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# Simultaneous Detection and Typing of Influenza Viruses A and B by a Nested Reverse Transcription-PCR: Comparison to Virus Isolation and Antigen Detection by Immunofluorescence and Optical Immunoassay (FLU OIA)

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**A nested reverse transcription (RT)-PCR was developed for simultaneous detection and typing of influenza viruses A and B. The detection limit for influenza virus A subtypes H1 and H3 and that for influenza virus B were between 1 and 4 target gene copies per reaction for each type. The clinical benefit of the RT-PCR method was evaluated by comparing the results with virus isolation and direct immunofluorescence (IF) assays on 215 nasopharyngeal aspirates from patients with suspected influenza virus infection. The RT-PCR detected 83 cases of influenza A, compared to 66 cases detected by virus isolation and 68 cases detected by IF assay. The corresponding figures for the detection of influenza B were 15, 12, and 11 cases, respectively. In total, 16 out of 98 RT-PCR-positive specimens were negative by virus isolation and IF. An optical immunoassay for rapid detection of influenza A and B (FLU OIA; Bio Star Inc., Boulder, Colo.) was compared to RT-PCR and IF on 105 nasopharyngeal aspirates and 79 swabs. The sensitivity for the OIA was 40.4% compared to PCR and 48.8% compared to IF assay, when nasopharyngeal aspirates were examined. The specificities were 94.3 and 93.9%, respectively. The sensitivity was higher for OIA on nasopharyngeal swabs, 77.5% and 86.6% compared to PCR and IF, respectively, while the specificity was lower, 82.0% and 75.5%, respectively. The RT-PCR provides a sensitive and specific method for detecting and typing influenza viruses A and B. The rapid OIA is useful as a complementary test, but it cannot replace established methods without further evaluation.**

Influenza viruses A and B are world-wide major causative agents of human acute respiratory infections. Infants, the elderly, and individuals with compromised cardiac, pulmonary, or immune systems are at greatest risk of serious complications from these viruses (8 and references therein). The availability of improved drug treatments with neuraminidase-blocking agents for influenza viruses (11) has emphasized the importance of a rapid laboratory diagnosis. Furthermore, unnecessary use of antibiotics for suspected bacterial infection and prevention of influenza outbreaks in hospitals and in the community are given increasing priority, which also require efficient diagnostic methods.

Current diagnostic methods include virus isolation, antigen detection, and serology. Major limitations of these techniques include prolonged time to completion, subjective evaluation, low sensitivity, and low specificity. Use of nucleic acid amplification techniques has made sensitive diagnosis of influenza virus infection feasible, with the possibility of type determination. New rapid tests for influenza virus detection are also available but need to be evaluated by other methods before they are introduced in clinical practice.

With the strategy to detect and distinguish normally prevalent types of human influenza viruses, we have developed and evaluated an in-house multiplex reverse transcription (RT)-PCR assay for influenza virus A types H1 and H3 and for influenza virus B. The method was compared to virus isolation and antigen detection by immunofluorescence (IF) assay. Furthermore, the FLU OIA test (Bio Star Inc, Boulder, Colo.), a rapid test for detection of influenza viruses A and B, was compared to virus isolation, PCR, and IF.

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## MATERIALS AND METHODS

**Clinical specimens.** Nasopharyngeal aspirates were collected from 289 patients with a suspected influenza virus infection. Twenty-one cases 7.3% were excluded from the study due to nonrepresentative samples (<50 nasopharyngeal epithelial cells per preparation). Specimens from 268 patients remained for analysis, and from 79 of these patients nasopharyngeal swabs were also collected. The patients were between 2 months and 83 years old.

Nasopharyngeal aspirates were collected by using a baby-feeding tube and an aspiration trap. After suction the feeding tube was rinsed with approximately 2 ml of sterile saline (17). Samples were transported to the lab immediately.

Nasopharyngeal swabs were collected in parallel with nasopharyngeal aspirates from 79 patients by using rayon-tipped swabs (Copan Italia, Brescia, Italy). The swabs were rubbed against the mucosal surface of the nasopharynx. They were then transported in sterile empty tubes to the lab and were processed within 6 h for use in the optical immunoassay (OIA).

