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Phylogenetic and antigenic analyses of coxsackievirus A6 isolates in Yamagata, Japan between 2001 and 2017

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ABSTRACT

Although coxsackievirus A6 (CV-A6) is generally recognized as a causative agent of herpangina in children, CV-A6 infections globally emerged as a new and major cause of epidemic hand-foot-and-mouth-diseases (HFMDs) around 2008. To clarify the longitudinal epidemiology of CV-A6, we carried out sequence and phylogenetic analyses for the VP1 and partially for the VP4-3D regions as well as antigenic analysis using 115 CV-A6 isolates and 105 human sera in Yamagata, Japan between 2001 and 2017. Phylogenetic analysis revealed that CV-A6 isolates were clearly divided into two clusters; strains in circulation between 2001 and 2008 and those between 2010 and 2017. Neutralizing antibody titers of two rabbit antisera, which were immunized with Yamagata isolates in 2001 and 2015, respectively, against 28 Yamagata representative strains as well as the prototype Gdula strain were 1:2560–1:5120 and 1:160–1:640, respectively. The neutralizing antibody titers among residents in Yamagata against the above two strains were similar. Our analyses revealed that there were cross-antigenicities among all analyzed CV-A6 strains, although the newly emerged strains were introduced into Yamagata around 2010 and replaced the previous ones. With regard to control measures, these findings suggest that we can prevent CV-A6 infections through the development of a vaccine that effectively induces neutralizing antibodies against CV-A6, irrespective of genetic cluster.

1. Introduction

Hand-foot-and-mouth disease (HFMD) is characterized by vesicular exanthema of the oral mucosa, peripheral extremities and sometimes on the buttocks or genitalia [1,2]. It predominately affects children younger than 10 years during the summer season in regions with temperate climates [1]. HFMD is primarily associated with human enterovirus species A (EV-A) serotypes such as coxsackievirus A4 (CV-A4), CV-A5, CV-A6, CV-A10, CV-A16, and EV-A71 [1]. CV-A16 and EV-A71 are the most common causes of HFMD, with EV-A71 in particular responsible for large outbreaks associated with severe central nervous system disease and deaths since 1997 across the Asia-Pacific region [1–3].

Österback et al. reported that CV-A6 infections may be emerging as a new and major cause of epidemic HFMD, which shows not only typical HFMD symptoms but demonstrated onychomadesis as a feature during an outbreak in the fall of 2008 in Finland [4]. Since then, this atypical form of HFMD caused by a novel CV-A6 strain, which is characterized by a wider distribution of skin lesions that are enlarged and vesiculated, especially in areas of eczematous skin, has been observed globally [1–3,5–13]. Aswathyraj et al. recently pointed out that the development of a globally representative multivalent HFMD vaccine could afford the best strategy as several etiologies of HFMD, such as emerging CV-A6, threaten infants and children these days [3].

In Japan, CV-A16 and EV-A71 have been commonly detected in patients with HFMD since the start of the National Epidemiological Surveillance of Infectious Diseases (NESID) in Japan in 1981 [14]. However, an increase in the detection rate of CV-A6 in HFMD was reported in 2009, and the most frequent type detected from

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the 2011 HFMD epidemic was CV-A6, which had been consistently associated with herpangina [13,14].

Enteroviruses have a genome of single-strand, positive-sense RNA of ~ 7,500 nt in length that encodes a single long open reading frame, flanked by non-translated regions at the 5′ and 3′ ends [15]. The coding regions are divided into three regions P1, P2 and P3; the P1 region encodes the viral capsid proteins (VP4, VP2, VP3 and VP1) and the non-structural proteins are encoded in the P2 regions (2A, 2B and 2C) and P3 regions (3A, 3B, 3C and 3D) [15]. Several previous reports indicated that phylogenetic analysis based on the VP1 regions of CV-A6 strains branched mainly into two clusters; those strains in circulation prior to around 2008 and those after around 2008, when the novel CV-A6-related HFMD emerged and spread [6–8,13,16–17]. Ogi et al. in particular, reported that CV-A6 strains collected in 1999–2009 and those in 2009–2013 were separately clustered by phylogenetic analysis based on the 5′UTR and 3D region as well as the VP1 region and concluded that HFMD outbreaks caused by CV-A6 were becoming more frequent in Japan [16].

In this study, to clarify the longitudinal epidemiology of CV-A6, we carried out molecular epidemiological analysis of the VP1 region of CV-A6 Yamagata isolates between 2001 and 2017, as well as the VP4-3D regions for several representative strains from different years. To compare antigenicity, which is critical to vaccine development, we prepared two post-infection rabbit antisera raised against Yamagata representative CV-A6 isolates in 2001 and 2015, and carried out antigenic analysis using several Yamagata isolates obtained during the study period. We further performed seroepidemiological study using sera of residents in Yamagata against the above two representative CV-A6 isolates in 2001 and 2015.

2. Materials and methods

2.1. Specimen collection, virus isolation and clinical data

Virus isolation was carried out by means of a microplate method from pharyngeal and nasal samples collected from children with herpangina, HFMD, respiratory illnesses and other conditions as described previously [18]. CV-A6 strains were isolated using the RD-18S cell line up to 2011. However, we also introduced the RD-A cell line, which is more sensitive to CV-A6 than the RD-18S cell line, from 2013 [19]. The clinical characteristics of children who tested positive for CV-A6 were obtained from their medical records.

