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Monitoring Epidemic Viral Respiratory Infections Using One-Step Real-Time Triplex RT-PCR Targeting Influenza A and B Viruses and Respiratory Syncytial Virus

Solesne Papillard-Marechal,[1] Vincent Enouf,[2,3,4,5] Aurélie Schnuriger,[6,7] Astrid Vabret,[8] Edouard Macheras,[9,10] Marie-Anne Rameix-Welti,[9,10] Bernard Page,[11] François Freymuth,[8] Sylvie van der Werf,[2,3,4,5] Antoine Garbarg-Chenon,[6,7] Bertrand Chevallier,[1] Jean-Louis Gaillard,[9,10] and Elyanne Gault[9,10]*

[1]*AP-HP, Ambroise Paré Hospital, Department of Pediatrics, Boulogne-Billancourt, France*
[2]*Institut Pasteur, Molecular Genetics of RNA Viruses, Paris, France*
[3]*CNRS URA3015, Paris, France*
[4]*Paris Diderot Paris 7 University, Paris, France*
[5]*National Centre for Influenza Viruses (Northern France), Paris, France*
[6]*AP-HP, Armand Trousseau Hospital, Department of Virology, Paris, France*
[7]*Pierre et Marie Curie University, ER_7, Paris, France*
[8]*Department of Human and Molecular Virology, National Centre for Measles and Other Respiratory Paramyxoviridae, Georges Clémenceau Universitary Hospital, Caen, France*
[9]*AP-HP, Ambroise Paré Hospital, Department of Microbiology, Boulogne-Billancourt, France*
[10]*Versailles St-Quentin-en-Yvelines University, UPRES EA3647, Guyancourt, France*
[11]*AP-HP, Ambroise Paré Hospital, Intensive Care Unit, Boulogne-Billancourt, France*

Rapid and specific diagnosis of influenza A/B and respiratory syncytial virus (RSV) viruses is needed for optimal management of patients with acute respiratory infections. In this study, a one-step triplex real-time RT-PCR assay was developed for rapid diagnosis of influenza A/B and RSV infections to optimize diagnosis efficiency of acute respiratory infections. Cell-culture supernatants and clinical samples were used to evaluate specificity and sensitivity of the assay. The assay was used routinely during two winter epidemics for testing respiratory specimens from 2,417 patients. The limit of detection in cell-culture supernatant was 1–10 plaque forming units/input (influenza A/B) and 2×10^{-2} 50% tissue culture infectious dose/input (RSV). In clinical samples, the assay was as sensitive as commercial molecular assays for the detection of each influenza A/B and RSV (Flu-A/B and RSV-A/B r-gene™) individually, and far more sensitive than antigen detection. During the winter 2008–2009, the assay identified 145 RSV, 42 influenza A, and one mixed RSV-influenza A infections among 298 patients. The next winter, the assay was used in two independent hospital laboratory settings. 776 patients were tested in one hospital and 1,343 in the other, resulting in 184 and 501 RSV, 133 and 150 influenza A, and 1 and 11 mixed RSV-influenza A infections, respectively, being detected. This new user-friendly assay allows rapid (within hours), effective molecular diagnosis of single or mixed infections involving influenza A (including seasonal A H1N1 and H3N2, and A(H1N1) 2009), influenza B, and RSV(A/B). The assay is very valuable for managing patients during winter epidemics when influenza and respiratory syncytial viruses co-circulate. ***J. Med. Virol. 83:695–701, 2011.***

KEY WORDS: orthomyxovirus; paramyxovirus; molecular diagnosis

INTRODUCTION

The seasonal epidemic circulation of influenza A, influenza B, and human respiratory syncytial virus

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Vincent Enouf and Aurélie Schnuriger contributed equally to this work.

