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Loïc Legrand, Astrid Vabret, Julia Dina, Joelle Petitjean-Lecherbonnier, Stephanie Gouarin, Delphine Cuvillon-Nimal, Valérie Tripey, Jacques Brouard, Francois Freymuth

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Epidemiological and phylogenic study of human metapneumovirus infections during three consecutive outbreaks in Normandy, France.

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Keywords:	Human metapneumovirus, epidemiological study, M gene, Phylogenic study

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Table 1: Virus repartition in positive respiratory samples from children hospitalized during three consecutive winters from November 2002 to March 2005.

		Winters			Total*
		2002-03*	2003-04**	2004-05*	
Identified viruses	hMPV	59 (42)	17	52 (39)	128 (98)
	hRSV	207 (187)	158	299 (237)	664 (582)
	influenza virus A	40 (15)	18	106 (54)	164 (87)
	influenza virus B	29 (10)	1	11 (3)	41 (14)
	PIV1/2/3	36 (26)	9	37 (22)	82 (57)
	Adenoviruses	37 (29)	5	36 (29)	78 (63)
	Rhinoviruses	66 (52)	91	28 (20)	185 (163)
	Enteroviruses	19 (13)	6	19 (13)	44 (32)
	Coronavirus OC43, 229E	5 (3)	11	54 (37)	70 (51)
	Total	498 (377)	316	642 (454)	1456 (1147)

* Data in brackets concern children less than two years old

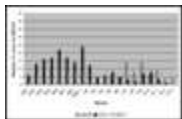
** These data were obtained only in children less than two years old.

Table 2: Analysis of the epidemiological study in the paediatric patient population during three consecutive winters from November 2002 to March 2005.

	Winter 2002-03	Winter 2003-04*	Winter 2004-05
N° of nasal aspirates	1040	263	1112
N° of positive nasal aspirates	439	188	590
N° of hMPV detected (% of negative specimens/% of total nasal aspirates)	59 (10/5.6)	17 (22.6/6.4)	52 (8.8/4.7)
Primers used for the RT-PCR	MPV.M1/ MPV.M2	MPV.M1/ MPV.M2	MPV.M1/ MPV.M2
Repartition in the child population (%):			
- 0-6 months	35.6	56.5	32.7
- 6-12 months	22	26.1	23.1
- 12-24 months	13.6	17.4	19.2
- >24 months	28.8	...	25
Male	27	16	27
Female	32	7	25

* These data were obtained only in children less than two years old.

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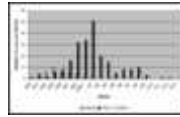


Weekly comparison of the outbreak of hRSV, influenza viruses A and B (MIA/B) and hMPV in a paediatric population. (a) from November to March, 2002-03; (b) from 2003-04; (c) from 2004-05

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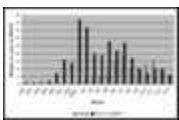


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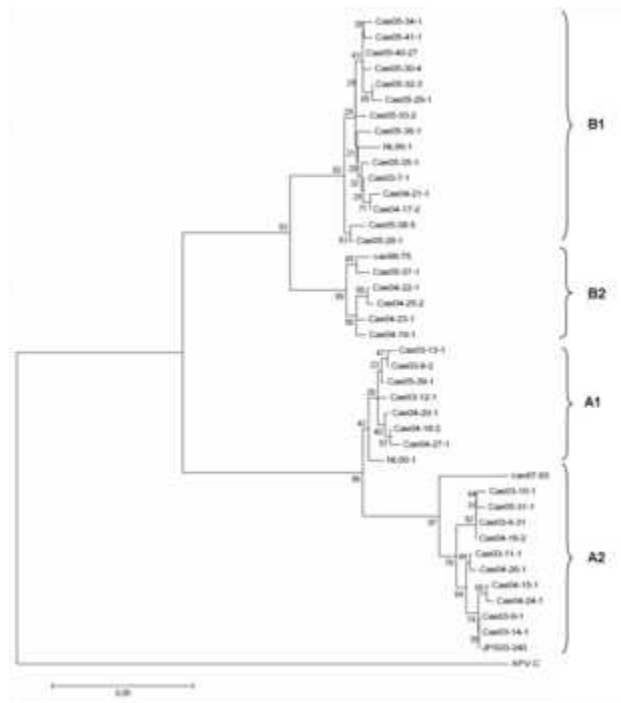
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Weekly comparison of the outbreak of hRSV, influenza viruses A and B (MIA/B) and hMPV in a paediatric population. (a) from November to March, 2002-03; (b) from 2003-04; (c) from 2004-05

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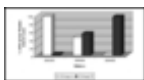
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Phylogenetic analysis performed for the M gene for 107 sequenced hMPV isolates in Normandy during three consecutive winters. The tree was built with Mega 3.1 software using the neighbour joining method. Bootstrap values (1000 replicates) are reported on branch nodes of the tree. Four genotypes are indicated. The strains obtained in Normandy are written as follows: Cae-isolation winter (A strain isolated during the winter 2002-03 is notified 03)-Number of strain-Quantity of sample containing this strain. The Genbank access numbers of the referenced strains are NL00-1(AF371337), NL99-1(AY525843), Can98-75(AY297748), Can97-83(AY297749), JPS03-240(AY530095).

