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Epidemiological and Phylogenic Study of Human Metapneumovirus Infections During Three Consecutive Outbreaks in Normandy, France

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Human metapneumovirus (hMPV) is responsible for respiratory tract disease, particularly in the young and elderly population. An epidemiological and phylogenic study was performed on children admitted to hospital with an acute lower respiratory tract infection (LRI). Data were obtained and analyzed over three consecutive winters, from 2002–2003 to 2004–2005. Each year during the winter period, from November to March, 2,415 nasal swabs were tested by a direct immunofluorescence assay (DFA) for influenza viruses A and B, respiratory syncytial virus, parainfluenza viruses, and adenoviruses. Rhinoviruses, enteroviruses, and coronaviruses OC43 and 229E were detected by RT-PCR. A RT-PCR designed for the M gene was performed on negative samples for hMPV detection and phylogenic analyses. For the three consecutive winters, hMPV represented 10%, 22.6%, and 8.8% of virus-negative samples, respectively. In most cases, clinical symptoms indicated a LRI with a final diagnosis of bronchiolitis. During the winter of 2003–2004, all viral clusters (A1, A2, B1, and B2) that circulated in France shifted progressively from the A group to the B group. This study determined the prevalence of hMPV in Normandy, its clinical impact and permitted the analysis of the molecular evolution during the successive outbreaks. ***J. Med. Virol. 83:517–524, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: human metapneumovirus; epidemiological study; M gene; phylogenic study

INTRODUCTION

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

Human metapneumovirus causes upper and lower respiratory tract diseases such as bronchiolitis or pneumonia [Boivin et al., 2002; Williams et al., 2004, 2006; Bouscambert-Duchamp et al., 2005; Gray et al., 2006; Sarasini et al., 2006]. Human infections occur mainly during the winter period and affect, in particular, young children, elderly people, and immunocompromised patients [Pelletier et al., 2002; Bastien et al., 2003a; Falsey et al., 2003]. It was shown serologically that children under 5 years old were the most vulnerable and susceptible to contracting the virus [van den Hoogen et al., 2001; Ebihara et al., 2003; Regev et al., 2006].

Furthermore, hMPV can be detected in respiratory samples by reverse transcription-PCR (RT-PCR) [Mackay et al., 2003; Maertzdorf et al., 2004; Bellau-Pujol et al., 2005] or real-time RT-PCR [Hopkins et al.,

Mononegavirales, Paramyxoviridae, Pneumovirinae, Metapneumovirus, Human metapneumovirus.

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2008; Kaida et al., 2008]. In addition, several monoclonal antibodies were developed for immunofluorescence assays (IFA) [Ebihara et al., 2005; Landry et al., 2005; Gerna et al., 2006; Aslanzadeh et al., 2008]. Molecular studies have shown that hMPV strains can be divided into two major groups: A and B, and 4 minor groups: A1, A2, B1, and B2 [Bastien et al., 2003b; Boivin et al., 2004; van den Hoogen et al., 2004].

This paper describes studies concerning hMPV infection and reports on a genetic analysis of hMPV strains that were detected in infected children during three consecutive winters in Normandy, France.

MATERIAL AND METHODS

Patients and Samples

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

Conventional Methods

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

Molecular Methods for Detection of hMPV

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

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

Sequencing and Phylogenic Analysis

An hemi-nested PCR was performed with the reverse primer MPV.M2 and a new forward primer MPV.M3 (5′-AGGCCAACACACCACCAG-3′). The amplification was realized through the use of an AmpliTaq (Applied Biosystem, Foster City, CA) with the following 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 72°C and a final elongation at 72°C for 10 min. The 405 pb product obtained was first purified with the enzyme ExoSap-IT (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions and then quantified on a spectrometer. Subsequently, the purified PCR product was sequenced on a Beckman Ceq™ 8000 genetic analysis system with the CEQ DTCS Quick start kit (Beckman Coulter, Brea, CA). The sequences were assembled and analyzed with BioEdit and ClustalW software. A phylogenic study was carried out using Mega 3.1 software with the neighbor joining method. A bootstrap was performed with a replicate rate of 1,000.

