From November 2004 to April 2007, specimens were obtained from 2,281 patients with acute respiratory tract illness in Normandy, France. Eighteen strains of influenza C virus were detected in these samples using a combined tissue culture/RT-PCR diagnostic method. Most patients with influenza C virus infection (13/18) were infants or young children (<2 years of age). The most frequent symptoms were fever and cough, and the clinical presentation of influenza C virus infection was similar to that of other respiratory viruses. Thirteen of the 18 infected patients were hospitalized; 3 presented with a severe lower respiratory infection. The hemagglutinin-esterase (HE) gene of 10 isolates was sequenced to determine the lineages of the circulating influenza C viruses. Phylogenetic analysis revealed that most of the isolated strains had an HE gene belonging to the C/Yamagata/26/81-related lineage. These results show that influenza C virus regularly circulates in Normandy and generally causes a mild upper respiratory infection. Because the differential clinical diagnosis of influenza C virus infection is not always easy, it is important to identify viral strains for both patient management and epidemiological purposes. J. Med. Virol. 80:1441–1446, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: respiratory infection; multiplex RT-PCR assay; phylogenetic analysis

INTRODUCTION

Influenza C virus usually produces upper respiratory tract infections in children or young adults [Manuguerra et al., 1992; Greenbaum et al., 1998]. Some lower respiratory infections caused by influenza C, such as bronchitis or pneumonia, have also been reported [Moriuchi et al., 1991; Calvo et al., 2006]. Serological findings indicate that the influenza C virus is distributed widely throughout the world, and that most people acquire antibodies to the virus early in life [Dykes et al., 1980; Homma et al., 1982; Manuguerra et al., 1992]. There are few studies concerning influenza C virus infection in Europe, both because few laborato-
distribution of the tested patients was as follows: 1,095 patients (48%) were <2 years of age, 388 patients (17%) were 2–5 years of age, 294 patients (13%) were 6–20 years of age, and 504 patients (22%) were >20 years of age. All patients, or their parents or legal guardians, consented to have their samples tested for respiratory viruses, including the influenza C virus. The clinical data of influenza C virus-infected patients were obtained retrospectively.

**Virus Isolation and Identification: Multiplex RT-PCR**

The collected samples were placed in 4 ml of viral transport medium. Samples were tested for influenza virus A and B, respiratory syncytial virus (RSV), parainfluenza virus 1, 2, 3, and 4, and adenovirus, using a direct immunofluorescence assay (IFA) with monoclonal antibodies. A total of 2,281 IFA-negative samples were inoculated onto a continuous epithelial cell line derived from a human hepatocarcinoma (HuH7 cells), as described previously [Freymuth et al., 2005; Vabret et al., 2006]. By day 4 post-infection, the HuH7 cultures showing extensive lysis were analyzed using amplification techniques. The RNA was extracted from 100 μl of HuH7 supernatant using the QIAamp RNA viral Minikit (QIAGen, Courtaboeuf, France) according to the manufacturer’s protocol. A multiplex RT-PCR method was used for the detection of influenza C virus as well as human rhinovirus and human coronavirus OC43 and 229E, according to a procedure described previously [Bellau-Pujol et al., 2005]. The RT-PCR amplification was performed using the OneStep RT-PCR kit (QIAGen) using primers that target specifically the influenza C virus HE gene (Table I). The amplification products of the influenza C virus HE gene were 485-bp length, and were visualized under UV light after electrophoresis on an ethidium bromide-stained 2% agarose gel.