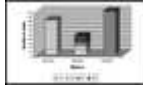
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Phylogenetic evolution of hMPV strains detected during the outbreaks circulating in Normandy for three consecutive winter to spring periods. (a) Prominence of hMPV group A and B during each winter; (b) Strain repartition between subgroup A1, A2, B1 and B2 during each winter (c) hMPV sequence analysis for each outbreak.
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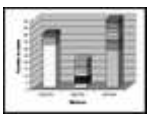
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Phylogenetic evolution of hMPV strains detected during the outbreaks circulating in Normandy for three consecutive winter to spring periods. (a) Prominence of hMPV group A and B during each winter; (b) Strain repartition between subgroup A1, A2, B1 and B2 during each winter (c) hMPV sequence analysis for each outbreak.
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Phylogenetic evolution of hMPV strains detected during the outbreaks circulating in Normandy for three consecutive winter to spring periods. (a) Prominence of hMPV group A and B during each winter; (b) Strain repartition between subgroup A1, A2, B1 and B2 during each winter (c) hMPV sequence analysis for each outbreak.
3x2mm (600 x 600 DPI)

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14 6 Stéphanie^a, Delphine Cuvillon^c, Valérie Tripey^c, Jacques Brouard^c, François Freymuth^{a**}
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Phylogenic study of human metapneumovirus

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3 26 Abstract:
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8 28 Human metapneumovirus (hMPV) is responsible for respiratory tract disease, particularly in
9
10 29 the young and elderly population. An epidemiological and phylogenic study was performed
11
12 30 on children admitted to hospital with an acute lower respiratory tract infection. Data was
13
14
15 31 obtained and analyzed over three consecutive winters, from 2002-03 to 2004-05.
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17 32 Each year during the winter period, from November to March, 2,415 nasal swabs were tested
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19
20 33 by a direct immunofluorescence assay (DFA) for influenza virus A and B, respiratory
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22 34 syncytial virus, parainfluenza viruses and adenoviruses. Rhinoviruses, enteroviruses and
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24 35 coronaviruses OC43 and 229E were detected by RT-PCR. A RT-PCR designed for the M
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26 36 gene was performed on negative samples for hMPV detection and phylogenic analyses. For
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29 37 the three consecutive winters, hMPV represented 10%, 22.6% and 8.8% of virus-negative
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31 38 samples, respectively. In most cases, clinical symptoms indicated a lower respiratory tract
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34 39 infection (LRI) with a final diagnosis of bronchiolitis. During the winter of 2003-04, all viral
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36 40 clusters (A1, A2, B1 and B2) that circulated in France shifted progressively from the A group
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39 41 to the B group. This study determined the prevalence of hMPV in Normandy, its clinical
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41 42 impact and permitted the analysis of the molecular evolution during the successive outbreaks.
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51 Keywords: Human metapneumovirus, epidemiological study, M gene, Phylogenic study

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101 Introduction

102 Human metapneumovirus (hMPV) was first isolated by a Dutch group in 2001 (van den
103 Hoogen et al., 2001). Since then, the virus has been detected in all continents (Nissen et al.,
104 2002; Peret et al., 2002; Stockton al., 2002; Freymuth et al., 2003; Peiris et al., 2003; Cuevas
105 et al., 2003; Fodha et al., 2004). Human metapneumovirus is the single human pathogen
106 within the genus *Metapneumovirus*, subfamily *Pneumovirinae*, family *Paramyxoviridae*.
107 Serological studies suggest that the virus has been circulating in humans for at least the past
108 60 years (van den Hoogen et al., 2001; Ebihara et al., 2003; Mejias et al., 2004). However,
109 recent evidence from molecular studies suggests that hMPV may have diverged from the
110 avian metapneumovirus type C as early as 200 years ago (De Graaf et al., 2008; Yang et al.,
111 2009).

112 Human metapneumovirus causes upper and lower respiratory tract diseases such as
113 bronchiolitis or pneumonia (Boivin et al., 2002; Williams et al., 2004; Bouscambert-Duchamp
114 et al., 2005; Williams et al., 2006; Gray et al., 2006; Sarasini et al., 2006). Human infections
115 occur mainly during the winter period and affect, in particular, young children, elderly people
116 and immunocompromised patients (Pelletier et al., 2002; Bastien et al., 2003a; Falsey et al.,
117 2003). It was shown serologically that children under five years old were the most vulnerable
118 and susceptible to contracting the virus (van den Hoogen et al., 2001; Ebihara et al., 2003;
119 Regev et al., 2006).

120 Furthermore, human metapneumovirus can be detected in respiratory samples by reverse
121 transcription-PCR (RT-PCR) (Mackay et al., 2003; Maertzdorf et al., 2004; Bellau-Pujol et
122 al., 2005) or real-time RT-PCR (Hopkins et al., 2008; Kaida et al., 2008). In addition, several
123 monoclonal antibodies were developed for immunofluorescence assays (IFA) (Ebihara et al.,
124 2005; Landry et al., 2005; Gerna et al., 2006; Aslanzadeh et al., 2008). Molecular studies have

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3 125 shown that hMPV strains can be divided into two major groups: A and B, and 4 minor groups:
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5 126 A1, A2, B1, B2 (Bastien et al., 2003b; Boivin et al., 2004; van den Hoogen et al., 2004).
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8 127 This paper describes studies concerning hMPV infection and reports on a genetic analysis of
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10 128 hMPV strains that were detected in infected children during three consecutive winters in
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12 129 Normandy, France.

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131 **Material and methods**

132 **Patients and samples**

133 The study was performed on 2,415 children admitted to the University hospital of Caen and
134 the hospital of Flers, for an acute lower respiratory tract infection from November to March
135 during three consecutive years (November 2002 to March 2004). All patients underwent a
136 nasal aspirate which was processed immediately by conventional methods (see below). The
137 remaining samples were frozen at -70°C and used for subsequent molecular analyses. All
138 samples were collected and tested in conformation with the ethical legislation in place.

139 **Conventional methods**

140 The conventional methods included a direct immunofluorescence assay (DFA). This
141 immunofluorescence assay used commercially available monoclonal antibodies (Imagen[®],
142 Dako, Glostrup, Denmark) and permitted the detection of influenza virus types A and B,
143 respiratory syncytial virus (RSV), parainfluenza virus types 1, 2 and 3, and adenoviruses.
144 When the DFA was negative, samples were inoculated onto HuH7 cell lines grown in 48-well
145 microplates as described previously (Freymuth et al., 2005). Briefly, after four days of
146 incubation, HuH7 cells were harvested and used for DFA with the same antiviral antibodies.
147 When a cytopathic effect was observed, supernatants of the relevant wells were harvested and
148 used in a multiplex PCR for the detection of rhinovirus, enterovirus and human coronaviruses
149 229E, OC43 (Bellau-Pujol et al., 2005).