**Virus isolation.** The nasopharyngeal aspirates were centrifuged at 1,000 × *g* for 10 min. The supernatant was used for inoculation of two tubes with MDCK cells, as has been described previously (17). The cell cultures were observed for cytopathic effect (CPE) for a total of 2 weeks. If no CPE was seen, hemadsorption with guinea pig red blood cells was performed. CPE or positive hemadsorption was confirmed by IF staining with influenza A and B monoclonal antibodies (Dako AS, Glostrup, Denmark).

**Antigen detection by IF assay.** Cells from centrifuged nasopharyngeal aspirates were washed and resuspended in phosphate-buffered saline and spotted onto multiwell slides (20 μl/well). The cells were air-dried and fixed in acetone, and multiple wells were incubated with 20 μl of fluorescein-labeled influenza A and B monoclonal antibodies (Imagen, Dako AS) as previously described (17).

TABLE 1. Characterization of primers in RT-PCR for detection of influenza viruses A and B

| Step, influenza virus strain, and primer | Sequence | 5′ Position (bp) | Product size | $T_m$[a] (°C) | Maximum annealing temp[b] (°C) | GenBank accession no. |
|---|---|---|---|---|---|---|
| First step | | | | | | |
| Influenza A/H1 | | | | | | |
| AH1A | 5′-CA(G/T)ATGCAGACACAATATGT-3′ | 76 | 1,013 | 54 | 60 | D13573 |
| AH1R | 5′-ACCGGCAATGGCTCCAAA-3′ | 1,088 | | 68 | | |
| Influenza A/H3 | | | | | | |
| H3OF | 5′-(T/C)CA(G/A)ATTGA(A/G)GTGACTAATGC-3′ | 173 | 892 | 57 | 60 | X05907 |
| H3OR | 5′-TCTAGTTTGTTT(T/C)TCTGGTACAT-3′ | 1,064 | | 58 | | |
| Influenza B | | | | | | |
| BOF | 5′-TGTGACTGGTGT(G/A)ATA(C/T)CACT-3′ | 153 | 905 | 59 | 60 | X00897 |
| BOR | 5′-A(A/G)GGTGTTTT(C/T)ACCCATAT-3′ | 1,057 | | 58 | | |
| Second step | | | | | | |
| Influenza A/H1 | | | | | | |
| AH1B | 5′-ATAGGCTACCATGCGAACAA-3′ | 96 | 944 | 63 | 62 | D13573 |
| AH1E2 | 5′-CTTAGTCCTGTAACCATCCT-3′ | 1,039 | | 55 | | |
| Influenza A/H3 | | | | | | |
| H3IF | 5′-CAGCAA(A/T)GCTT(T/A)CAGCAACT-3′ | 347 | 592 | 63 | 61 | X05907 |
| H3IR | 5′-GCTTCCATTTGGAGTGATGC-3′ | 938 | | 64 | | |
| Influenza B | | | | | | |
| BIF | 5′-(A/G)ATC(T/C)CAT(T/C)TTG(C/G)AAATCTCA-3′ | 191 | 769 | 60 | 62 | X00897 |
| BIR | 5′-AGGCA(A/G)TCT(G/C)(G/C)TTC(A/G)CCAA-3′ | 959 | | 62 | | |

[a] As determined by nearest-neighbor method.
[b] Highest temperature giving PCR product without decreased band intensity on gel.

The slides were incubated in a moist chamber at 37°C for 30 min and washed three times with phosphate-buffered saline. Examination was performed in a fluorescence microscope at 200 to 400 × magnification. A specimen was considered representative if more than 50 nasopharyngeal epithelial cells per preparation was present. The presence of at least two cells (or cell nuclei) with a typical distinct staining was regarded as a positive result.

**Antigen detection by the OIA test.** For the OIA test, the influenza virus antigens were extracted from two types of specimens according to specifications from the manufacturer: (i) rayon-tipped swabs dipped into concentrated cells obtained from centrifuged nasopharyngeal aspirates (the same preparations as were used for IF slide preparation) and (ii) rayon-tipped swabs rubbed against the mucosal surface of the nasopharynx.

The OIA test uses a mirrorlike surface of a silicon wafer coated with optical molecular thin film and a capture antibody specific for influenza A and B. White light reflected through this surface appears as a gold-colored background.

The extracted material is incubated with a horseradish peroxidase-conjugated antibody specific for influenza A and B virus. The resulting antigen-antibody complex binds to the solid phase with influenza-specific antibodies and is thereafter visualized with horseradish peroxidase substrate. The obtained mass enhancement of the antigen-capturing optical surface of the silicon wafer develops a purple spot, indicating detection of influenza virus without differentiation between types A and B. If no binding occurs, the original molecular thickness remains unchanged, and the test surface retains the gold color, indicating a negative result. The test procedure for one sample is completed within 15 to 20 min. In our study positive test results were scored from 1+ to 4+.

