20 to 1/40960. The titre of IgG class influenza antibodies was defined as the dilution in which the absorbance given by the serum tested was three times that of a negative control serum or at least 0.1 absorbance units. The titres of IgM class antibodies were determined in a similar way. IgM antibodies were accepted as positive if a titre of 320 or higher was detected, provided the absorbances given by antigen coated wells were at least twice those of the corresponding control antigen-coated wells.

All the pairs of sera which showed significant increases in complement-fixing antibodies to influenza A in the preliminary testing also gave significant increases in antibody titres by EIA, but the titre in the first specimen of each pair varied widely (ranging from 40 to 5120). In an attempt to develop an EIA using a single dilution of the patients' serum which would reliably recognize changes in antibody

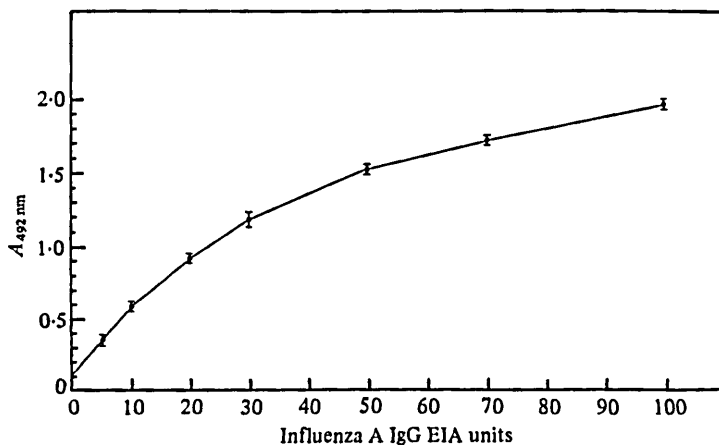


Fig. 2. Standard curve of the single dilution influenza A IgG enzyme immunoassay. Absorbances at 492 nm plotted against the proportion of the positive serum pool (with an assigned value of 100 influenza A IgG EIA units) added to a pool of influenza A IgG negative control serum.

titres from material with wide variations we summarized the titration curves in one graph (Fig. 1). From this a serum dilution of 1/1000 was selected because it is in the linear part of the titration curves of most sera and this minimizes the chance of failing to detect increases in antibody titres due to single dilution measurements being made in the plateau or prozone regions. Titration curves for influenza B antibodies were analysed in the same way, and a dilution of 1/100 was found to be optimal for a single dilution influenza B IgG enzyme immunoassay.

In the single dilution EIA the antibody content of sera were estimated relative to a standard rather than the absolute antibody titre by constructing a standard curve (Fig. 2). An arbitrary value of 100 EIA units was given to a serum pool with a high level of influenza A antibody. This pool was diluted with an influenza A antibody negative serum pool (0 EIA units) to obtain standards containing 0, 5, 10, 20, 30, 50, 70 and 100 EIA units, respectively. Duplicates of these standards (1/1000 dilution) were included in each test series and the results of the test sera were read from the standard curve of absorbance values versus EIA units. Standards for influenza B IgG antibody assay were prepared in a similar way.

Viral antigen detection

Influenza A and B virus antigen detection in nasopharyngeal secretions was done as described earlier by Sarkkinen, Halonen & Salmi (1981).

Complement-fixation test

Complement fixation for influenza A and B virus antibodies was done as described by Chanock (1979) using soluble antigen from infected allantoic fluid.

RESULTS

Reproducibility of the single-dilution IgG enzyme immunoassay

The reproducibility of the single-dilution IgG enzyme immunoassay was determined using three pools of sera containing different amounts of influenza IgG

