Evaluation of the One-Step Multiplex Real-Time Reverse Transcription-PCR ProFlu-1 Assay for Detection of Influenza A and Influenza B Viruses and Respiratory Syncytial Viruses in Children

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We evaluated the one-step multiplex real-time reverse transcription-PCR ProFlu-1 assay for the detection of influenza A and influenza B viruses and respiratory syncytial viruses from 353 pediatric nasopharyngeal aspirates. As assessed by comparison with the results of immunofluorescence testing and cell culture, the specificity and the sensitivity of the ProFlu-1 assay ranged from 97% to 100%. In addition, the ProFlu-1 assay amplified 9% of samples not detected by conventional methods.

Respiratory syncytial viruses (RSVs), influenza virus types A (FluA) and B (FluB), and parainfluenza viruses (PIVs) are the leading causes of viral lower respiratory tract infections in children (9). In clinical practice, rapid immunochromatographic antigen tests and immunofluorescent tests (IFs) are often the first diagnostic tests used. Due to the low sensitivities of some of these tests, viral culture should be performed with negative specimens (4, 13, 14); but the results, which are often available only after the child has been discharged, may have little impact on patient care. Despite the better sensitivities of molecular assays, several separate assays are required because of the number of viral targets, resulting in increased costs. Multiplex reverse transcription-PCR (RT-PCR) assays with enzyme hybridization probes have been available for the detection of RSV types A and B; FluA and FluB; and PIV 1, 2, and 3 (3, 5, 8, 11, 12). Despite their overall excellent sensitivities and specificities, these assays have long turnaround times, requiring multiple steps and PCR product manipulation. In order to overcome these limitations, a one-step multiplex real-time RT-PCR assay (the ProFlu-1 real-time assay; Prodesse, Waukesha, WI) was developed for the rapid detection of RSV, FluA, and FluB nucleic acids in a single test. We evaluated the ProFlu-1 assay with samples from a cohort of children with acute respiratory disease during the 2005–2006 winter season.

Nasopharyngeal aspirates (NAs) were collected from children <15 years old admitted to Saint Vincent de Paul Hospital with acute respiratory disease between October 2005 and April 2006. Specimens were tested by IF with a pool of monoclonal fluorescein isothiocyanate (FITC)-labeled antibodies directed against adenoviruses; FluA and FluB; and PIV 1, 2, and 3 (Argene, Varilhes, France) and an FITC-labeled monoclonal antibody directed against RSV (Dako, Trappes, France). When IF with the pooled antibodies was positive, identification was carried out by using specific individual monoclonal antibodies (Argene). All specimens except those found to be positive for RSV by IF during the epidemic period were inoculated into the HuH7 and A549 cell lines, as described previously (6). The specimens were then stored at 2 to 8°C for up to 24 h and then frozen at −80°C. Samples to be tested by the ProFlu-1 assay were selected as follows: among the samples with enough volume for testing by the ProFlu-1 assay, we tested all samples positive for FluA (n = 21); FluB (n = 11); or a virus other than FluA, FluB, or RSV (n = 26) and a random selection of 187 (of 260) RSV-positive samples and 108 RSV-negative samples. Two hundred-microliter aliquots of nasal aspirates were spiked with the ProFlu-1 assay internal control (IC) and were incubated for 1 h at 56°C with 20 μl of proteinase K (Qiagen, Courtaboeuf, France). This was followed by nucleic acid extraction and elution in a 55-μl volume by using the EasyMag system (Biomérieux, Marcy l’Etoile, France). Five microliters of the nucleic acid extract was then mixed with murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA); Platinum Taq polymerase (Invitrogen, Carlsbad, CA); oligonucleotide primers complementary to highly conserved regions of the nonstructural genes for FluA and FluB and the polymerase gene for RSV; and dually labeled oligonucleotide probes for RSV (6-carboxyfluorescein [FAM], BHQ1), FluA (Cal Orange, BHQ1), FluB (Texas Red, BHQ2), and IC (Q670, BHQ2). Amplification was performed on an ABI 7500 (Applied Biosystems) real-time thermocycler according to the following protocol: 30 min at 42°C, 5 min at 95°C, and 40 cycles of 5 s at 95°C and 60 s at 55°C. The fluorescent signals transmitted by Cal Orange and Q670 were read on the JOE and Cy5 channels, respectively. Real-time fluorescence measurements were taken, and a
threshold cycle \( (C_T) \) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit. For a valid run, RNA controls should be detected above the threshold before cycle 33 for FluA, FluB, and RSV and before cycle 37.5 for IC (Fig. 1). The detection of the IC in the Cy5 detection channel is not required for a positive result (Fig. 1). A high viral load can lead to a reduced or absent IC signal. All samples with discrepant results (between IF/cell culture [CC] and the ProFlu-1 assay) were retested by an in-house one-step real-time RT-PCR assay which detects a different region of the genome, as described previously (7, 10, 15).