150 **Molecular methods for detection of hMPV**

151 RNA was extracted from nasal aspirates with the QIAamp Viral RNA minikit (Qiagen,
152 Hilden, Germany) according to the manufacturer's instructions. RT-PCR was likewise
153 performed with the one-step RT-PCR kit (Qiagen) using primers designed for the M gene.
154 The forward primer hMPV.M1 (5'-CCCTTTGTTTCAGGCCAAC-3') and the reverse primer
155 hMPV.M2 (5'-GCAGCTTCAACAGTAGCTG-3') provided a 416 pb PCR product. The
156 thermocycler conditions were 30 min at 50°C, 15 min at 95°C following by 40 cycles of 1
157 min at 95°C, 1 min at 60°C and 1 min at 72°C. Finally, an extension at 72°C was performed
158 during 10 min. The result was analysed on 2% agarose gel.

159 The PCR sensitivity for each group was established using two fragments of the M gene
160 containing the amplification product (Eurofin-MWG-Operon, Ebersberg, Germany)
161 representing NL00-1 (AF371337) and NL99-1 (AY525843), respectively. NL00-1 was
162 considered like group A prototype sequence and NL99-1 like group B prototype sequence.
163 Synthesized DNA were cloned in a pCR2.1 vector and transcription was performed using T7
164 polymerase (Applied Biosystems/Ambion, Courtaboeuf, France). After DNase treatment
165 (Applied Biosystems/Ambion, Courtaboeuf, France), RNA concentration was determined by
166 measuring the absorbance at 260 nm with the Nanodrop spectrophotometer system. Serial 10-
167 fold dilutions were performed to determine the sensitivity of the assay in copy of the viral
168 RNA per reaction.

169 **Sequencing and phylogenic analysis**

170 An hemi-nested PCR was performed with the reverse primer MPV.M2 and a new forward
171 primer MPV.M3 (5'-AGGCCAACACACCACCAG-3'). The amplification was realized
172 through the use of an AmpliTaq (Applied Biosystem, Foster City, CA) with the following
173 program: 5 min at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 45 sec at
174 72°C and a final elongation at 72°C for 10 min. The 405 pb product obtained was first

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3 175 purified with the enzyme ExoSap-IT (Amersham Biosciences, Uppsala, Sweden) according to
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5 176 the manufacturer's instructions and then quantified on a spectrometer. Subsequently, the
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8 177 purified PCR product was sequenced on a Beckman CeqTM 8000 genetic analysis system with
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10 178 the CEQ DTCS Quick start kit (Beckman coulter, Brea, CA). The sequences were assembled
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13 179 and analyzed with BioEdit and ClustalW software. A phylogenic study was carried out using
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15 180 Mega 3.1 software with the neighbour joining method. A bootstrap was performed with a
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17 181 replicate rate of 1000.

182 **Nucleotide Sequence Accession Numbers**

183 The nucleotide sequences reported in this study were submitted to the GenBank under
184 accession numbers H384124 to H384159.

185 **Results**

186 **hMPV infections**

187 The RT-PCR allowed detection of about 30 and 50 copies per reaction for the A and B group,
188 respectively. This method was performed on virus-negative nasal aspirates. The frequency of
189 hMPV infection in hospitalized children could be assessed only during the first and last
190 winters because the study performed during the winter of 2003-04 was limited to children
191 under 2 years of age; this study was described by Freymuth et al (2006). In order to compare
192 the hMPV prevalence to the other common cold viruses, the period from November to March
193 was studied exclusively. In 2002 and 2004, there were 498 and 642 samples, respectively,
194 that contained a respiratory virus out of the 1040 and 1112 nasal aspirates tested (Table 1).
195 Human metapneumovirus was detected in 59 (10%) and 52 (8.8%) of the virus-negative
196 specimens analysed, respectively. As a function of age, there were 34.2%, 22.5%, 16.4% and
197 26.9% cases of infected children between 0-6 months, 6-12 months, 12-24 months and older
198 than 24 months, respectively (Table 2).

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3 199 During the winters of 2002 to 2004, other viruses were scrutinized for by a DFA test or by
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5 200 using culture models or molecular tools. Human metapneumovirus was the fourth most
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7 201 commonly detected virus (8.8%). Human respiratory syncytial virus (hRSV) was the most
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9 202 predominant and represented 45.6% of virus-positive specimens followed by rhinoviruses
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11 203 (12.7%) and the influenza A virus (11.3%). The other detected viruses were parainfluenza
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13 204 viruses 1, 2 and 3 (5.6%); adenoviruses (5.3%); coronaviruses (4.8%); enteroviruses (3%) and
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15 205 the influenza B virus (2.8%). In children under two year old, hMPV (8.5%) became the third
16
17 206 most frequently encountered virus after hRSV (50.7%) and rhinoviruses (14.2%). Influenza A
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19 207 virus represented about 7.7% of the cases studied; adenoviruses 5.5%; parainfluenza viruses
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21 208 1, 2 and 3 5%; coronaviruses 4.4%; enteroviruses 2.8% and influenza B virus 1.2% (Table 1).
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23 209 Clinical information was available from 46 out of the 52 hMPV-infected children during the
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25 210 winter of 2004. The mean age of the infected children was 21 months (Table 2) and the
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27 211 male/female ratio was equal. Children were hospitalized about 3.5 days after the onset of
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29 212 symptoms. The most frequent clinical signs at admission were rhinitis (84.8%), cough
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31 213 (71.7%), respiratory distress syndrome (30.4%) and otitis (17.4%). Nineteen children (41.3%)
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33 214 presented with an upper respiratory tract infection and 26 children (56.5%) with a lower
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35 215 respiratory tract infection. The final clinical diagnosis was bronchiolitis in 17 cases (37%),
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37 216 broncho-pneumonia in six cases (13%) and acute exacerbation of asthma in three cases
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39 217 (6.6%). Signs of gastroenteritis were present in 17 (30.4%) of hMPV-infected children and
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41 218 rotavirus or adenovirus were present in nine cases. Seven hMPV-infected children (15.2%)
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43 219 required oxygen therapy.
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52 **Epidemics of hMPV infections**

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55 221 During the winter of 2002-03, the hMPV and hRSV epidemics appeared at the end of
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57 222 November and they peaked almost immediately at three to four weeks later. In
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59 223 contradistinction, during the winters of 2003-04 and 2004-05, the hMPV and hRSV epidemics

224 appeared later and continued more slowly. Human metapneumovirus seemed to have a
225 constant presence without any obvious peak in activity (Figure 1).