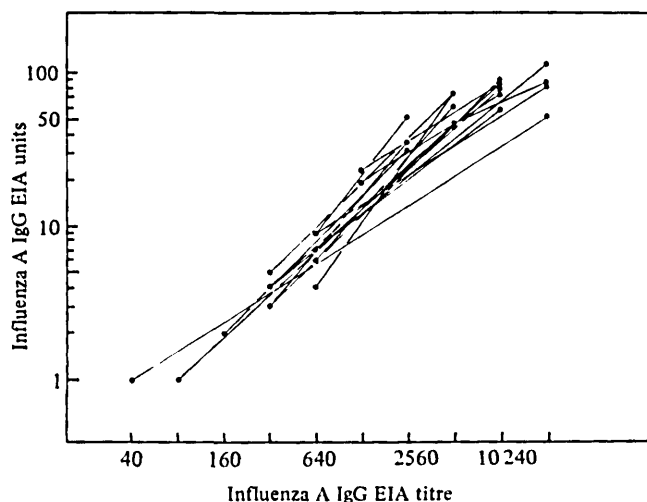


Fig. 3. Increases of influenza A IgG EIA titres and EIA units in 17 pairs of sera with significant increase in complement fixing antibodies. Log-transformed values gave the regression equation: $\log \text{EIA unit} = 0.82 \times \log \text{EIA titre} - 1.45$, $r = 0.97$. A fourfold increase in EIA titres corresponds to a 3.1-fold increase in EIA units.

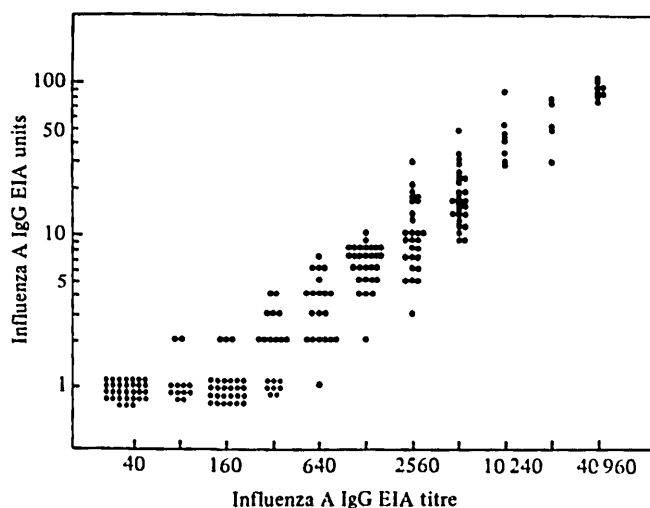


Fig. 4. Correlation of influenza A IgG EIA titres and EIA units in the sera from 107 patients. Log-transformed values gave the regression equation: $\log \text{EIA unit} = 0.69 \times \log \text{EIA titre} - 1.35$, $r = 0.94$. A fourfold increase in EIA titres corresponds to 2.6-fold increase in EIA units.

antibodies. In the influenza A EIA the test pools gave mean absorbances 0.340, 1.019 and 1.506 ($n = 48$ for each series) corresponding to 4, 24 and 48 EIA units. The coefficients of variation of the absorbance were 5.3, 4.6 and 4.6%, respectively. The reproducibility of the influenza B IgG EIA was determined in a similar way, the three serum pools giving mean absorbances 0.211, 0.966 and 1.215 corresponding to 2, 38 and 68 EIA units. The coefficients of variation of the absorbances were 9.2, 8.6 and 5.6%, respectively.

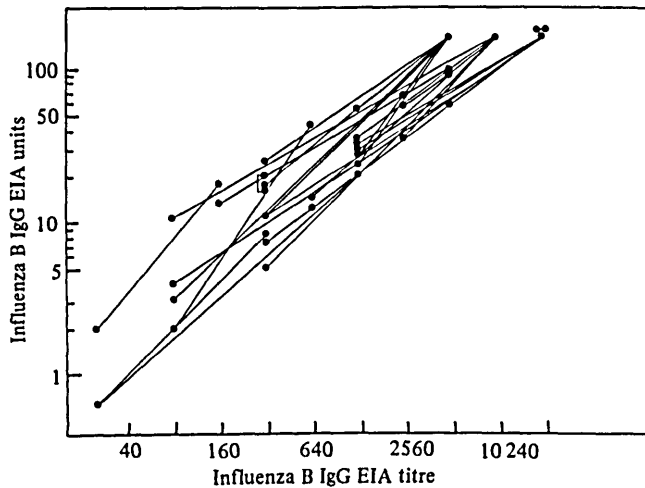


Fig. 5. Increases of influenza B IgG EIA titres and EIA units in 29 pairs of sera with a significant increase in complement-fixing antibodies. Log-transformed values gave the regression equation: $\log \text{EIA unit} = 0.57 \times \log \text{EIA titre} - 0.32$, $r = 0.89$. A fourfold increase in EIA titres corresponds to a 2.2-fold increase in EIA units.