Upper respiratory infection includes the common cold, which is an acute illness with nasal stuffiness, rhinitis, no objective evidence of pharyngitis, and no or minimal fever [20], and pharyngitis, which was defined by a red pharynx and an accompanying lack of any vesicles or tonsillitis [21]. Tonsillitis was defined by red, swollen tonsils with white exudate [21]. Herpangina was defined by papular, vesicular, and ulcerative lesions on the soft palate, uvula, or other parts of the oropharynx [21]. HFMD was defined by vesicular exanthema of the oral mucosa and peripheral extremities as described above. Nonpolio EVs are a common cause of a variety of skin manifestations such as maculopapular rashes [20] and these cases were defined as viral exanthema due to enterovirus infections. Patients with fever and/or symptoms of

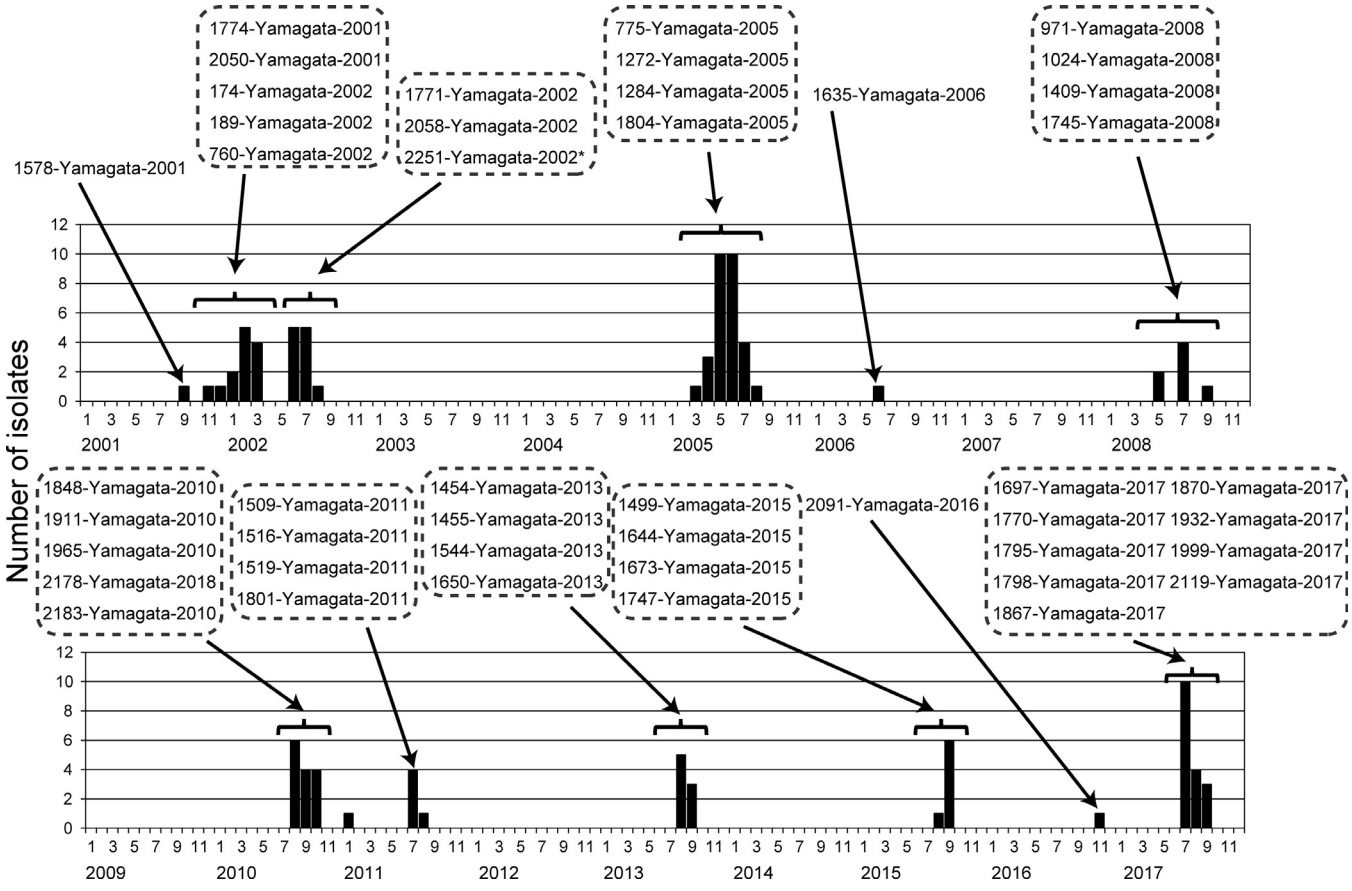

Fig. 1. Monthly isolation of CV-A6 strains from children in Yamagata, Japan, between 2001 and 2017. The representative Yamagata strains, for which VP4-3D regions were analyzed, are also indicated by strain name. *VP1 and 3D sequences are only available for 2251-Yamagata-2002.

the common cold during the winter season were clinically diagnosed as influenza-like illness. Informed consent was obtained from their guardians and this work was approved by the Ethics Committee of the Yamagata Prefectural Institute of Public Health (YPIPHEC 18–02 and 18–03).

2.2. Sequence and phylogenetic analyses

We analyzed the VP1 region in all 115 isolates and the VP4-3D regions in 45 isolates (Fig. 1). For reverse transcription–PCR (RT-PCR), viral RNA was extracted from 200 μl of viral fluid, using a High Pure Viral RNA kit (Roche Diagnostics, Manheim, Germany) according to the manufacturer's instructions and then transcribed into cDNA using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). The target region was amplified by PCR with 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 10 min. We used a mixture of CoxA6-2317F and CoxA6-3408R primers (Supplementary Table 1) for the amplification of the VP1 region. We then carried out sequence analysis using a 3130 Genetic analyzer or 3730 DNA analyzer (Thermo Fisher Scientific, Tokyo, Japan). For sequencing, we used primers CoxA6-OuterF and CA6-VP1-R. For amplification and sequence analysis for parts other than the VP1 region, we used primers published elsewhere as well as our original primers shown in Supplementary Table 1. Sequence data were registered with GenBank (Accession numbers LC421542-LC421657).