**Nucleotide Sequencing and Phylogenetic Analysis**

The HE genes of 10 influenza C virus strains were sequenced. Amplification was performed in two segments using four published primers [Kimura et al., 1997] (Table I). Briefly, viral RNA was extracted from the influenza C virus-infected respiratory specimens using the QIAamp Viral RNA Minikit (QIAGen, Courtaboeuf, France). The viral RNA was then amplified in two RT-PCR reactions using the OneStep RT-PCR kit (QIAGen). These reactions were performed in a 25 μl final reaction volume containing 2.5 μl RNA extract, 5 μl of OneStep RT-PCR Buffer, 1 μl of 10 mmol/L deoxy-nucleoside triphosphate (dNTPs), 1 μl OneStep RT-PCR Enzyme Mix, and 1.2 μl of 10 μmol/L reverse and forward primers. The reaction was carried out in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA) with an initial reverse transcription step at 50°C for 30 min, followed by 40 cycles of amplification (30 sec at 95°C, 30 sec at 53°C, 1 min at 72°C) and a final extension step at 72°C for 10 min. Each RT-PCR test included water controls that were treated identically to the specimens throughout; reactions were performed with care to avoid cross-contamination. The resulting PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced using the ET Dye Terminator Sequencing kit on a MegaBACE 1000 automatic sequencer (GE Healthcare, Buckinghamshire, UK). The region from nucleotide 64 to 1,792 was analyzed. BioEdit software (Tom Hall, Ibis Biosciences, Carlsbad, CA) was used for nucleotide sequence alignments. The sequences of the 10 influenza C virus HE genes were analyzed along with 18 sequences that were published previously [Buonaguro et al., 1985; Matsuzaki et al., 1994, 2000, 2002, 2003, 2007; Muraki et al., 1996, 2004]. The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were determined by 1,000 replicates using the Phylowin software [Galtier et al., 1996].

**Nucleotide Sequence Accession Numbers**

The 10 nucleotide sequences determined in this study were submitted to the Genbank database and were assigned accession numbers EU086908 to EU086917.

**RESULTS**

**Characterization of Influenza C Virus-Infected Patients**

Influenza C virus was detected in 18 of the 2,281 respiratory specimens collected from November 2004 to April 2007. The age distribution of influenza C virus-infected patients was as follows: 13 were <2 years of age, the youngest being 4 months old; two patients were

**TABLE I. Nucleotide Sequences of Oligonucleotide Primers Used in the Multiplex RT-PCR Experiments and in the Two RT-PCR Sequencing Experiments**

| Primers in influenza C virus | Sequence (5'-3') | References |
|-----------------------------|------------------|------------|
| Multiplex RT-PCR            |                  |            |
| CHAA                        | ACA CTT CCA ACC CAAT TTT GG | Bellau-Pujol et al. [2005] |
| CHAD                        | CCT GAC AGC AAC TCC CTC AT | Bellau-Pujol et al. [2005] |
| Nucleotide sequencing RT-PCR no. 1 |                  |            |
| MIC1S                       | ATA ATG TTT TTC TCA TTA CT | Kimura et al. [1997] |
| MIC1AS                      | CAA CAT GCA CCC TGG AGT GC | Kimura et al. [1997] |
| Nucleotide sequencing RT-PCR no. 2 |                  |            |
| MIC2S                       | GTC ACT GCT GTC CAA | Kimura et al. [1997] |
| MIC2AS                      | CCC AGT AAA AAG GAT CTG | This study  |

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2–5 years of age; one patient was 6–20 years of age; and two patients were >20 years of age, the oldest being 74 years old. Thirteen of the 18 influenza C virus-positive samples were from hospitalized patients and 5 were from patients seen by general practitioners. Three patients were co-infected with a rhinovirus. The seasonal distribution of influenza C virus isolates is shown in Figure 1. Positive specimens were collected principally during the winter months, from December to April. Only three strains were recovered during the summer period.

Clinical Manifestations

The medical records of 14 influenza C virus-infected patients were examined retrospectively; the records of 4 were unavailable. There was no difference in the clinical symptoms of hospitalized and non-hospitalized patients. The most frequent symptoms were fever (93%, \( n = 13 \)) and cough (71%, \( n = 10 \)). Maximum body temperatures were 38–38.9°C in six patients and 39–39.9°C in eight patients. The duration of hospitalization was less than 24 hr for three patients, from 2 to 4 days for seven, and up to 5 days for four patients. All influenza C virus-infected patients had a clinical diagnosis of flu, and there were no symptoms that distinguished influenza C virus infection from influenza A or B virus infection. Three influenza C virus-infected patients had a lower respiratory tract illness. One 3-year-old patient with a family history of atopic allergy presented with an acute exacerbation of asthma. Pneumonia was reported in one cystic fibrosis patient (age 21 months), and bronchiolitis was reported in a 10-month-old infant without any other associated pathology. Gastrointestinal symptoms were present in 5 of the 18 influenza C virus-infected patients (28%). Rotavirus was isolated from the feces of two infected patients with diarrhea, and in one case the patient had both adenovirus and rotavirus infections. In the two other infected patients with gastrointestinal symptoms, no other infectious agent was recovered from the feces. These data are summarized in Table II.