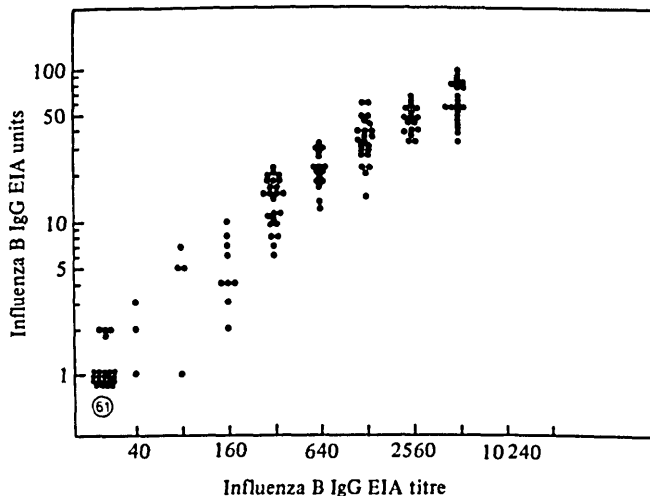


Fig. 6. Correlation of influenza B IgG EIA titres and EIA units in the sera from 98 patients (9 patients omitted due to shortage of sera). Log-transformed values gave the regression equation: $\log \text{EIA unit} = 0.47 \times \log \text{EIA titre} - 0.02$, $r = 0.97$. A fourfold increase in EIA titres corresponds to 1.9-fold increase in EIA units.

Comparison of multiple- and single-dilution IgG enzyme immunoassays

The influenza A IgG EIA titres and EIA units were determined separately for the 17 paired sera with significant increases in complement fixing antibodies (Fig. 3) and for the specimens from the 107 patients (Fig. 4). After logarithmic transformation of both EIA titres and EIA units a significant correlation was found between the two variables ($r = 0.97$ and 0.94 for the preliminary test series and the series of 107 patients, respectively). Using linear regression analysis a fourfold

Table 1 *Distribution of 107 patients according to IgG and IgM enzyme immunoassay results, complement fixation serology and positive/negative influenza A antigen detection in nasopharyngeal secretions*

	Significant increase in IgG EIA antibodies		No significant increase in IgG EIA antibodies	
	IgM positive	IgM negative	IgM positive	IgM negative
Antigen detection positive	15 (5)*	12 (1)	0 (0)	2 (1)
Antigen detection negative	6 (2)	4 (0)	11 (2)	57 (0)

* Figures indicate the total number of patients in each category, figures in parentheses the number of patients with a significant increase in complement-fixing antibodies against influenza A virus.

increase in EIA titres was found to correspond to 3.1-fold increase in EIA units in the first series (Fig. 3) and 2.6-fold increase in the second series (Fig. 4). A similar 3.1-fold increase was also found when the pairs of sera with significant increases in the EIA titres in the series of 107 patients were analysed separately. We therefore selected a threefold increase in EIA units as the threshold for a significant increase. The increases of EIA units were less than threefold in 2 out of the 37 pairs of sera with fourfold or greater increases in the EIA titres (an increase in titre from 80 to 320 corresponded to an increase from 2 to 3 EIA units and an increase in titre from 2560 to 10240 corresponded to a stable value of 28 units in both specimens, respectively). No threefold or greater increases in EIA units were found among sera with less than fourfold increases in EIA titres.

Influenza B IgG enzyme immunoassay titres and units were also analysed. Fourfold increase in EIA titres was found to correspond to 2.2-fold increase in EIA units ($r = 0.89$, Fig. 5) in the series of 29 pairs of sera with significant increases in CF antibody titres, and to 1.9-fold increase in EIA units in the series of 107 patients ($r = 0.97$, Fig. 6). In the first series no pairs of sera gave inconsistent results when calculated in titres or units.

Comparison of the IgG and IgM EIAs with CF serology and with antigen detection in the diagnosis of influenza A infections

The material for this part of the study was collected during two influenza A epidemics in Finland as described in Materials and Methods. During these periods only a few influenza B infections were diagnosed. Therefore we have restricted our evaluation to influenza A infections alone.

The results from the 107 patients of IgG and IgM EIAs, and CF serology and positive antigen detection for influenza A are shown in Table 1. Only 22% of the significant increases detected by EIA IgG serology were positive by the complement fixation technique.

