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A simple double immunodiffusion test for typing influenza viruses

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The identification of influenza virus type has traditionally been based on the characterization of internal nucleoprotein (NP) antigens by the complement fixation (CF) test. Because this test is complex and time-consuming, it is used only infrequently. In this report we describe a double immunodiffusion (DID) test, which is proposed as a replacement for the CF test for the typing of influenza viruses. The DID test is simple and requires only the allantoic fluid content of a single infected embryonated egg. Virus from the infected fluid is precipitated with mild acid and disrupted by detergent. Anti-NP or anti-matrix protein (MP) antibody is equally effective in most instances for typing influenza isolates by this test. The latter is preferred, however, since it seemed to be slightly more sensitive than anti-NP antibody.

Influenza viruses possess two antigenically stable, type-specific internal antigens: the nucleoprotein (NP) and the matrix protein (MP). In addition, there are two strain-specific surface antigens: the haemagglutinin (HA) and the neuraminidase (NA). The identification of influenza viruses as to types and subtypes is based on the characterization of both the internal and the surface antigens (1). The identification of the antigenic type of new influenza virus isolates has traditionally been undertaken by complement fixation (CF) tests with anti-NP sera (2). However, since the CF test is complex and time-consuming and requires highly specific and expensive reagents, it is used only infrequently to identify influenza virus types. Indeed, since influenza viruses are usually the only human myxoviruses that can be isolated in embryonated eggs 10–11 days old, the usual laboratory procedure is to carry out direct subtype identification of isolates by the haemagglutination-inhibition (HI) test with antisera prepared against current influenza A and B (or C, if indicated) strains. The identification of haemadsorbing or haemagglutinating agents in primary cell cultures is somewhat more time-consuming, since paramyxoviruses that are not isolated in eggs may be isolated in such cultures. Here, too, the usual procedure is to carry out directly an HI or haemadsorption-inhibition test with antisera for all suspected agents. Failure of available HI antisera to inhibit haemagglutination is infrequent, but may indicate the presence of bacteria, avian mycoplasma, parainfluenza viruses of birds or man, or a mixture of haemagglutinating agents. It may also indicate an antigenically novel influenza strain, or influenza A viruses of one of the 11 haemagglutinin subtypes that may be isolated from horses, pigs, and birds. An effective and simple serological test for the typing of influenza virus is important for influenza surveillance and for epidemiological studies. An agar gel immunoprecipitin test for the identification of NP antigen from extracts of infected chorioallantoic membranes has been described by Beard (3). In this paper we describe a similar double immunodiffusion (DID) test, which is performed with acid-precipitated virus from infected allantoic or tissue culture supernatant fluids. The DID test requires type-specific NP or MP antisera. It necessitates no special equipment or expensive reagents and may be easily performed in laboratories with the minimum of resources.

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MATERIALS AND METHODS

Viruses

All influenza type A and B strains were obtained from the collection of the WHO Collaborating Centres for Influenza, Atlanta and London.

Reference antigens

Type A and B reference antigens consisted of whole virus concentrates of A/England/42/72(H3N2) and B/Hong Kong/5/72. Viruses were grown in embryonated eggs and concentrated by centrifugation at 80 000 *g* for 1 h. Concentrates were partly purified by centrifugation on a 10–40% sucrose linear gradient for 1 h at approximately 40 000 *g*. The resulting virus band was removed, diluted with saline, and centrifuged at 40 000 *g* for 1 h. The pellets were resuspended and dialyzed against frequent changes of saline. Final preparations contained approximately 10^7 haemagglutinating (HA) units per millilitre.

Reference antiserum

Type A MP immunizing antigen consisted of M protein isolated by electrophoresis on cellulose acetate membranes from recombinant virus A/Aichi/2/68(H3)-Bel/42(N1) [a] disrupted by sodium dodecyl sulfate. This method was originally described by Laver (4) and was modified by Schild (5) for the isolation of M protein by substituting a phosphate buffer pH 6.6 for electrophoresis. Type A antiserum was prepared in rabbits. Equal volumes of purified MP antigen (approximately 150 μg per ml) and Freund's complete adjuvant were mixed and the rabbits were given a 1-ml injection of the mixture in each thigh on day 0 and again 3 weeks later. Six weeks after the initial injection, 75 μg of MP antigen mixed with Freund's incomplete adjuvant were injected into each thigh. Beginning 2 weeks after the final injection, the animals were bled weekly until the precipitin titre decreased.

