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Comparison of SmartCycler Real-Time Reverse Transcription-PCR Assay in a Public Health Laboratory with Direct Immunofluorescence and Cell Culture Assays in a Medical Center for Detection of Influenza A Virus

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A single-tube real-time (fluorogenic) reverse transcription (RT)-PCR with the SmartCycler instrument (SmartCycler RT-PCR) for influenza A virus detection was evaluated with 238 respiratory specimens. Direct immunofluorescence antibody staining (DFA) and primary rhesus monkey kidney cell culture were performed on-site at Yale-New Haven Hospital. Specimens were transported to the Connecticut Department of Public Health Laboratory for real-time RT-PCR. Cell culture detected influenza A virus in all 150 influenza A virus-positive specimens, DFA detected the virus in 148 influenza A virus-positive specimens, and SmartCycler RT-PCR detected the virus 143 influenza A virus-positive specimens. The sensitivity and specificity of RT-PCR were 95.3 and 100%, respectively. The high sensitivity and specificity and the rapid turnaround time made the SmartCycler RT-PCR valuable for the rapid diagnosis of influenza A, especially in a public health laboratory. The closed real-time RT-PCR system avoided cross-contamination possible with RT-PCR and the excessive manipulations required for conventional RT-PCR analysis and saved time and labor as well. In a medical center, rapid diagnosis by DFA was labor intensive but was 98.7% sensitive and 100% specific compared to the results of culture and provided results within 2 h throughout operating hours, helping with bed allocation on admission and patient management.

Influenza epidemics in the United States cause approximately 114,000 hospitalizations and 20,000 deaths annually (5). Influenza is often underdiagnosed and affects individuals of all ages but is more severe in very young, aged, and immunocompromised individuals. The disease has a rapid onset and a myriad of symptoms, including fever, headache, malaise, anorexia, cough, chills, myalgia, and sore throat. Other respiratory viruses and bacteria also cause influenza-like illnesses, defined as cough or sore throat and a temperature of ≥100°F (37.8°C). At the peak of an influenza season, approximately one-third of patients with influenza-like illnesses are positive for influenza A virus. Successful treatment of influenza depends on the initiation of antiviral therapy within the first 2 days of illness; thus, rapid diagnosis is of benefit (7). In addition to early antiviral treatment, rapid diagnosis of viral respiratory infections is associated with more judicious antibiotic use, prevention of nosocomial spread, reduced lengths of hospital stay, and reduced costs (2, 25).

Classic diagnostic techniques, such as cell culture and serologic testing, require 2 days to 2 weeks for results and thus are less useful in making therapeutic and infection control decisions. Although rapid shell vial culture is more rapid than standard cell culture, it still requires 2 to 3 days for completion (11, 20). Rapid diagnostic methods such as membrane enzyme immunoassay (EIA) and optical immunoassay can provide results in 30 min or less and are easy to perform. Unfortunately, these assays have suboptimal sensitivities and, in some cases, suboptimal specificities as well (4, 6, 12, 13, 18, 20, 22).

Direct immunofluorescence antibody staining (DFA) of respiratory epithelial cells can achieve a sensitivity comparable to that of cell culture in expert laboratories (2, 14). DFA reagents are also available as a pool of monoclonal antibodies for the detection of influenza A and B viruses, respiratory syncytial virus (RSV), parainfluenza virus (PIV) types 1 to 3, and adenovirus in a single cell spot. At Yale-New Haven Hospital (YNHH), DFA is the mainstay of respiratory virus detection since DFA can be performed continuously 18 h a day during the respiratory virus season, with results obtained in 1 to 2 h, and detects seven viruses in a single cell spot (13). However, DFA requires samples with adequate numbers of target cells, high-quality equipment, and expertise in microscopic slide preparation and reading; is labor-intensive; and is, ultimately, subjective. For all these reasons, the results of DFA are highly variable among laboratories and DFA is less suitable for use in reference laboratories.

