*Acta virol.* **29**: 19—24, 1985

# DETECTION OF TYPE A AND B INFLUENZA VIRUSES IN CLINICAL MATERIALS BY IMMUNOELECTRONMICROSCOPY

M. PTÁKOVÁ, B. TŮMOVÁ

Institute of Hygiene and Epidemiology, 100 42 Prague, Czechoslovakia

*Received March 9, 1984*

*Summary.* — Direct immunoelectronmicroscopy (IEM) was used for detecting influenza subtype A(H1N1), A(H3N2) and type B viruses in nasopharyngeal washings or swabs collected during three consecutive periods of enhanced influenza incidence. Virus identification was performed with immune rat sera and in the case of the A(H3N2) subtype also with convalescent human sera. In all the materials examined influenza virus was demonstrated by isolation in chick embryos or by immunofluorescence in infected tissue cultures. IEM detected subtype A(H1N1), A(H3N2) and type B viruses in 6 of 13, all 5 and 3 of 8 washings, respectively. Immune complexes were observed in only those materials from which virus was isolated already in the first chick embryo passage, which was evidence that a positive IEM result depended on the amount of virus present in the material. The use of immune sera against two antigenically distinct A(H1N1) strains, A/Khabarovsk/77 and A/England 403/80, did not considerably influence the IEM result.

*Key words: electron microscopy; immune complexes; influenza virus type A, B; rapid diagnosis*

## *Introduction*

One of the present methods for rapid diagnosis of viral diseases is the IEM with negative staining (first employed by Brenner and Horne, 1959). Detection of influenza viruses by this method has been attempted for over 20 years (Lafferty and Oertelis, 1961, 1963; Bayer and Manweiler, 1963; Kelen and McLeod, 1974), but the situation is complicated since influenza viruses undergo frequent antigenic changes and their concentration in nasopharyngeal washings varies being usually rather low. Influenza virus has been demonstrated in clinical material by indirect IEM by Edwards (1975). We have used the methods of direct and indirect IEM to determine the optimal virus to antibody concentration ratio in infected chick embryo allantoic fluid (Ptáková and Tůmová, 1985) and have found the indirect method more sensitive.

This report is concerned with detection of influenza viruses by the direct IEM method in washings and swabs collected from patients suffering of

acute respiratory disease during periods of enhanced influenza incidence in 1980, 1981, and 1982. An attempt was made to determine whether and under what conditions this method could be practically utilized for rapid influenza diagnosis during epidemics.

## *Materials and Methods*

*Clinical material*, Twenty-six samples of nasopharyngeal washings or nasal and pharyngeal swabs collected from patients with acute febrile respiratory disease were used in attempts at virus detection by IEM. Each washing was made with 10 ml of physiological saline and mixed with an equal volume of sampling medium, Veal Infusion Broth Difco supplemented with 0.5 % of bovine albumin. Nasal and pharyngeal cotton-wool swabs were collected from child patients and each pair was immediately immersed into 6 ml of sampling medium and agitated. All samples were subjected to virus isolation attempts on chick embryos (E), monkey-kidney (MK) and dog-kidney (MDCK) tissue cultures. Virus was demonstrated by haemagglutination and haemadsorption in all these systems and was identified by haemagglutination-inhibition test (E, MDCK) or the method of indirect immunofluorescence (MK, MDCK). Virus identification was done with specific immune sera (Tůmová, 1985).

*Immune sera*. For virus detection by IEM the following hyperimmune sera were used: A/Khabarovsk/77(H1N1), haemagglutination-inhibition titre 1 : 1028; A/England 403/80(H1N1), titre 1 : 160; A/Texas/77(H3N2), titre 1 : 80; B/Bratislava 11/81, titre 1 : 160. Subtype A(H3N2) virus detection was performed apart from rat serum, also with acute and convalescent human sera collected from the same patients from whom the samples had been collected. Rat hyperimmune sera used were diluted in physiological buffered saline (PBS), pH 6.8; the dilutions were 1 : 200 and 1 : 400 for detecting type A viruses and 1 : 200 for detecting type B virus (in view of the small amounts of clinical material in hand). Human sera were diluted 1 : 10 and 1 : 20. All sera were inactivated at 56 °C for 30 min before experiment.

