Isolation and characterization of influenza C viruses in the Philippines and Japan.

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Running title: Isolation of influenza C in the Philippines and Japan

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Abstract

From November 2009 to December 2013, 15 influenza C viruses were isolated, using MDCK cells, from specimens obtained from children with severe pneumonia and influenza-like illness (ILI), in the Philippines. 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 Philippines and 7 from Japan) were similar to the reference strain C/Sao Paulo/378/82(SP82). Phylogenetic analysis of the HE gene showed that strains from the Philippines and Japan formed distinct clusters within an SP82-related lineage. The clusters including the Philippine and Japanese strains were shown to have diverged from a common ancestor around 1993. In addition, phylogenetic analysis of 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 could not be determined.
Influenza C virus usually causes a 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 that recurrent infection with this virus occurs frequently in children, as well as in adults (7). However, the virus has been isolated only occasionally by cell culture, and long-term monitoring of influenza C viruses is rarely conducted. Monitoring of influenza C among children in 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 from 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 (PB2, PB1, and P3), the hemagglutinin–esterase glycoprotein (HE), the nucleoprotein (NP), the matrix protein (M), the CM2 protein, and two nonstructural proteins (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 showed a high degree of cross-reactivity among all the isolates examined so far (17, 19-21), indicating that influenza C virus is antigenically more homogenous than 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(AI181), C/Yamagata/26/81(YA2681), and C/Sao Paulo/378/82(SP82) (19), and that influenza C viruses belonging to different lineages can be co-circulating in a single community (10, 17). Thus, mixed infections with influenza C viruses belonging to different lineages may be occurring in a single host, resulting in the emergence of reassortant viruses, characterized by exchange of genomic segments between two different strains (19, 24).

Long-term surveillance studies carried out in Yamagata and Miyagi Prefectures in Japan also revealed that reassortment between viruses of different lineages had occurred frequently, and that newly emerged reassortant viruses had replaced previously circulating viruses (10). The virus antigenically similar to KA176, which reemerged in
Miyagi Prefecture in 1996 for the first time in 20 years and subsequently spread throughout Japan, acquired its internal genes from the previous epidemic virus, which belongs to the YA2681 lineage, through a reassortment event (10). These observations indicate that the genome composition of influenza C viruses may affect their ability to spread among humans, and that reassortment events can be 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 Philippines, 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 circulating influenza C viruses in these two countries. This characterization included sequence analysis of all seven RNA segments.

Materials and methods

Virus isolation

We conducted two prospective studies of respiratory viruses 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 pediatric pneumonia study was expanded to include Biliran Provincial
Hospital (BPH) in Naval in Biliran Island and Ospital ng Palawan (ONP) in Puerto
Princesa City on Palawan Island (Fig. S1). 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 specimens were collected
following the Integrated Management of Childhood Illness (IMCI) guideline, from
hospitalized children with the clinical diagnosis of severe pneumonia, and from the
patients who visited the outpatient clinic of LPH, TCHCC or TRHU with ILI; ILI was
defined as fever \( \geq 38 ^\circ C \) or, feverish and either cough or nasal discharge. Details of the
study design have already been described (25).

From November 2009 to December 2013, a total of 5,343 nasopharyngeal swab
specimens were collected from children with severe pneumonia or ILI (age range,
0.0–14.9 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, VeroE6, and human fetal lung fibroblast cells by using the microplate method for isolation of viruses (26). These cell lines were used to detect common respiratory viruses, such as influenza A, B, and C viruses, enteroviruses, human metapneumovirus (hMPV), respiratory syncytial virus (RSV), and human adenoviruses (HAdV). When cytopathic effects (CPE) were observed in MDCK cells, the culture supernatant was tested with the hemagglutination (HA) test using chicken or turkey erythrocytes. Influenza C virus causes agglutination in the chicken or turkey erythrocytes, but does not cause agglutination of the 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. Then, the presence of influenza C in these isolates was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) method using 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 (Fig. S2). A total 1,845 nasopharyngeal and throat swab specimens were collected from children with ARI (age range 0.1–14.9 years, mean age 5.3), and the samples were transferred to Tohoku University Graduate School of Medicine (Sendai, Japan) for virus isolation. The isolation of influenza C virus was performed using same method that was used in the studies in the Philippine.
Hemagglutination inhibition (HI) test for antigenic analysis

