Isolation and Characterization of Influenza C Viruses in the Philippines and Japan

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From November 2009 to December 2013 in the Philippines, 15 influenza C viruses were isolated, using MDCK cells, from specimens obtained from children with severe pneumonia and influenza-like illness (ILI). This is the first report of influenza C virus isolation in the Philippines. In addition, from January 2008 to December 2013, 7 influenza C viruses were isolated from specimens that were obtained from children with acute respiratory illness (ARI) in Sendai city, Japan. Antigenic analysis with monoclonal antibodies to the hemagglutinin-esterase (HE) glycoprotein showed that 19 strains (12 from the Philippines and 7 from Japan) were similar to the influenza C virus reference strain C/Sao Paulo/378/82 (SP82). Phylogenetic analysis of the HE gene showed that the strains from the Philippines and Japan formed distinct clusters within an SP82-related lineage. The clusters that included the Philippine and Japanese strains were shown to have diverged from a common ancestor around 1993. In addition, phylogenetic analysis of the internal genes showed that all strains isolated in the Philippines and Japan had emerged through reassortment events. The composition of the internal genes of the Philippine strains was different from that of the Japanese strains, although all strains were classified into an SP82-related lineage by HE gene sequence analysis. These observations suggest that the influenza C viruses analyzed here had emerged through different reassortment events; however, the time and place at which the reassortment events occurred were not determined.

Influenza C virus usually causes mild upper respiratory illness, but it can also cause lower respiratory infections, such as bronchitis and pneumonia (1). Seroepidemiological studies have revealed that influenza C virus is widely distributed throughout the world (2–6), and recurrent infection with this virus occurs frequently in children and adults (7). However, the virus has been isolated by cell culture only occasionally, and long-term monitoring of influenza C viruses is rarely conducted. The monitoring of influenza C virus among children in the Yamagata and Miyagi Prefectures in Japan since 1988 has revealed that outbreaks of influenza C virus occur in winter or early summer at 1- or 2-year intervals (8–10). Influenza C virus infections, detected using molecular detection methods, have recently been reported in several countries, including Spain, France, Cuba, Canada, Italy, India, and Finland (7, 11–16). A serological study conducted in the Philippines in 1984 indicated the existence of influenza C viruses, but the viruses themselves were not detected.

The genome of influenza C virus consists of seven RNA segments that encode three polymerase proteins (polymerase basic 2 [PB2], PB1, and polymerase 3 [P3]), hemagglutinin–esterase glycoprotein (HE), nucleoprotein (NP), matrix protein (M), CM2 protein, and two nonstructural proteins (nonstructural 1 [NS1] and NS2). Antigenic variation exists among influenza C virus isolates, as demonstrated by antigenic analysis with anti-HE monoclonal antibodies (MAbs) (17–19). However, analysis with polyclonal immune sera has shown a high degree of cross-reactivity among all the isolates examined so far (17, 19–21), indicating that the influenza C virus is antigenically more homogenous than are the human influenza A and B viruses. Early studies analyzing the molecular characteristics of isolates have suggested that influenza C virus epidemiology might be characterized by the presence of multiple lineages (22, 23). Antigenic and sequence analyses of the HE gene revealed the existence of six lineages, which are represented by C/Taylor/1233/47 (Taylor/47), C/Kanagawa/1/76 (KA176), C/MISSISSIPPI/80 (MS80), C/Aichi/1/81 (A1181), C/Yamagata/26/81 (YA2681), and C/Sao Paulo/378/82 (SP82) (19), and influenza C viruses belonging to different lineages can cocirculate in a single community (10, 17). Thus, mixed infections with influenza C viruses belonging to different lineages may occur in a single host, resulting in the emergence of reassortant viruses, characterized by the exchange of genomic segments between two different strains (19, 24).

Long-term surveillance studies carried out in the Yamagata and Miyagi Prefectures in Japan also revealed that reassortment between viruses of different lineages had occurred frequently, and newly emerged reassortant viruses had replaced previously circulating viruses (10). The virus that is antigenically similar to KA176, which reemerged in the Miyagi Prefecture in 1996, for the first

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time in 20 years, and subsequently spread throughout Japan, ac-
quired its internal genes from the previous epidemic virus, which
belongs to the YA2681 lineage, through a reassortment event (10).
These observations indicate that the genomic compositions of in-
fluenza C viruses may affect their ability to spread among humans,
and reassortment events can be a means of evolution for influenza
C viruses.

