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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

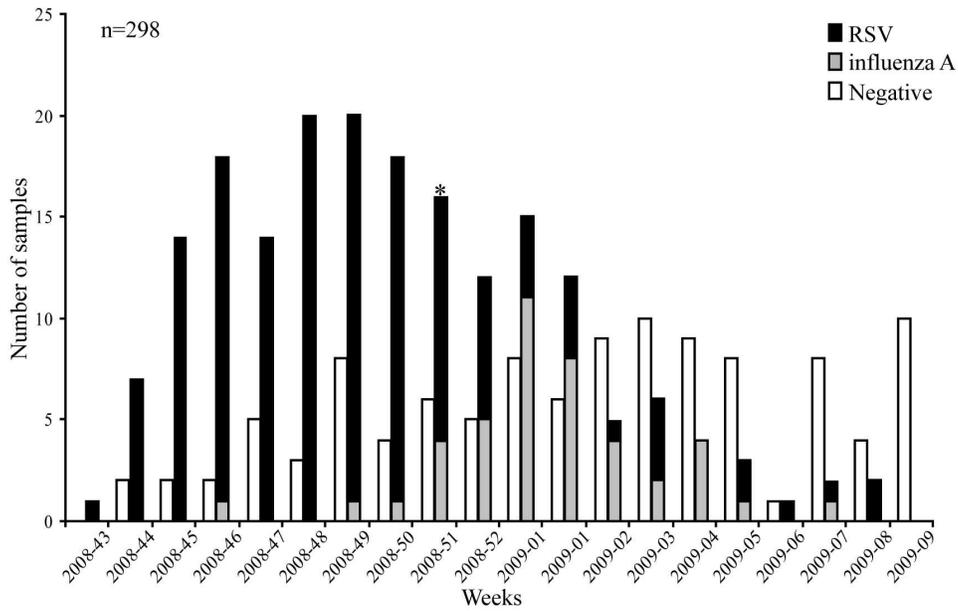
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Keywords:	orthomyxovirus, paramyxovirus, molecular diagnosis

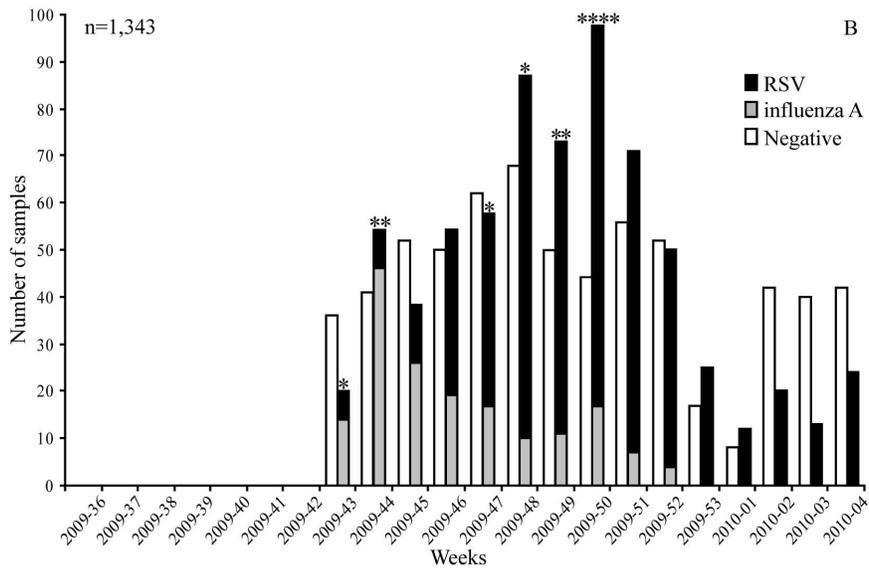
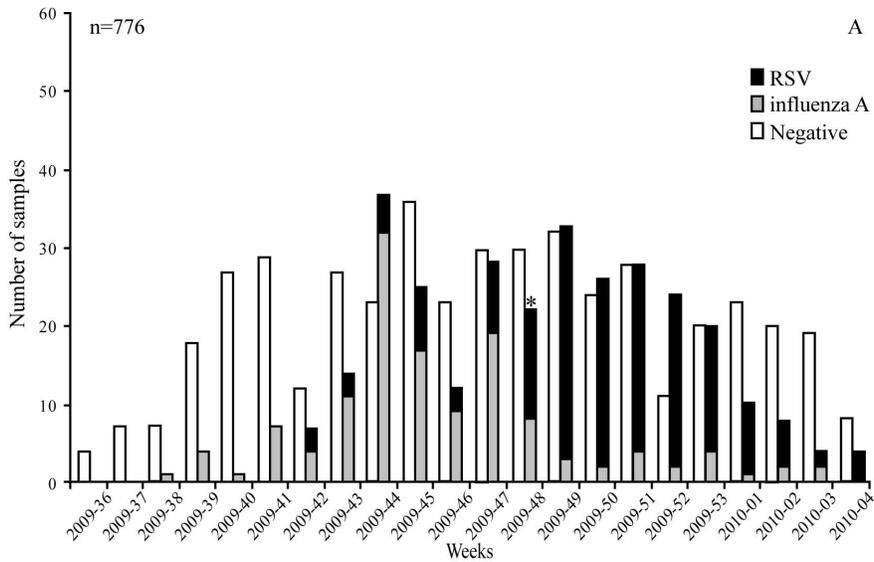
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Temporal distribution of virus detection during the winter 2008-2009. The number of positive (respiratory syncytial virus (RSV) in black, influenza A in grey) and negative (in white) samples is reported for each week. One mixed infection, indicated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