Recently, molecular diagnosis of influenza by reverse transcription (RT)-PCR has provided improved sensitivity and a shorter time to results than cell culture (1, 22, 24) and has facilitated the typing and subtyping of influenza viruses (19). Multiplex RT-PCR has allowed the detection of several viruses simultaneously (10, 12, 16). In the previous studies, however, RT-PCR was followed by nested PCR, agarose gel electrophoresis, sequencing, slot blot or microplate hybridization, EIA, or PCR-heteroduplex mobility assay for amplicon identification.

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The introduction of real-time RT-PCR in clinical laboratories can reduce the time to results as well as the number of false-positive results due to potential amplicon carryover. Precautions against cross-contamination and RNase contamination still need to be observed during the RNA extraction step and RT-PCR setup. Schweiger et al. (21) and van Elden et al. (23) described real-time TaqMan RT-PCR assays that were more sensitive than culture for the detection of influenza viruses in respiratory samples. Both groups performed the RT and PCR steps in separate tubes.

Public health laboratories and other reference laboratories that have large test volumes and that analyze samples that have been transported some distance can especially benefit from the use of real-time RT-PCR. At the Connecticut Department of Public Health (DPH) Laboratory, EIA has served as the rapid test, with cell culture used as the "gold standard." However, real-time RT-PCR with the SmartCycler instrument (SmartCycler RT-PCR) has recently replaced cell culture for the detection of West Nile virus at the DPH Laboratory (3). Consequently, a collaborative project to establish a SmartCycler RT-PCR for influenza A virus detection was initiated in which samples collected and tested at YNHH for influenza virus during the 2002 influenza season were retested by real-time RT-PCR at the DPH Laboratory.

We performed the RT-PCR assay for detection of influenza A virus described by Schweiger et al. (21). However, we adapted it for use with the SmartCycler instrument and used one tube for both RT and PCR. The performance of the assay was evaluated by comparing its results with those obtained by DFA and primary rhesus monkey kidney (RhMK) cell culture.

MATERIALS AND METHODS

Patients and specimens. In total, 238 specimens submitted to the Clinical Virology Laboratory at YNHH between December 2001 and February 2002 for testing for respiratory viruses were included in the study. Nasopharyngeal swabs collected in viral transport medium (M4 medium; MicroTest, Inc, Lilburn, Ga.) accounted for 145 of the specimens tested. The other specimens included 87 nasopharyngeal aspirates, 3 bronchial washings, 2 postmortem lung biopsy specimens, and 1 throat swab specimen. Specimens were obtained from 235 patients (122 females and 113 males) ranging in age from 22 days to 92 years (mean and median ages, 29 and 14 years, respectively). One hundred three patients were hospitalized, and 132 were treated as outpatients. Samples were transported from the Emergency Department, clinics, and hospital wards to the laboratory within 1 to 2 h of collection. All specimens were tested by DFA and cell culture at YNHH. Specimens were batched and tested by RT-PCR at the DPH Laboratory as described below.

DFA. The DFA procedure used was that described previously (14). Briefly, samples were centrifuged at 700 × *g* for 5 min to pellet the cells for DFA. The cell pellets were resuspended in a small amount of phosphate-buffered saline, and 200 μl was applied to each slide, by cytocentrifugation (Cytospin 3; Shandon Inc., Pittsburgh, Pa.) at 800 rpm for 4 min. The slides were air dried and then fixed in cold acetone for 10 min. Cell spots were stained with 40 μl of SimulFluor Respiratory Screen reagent (Chemicon International, Temecula, Calif.) for 15 min at 37°C. Following a 30-s wash in phosphate-buffered saline, the slides were mounted in glycerol and examined with a fluorescein filter for fluorescein-labeled cells (influenza A and B viruses, PIV types 1 to 3, and adenovirus) and then reexamined with a rhodamine filter for rhodamine-labeled cells (RSV). For samples exhibiting fluorescein-positive staining, additional slides were stained with dual DFA reagents (SimulFluor Influenza A/B; SimulFluor Parainfluenza 1, 2, 3; and Adenovirus; Chemicon International) to identify the infecting virus.