From 2011 to 2013, we isolated influenza C viruses from cases
with severe pneumonia and influenza-like illness (ILI) in the Phil-
ippines, for the first time. We also isolated influenza C viruses
through acute respiratory illness (ARI) surveillance conducted in
Sendai city, Miyagi, Japan, from 2008 to 2013. In this study, we
analyzed the influenza C strains collected in the Philippines and
Japan to characterize the circulating influenza C viruses in these
two countries. This characterization included a sequence analysis
of all seven RNA segments.

MATERIALS AND METHODS

Virus isolation. We conducted two prospective studies of respiratory vi-
ruses in the Philippines. One of these studies is a pediatric pneumonia
study conducted at Eastern Visayas Regional Medical Center (EVRMC)
on Leyte Island, since January 2010. Beginning in August 2012, the pedi-
atrian pneumonia study was expanded to include Biliran Provincial Hos-
pital (BPH) in Naval on Biliran Island and Osipalng Palawan (ONP) in
Puerto Princesa City on Palawan Island (see Fig. S1 in the supplemental
material). The other is an ILI study conducted in the outpatient clinics
of three medical facilities on Leyte Island: Leyte Provincial Hospital (LPH),
Tacloban City Health Catchment Center (TCHCC), and Tanauan Rural
Health Unit (TRHU), since November 2009. Nasopharyngeal swab spec-
imens were collected, according to the Integrated Management of Child-
hood Illness (IMCI) guideline, from hospitalized children with a clinical
diagnosis of severe pneumonia, and from the patients who visited the
outpatient clinics of LPH, TCHCC, and TRHU, with ILI. ILI was defined as
a fever of ≥38°C or feverish and either cough or nasal discharge. The
details of the study design have already been described (25).

From November 2009 to December 2013, a total of 5,343 nasopharyn-
geal swab specimens were collected from children with severe pneumonia
or ILI (age range, 0.0 to 14.9 years; mean age, 1.2 years), and the specimens
were transferred to the Research Institute of Tropical Medicine (RITM),
Metro Manila, the Philippines, for virus isolation. All specimens were
inoculated into Madin–Darby canine kidney (MDCK), HEP-2, Vero E6,
and human fetal lung fibroblast cells by using the microplate method for
isolating viruses (26). These cell lines were used to detect common respi-
atory viruses, such as influenza A, B, and C viruses, enteroviruses, human
metapneumovirus (hMPV), respiratory syncytial virus (RSV), and hu-
man adenosviruses (HADV). When cytopathic effects (CPE) were observed
in the MDCK cells, the culture supernatant was tested with a hemagglu-
tination (HA) test using chicken or turkey erythrocytes. Influenza C virus
causes agglutination in chicken or turkey erythrocytes but does not cause
agglutination of guinea pig cells (27). If HA tests were positive with
chicken or turkey erythrocytes but negative with guinea pig erythrocytes,
influenza C virus isolation was suspected. Next, the presence of influenza
C virus in these isolates was confirmed by reverse transcription-PCR (RT-
PCR) using an influenza C virus-specific primer (28).

We also conducted an ARI study at the outpatient pediatric clinic from
January 2008 to December 2013 in Sendai city, Japan (see Fig. S2 in the
supplemental material). A total of 1,845 nasopharyngeal and throat swab
specimens were collected from children with ARI (age range, 0.1 to 14.9
years, mean age, 5.3 years), and the samples were transferred to Tohoku
University Graduate School of Medicine (Sendai, Japan) for virus iso-
lation. The isolation of influenza C virus was performed using same method
that was used in the studies in the Philippines.