Phylogenetic Analysis of the Influenza C Virus Isolates

The sequence of the HE gene (nucleotides 64–1,792) was determined for 10 of the 18 isolates (CaenBO1/2004, CaenJO2/2004, CaenME1/2005, CaenHE2/2005, CaenMA3/2005, CaenCA4/2005, CaenMA5/2005, CaenBR1/2006, CaenLE2/2006, and CaenBA1/2007). A phylogenetic tree was constructed using these 10 sequences as well as 18 sequences that were published previously [Buonagurio et al., 1985; Matsuzaki et al., 1994, 2000, 2002, 2003, 2007; Muraki et al., 1996; Muraki et al., 2004]. As shown in Figure 2, the HE gene of the influenza C viruses has been classically divided into six lineages, represented by C/Taylor/47, C/Aichi/1/81, C/Sao Paulo/378/82, C/Kanagawa/1/76, C/Yamagata/26/81, and C/Mississippi/80. Phylogenetic analysis of the HE genes revealed that the 10 sequenced strains were clustered in two lineages. The two strains isolated in 2004 (CaenBO1/2004 and CaenJO2/2004) were located within the C/Yamagata/26/81-related lineage, and the other eight strains, which were isolated from 2005 to 2007 (CaenME1/2005, CaenHE2/2005, CaenMA3/2005, CaenCA4/2005, CaenMA5/2005, CaenBR1/2006, CaenLE2/2006, and CaenBA1/2007), were located within the C/Sao Paulo/378/82-related lineage. The two strains isolated in 2004 had 100% sequence identity. The nucleotide sequences of the HE genes of the eight 2005–2007 isolates were similar or identical, with sequence identity ranging from 99.3% to 100%. The nucleotide sequence identity between the two 2004 isolates and the eight 2005–2007 isolates ranged from 94.0% to 94.2%.

DISCUSSION

Seroepidemiological studies of influenza C virus infection indicate that most people acquire antibodies to influenza C early in life, and up to 80% of humans have antibodies to the influenza C virus by age 7–10 years [O’Callaghan et al., 1980; Homma et al., 1982;
TABLE II. Clinical Features Associated With Influenza C Virus Infection

| Symptom or sign                | Hospitalized patients (n = 12) | Non-hospitalized patients (n = 2) | Total (n = 14) |
|-------------------------------|--------------------------------|----------------------------------|----------------|
| Fever                         | 11                             | 2                                | 13             |
| Cough                         | 8                              | 2                                | 10             |
| Rhinitis                      | 6                              | 2                                | 8              |
| Pharyngitis                   | 7                              | 1                                | 8              |
| Wheeze/stridor                | 2                              | 0                                | 2              |
| Pneumonic infiltration        | 1                              | 0                                | 1              |
| Otitis                        | 1                              | 0                                | 1              |
| Difficulty feeding            | 6                              | 0                                | 6              |
| Vomiting                      | 4                              | 1                                | 5              |
| Diarrhea                      | 4                              | 0                                | 4              |

Fig. 2. Phylogenetic tree of influenza C viruses based on a 1,729-bp region of the HE gene. Viruses isolated in the present study (Caen, France) are marked with asterisks. The other sequences have been described elsewhere [Buonagurio et al., 1985; Matsuzaki et al., 1994, 2000, 2002, 2003, 2007; Muraki et al., 1996, 2004]. The numbers above the branches are the bootstrap values of each branch (%).
Influenza C Virus Infection in France