*Correspondence to: Elyanne Gault, Laboratoire de Microbiologie, Hôpital A. Paré, 9, Ave Charles de Gaulle, 92100 Boulogne-Billancourt, France. E-mail: elyanne.gault@apr.aphp.fr

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(RSV) viruses causes a large part of the burden of viral respiratory infections in the northern hemisphere, especially that involving infants under 96 weeks of age and elderly patients [Simoes, 1999; Neuzil et al., 2000; Simonsen et al., 2000; Poehling et al., 2006; Nair et al., 2010]. Rapid identification of the viral cause of acute respiratory infection has consequences for patient monitoring and care, including hospital admission or discharge, administration of antibiotics and antiviral therapy, and reduction of diagnosis investigation [Adcock et al., 1997; Barenfanger et al., 2000]. Antigen detection by immunofluorescence (IF) or immunochromatography is the usual first-line diagnostic approach, although it lacks sensitivity [Steininger et al., 2002; Louie et al., 2010]. An alternative is to test for viral genomes by molecular means. Although various in-house and commercial assays are available, first-line molecular detection is rarely routinely practiced, either because of high cost (commercial assays) or because few assays are multiplex and have short turnaround times [Bellau-Pujol et al., 2005; Gunson et al., 2005; Kuypers et al., 2006; Bonroy et al., 2007; van de Pol et al., 2007; Brittain-Long et al., 2008].

In this study, a simple, sensitive, and specific one-step triplex real-time RT-PCR assay was developed to detect the genomes of RSV, influenza A (including the pandemic influenza A(H1N1) 2009 virus) and influenza B simultaneously in respiratory samples. This assay was performed daily during the 2008–2009 and 2009–2010 seasons in two independent teaching-hospital laboratories, and 2,417 samples from adults and children were tested. As compared to IF, identification of viral infections was rapid and accurate and allowed an improvement in the management of patients presenting with acute respiratory infection.

MATERIALS AND METHODS

Viruses

Various influenza A, influenza B, and RSVA/B strains were used to determine sensitivity and efficiency of the assay (Table I). RSV-A of genotypes 1–5 and RSV-B of genotypes 1–6, isolated from clinical specimens and collected between 1986 and 2003 at the Hôpital Clémenceau, Caen, France were tested. RSV genotypes were determined by phylogenetic analysis of the G gene, according to the protocol established by the National Paramyxoviridae Centre (AV and FF, unpublished data) using primers described previously [Zambon et al., 2001]. Viruses were propagated in cell culture (MDCK for influenza A and influenza B, MRC5 for RSV) and virus titers are reported as plaque forming units (pfu) (influenza A and B) or the 50% tissue culture infectious dose ($TCID_{50}$)/ml (RSV).

Undiluted culture supernatants of PIV−1, −2, −3, and −4, hMPV-B, adenovirus type-1, echovirus type-30, rhinovirus type-31, and coronavirus OC43 and 229E were used to assess the specificity of the assay.

TABLE I. Viral Strains Used to Assess Sensitivity and Efficiency of the Assay

Viral strains	Titers/ml	Limit of detection per input (C_T value)	Mean RT-PCR efficiency (%)[a]
Influenza A			88
A/Brisbane/10/07(H3N2)	1.5×10^8 pfu	10 pfu (38.10)	
A/Wisconsin/67/05(H3N2)	3.7×10^8 pfu	10 pfu (38.80)	
A/California/7/04(H3N2)	2.5×10^8 pfu	1 pfu (40.06)	
A/Solomon Islands/03/06(H1N1)	2.2×10^6 pfu	1 pfu (38.69)	
A/New Caledonia/20/99(H1N1)	3.5×10^8 pfu	10 pfu (39.55)	
Influenza B			92
B/Florida/4/06	2.0×10^6 pfu	1 pfu (41.38)	
B/Jiangsu/10/03	1.2×10^7 pfu	10 pfu (39.28)	
B/Malaysia/2506/04	1.5×10^8 pfu	1 pfu (42.78)	
RSV-A			85
ATCC VR-26 A2 long	10^4 $TCID_{50}$	2×10^{-2} $TCID_{50}$ (43.23)	
Ga1/Caen/CHE/86[b]	ND	ND	
Ga2/Caen/BOU/00			
Ga3/Caen/TIS/97			
Ga4/Caen/CAS/01			
Ga5/Caen/PAT/93			
RSV-B			88
ATCC strain B1	ND	ND	
Gb1/Caen/WIS/03[c]			
Gb2/Caen/VER/02			
Gb3/Caen/GAU/02			
Gb4/Caen/MAR/01			
Gb5/Caen/LEN/99			
Gb6/Caen/GOD/01			

ND, not determined.
[a]RT-PCR efficiency was calculated for each strain, as previously described, by testing tenfold serial dilutions of the corresponding RNAs [Gault et al., 2001; Le Gal et al., 2005].
[b]Ga1 to Ga5 are RSV-A strains of genotypes 1–5.
[c]Gb1 to Gb6 are RSV-B strains of genotypes 1–6.