**Nucleic acid extraction.** RNA in 140 μl of the crude nasopharyngeal aspirate was prepared by binding to a silica matrix followed by a spin column purification with a final eluate of 60 μl according to the instructions of the manufacturer of the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). To monitor for cross-contamination during the RNA preparation and the subsequent PCR, every fourth RNA extraction was a negative water control.

**Design of RT-PCR.** Target sequences were from the hemagglutinin genes of influenza virus type A subtype H1 and subtype H3 and influenza virus type B. Primers were selected after analysis with OLIGO primer analysis software (version 5.0; National Biosciences Inc.) and compared with known sequences in both GenBank (http://www.ncbi.nlm.nih.org/BLAST) and results from a previous study (23; see Table 1).

Optimization of a thermal amplification profile and concentration of deoxynucleotide triphosphates (dNTPs) and $Mn(OAc)_2$ (step one) or $MgCl_2$ (step two) were performed for each primer set.

For the analysis of clinical samples, 10 μl of the eluted influenza RNA was converted into complementary DNA (cDNA) and subsequently amplified by PCR using 5 U of r*Tth* DNA polymerase in EZ-buffer (50 mM bicine, 115 mM potassium acetate, 8% glycerol [pH 8.2]), purchased from PE Biosystems, with 2.5 mM $Mn(OAc)_2$, 0.1 mM dNTP, and 0.3 μM each primer (Table 1). The reaction volume was 50 μl, and the optimized profile in the thermal cycler (Biometra T3; Biometra, Göttingen, Germany) in step one was 37°C for 2 min, 58°C for 30 min, and 94°C for 2 min during the RT reaction, followed by 40 amplification cycles (denaturation at 94°C for 30 s, annealing at 62 to 58°C for 40 s, and synthesis at 72°C for 40 s). To enhance specificity the annealing temperature was 62°C for the initial five cycles, 60°C for the next five cycles, and 58°C for the remaining 30 cycles. Amplification was completed with a prolonged synthesis at 72°C for 5 min.

In the nested PCR step, 3 μl of the initial reaction product was added to a second PCR reaction mixture of 50 μl containing 1.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) in 1.5 mM $MgCl_2$–50 mM KCl–10 mM Tris-HCl (pH 8.3)–0.2 mM dNTP–0.4 μM each primer (Table 1). The optimized cycle profile comprised an initial denaturation at 95°C for 1 min followed by 40 cycles of amplification and final synthesis as in step one above. Obtained PCR products were detected by electrophoresis on 1% ethidium bromide-stained agarose gels.

**Cloning of plasmids for sensitivity determination of RT-PCR.** Products obtained after primary RT-PCR reactions for influenza A/Stockholm/01/96 H1N1, influenza A/Wuhan 359/95 H3N2, and influenza B/Sweden/2724/99 were inserted into plasmid pCR 2.1-TOPO and propagated in *Escherichia coli* strain TOP 10 according to the directions of the manufacturer (TOPO TA cloning kit; Invitrogen, Carlsbad, Calif.). Selected plasmid clones were purified by using a Wizard Plus Minipreps DNA Purification System (Promega, Madison, Wis.), and the DNA concentration was determined by using luciferase measurement of restriction enzyme-cleaved plasmid (DNAquant; Promega, Madison, Wis.). The calculated plasmid copy number was used in end-point titration of the sensitivity for each primer pair. A stock solution of each plasmid was diluted in TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA) in half-log dilution steps at each run. For each dilution, one measure point was recorded, and three sensitivity determinations were performed for each primer pair.

## RESULTS

**Design of RT-PCR method.** Three sets of oligonucleotide primers were selected from the hemagglutination genes to differentiate influenza viruses type A subtype H1, type A subtype H3, and type B. In order to optimize annealing properties, the primers were aligned with known nucleotide sequences from GenBank, and at ambigious positions mixed nucleotides

TABLE 2. Detection limits for one-step RT-PCR and nested RT-PCR as determined by end-point titration of plasmids containing the target sequences for influenza viruses A subtype H1, A subtype H3, and type B

| Target; plasmid | No. of copies detected by method | |
|---|---|---|
| | One-step RT-PCR | Nested RT-PCR |
| Influenza A/H1; CL-A/H1 | 100 | 1 |
| Influenza A/H3; CL-A/H3 | 400 | 4 |
| Influenza B; CL-B | 300 | 3 |

were incorporated. The assay design was aimed at using annealing temperatures of approximately 60°C, which avoid unspecific amplification.