151x93mm (300 x 300 DPI)



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 (respiratory syncytial virus (RSV) in black, influenza A in grey) and negative
 (in white) samples is reported for each week. Mixed infections are indicated by asterisks.

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1 **Monitoring epidemic viral respiratory infections using one-step real-time triplex RT-**
2 **PCR targeting influenza A and B viruses and respiratory syncytial virus**

3 **Running title:** Real-time triplex PCR for influenza A/B and RSV

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ABSTRACT

Rapid and specific diagnosis of influenza A/B and respiratory syncytial (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 optimise 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 pfu/input (influenza A/B) and 2×10^{-2} TCID₅₀/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™, Argene) 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.

Keywords: Orthomyxovirus, Paramyxovirus, Molecular diagnosis

60 INTRODUCTION

61 The seasonal epidemic circulation of influenza A, influenza B and human respiratory
62 syncytial virus (RSV) viruses causes a large part of the burden of viral respiratory infections
63 in the northern hemisphere, especially that involving infants under 96 weeks of age and
64 elderly patients [Nair et al., 2010; Neuzil et al., 2000; Poehling et al., 2006; Simoes, 1999;
65 Simonsen et al., 2000]. Rapid identification of the viral cause of acute respiratory infection
66 has consequences for patient monitoring and care, including hospital admission or discharge,
67 administration of antibiotics and antiviral therapy, and reduction of diagnosis investigation
68 [Adcock et al., 1997; Barenfanger et al., 2000]. Antigen detection by immunofluorescence
69 (IF) or immunochromatography is the usual first-line diagnostic approach, although it lacks
70 sensitivity [Louie et al., 2010; Steininger et al., 2002]. An alternative is to test for viral
71 genomes by molecular means. Although various in-house and commercial assays are
72 available, first-line molecular detection is rarely routinely practiced, either because of high
73 cost (commercial assays) or because few assays are multiplex and have short turnaround
74 times [Bellau-Pujol et al., 2005; Bonroy et al., 2007; Brittain-Long et al., 2008; Gunson et al.,
75 2005; Kuypers et al., 2006; van de Pol et al., 2007].

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

83 MATERIALS AND METHODS

84 Viruses

85 Various influenza A, influenza B and RSVA/B strains were used to determine sensitivity and
86 efficiency of the assay (table 1). RSV-A of genotypes 1 to 5 and RSV-B of genotypes 1 to 6,
87 isolated from clinical specimens and collected between 1986 and 2003 at the Hôpital
88 Clémenceau, Caen, France were tested. RSV genotypes were determined by phylogenetic
89 analysis of the G gene, according to the protocol established by the National Paramyxoviridae
90 Centre (AV and FF, unpublished data) using primers described previously [Zambon *et al.*,
91 2001]. Viruses were propagated in cell culture (MDCK for influenza A and influenza B,
92 MRC5 for RSV) and virus titres are reported as plaque forming units (pfu) (influenza A and
93 B) or the 50% tissue culture infectious dose (TCID₅₀) /mL (RSV).