Nucleotide Sequence Accession Numbers

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

RESULTS

hMPV Infections

The RT-PCR allowed detection of about 30 and 50 copies per reaction for the A and B group, respectively. This method was performed on virus-negative nasal aspirates. The frequency of hMPV infection in hospitalized children could be assessed only during the first and last winters because the study performed during the winter of 2003–2004 was limited to children under 2 years of age; this study was described by Freymuth et al. [2006]. In order to compare the hMPV prevalence to the other common cold viruses, the period from November to March was studied exclusively. In 2002 and 2004, there were 498 and 642 samples, respectively,

TABLE I. Virus Repartition in Positive Respiratory Samples From Children Hospitalized During Three Consecutive Winters From November 2002 to March 2005

Identified viruses	Winters			Total[a]
	2002–2003[a]	2003–2004[b]	2004–2005[a]	
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)	1,456 (1,147)

[a]Data in brackets concern children less than 2 years old.
[b]These data were obtained only in children less than 2 years old.

that contained a respiratory virus out of the 1,040 and 1,112 nasal aspirates tested (Table I). Human metapneumovirus was detected in 59 (10%) and 52 (8.8%) of the virus-negative specimens analyzed, respectively. As a function of age, there were 34.2%, 22.5%, 16.4%, and 26.9% cases of infected children between 0 and 6 months, 6–12 months, 12–24 months, and older than 24 months, respectively (Table II).

During the winters of 2002–2004, other viruses were scrutinized for by a DFA test or by using culture models or molecular tools. Human metapneumovirus was the fourth most commonly detected virus (8.8%). Human respiratory syncytial virus (hRSV) was the most predominant and represented 45.6% of virus-positive specimens followed by rhinoviruses (12.7%) and the influenza A virus (11.3%). The other detected viruses were parainfluenza viruses 1, 2, and 3 (5.6%); adenoviruses (5.3%); coronaviruses (4.8%); enteroviruses (3%); and the influenza B virus (2.8%). In children under 2 years old, hMPV (8.5%) became the third most frequently encountered virus after hRSV (50.7%) and rhinoviruses (14.2%). Influenza A virus represented about 7.7% of the cases studied; adenoviruses 5.5%; parainfluenza viruses 1%, 2%, and 3 5%; coronaviruses 4.4%; enteroviruses 2.8%; and influenza B virus 1.2% (Table I).

Clinical information was available from 46 out of the 52 hMPV-infected children during the winter of 2004. The mean age of the infected children was 21 months (Table II) and the male/female ratio was equal. Children were hospitalized about 3.5 days after the onset of symptoms. The most frequent clinical signs at admission were rhinitis (84.8%), cough (71.7%), respiratory distress syndrome (30.4%), and otitis (17.4%). Nineteen children (41.3%) presented with an upper respiratory tract infection and 26 children (56.5%) with a LRI. The final clinical diagnosis was bronchiolitis in 17 cases (37%), broncho-pneumonia in six cases (13%), and acute exacerbation of asthma in three cases (6.6%). Signs of gastroenteritis were present in 17 (30.4%) of hMPV-infected children and rotavirus or adenovirus were present in nine cases. Seven hMPV-infected children (15.2%) required oxygen therapy.

Epidemics of hMPV Infections

During the winter of 2002–2003, the hMPV and hRSV epidemics appeared at the end of November and they peaked almost immediately at 3–4 weeks later. In contradistinction, during the winters of 2003–2004 and 2004–2005, the hMPV and hRSV epidemics appeared

TABLE II. Analysis of the Epidemiological Study in a Pediatric Patient Population During Three Consecutive Winters From November 2002 to March 2005

	Winter 2002–2003	Winter 2003–2004[a]	Winter 2004–2005
No. of nasal aspirates	1,040	263	1,112
No. of positive nasal aspirates	439	188	590
No. 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

[a]These data were obtained only in children less than 2 years old.

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

Phylogenetic Analysis

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

Distribution of hMPV Groups and Subgroups

Human metapneumovirus strains of different groups and subgroups were associated with each outbreak (Fig. 3a). However, there was often one dominant subgroup in each epidemic. Human metapneumovirus belonging to the A2 subgroup and B1 subgroup were predominant during the winters of 2002–2003 and 2004–2005, respectively (Fig. 3b). For both these periods, two strains (Cae03-6-31 and Cae05-40-27) were more prominent and represented greater than 50% of the isolated hMPV. During the winter of 2003–2004, the four subgroups were present equally (Fig. 3c).