Type B MP immunizing antigen was prepared from virus B/Hong Kong/5/72 by electrophoresis on cellulose acetate membranes as described above for type A MP antigens. Type B MP antiserum was prepared in a goat [b] according to the method of R. G. Webster (personal communication, 1973). Purified MP antigen (approximately 300 μg of protein per ml) and Freund's complete adjuvant were mixed in equal volumes. Then 1 ml of the mixture was injected into each thigh and 1 ml was injected intramuscularly into the tail. After 30 days, the injections into each thigh were repeated and 1 ml of the aqueous antigen was injected into the jugular vein. The animal was bled 7 days after the final inoculation.

Type A NP antiserum was obtained from the Biological Products Division, Center for Disease Control, and consisted of hyperimmune-mouse ascitic fluid prepared by multiple injections of NP antigen derived from mammalian cell culture according to the method of White et al. (6).

Type B NP antiserum was produced in a goat in accordance with the schedule described above for the type B MP antiserum. The immunizing antigen was derived from electrophoresis of SDS-disrupted B/Hong Kong/5/72 virus on cellulose acetate membranes. This antigen was isolated by the same procedure as that described for the B MP antigen, except that the protein band representing NP was located between the MP antigen and the origin.

Haemagglutination (HA) tests

Assays for the presence of haemagglutination were performed in microtitre as described by Hierholzer et al. (7).

Preparation of isolates for typing

The isolates to be typed were inoculated into embryonated chicken eggs 10–11 days old and incubated for 48–72 h at 35°C. Two uninoculated eggs of the same age were included for the preparation of negative control antigens. After incubation, the eggs were chilled overnight at 4°C. Approximately 8–10 ml of infective and control allantoic fluid harvests were placed in 15-ml conical centrifuge tubes and held at 4°C. Haemagglutination titres were determined. Cold test fluids were acidified by adding 1N hydrochloric acid to obtain a final pH of 4.0±0.5. Usually, 0.03 ml of 1N hydrochloric acid was required for each millilitre of allantoic fluid. The approximate pH was determined by colorimetric pH paper. The acidified test fluids were chilled in an ice bath for 30–60 min and then centrifuged at 1 000 *g* for 10 min at 4°C. The supernatant fluid was discarded and the tubes were drained on absorbent paper. The packed precipitates were resuspended by adding 0.01 ml of " glycine–sarcosyl " reagent (0.5 mol/l glycine–sodium hydroxide buffer, pH 9, containing 100 g/l sodium (methylamino)acetyl sulfate [c]) for each millilitre of original allantoic fluid.

[a] Provided by Dr R. G. Webster, St Jude Children's Hospital, Memphis, Tenn., USA.

[b] Type B MP antiserum may also be prepared in rabbits as described for type A. The goat was used in order to obtain a larger volume of antiserum.

[c] " Sodium sarcosyl sulfate ", available commercially as " Sarkosyl NL-97 " from Geigy Industrial Chemicals, Division of Geigy Chemical Corp., Ardsley, N.Y. 10502, USA.

Precipitates were mixed thoroughly with a Vortex mixer or a small rod. All precipitate does not dissolve.

Double immunodiffusion (DID) tests

Immunodiffusion tests were performed in 1–1.5% agarose,[a] or 1% ion agar.[b] Commercially available prepackaged plates containing 2% agar [c] were also satisfactory. Each agar plate contained 1–3 six-well patterns. The wells were 3 mm in diameter. Each of the 5 wells in the outer circle was spaced 7 mm from the centre well. Reagents were added to the wells in a consistent pattern (Fig. 1, 2). Approximately 10 μl of each reference antiserum and of each reference antigen were added to the designated well. Slides were allowed to stand at room temperature for 15–20 min in order to permit the antisera to begin diffusion before disruption and release of viral antigenic components by addition of the detergent. Approximately 10 μl of the isolate were added to the centre well and 10 μl of the negative control antigen were placed in the designated well. Approximately 5 μl of 100 g/l sodium sarcosyl sulfate were then added to wells containing the whole virus reference antigens. Slides were incubated in a moist atmosphere at room temperature. Readings were made after overnight incubation.