Hemagglutination inhibition test for antigenic analysis. All hemagglu-
tination inhibition (HI) tests were performed using isolates that were
propagated in embryonated hen eggs, because previous research has
shown that viruses with high HA titers can be obtained from embryonated
eggs without an alteration in antigenicity (29). Therefore, clinical speci-
mens testing positive for influenza C virus were reisolated into the
amniotic cavities of 9-day-old embryonated hen eggs, and amniotic
fluid was used in the following analysis. Four previously characterized anti-HE
Mabs (J14, Q5, U4, and MS2) (30, 31) were used for antigenic analysis
during HI testing. Briefly, 50 μl of virus suspension (8 HA units/50 μl)
was added to each well of a microtiter plate containing 50 μl of 2-fold-
diluted Mabs. After incubation for 30 min at room temperature, 100 μl of
0.5% chicken erythrocytes was added to each well, and the plates were
stored at 4°C for 60 min. The HI titer was expressed as the reciprocal of
the highest antibody dilution that completely inhibited hemagglutination (9).

Nucleotide sequencing and phylogenetic analysis. Nucleotide se-
quencing and analyses were carried out as previously described (9, 10).
Briefly, viral RNA was extracted from 100 μl of the virus-containing am-
niotic fluid using the RNeasy minikit (Qiagen, Hilden, Germany). Viral RNA
was then transcribed into cDNA with a primer complementary to
positions 1 to 12 at the 3′ end of the RNA containing all of the influenza C
virus RNA segments (18). Using this synthesized cDNA as a template,
the individual segments were amplified using gene-specific primers (18).
The coding region of the HE gene, corresponding to nucleotide positions 64 to
1989, was sequenced using the BigDye Terminator version 3.1 kit (Ap-
plied Biosystems, Foster City, CA) and an ABI Prism 3130 sequencer
(Applied Biosystems). In addition to the HE gene, the partial nucleotide
sequences of the PB2, PB1, P3, and NP genes, as well as the complete
coding region of the M and NS genes, were determined. The oligonucleo-
tide primers used for sequencing were described previously (10, 18, 32,
33). The sequence data were analyzed with the MEGA software (version
5.1), and phylogenetic trees of the individual genes were constructed using
the neighbor-joining method (34), with p-distance as a substitution
model, and 1,000 bootstrapped replicates together with previously re-
ported sequences (9, 10, 19, 21, 24, 32, 35, 36), using the same software.
The divergence times for the influenza C viruses detected in the Philip-
ines and Japan were estimated using the Bayesian Markov chain Monte
Carlo (MCMC) approach implemented in the BEAST package version
1.8.1. Lognormal relaxed clock (uncorrected) was used with a tree prior of
Bayesian skyline, and the general-time-reversal (GTR) substitution model
with gamma-distributed and invariant sites was the site heterogeneity
model. The MCMC chains were run for two billion iterations, with sam-
pling at every 250,000 iterations, and the first 1,000 trees were discarded as
burn-in. The MCMC process was analyzed using TRACER version 1.5. A
maximum clade credibility tree was generated using FigTree version 1.3.1.

Nucleotide sequence accession numbers. The nucleotide sequences
determined in this study have been submitted to the DDBJ/GenBank da-
bases and assigned accession no. AB978548 to AB978566.

RESULTS

Isolation of influenza C viruses. A total of 15 (0.28%) strains of
influenza C virus were isolated from 5,343 specimens collected in the
Philippines between 2009 and 2013, including 6 strains in 2011
(6 of 1,324 [0.45%]) and 9 strains in 2013 (9 of 1,694 [0.53%]) (Fig. 1A).
Influenza C Virus was isolated as the single etiological
agent in 14 patients. However, respiratory syncytial virus was also
isolated from one patient with ILI, using HEP-2 cells. In Japan, 7
(0.38%) strains of influenza C virus were isolated from children
(age range, 0.9 to 7.9 years), including 5 strains in 2008 (5/358
[1.40%]) and 2 strains in 2012 (2/351 [0.57%]) (Fig. 1B). No other
respiratory viruses, such as influenza A and B viruses, enterovi-
ruses, hMPV, RSV, and HADV, were isolated from any of these
seven patients.