Multiple-sequence alignment of the CV-A6 isolates and reference strains was conducted using Clustal W, and phylogenetic analyses were performed with the neighbor-joining method as previously described [22].

2.3. Preparation of the CV-A6 virus as an immunogen, rabbit immunization and antigenic analysis

Preparation of the CV-A6 virus as an immunogen and rabbit immunization were carried out based on our previously reported methods [22,23]. Animal experiments were carried out with approval from the Committee for Animal Experiments of Yamagata University (Project license number 29012). The representative strain for CV-A6 prior to 2009 (2050-Yamagata-2001) and that after 2009 (1644-Yamagata-2015) were passaged 6–7 times in RD-18S and/or RD-A cell lines and used as immunogens to immunize rabbits. The virus in the cultured fluid was purified by precipitation with 8% polyethylene glycol 6000 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and then centrifuged on a 40% sucrose gradient at 40,000 rpm for 3 h. The virus titer was determined as the tissue culture infective dose 50 ($TCID_{50}$) on RD-A cells. One specific pathogen-free 11-week-old female New Zealand White (Kumagai-shigeyasu Co.,Ltd, Sendai, Japan) was independently used for 2050-Yamagata-2001 and 1644-Yamagata-2015 immunogens, respectively. The animals were subcutaneously injected with 1.6 ml of virus fluid (approximately 10^7–10^8 $TDID_{50}$) inactivated with formalin and the same volume of adjuvant incomplete FREUND (DIFCO Laboratories, Detroit Michigan) at an interval of 1 week. Four weeks after the 4th immunization, the rabbits were boosted by one subcutaneous injection of the identical virus with the adjuvant. A combination anesthetic (5.25 ml) was prepared with 0.3 mg of medetomidine, 4.0 mg of midazolam, and 5.0 mg of butorphanol and serum samples were collected 1-week later under anesthesia (0.525 ml/kg) and then stored at −20 °C after heat inactivation (56 °C for 30 min). Antigenic analysis were carried out using these rabbit sera and representative CV-A6 isolates for each year as well as the CV-A6 prototype Gdula strain, which was purchased from the American Type Culture Collection (ATCC).

2.4. Seroepidemiological survey

Serum samples were collected from residents in Yamagata in 2017, from whom we had obtained informed consent (either from the individual or a guardian) for the National Epidemiological Surveillance of Vaccine-preventable Diseases led by the Ministry of Health, Labour and Welfare, Japan. Among them, a total of 105 serum samples (0–4 years, 37; 5–9 years, 32; 10–14 years, 25; 15–19 years, 11) were enrolled for this study. The neutralization titers against two CV-A6 strains (2050-Yamagata-2001 and 1644-Yamagata-2015), which were used for rabbit immunization, were measured using a microneutralization test with the RD-A cell line as described previously [24]. Briefly, samples were inactivated and serially diluted two-fold from 1:8 to 1:8192-fold. We mixed and incubated (at 37 °C for 60 min) each diluted sample with viral fluid containing 100 times the $TCID_{50}$ of the each CV-A6 strain, and then

Table 1
Yearly distribution by age and clinical diagnosis of patients, from whom CV-A6 was isolated in Yamagata, Japan between 2001 and 2017.

(A) Age

	2001	2002	2005	2006	2008	2010	2011	2013	2015	2016	2017	Total
0	0	1	2	0	1	2	1	0	0	0	0	7
1	0	7	5	0	2	3	2	1	0	1	7	28
2	0	1	4	0	2	3	0	2	1	0	1	14
3	3	2	5	0	1	4	2	1	0	0	5	23
4	0	3	5	0	0	1	1	1	3	0	2	16
5–9	0	7	7	1	1	1	0	2	3	0	1	23
10–14	0	1	1	0	0	0	0	0	0	0	0	2
>14	0	0	0	0	0	0	0	1	0	0	1	2
Total	3	22	29	1	7	14	6	8	7	1	17	115

(B) Clinical diagnosis

	2001	2002	2005	2006	2008	2010	2011	2013	2015	2016	2017	Total
Upper respiratory infection	1	16	22	0	3	7	1	2	4	0	3	59
Herpangina	0	1	2	0	0	1	3	3	0	0	6	16
Hand-foot-mouth disease	1	0	1	0	1	2	0	1	1	1	6	14
Tonsillitis	0	0	2	0	3	0	0	2	2	0	2	11
Exanthema	1	1	0	1	0	4	0	0	0	0	0	7
Influenza-like illness	0	3	0	0	0	0	0	0	0	0	0	3
Others	0	1	2	0	0	0	2	0	0	0	0	5
Total	3	22	29	1	7	14	6	8	7	1	17	115