Clinical Samples

Specimens (nasopharyngeal aspirates or swabs) were collected from patients with acute respiratory infection or influenza-like syndrome and tested daily either at Hôpital Ambroise Paré, a general teaching hospital, or at Hôpital Trousseau, a pediatric teaching hospital.

One-Step Real-Time Triplex RT-PCR for Influenza A, Influenza B, and RSVA/B Genome Detection in Respiratory Samples

RNA was extracted with the QIAamp Viral-RNA Mini-Kit (Qiagen, Courtaboeuf, France) from 140 μl of fresh or thawed specimen. Aliquots of the resulting RNA preparation (7.5 μl of 60 μl eluted) were included in 25 μl of RT-PCR mixture containing primers and probes (Table II), and the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applera, Courtaboeuf, France). Each run included a negative control and a triplex positive control; the positive controls contained 2.5 μl of each target RNA extracted from culture supernatants and diluted to give a positive signal of 32 C_Ts, corresponding to an input of approximately 1,000 pfu for influenza A and B, and 20 TCID50 for RSV (Table I). The reaction consisted of 30 min of reverse-transcription at 48°C, 10 min of activation at 95°C, and 45 cycles of amplification (95°C for 15 sec and 59°C for 90 sec). A 7900HT Fast Real-Time PCR System (Applera) was used for real-time RT-PCR. Reaction giving C_T values $\leq$44 were considered to be positive.

Other Virological Methods

IF antigen detection. IF tests were performed on freshly sampled nasopharyngeal aspirates. Anti-influenza A, -influenza B, and -RSV monoclonal antibodies (Argene S.A., Verniolle, France) were used as recommended by the manufacturer.

Commercial real-time RT-PCR assays. Primers and probes of the Flu-A/B r-gene™ and RSV-A/B r-gene™ (Argene S.A.) assays were used in two independent RT-PCR targeting either influenza A/B or RSV-A/RSV-B. Aliquots of RNA preparations (10 μl, see procedure above) were added to two RT-PCR mixtures containing 14 μl of primers and probes and 1 μl of OneStep RT-PCR mix (Qiagen). Internal control IC2 (DICO Extra r-gene™, Argene S.A.) was added to the sample prior to extraction, to monitor the extraction step and to detect PCR inhibitors. An additional RT-PCR mixture containing 10 μl of RNA extract, 15 μl of the DICO Extra r-gene premix and 0.17 μl of the OneStep RT-PCR mix (Qiagen) was prepared. The cycling conditions for real-time RT-PCR were those recommended by Argene.

Sub-typing of influenza A viruses. Samples were used to inoculate MDCK. Influenza viruses were detected by hemagglutination assay with guinea-pig erythrocytes. Isolate subtype was determined by hemagglutination-inhibition, as previously described [Kendal et al., 1979].

H1 and N1 subtype-specific RT-PCR assays were used to characterize pandemic influenza A(H1N1) 2009 viruses, following the protocol established by the National Influenza Centre, Northern France (http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf).

Statistical Analyses

The nonparametric Mann–Whitney *U*-test was used for statistical comparisons. Differences were considered significant at P values <0.05.

RESULTS

Specificity, Sensitivity, and Efficiency of the One-Step Real-Time Triplex RT-PCR Assay

The specificity of the triplex assay was confirmed by the negative results obtained with viral genomes of 10 other respiratory viruses. The triplex positive control gave repeatedly positive signals for the three viruses targeted. No cross-amplification was observed in the triplex assay when tested with individual control influenza A, influenza B, and RSV genomes. The sensitivity of the assay varied according to the strain, from 1 to 10 pfu/input for influenza viruses and was 0.02 $TCID_{50}$/input for RSV (Table I).