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

97 Clinical samples

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

101 One-step real-time triplex RT-PCR for influenza A, influenza B and RSVA/B genome 102 detection in respiratory samples

103 RNA was extracted with the QIAamp Viral-RNA Mini-Kit (Qiagen, Courtaboeuf, France)
104 from 140µL of fresh or thawed specimen. Aliquots of the resulting RNA preparation (7.5µL
105 of 60µL eluted) were included in 25µL of RT-PCR mixture containing primers and probes
106 (table 2), and the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied
107 Courtaboeuf, France). Each run included a negative control and a triplex positive control; the

1
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3 108 positive controls contained 2.5µL of each target RNA extracted from culture supernatants and
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5 109 diluted to give a positive signal of 32 C_Ts, corresponding to an input of approximately 1,000
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8 110 pfu for influenza A and B, and 20 TCID₅₀ for RSV (table 1). The reaction consisted of 30
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10 111 min of reverse-transcription at 48°C, 10 min of activation at 95°C, and 45 cycles of
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12 112 amplification (95°C for 15 s and 59°C for 90 s). A 7900HT Fast Real-Time PCR System
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15 113 (Applied) was used for real-time RT-PCR. Reaction giving C_T values ≤44 were considered to
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17 114 be positive.

115 **Other virological methods**

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

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

128 Sub-typing of influenza A viruses:

129 Samples were used to inoculate MDCK. Influenza viruses were detected by
130 haemagglutination assay with guinea-pig erythrocytes. Isolate subtype was determined by
131 haemagglutination-inhibition, as described previously [Kendal et al., 1979].

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3 132 H1 and N1 subtype-specific RT-PCR assays were used to characterise pandemic influenza
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5 133 A(H1N1) 2009 viruses, following the protocol established by the National Influenza Centre,
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8 134 Northern France, available at:
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10 135 http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_Recommendations
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13 136 H1N1_20090521.pdf.

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15 137 **Statistical analyses**

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17 138 The nonparametric Mann-Whitney U-test was used for statistical comparisons. Differences
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20 139 were considered significant at *P* values less than 0.05.
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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 ten 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 TCID₅₀/input for RSV (table 1).

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 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 (ten 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 of these samples were positive with the triplex assay, with a range of CTs similar to those of samples positive for epidemic H1N1 strains.

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6 167 **Use of the one-step real-time triplex RT-PCR assay for routine monitoring of viral**
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8 168 **respiratory infections**

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10 169 From week 2008-43 to 2009-10, samples were routinely collected from patients at Hôpital
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12 170 Ambroise Paré and tested with the in-house triplex assay for the genomes of influenza A,
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15 171 influenza B and RSV (table 3). The temporal distribution of virus detection is shown in figure
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17 172 1. Of the 298 patients tested, 188 (63%) were positive: 145 (78%) for RSV, 42 (22%) for
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19 173 influenza A and one for both RSV and influenza A. No influenza B infection was detected
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21 174 during this period. For influenza A, cell-culture isolation and subsequent sub-typing was
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23 175 performed for 23 (56%) samples: all corresponded to the epidemic strain current at that time:
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25 176 A/Brisbane/10/2007 (H3N2). Positive cell-culture isolation was significantly correlated with
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27 177 viral load in the specimen (viral load being estimated from C_T values). In cell-culture positive
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29 178 specimens, the median C_T value was 27.09, whereas in cell-culture negative specimens it was
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31 179 32.57 ($P < 0.0001$).