All HI tests were performed using isolates that were propagated in embryonated hen’s 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 specimens testing positive for influenza C virus were re-inoculated into the amniotic cavity of 9-day-old embryonated hen’s 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 suspensions (8 HA units/50 μl) were added to each well of a microtiter plate containing 50 μl of two-fold-diluted MAbs. After incubation for 30 min at room temperature, 100 μl of 0.5% chicken erythrocytes were added to all wells, and the plates were stored at 4°C for 60 min. HI titer was expressed as the reciprocal of the highest antibody dilution that completely inhibited hemagglutination. (9).

Nucleotide sequencing and phylogenetic analysis

Nucleotide sequencing and analyses were carried out as previously described (9, 10). Briefly, viral RNA was extracted from 100 μl of the virus-containing amniotic fluid by
using an RNeasy mini kit (Qiagen, Hilden, Germany). Viral RNA was then transcribed into cDNA with a primer complementary to positions 1–12 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 from 64 to 1989, was sequenced by using the BigDye Terminator v3.1 Kit (Applied 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 oligonucleotide primers used for sequencing were previously described (10, 18, 32, 33). Sequence data were analyzed with MEGA software (version 5.1), and phylogenetic trees of individual genes were constructed by the neighbor-joining method (34), with p-distance as a substitution model, bootstrapped 1,000 replicates together with previously reported sequences (9, 10, 19, 21, 24, 32, 35, 36) using the same software. The divergence time for influenza C viruses detected in the Philippines and Japan was estimated by using the Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the BEAST package v1.8.1. 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 as site heterogeneity model. The MCMC chains were run for two billion iterations,
with sampling at every 250,000 and the first 1,000 trees were discarded as burn-in. The
MCMC process was analyzed by using TRACER v1.5. A maximum clade credibility
tree was generated by using FigTree v1.3.1. The nucleotide sequences determined in
this study have been submitted to the DDBJ/GenBank databases and assigned accession
numbers AB978548 to AB978566.

Results

Isolation of influenza C viruses

A total of 15 (0.28%) strains of influenza C were isolated from 5,343 specimens
collected in the Philippines between 2009 and 2013, including 6 strains in 2011 (6 of
1324; 0.45%) and 9 strains in 2013 (9 of 1694; 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 were isolated from children (age range, 0.9–5.7 years),
including 5 strains in 2008 (5/358, 1.40%) and 2 strains in 2012 (2/351, 0.57%) (Fig.
13B). No other respiratory viruses, such as influenza A and B viruses, enteroviruses, hMPV, RSV, and HAdV, were isolated from any of these seven patients.

Clinical manifestations of influenza C virus-positive patients are shown in Table 1. In the Philippines, 7 (7 of 3118; 0.22%) strains of influenza C virus were isolated from severe pneumonia patients (age range, 0.4–5.5 years), and 8 were isolated from ILI patients (8 of 2225; 0.36%) (age range, 0.4–2.9 years) (Table 1). Among influenza C virus-positive children with severe pneumonia, chest indrawing was seen in all cases, while difficulty in breathing and fever over 38°C was seen in 6 cases. Regarding outcome of these cases, six were discharged, and one refused admission. The primary symptoms seen in influenza C virus-positive ILI cases in the Philippines and 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 re-inoculation into the amniotic cavity of embryonated eggs. Influenza C viruses were isolated from embryonated eggs inoculated with each of these 12 specimens. For Japanese specimens, influenza C viruses were isolated from embryonated eggs inoculated with 7 specimens,
including 5 from 2008 and 2 from 2012. The antigenicity 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 MAbs J14, Q5, and U4, but were unreactive or very weakly reactive with MS2. The reactivity patterns of all isolates analyzed 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 sequence of the HE gene from each of the 19 strains (nucleotide 64 to 1989) was 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 (99.3–100% nucleotide identity). The seven strains isolated in 2008 and 2012 in Japan were also highly homologous (99.3–100% nucleotide identity). The strains isolated in the Philippines and Japan formed distinct clusters within the SP82-related lineage;
although, the sequences within this branch, including those from the Philippines and Japan, shared high sequence homology (98.2–98.8% nucleotide identity).