The ability of the one-step real-time triplex RT-PCR assay to detect viral genomes in nasopharyngeal samples was assessed on a selected panel of 182 samples that were collected and tested by IF in 2007–2008 and stored at −80°C. These samples were tested both by the in-house triplex RT-PCR assay and the commercial Flu A/B and RSV A/B r-gene™ assays. The DICO Extra r-gene™ control was used to detect PCR inhibitors. All

TABLE II. Primers and Probes Used for the One-Step Real-Time Triplex RT-PCR

Target	Amplicon size (bp)	Primers and probes	5′–3′ Nucleotide sequences (final concentration in nmol/L)
Influenza A segment 7	85	Forward	AGGCTCTCATGGAA/GTGGCTAAAG[a] (300)
		Reverse	ACGGTGAGCGTGAACACAAA[a] (300)
		Probe	VIC-TGTCACCTCTGACTAAGG-MGB[a] (100)
Influenza B segment 7	65	Forward	TGTCGCTGTTTGGAGACACAA (100)
		Reverse	TGCTTTGCCTTCTCCATCTTC (100)
		Probe	NED-TGCCTACCTGCTTTCA-MGB (200)
RSV-A/B L gene	149	Forward	GTGGAACTTCATCCTGACATAAGATATATT (900)
		Reverse	GTTGCATCTGTAGCAGGAATGGT (900)
		Probe	FAM-ATTGCAATGATCATAGTTTACCT-MGB (200)

[a]Modified from Kuypers et al. [2006] and van de Pol et al. [2007].

samples positive by IF (n = 37) for influenza A (n = 8), influenza B (n = 3) or RSV (n = 26) were found positive for the respective viruses with both molecular tests. Furthermore, of 145 IF-negative samples, 38 (26%) were positive by both in-house and r-gene™ assays with 100% concordance between the two tests (10 positive for influenza A, seven for influenza B, and 21 for RSV). No PCR inhibitor was detected. These various results indicate that the ability of the in-house triplex assay to detect viral genomes was similar to that of the individual Flu-A/B and RSV-A/B r-gene™ assays. The assay was initially designed to detect current epidemic H1N1 and H3N2 viral strains. Thus, its sensitivity was re-assessed when the pandemic H1N1(2009) strain emerged. Twenty-five clinical isolates, positive for the H1N1 (2009) virus with the H1 and N1 subtype-specific RT-PCR assays, were provided by the Influenza National Reference Centre. All these samples were positive with the triplex assay, with a range of CTs similar to those of samples positive for epidemic H1N1 strains.

Use of the One-Step Real-Time Triplex RT-PCR Assay for Routine Monitoring of Viral Respiratory Infections

From week 2008-43 to 2009-10, samples were routinely collected from patients at Hôpital Ambroise Paré and tested with the in-house triplex assay for the genomes of influenza A, influenza B, and RSV (Table III). The temporal distribution of virus detection is shown in Figure 1. Of the 298 patients tested, 188 (63%) were positive: 145 (78%) for RSV, 42 (22%) for influenza A, and one for both RSV and influenza A. No influenza B infection was detected during this period. For influenza A, cell-culture isolation and subsequent sub-typing was performed for 23 (56%) samples: All corresponded to the epidemic strain current at that time: A/Brisbane/10/2007 (H3N2). Positive cell-culture isolation was significantly correlated with viral load in the specimen (viral load being estimated from C_T values). In cell-culture positive specimens, the median C_T value was 27.09, whereas in cell-culture negative specimens it was 32.57 ($P < 0.0001$).

