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Note

Evaluation of a multiplex real-time polymerase chain reaction assay for the detection of influenza and respiratory syncytial viruses

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ABSTRACT

Nasopharyngeal swabs from 424 children were used to compare the performances of the new multiplex real-time polymerase chain reaction (RT-PCR) RIDA®GENE Flu & RSV kit and monospecific RT-PCR assays in detecting respiratory syncytial and influenza viruses. The easy-to-use kit was highly sensitive and specific and is recommended for routine practice.

Respiratory syncytial virus (RSV) and influenza viruses are among the main causes of upper and lower respiratory tract infections in children and adults, particularly during the winter (Kidd, 2014; Meng et al., 2014). The peak infective periods of the viruses may differ but, as they circulate simultaneously for several weeks during epidemic months, it is epidemiologically, preventively, and therapeutically essential to differentiate the 2 infections (Brooks et al., 2004; Mills et al., 2011), which, unfortunately, have quite similar signs and symptoms (Esposito et al., 2013; Falsey et al., 2014). Traditional methods of viral identification may take days to provide reliable results (Zumla et al., 2014) or are poorly sensitive even when they are sufficiently specific (Principi and Esposito, 2009), and so other molecular biology-based methods have been developed. Real-time polymerase chain reaction (RT-PCR) assays are rapid, sensitive, and specific in identifying respiratory viruses, including influenza viruses and RSV (Caliendo, 2011), but monospecific RT-PCR assays are unsatisfactory because the need to use multiple assays is potentially expensive, resource intensive, and significantly time consuming, whereas multiplex RT-PCR assays are cheaper, provide more rapid results, and improve clinical management (Beck et al., 2010; Caliendo, 2011; Zumla et al., 2014). The main aim of this study was to evaluate the sensitivity, specificity, and positive and negative predictive values of a new multiplex RT-PCR assay for the direct, qualitative detection and differentiation of influenza viruses and RSV in human nasopharyngeal swabs.

The study involved all of the children aged 6–60 months admitted to the Pediatric Highly Intensive Care Unit of the University of Milan's Department of Pathophysiology and Transplantation between March 15, 2015, and May 31, 2015, because of signs and symptoms of upper or lower respiratory infection. The study protocol was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, and written informed consent was obtained from the parents of the enrolled children. A nasopharyngeal sample was collected from each child using pediatric mid-turbinated nasal flocked swabs (Copan, Brescia, Italy; code 56750CS01 for children aged ≤2 years; code 56380CS01 for older children) and processed as soon as it was received by the laboratory. Viral RNA was extracted using an automated Nuclisens EasyMAG extraction system (bioMérieux, Craponne, France) as previously described (Esposito et al., 2010): some of the extract was used to detect influenza A and B viruses and RSV simultaneously but separately with traditional methods as previously described (Esposito et al., 2010, 2015), and some was used to detect the same viruses with the RIDA®GENE Flu & RSV kit (R-Biopharm AG, Darmstadt, Germany), which allows the simultaneous detection of influenza A/H1N1, A/H3N2, B viruses, and RSV in about 2 hours using a 1-step RT-PCR format in which reverse transcription is followed by RT-PCR in the same reaction tube. The traditional PCR assays were in-house but largely validated in several studies published by our groups (Bosis et al., 2008; Esposito et al., 2010, 2015) and other authors (Gerna et al., 2008: Rahamat-Langendoen et al., 2012). They are considered the gold standard for the diagnosis of influenza and RSV infection. RIDA®GENE Flu & RSV kit has been approved by European Community (CE mark), but there aren't studies already published on the validation of this kit. This commercial kit contains an internal control RNA (ICR) to check sample preparation and possible PCR inhibition and a positive control based on a mix of 3 different synthetic

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RNA fragment (influenza A, influenza B, and RSV) in a concentration of 10^3 copies/μL of each. The amplified targets (influenza A/B: M-gene and NP1-gene; RSV: F-gene) were detected by means of hydrolysis probes labeled with a quencher at one end and a fluorescent reporter dye (fluorophore) at the other, and the emitted fluorescent signals were analyzed by the optical unit of a Stratagene Mx3005P equipped with FAM, HEX, ROX, and Cy5 channels that, respectively, detect RSV, ICR, influenza B, and influenza A. The sensitivity of the method calculated by the producer using synthetic RNA fragment for all of the viruses is ≥50 RNA copies per reaction or 10 copies/μL. Positive and negative controls (PCR water) were always included in each assay run in order to determine a valid run. A sample was evaluated positive if both the sample and the controls (ICR and positive control) show an amplification signal in the detection system. In both multiplex and monospecific RT-PCR assays, we determined a threshold cycle of 40 using serially diluted 10-fold positive control because, as with all PCR-based in vitro diagnostic tests, extremely low levels of target below the limit of detection may be detected, but results may not be reproducible.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Cohen's kappa coefficients with 95% confidence intervals (95% CI) of the kit were calculated. A kappa coefficient higher than 0.80 was considered adequate. The analyses were performed using SAS version 9.2 (Cary, NC, USA).