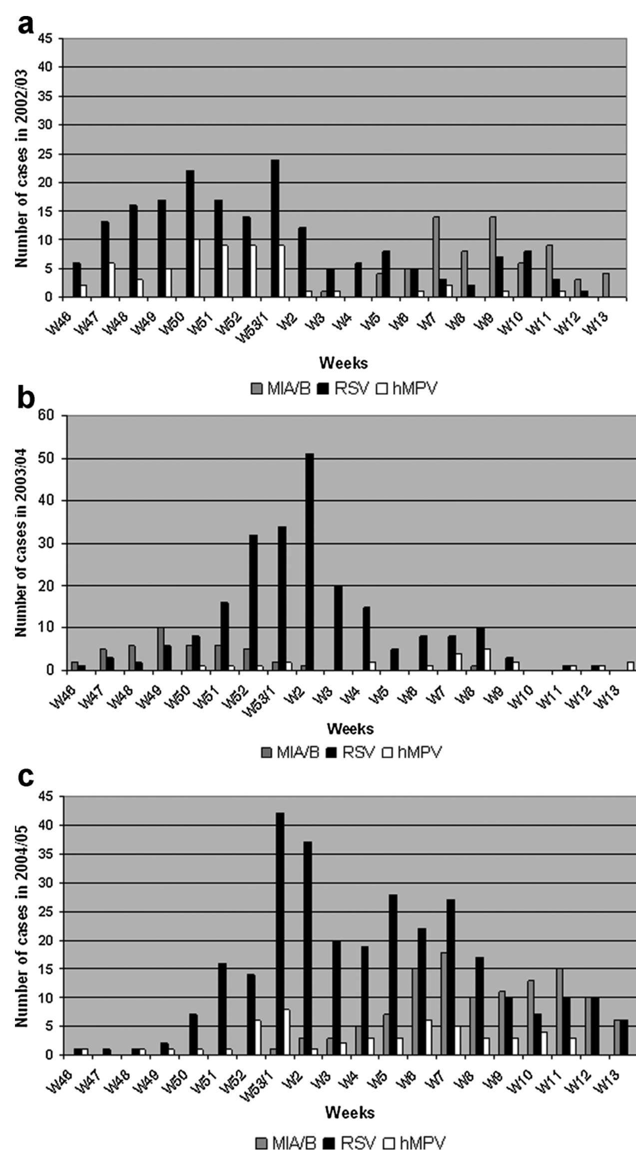

Fig. 1. Weekly comparison of the outbreak of hRSV, influenza viruses A and B (MIA/B) and hMPV in a pediatric population. (**a**) From November to March, 2002–2003; (**b**) from 2003–2004; (**c**) from 2004–2005.

DISCUSSION

The present study reports on an epidemiological, clinical, and phylogenic study of hMPV outbreak during three consecutive winters in Normandy, France. Human metapneumovirus infection was assessed in nasal aspirates received in the laboratory during the months of November to March in order to perform a comparison of hMPV rate with the other common cold viruses. It was reported that, the hMPV infection season is longer than that of hRSV or influenza viruses [van den Hoogen et al., 2003; Esper et al., 2004; Mackay et al., 2006]. As such, the number of positive cases may have been underestimated although it was shown previously that hMPV circulates predominantly during the winter–spring period [Bastien et al., 2003a; van den Hoogen et al., 2003; Esper et al., 2004; Williams et al., 2004; Pitoiset et al., 2010]. The number and the percentage of hMPV detected in virus-negative samples during the epidemics of 2002–2003, 2003–2004, and 2004–2005 were 59 (10%), 17 (22.6%), and 52 (8.8%), respectively. The incidence of hMPV infections are reported in many other studies [Boivin et al., 2002; van den Hoogen et al., 2004; Williams et al., 2004]. It is also suggested that the number of hMPV infections is variable from one winter to another [Falsey et al., 2003; Mackay et al., 2006; Williams et al., 2006].

Human metapneumovirus was the fourth most prominent virus encountered during the present study and became the third most prominent if the results are focused only on children under 2 years old. Three-quarters of the hMPV were detected on children under 2 years old and more than one third on those less than 6 months old. Human respiratory syncytial virus was the most predominant virus observed with an increase in prevalency in children under 2 years old. Influenza A virus was more commonly encountered in the population of over 2 years old. Unlike the hMPV and the other isolated viruses, the influenza A and B viruses show a distinct prevalence between children under 2 years old and those over 2 years old.