The period between week 2009-36 and 2010-04 was characterized by the pandemic circulation of influenza A(H1N1) 2009. From week 2009-43 to 2010-04, the test was also used in the Hôpital Trousseau laboratory (Table III). Of 776 patients (adults and children) tested at Hôpital Ambroise Paré, 316 (41%) were positive: 183 (58%) for RSV, 132 (22%) for influenza A and one for both RSV and influenza A (Fig. 2A). Of 1,343 patients (only children) tested at Hôpital Trousseau, 663 (49%) were positive: 501 (76%) for RSV, 151 (23%) for influenza A, and 11 for mixed RSV-influenza A infections (Fig. 2B). Of the 283 influenza A genomes detected, 175 were further characterized with the H1 and N1 subtype-specific RT-PCR assays. All 283 were A(H1N1) 2009 virus. No influenza B infection was detected during the period.

DISCUSSION

The detection of RSV infection in infants under 6 weeks old, or presenting with a medical condition such as prematurity or underlying cardiopulmonary disease, leads to hospital admission to monitor the risk of rapid respiratory aggravation or apnea [Hall, 2000; Tregoning and Schwarze, 2010]. Also, children with influenza often present with alarming neurological symptoms associated with respiratory disease, but will not need further neurological investigation if influenza virus is detected rapidly [2009b]. Similarly, if a viral infection is diagnosed in a newborn with isolated fever, investigations for neonatal bacterial infection can be limited. Moreover, early administration of neuraminidase inhibitors in cases of influenza may prevent severe forms of infection, as reported during the influenza A(H1N1) 2009 pandemic [2009a,c]. Finally, when RSV and influenza viruses co-circulate at an epidemic rate (as was the case in 2008–2009 and 2009–2010), it may not be possible to distinguish infection by one virus from that by the other on the basis of clinical presentation

TABLE III. Routine Monitoring of Viral Respiratory Infections in 2008–2009 and 2009–2010 by the Use of the One-Step Real-Time Triplex RT-PCR Assay

Weeks (hospital)	2008-43/2009-10 (Ambroise Paré)		2009-36/2010-04 (Ambroise Paré)		2009-44/2010-04 (Trousseau) (children)
	Children	Adults	Children	Adults	
Number of patients	257	41	475	301	1,343
Median age (IQR)	13w (6–36)	61y (51–73)	56w (12–179)	44y (32–66)	62w (16–203)
Influenza A					
Number (%)	25 (10)	17 (42)	75 (16)	57 (19)	151 (11)
Median age (IQR)[a]	37w (6–60)	57y (51–73)	81w (20–192)	42y (30–60)	201w (90–421)
RSV					
Number (%)	140 (55)	5 (12)	173 (36)	10 (4)	501 (38)
Median age (IQR)	11w (6–24)	72y (69–80)	38w (10–133)	46y (33–66)	27w (9–74)
Mixed RSV-influenza A					
Number (%)	1 (0.4)	0	1 (0.2)	0	11 (8)
Median age (IQR)	8w (NA)	NA	23w (NA)	NA	90w (28–209)

w, weeks; y, years; NA, not applicable.
[a]Children infected by influenza A were significantly older than those infected by RSV ($P < 0.0001$).