**Detection limits of the RT-PCR assays.** The amplification products of the three outer primer pairs for influenza viruses type A subtype H1, type A subtype H3, and type B were inserted in plasmid pCR 2.1-TOPO and propagated in *E. coli* strain TOP 10. The sensitivity of the three one-step RT-PCR assays was determined by end-point titration of purified plasmid stocks with defined copy numbers. Detection limits between 100 and 400 copies per reaction were obtained (Table 2), and with nested PCR the sensitivities for the three methods were in the range of 1 to 4 copies per reaction. The variation in sensitivity limits was not more than one half-log dilution within any of the six different reactions.

**Evaluation of RT-PCR on clinical specimens.** The diagnostic outcome of RT-PCR was compared to virus isolation and IF. Sixty-three out of 66 (95.4%) influenza A culture-positive specimens were detected by RT-PCR, and 60 of 66 (90.9%) were positive by IF assay. Similarily, 13 out of 14 (92.9%) influenza B isolates were detected by RT-PCR, and 11 of 14 (78.6%) were positive by IF assay. In total, 27% more samples were positive by RT-PCR (99) than by culture (78). Discrepant test results of culture, RT-PCR, and IF assay are shown in Table 3. Noteworthy were 16 samples positive only in RT-PCR. Of the six PCR-negative samples that were positive in culture or IF, four became positive upon repeated analysis with RT-PCR. All cases of influenza A virus detected by RT-PCR were of subtype H3.

Using culture as the standard, the specificities of RT-PCR for influenza viruses A and B were 86.6% and 98.5%, respectively. An extended golden standard was also used, and a true positive result was considered as any virus isolation-positive sample or any sample positive in both PCR and IF. For detection of influenza A virus, the sensitivity of RT-PCR was 95.9%, and the specificity was 90.8%. For influenza B virus the use of an extended standard did not change the sensitivity or specificity values for the RT-PCR method.

TABLE 3. Analysis of 37 discrepant test results comparing virus isolation. IF and RT-PCR methods

| Test results[a] | No. of isolates showing test results for influenza type(s) | | |
|---|---|---|---|
| | A | B | A and B |
| Isolation +, IF +, PCR− | 2 | 1 | 3 |
| Isolation +, IF −, PCR− | 1 | 0 | 1 |
| Isolation +, IF −, PCR+ | 6 | 2 | 8 |
| Isolation −, IF +, PCR+ | 7 | 0 | 7 |
| Isolation −, IF −, PCR+ | 13 | 3 | 16 |
| Isolation −, IF +, PCR− | 1 | 1 | 2 |
| Total | 30 | 7 | 37 |

[a] +, positive test result; −, negative test result.

TABLE 4. Comparison of diagnostic sensitivity for RT-PCR with single primer pairs and a multiplex assay with three primer pairs after one step and after two steps of amplification[a]

| Primers used in assay | No. of cases detected after step | |
|---|---|---|
| | 1 | 2 |
| Influenza A/H3 primer pair | 48 | 70 (46[b]) |
| Influenza B primer pair | 4 | 13 (225[b]) |
| Influenza A/H1, A/H3, B primer pairs | 20 type A<br>2 type B | 65 type A (225[b])<br>8 type B (300[b]) |

[a] The comparison is based on the extended standard defining a true influenza virus case as positive in culture or positive in both PCR and IF.
[b] Increase (%) in number of detected cases compared to step 1.

The diagnostic sensitivity of RT-PCR using single primer pairs was compared with the multiplex assay, including three primer pairs for detection of influenza virus types A/H1, A/H3, and B (Table 4). Based on the extended standard for defining true positive cases, the uniplex performance of the assays showed a higher detection rate for influenza virus A/H3 (8%) and influenza virus B (62%) compared to the multiplex system. The importance of a second amplification step was evaluated, and the detection rate was increased between 46 and 300% in the assays for influenza A and B viruses.