The clinical manifestations of influenza C virus-positive
patients are shown in Table 1. In the Philippines, 7 (of 3,118
[0.22%]) strains of influenza C virus were isolated from patients

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with severe pneumonia (age range, 0.4 to 5.5 years), and 8 (of 2,225 [0.36%]) were isolated from ILI patients (age range, 0.4 to 2.9 years) (Table 1). Among influenza C virus-positive children with severe pneumonia, chest indrawing was seen in all cases, while difficulty breathing and fever of >38°C were seen in 6 cases. Regarding the outcomes of these cases, six were discharged, and one refused admission. The primary symptoms seen in the influenza C virus-positive ILI cases in the Philippines and the ARI cases in Japan were cough, nasal discharge, and fever.

**Antigenic analysis of influenza C viruses isolated in the Philippines and Japan.** Among 15 positive specimens in the Philippines, only 12 specimens, including 5 specimens collected in 2011 and 7 in 2013, were available for reinoculation into the amniotic cavities of the embryonated eggs. The influenza C viruses were isolated from embryonated eggs inoculated with each of these 12 specimens. For the Japanese specimens, influenza C viruses were isolated from embryonated eggs inoculated with 7 specimens, including 5 from 2008 and 2 from 2012. The antigenicities of all 19 strains and the HI titers of reference strains belonging to five antigenic groups (KA176, MS80, AI181, YA2681, and SP82) are shown in Table 2. All the strains isolated in this study were highly reactive with MAb J14, Q5, and U4 but were unreactive or very weakly reactive with MS2. The reactivity patterns of all analyzed isolates were similar to that of SP82.

**Phylogenetic analyses of individual RNA segments of influenza C virus strains.** In order to confirm the results of the antigenic analyses, the sequences of the HE gene from each of the 19 strains (nucleotides 64 to 1989) were determined. Previous studies revealed that the HE genes of influenza C viruses could be classified into six discrete lineages, represented by Taylor/47, KA176, YA2681, AI181, SP82, and MS80. As shown in Fig. 2, all strains isolated in the Philippines and Japan were classified as members of the SP82-related lineage. The nucleotide sequences of the HE genes of strains isolated in the Philippines in 2011 and 2013 were highly homologous (99.3 to 100% nucleotide identity). The seven strains isolated in 2008 and 2012 in Japan were also highly homologous (99.3 to 100% nucleotide identity). The strains isolated in the Philippines and Japan formed distinct clusters within the SP82-related lineage; however, the sequences within this branch, including those from the Philippines and Japan, shared high sequence homology (98.2 to 98.8% nucleotide identity).

To determine the genomic compositions of the strains isolated in the Philippines and Japan, and to determine the occurrence of reassortment event(s), the nucleotide sequences of the internal genes were also determined for the 10 strains from the Philippines (C/Leyte/1/2011, C/Leyte/2/2011, C/Leyte/3/2011, C/Leyte/1/2013, C/Leyte/2/2013, C/Leyte/3/2013, C/Biliran/1/2013, C/Biliran/2/2013, C/Biliran/3/2013, and C/Palawan/1/2013) and the 6 strains from Japan (C/Sendai/TU1/2008, C/Sendai/TU2/2008, C/Sendai/TU3/2008, C/Sendai/TUS/2008, C/Sendai/TU1/2012, and C/Sendai/TU2/2012).
and C/Sendai/TU2/2012). As shown in Fig. 3, all of the 10 strains isolated in the Philippines that had an HE gene belonging to the SP82-related lineage were classified into the YA2681-related lineage based upon the phylogenetic trees constructed with PB1, M, and NS, but they were classified into the MS80-related lineage in the phylogenetic trees constructed with P3. Moreover, in the phylogenetic tree constructed using PB2, all strains from the Philippines were classified into the C/Pig/Beijing/115/81 (PB11581)-related lineage. On the other hand, all six strains isolated in Japan that had an HE gene belonging to the SP82-related lineage were classified into the YA2681-related lineage using trees constructed with the PB2 gene, as were the Philippine strains. Interestingly, in the phylogenetic trees constructed with the six internal genes, all the Philippine strains were closely related to C/Miyagi/9/96, C/Miyagi/2/2000, C/Saitama/3/2000, and C/Hiroshima/246/2000, which have HE genes belonging to the KA176-related lineage, and they were detected in Japan between 1996 and 2000 (10, 37). The genome compositions of the strains in this study, which were determined by phylogenetic analyses, are summarized in Table 3. These results confirmed that all the strains isolated in the Philippines and Japan are reassortant viruses, and the compositions of the internal genes of the Philippine strains are different from those of the Japanese strains.