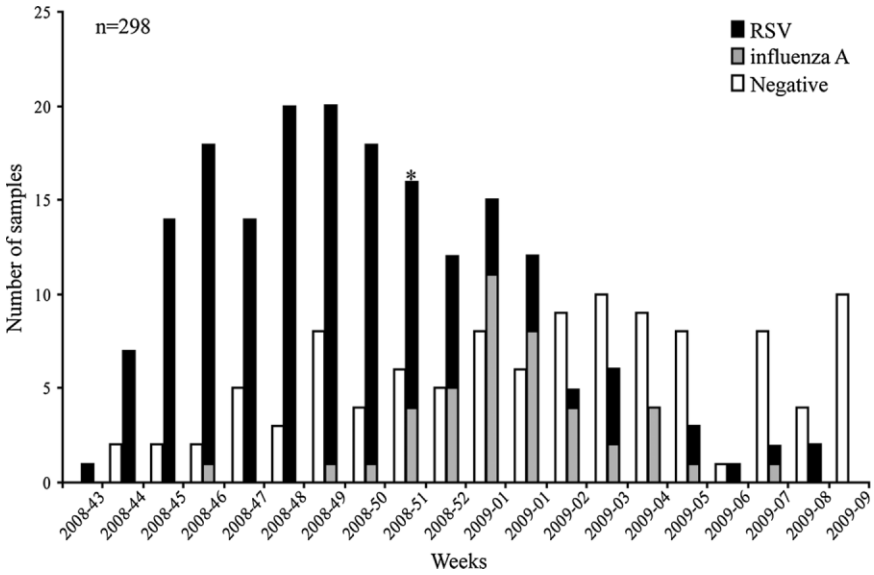

Fig. 1. Temporal distribution of virus detection during the winter 2008–2009. The number of positive (RSV in black, influenza A in gray) and negative (in white) samples is reported for each week. One mixed infection, indicated by an asterisk, was detected during week 2008-51.

[Bosis et al., 2008; Bourgeois et al., 2009]. Thus, rapid and sensitive tools are needed specifically to detect influenza and RSV infections, and to identify mixed infections. The diagnostic value of rapid antigen detection tests for influenza and RSV infections remains a subject of debate: Although technically well adapted to the management of acute respiratory infections, the currently available tests lack sensitivity and specificity [Steininger et al., 2002; Louie et al., 2010].

The aim of this study was to improve the efficiency of diagnosis of acute respiratory infections, in a context of high rates of infection by three major pathogens, which can be involved in mixed infections. The higher sensitivity of RT-PCR compared to antigen detection prompted us to use this technology to develop an inexpensive (<10.00€/test), rapid (<4 hr), specific and sensitive assay, targeting the genomes of influenza A, influenza B, and RSV. Triplex RT-PCR is cheaper and faster than three simplex assays. To facilitate use in daily practice, the procedure was simplified by adapting the extraction step to the MagNA Pure Compact system (Roche Diagnostics, Meylan, France), and the assay was successfully transferred to a different laboratory setting, where a different real-time PCR thermocycler was used (ABI PRISM® 7500 Real Time PCR System – Applera). Although not to be compared to rapid antigen testing, the assay was efficient enough to provide clinicians with specific and sensitive results daily: For samples made available at 13:00, the results were returned by 16:30.

The assay was designed to detect simultaneously the presence of the three viral genomes, and could thus identify mixed infections. Its ability to detect various strains of viruses was assessed with a panel of viruses isolated in cell-culture. With this panel, the sensitivity and efficiency of the triplex RT-PCR were found to be slightly lower than those obtained with the equivalent simplex assays, although the difference was not significant (results not shown), as previously reported by others for multiplex RT-PCR [Bonroy et al., 2007]. However, the sensitivity and efficiency of the assay were suitable for diagnosis. Indeed, the results were concordant for 100% of the 75 samples found positive with commercial RT-PCR assays, and the triplex assay successfully detected mixed infections. The 7900HT thermocycler allows no more than three different fluorescent dyes in real-time PCR assays, so the triplex assay could not also include an internal control. However, no PCR inhibitors were detected in 132 samples tested by the Argene method, indicating that the risk of missing a positive sample was small. If necessary for routine diagnosis, an internal control could be added to the sample prior to extraction, and the corresponding control RT-PCR could then be performed in parallel during the same run. The results obtained with this assay in daily practice during the 2008–2009 and 2009–2010 seasons were consistent with the data provided by the national influenza-monitoring network of GPs and pediatricians GROG (Groupes Régionaux d'Observation de la Grippe–Regional Influenza Surveillance Group; http://www.grog.org), and by the National Influenza Centre (Northern France), indicating, in particular, that circulation of influenza B was sporadic or absent in France during the periods studied. As previously described [Bourgeois et al., 2009], RSV infection was more frequent than influenza in young children, as evidenced by the higher percentage of RSV-positive specimens, even during the influenza A(H1N1) pandemics. Finally, the availability of a molecular tool allowing to distinguish rapidly influenza A from RSV in two general hospital settings in the context of the influenza A(H1N1) pandemics was of utmost value.