**Evaluation of the OIA test on clinical specimens.** The OIA test was evaluated on 184 representative specimens, of which 105 were from concentrated nasopharyngeal aspirates and 79 were from nasopharyngeal swabs exclusively used for OIA testing. For detection of influenza viruses A and B, the OIA test was compared to PCR, IF, and virus isolation (Table 5). Seventy-nine out of 184 specimens were RT-PCR positive for influenza virus A, and 13 were positive for influenza virus B. The overall sensitivity of the OIA test for detection of influenza viruses A and B was 55.7% compared to the combined result of PCR, IF, and virus isolation methods. RT-PCR confirmed 52 out of 62 OIA test-positive samples, and two further samples were confirmed as influenza virus positive by both IF assay and isolation methods. The specificity for the OIA test compared to the combination of PCR, IF, and isolation methods was 90.8%. If only OIA results with a score of ≥2+ were considered as true positive results, 48 cases of influenza A or B were detected, of which 45 were confirmed by PCR, IF, or isolation methods. The recalculated overall specificity for the OIA test was 96.6%, and the corresponding sensitivity was 45.3%.

## DISCUSSION

**Design of RT-PCR method.** One aim of the described RT-PCR method for influenza virus is to have a user-friendly design for clinical diagnostics. The QIAamp viral RNA mini kit is convenient for extraction of viral RNA in nasopharyngeal aspirates and has been documented to be reproducible and sensitive for viral RNA in plasma (7).

TABLE 5. Comparison of OIA, PCR, IF, and virus isolation methods for the detection of influenza viruses A and B in 184 nasopharyngeal specimens

| Specimen type (*n*) | No. of cases detected by method | | | | OIA versus PCR (%) | | OIA versus IF (%) | | OIA versus isolation (%) | | OIA versus combined results of PCR, IF, and isolation (%)[a] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PCR | IF | Isolation | OIA | Sensitivity | Specificity | Sensitivity | Specificity | Sensitivity | Specificity | Sensitivity | Specificity |
| Aspirates (105) | 52 | 39 | 41 | 24 | 40.4 | 94.3 | 48.8 | 93.9 | 46.3 | 90.8 | 39.3 | 95.9 |
| Swabs (79) | 40 | 30 | | 38 | 77.5 | 82.0 | 86.6 | 75.5 | | | 78.6 | 84.2 |
| Total (184) | 92 | 69 | | 62 | 56.5 | 89.1 | 64.4 | 86.1 | | | 55.7 | 90.8 |

[a] Virus isolation not performed for swabs. A true positive case is defined as any specimen positive in at least two tests.

The r*Tth* enzyme has both RT and DNA polymerase activity and enables the RT reaction to be combined with the first PCR amplification in a single tube, without manipulation between the two reactions. Furthermore, r*Tth* has previously been shown to be less sensitive to inhibitory components in nasopharyngeal secretions when compared to *Taq* DNA polymerase-based PCR amplification of influenza virus (15). Another advantage of r*Tth* DNA polymerase is its RT activity at temperatures up to 60°C, which decreases unspecific primer annealing and cDNA synthesis.

**Detection limit and specificity of RT-PCR.** The detection limit of the RT-PCR method was measured by using a defined number of target sequence copies inserted in plasmids. The obtained sensitivities for the three different RT-PCR assays ranged between 1 and 4 target copies per reaction after the second amplification step. This may, however, not reflect the true detection limit of the assay since the efficiency of the RT reaction is not measured. RNA transcripts synthesized from plasmids have also been used, and sensitivity levels between 3 and 10 RNA copies per reaction have been found (6, 10). Other evaluations have used 50% tissue culture infectious dose ($TCID_{50}$) (1, 2, 12), plaque forming units (19, 21, 22), and hemagglutination units (16) to determine the sensitivity of the RT-PCR methods. These may give correct calculations, but the quantification of the infectious dose is difficult to standardize and does not measure the number of target copies. Regardless, of the method chosen for sensitivity determination, it is difficult to ascertain the minimum number of target viruses needed for a detectable PCR product.

The specificity of our RT-PCR assay was tested, and we detected no cross-reactivity between influenza viruses A/H1 (Wuhan 359/95), A/H3 (A/Stockholm/01/96), and B (Sweden/2724/98). The fact that the PCR assay is based on a nested system excludes the risk of unspecific reaction with more distantly related organisms.

