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# INFLUENZA VIRUS DETECTION IN CLINICAL SPECIMENS

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*Summary.* — The authors compared the results of influenza A (H1N1) and influenza A (H3N2) virus detection in nasopharyngeal swabs from flu patients by molecular hybridization (MH), ELISA, virus isolation and seroconversion. Using the immunofluorescence (IF) technique influenza virus was detected in cell suspensions from the first chick embryo passage. Altogether 63 swabs from various epidemic seasons were separated into 3 groups according to specimen sampling and storage. It was shown that influenza virus RNA could be found in 16 out of 22 swab specimens (72%) stored at —70 °C without thawing and that ELISA revealed the influenza virus antigen in 19 cases (86%); in contrast, IF was positive in 6 (27%) and virus isolation in 5 (22%) cases only. However, the positive rate of MH decreased to 9% in 21 swab specimens repeatedly thawed and stored at —20 °C and was completely negative after prolonged storage of repeatedly thawed samples. Despite these conditions, ELISA was still successful in both latter sample groups (71—80%). For specificity control, 29 samples coming from patients with influenza B virus and other respiratory virus diseases (adeno- and respiratory syncytial virus) were used.

*Key words*: *DNA/RNA hybridization*; *ELISA*; *immunofluorescence*; *influenza A and B viruses*

## *Introduction*

Attempts for a more reliable and quicker laboratory diagnosis of acute respiratory diseases (ARD) especially of influenza have been promoted by still increasing demands of clinicians aiming at correct antibiotic therapy especially in children, by new progression of antiviral chemotherapy and chemoprophylaxis and last but not least by the importance of early anti-epidemic measures. Therefore, in the course of last years several new diagnostic methods were introduced (Berg *et al.*, 1980; Halonen *et al.*, 1983; Ptáková and Tůmová, 1986; Walls *et al.*, 1986*a*; Anestad *et al.*, 1988). However, no generally accepted technique has been recommended to replace the up to now used classical — not always optimal and certainly time consuming —

procedures of virus isolation and the serologic assays. ELISA and IF techniques are widely used for virus as well as antibody detection. Hybridization has been less extensively used and its efficacy is under investigation (Kulski and Norval, 1985; Baurin *et al.*, 1985; Bukrinskaya *et al.*, 1986; Pljusnin *et al.*, 1987; 1988). Here we report of influenza virus RNA detection by means of a DNA probe in routinely sampled clinical specimens (nasopharyngeal swabs) as compared with results of ELISA, immunofluorescence staining, virus isolation in chick embryos, and serology. The aim of this work which is still being continued is the choice of more suitable and reliable method (or methods) which could be standardized for clinical practice and for specialized laboratories.

## *Materials and Methods*

*Clinical specimens.* Swabs were taken from the hind pharyngeal wall and nasal ducts into 5 ml medium (Veal infusion Broth Difco supplemented with 0.5% BSA and antibiotics); material was collected in the epidemic seasons 1987—88 and 1983—86. From these 22 specimens were immediately frozen and stored at —70°C until use. Another 41 specimens from seasons 1983—88 were stored either at —70°C or at —20°C and thawed several times for different assays. Hybridization was made as last. For ELISA 0.2 ml, for hybridization on filters 0.4—0.5 ml of the specimen was taken.

*Immune sera* were raised in guinea pigs and ferrets by intranasal inoculation of 0.2 ml freshly grown virus under deep ether anesthesia on 2 subsequent days. Booster doses were given in the case of low haemagglutination inhibition and anti-RNP antibody titres 3—4 weeks after the first dosis by intranasal (guinea pig) and intraperitoneal (ferret) administration of virus purified on sucrose gradient.