A positive result was indicated by the presence of two or more intact cells exhibiting specific fluorescence. A single positive cell required reexamination by a supervisor before the result was reported as positive. A negative result was indicated by the absence of fluorescence in a sample with a minimum of 25 respiratory epithelial cells. Samples containing less than 25 ciliated respiratory epithelial cells were considered inadequate for DFA.

Virus isolation. For isolation of influenza virus, primary RhMK cell monolayers (Viromed Laboratories, Minneapolis, Minn.) in roller tubes were rinsed twice with serum-free Eagle's minimum essential medium and then inoculated with 0.2 ml of sample. After adsorption in a stationary rack at 35°C for 1 h, 1.5 ml of serum-free Eagle's minimum essential medium was added. RhMK cultures were incubated at 35°C in a rotating drum for up to 2 weeks and were examined daily for cytopathic effects (CPEs). Hemadsorption was performed with a 0.5% suspension of guinea pig red blood cells as soon as a CPE was noted or, in the absence of a CPE, on days 7 and 14. Influenza virus isolates were identified by staining with monoclonal antibodies. Positive supernatants were saved and stored at −70°C.

RNA extraction. An aliquot of each clinical sample was either stored for 1 to 3 days at 4°C until RNA extraction or stored at −70°C for batch processing. RNA was extracted with the QIAmp Viral RNA Mini kit (Qiagen Inc., Valencia, Calif.) according to the directions of the manufacturer. Briefly, 140 μl of sample was thoroughly mixed with 560 μl of lysis buffer, and the mixture was incubated at room temperature for 10 min. Ethanol was added to the mixture, and the mixture was loading onto a spin column and washed with buffer. The RNA was then eluted in 60 μl of buffer and stored at 4°C for 1 to 3 days or at −70°C if it was to be held for longer periods.

SmartCycler RT-PCR. The primers and probe used for influenza A virus detection were those described by Schweiger et al. (21) and were obtained from Operon (Qiagen Inc.). The primers and probe consisted of primer AM-151 (5′-CATGGAATGGCTAAAGACAAGACC; positions 151 to 174), primer AM-397 (5′-AAGTGCACCAGCAGAATAACTGAG; positions 374 to 397), and probe AM-245 (5′-CTGCAGCGTAGACGCTTTGTCCAAAATG; positions 245 to 272) (the primer and probe positions correspond to those of the matrix gene [AM] of influenza virus A/Bangkok/1/79). The primers were desalted and diluted prior to use. The probe was purified by high-pressure liquid chromatography and diluted prior to use. The primers amplified a 246-bp segment of the matrix gene, which is conserved for influenza A virus and which is substantially different from those of influenza B viruses. The oligonucleotide probe contained the reporter dye 6-carboxyfluorescein on its 5′ end and the quencher dye 6-carboxytetramethylrhodamine on its 3′ end. The 25-μl reaction mixture used in the single-tube test consisted of 2.5 μl of RNA eluate and 22.5 μl of the master mixture. The latter was prepared by using the Perkin-Elmer master mixture (Applied Biosystems, Foster City, Calif.) supplemented with 1 μM each primer, 0.15 μM probe, and 2.5 U of AmpliTaq enzyme. The assay was performed with the Cepheid (Sunnyvale, Calif.) SmartCycler instrument in a protocol comprising RT and PCR: 30 min at 50°C for RT and then 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 62°C for 1 min.

Data analysis was performed with Cepheid software. Both the threshold cycle (C_T) and maximum fluorescence (Fl) were used for interpretation of the results, as follows: positive result, $C_T \leq 38$ and Fl ≥ 50; negative result, $C_T > 38$ and Fl < 50; and indeterminate result, either $C_T \leq 38$ and Fl < 50 or $C_T > 38$ and Fl ≥ 50. Extraction controls and RT-PCR controls were included in each run.