**Evaluation of RT-PCR on clinical samples.** By using the culture method as the standard, the RT-PCR method detected 92.9% to 95.4% of the isolated influenza strains. Other evaluations of RT-PCR methods on clinical specimens have reported a sensitivity similar to (16) and even less than (21) culture, but in the latter case they used only one amplification step. Introduction of an extended standard is controversial (14 and references therein), but it may be used, and in our study it only slightly improved the sensitivity, but it increased the specificity of the RT-PCR.

Moreover, 16 cases were RT-PCR positive for influenza viruses A and B without confirmation by other methods. This indicates that several influenza cases were detected only by the RT-PCR method. False positivity due to cross contamination is well known, especially when nested PCR assays are performed. Although it can not be completely excluded, contamination is unlikely to explain cases that were positive only by RT-PCR. Out of more than 400 negative water controls subjected to an RNA extraction procedure and nested RT-PCR, only 3 were positive. An alternative explanation to false-positive results could be unspecific primer annealing and subsequent amplification. However, it is improbable that these factors would result in a PCR product when a nested method is used, as in the present study.

When using our multiplex RT-PCR, the total detection rate for detection of influenza A and B was 27% higher than that of culture. This was clearly higher than was found in a recent evaluation of a nested multiplex RT-PCR method with the hemagglutinin gene as the target (12) but lower than reported from a study using the matrix gene as the target (20).

Using an extended standard for case definition of influenza infections, our uniplex assays showed higher sensitivity compared to multiplex PCR for detection of influenza A (8%) and influenza B (62%). Such differences may be due to interaction of the selected primers. The RT-PCR method evaluated by Magnard et al. (12) was previously described by Ellis et al. (5); their sensitivity determination by end-point titration of different targets showed no difference for uniplex and multiplex RT-PCR. However, the multiplex and uniplex assays were not compared by using clinical samples in their study.

Six samples were positive in culture or IF assay but initially negative in the RT-PCR. This difference could be due to inhibitors in the PCR reaction, which has been reported to occur in about 2% of samples from the respiratory tract (18). However, after repeated RNA extraction, 4 out of 6 samples became positive in the PCR method, which may indicate limited reproducibility of the RNA extraction method used.

**Evaluation of OIA on clinical samples.** RT-PCR, IF, and virus isolation methods were used as references for evaluation of the OIA test. For the detection of influenza A virus in concentrated cells from nasopharyngeal aspirates, the OIA test had a significantly lower sensitivity than the three reference methods. However, for nasopharyngeal swabs the difference was less evident, and the OIA test was only slightly less sensitive than IF. We expected that influenza virus would be detected in the concentrated cells from centrifuged nasopharyngeal aspirates to the same degree as from swabs (4, 13). The superiority we found for swabs may indicate that free-virus

particles may be washed away in the preparation step of aspirates.

Our findings of different outcomes for nasopharyngeal aspirates and nasopharyngeal swabs are somewhat in contrast to a previous report, where the OIA test showed a similar sensitivity for nasal aspirates (88%) and nasopharyngeal swabs (83%) compared to culture (3). There is no obvious reason for nasal aspirates to contain more influenza virus than nasopharyngeal aspirates. In a recent report (9), the FLU OIA test had a sensitivity of 48% for detection of influenza viruses A and B, when compared with culture. This is similar to our results, but since the numbers of samples were few and originated from different localities (nose, throat, and sputum), a reliable comparison was not possible.

Analysis of the scored results in the OIA test showed that weakly positive (1+) specimens were frequently negative by the reference methods. This indicates that false-positive results occur and render a specificity that limits the use of the test. The positive predictive value would be unacceptably low when using the test in a nonepidemic situation where the prevalence of influenza virus is low. This can be partly compensated for by raising the cut-off level for a positive result and regarding scores of 1+ as negative. However, such a reading system requires experience and access to a test panel. The user-friendly design and the rapidity of the FLU OIA test make it valuable for preliminary diagnosis of influenza virus infection when other methods are too laborious and time consuming.

Evaluation of the OIA assay for detection of influenza B was not feasible due to the low virus prevalence during the test period. The limited results that were available indicated that the OIA test may have a detection performance for influenza virus B that is similar to that for influenza virus A.

In summary, the developed RT-PCR provides a sensitive and specific method for detection of influenza viruses A and B and discriminates between virus subtypes. RT-PCR may replace culture as the primary reference method, but it is still too resource demanding to be an alternative to the IF assay in routine diagnostics. The OIA test is a convenient and rapid assay for the detection of influenza viruses A and B, but it must be further evaluated before becoming an alternative to other established methods.

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