226 **Phylogenetic analysis**

227 A phylogenetic analysis was carried out on the M gene of 107 hMPV strains isolated during
228 the three consecutive outbreaks (Figure 2). The hMPV strains were distributed into two
229 groups, A and B, and into four relevant subgroups: A1, A2, B1, and B2. The percentages of
230 identity in the M gene between the hMPV group A and B strains varied from 84.2% to 88.8%.
231 The percentages of genetic similitude between the strains of subgroups A1 and A2 and
232 between the strains of B1 and B2 subgroups were 94.1% to 96.3% and 93.8% to 95.7%,
233 respectively.

234 **Distribution of hMPV groups and subgroups**

235 Human metapneumovirus strains of different groups and subgroups were associated with each
236 outbreak (Figure 3a). However, there was often one dominant subgroup in each epidemic.
237 Human metapneumovirus belonging to the A2 subgroup and B1 subgroup were predominant
238 during the winters of 2002-03 and 2004-05, respectively (Figure 3b). For both these periods,
239 two strains (Cae03-6-31 and Cae05-40-27) were more prominent and represented greater than
240 50% of the isolated hMPV. During the winter of 2003-04, the four subgroups were present
241 equally (Figure 3c).

242 **Discussion**

243 The present study reports on an epidemiological, clinical and phylogenetic study of hMPV
244 outbreak during three consecutive winters in Normandy, France. Human metapneumovirus
245 infection was assessed in nasal aspirates received in the laboratory during the months of
246 November to March in order to perform a comparison of hMPV rate with the other common
247 cold viruses. It was reported that, the hMPV infection season is longer than that of hRSV or
248 influenza viruses (van den Hoogen et al., 2003; Esper et al., 2004; Mackay et al., 2006). As

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2
3 249 such, the number of positive cases may have been underestimated although it was shown
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5 250 previously that hMPV circulates predominantly during the winter-spring period (van den
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8 251 Hoogen et al., 2003; Bastien et al., 2003a; Williams et al., 2004; Esper et al., 2004; Pitoiset et
9
10 252 al., 2010). The number and the percentage of hMPV detected in virus-negative samples
11
12 253 during the epidemics of 2002-03, 2003-04 and 2004-05 were 59 (10%), 17 (22.6%) and 52
13
14 254 (8.8%), respectively. The incidence of hMPV infections are reported in many other studies
15
16 255 (Boivin et al., 2002; van den Hoogen et al., 2004; Williams et al., 2004). It is also suggested
17
18 256 that the number of hMPV infections is variable from one winter to another (Falsey et al.,
19
20 257 2003; Mackay et al., 2006; Williams et al., 2006).

21
22
23 258 Human metapneumovirus was the fourth most prominent virus encountered during the present
24
25 259 study and became the third most prominent if the results are focused only on children under
26
27 260 two years old. Three-quarters of the hMPV were detected on children under two years old and
28
29 261 more than one third on those less than six months old. Human respiratory syncytial virus was
30
31 262 the most predominant virus observed with an increase in prevalency in children under two
32
33 263 years old. Influenza A virus was more commonly encountered in the population of over two
34
35 264 year olds. Unlike the hMPV and the other isolated viruses, the influenza A and B viruses
36
37 265 show a distinct prevalence between children under two years old and those over two years
38
39 266 old.

40
41 267 In comparison with hRSV and influenza viruses A and B, the outbreak of hMPV occurs over
42
43 268 the same period and appears to be closely related to the hRSV epidemic profile. In fact,
44
45 269 hMPV and hRSV outbreaks showed the same variation during the winter months (Boivin et
46
47 270 al., 2003; Esper et al., 2004). The influenza epidemic profile, which might be a more
48
49 271 independent entity, did not correlate with hMPV or hRSV. During these three winters, the
50
51 272 influenza A virus is more prominent than influenza B virus and the percentage was
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53 273 appreciatively the same for that described in Europe (Paget et al., 2003; Meijer et al., 2006).