Analytical sensitivity and specificity. To test for the analytical sensitivity of the SmartCycler RT-PCR, RNA was also extracted from serial 10-fold dilutions of clinical H1N1 and H3N2 influenza A virus cell culture isolates from the 2001 influenza season. The specificity of the PCR assay was tested in the presence of different respiratory viruses and bacteria. Clinical isolates of influenza A virus (H1N1, H3N2), influenza B virus, RSV, PIV, adenovirus, rhinovirus, herpes simplex viruses types 1 and 2, cytomegalovirus (CMV), enterovirus, echovirus, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Haemophilus influenzae* as well as the *Bacillus anthracis* vaccine strain were tested. The non-influenza A virus isolates were also tested in the presence of an H3N2 influenza A virus isolate to investigate interference with the assay.

Resolution of discordant results. Samples with results not in agreement by all three methods were retested. RNA extracts of PCR-indeterminate and PCR-negative but otherwise positive samples were thawed and reamplified. In a few cases, when original sample remained, a reextraction was performed. An inhibitor effect was also evaluated for repeatedly PCR-negative but otherwise positive samples by spiking the PCR mixture with 10 to 100 50% tissue culture infective doses ($TCID_{50}$s) of influenza A virus-positive RNA extract. Virus isolates from samples positive by cell culture but with false-negative (FN) results by RT-PCR were also tested by RT-PCR to rule out genetic variability as a cause of the FN results by RT-PCR. All slides of samples with discordant results by DFA were restained and reexamined.

TABLE 1. Initial results of detection of influenza A virus by the three diagnostic techniques

Assay	No. of samples with the following results:			
	Positive	Negative	Inadequate cells[a]	Indeterminate[b]
Culture	150	88	NA[c]	NA
DFA	148	78	12	NA
RT-PCR	132	97	NA	9

[a] Inadequate cells for DFA was defined as less than 25 respiratory epithelial cells.
[b] An indeterminate result by RT-PCR was defined as either a C_T of ≤38 and an FI of <50 or a C_T of >38 and an FI of ≥50.
[c] NA, not applicable.

TABLE 2. Resolution of discordant results by RT-PCR for influenza A virus

Initial discordant RT-PCR result (no. of samples)	No. of samples with the following final RT-PCR results:			
	True positive	Indeterminate	FN	True negative
FN (13)	7	1	5	NA[a]
Indeterminate (9)	4	0	1	4
Total (22)	11	1	6	4

[a] NA, not applicable.

RESULTS

Analytical sensitivity and specificity of SmartCycler RT-PCR protocol. We initially studied the analytical sensitivities as well as the specificities of the primers and probe for influenza A virus detection by RT-PCR. Both H1N1 and H3N2 influenza A viruses were amplified successfully. The analytical sensitivity of the RT-PCR assay, as determined by titration of influenza virus stocks on primary RhMK cells as described in Materials and Methods, was determined to be <0.5 $TCID_{50}$. None of the respiratory viruses or bacteria (influenza B virus, RSV, PIV, adenovirus, rhinovirus, herpes simplex virus types 1 and 2, CMV, enterovirus, echovirus, *S. aureus*, *S. pneumoniae*, *S. pyogenes*, *H. influenzae*, and *B. anthracis* vaccine strain) tested was PCR positive or interfered with the assay. The results for the RNA extracts of six influenza A virus isolates harvested from cell cultures were reproducible on three PCR runs. In no PCR run was the RNA extraction control or the negative PCR control positive.

Detection of influenza A virus in respiratory samples. The initial results of cell culture, DFA, and RT-PCR for detection of influenza A virus are shown in Table 1. One hundred fifty samples were positive by culture, 148 were positive by DFA, and 132 were positive by RT-PCR. Twelve specimens (5%) were deemed to have inadequate cells for DFA, and the results for 9 samples (3.8%) were indeterminate by RT-PCR. The results of the three tests were in agreement for 216 specimens.