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36 180 The period between week 2009-36 and week 2010-04 was characterized by the pandemic
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38 181 circulation of influenza A(H1N1) 2009. From week 2009-43 to 2010-04, the test was also
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40 182 used in the Hôpital Trousseau laboratory (table 3). Of 776 patients (adults and children) tested
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42 183 at Hôpital Ambroise Paré, 316 (41%) were positive: 183 (58%) for RSV, 132 (22%) for
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44 184 influenza A and one for both RSV and influenza A (figure 2A). Of 1,343 patients (only
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46 185 children) tested at Hôpital Trousseau, 663 (49%) were positive: 501 (76%) for RSV, 151
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48 186 (23%) for influenza A, and eleven for mixed RSV-influenza A infections (figure 2B). Of the
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50 187 283 influenza A genomes detected, 175 were further characterized with the H1 and N1
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52 188 subtype-specific RT-PCR assays. All 283 were A(H1N1) 2009 virus. No influenza B
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54 189 infection was detected during the period.

DISCUSSION

The detection of RSV infection in infants under six 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 apnoea [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; 2009c]. 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 [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 [Louie *et al.*, 2010; Steininger *et al.*, 2002].

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 (<4h), 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

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3 215 daily practice, the procedure was simplified by adapting the extraction step to the MagNA
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5 216 Pure Compact system (Roche Diagnostics, Meylan, France), and the assay was successfully
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7 217 transferred to a different laboratory setting, where a different real-time PCR thermocycler was
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9 218 used (ABI PRISM® 7500 Real Time PCR System – Applied). Although not to be compared
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11 219 to rapid antigen testing, the assay was efficient enough to provide clinicians with specific and
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13 220 sensitive results daily: for samples made available at 13:00, the results were returned by
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18 222 The assay was designed to detect simultaneously the presence of the three viral genomes, and
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20 223 could thus identify mixed infections. Its ability to detect various strains of viruses was
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22 224 assessed with a panel of viruses isolated in cell-culture. With this panel, the sensitivity and
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24 225 efficiency of the triplex RT-PCR were found to be slightly lower than those obtained with the
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26 226 equivalent simplex assays, although the difference was not significant (results not shown), as
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28 227 previously reported by others for multiplex RT-PCR [Bonroy *et al.*, 2007]. However, the
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30 228 sensitivity and efficiency of the assay were suitable for diagnosis. Indeed, the results were
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32 229 concordant for 100% of the 75 samples found positive with commercial RT-PCR assays, and
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34 230 the triplex assay successfully detected mixed infections. The 7900HT thermocycler allows no
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36 231 more than three different fluorescent dyes in real-time PCR assays, so the triplex assay could
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38 232 not also include an internal control. However, no PCR inhibitors were detected in 132
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40 233 samples tested by the Argene method, indicating that the risk of missing a positive sample
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42 234 was small. If necessary for routine diagnosis, an internal control could be added to the sample
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44 235 prior to extraction, and the corresponding control RT-PCR could then be performed in
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46 236 parallel during the same run. The results obtained with this assay in daily practice during the
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48 237 2008-2009 and 2009-2010 seasons were consistent with the data provided by the national
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50 238 influenza-monitoring network of GPs and paediatricians GROG (Groupes Régionaux
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52 239 d'Observation de la Grippe - Regional Influenza Surveillance Group - <http://www.grog.org>),

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3 240 and by the National Influenza Centre (Northern France), indicating, in particular, that
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5 241 circulation of influenza B was sporadic or absent in France during the periods studied. As
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7 242 previously described [Bourgeois et al., 2009], RSV infection was more frequent than
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10 243 influenza in young children, as evidenced by the higher percentage of RSV-positive
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12 244 specimens, even during the influenza A(H1N1) pandemics. Finally, the availability of a
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15 245 molecular tool allowing to distinguish rapidly influenza A from RSV in two general hospital
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17 246 settings in the context of the influenza A(H1N1) pandemics was of utmost value.
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19 247 In conclusion, the user-friendly in-house triplex RT-PCR reported here was efficient for
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21 248 testing nasopharyngeal specimens for current epidemic strains of influenza A (including
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23 249 seasonal H1N1, predominant in 2007-2008 [Vaux, 2008], seasonal H3N2, as sub-typed by
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25 250 cell-culture during the 2008-2009 season and pandemic influenza A(H1N1) 2009), influenza
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27 251 B, RSV-A and RSV-B. Its ability to detect mixed infections also facilitates investigations to
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29 252 increase our knowledge about the incidence and severity of influenza A and RSV co-
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33 253 infections [Coiras et al., 2004; Kehl et al., 2001].
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10 257 No conflict of interest is to be reported for any of the authors.
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15 259 Clinical data collection and nasopharyngeal sampling for respiratory virus testing are part of
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17 260 current management of patients with acute respiratory infection or influenza-like illness. No
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20 261 ethical approval was required for this study.
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352 TABLES

