Direct Detection of Influenza Virus Antigen in Nasopharyngeal Specimens by Direct Enzyme Immunoassay in Comparison with Quantitating Virus Shedding

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We developed a direct enzyme immunoassay [EIA; Enzygnost Influenza A(Ag) and Enzygnost Influenza B(Ag)] for the direct detection of influenza A and B virus antigens in nasopharyngeal secretion specimens (NPS). The test is performed without sonication of specimens, and results are obtained within 4 h. A direct comparison between direct EIA and quantitation of virus shedding for influenza A and B virus antigen detection was carried out. A total of 210 NPS and 98 nasopharyngeal wash specimens (NPW) were investigated. We isolated influenza A viruses from 79 (37.6%) of 210 NPS; of these 79 cell-culture-positive NPS, 70 (88.6%) were also positive by direct EIA. Of 29 (13.8%) NPS from which influenza B virus was isolated, 24 (82.8%) NPS were positive by direct EIA. Virus shedding was determined quantitatively in 48 NPS from patients with influenza A and in 24 NPS from patients with influenza B. Only a crude correlation between optical density values and virus concentrations was observed. Detection of influenza virus antigens in NPS by direct EIA showed sensitivities of 89.7% for influenza A virus and 87.9% for influenza B virus and specificities of 99.3% for influenza A virus and 100% for influenza B virus. With direct EIA, all NPW were negative for influenza A virus, although virus was isolated from 21 (21.4%) NPW. Of 15 NPW from which influenza B virus was isolated, 7 showed positive results in direct EIA. In addition, direct EIA is suitable for detecting influenza A and B viruses in cell cultures before the appearance of any cytopathic effects and can be used as a cell culture confirmation test.

Since it is not possible to differentiate influenza clinically from other acute respiratory diseases, etiologic diagnosis can be established only by virological methods. Rapid diagnosis of an influenza A or B virus infection may be essential because amantadine prophylaxis for influenza A infections (and eventually antiviral therapy) is possible and can be started early. Additionally, early diagnosis is also important for initiation of further control measures.

Until now, many test systems (e.g., immunofluorescence test, radioimmunoassay, enzyme immunoassay [EIA], and enzyme immunomembrane filter assay) have been described for rapid detection of influenza A and B virus infections (3-5, 7, 9, 11-14, 16-19, 21-25). However, these tests (except for the recently described enzyme immunomembrane filter assay) have certain drawbacks for practical application, such as the need for sonification of the specimens, overnight incubation of specimens, and the use of cell cultures. The application of cell culture techniques is work-intensive and time-consuming; therefore, in most routine diagnostic laboratories virus isolation services are not available.

We describe here a simple and rapid EIA [direct EIA; Enzygnost Influenza A(Ag) and Enzygnost Influenza B(Ag), research products of Behringwerke AG, Marburg, Germany] for the direct detection of influenza A and B virus antigens in nasopharyngeal secretion specimens (NPS) and compare it with quantitation of virus shedding. Furthermore, EIA allows detection of influenza A and B virus antigens in infected cells before the appearance of any cytopathic effects (CPE).

**MATERIALS AND METHODS**

**Specimens.** Patients exhibiting the sudden onset of (i) high fever, (ii) myalgia, arthralgia, or cephalalgia, and (iii) sore throat, cough, or rhinitis were studied. Between January 1989 and April 1991, 210 NPS were collected from subjects aged 1 to 16 years with Mucus Extractors (Pharma Plast, Værløse, Denmark) by means of a hand vacuum pump (Nalgene Company, Rochester, N.Y.). Between January 1989 and April 1991, 98 nasopharyngeal washes (NPW) were obtained from persons aged 17 to 77 years. Until transported to the laboratory, specimens were stored at 4°C, and virus isolation and direct EIA were carried out no later than 24 h after collection. Both virus isolation and direct EIA were initiated at the same time.

**Pretreatment of specimens for direct EIA.** NPS were diluted 1:2 in commercially available sample buffer STD (catalog no. OUWO; Behringwerke AG). NPW were concentrated by centrifugation (800 x g, 30 min, 4°C) and stored at 4°C. The supernatant was diluted 1:2 in sample buffer STD.

**Direct EIA for detection of influenza virus antigens.** For EIA for direct antigen detection (direct EIA), 96-well polystyrene microtitration plates were coated with polyclonal antibodies (from rabbits) directed against influenza A and B virus as catching antibodies. Propagation and purification of influenza virus for immunization were done as described previously (20). Immunization of rabbits with whole virus was performed by standard procedures and a combination of subcutaneous and intravenous injections over 8 weeks, with bleeding performed in week 9. Samples (150 μl) of NPS (diluted 1:2 in sample buffer STD) were added to the wells
and incubated for 1 h at 37°C in a moist chamber. After four washings, bound influenza virus antigens were detected by the addition of 100 μl of peroxidase (POD)-labeled monoclonal antibodies for 0.5 h at 37°C in a moist chamber. Monoclonal antibodies described elsewhere (23) were obtained from Centers for Disease Control, Atlanta, Ga. The wells were washed again four times with washing solution, and bound POD-labeled antibodies were detected by the addition of 100 μl of chromogen tetramethyl benzidine dihydrochloride (TMB) for 0.5 h at 18 to 25°C. The POD-substrate reaction was stopped by the addition of 100 μl of H₂SO₄ (stopping solution), and the reactions were measured in a Behring ELISA processor II with a wavelength of 450 nm. Commercially available supplementary reagents for Enzygnost TMB (catalog no. OUVP; Behringwerke AG) (washing solution POD, buffer/substrate TMB, chromogen solution) were used.