In total, 22 samples had discordant results. One culture-positive sample had inadequate numbers of cells for DFA and one was DFA negative. Eighteen culture-positive samples were either RT-PCR indeterminate (5 samples) or RT-PCR negative (13 samples). Four samples negative by both DFA and culture were indeterminate by RT-PCR.

Detection of other viruses by DFA and culture. Seven samples with dual RSV and influenza A virus infections were detected by DFA among the influenza A virus-positive samples. In addition, DFA identified one adenovirus, one influenza B virus, and seven RSV infections among the influenza A virus-negative samples. Cell culture identified one case each of influenza B virus, CMV, adenovirus, and PIV type 4 infection among the influenza A-negative specimens.

Resolution of discordant results. Twenty-two samples showed discordant results for influenza A virus. The results obtained after retesting of these samples are shown in Table 2.

Four samples with indeterminate results by RT-PCR that were negative by both culture and DFA were RT-PCR negative on repeat amplification. Eleven samples, including 7 RT-PCR-negative and 4 RT-PCR-indeterminate samples, were RT-PCR positive after reextraction (1 sample) or repeat amplification (10 samples). One RT-PCR-negative sample became indeterminate on retesting, and six remained RT-PCR negative. Spiking of these seven RNA extracts with 10 to 100 $TCID_{50}$s of influenza A virus showed clear evidence of inhibition for two of the samples. Unfortunately, no original sample remained for reextraction of these seven samples with FN results for RT-PCR.

Evaluation of these seven samples by DFA revealed that all either were poor samples (one had inadequate numbers of cells for DFA) or had only one to a few influenza A virus-positive cells. Four samples consisted of swab samples obtained from adults 31, 42, 76, and 80 years of age, respectively.

Restaining of the two slides with FN results by DFA confirmed the original DFA-negative results. One slide had an inadequate number of cells for DFA; however, the other slide had an adequate number of cells for DFA. The latter sample with FN results by DFA was culture positive and on retesting was RT-PCR positive as well. Thus, the final results (Table 3) showed that 150 samples had true-positive results, of which cell culture detected influenza A virus in 150 samples (100%), DFA detected the virus in 148 samples (98.7%), and RT-PCR detected the virus in 143 samples (95.3%).

DISCUSSION

A number of recent studies have compared the nested or TaqMan RT-PCR with culture for the diagnosis of influenza (1, 4, 9, 17, 19, 22). Several studies have also evaluated a

TABLE 3. Final results for detection of influenza A virus by RT-PCR, culture, and DFA

RT-PCR result (no. of samples)	No. of samples with the indicated result by:				
	Culture		DFA		
	Positive	Negative	Positive	Negative	Inadequate cells[a]
Positive (143)	143	0	142	1	0
Negative (94)	6	88	5	77	12
Indeterminate[b] (1)	1	0	1	0	0
Total (238)	150	88	148	78	12

[a] Inadequate cells for DFA was defined as less than 25 respiratory epithelial cells.
[b] An indeterminate RT-PCR result was either a C_T of ≤38 and an FI of <50 or a C_T of >38 and an FI of ≥50.

commercial RT-PCR (Hexaplex) that detects PIV types 1, 2, and 3 and RSV as well as influenza A and B viruses (10, 12, 16). Previous reports of real-time RT-PCR for the diagnosis of influenza have described the use of two separate reaction tubes for the RT and PCR steps (21, 23). In our study, real-time RT-PCR was performed in one tube with the SmartCycler instrument.