In comparison with hRSV and influenza viruses A and B, the outbreak of hMPV occurs over the same period and appears to be closely related to the hRSV epidemic

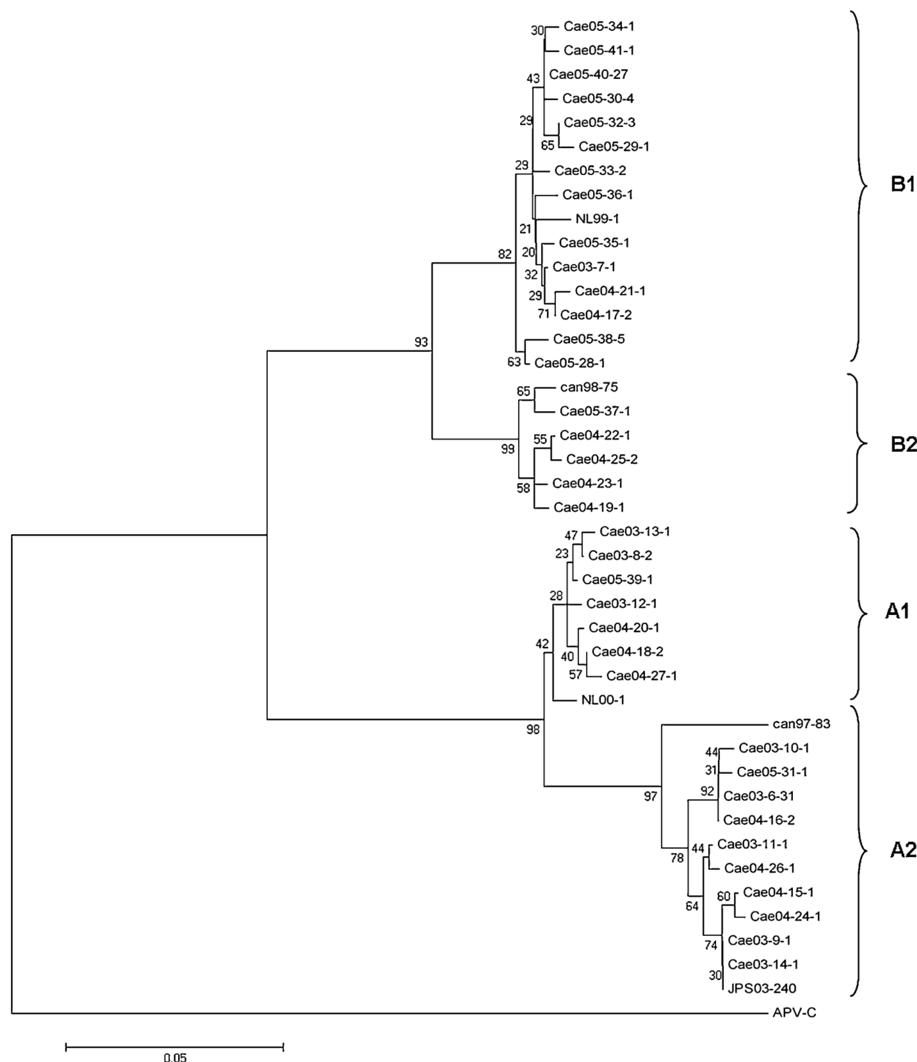

Fig. 2. Phylogenic analysis performed for the M gene for 107 sequenced hMPV isolates in Normandy during three consecutive winters. The tree was built with Mega 3.1 software using the neighbor joining method. Bootstrap values (1,000 replicates) are reported on branch nodes of the tree. Four genotypes are indicated. The strains obtained in Normandy are written as follows: Cae-isolation winter (A strain isolated during the winter 2002–2003 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).

profile. In fact, hMPV and hRSV outbreaks showed the same variation during the winter months [Boivin et al., 2003; Esper et al., 2004]. The influenza epidemic profile, which might be a more independent entity, did not correlate with hMPV or hRSV. During these three winters, the influenza A virus is more prominent than influenza B virus and the percentage was appreciatively the same for that described in Europe [Paget et al., 2003; Meijer et al., 2006].