Two controls were included (1) IEM of washings mixed with normal, influenza-nonimmune rat sera prepared in the same way as the hyperimmune sera; (2) direct electron microscopy of washings without addition of serum.

*Immunoelectronmicroscopy*. Equal 0.1 ml volume of the sample mixed with 0.1 ml of diluted antiserum was incubated at 37 °C for 1 hr under occasional agitation. The mixtures in special small-volume tubes were placed in specially designed adapters and spun down in a Beckmann centrifuge at 15 000 rev/min (27 000 g) for 1 hr (see Almeida and Goffé, 1965; Almeida and Waterson, 1969). The supernatant was discarded, the sediment resuspended in two drops of distilled water, and one drop of this suspension was instilled on 0.8 % ionoagar No. 2 Oxoid (Kelen and McLeod, 1974). An electronmicroscopic grid with a carbon-coated formvar membrane was applied on top of the drop. Following 30-min diffusion, the grids were washed by floating on a drop of distilled water and negatively stained with 2 % phosphotungstic acid (PTA) pH 7.0 for 30 sec. Specimens were examined in a Philips EM 300 electron microscope at acceleration voltage 80 kV.

## *Results*

Influenza virus A(H3N2) was demonstrated with 1 : 400 diluted rat sera in all the corresponding 5 washings and swabs collected (Table 1). Using paired human sera the virus was also detected in all the 5 materials, a marked difference being evident between the results with acute and convalescent sera. Only acute sera No. 3938/106 and 3950/118 gave 1 : 10 titres in the haemagglutination-inhibition test, this being evidence of the patients previous contact with a virus of the A(H3N2) subtype.

Influenza virus A(H1N1) was demonstrated in 6 of 13 samples, namely in those where it was also demonstrable already in the first chick embryo amniotic passage, albeit at very low titre (1 : 2—1 : 4) that necessitated one

**Table 1. Demonstration of subtype A(H3N2) virus**

| Material No. | Age | Dates of onset/ sampling | Virus isolation | Rat serum A/Texas/77 1:200 | Rat serum A/Texas/77 1:400 | Human sera acute 1:10 | Human sera acute 1:20 | Human sera convalescent 1:10 | Human sera convalescent 1:20 | HIT ac. | HIT con. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3903/56 | 59 | 30/31. 1. | E1 (E2) | 0 | + a.e. | 0 | 0 | ± | 0 | 0 | 10 |
| 3921/79 | 30 | 1/4. 2. | E1 (E2) | ±/+ | + | 0 | 0 | ±/+ | ± | 0 | 20 |
| 3924/82 | 43 | 3/4. 2. | E1 | ± | + | 0 | 0 | ± | ± | 0 | 20 |
| 3938/106 | 38 | 3/7. 2. | E1 | ± | ± | ± | ± | + | ±/+ | 10 | 40 |
| 3950/118 | 18 | 10/11. 2. | E1 (E2) | ±/+ a.e. | + | − | ± | + | ± | 10 | 80 |

0 = virus not detected; ± = isolated antibody-coated virions; + = isolated immune complexes; ++ = fairly many immune complexes; ±/+ = isolated virions and immune complexes; E1 (E2) = number of chick-embryo passage in which virus was detected (in parentheses, passage in which identification was possible); a.e. = antibody excess; HIT = haemagglutination-inhibition test

more passage before identification of the virus was possible (Table 2). Where virus was first detected in the second or third amniotic passage at low titre, IEM was not successful.

Similar evidence of a dependence between amount of virus in sample and positive IEM was obtained with type B influenza virus (Table 3). The virus was demonstrated in 3 of 8 samples. Isolation of type B virus on chick