The analytical sensitivity of our RT-PCR assay was determined to be <0.5 $TCID_{50}$, and, with no false-positive results, the specificity of the RT-PCR was 100%. The analytical sensitivities reported in the various studies, which in some cases were as low as <0.01 $TCID_{50}$, are difficult to compare (1, 4, 9, 10, 12, 16, 17, 19, 21–23). The calculation formulas are not stated, there is no universal standard, and the comparator culture conditions (or the RNA transcripts for the Hexaplex assay) have varied tremendously. The same virus stock titrated under various conditions can yield 10- to 100-fold differences in $TCID_{50}$s, thus making the RT-PCR appear to be more or less sensitive. Likewise, the application of culture techniques to clinical specimens can result in substantial differences in recovery rates. Culture with primary RhMK cells, rinsed to remove serum, has been reported to be the most sensitive culture system for the detection of influenza virus (8), but primary RhMK cells are often not used due to expense or concern about contamination with monkey viruses.

In the analysis of clinical samples, the sensitivity of RT-PCR has either approached or exceeded the sensitivity of culture. Cell culture was disadvantaged, however, since samples were transported a distance and inoculation into cell culture was delayed. Furthermore, despite calculated analytical sensitivities that exceeded culture by 1 to 3 $\log_{10}$, RT-PCR has failed to detect virus in some culture-positive samples (1, 10, 17, 19, 22). In our study, culture detected virus in all virus-positive samples and no samples were positive by RT-PCR only. In contrast to other published reports, all cultures in the present study were done on-site at YNHH, where the samples were collected, and optimal cell cultures and procedures were used. The primary RhMK cells used for culture were rinsed to remove serum prior to inoculation, incubated in serum-free medium on roller drums, observed for 2 weeks, and screened by hemadsorption before termination. On-site inoculation of cell cultures likely enhanced the recovery of infectious virus. Conversely, the results obtained when samples are transported to a distant site would be expected to favor molecular methods, since a greater decline in the amount of infectious virus than in the amount of viral nucleic acid would be anticipated.

In this study of 238 clinical specimens, the SmartCycler RT-PCR detected influenza A virus in 132 of 150 (88%) influenza A virus-positive samples on initial testing. After retesting of samples with discordant results, 143 of 150 (95.3%) samples were found to be positive. The final sensitivity of 95.3% is comparable to those reported in other studies (1, 22). Nevertheless, our data raise concern about the frequency of inhibition and loss of influenza virus RNA either during the extraction process or due to the presence of RNases in the samples. The results for 18 culture-positive samples were either FN (13 samples) or indeterminate (5 samples) by RT-PCR on initial testing. While storage of samples at 4°C prior to extraction could have contributed to RNA degradation, for 12 of these 18 samples, including 4 of 6 samples negative after repeat amplification, extraction was done within 24 h of collection. On repeat amplification of the frozen RNA extracts, 10 samples with negative results were RT-PCR positive on retesting and 1 sample that was negative was RT-PCR indeterminate on retesting. One additional sample was RT-PCR positive after reextraction of RNA from the original sample. Fourteen of the samples were swab samples, and 11 samples were from teenagers or adults, who are known to shed virus at lower titers than young children. Fifteen of these 18 samples were minimally positive by DFA, and 1 was deemed inadequate for DFA. Thus, most of these samples had low-positive results, and virus could have been masked by inhibitors.

In published studies on RT-PCR for influenza A virus detection, controls for inhibitors are strikingly absent. One could argue that for public health surveillance, especially if the RT-PCR results are superior to culture results, testing for inhibitors would add unnecessary expense with negligible benefit. However, for the diagnosis of an individual case, such as in the hospital setting, ensuring that adequate amplification has occurred should be standard, as it is for other assays. In our laboratory, inhibitors have been a significant problem in nasopharyngeal and throat swab specimens tested for enterovirus by nucleic acid sequence-based amplification (15). Freezing and thawing of the RNA extracts often reduce the levels of inhibition of amplification (15), as was seen in the present study.

Of note, in our RT-PCR assay we used 2.5 μl of RNA extract in the reaction mixture instead of the 5 to 10 μl routinely used in other studies (17, 19, 22). The SmartCycler protocol for the detection of West Nile virus in use at the DPH Laboratory for the testing of bird brains uses 2.5 μl of extract (3). Initial studies in the DPH Laboratory with influenza A virus indicated that 2.5 μl yielded the best results (data not shown). However, respiratory samples may contain low titers of influenza virus, and the lower sample input could have contributed to the FN RT-PCR results.