RESULTS

Allantoic fluids

The DID test with anti-NP and anti-MP antisera was evaluated with initial embryonated egg passages of current type A and B isolates, as well as with H0N1, H1N1, H2N2, and type B stock strains from previous years (Table 1). Of the 84 strains, 79 were identified as type A or B with anti-NP antisera and 82 were identified with MP antisera. Typical precipitin patterns are shown in Fig. 1 for anti-NP and in Fig. 2 for anti-MP.

The two strains that were not identified by anti-MP or anti-NP antisera had initial allantoic fluid HA titres of 8 (influenza A) and of 16 (influenza B), respectively. The strains were successfully identified by DID after a second allantoic passage when HA titres were $\geq$64. Other strains with HA titres of 16 and all strains with titres of $\geq$32 were successfully identified by anti-MP antisera. The remaining three type A strains that were positive with MP antisera but negative with NP antisera had allantoic fluid HA titres of 16, 64, and 128. However, all other strains that had HA titres of $\geq$32 were successfully typed.

Table 1. Type-specific identification of influenza viruses from allantoic fluids by double immunodiffusion tests with anti-NP and anti-MP antisera

Influenza viruses	No. tested	No. identified	
		anti-NP	anti-MP
Type A:			
H0N1	5	5	5
H1N1	8	8	8
H2N2	7	6	7
H3N2	32	31	32
Avian	4	4	4
Equine	4	2	3
Swine	2	2	2
Type B	22	21	21
Total	84	79	82

Chorioallantoic membranes

Because infected chorioallantoic membrane is recognized as being rich in viral NP, this tissue was investigated as an alternative to allantoic fluid for the antigen source for the DID test. Extracts from infected egg chorioallantoic membranes were prepared with a modification of the method of Beard (3). First, 2–3 infected membranes were washed with saline, drained on absorbent paper, and ground in a mortar with a small amount of alundum. The contents of the mortar were transferred to a centrifuge tube. After 3 freeze–thaw cycles to disrupt the remaining cells, the debris was packed by centrifugation at 1 000 *g* for 10 min. Without further treatment the supernatant fluid was used in the DID test. Chorioallantoic membrane extracts from infected eggs were tested by DID with anti-NP and anti-MP antisera simultaneously with allantoic fluid precipitates; 33 human influenza A viruses, which included all subtypes, were successfully typed from allantoic fluid precipitates by anti-MP antiserum and 31 were typed by anti-NP antiserum. Of the 33 strains 12 were typed from chorioallantoic membrane extracts with both antisera.

[a] Kallestad Laboratories, Minneapolis, Minn., USA.

[b] Colab, Inc., Glenwood, Ill., USA.

[c] Hyland Laboratories, Inc., Costa Mesa, Calif. 92626, USA.