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3 274 Only the negative samples were taken because a low percentage of co-infection was reported
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5 275 previously (Peiris et al., 2003; van den Hoogen et al., 2003; Mullins et al., 2004) and thus,
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7
8 276 even if this strategy introduced a selection bias, it allows a more reliable evaluation of the
9
10 277 clinical impact of hMPV in affected children. Unlike upper respiratory tract infections, human
11
12 278 metapneumovirus infection is normally associated with an acute lower respiratory tract
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15 279 infection as observed in the present study and in other previous reports (Boivin et al., 2002;
16
17 280 Freymuth et al., 2003; Williams et al., 2004). Indeed, some patients present with only
18
19 281 gastroenteritis in the absence of altered respiratory signs. No difference in severity of clinical
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21 282 manifestation was observed between the infection with group A or group B.
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23
24 283 Cultivation of hMPV is very difficult to achieve and, at the start of the present study, no direct
25
26 284 immunofluorescence assay test was available commercially. The RT-PCR was designed for
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28
29 285 the M gene to detect hMPV and to have an equivalent sensitivity for both genotypes. The M
30
31 286 gene appeared to be suitable for hMPV detection, since it has already been validated (Bellau-
32
33 287 Pujol et al., 2005; Chano et al., 2005) and a genetic discrimination has been determined (van
34
35 288 den Hoogen et al., 2001; Bastien et al., 2003b; Carr et al., 2005; Chano et al., 2005).
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38 289 Moreover, the M gene seems to be a good candidate for the detection of other viruses like
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40 290 influenza viruses or coronaviruses (Donofrio et al., 1992; Vabret et al., 2001). This PCR
41
42 291 appears to be adapted to hMPV detection and characterization since the four hMPV clusters
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44 292 described were amplified successfully (van den Hoogen et al., 2004; Mackay et al., 2004).
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46
47 293 In the present study, four hMPV subtypes termed, “A1”, “A2”, “B1” and “B2” were detected
48
49 294 by RT-PCR. The rate of the different subgroups was not the same for each winter and the
50
51 295 strain composition varied during each outbreak. The strains circulating in France were
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53 296 described in four previous studies (Freymuth et al., 2003; Bouscambert et al., 2005; Foulongne
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55 297 et al., 2006; Pitoiset et al., 2010). The first two studies reported only A1 and A2 subtypes of
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57 298 hMPV, while Foulongne and colleagues (2006) detected four subtypes of the virus with an A1
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3 299 subgroup in predominance. The last study (Pitoiset et al., 2010) reports on a phylogenetic
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5 300 analysis carried out on 100 strains isolated over eight years, from 2002 to 2009 with
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8 301 alternative subgroup predominance. During the first winter 2002-03, the A group was the
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10 302 most prevalent, with 87.5% of A2 subgroup being the most prominent. This repartition was
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12 303 also found in many other parts of the world (Mackay et al., 2004; Esper et al., 2004;
13
14 304 Ludewick et al., 2005. Pitoiset et al., 2010). Two studies described another pattern of
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16 305 circulation of the virus during the winter of 2002-03 in which a prevalent B1 subgroup was
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18 306 noted (Gerna et al., 2005; Gray et al., 2006). For the winter of 2003-04, no predominant group
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20 307 could be identified. The incidence of groups A and B were approximately the same, although
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22 308 the number of strains belonging to group B and A were nine and eight, respectively. The B2
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24 309 and A2 subgroup were the most frequently identified with five strains for each cluster. During
25
26 310 this same period in Italy (winter of 2003-04), the A2 subtype was also the most prominent
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28 311 strain, even if other strains were present (Sarasini et al., 2005; Gerna et al., 2005). The rate of
29
30 312 occurrence of group B was confirmed during the winter of 2004-05 and represented 96% of
31
32 313 all hMPV detected, including one hMPV B2 strain. Over the three winter periods, 46.5% of
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34 314 B1, 37.5% of A2, 8% of A1 and 8% of B2 strains were observed. These data are closely
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36 315 related to those proposed by Mackay et al. (2006) in a study carried out in Australia during
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38 316 the same period. In addition, both predominant subgroups found in 2002 and 2004 were the
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40 317 same (viz., A2 and B1).

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42 318 The RNA fragment studied is small (405bp), but some mutations that were found in this gene
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44 319 allowed us to discriminate between, and compare, the different isolates. The sequence
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46 320 analysis for the three epidemic periods showed two distinct genetic profiles. The first profile,
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48 321 found during the winter of 2002-03 and 2004-05, seemed to be composed of two predominant
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50 322 strains showing exactly the same sequence (Cae03-6-31 and Cae05-40-27) which represented
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52 323 at least 52% of hMPV detected, increasing to 79.5% in 2002-03. During these two winters
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3 324 (2002-03 and 2004-05), the foremost strains belonged to the A2 and B1 subgroups,
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5 325 respectively. This finding corroborates previous observations (Mackay et al., 2006). For the
6
7 326 winter period 2003-04, the isolated strains belonged to the four subgroups and, at the most,
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10 327 two identical sequences were found. Even if it is impossible to consider these isolates like
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12 328 different strains because the analysis was assessed on too short a RNA fragment, such
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15 329 heterogeneity was not observed during the other winter periods and might be considered as
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17 330 the beginning of a shift from group A to group B as confirmed by the data of 2004. A shift of
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19 331 this nature was reported previously (Agapov et al., 2006; Mackay et al., 2006; Oliveira et al.,
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21 332 2009). However, no evolution can be established from the heterogenic composition of hMPV
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23 333 infection during the winter of 2003-04 to the subgroup B1 predominance during the winter of
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25 334 2004-05. The B1 strain responsible for 52% of hMPV infection during the winter of 2004-05
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27 335 was not found during the previous winter of 2003-04.

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29 336 Despite the fact that the 2003-04 study is incomplete and that the period studied was only
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31 337 from November to March, the rate of hMPV can still be evaluated each year and the
32
33 338 molecular evolution of the viruses can be analyzed. Human metapneumovirus is circulating in
34
35 339 Normandy at a rate equivalent to that described previously (Peret et al., 2002; Bastein et al.,
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37 340 2003; Bovin et al., 2003; Esper et al., 2004). The present clinical study demonstrates that
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39 341 hMPV is responsible for lower respiratory tract infection in the majority of cases studied. All
40
41 342 the reported hMPV subgroups are present in France but not with the same incidence each
42
43 343 year. The results confirm the genetic evolution of hMPV in Normandy with a switch from the
44
45 344 A group to the B group being noted during the winter of 2003-04. Finally, molecular tools
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47 345 developed for the M gene appear to be useful for diagnosis and molecular analysis, since the
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49 346 four subgroups are detected and can be discriminated between in a significant manner.
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Tables**Table 1:** Virus repartition in positive respiratory samples from children hospitalized during three consecutive winters from November 2002 to March 2005.