*Molecular hybridization.* The sample solution (0.4—0.5 ml) was deproteinized with proteinase K (Fluka AG, CH 9470 — Busch), phenol and a phenol-chloroform (1 : 1) mixture. The RNA was precipitated with alcohol, centrifuged and diluted in TE buffer (0.01 mol/l Tris-HCl buffer, pH 8.0, 1 mmol/l EDTA). Denaturation was made in 0.1 mol/l natrium phosphate buffer containing 6—7% formaldehyde at 60°C for 5—10 min. After cooling to 0°C the samples were spotted in parallel to a nylon filter previously moistured with 6×SSC (1×SSC : 0.15 mol/l NaCl, 0.015 mol/l sodium citrate, pH 7.4). Immobilization was made in vacuum at 80°C for 90 min. Then the membranes were prehybridized in 5×SSC containing 50% formamide (Fluka AG Busch), 0.25% sodium dodecylsulphate, (SDS) 50 mmol/l natrium phosphate, 0.1% bovine serum albumin, Ficoll and polyvinylpyrrolidon for 4 hr at 37°C. Finally, 400 μg/ml denatured (100°C for 3 min) carrier DNA was added. The hybridization mixture contained, in addition, the $^{32}$P-labelled denatured DNA probe (nick-translated DNA copies of P, NP, and M genes of influenza A/PR8/34 virus cloned in plasmids pBR 322 and/or pBR 327). The activity of the probe DNA mixture was 3 MBq, specific activity $1.8 \times 10^8$ cpm/μg DNA. Finally, 10 mmol/l EDTA was added to the hybridization mixture. Incubation proceeded for 66 hr at 37°C. The membranes were washed for 40—60 min at hybridization temperature in 1×, 0.5×, 0.2×, and 0.1×SSC-SDS and autoradiography was performed (Pljusnin *et al.*, 1987).

*Immunofluorescence staining.* Amniotic cells sedimented at initial passages in chick embryos(CE) were stained by indirect method (Fedová and Zelenková, 1965*a*, *b*). Guinea pig sera to influenza A and B viruses were used in the first layer and in anti-guinea pig FITC labelled conjugate in the second (SwAGp IFITC, Sevac, Prague). The smears were counterstained with Evans blue (1 : 10,000).

*Enzyme-linked immunoassay.* The indirect double antibody sandwich modification was used for antigen detection. The wells were coated with ferret influenza A and B antiserum (A/Singapore/6/86, B/Prague/17/84). The second layer antiserum was raised in guinea pigs (A/Singapore/6/86; B/Buenos Aires/37/71). The optimal serum dilutions were determined by chess-board titrations. The test was performed as described by Shekarchi and Sever (1986). In the third layer SwA Gp/Px (peroxidase labelled) conjugate prepared by Sevac, Prague, was used; the substrate

was 5-amino-salicylic acid. The results were read at 405 nm (MR 580 Microelisa Auto Reader, Dynatech).

*Virus assay.* Virus isolations were made in 10-day-old chick embryos.

*Serologic tests.* Paired sera were tested by haemagglutination inhibition and immunoprecipitation in gel as recommended by WHO (Palmer *et al.*, 1975). As positive was regarded a double increase of virus titre or the appearance of a distinct precipitation line with the convalescent serum.

## *Results*

The tested 63 swab specimens from patients with diagnosis of influenza were divided into 3 groups: Group 1 consisted of 22 samples taken during the first 72 hr since the onset of disease and stored at −70 °C until assayed. Group 2 contained 21 clinical specimens shipped from other virological laboratories and Hygiene Stations were coming from similar patients, but the samples underwent several thawings during earlier investigations and were stored at −20 °C. The third group of specimens were selected from nasopharyngeal swabs of patients who became sick during epidemics A (H1N1) and A (H3N2) in 1983—1988; these samples were stored at −70 °C and they also were thawed several times. As negative control of these investigations we included 21 swabs from influenza B and 8 swabs from adenovirus and/or respiratory syncytial virus-infected patients. The results are shown in Tables 1 and 2.

As compared to the results of virus isolation attempts and immunofluorescence tests performed in the first CE passages, ELISA and hybridization were more sensitive and quicker when performed with the freshly collected swab specimens (group 1). Out of 22 samples of this group influenza A virus antigen was detected by ELISA in 19 cases (86%) and influenza virus RNA in 16 cases (72%). In contrast, influenza virus was isolated in 5 cases (22%) only at passage levels 2—6,which corresponded to 1—3 week delay. By IF staining the detection of virus antigen was quicker but it was positive in 6 swabs only (27%). It seems therefore, that even the IF technique may be certainly quicker and probably more sensitive than classical virus isolation.

Results with the group 2 materials were similar in ELISA, 15 swabs out of 21 being positive (71%). No IF staining was made because the classical techniques were performed outdoors. By inoculation into CE, 4 strains were isolated only (identified in the Reference laboratory), although serologic assay indicated seroconversion to influenza A (H1N1) in 20 patients (95%). From these, hybridization confirmed the virus in 2 cases (9%) (Fig. 1-b). We ascribe this discrepancy to the fact that the material was thawed several times for other assays. The viral RNA is labile and in the case of repeated thawing it is difficult to prevent degradation by RNases present in the sample. Therefore, the ELISA working under field conditions seems the most promising method for routine examination.