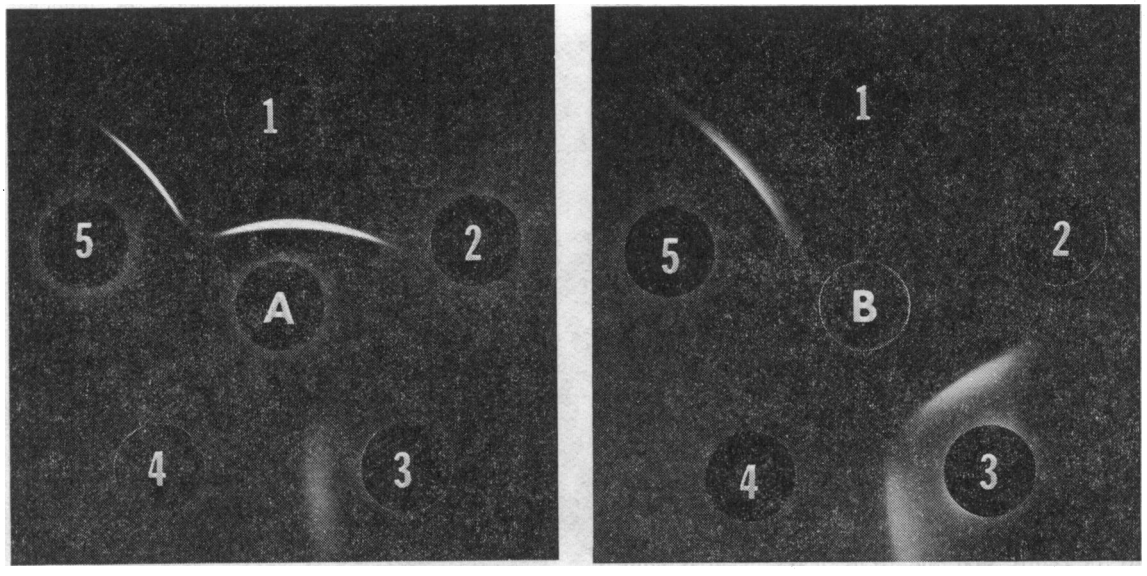

Fig. 1. Identification of influenza virus type by double immunodiffusion (DID) tests with anti-nucleoprotein (NP) antisera (1 = type A NP antiserum; 2 = control allantoic fluid precipitate; 3 = type B NP antiserum; 4 = type B reference antigen; 5 = type A reference antigen; A = type A isolate; B = type B isolate).

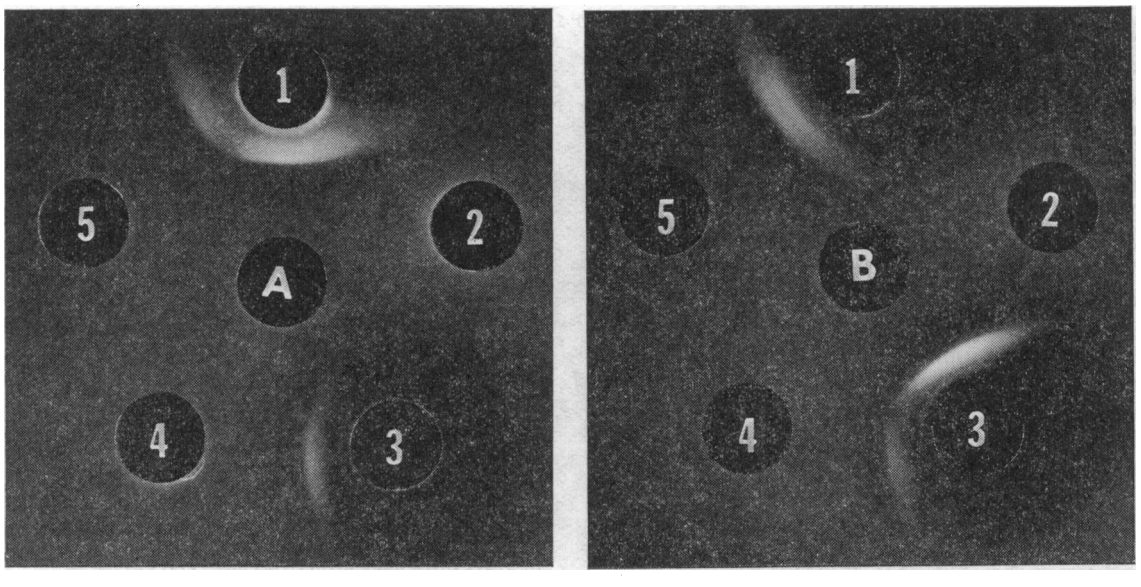

Fig. 2. Identification of influenza virus type by double immunodiffusion (DID) tests with anti-matrix protein (MP) antisera (1 = type A MP antiserum; 2 = control allantoic fluid precipitate; 3 = type B MP antiserum; 4 = type B reference antigen; 5 = type A reference antigen; A = type A isolate; B = type B isolate).

Cell cultures

The DID test was also applicable to identification of isolates from primary cell cultures. Thus 10 recent influenza A and 10 influenza B strains that had previously been passaged only in rhesus monkey kidney cells were inoculated into rhesus monkey kidney cell cultures containing Eagle's MEM without serum. After 5 days at 35°C, cultures were harvested by 2 freeze–thaw cycles. Fluids were acidified with 1N hydrochloric acid and treated with detergent in the same manner as described for allantoic fluid harvests. Several uninoculated cell cultures were included as a control. In spite of low HA titres (usually 4–32) of the infected cell culture harvest, 16 of the 20 isolates were typed with MP or NP antisera without requiring further passage.