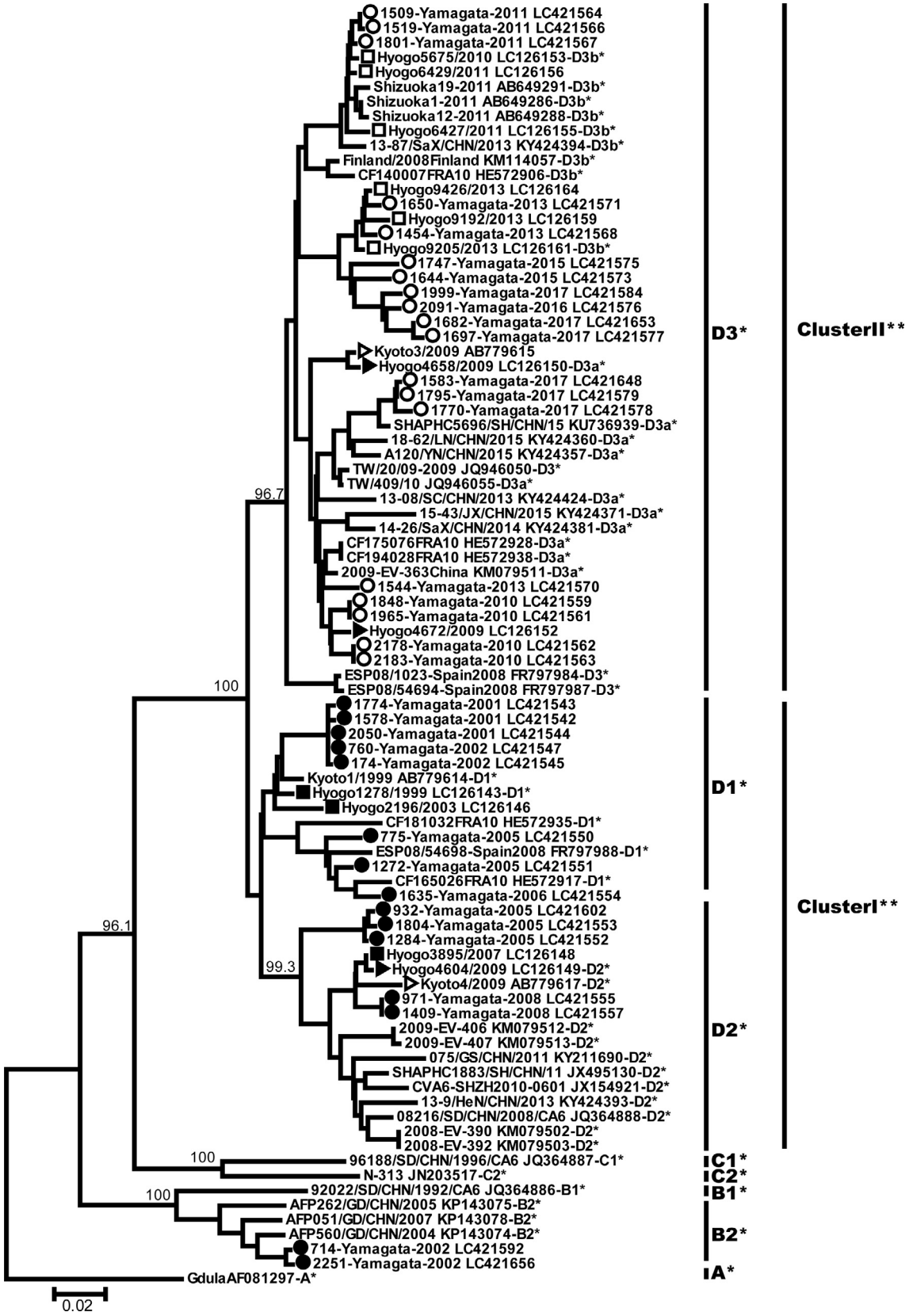

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method for the 915 bps sequence of the VP1 region of CV-A6 in Yamagata and reference strains, which were based on Song et al. [25]. Yamagata and Hyogo strains prior to 2009 are indicated by closed circles and closed squares, respectively, while those after 2010 are indicated by open circles and open squares, respectively. Hyogo strains and Kyoto strains circulating in 2009 are indicated by closed and open triangles, respectively. Genetic distances between sequences were calculated by using the Kimura parameter method. Bootstrap values from 1000 replicates are shown near the branches (%). The branch lengths are proportional to differences in the number of nucleotides. The marker denotes the measurement of relative phylogenetic distance. The GenBank accession numbers are also shown. *Subgenotypes proposed by Song et al. [25]. **Clusters proposed by Ogi et al. [16].