Nishimura et al., 1987]. Despite its extensive circulation, influenza C virus infection in the population is rarely documented. This is likely due both to its mild viral pathogenicity and to the difficulty in detecting it. The first seroepidemiological study carried out in France indicated that 61–70% of the tested population had been exposed to the virus [Manuguerra et al., 1992]. RT-PCR for the detection of influenza C virus has been described elsewhere [Claas et al., 1992; Hirsiela et al., 2001]. Recently, the multiplex RT-PCR assay has been used to detect several respiratory viruses simultaneously [Eugene-Ruellan et al., 1998; Coiras et al., 2003; Templeton et al., 2004; Bellau-Pujol et al., 2005]. In this study, multiplex RT-PCR assay was combined with a technique that isolates influenza C virus in infected HuH7 cell supernatant [Freymuth et al., 2005]. During a 30-month survey, 18 influenza C virus isolates were found in 2,281 IFA-negative respiratory samples. These isolates were detected in both hospitalized and non-hospitalized patients, principally in young infants but also in some adults. The isolation rate of influenza C virus in acute respiratory infections in this study was very low (0.79%), in agreement with other reports [Moriuchi et al., 1991; Calvo et al., 2006; Matsuzaki et al., 2006]. Most of the influenza C virus infections were detected during the winter, similar to outbreaks of influenza A or B viruses; however, we also confirmed the existence of summer infections, as reported previously by others [Katagiri et al., 1983; Moriuchi et al., 1991; Greenbaum et al., 1998; Matsuzaki et al., 2002].

Coinfections with other respiratory viruses such as RSV, adenovirus, or the influenza A or B viruses are often associated with influenza C virus infection [Matsuzaki et al., 2006; Calvo et al., 2006]. This study did not assess coinfection with RSV, adenovirus, or influenza A or B, but the multiplex RT-PCR carried out using HuH7 cell supernatant found three coinfections with rhinovirus. These coinfections were not associated with more severe symptoms. Previous studies showed that the rate of viral coinfection among rhinovirus infections may be high [Guittet et al., 2003; Bellau-Pujol et al., 2005], probably because this virus can persist in the respiratory tract for more than 2 weeks after the acute phase [Jartti et al., 2004].

Medical records were available for 14 infected patients and were used to determine the clinical features of influenza C virus infection. The high fever and upper respiratory symptoms in patients in this study were similar to those described in other studies [Katagiri et al., 1983; Moriuchi et al., 1991; Matsuzaki et al., 2006]. The clinical symptoms of influenza C virus infection are not different from those observed in influenza A and B infections. However, a recent study reported a lower maximum temperature and a shorter duration of fever in influenza C virus infections compared to influenza A virus infections [Matsuzaki et al., 2006].

Although the majority of influenza C infections appeared to be mild and were associated with no or short hospitalization time, three cases of severe lower respiratory infections (bronchiolitis and pneumonia) were observed in this study. These severe infections were in infants presenting with a chronic pulmonary disease. Other authors have also reported severe lower respiratory complications in patients infected with the influenza C virus [Moriuchi et al., 1991; Calvo et al., 2006]. Digestive problems were noted in approximately one third of the patients in this study. The role of influenza C virus as a causative agent of diarrhea is difficult to establish because of the high incidence of coinfections with rotavirus and/or adenovirus. To date there is no evidence that influenza C virus can replicate in enteric tract tissue. However, Matsuzaki et al. [2006] also described a high incidence of gastrointestinal symptoms in influenza C virus-infected children <6 years old.

The HE gene of the influenza C virus was categorized previously into six genetically distinct groups of viruses represented by the following strains: C/Taylor/47, C/Kanagawa/1/76, C/Yamagata/26/81, C/Aichi/1/81, C/Sao Paulo/378/82, and C/Mississippi/80. Genetic analysis of the HE gene of the 10 influenza C virus strains sequenced in this study revealed that viruses belonging to two of these groups were co-circulating in Normandy from 2004 to 2007: the C/Yamagata/26/81-virus group in 2004, and the C/Sao Paulo/378/82-virus group from 2005 to 2007. The presence of a dominant genetic group in a community for several years, and its replacement by another dominant group, was reported previously in Japan [Matsuzaki et al., 2003]. It would be interesting to analyze retrospectively respiratory samples from an earlier period in Normandy in order to define their lineages, and also to study whether influenza C viruses with similar genetic composition spread in other areas of France during the same time.

To summarize, the influenza C virus can be found in patients with both upper and lower respiratory tract illness. This is the first survey of influenza C virus infection in Normandy, France, and the data suggest that influenza C virus detection should be included in the routine panel for diagnosing viral infections in clinical respiratory samples. Future studies will yield more accurate information about the circulation of the influenza C virus in France, and viral detection should be expanded to additional areas and to samples taken from patients seen by general practitioners.

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