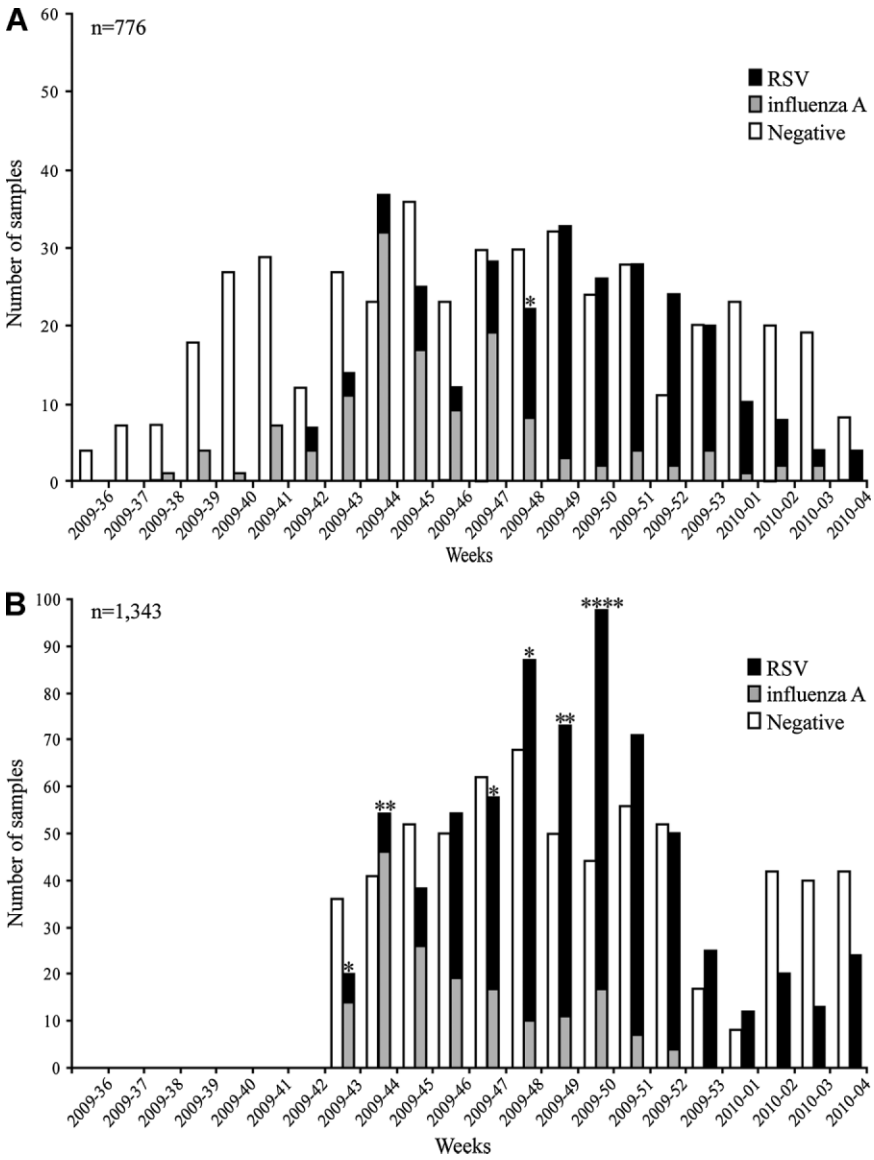

Fig. 2. Temporal distribution of virus detection during the winter 2009–2010. Patients (adults and children) from Ambroise Paré hospital (**A**) and patients (children) from Trousseau hospital (**B**) The number of positive (RSV in black, influenza A in gray) and negative (in white) samples is reported for each week. Mixed infections are indicated by asterisks.

In conclusion, the user-friendly in-house triplex RT-PCR reported here was efficient for testing nasopharyngeal specimens for current epidemic strains of influenza A (including seasonal H1N1, predominant in 2007–2008 [Vaux, 2008], seasonal H3N2, as sub-typed by cell-culture during the 2008–2009 season and pandemic influenza A(H1N1) 2009), influenza B, RSV-A, and RSV-B. Its ability to detect mixed infections also facilitates investigations to increase our knowledge about the incidence and severity of influenza A and RSV co-infections [Kehl et al., 2001; Coiras et al., 2004].

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