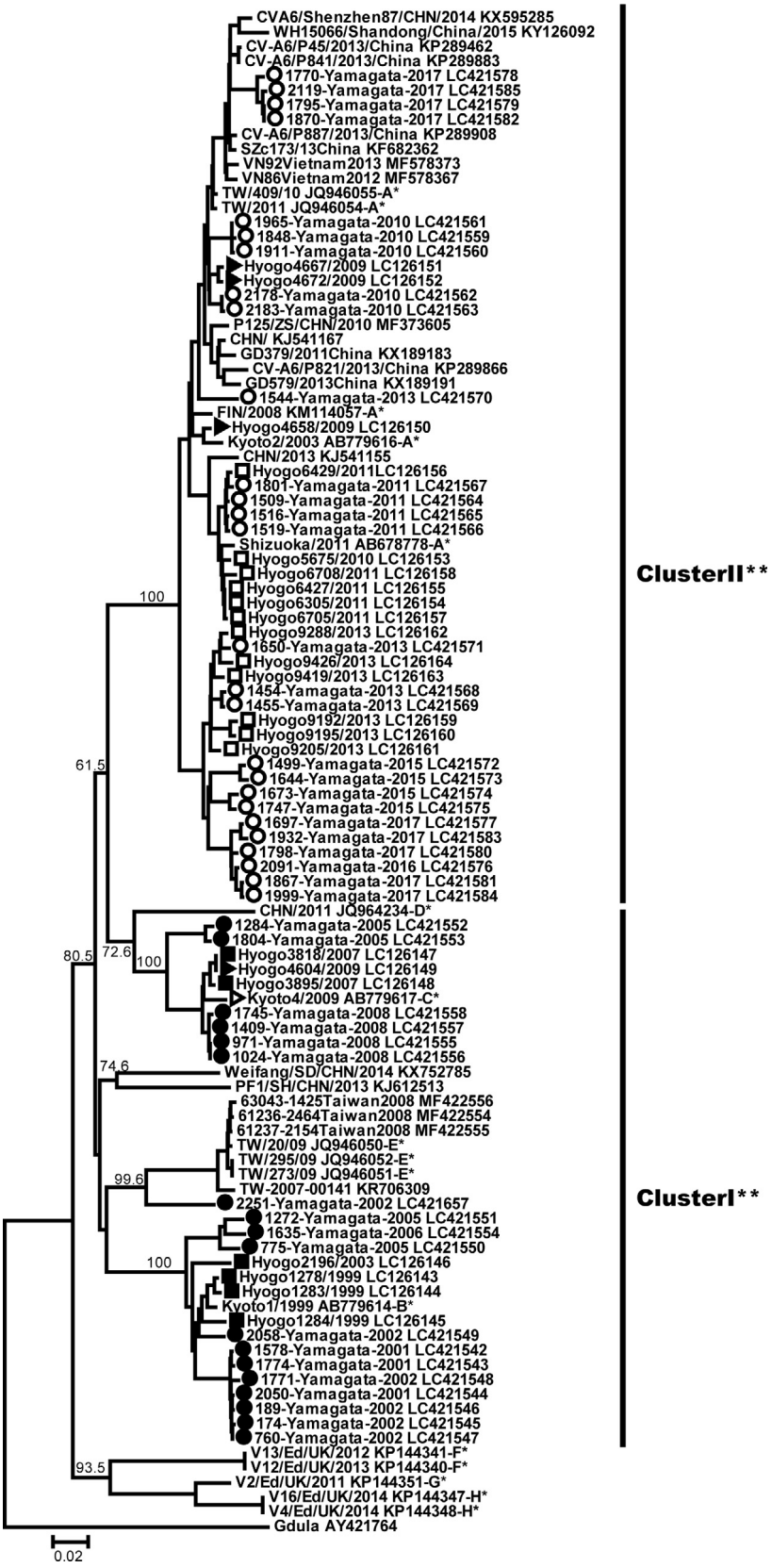

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method for the 1386 bps sequence of the 3D region of CV-A6 in Yamagata and reference strains. Yamagata, and Hyogo strains prior to 2009 are indicated by closed circles and closed squares, respectively, while those after 2010 are indicated by open circles and open squares, respectively. Hyogo strains and Kyoto strains circulating in 2009 are indicated by closed and open triangle, respectively. Genetic distances between sequences were calculated by using the Kimura parameter method. Bootstrap values from 1000 replicates are shown near the branches (%). The branch lengths are proportional to differences in the number of nucleotides. The marker denotes the measurement of relative phylogenetic distance. The GenBank accession numbers are also shown. *Recombinant forms proposed by Gaunt et al. [26] **Clusters proposed by Ogi et al. [16].

we inoculated each incubated virus-serum mixture into each of two RD-A cell cultures. The reciprocal value of the highest dilution of each serum resulting in no or only a weak cytopathic effect as compared to the control was taken to be the titer. Seropositivity was defined as a serotiter ≥1:8.

3. Results

3.1. Virus isolation, age, clinical diagnoses and yearly distribution

Although we isolated a total of 3055 enteroviruses including CV-A2, -A3, -A4, -A5, -A6, -A9, -A10, -A14, -A16, -B1,-B2, -B3, -B4, -B5, EV-A71, echoviruses 3, 6, 7, 9, 11, 13, 16, 18, 21, 25, 30, EV-D68, polio 1, polio 2, polio 3 and untyped enteroviruses (Supplementary Table 2) during the study period, we here focus on CV-A6. Monthly isolation of CV-A6 strains from children in Yamagata, Japan, between 2001 and 2017 is shown in Fig. 1. Among the 115 CV-A6-positive patients, 88 (76.5%) were aged < 5 years old, 23 (20.0%) aged 5–9 years old and 4 (3.5%) aged over 9 years old (Table 1). Fifty nine (51.3%) patients were diagnosed with upper respiratory infections, 16 (13.9%) with herpangina, 14 (12.2%) with HFMD, 11 (9.6%) with tonsillitis, 7 (6.1%) with exanthema and 8 (6.9%) with other conditions (Table 1). One to 29 CV-A6 viruses were isolated each year in the following years 2001–2, 2005–6, 2008, 2010–11, 2013, and 2015–7 (Fig. 1 and Table 1).

3.2. Sequence and phylogenetic analyses

Amino acid homologies among the VP1 regions of Yamagata strains as well as those for the 3D region were 94–100% among the analyzed strains (Supplementary Table 3).

Phylogenetic analysis based on the VP1 region is shown in Fig. 2 and indicated that the majority of the CV-A6 viruses from Yamagata belonged to genotype D (subgenotypes D1, D2 and D3) based on the genotyping method of Song et al. [25], whereas 714-Yamagata-2002 and 2251-Yamagata 2002 belonged to subgenotype B2. Yamagata isolates obtained between 2001 and 2008 and reference strains, which belonged to subgenotypes D1 or D2, formed a cluster identical to cluster I proposed by Ogi et al. [16]. Those obtained between 2010 and 2017 and reference strains, which belonged to subgenotype D3, formed a cluster identical to cluster II proposed by Ogi et al. [16]. Among the cluster I strains, subgenotype D1 strains circulated between 2001 and 2006, whereas subgenotype D2 strains circulated between 2005 and 2008. No CV-A6 strains belonging to other subgenotypes (A, B1, C1 or C2) were found. Although we did not observe any CV-A6 strains in 2009, several Japanese strains in 2009 were classified into subgenotype D3 (Kyoto3/2009, Hyogo4658/2009 and Hyogo4672/2009) and D2 (Hyogo4604/2009 and Kyoto4/2009).