		Winters			Total*
		2002-03*	2003-04**	2004-05*	
Identified viruses	hMPV	59 (42)	17	52 (39)	128 (98)
	hRSV	207 (187)	158	299 (237)	664 (582)
	influenza virus A	40 (15)	18	106 (54)	164 (87)
	influenza virus B	29 (10)	1	11 (3)	41 (14)
	PIV1/2/3	36 (26)	9	37 (22)	82 (57)
	Adenoviruses	37 (29)	5	36 (29)	78 (63)
	Rhinoviruses	66 (52)	91	28 (20)	185 (163)
	Enteroviruses	19 (13)	6	19 (13)	44 (32)
	Coronavirus OC43, 229E	5 (3)	11	54 (37)	70 (51)
Total		498 (377)	316	642 (454)	1456 (1147)

* Data in brackets concern children less than two years old

** These data were obtained only in children less than two years old.

Table 2: Analysis of the epidemiological study in a paediatric patient population during three consecutive winters from November 2002 to March 2005.

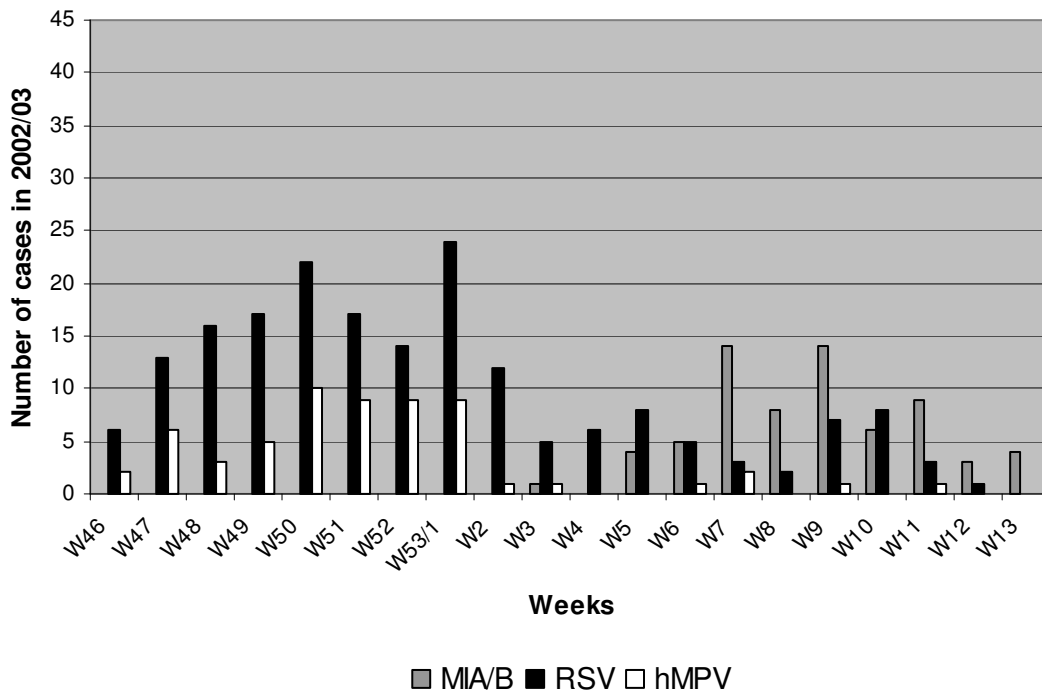
	Winter 2002-03	Winter 2003-04*	Winter 2004-05
N° of nasal aspirates	1040	263	1112
N° of positive nasal aspirates	439	188	590
N° of hMPV detected (% of negative specimens/% of total nasal aspirates)	59 (10/5.6)	17 (22.6/6.4)	52 (8.8/4.7)
Primers used for the RT-PCR	MPV.M1/ MPV.M2	MPV.M1/ MPV.M2	MPV.M1/ MPV.M2
Repartition in the child population (%):			
- 0-6 months	35.6	56.5	32.7
- 6-12 months	22	26.1	23.1
- 12-24 months	13.6	17.4	19.2
- >24 months	28.8	...	25
Male	27	16	27
Female	32	7	25

* These data were obtained only in children less than two years old.

615 **Figure 1:** Weekly comparison of the outbreak of hRSV, influenza viruses A and B (MIA/B) and hMPV
 616 in a paediatric population. (a) from November to March, 2002-03; (b) from 2003-04; (c) from 2004-05