The phylogenetic analyses revealed that different reassortment events occurred in the Philippine and Japanese strains. On the HE gene tree, the Philippine and Japanese strains formed distinct clusters within the SP82-related lineage. A Bayesian evolutionary tree of the HE gene was generated to determine when these clusters diverged from their last common ancestor. As shown in Fig. 4, the clusters that included the strains isolated in the Philippines and Japan diverged from their last common ancestor at an estimated node age of 1993.

**DISCUSSION**

This study is the first report of influenza C virus isolation in the Philippines. During this study period in the Philippines, 15 strains (0.28%) of influenza C virus were isolated from children with severe pneumonia and ILI. In contrast, seven strains (0.38%) of influenza C virus were isolated from children with ARI in Japan. The detection rates, which were similar in the Philippines and Japan, are also in agreement with those of other studies, which reported positive rates from 0.18 to 0.79% (1, 11, 13, 38). In the Philippines, although the overall influenza C virus detection rate was low, the detection rate in pediatric severe pneumonia cases (0.22%) was not significantly different than that in ILI cases (0.36%). The symptoms of hospitalized pediatric patients included chest indrawing and difficulty breathing, suggesting that

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**TABLE 2 Antigenic analysis of influenza C virus isolates, including representative strains, using the HI test performed with monoclonal antibodies against the HE**

<table>
<thead>
<tr>
<th>Antigenic group</th>
<th>Virus strain</th>
<th>HI titer of anti-HE MAbs:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>J14</td>
</tr>
<tr>
<td>KA176</td>
<td>C/Kanagawa/1/76</td>
<td>512,000</td>
</tr>
<tr>
<td>MS80</td>
<td>C/Mississippi/80</td>
<td>409,600</td>
</tr>
<tr>
<td>AI181</td>
<td>C/Aichi/1/81</td>
<td>1,024,000</td>
</tr>
<tr>
<td>YA2681</td>
<td>C/Yamagata/26/81</td>
<td>128,000</td>
</tr>
<tr>
<td>SP82</td>
<td>C/Sao Paulo/378/82</td>
<td>256,000</td>
</tr>
</tbody>
</table>

* HI, hemagglutination inhibition; HE, hemagglutinin-esterase. The strains isolated in this study are indicated in bold type.

* KA176, C/Kanagawa/1/76; MS80, C/Mississippi/80; AI181, C/Aichi/1/81; YA2681, C/Yamagata/26/81; SP82, C/Sao Paulo/378/82.

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Phylogenetic tree of influenza C virus hemagglutinin-esterase (HE) genes. The region between nucleotide positions 64 and 1989 was used for this analysis. The numbers above the branches are the bootstrap probabilities (%) for each branch, determined using the MEGA software (version 5.1). Each showed a value of >75%. The Philippine strains are marked with squares; the strains isolated in 2011 are blue, and the strains isolated in 2013 are purple. The Japanese strains are marked circles; the strains isolated in 2008 are green, and the strains isolated in 2012 are red.

FIG 2  Phylogenetic tree of influenza C virus hemagglutinin-esterase (HE) genes. The region between nucleotide positions 64 and 1989 was used for this analysis. The numbers above the branches are the bootstrap probabilities (%) for each branch, determined using the MEGA software (version 5.1). Each showed a value of >75%. The Philippine strains are marked with squares; the strains isolated in 2011 are blue, and the strains isolated in 2013 are purple. The Japanese strains are marked circles; the strains isolated in 2008 are green, and the strains isolated in 2012 are red.
FIG 3 Phylogenetic trees for the PB2 (A), PB1 (B), P3 (C), NP (D), M (E), and NS (F) genes of influenza C virus isolates. The nucleotide sequences of the following regions were used for analysis: nucleotide positions 52 to 520 for the PB2 gene, 50 to 425 for the PB1 gene, 49 to 420 for the P3 gene, 71 to 670 for the NP gene, 26 to 1147 for the M gene, and 28 to 889 for the NS gene. The numbers below or above the branches are the bootstrap probabilities (%) of each branch, determined using the MEGA software (version 5.1), and values of <75% are hidden. The Philippine strains are marked with squares; the strains isolated in 2011 are blue, and the strains isolated in 2013 are purple. The Japanese strains are marked with circles; the strains isolated in 2008 are green, and the strains isolated in 2012 are red.
Influenza C Virus in the Philippines and Japan