Only the negative samples were taken because a low percentage of co-infection was reported previously [Peiris et al., 2003; van den Hoogen et al., 2003; Mullins et al., 2004] and thus, even if this strategy introduced a selection bias, it allows a more reliable evaluation of the clinical impact of hMPV in affected children. Unlike upper respiratory tract infections, human metapneumovirus infection is normally associated with an acute LRI as observed in the present study and in other previous reports [Boivin et al., 2002; Freymuth et al., 2003; Williams et al., 2004]. Indeed, some patients present with only gastroenteritis in the absence of altered respiratory signs. No difference in severity of clinical manifestation was observed between the infection with group A or group B.

Cultivation of hMPV is very difficult to achieve and, at the start of the present study, no DFA test was available commercially. The RT-PCR was designed for the M gene to detect hMPV and to have an equivalent sensitivity for both genotypes. The M gene appeared to be suitable for hMPV detection, since it has already been validated

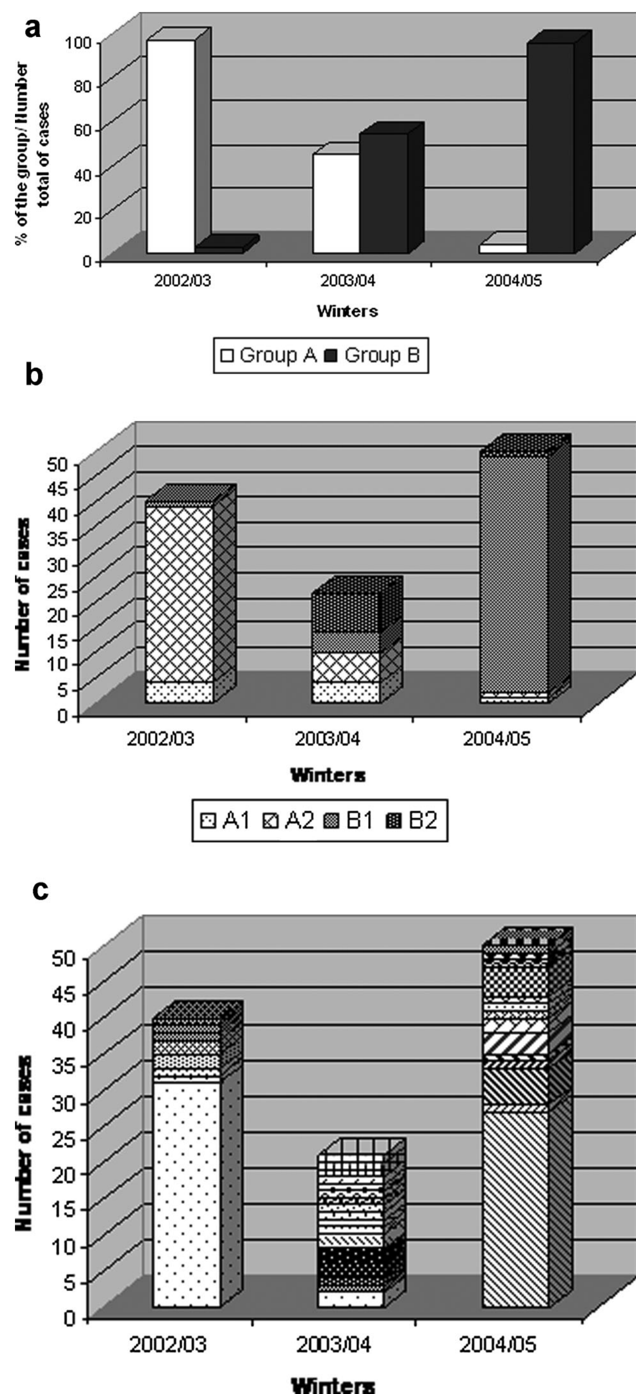

Fig. 3. Phylogenic evolution of hMPV strains detected in Normandy for three consecutive winter to spring periods. (**a**) Prominence of hMPV group A and B during each winter; (**b**) Strain repartition between subgroup A1, A2, B1, and B2 during each winter; and (**c**) hMPV sequence analysis for each outbreak.

[Bellau-Pujol et al., 2005; Chano et al., 2005] and a genetic discrimination has been determined [van den Hoogen et al., 2001; Bastien et al., 2003b; Carr et al., 2005; Chano et al., 2005]. Moreover, the M gene seems to be a good candidate for the detection of other viruses like influenza viruses or coronaviruses [Donofrio et al., 1992; Vabret et al., 2001]. This PCR appears to be adapted to hMPV detection and characterization since the four hMPV clusters described were amplified successfully [van den Hoogen et al., 2004; Mackay et al., 2004].