The results of the ProFlu-1 assay according to the conventional diagnosis are depicted in Table 1. The ProFlu-1 assay detected the virus in 188 RSV-positive samples, including 5 samples found to be negative by IF and CC, which corresponded to 4.6% of the negative samples. In contrast, the viruses in three RSV-positive samples detected by IF were not detected by the ProFlu-1 assay. The result for one RSV culture-positive specimen was unresolved by the ProFlu-1 assay since the IC failed to be amplified. The ProFlu-1 assay detected the viruses in all samples positive for FluA and FluB by IF or CC and two additional samples positive for FluA and three additional samples positive for FluB, which corresponded to 4.6% of negative samples. One sample that was positive for FluB by the ProFlu-1 assay but negative by IF was also infected with an adenovirus, as detected by culture. Except for this dually infected sample, the NA samples positive for other viral pathogens were negative by the ProFlu-1 assay. The in-house real-time RT-PCR carried out with discrepant spec-
imens (11 ProFlu-1 assay-positive/IF-CC-negative samples, 3 ProFlu-1 assay-negative/IF-positive samples) confirmed the ProFlu-1 assay results for all samples and thus did not detect the three samples positive for RSV by IF only, suggesting a possible misinterpretation of the IF results. On the basis of only the results obtained by conventional diagnostic methods (a FluA-, FluB-, or RSV-positive result by IF and/or culture), the specificity and the sensitivity of the ProFlu-1 assay were 99.4% (95% confidence interval [CI], 98.6% to 100%) and 100%, respectively, for FluA; 98.8% (95% CI, 97.6% to 100%) and 100%, respectively, for FluB; and 97.0% (95% CI, 94.3% to 99.5%) and 97.8% (95% CI, 95.7% to 99.9%), respectively, for RSV. After the analysis of the discrepant results, the specificity of the ProFlu-1 assay was 100% for the three viral targets. The ProFlu-1 assay simultaneously amplifies and detects three viral targets and an IC in a single closed-tube reaction for a cost of $46.5, including the costs of enzymes, which are not provided in the assay kit. The ProFlu-1 assay has a turnaround time of 2.5 h once the nucleic acids are purified, and viral nucleic extraction requires 1 h for 24 specimens with the EasyMag system. Due to the rapid test results and the reduced hands-on time, the implementation of the ProFlu-1 assay may positively influence patient care by reducing hospital stays, curtailing or preventing antibiotic therapy, preventing nosocomial spread, and offering specific antiviral therapy (1, 2, 16). However, this procedure requires a real-time PCR machine and an automated extractor and would mainly be used by laboratories receiving large series of samples on a daily basis. In the present analysis of a series of pediatric patients with acute respiratory disease, the overall performance of the ProFlu-1 assay compared with that of IF and viral culture methods was excellent. IF testing allowed the detection of most viruses in respiratory specimens from children. J. Clin. Virol. 78:228–239.

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REFERENCES