Phylogenetic analysis based on the 3D region is shown in Fig. 3 and indicated that most of the Yamagata isolates obtained between 2001 and 2002 were closely related to recombination form B (RF-B) based on the proposal by Gaunt et al. [26], whereas 251-Yamagata-2002 was closer to several Taiwanese strains, which belong to RF-E. Yamagata strains obtained between 2005 and 2006 were closely related to RF-B and RF-C, and those in 2008 belonged to RF-C. All Yamagata strains obtained between 2010 and 2017 belonged to RF-A. Phylogenetic analysis based on the 3D region also showed that Yamagata strains in circulation before and after 2009 were separately clustered similar to the results for the VP1 region, into Cluster I and Cluster II, as suggested by Ogi et al. [16] (Fig. 3). Similar amino acid sequence differences between CV-A6 strains in circulation before and after 2009 in Hyogo prefecture [16] were observed in Yamagata, except for several additional changes in the VP4-3D regions (Supplementary Table 4).

3.3. Antigenic analysis

The neutralization titers of rabbit antisera raised against 2050-Yamagata-2001 and 1644-Yamagata-2015 were measured against 28 Yamagata isolates in different years as well as the prototype Gdula strain. The titers obtained were 1:2560–1:5120 and 1:160–:640 (homo-titer 1:5120 and 1:640), respectively (Table 2). Two minor strains isolated in 2002 (714-Yamagata-2002 and 2251-Yamagata-2002), which belonged to subgenotype B2 based on VP1 phylogenetic analysis (Fig. 2.), were neutralized similarly to the other Yamagata strains. We failed to neutralize one strain isolated in 2006 (1635-Yamagata-2006), as the viral fluid included another enterovirus that was able to grow on the RD-A cell line.

3.4. Seroepidemiological survey

The neutralization titers for 105 residents in Yamagata in 2017 against the two representative CV-A6 strains are shown in Table 3. Sero-positive rates were 40.5% (15/37), 75% (24/32), 80% (20/25) and 81.8% (9/11) against the 2050-Yamagata-2001 strain among the residents under the age of 5, between the ages of 5 and 9, between the ages of 10 and 14 and between the ages of 15 and 19, respectively. Those against the 1644-Yamagata-2015 strain were 37.8% (14/37), 75% (24/32), 76% (19/25), and 81.8% (9/11), respectively. The seroprevalence against 2050-Yamagata-2001 and 1644-Yamagata-2015 were 64.8% (68/105) and 62.9% (66/105) for all age groups, respectively. Two residents (02008 and 10024) were neutralization antibody positive (1:8) against

Table 2
Neutralizing antibody titers of two rabbit antisera against the representative CV-A6 strains in Yamagata, Japan isolated between 2001 and 2017 and the prototype Gdula strain.

CV-A6 strains	Serum from rabbits immunized with:	
	2050-Yamagata-2001	**1644-Yamagata-2015**
Prototype Gdula strain (A**)	1:2560	1:640
1578-Yamagata-2001 (D1**)	1:5120	1:320
1774-Yamagata-2001 (D1)	1:5120	1:640
2050-Yamagata-2001 (D1)	**1:5120**	1:640
174-Yamagata-2002 (D1)	1:5120	1:160
714-Yamagata-2002 (B2**)	1:5120	1:320
2251-Yamagata-2002 (B2)	1:2560	1:320
775-Yamagat-2005 (D1)	1:5120	1:320
1052-Yamagata-2005	1:5120	1:320
1272-Yamagata-2005 (D1)	1:5120	1:320
1635-Yamagata-2006* (D1)	–	–
971-Yamagata-2008 (D2**)	1:5120	1:320
1409-Yamagat-2008 (D2)	1:5120	1:160
1745-Yamagata-2008	1:2560	1:160
1848-Yamagata-2010 (D3**)	1:5120	1:640
1911-Yamagata-2010	1:5120	1:320
1965-Yamagata-2010 (D3)	1:2560	1:640
1516-Yamagata-2011	1:2560	1:320
1519-Yamagata-2011 (D3)	1:5120	1:320
1801-Yamagata-2011 (D3)	1:2560	1:320
1454-Yamagata-2013 (D3)	1:2560	1:160
1544-Yamagata-2013 (D3)	1:5120	1:320
1650-Yamagata-2013 (D3)	1:5120	1:320
1644-Yamagata-2015 (D3)	1:5120	**1:640**
1673-Yamagata-2015	1:2560	1:160
1747-Yamagata-2015 (D3)	1:2560	1:640
2091-Yamagata-2016 (D3)	1:2560	1:320
1823-Yamagata-2017	1:2560	1:160
1932-Yamagata-2017	1:2560	1:160
1999-Yamagata-2017 (D3)	1:2560	1:320

* The strain "1635-Yamagata-2006" was not neutralized as the viral fluid included another enterovirus that was able to grow in the RD-A cell line.
** Subgenotype based on Fig. 1.

2050-Yamagata-2001 and negative (<1:8) against 1644-Yamagata-2015, respectively. Excluding these two residents, the titers against the two strains were within 2–4-fold.