In the present study, four hMPV subtypes termed, "A1," "A2," "B1," and "B2" were detected by RT-PCR. The rate of the different subgroups was not the same for each winter and the strain composition varied during each outbreak. The strains circulating in France were described in four previous studies [Freymuth et al., 2003; Bouscambert-Duchamp et al., 2005; Foulongne et al., 2006; Pitoiset et al., 2010]. The first two studies reported only A1 and A2 subtypes of hMPV, while Foulongne et al. [2006] detected four subtypes of the virus with an A1 subgroup in predominance. The last study [Pitoiset et al., 2010] reports on a phylogenic analysis carried out on 100 strains isolated over 8 years, from 2002 to 2009 with alternative subgroup predominance. During the first winter 2002–2003, the A group was the most prevalent, with 87.5% of A2 subgroup being the most prominent. This repartition was also found in many other parts of the world [Esper et al., 2004; Mackay et al., 2004; Ludewick et al., 2005; Pitoiset et al., 2010]. Two studies described another pattern of circulation of the virus during the winter of 2002–2003 in which a prevalent B1 subgroup was noted [Gerna et al., 2005; Gray et al., 2006]. For the winter of 2003–2004, no predominant group could be identified. The incidence of groups A and B were approximately the same, although the number of strains belonging to group B and A were nine and eight, respectively. The B2 and A2 subgroup were the most frequently identified with five strains for each cluster. During this same period in Italy (winter of 2003–2004), the A2 subtype was also the most prominent strain, even if other strains were present [Sarasini et al., 2006; Gerna et al., 2005]. The rate of occurrence of group B was confirmed during the winter of 2004–2005 and represented 96% of all hMPV detected, including one hMPV B2 strain. Over the three winter periods, 46.5% of B1, 37.5% of A2, 8% of A1, and 8% of B2 strains were observed. These data are closely related to those proposed by Mackay et al. [2006] in a study carried out in Australia during the same period. In addition, both predominant subgroups found in 2002 and 2004 were the same (viz., A2 and B1).

The RNA fragment studied is small (405 bp), but some mutations that were found in this gene allowed us to discriminate between, and compare, the different isolates. The sequence analysis for the three epidemic periods showed two distinct genetic profiles. The first profile, found during the winter of 2002–2003 and 2004–2005, seemed to be composed of two predominant strains showing exactly the same sequence (Cae03-6-31 and Cae05-40-27) which represented at least 52% of hMPV detected, increasing to 79.5% in 2002–2003. During these two winters (2002–2003 and 2004–2005), the foremost strains belonged to the A2 and B1 subgroups, respectively. This finding corroborates previous observations [Mackay et al., 2006]. For the winter period 2003–2004, the isolated strains belonged

to the four subgroups and, at the most, two identical sequences were found. Even if it is impossible to consider these isolates like different strains because the analysis was assessed on too short a RNA fragment, such heterogeneity was not observed during the other winter periods and might be considered as the beginning of a shift from group A to group B as confirmed by the data of 2004. A shift of this nature was reported previously [Agapov et al., 2006; Mackay et al., 2006; Oliveira et al., 2009]. However, no evolution can be established from the heterogenic composition of hMPV infection during the winter of 2003–2004 to the subgroup B1 predominance during the winter of 2004–2005. The B1 strain responsible for 52% of hMPV infection during the winter of 2004–2005 was not found during the previous winter of 2003–2004.

Despite the fact that the 2003–2004 study is incomplete and that the period studied was only from November to March, the rate of hMPV can still be evaluated each year and the molecular evolution of the viruses can be analyzed. Human metapneumovirus is circulating in Normandy at a rate equivalent to that described previously [Peret et al., 2002; Bastien et al., 2003a; Boivin et al., 2003; Esper et al., 2004]. The present clinical study demonstrates that hMPV is responsible for LRI in the majority of cases studied. All the reported hMPV subgroups are present in France but not with the same incidence each year. The results confirm the genetic evolution of hMPV in Normandy with a switch from the A group to the B group being noted during the winter of 2003–2004. Finally, molecular tools developed for the M gene appear to be useful for diagnosis and molecular analysis, since the four subgroups are detected and can be discriminated between in a significant manner.

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