In 73% of patients with significant increases in EIA IgG titres influenza A antigen was detected in the nasopharyngeal secretions. In two patients without an increase in EIA IgG antibody, antigen detection was positive, and the sensitivity of antigen detection was found to be dependent on the time of specimen collection.

Thus antigen detection was positive in 84% of specimens collected at day 0-2 after onset of symptoms, but in only 69 and 43% of specimens collected at 3-6 and 8-18 days after onset of symptoms, respectively.

In 57% of patients with a significant increase in EIA IgG antibodies EIA IgM serology was also positive. In addition, it was positive in 16% of patients who had no increase in EIA IgG antibody. In most patients, however, only moderately low titres of IgM antibodies (≤ 2560) were detected.

DISCUSSION

The advantages of enzyme immunoassay serology over complement fixation are clear. The sensitivity of EIA has been found in several reports to be greater than CF (Bishai & Galli, 1978; Lambre & Kasturi, 1979; Leinikki & Pässilä, 1976; Madore, Reichman & Dolin, 1983; Masihi & Lange, 1980) or at least comparable (Hammond, Smith & Noble, 1980). In the present study only a small fraction of individuals with a significant increase in IgG antibody detected with EIA could have been detected by complement fixation. In addition, the finding that in the majority of individuals with an increase in EIA IgG antibody influenza A antigen was also detected, further substantiates the reliability of the EIA results. The enzyme immunoassay is technically uncomplicated and less expensive than complement fixation test if the time required to do the test is taken into consideration as well as the materials. Furthermore, the objective and reproducible recording of a quantitative results from the EIA is easily arranged.

In the present study we used two modifications of the EIA IgG assays. The major advantage of the single dilution assay is that even large series of sera can be easily tested at a time even when each is analysed in duplicate which is necessary to exclude random technical errors. The need for meticulous quality control should not be overlooked in this type of test as pointed out earlier (de Savigny & Voller, 1980).

The correspondence between EIA titres determined by multiple titration curves of different sera are approximately parallel. Here the correlation between EIA titres and EIA units is good, and most of the significant increases in titres were detected by the single dilution assay as well. The absorbance value obtained at a single dilution is a product of both the affinity and binding capacity of the antibody, providing less information when compared to that given by an entire titration series. Nevertheless for the highest economy and efficiency, however, a test based on a single serum dilution may be applied in routine serology (de Savigny & Voller, 1980).

Another problem associated with the use of the single dilution assay is standardization. As long as generally recognized standards are not available the units determined are arbitrary and not comparable between laboratories. Great care should be taken even when comparing the results obtained between different assays in the same laboratory despite the use of the same standards in each assay. Only results obtained within one assay plate are reliably comparable to one another. The correspondence between the limits of a significant increase in the antibody content in paired sera between the two assay types (in the present test a fourfold increase in titre vs. a threefold increase in units) is likely to be dependent

on the assay design and should be determined separately for different antibody assays.

The contribution of the IgM test seems to be minor when compared to IgG EIA serology in the diagnosis of influenza A infections. Similar observations have been reported recently (Julkunen, Kleemola & Hovi, 1984). Eleven individuals in this study had isolated EIA IgM titres of ≥ 320 , which were regarded as positive. Most of these patients also had high IgG titres in both specimens which often were taken more than 1 week after the onset of illness.

Influenza A virus antigens were not detected in nasopharyngeal secretions in 27% of individuals with significant increases in IgG EIA antibodies. Only two individuals without IgG rises by EIA were positive by antigen detection, but one of these two had a fourfold increase in complement-fixing antibodies. A factor which probably limits the usefulness of the antigen detection in the diagnosis of influenza A infections is that influenza A does not generally induce pronounced mucus secretion in the upper respiratory tract, particularly in adults, and this hampers adequate sampling.

In conclusion we recommend the IgG enzyme immunoassay instead of complement fixation in the serological diagnosis of influenza A infections. The antigen detection test should be done first, since this is the most rapid method of diagnosis in the majority of infections. If the antigen detection test is negative, an infection may still be diagnosed by IgG serology on paired sera. As a matter of principle the most reliable approach is to titrate the antibody content by using several serum dilutions in the test. For an increased efficacy in routine testing the simplified single dilution version may be applied yielding essentially the same diagnostic information.

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