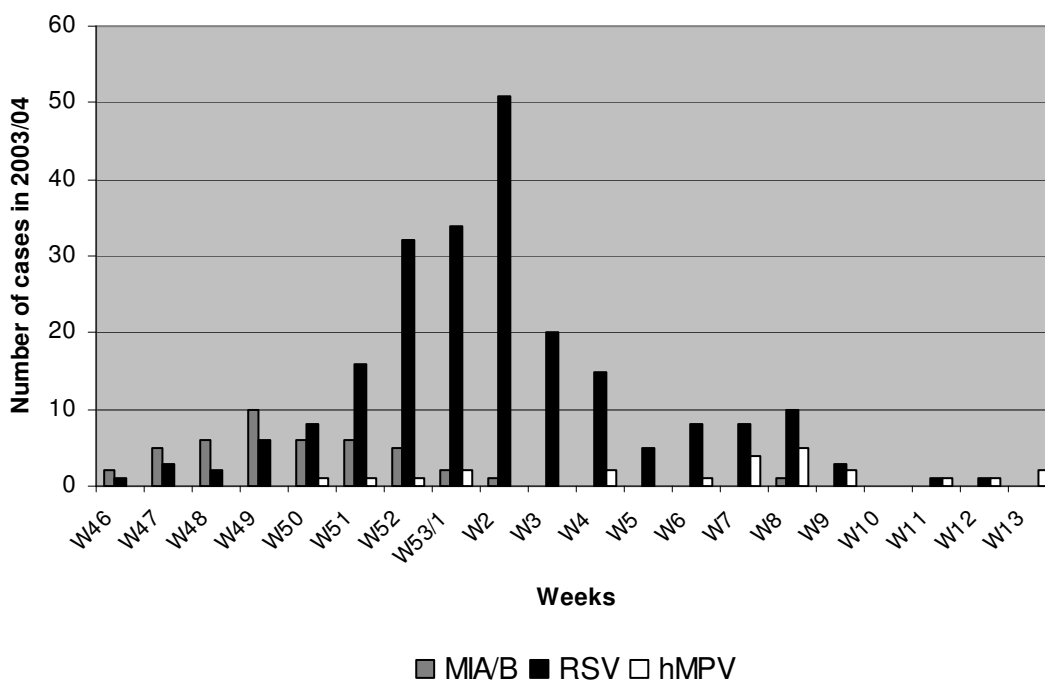
617 **2002-03**

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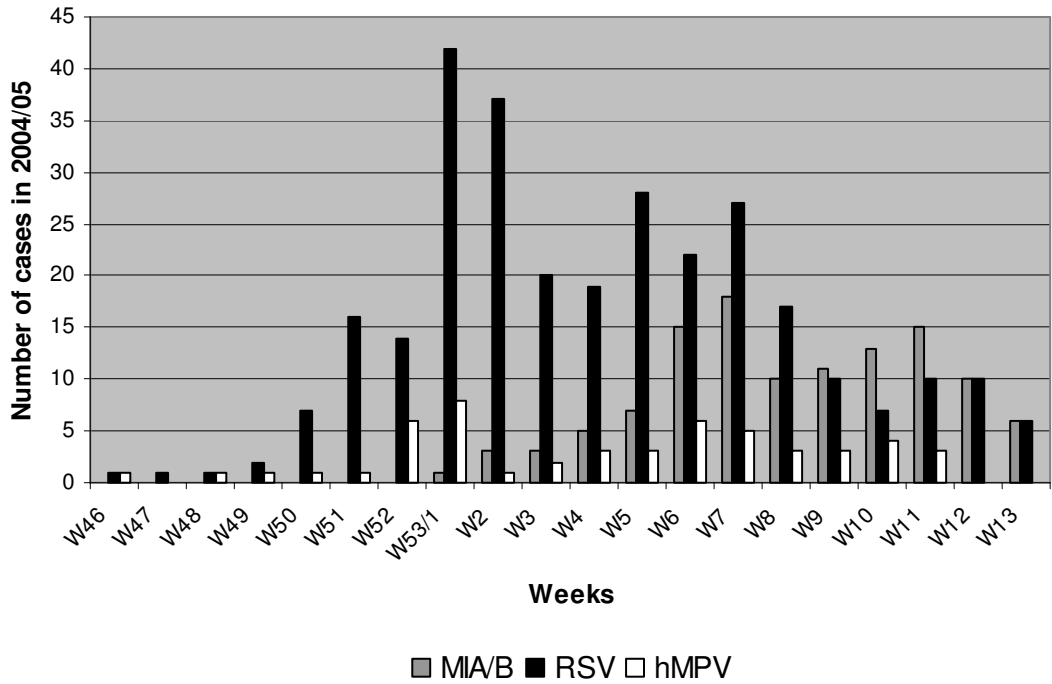
619 **2003-04**

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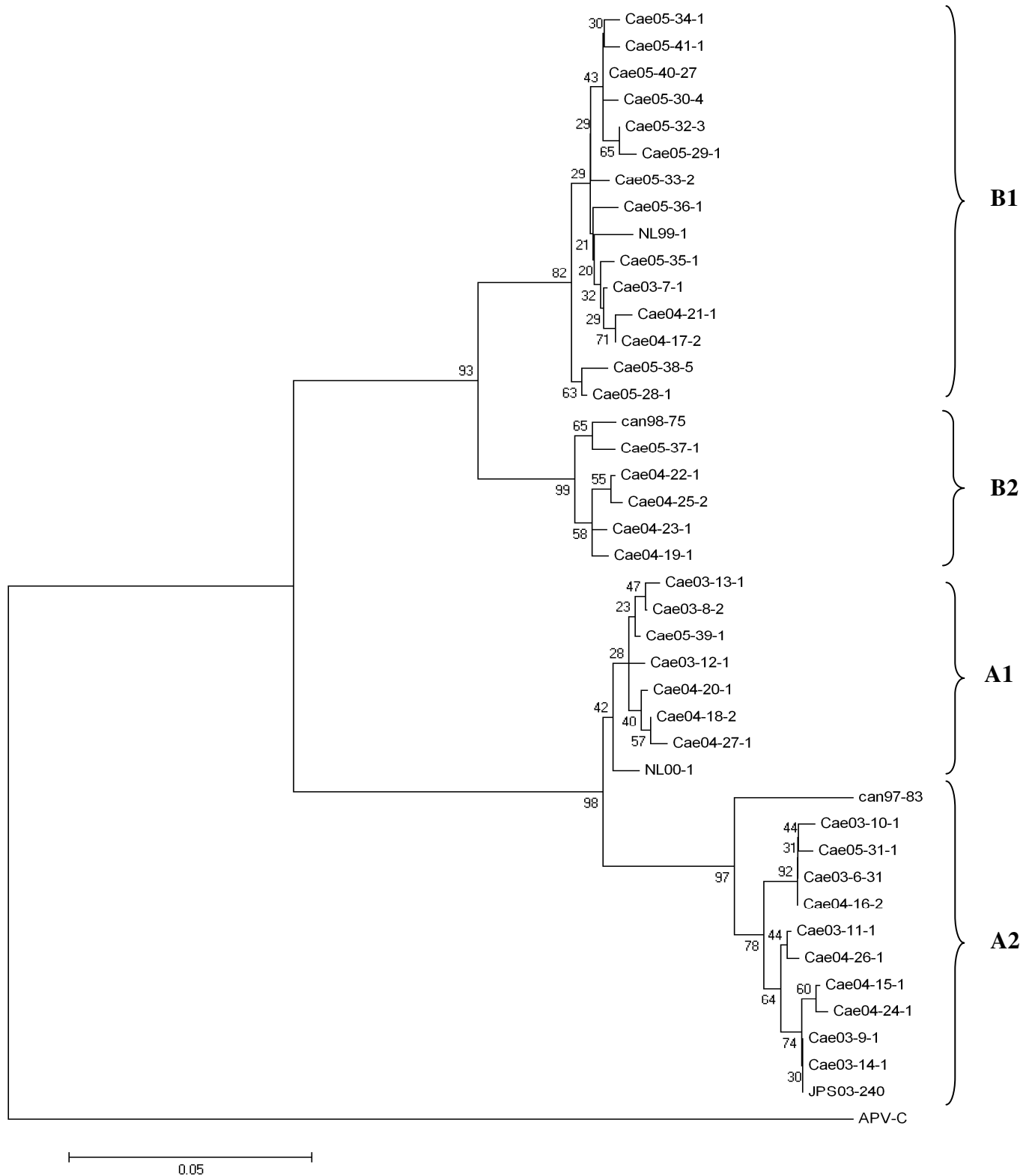
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2004-05