FIG 3 continued
influenza C virus may also cause severe lower respiratory tract infections in children.

The long-term surveillance of influenza C virus infections in Japan has revealed that biennial influenza C virus epidemics have occurred during the even-numbered years since 1996 (8–10). In the Philippines, influenza C virus was detected in 2011 and 2013, which suggests that epidemics of the virus may be occurring in odd-numbered years. A previous report revealed that seasonal
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peaks of influenza C are different from those of influenza A, and epidemics of influenza C usually occur after peaks of influenza A in Japan (8). In the Philippines, influenza A virus circulates almost throughout the year without clear seasonality (39). However, influenza C viruses were isolated mainly between January and July in the Philippines (Fig. 1). Therefore, there is a possibility that there is some seasonality for influenza C in the Philippines. Long-term studies should be conducted to define the epidemic interval and seasonality of influenza C virus infections in the Philippines.

Regarding antigenicity, all 19 strains detected in the Philippines and Japan belong to the SP82 antigenic group (Table 2), suggesting that antigenically similar influenza C viruses may be circulating throughout the region. Previous studies reported that the KA176 antigenic group and the SP82 antigenic group were dominant strains in Yamagata, Japan, between 2002 and 2004 and between 2006 and 2012, respectively (8, 40); also, the strains of the KA176 and SP82 antigenic groups were cocirculating in 2012. However, no strains belonging to the KA176 antigenic group were isolated in the Philippines and Japan during the study period.

For influenza C virus, the phylogenetic classification based upon the HE gene corresponds with the antigenic classification. Our results also confirmed that both antigenic phylogenetic analyses identified all isolates from the Philippines as SP82-like viruses. Influenza C viruses belonging to the KA176-related lineage were identified in Singapore in 2006. In Caen, France, it was reported that influenza C viruses detected from 2004 to 2007 were classified into two lineages, a YA2681-related lineage and an SP82-related lineage (11). Additionally, in 2011, an influenza C virus with an HE gene belonging to the YA2681-related lineage was detected in eastern India (16). The cocirculation of strains classified into the KA176-related and the SP82-related lineages have been reported in Catalonia, Spain (2009-2010 season) (13), Milan, Italy (2008-2009 and 2009-2010 seasons) (15), Alberta, Canada (2010-2011 season) (41), and Yamagata, Japan (2011-2012 season). In our study, all of the strains isolated in the Philippines and Japan were classified into the SP82-related lineage. In 2012, strains of the SP82-related lineage were also detected in Mie Prefecture, Japan (42). These studies suggest that similar influenza C viruses are circulating worldwide, and viruses classified into the SP82-related lineage may be increasing.

One of the aims of our study was to clarify the evolutionary process of influenza C virus through an analysis of reassortment events. A previous report demonstrated that most of the strains isolated in various regions of the world in the 1970s and 1980s had the same genomic compositions as those of contemporary Japanese strains and suggested the possibility that genetically similar influenza C viruses were circulating all over the world (37). Although all strains in the Philippines and Japan were classified into the SP82-related lineage based upon the phylogenetic tree constructed with the HE gene, the internal gene compositions of the strains detected in the Philippines and Japan were different (Table 3). This finding suggests that different reassortment events occurred for the strains detected in these countries. A Bayesian evolutionary tree of the HE gene revealed that the strains in the Philippines and Japan diverged from a common ancestor around 1993 (Fig. 4), suggesting that the strains isolated in the Philippines and