353 Table 1

Table 1: Viral strains used to assess sensitivity and efficiency of the assay

Viral strains	Titres /mL	Limit of detection per input (C _T value)	Mean RT-PCR efficiency ^a
influenza A			
A/Brisbane/10/07(H3N2)	1.5x10 ⁸ pfu	10 pfu (38.10)	
A/Wisconsin/67/05(H3N2)	3.7x10 ⁸ pfu	10 pfu (38.80)	
A/California/7/04(H3N2)	2.5x10 ⁸ pfu	1 pfu (40.06)	88%
A/Solomon Islands/03/06(H1N1)	2.2x10 ⁶ pfu	1 pfu (38.69)	
A/New Caledonia/20/99(H1N1)	3.5x10 ⁸ pfu	10 pfu (39.55)	
influenza B			
B/Florida/4/06	2.0x10 ⁶ pfu	1 pfu (41.38)	
B/Jiangsu/10/03	1.2x10 ⁷ pfu	10 pfu (39.28)	92%
B/Malaysia/2506/04	1.5x10 ⁸ pfu	1 pfu (42.78)	
RSV-A			
ATCC VR-26 A2 Long	10 ⁴ TCID ₅₀	2x10 ⁻² TCID ₅₀ (43.23)	
Ga1/Caen/CHE/86 ^b			
Ga2/Caen/BOU/00			
Ga3/Caen/TIS/97	ND ^d	ND	85%
Ga4/Caen/CAS/01			
Ga5/Caen/PAT/93			
RSV-B			
ATCC strain B1			
Gb1/Caen/WIS/03 ^c			
Gb2/Caen/VER/02			
Gb3/Caen/GAU/02	ND	ND	88%
Gb4/Caen/MAR/01			
Gb5/Caen/LEN/99			
Gb6/Caen/GOD/01			

^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 to 5.

^c Gb1 to Gb6 are RSV-B strains of genotypes 1 to 6.

^d ND: not determined

354 **Table 2**

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Table 2: 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	TGCTTTCCTTCTCCATCTTC (100)
		Probe	NED-TGCCTACCTGCTTTCA-MGB (200)
RSV-A/B L gene	149	Forward	GTGGAACCTTCATCCTGACATAAGATATATT (900)
		Reverse	GTTGCATCTGTAGCAGGAATGGT (900)
		Probe	FAM-ATTGCAATGATCATAGTTTACCT-MGB (200)

^a Modified from [Kuypers et al., 2006; van de Pol et al., 2007]

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361 **Table 3**

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Table 3: 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	Adults	Children	Adults	Children
Number of patients	257	41	475	301	1,343
Median age (IQR)	13w ^a (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) ^b	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 (.4)	0	1 (.2)	0	11 (.8)
median age (IQR)	8w (NA ^c)	NA	23w (NA)	NA	90w (28-209)

^a w: weeks, y: years^b children infected by influenza A were significantly older than those infected by RSV ($P < 0.0001$)^c NA: not applicable

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3 367 **FIGURE LEGENDS**

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5 368 **Figure 1**

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8 369 **Temporal distribution of virus detection during the winter 2008-2009**

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10 370 The number of positive (respiratory syncytial virus (RSV) in black, influenza A in grey) and
11 371 negative (in white) samples is reported for each week. One mixed infection, indicated by an
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13 372 asterisk, was detected during week 2008-51.
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19 374 **Figure 2**

20 375 **Temporal distribution of virus detection during the winter 2009-2010**

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23 376 Patients (adults and children) from Ambroise Paré hospital (A) and patients (children) from
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25 377 Trousseau hospital (B)
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28 378 The number of positive (respiratory syncytial virus (RSV) in black, influenza A in grey) and
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30 379 negative (in white) samples is reported for each week. Mixed infections are indicated by
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32 380 asterisks.
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