Antigen stability in sample buffer STD at different storage temperatures. For the evaluation of antigen stability in sample buffer STD, we stored samples of influenza A/Singapore/6/86 (A/H1N1) at room temperature at 22°C and in a refrigerator at 4°C. Phosphate-buffered saline (PBS), pH 7.2, plus 1% (wt/vol) bovine serum albumin (BSA) were used for a control for the same storage conditions. The samples were examined by the direct EIA on days 0, 1, 3, 5, and 7.

Virus isolation and identification. Virus isolation was performed in MDCK monolayers with serum-free minimum essential Earle’s medium containing 1 μg of trypsin per ml. Identification was performed by the immunofluorescence test as described earlier (7).

Direct EIA for antigen detection in cells. All specimens that gave negative results in direct EIA were cultured in MDCK cells for detection of influenza virus antigens before the appearance of CPE. At 1, 2, and 3 days after inoculation in MDCK cells, supernatants were decanted and cells were cultured with sample buffer STD to lyse the cells for direct examination of virus content by direct EIA.

Determination of virus shedding in specimens from patients. For determination of infectivity, the NPS and NPW were titrated in MDCK monolayers by inoculating six wells per dilution of a 48-well microtiter plate (catalog no. 3548; Costar Cambridge, Mass.) in 10-fold dilution steps from undiluted to 10⁻⁸. After 7 days of incubation, each well was screened for the presence of influenza virus surface antigens by hemagglutination test and for the presence of influenza virus antigens by direct EIA. The 50% tissue culture infective dose per milliliter was calculated by the method of Kärber (15).

Hemagglutination test. Hemagglutinating antigen in MDCK cell supernatants was detected by the hemagglutination test in microtiter plates by standard procedures (8).

Specificity and sensitivity. Specificity and sensitivity of direct EIA for influenza A and B virus antigen detection were calculated and compared with values found by virus isolation in cell cultures (10).

Calculation of precision. Intraassay coefficient of variation for influenza A and B virus EIA was calculated by 192 values (2).

RESULTS

Antigen stability. The influenza virus antigen stability was examined in PBS-BSA and in sample buffer STD at 4°C and at room temperature for 7 days. No significant differences were observed after storage of influenza virus at 4°C in different buffers and at room temperature in PBS-BSA buffer. A 15% decrease of detectability was observed when influenza virus was stored in STD buffer at room temperature over 4 days.

EIA for direct detection of influenza A virus antigen in NPS. Of 210 NPS, influenza A viruses were isolated from 79 (37.6%). Of 79 cell-culture-positive NPS, 70 (88.6%) also were positive in direct EIA, and 9 (11.4%) were negative. Additionally, seven of these negative samples were examined by culturing in MDCK cells (Table 1). Of these seven NPS which were influenza A virus negative by direct EIA, four were positive after being cultured on MDCK for 1 day and the remaining three were positive after 3 days of being cultured on MDCK cells. In 5 NPS, high virus concentrations were determined (Table 1).

Determination of quantitative virus shedding in NPS. Virus shedding was determined quantitatively from 48 NPS. Results for influenza A virus are shown in Fig. 1. As expected, there were considerable individual differences in the quantity of virus shed. Only a crude correlation between optical density values and infectious virus concentration was observed (Fig. 1).

EIA for detection of influenza A virus antigen in infected cells. All 98 NPW tested were negative for influenza A virus antigen in direct EIA. All 98 NPW were inoculated into MDCK cells and tested by direct EIA. Influenza A virus was isolated in 21.4% (n = 21). Results are shown in Table 1. Of these influenza A isolations, 57% (n = 12) could be detected by direct EIA after 1 day of culture, whereas 9.5% (n = 2) remained negative after 3 days. Virus concentrations were determined for seven specimens and high virus concentrations (50% tissue culture infective dose of ≥2.2) were found in five specimens (Table 1).

EIA for direct detection of influenza B virus antigen in NPS and NPW. Since January 1989, we isolated influenza B virus from 29 NPS and 15 NPW. Twenty-four (82.8%) NPS and seven (46.7%) NPW were positive in direct EIA, and five (17.2%) NPS and eight (53.3%) NPW were negative in direct EIA. The direct EIA-negative specimens were inoculated in MDCK cells and tested 1, 2, and 3 days postinoculation in direct EIA. Two NPS and six NPW were positive after 1 day, one NPS and one NPW were positive after 2 days, and two NPS and one NPW were positive after 3 days. The influenza infection was diagnosed for 10 specimens before the appearance of CPE.