### TABLE 3 Genomic compositions of previous isolates and representative influenza C viruses isolated in this study

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Virus strain</th>
<th>RNA segment^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PB2</td>
</tr>
<tr>
<td>AI181-related</td>
<td>C/Aichi/181</td>
<td>A</td>
</tr>
<tr>
<td>MS80-related</td>
<td>C/Alabama/1980</td>
<td>M</td>
</tr>
<tr>
<td>YA2681-related</td>
<td>C/Yamagata/26/81</td>
<td>Y</td>
</tr>
<tr>
<td>KA176-related</td>
<td>C/Kanagawa/1/76</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>C/Miyagi/9/96</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Miyagi/2/2000</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Saitama/3/2000</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Hiroshima/246/2000</td>
<td>P</td>
</tr>
<tr>
<td>SP82-related</td>
<td>C/Sao Paulo/378/82</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>C/Leyte/1/2011</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Leyte/2/2011</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Leyte/3/2011</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Leyte/1/2013</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Leyte/2/2013</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Leyte/3/2013</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Biliran/1/2013</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Biliran/2/2013</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Biliran/3/2013</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Palawan/1/2013</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>C/Sendai/TU1/2008</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>C/Sendai/TU2/2008</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>C/Sendai/TU3/2008</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>C/Sendai/TU5/2008</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>C/Sendai/TU1/2012</td>
<td>K</td>
</tr>
<tr>
<td></td>
<td>C/Sendai/TU2/2012</td>
<td>P</td>
</tr>
</tbody>
</table>

^a Strains isolated in this study are indicated in bold type.
^b A, AI181-related lineage; S, SP82-related lineage; Y, YA2681-related lineage; K, KA176-related lineage; M, MS80-related lineage; P, PB11581-related lineage.
Japan evolved with reassortment events independently after divergence from a common ancestor. Interestingly, different strains identified in Catalonia, Spain, in 2009 were classified into different clusters that included Philippine and Japanese strains in the phylogenetic tree constructed with the HE gene, for example, C/Catalonia/1430/2009 in the Philippine cluster and C/Catalonia/1284/2009 in the Japanese cluster (Fig. 2). This suggests that viruses in these clusters might be circulating not only in Asia but also in other parts of the world.

The studies in the Philippines were carried out on three different islands. Although Leyte Island and Biliran Island are geographically close, Palawan Island is far from these islands (see Fig. S1 in the supplemental material). However, one strain isolated in Palawan Island in 2013 was highly homologous with the strains detected on Leyte Island and Biliran Island in all genes (Fig. 2 and 3). This suggests that an almost identical strain was circulating throughout the Philippines. In Japan, C/Sendai/TU2/2012 has a different internal genetic composition than those of the other strains isolated in our study (Table 3).
Although the bootstrap value of the internal gene tree was low, C/Sendai/TU2/2012 was assigned to different clusters in the trees constructed with the PB1 and P3 genes (Fig. 3B and C). Therefore, our observation suggests that multiple reassortment events might have occurred in the same population, which generated viruses with different gene compositions.

The biological significance of these reassortment events is unresolved. However, interestingly, the internal genomic composition of all of the Philippine strains was the same as that of the strains having HE genes belonging to the KA176-related lineage isolated in Japan between 1996 and 2000, such as C/Miyagi/9/96, C/Miyagi/2/2000, C/Saitama/3/2000, and C/Hiroshima/246/2000 (Table 3). The KA176-related lineage reemerged in 1996, for the first time in 20 years, by acquiring these internal genes through reassortment events. It had been dominantly circulating in Japan until 2004. This fact suggests that there is a possibility that this internal gene composition may confer some advantage for viral fitness. Reassortment might play a role in the acquisition of such advantageous internal genes.

In conclusion, we isolated influenza C viruses in both Japan and the Philippines. This was the first report of influenza C virus isolation in the Philippines. Epidemiological information for influenza C virus is still limited, especially in tropical countries. Therefore, our data are a valuable contribution to our understanding of the epidemiology of influenza C virus in the tropics. However, long-term monitoring of the virus is necessary to define the etiological significance and epidemiology of influenza C virus in the Philippines. We also revealed that strains in the Philippines and Japan have emerged through different reassortment events. However, the role of reassortment events in the evolution influenza C virus has yet to be defined.

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