For in-house testing at YNHH, the DFA for the screening of respiratory viruses detected influenza A virus in 98.7% of culture-positive specimens and had no false-positive results. In addition, DFA results were routinely available within 1.5 to 2 h of the time of arrival of the sample in the laboratory, and the screening detected additional non-influenza viruses. In our laboratory, DFA is done 7 days a week throughout operating hours in the influenza season and is used to assign beds on admission and implement infection control practices, as well as to initiate antiviral therapy when needed. Of note, studies documenting the cost-effectiveness of rapid testing for respiratory infections for inpatient management have used DFA and not RT-PCR (2, 25). If testing is done once a day with batching of samples, it would lose much of its usefulness in the hospital setting, and it would not be practical to do RT-PCR more than once a day in most hospital laboratories.

Although EIA is the most commonly used rapid test methodology for the detection of influenza viruses, it was not included in this study. Due to in-house sensitivities of only 50 to 75%, EIA for the detection of influenza viruses was replaced in 1997 by DFA at YNHH. However, following a death from anthrax in Connecticut in 2001, a rapid EIA for the detection of influenza viruses was introduced into the Chemistry Laboratory on the night shift to provide 24-h coverage when the Clinical Virology Laboratory was closed. When samples were

retested in the Clinical Virology Laboratory in the morning by DFA, it was determined that only 16 of 31 (52%) specimens positive for influenza A virus by DFA were positive by EIA. In addition, DFA detected RSV in three specimens, adenovirus in two specimens, and parainfluenza virus in one specimen (14a). Furthermore, evaluation of a new rapid test for the detection of influenza A and B viruses in the winter of 2002-2003 showed that it had an equivalently poor sensitivity, as well as several false-positive results for influenza B virus (D. Ferguson, S. Cohen, H. Boyde, R. Garner, M. Owen, C. Blake, L. Voglesong, and M. L. Landry, Abstr. 19th Annu. Clin. Virol. Symp., abstr. S37, 2003).

Nevertheless, the decision to perform DFA cannot be taken lightly. For sensitive and accurate DFA results, significant effort is required to train personnel and monitor quality. Furthermore, cytocentrifugation is used in our laboratory to improve slide quality and cell morphology and enhance the results (13, 14), and all slides are saved at 4°C for 1 month to allow correlation of the results with those of culture and to reexamine discrepancies. Without this commitment and attention to detail, DFA results will be suboptimal in terms of both sensitivity and specificity.

In a reference laboratory, there is much less control over sample quality, delays in specimen transport adversely affect both culture and DFA methods (20), and automated techniques are preferred for the handling of large sample numbers. In this setting, real-time RT-PCR has tremendous advantages.

For the DPH Laboratory, an EIA is performed for rapid diagnosis, followed by virus isolation in cell culture. In addition to examination of cultures for CPE for 2 weeks, repeated hemadsorptions are performed, which are very labor-intensive. With real-time RT-PCR, large numbers of samples can be rapidly tested and sensitive results can be provided within a day of sample receipt. The SmartCycler protocol offers a single, closed-tube format, which prevents contamination of the work space with amplified product and which decreases the time to results by simultaneous amplification and detection. Consequently, the DPH Laboratory is setting up a multiplex RT-PCR on the SmartCycler instrument for the detection of both influenza A and B viruses, with the intention of replacing both EIA and culture during influenza season.

In conclusion, in this first attempt to implement a real-time RT-PCR for influenza A detection with the SmartCycler instrument for the DPH Laboratory, a sensitivity of 95.3% was achieved. In the future, reevaluation of the optimal sample RNA input, detection of inhibitors, stabilization of the RNA in samples, and implementation of a multiplex PCR for the detection of influenza B virus will be priorities.

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