To determine the genomic compositions of the strains isolated in the Philippine and Japan, and to determine the occurrence of reassortment event(s), the nucleotide sequences of 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/TU5/2008, C/Sendai/TU1/2012, and C/Sendai/TU2/2012). As shown in Fig. 3, all of the 10 strains isolated in the Philippines that had an HE gene of the SP82-related lineage, were classified into the YA2681-related lineage based upon the phylogenetic trees constructed with PB1, M, and NS, but classified into the MS80-related lineage in the phylogenetic trees constructed with P3 and NP. 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 of the SP82-related lineage were classified into the YA2681-related lineage based upon the phylogenetic trees constructed with PB1, M,
and NS. They were also classified into the MS80-related lineage using phylogenetic
trees constructed with the P3 gene; the same lineage as the Philippine strains. However,
they were classified into the PB11581-related lineage based upon phylogenetic trees
constructed with the NP gene, and into the KA176-related lineage using trees
constructed with the PB2 gene. One strain (C/Sendai/TU2/2012) was classified into the
PB11581-related lineage based upon a tree 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 were detected in Japan between 1996 and
2000 (10, 37). The genome compositions of the strains in this study, 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 that the
composition of the internal genes of the Philippine strains is different from those of the
Japanese strains.

The phylogenetic analyses revealed that different reassortment events occurred in the
Philippine and Japanese strains. And on 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 the 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 other studies, which reported positive rates from 0.18 to 0.79% (1, 11, 13, 38). In the Philippines, although the overall influenza C 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 influenza C virus may also cause severe lower respiratory tract infections in children.

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 peaks of influenza C are different from those of influenza A, and that epidemics of influenza C usually occur after peaks of influenza A in Japan (8). In the Philippines, influenza A virus is circulating 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 might be 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); and that, the strains of the KA176 and SP82 antigenic groups were co-circulating in 2012. However, no strains belonging to the KA176 antigenic group were isolated in the Philippines or
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 and Japan 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 a SP82-related lineage (11). Additionally, in 2011, an influenza C virus with an HE gene of the YA2681-related lineage was detected in Eastern India (16).

Co-circulation of strains classified into the KA176-related lineage and the SP82-related lineage have been reported in Catalonia, Spain (2009-2010 season) (13); Milan, Italy (2008-2009 and 2009-2010 season) (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 that 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 the 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 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 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 Japan evolved with
reassortment events independently after divergence from a common ancestor.
Interestingly, different strains identified in Catalonia in 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/1318/2009 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 the other parts of the world.
The studies in the Philippines were carried out on three different islands. Although Leyte and Biliran Islands are geographically close, Palawan Island is far from these islands (Fig. S1). However, one strain isolated in Palawan Island in 2013 was highly homologous with strains detected on Leyte 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 had a different internal genetic composition than 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 3C). Therefore, our observation suggests that multiple reassortment events could have occurred in the same population and generated viruses with different gene compositions.

The biological significance of these reassortment events remains unresolved. However, interestingly, the internal genomic composition of all of the Philippine strains was same as that of strains having HE genes of 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 virus fitness. Reassortment might play a role in the acquisition of such advantageous internal genes.