4. Discussion

We carried out sequence and phylogenetic analyses using Yamagata strains, including those isolated before and after 2009, when the new HFMD-associated CV-A6 was reported for the first time in Japan [13,14]. Yamagata strains were clearly branched into subgenotypes D1 or D2 (cluster I) and subgenotype D3 (cluster II), and the switch from the former into the latter was observed around 2009 in agreement with the report by Ogi et al. [16], although subgenotype D3 strains were not always associated with HFMD in Yamagata (Table 1). In 2009, when no CV-A6 was isolated in Yamagata, Ogi et al. reported that three strains from Hyogo (Hyogo4658/2009, Hyogo4667/2009 and Hyogo4672/2009) and Kyoto3/2009 branched into cluster II, whereas Hyogo4604/2009 and Kyoto4/2009 belonged to cluster I (Fig. 2) [16]. These findings suggested that the subgenotype D3 strains were introduced into Japan around 2009, that subgenotype D2 and subgenotype D3 strains were co-circulating in 2009, and then subgenotype D3 strains spread throughout Japan, including Yamagata, in 2010, at which time subgenotypes D1 and D2 strains disappeared. Thus, the newly emerged subgenotype D3 strains, which were first identified as a possible causative agent of new HFMD in Finland in 2008, appeared in Japan the following year. We observed a re-emergence of echovirus type 13 infections in 2002 in Yamagata, Japan, following reports of this re-emergence in Europe and the United States in 2000–2001 [27]. These findings suggested that a newly emerging or re-emerging enterovirus strains appears in Japan within 1–2 years due to the rapid global circulation of enteroviruses.

Österback et al. reported that CV-A6 strains in Finland in 2008 branched independently from Japanese strains detected between 1999 and 2003 based on phylogenetic analysis using 289 bps of the VP1 region [4]. In China, CV-A6, together with EV-A71 and CV-A16, was one of the major circulating enteroviruses from 2009 to 2013 among HFMD patients [10]. Zeng et al. classified CV-A6 strains into eight clusters (A–G) based on the VP1 sequences and they found that strains circulating in China from 2009 to 2011 were dispersed into clusters A and D, whereas almost all strains circulating between 2012 and 2013 were segregated into one major cluster A [10]. Their cluster A displayed a close relationship with the 2008 Finland strain, 2009/2010 Taiwan strains and 2011 Japanese strains [10], and our analysis supported the notion that their cluster A coincided with cluster II by Ogi et al. [16], which was proposed as subgenotype D3 by Song et al. [25], in this study (Fig. 2). These findings revealed that subgenotype D3 (cluster II) strains have spread widely across Asian countries since around 2009.

CV-A6 has become one of the major pathogens of HFMD and the number of severe cases has increased recently [11]. Thus, the development of a vaccine against CV-A6 appears to be an effective method of controlling and preventing HFMD, with several vaccine-related studies currently underway, and the development of a vaccine against CV-A6 already started [3,28]. To this end, not only genomic but also antigenic analysis is quite important. In this study, sera from rabbits, immunized with the subgenotype D1 and subgenotype D3 strains, indicated cross-antigenicity among strains from subgenotypes A, B2, and D1-3, suggesting that the eliciting of neutralizing antibodies against strains from one subgenotype strain is likely to confer cross-neutralization even against strains from other subgenotype (Table 2). These findings support the notion that passive transfer of antisera from mice immunized with a CV-A6 virus-like particle (VLP)-based vaccine and the active immunization of mice with CA-V6 VLP-based vaccine efficiently conferred protection against both homologous and heterogeneous CV-A6 infection [28].

Seroepidemioligical analysis using residents in Yamagata in 2017 indicated that infants and children are affected with CV-A6 at a young age, such as between 2 and 4 years old, and the

Table 3
Neutralizing antibody titers against two representative strains of CV-A6 among residents in Yamagata, Japan in 2017.