Traditional RT-PCR methods showed that 124 (29.2) of the 424 enrolled children (220 males, 52.8%; mean age 3.3 ± 3.2 years) were positive for 1 influenza virus, 150 (35.4%) were positive for RSV, and 150 (35.4%) were negative. Table 1 compares these findings with the results obtained using the RIDA®GENE Flu & RSV kit. The kappa coefficient of the kit was 0.96 for influenza viruses (95% CI 0.93–0.995), 0.91 for RSV (95% CI 0.86–0.95), and 0.91 for both (95% CI 0.86–0.95).

Multiplex molecular methods can rapidly differentiate influenza and RSV infections, which not only allows the prompt initiation of effective prophylactic and therapeutic measures but can also improve physician–patient communications (Principi and Esposito, 2009). This study compared the new commercial RIDA®GENE Flu & RSV kit (specifically designed for the simultaneous identification of influenza A and B viruses and RSV) with traditional RT-PCR assays for their separate detection in children with respiratory symptoms admitted to a pediatric highly intensive care unit. In comparison with traditional monospecific RT-PCR, the kit had a specificity of 100% for all the studied viruses, a sensitivity that was never lower than 90%, and a very high kappa index, thus making it apparently equivalent to the Prodess ProFlu+™ and Simplexa™ Flu A/B & RSV commercial kits, which have been found to be similarly efficient in detecting influenza viruses and RSV with a marginal risk of false-negative results (Selvaraju et al., 2014a; Svensson et al., 2014). However, the RIDA®GENE Flu & RSV kit offers greater flexibility since it has been validated on different thermocyclers and with different extraction systems, adapting well to the instruments usually present in research laboratories. On the contrary, Prodess ProFlu+™ and Simplexa™ Flu A/B & RSV require dedicated laboratory instruments. Moreover, a possible limitation of such assays is that they require nucleic acid extraction, which increases both the complexity and cost of the evaluation. However, a study of a recently developed RT-PCR assay for the detection of influenza viruses and RSV without RNA extraction found that it was less sensitive and specific than all the traditional methods used for comparison (Selvaraju et al., 2014b). Furthermore, although more accurate than their predecessors, the most recently developed point-of-care rapid antigen detection tests for RSV and influenza are still significantly less sensitive than desired and cannot be considered suitable alternatives (Bruning et al., 2014).

In conclusion, the RIDA®GENE Flu & RSV assay, which is designed to run on standard molecular diagnostic equipment, has a simple workflow that, together with its positive and negative predictive values, makes it easy to validate and use in routine practice. Although it should be evaluated also in patients with mild symptoms, in those hospitalized for respiratory tract infection, it provides reliable results more rapidly than repeated conventional monospecific RT-PCR (in about 2 hours) and is more specific and sensitive than the simpler or faster tests currently available.

Table 1
Comparison of RIDA®GENE Flu & RSV kit and RT-PCR results.

	RIDA®GENE Flu & RSV kit results		
RT-PCR results	Negative	Positive	Total
Influenza A/H1N1			
Negative (%)	150 (75.8)	0 (0.0)	150 (75.8)
Positive (%)	2 (1.0)	46 (23.2)	48 (24.2)
Total (%)	152 (76.8)	46 (23.2)	198 (100.0)
Sensitivity	95.8%		
Specificity	100%		
PPV	100%		
NPV	98.7%		
Influenza A/H3N2			
Negative (%)	150 (73.2)	0 (0.0)	150 (73.2)
Positive (%)	2 (1.0)	53 (25.8)	55 (26.8)
Total (%)	152 (74.2)	53 (25.8)	205 (100.0)
Sensitivity	96.4%		
Specificity	100%		
PPV	100%		
NPV	98.7%		
Influenza B			
Negative (%)	150 (87.7)	0 (0.0)	150 (87.7)
Positive (%)	1 (0.6)	20 (11.7)	21 (12.3)
Total (%)	151 (88.3)	20 (11.7)	171 (100.0)
Sensitivity	95.2%		
Specificity	100%		
PPV	100%		
NPV	99.3%		
Combined influenza A and B			
Negative (%)	150 (54.7)	0 (0.0)	150 (54.7)
Positive (%)	5 (1.8)	119 (43.4)	124 (45.3)
Total (%)	155 (56.6)	119 (43.4)	274 (100.0)
Sensitivity	96.0%		
Specificity	100%		
PPV	100%		
NPV	96.8%		
RSV			
Negative (%)	150 (50.0)	0 (0.0)	150 (50.0)
Positive (%)	14 (4.7)	136 (45.3)	150 (50.0)
Total (%)	164 (54.7)	136 (45.3)	300 (100.0)
Sensitivity	90.7%		
Specificity	100%		
PPV	100%		
NPV	91.5%		

Conflict of interest

None of the authors has any conflict of interest to declare. The manufacturer of RIDA®GENE Flu & RSV kit did not participate in the study.

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