Review

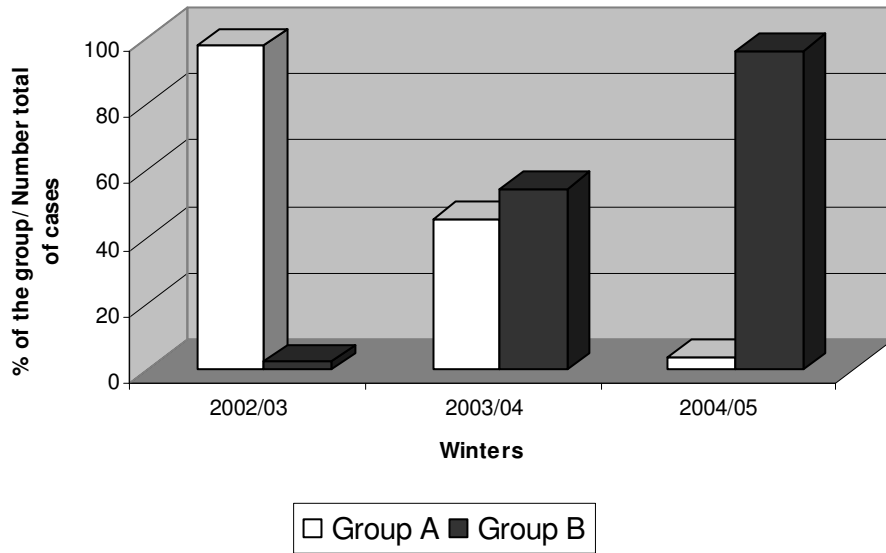
653 **Figure 2:** Phylogenetic analysis performed for the M gene for 107 sequenced hMPV isolates in Normandy
 654 during three consecutive winters. The tree was built with Mega 3.1 software using the neighbour joining
 655 method. Bootstrap values (1000 replicates) are reported on branch nodes of the tree. Four genotypes are
 656 indicated. The strains obtained in Normandy are written as follows: Cae-isolation winter (A strain
 657 isolated during the winter 2002-03 is notified 03)-Number of strain-Quantity of sample containing this
 658 strain. The Genbank access numbers of the referenced strains are NL00-1(AF371337), NL99-
 659 1(AY525843), Can98-75(AY297748), Can97-83(AY297749), JPS03-240(AY530095).



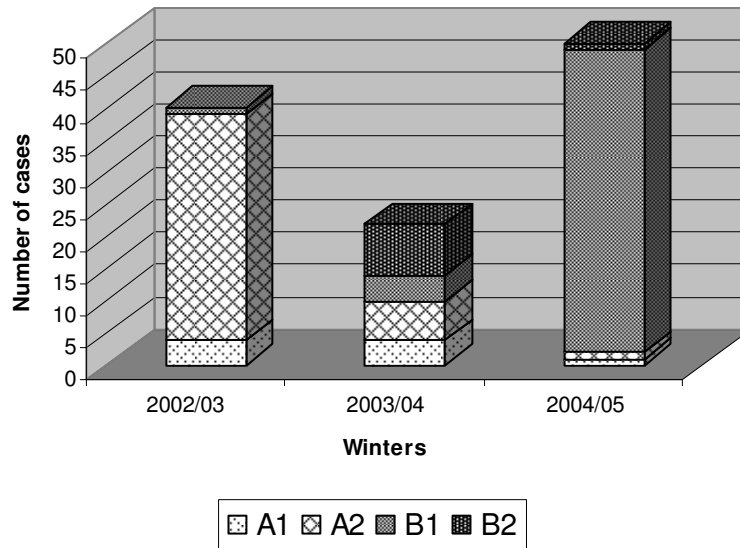
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661 **Figure 3:** Phylogenetic evolution of hMPV strains detected during the outbreaks circulating in Normandy
 662 for three consecutive winter to spring periods. (a) Prominence of hMPV group A and B during each
 663 winter; (b) Strain repartition between subgroup A1, A2, B1 and B2 during each winter (c) hMPV
 664 sequence analysis for each outbreak.

665 (a)

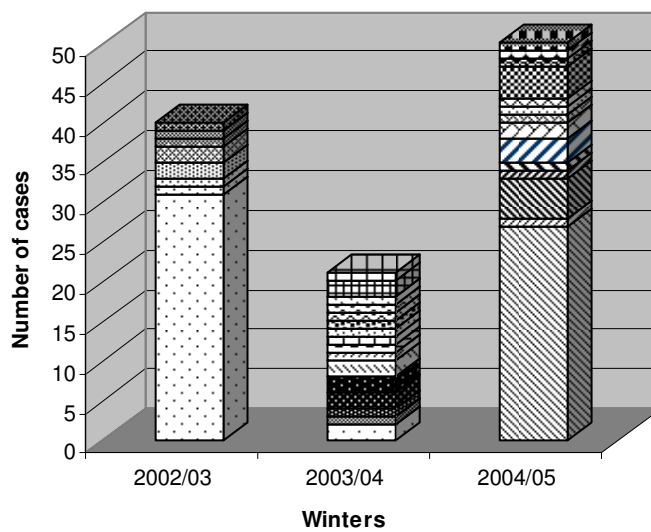


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680 (c)
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Peer Review