In the 3rd group of swabs virus antigen could be detected in 16 out of 20 (80%) cases by ELISA despite of the long term storage. This confirmed that molecular hybridization was not suitable for detection of viral RNA in repeatedly thawed clinical samples after prolonged storage. Because the virus antigens are much more resistant than viral RNA, hybridization is rather suitable for immediate diagnosis.

**Table 1. Influenza virus detection in clinical material by molecular hybridization, ELISA, immunofluorescence, and classical viral diagnostic methods**

| Nasopharyngeal swabs in the season | No. of specimens | No. of positive tests | | | | No. of isolated strains |
|---|---|---|---|---|---|---|
| | | MH | ELISA | IF* | Seroconversion | |
| 1987/83[1] | 22 | 16 (72%) | 19 (86%) | 6 (27%) | 21 (93%) type A | 5 (22%) A (H1N1) |
| 1987/83[2] | 21 | 2 (9%) | 15 (71%) | NT | 20 (95%) type A | 4 (19%) A (H1N1) |
| 1988/86 | 20 | negat | 16 (80%) | NT | 20 (100%) type A | 12 (60%) A (H1N1) A (H3N2) |
| Controls | 21** | negat | 14 (66%) | NT | 18 (85%) | 7 (33%) type B |
| | 8 *** | negat | negat | NT | none to influenza antigens | Adeno and RS |

[1] stored at −70°C [2] stored at −20 °C and repeatedly thawed

NT = not tested

* epithelium cell smears from initial egg passages

** specimens from influenza B patients. In ELISA and for demonstration of seroconversion B type sera and antigesn were used; tests for A type antigen and antibody were negative

*** ARD of non influenza aetiology

**Table 2. Demonstration of influenza virus in clinical specimens from a local epidemics in the seson 1987/88**

| Swab No. | MH | ELISA | IF | Virus isolation | Seroconversion |
|---|---|---|---|---|---|
| 9327 | − | + | − | − | + |
| 9328 | − | + | − | − | + |
| 9329 | + | + | − | − | + |
| 9330 | + | + | $E_2$, $E_3$+ | $E_3$+ | + |
| 9331 | + | + | $E_2$, $E_3$+ | $E_3$+ | + |
| 9332 | + | + | − | − | + |
| 9333 | | + | − | − | + |
| 9334 | + | + | − | − | + |
| 9335 | + | − | − | − | + |
| 9336 | + | − | − | − | + |
| 9337 | + | + | $E_1$, $E_2$+ | $E_2$+ | + |
| 9338 | + | + | $E_2$+ | − | + |
| 9339 | + | + | − | − | + |
| 9340 | + | + | − | − | + |
| 9341 | + | + | $E_2$+ | − | + |
| 9342 | + | + | − | − | + |
| 9343 | − | + | − | − | + |
| 9344 | − | − | − | − | + |
| 9345 | + | + | − | − | + |
| 9354 | − | + | − | $E_6$+ | + |
| 9297 | + | + | − | $E_2$+ | + |
| 9279 | + | + | $E_1$+ | − | NT |
| Total No. of patients 22 | 16 (72.7) | 19 (86.3) | 6 (27.2) | 5 (22.7) | 21 (95.4) |

\+ virus detection positive NT = not tested
− virus detection negative
$E_1$ to $E_6$ = egg passage number allowing virus identification

Virus detection directly depends on the number of infected cells and/or free virus particles in the collected material. Using fresh swabs, hybridization is highly sensitive as it allows to detect as little as 1 pg viral RNA. Fig. 1-a shows that the results could be correctly interpreted when the amount of RNA present in the samples ranged between 1—10 pg. The sensitivity and specificity of the hybridization test was defined according to the criteria of Weiss *et al.* (1985). In all 3 groups the sensitivity correlated with the number of positive samples. The specificity of hybridization was checked in group 4 (Fig. 1-c) using influenza B virus, adenovirus, and respiratory syncytial virus-containing specimens: none of these revealed false positive reactions, specificity being 100%. ELISA with adenovirus and respiratory syncytial virus containing swabs gave identical results.