**Table 2. Demonstration of subtype A(H1N1) virus**

| Material No. | Age | Date of onset/sampling | Virus isolation | Immune serum dilution A/Khabarovsk/77 1 : 200 | A/Khabarovsk/77 1 : 400 | A/England/403/80 1 : 200 | A/England/403/80 1 : 400 |
|---|---|---|---|---|---|---|---|
| 4389/952 | 15 | 23/25. 12. | E1 (E2) | ±/+ | + | ++ | + |
| 4544/32 | 6 | 11/12. 1. | E2 (E3) | 0 | 0 | 0 | 0 |
| 4573/91 | 6 | 16/19. 1. | E3 (E4) | 0 | 0 | 0 | 0 |
| 4615/123 | 14 | 29/29. 1. | E2 | 0 | 0 | 0 | 0 |
| 4617/125 | 4 | 28/29. 1. | E1 (E2) | ±/+ a. e. | + | ±/+ | + |
| 4632/144 | 16 | 2/3. 2. | E1 (E2) | ± | + | ±/+ | + |
| 4652/170 | 16 | 5/6. 2. | E1 (E3) | ± | ± | ± | ± |
| 4653/171 | 15 | 5/6. 2. | E2 (E3) | ± | 0 | 0 | 0 |
| 4666/201 | 7m | 7/11. 2. | E2 | 0 | 0 | ± | 0 |
| 4684/217 | 15 | 12/12. 2. | E1 (E2) | ±/+ | + | ± | + |
| 4626/132 | 24 | 30/2. 2. | E2 (E3) | 0 | 0 | 0 | 0 |
| 4622/128 | 14 | 30/2. 2. | E2 (E3) | 0 | 0 | 0 | 0 |
| 350-S/77 | 22 | 17/20. 1. | E2 (E3) | 0 | 0 | 0 | 0 |

**Table 3. Demonstration of type B virus**

| Material No. | Age | Onset/sampling | Virus detection (identification) | Rat serum B/Bratisl. 11/81 1 : 200 |
|---|---|---|---|---|
| 5527/167 | 4 | 23/25. 2. | E2 (E3) | 0 |
| 5528/168 | 3 | 21/25. 2. | *MDCK 1 | ± |
| 5540/180 | 9 | 27/3. 3. | *MDCK 1 (E1) | ± |
| 5550/188 | 10 | 7/9. 3. | *MDCK 2 MK2 | 0 |
| 5558/200 | 40 | 10/11. 3. | E3 | 0 |
| 5562/204 | 2 | 11/12. 3. | E2 | 0 |
| 5564/205 | 4 | 12/15. 3. | E1 | 0 |
| 5585/241 | 14 | 23/25. 3. | E1 (E2) | ± |

* In indicated type(s) of cells virus was isolated and/or identified by indirect immunofluorescence

embryos is more difficult than of type A: very often the virus is detected sooner on MK or MDCK tissue cultures, where its type can be identified by the immunofluorescence method (Tůmová, unpublished data). Even with the type B virus, IEM was only successful where the sample was positive in the first passage in one of the cell systems used. Sample No. 5564/205 was the only one positive in the first egg passage but negative in IEM.

In IEM, the specimens displayed either individual virions or virion pairs covered by antibody molecules (Fig. 1), or antibody-bound aggregates of virus particles, so-called immune complexes (Figs 2—4). However, immune complexes only used to be found after checking about 30 fields on an average, which was additional evidence of very low virus concentration in the samples.

No influenza virus particles were found in samples not mixed with immune sera or mixed with control rat sera.

## *Discussion*

Using the method of direct IEM we succeeded in demonstrating and identifying (with specific rat sera) influenza viruses of two A subtypes and type B in samples of nasopharyngeal washings and nasal-pharyngeal swabs collected from patients during three consecutive influenza seasons. A necessary condition for IEM detection of virus whether as individual antibody-coated particles or aggregates of virions held together by antibody-molecule bridges is the sufficient virus concentration in sample. This has been demonstrated by Anderson and Doane (1973), Rýc (1981), and has been verified in a previous work of ours studying optimal virus dilution in allantoic fluid and optimal immune serum dilution for the formation of immune complexes (Ptáková and Tůmová, 1985).

Our present study also showed that influenza virions can be demonstrated by IEM in washings or swabs after concentrating the virus-antibody mixtures by centrifugation, since antibody attached to virus increases the virus-

particle size, while direct electronmicroscopic visualization of virions in the absence of antibody fails.

A positive virus-isolation result depends not only on the number of live virus particles present in the sample but also on their ability to grow in the particular cell system, as we found for B influenza viruses, whereas a positive IEM result is conditioned by the virion concentration only. The dependence of the IEM result on the virus concentration in our materials was shown by comparison of positive IEM findings with positive virus isolations: virus detection by IEM was only successful where virus was isolated already in the first egg passage or was demonstrated in the first cell-culture passage. Accordingly, one point of high importance for IEM seems to be the manner of collecting nasal and pharyngeal swabs and concentrating them into small amounts of sampling medium.