In conclusion, we isolated influenza C viruses in both in 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 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 is still to be defined.
Acknowledgements

This work was supported by a grant-in-aid from the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, Science and a Technology Research Partnership for Sustainable Development (SATREPS) from the Japan Science and Technology Agency (JST), Japan International Cooperation Agency (JICA) and JSPS KAKENHI Grant Number 24249041.

We thank the staff of health facilities in the Philippines and pediatricians in Sendai city, Miyagi, Japan for providing specimens for virus isolation.
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Table 1 Clinical manifestations of influenza C virus-positive patients.

Table 2 Antigenic analysis of influenza C virus isolates, including representative strains, using the hemagglutinin inhibition (HI) test performed with monoclonal antibodies against the hemagglutinin-esterase (HE).

Table 3 Genome compositions of previous isolates and representative influenza C viruses isolated in this study.
Figure Legends

Fig. 1. Monthly distribution of numbers of specimens tested and influenza C viruses isolated in the Philippines (A) and Sendai city, Japan (B).

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 MEGA software (version 5.1). Each showed a value greater than 75%. The Philippine strains are marked (■); strains isolated in 2011 are blue and strains isolated in 2013 are purple. The Japanese strains are marked (●); strains isolated in 2008 are green and strains isolated in 2012 are red.

Fig. 3. Phylogenetic trees for the PB2 (A), PB1 (B), P3 (C), NP (D), M (E), NS (F) genes of influenza C isolates. The nucleotide sequences of the following regions were used for analysis: nucleotide positions from 52 to 520 for the PB2 gene, from 50 to 425 for the PB1 gene, from 49 to 420 for the P3 gene, from 71 to 670 for the NP gene, from 26 to 1147 for the M gene, and from 28 to 889 for the NS gene. Numbers below or above the branches are the bootstrap probabilities (%) of each branch, determined using...
the MEGA software (version 5.1) and hide value lower than 75%. The Philippine strains are marked (■); strains isolated in 2011 are blue and strains isolated in 2013 are purple. The Japanese strains are marked (●), strains isolated in 2008 are green and strains isolated in 2012 are red.

Fig. 4. Bayesian evolutionary tree of influenza C virus based on the nucleotide sequence of the HE gene. The Philippine strains are marked (■); strains isolated in 2011 are blue and strains isolated in 2013 are purple. The Japanese strains are marked (●); strains isolated in 2008 are green and strains isolated in 2012 are red. This Bayesian tree was generated using BEAST software.
Table 1 Clinical manifestations of influenza C virus-positive patients.

<table>
<thead>
<tr>
<th>Age in years, median (range)</th>
<th>The Philippines</th>
<th>Japan*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pediatric pneumonia (n = 7)</td>
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<td>1.1 (0.4 – 2.9)</td>
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<tr>
<td>Influenza like illness (n = 8)</td>
<td>2.9 (0.9 – 7.9)</td>
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<td>Acute respiratory infection (n = 5)</td>
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<table>
<thead>
<tr>
<th>Male gender</th>
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<td>Sign and symptoms</td>
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<td>Cough</td>
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<td>8</td>
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<tr>
<td>Nasal discharge</td>
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</tr>
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<td>&gt;38.0°C</td>
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*Clinical information was available for only 5 of 7 patients.
Table 2 Antigenic analysis of influenza C virus isolates, including representative strains, using the hemagglutinin inhibition (HI) test performed with monoclonal antibodies against the hemagglutinin-esterase (HE).

<table>
<thead>
<tr>
<th>Antigenic group</th>
<th>Virus strain</th>
<th>J14</th>
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<th>U4</th>
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Strains isolated in this study are indicated in boldface.

Abbreviations: 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.
Table 3 Genome compositions of previous isolates and representative influenza C viruses isolated in this study.

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Strains isolated in this study are indicated in boldface.
Abbreviations: A, AI181-related lineage; S, SP82-related lineage; Y, YA2681-related lineage; K, KA176-related lineage; M, MS80-related lineage; P, PB11581-related lineage.