Virus shedding was determined for 24 NPS. The influenza B virus antigen results were similar to shedding of infectious

### Table 1. Influenza A virus antigen detection

<table>
<thead>
<tr>
<th>Virus concn (log₁₀ TCID₅₀/ml)</th>
<th>Positive on day 1 post-infection</th>
<th>Positive on day 2 post-infection, 4 NPW</th>
<th>Positive on day 3 post-infection</th>
<th>Negative on day 3 post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown (not titrated)</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>&lt;2.20</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2.2-4.0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.1-6.0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>≥6.1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

NPW from 21 adults and 7 NPS from subjects aged 1 to 11 years were studied. The seven NPS were negative by direct EIA.
virus for influenza A virus antigen (Fig. 2). Influenza B virus antigen detection was also successful in 7 NPW.

Specificity and sensitivity. The specificities for direct EIA were 99.3% (n = 131) for influenza A virus antigen detection and 100% (n = 181) for influenza B virus antigen detection; the sensitivities were 89.7% (n = 79) for influenza A virus antigen detection in NPS and 87.9% (n = 29) for influenza B virus antigen detection in NPS.

Precision. The intraassay coefficients of variation were 7.8% for influenza A virus detection and 8.0% for influenza B virus detection.

**DISCUSSION**

Detection of respiratory viruses (e.g., respiratory syncytial virus, influenza A virus, influenza B virus, adenovirus) in clinical specimens has gained increasing importance in recent years. Rapid detection of influenza A infection may have immediate consequences for chemotherapy, e.g., reduction of antibiotics, chemoprophyaxis, and clinical management. In addition, the identification of viruses is important for epidemiological aspects.

In this article, we report on the use of a direct EIA for rapid diagnosis of influenza A and B infection. The basis of successful influenza virus antigen detection in direct EIA is the collection of NPS by suction. In this study, a small hand vacuum pump was used. In contrast to earlier studies in which NPS were obtained by oral suction or by electric-powered motor suction no suction-related problems were encountered (6). Another suitable hand-operated airway suction device for use in newborns was described elsewhere (1). Collecting NPS from children proved now to be very easy.

Several test systems for rapid detection of influenza virus antigens have been described. Our aim was to overcome certain requirements such as (i) overnight incubation, (ii) no limitation by viscosity of mucus, (iii) need for sonification of specimens, and (iv) use of cell cultures. We were able to replace sonification by the detergent-containing sample buffer STD, which simplifies routine diagnosis. We were able to demonstrate that the sample buffer STD did not influence influenza virus antigen detection by direct EIA during the observation time of 1 week when specimens were stored at 4°C.

The titration of virus in NPS showed considerable individual differences. Only a crude relationship between optical density values and virus infectivity was observed (Fig. 1 and 2). We observed the presence of infectious virus and virus antigen up to 5 days after the onset of symptoms. More than 5 days after infection, antigen detection was no longer successful. Additionally, we tried to find a correlation (70 NPS) between direct influenza A virus antigen detection and the onset of symptoms, but optical density values and the onset of clinical symptoms did not strictly correlate (data not shown). However, as expected, the highest values were obtained at day 1 and 2 after the onset of symptoms. In our study, nine NPS (11.4%) were negative in direct EIA despite the isolation of influenza A virus. Strikingly, in five of these direct EIA-negative NPS, high virus concentrations were
found by titration of specimens in MDCK cell cultures (Table 1). Similar results were reported by other researchers, who isolated influenza viruses from NPS, whereas direct EIA gave negative results (13). In contrast, with the enzyme immunomembrane filter assay, 37 specimens were reported to be positive, although only 23 of these specimens yielded influenza A virus in cell cultures (24). The number of specimens was too small and the methods and materials used were too different to allow a meaningful comparison of these studies. In direct EIA-negative specimens, it was very helpful to use the same direct EIA as a cell culture confirmation test with infected MDCK cells for the detection of an influenza infection (Table 1). The influenza infection could be diagnosed before the appearance of CPE.

In this study, we also investigated NPW for influenza A and B virus antigen detection by direct EIA. To our surprise, we could not detect influenza virus A antigen in 21 NPW from adults, although in 5 specimens high virus concentrations comparable to those found in NPS were present. These results suggest the presence of inhibitory factors in the NPW. In these cases, using direct EIA as a cell culture confirmation assay with infected MDCK cells was helpful in 19 of 21 NPWs, because we could detect the influenza A or B infection after 1 (n = 12 for influenza A/m = 6 for influenza B), 2 (n = 4/m = 1), or 3 days (n = 3/m = 1) of culturing before the appearance of CPE. Two NPWs remained negative after 3 days.

We described an EIA for direct detection of influenza A and B virus antigens in NPS. Test results are available within 4 h, and specificity and sensitivity are comparable with those published by other researchers (11, 13).

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REFERENCES