Besides specific rat serum, paired human sera were used for IEM detection of A(H3N2) virus. The human sera showed a rise in antibody from acute to convalescent serum by conversion from negative to positive reaction or by better immune-complex formation. This also was evidence of aetiological relationship between the subtype A(H3N2) virus and the illnesses examined.

Anti-A(H1N1) Khabarovsk/77 and anti-A(H1N1) England 403/80 rat sera were used for detecting A(H1N1) virus by IEM, with the latter serum standing antigenically closer to the virus being sought. The results were similar with either serum, but with the anti A/England serum a greater number of virions were visible, and larger immune complexes were formed. These results apparently reflected the fact that both strains differ antigenically only in part and hence both sera partially share the same antibodies, which in the A/Khabarovsk antiserum were present in a much higher titre. The finding indicates that immune-complex detection may be performed even with serum against a relatively old virus variant without the result of the reaction being affected. This has important practical implications for the use of IEM in rapid influenza diagnosis.

Although IEM cannot fully replace virus isolation in laboratory diagnosis, it is a method capable of providing influenza virus type or subtype identification 1—3 weeks ahead of the isolation result, being particularly valuable when virus cultivation is tedious and time-consuming. Thus, IEM may become a highly valuable and relatively simple tool for the rapid identification of the aetiology of commencing epidemics and local outbreaks.

## References

Almeida, J. D., and Goffeé, A. P. (1965): Antibody to wart virus in human sera demonstrated by electron microscopy and precipitation tests. *Lancet* **II**, 1205—1207.

Almeida, J. D., and Waterson, A. P. (1969): The morphology of virus-antibody interaction. *Adv. Virus Res.* **15**, 307—338.

Anderson, N., and Doane, F. W. (1973): Specific identification of enteroviruses by immuno--electron microscopy using a serum-in-agar method. *Can. J. Microbiol.* **19**, 585—589.

Bayer, M. E., and Manweiler, E. (1963): Antigen-antibody reactions of influenza virus as seen in the electron microscope. *Arch. ges. Virusforsch.* **13**, 541—547.

Brenner, M. E., and Horne, R. W. (1959): A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta* **34**, 103—110.

Edwards, E. A., Valters, W. A., Boehm, L. G., and Rosenbaum, M. J. (1975): Visualisation by immune electron microscopy of viruses associated with acute respiratory diseases. *J. immunol. Meth.* **8**, 159—168.

Kelen, A. E., and McLeod, D. A. (1974): Differentiation of myxoviruses by electron microscopy and immunoelectron microscopy, pp. 257—275. In E. Kurstak, R. Morisset (Eds): *Viral Immunodiagnosis*, Acad. Press, New York.

Lafferty, K. J., and Oertelis, S. J. (1961): Attachment of antibody to influenza virus. *Nature (Lond.)* **192**, 764—765.

Lafferty, K. J., and Oertelis, S. J. (1963): The interaction between virus and antibody. *Virology* **21**, 61—90.

Ptáková, M., and Tůmová, B. (1985): The demonstration of the influenza virus by the method of the immune electron microscopy. *J. Hyg. microbiol. Epidem. (Praha)* (in press).

Rýc, M. (1981): Electron microscopy in virus diagnosis (in Czech). *Čs. Epidem. Mikrobiol. Imunol.* **30**, 297—309.

Tůmová, B. (1985): Laboratory diagnosis of influenza (in Czech). In L. Syrůček (Ed.): *Laboratorní diagnostika vírových nákaz dýchacích cest*, Avicenum, Prague.

*Explanation of Electron Micrographs* (Plates VI—VII):

*Fig. 1.* A(H3N2) influenza virus and specific rat anti-A(H3N2) Texas/77 serum diluted 1 : 400. Material No, 3950. Antibody excess veil virion structure, bar 100 nm.

*Fig. 2.* Aggregate of A(H3N2) virus and rat anti-(H3N2) Texas/77 serum diluted 1 : 400. Material No. 3903, bar 100 nm.

*Fig. 3.* Immune complex of A(H1N1) virions and rat anti-A(H1N1) Khabarovsk serum. Material No. 4389, serum diluted 1 : 200, bar 100 nm.

*Fig. 4.* Immune complex of A(H1N1) virions and rat anti-A(H1N1) England 403/80 serum. Material No. 4632, serum diluted 1 : 200, bar 100 nm.