Enrolled ID	Age (year)	2050-Y*-2001	1644-Y-2015	Enrolled ID	Age (year)	2050-Y-2001	1644-Y-2015	Enrolled ID	Age (year)	2050-Y-2001	1644-Y-2015	Enrolled ID	Age (year)	2050-Y-2001	1644-Y-2015
00001	0	<1:8	<1:8	03004	3	<1:8	<1:8	05015	7	1:128	1:128	10022	11	<1:8	<1:8
00002	0	<1:8	<1:8	03005	3	1:512	1:1024	05023	7	<1:8	<1:8	10024	11	1:8	<1:8
00003	0	<1:8	<1:8	03006	3	<1:8	<1:8	05024	7	<1:8	<1:8	10026	11	1:16	1:32
00005	0	<1:8	<1:8	03007	3	1:512	1:1024	05025	7	<1:8	<1:8	10007	12	<1:8	<1:8
00006	1	<1:8	<1:8	03008	3	1:128	1:128	05026	7	1:2048	1:2048	10010	12	1:512	1:1024
00007	1	<1:8	<1:8	03009	3	1:256	1:512	05027	7	1:256	1:128	10011	12	1:256	1:128
01001	1	<1:8	<1:8	04001	4	<1:8	<1:8	05032	7	1:256	1:512	10014	12	<1:8	<1:8
01002	1	<1:8	<1:8	04002	4	1:64	1:128	05004	8	1:16	1:32	10017	13	1:128	1:256
01003	1	<1:8	<1:8	04003	4	1:64	1:128	05006	8	1:256	1:256	10002	14	<1:8	<1:8
01004	1	<1:8	<1:8	04004	4	<1:8	<1:8	05016	8	1:32	1:16	10008	14	1:16	1:16
01005	1	<1:8	<1:8	05001	5	1:256	1:512	05019	8	<1:8	<1:8	10012	14	1:128	1:256
01006	1	<1:8	<1:8	05002	5	<1:8	<1:8	05012	9	1:32	1:32	10016	14	1:64	1:128
01007	1	1:128	1:256	05003	5	<1:8	<1:8	05013	9	<1:8	<1:8	10021	14	1:256	1:512
01008	1	1:32	1:32	05007	5	1:32	1:32	05028	9	1:512	1:256	15002	15	<1:8	<1:8
01009	2	1:1024	1:512	05014	5	1:256	1:1024	05029	9	1:64	1:16	15005	15	1:64	1:32
01010	2	<1:8	<1:8	05017	5	1:64	1:256	10001	10	1:32	1:64	15006	15	1:512	1:1024
02001	2	<1:8	<1:8	05030	5	1:128	1:256	10004	10	<1:8	<1:8	15007	15	1:128	1:64
02002	2	<1:8	<1:8	05005	6	1:32	1:32	10005	10	1:128	1:128	15001	16	1:512	1:1024
02003	2	1:256	1:512	05008	6	<1:8	<1:8	10006	10	1:64	1:32	15003	17	1:16	1:32
02004	2	1:512	1:512	05009	6	1:128	1:64	10015	10	1:16	1:16	15004	17	1:64	1:128
02005	2	1:512	1:256	05010	6	1:1024	1:512	10018	10	1:32	1:32	15008	17	1:64	1:128
02006	2	1:1024	1:2048	05018	6	1:128	1:256	10019	10	1:32	1:128	15011	17	<1:8	<1:8
02007	3	1:512	1:1024	05020	6	1:32	1:64	10020	10	1:64	1:128	15009	18	1:32	1:64
02008	3	1:8	<1:8	05021	6	1:64	1:32	10025	10	1:16	1:32	15010	18	1:128	1:256
03001	3	<1:8	<1:8	05031	6	1:128	1:512	10003	11	1:16	1:16				
03002	3	<1:8	<1:8	05033	6	1:64	1:64	10009	11	1:256	1:1024				
03003	3	<1:8	<1:8	05011	7	1:128	1:128	10013	11	1:128	1:256				

* Yamagata.

sero-positive rates increase by age to reach approximately 80% by the age of 10 (Table 3). Similar neutralizing antibody titers against 2050-Yamagata-2001 (subgenotype D1) and 1644-Yamagata-2015 (subgenotype D3) among residents in Yamagata were observed. Residents under 7 years old in Yamagata in 2017, in particular, could be affected with only subgenotype D3 strains, as subgenotype strains other than D3 had disappeared by 2009 in Yamagata, and their neutralizing antibodies reacted similarly with subgenotype D1 strain (2050-Yamagata-2001), strongly supporting the cross-antigenicity among different subgenotype CV-A6 strains observed in our analysis using rabbit antisera.

An increase in the detection rate of CV-A6 in atypical HFMD cases was reported in 2009, and a HFMD outbreak due to CV-A6 was observed in Japan in 2011 [13,14]. Gaunt E et al. [26] reported that host factors such as herd immunity could influence the underlying background in clinical phenotype of CV-A6, but the evidence for its rapid emergence argues strongly against population wide changes in host disease susceptibility to CV-A6. Pons-Salort M et al. estimated that an increase in pathogenicity in 2010 in Japan provided a better fit to the data than models with a change in transmissibility or antigenicity, based on their stochastic transmission model analysis [29]. Our seroepidemiological data clearly revealed that the antigenicity of CV-A6 did not change before and after 2009, suggesting that there was no herd immunity change before or after the emergence of subgenotype D3 strains. Although the cyclical patterns of EV-A71 epidemics are reportedly associated with changes in EV-A71 seroprevalence [30], this is not a case for CV-A6. Gaunt E et al. also reported that two novel RFs of CV-A6 (RF-H and RF-A) were associated specifically with the recent atypical HFMD cases [26]. Although we have not analyzed recombination events, the recent outbreak in Japan was possibly caused by RF-A (Fig. 3), and this observation supports the notion that "RF-A" could be a causative factor for the recent CV-A6 outbreak in Japan. Our findings in Japan, that the RD-A cell line is more sensitive to recent CV-A6 strains than is the RD-18S cell line [19], are interesting as they suggest a change in the relationship between the virus and host cell. We expect the mechanism of the recent worldwide outbreak due to newly emerged subgenotype D3 CV-A6 strains will be clarified in the near future.

5. Conclusions

Our phylogenetic analysis and the previous study revealed that newly emerged HFMD-related CV-A6 strains were introduced to Japan around 2009 and have since spread throughout Japan, including Yamagata, as well as across other Asian countries. On the other hand, our antigenic and seroepidemiological analyses suggested that CV-A6 strains isolated between 2001 and 2017 as well as the prototype Gdula strain have cross-antigenicities. These findings suggested that we can prevent CV-A6 infections through the development of a vaccine that effectively induces neutralizing antibodies against CV-A6, irrespective of genetic cluster/subgenotype.

Declaration of interest

All authors have no conflict of interest.

Author's contributions

The Yamagata Prefectural Institute of Public Health team (K. Mizuta, S. Tanaka, Y. Matoba, Y. Aoki, and T. Ikeda) performed virus isolation, sequence and phylogenetic analyses, and antigenic analysis. The pediatric team (T. Itagaki, F. Katsushima, Y. Katsushima, H. Yoshida and S. Ito) provided clinical data processing and analysis. Y. Matsuzaki was responsible for the rabbit immunization experiments. All authors performed critical data review and contributed to drafting the manuscript.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.12.065.

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