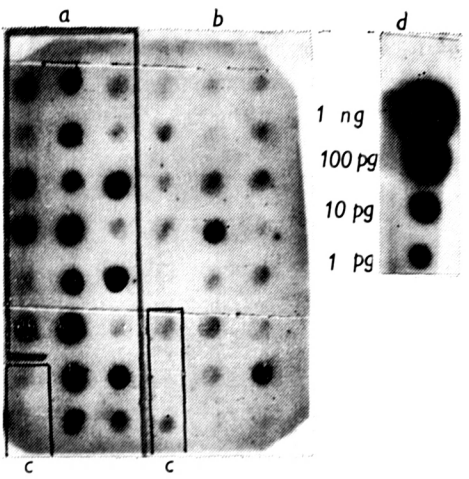


**Fig. 1**

Detection of influenza virus in nasopharyngeal swabs by molecular hybridization
a — group 1 specimens
b — group 2 specimens
c — group 3 (negative) control specimens
d — recombinant DNA specimens equivalent to given RNA concentrations (positive controls)
For further details see Results.

## *Discussion*

Identification of influenza virus by a simple technique replacing the time consuming virus isolation procedure has been since many years a matter of investigation, but no standardized method was recommended for general use in laboratory practice. The oldest alternative is doubtless the IF technique (Liu, 1956; Tateno *et al.*, 1962; Blaškovič *et al.*, 1963*a*; 1963*b*; Fedová and Zelenková, 1965*b*; McQuillin *et al.*, 1970; 1985) which is the method of choice in cell smears prepared directly from the swab sample. This technique allowing quick diagnosis is not used routinely due to difficulties with the frequently occurring nonspecific staining reactions. IF needs high degree experience, but in combination with chick embryo inoculation (Fedová and Zelenková, 1965*a*; Evans and Olson, 1982) it allows the virus identification before virus release from cells and also in the case of low haemagglutination titres of the amniotic fluid (1 : 2, 1 : 4). Therefore, we have also used IF for virus identification at individual egg passage levels.

Recently ELISA has been most frequently used in various modifications, although this assay is also not devoid of technical problems such as variations due to the quality of immune sera, lack of standardized test conditions and the possible occurrence of heterologous immune reactions interfering with the reading of results.

Of further methods the time resolved fluoroimmunoassay is worth mentioning (Halonen *et al.*, 1983; Wall *et al.*, 1986). This technique is highly sensitive but needs expensive equipment. Also the classical radioimmunoassay may be used for antigen detection in the allantoic fluid (Kostolanský *et al.*, 1986). Finally, detection of immunocomplexes can serve direct virus identification in the nasopharyngeal swabs, but its reliability is limited by the quality of the sample and technical conditions of sampling (Ptáková and Tůmová, 1985).

Although MH is the less frequently used method for influenza virus identification in clinical specimens, it was reported by several authors (Baurin *et al.*, 1985; Bukrinska *et al.*, 1985; Pljusnin *et al.*, 1987; 1989). Hybridization is sensitive — 1 pg RNA can be detected — and also highly specific for determination of positive and negative specimens. According to our experience fresh and/or correctly stored (at −70 °C) material is needed for successful and precise diagnosis. Hybridization seems relatively time consuming and expensive, thus it does not fulfil the criteria of a simple and quick diagnostic assay.

From our work, which still continues, we conclude that each alternative method described here was quicker than the classical chick embryo inoculation, but any of it depended on the quality of the collected sample, namely the amount of actually present cell-associated or free virus. Therefore, many authors recommend aspirates (Grandien *et al.*, 1985). Of aspirated specimens Ahluwalia *et al.* (1987) isolated respiratory syncytial virus in 23 or 32 cases (72%) as compared to 15 (47%) positive isolations from nasopharyngeal swabs. In such case, the positive rate of ELISA and IF test was higher as well. For outpatients, however, collection of aspirates cannot be always performed; material should be in each case swabbed to achieve the highest possible cell concentration closely approaching that of the aspirated samples.

Our results confirm that the most suitable diagnostic method was ELISA which can be also used for additional diagnosis omitting virus cultivation, not inevitable for clinical practice. Further improvements of this method may be achieved using monoclonal antibodies (Walls *et al.*, 1986*b*). Summing up, for selected laboratories ELISA and MH are equally suitable because aetiologifal diagnosis was made with these methods practically in each case.

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