DISCUSSION

The NP antigens of influenza A and B viruses were demonstrated by Styk & Hana (8), Hana & Hoyle (9), and Schild & Pereira (10) to be type-specific by the DID test. The MP antigens, which were recognized more recently, were shown by Schild (5) to be type-specific. In this study we describe the application of the DID test as a routine method for typing influenza A and B isolates. The main advantage of this test is its simplicity. Concentration of virus by high-speed centrifugation or other eleborate techniques requiring many infected eggs is unnecessary. The virus content from the allantoic fluid of a single infected embryonated egg is adequate, and concentration is achieved by a highly efficient one-step precipitation of virus by acid (11). Other "mild" procedures for protein precipitation, e.g., with 5% trichloroacetic acid, may also be used. The internal NP and MP antigens are released by disruption of the virus with detergent. In this study the reference antigens were prepared by high-speed centrifugation on sucrose gradients. This is not essential. Reference antigens may be easily prepared from allantoic fluid suspensions of any known type A or B strain by precipitation with acid, as described for the unknown isolates.

Allantoic fluids containing high haemagglutination titres are not prerequisites for successful typing by DID. Although allantoic fluids with HA titres of $\geqslant 32$ are recommended for the test, some isolates can be typed from fluids with HA titres as low as 8. In most instances, viruses were typed equally well with anti-NP or anti-MP antisera, but the anti-MP test is preferred. This system appears to be slightly more sensitive than the anti-NP, presumably because of the greater abundance of the MP antigen in the virion (13). Also, less time is required for the MP precipitin line to develop; the antigen of lighter molecular weight (20 000) migrates more rapidly than the NP antigen in agar gels (5).

Influenza-virus-infected extracts of chorioallantoic membrane were shown by Beard (3) to be an excellent source of NP antigen for serologic tests with avian and mammalian sera. Beard & Helfer (12) also reported this method to be suitable for typing viruses. In the present study we found chorioallantoic membrane extracts to be less useful than allantoic fluid precipitates. Our results with the method possibly could be improved, but acid precipitation of allantoic fluid offers simplicity of antigen preparation, which is not apparent in the processing of chorioallantoic membranes from single eggs.

The DID test was reasonably sensitive for typing influenza viruses from primary rhesus monkey kidney cell cultures, but the low virus titres often associated with early virus passages may limit its use. A subpassage in embryonated eggs may sometimes be required for identification of cell culture isolates.

The DID test with MP antisera, as described here, has been in use in both WHO Reference Centres for nearly 1 year. Because of its sensitivity and simplicity, we recommend that this test be considered as a replacement for the conventional CF test for typing influenza A and B isolates.

Reference reagents for the DID test may be obtained by writing to the WHO Collaborating Centre for Influenza, Virology Division, Center for Disease Control, Atlanta, Ga. 30 333, USA, or to the WHO Collaborating Centre for Influenza, National Institute for Medical Research, Mill Hill, London NW7 1AA, England.

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RÉSUMÉ

UNE ÉPREUVE SIMPLE DE DOUBLE IMMUNODIFFUSION POUR LE TYPAGE DES VIRUS GRIPPAUX

L'identification du type des virus grippaux est classiquement basée sur la caractérisation des antigènes nucléoprotéiques internes (NP) par l'épreuve de fixation du complément (FC). Ce test n'est cependant pas d'application courante en raison de sa complexité et du temps requis pour son exécution.

Dans le présent article, les auteurs décrivent une épreuve de double immunodiffusion destinée à remplacer l'épreuve FC pour le typage des virus grippaux. Elle est d'exécution facile et se pratique sur le liquide allantoïde d'un seul œuf fécondé. Le virus présent dans le liquide allantoïde infecté est précipité par un acide faible et désagrégé à l'aide d'un détergent. Les anticorps anti-NP ou anti-protéine matricielle (MP) font preuve dans la plupart des cas d'une égale efficacité en ce qui concerne le typage des isolats de virus grippaux A et B. Il semble cependant que les anticorps anti-MP soient légèrement plus sensibles que les anticorps anti-NP.

Les réactifs de référence permettant d'exécuter l'épreuve de double immunodiffusion peuvent être obtenus sur demande adressée au Centre collaborateur de l'OMS pour la grippe, Virology Division, Center for Disease Control, Atlanta, Ga. 30333, Etats-Unis d'Amérique, ou au Centre collaborateur de l'OMS pour la grippe, National Institute for Medical Research, Mill Hill, London NW7 1AA, Angleterre.

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