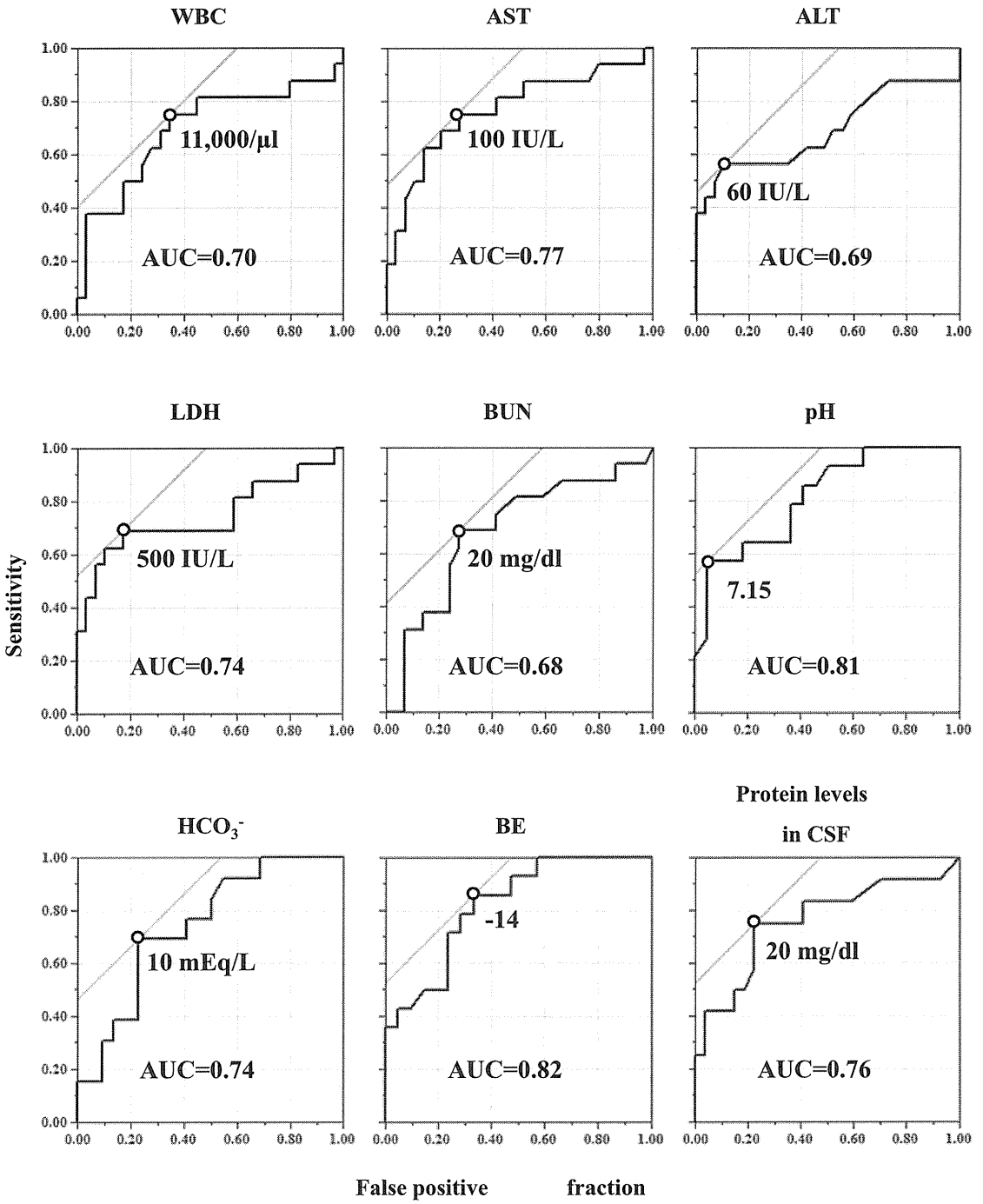

Figure 1. The panels show the receiver-operating curves for the nine variables that were found to be significantly different between the patients with poor prognosis and those without sequelae. Small circles indicate appropriate cutoff values. AUC; area under the curve.

hospitals was independent of the frequency of patients, then the number of annual cases of the rotavirus-associated encephalitis/encephalopathy and sudden unexpected death were estimated to be 41.1 and 5.0 patients/year, respectively. The assumption has to be validated because the response rate of the questionnaire was 70.5%. Although no postmortem analysis for confirmation of encephalitis was carried out in these subjects, some of the patients had mild pleocytosis suggesting inflammation of central nervous system (Table 3). Therefore, we used the terminology of "encephalitis/encephalopathy" in this study.

A recent nationwide survey for childhood encephalitis/encephalopathy in Japan determined that rotavirus was the third most common pathogen and annual cases of rotavirus-associated encephalitis/encephalopathy was estimated to be approximately 21.6 cases/years [16]. Our data is almost consistent with this previous survey for

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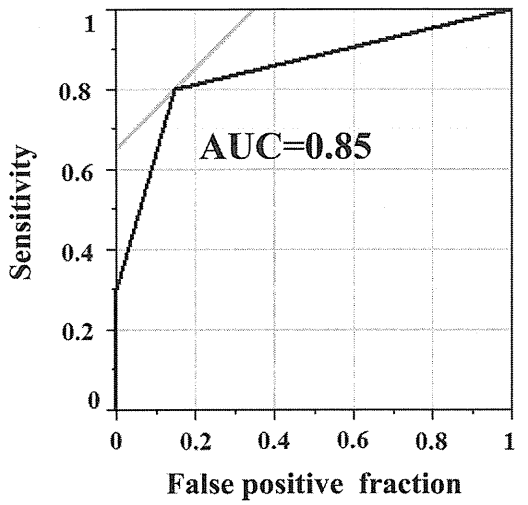

Figure 2. In order to identify the optimal prognostic factors from the 9 variables as shown in Figure 1, a backward elimination method in logistic regression was carried out. Based on this analysis, LDH (>500 IU/L) and pH (<7.15) were selected. The panel shows the receiver-operating curves for the two variables. AUC; area under the curve.

childhood encephalitis/encephalopathy, suggesting that the estimation of the annual cases of rotavirus-associated encephalitis/encephalopathy by the present survey was reliable. Additionally, data from the present survey was sufficient to estimate the incidence of rotavirus-associated sudden unexpected death. Although an autopsy was not performed for most of the cases, none of the cases had any history that suggested underlying diseases, such as an immunodeficiency. Moreover, previous studies have suggested an association between rotavirus infection and sudden unexpected death, including cases with myocarditis [5,6]. Further studies are needed to elucidate the pathogenesis of the sudden unexpected death caused by rotavirus infection. Therefore, postmortem pathological analyses should be carried out in all of the fatal cases caused by rotavirus infection in future.

Previous studies have determined an estimated fatality rate of 1 death per 1616 rotavirus-coded hospitalizations in the United States [14] and 1 death per 984 children with rotavirus gastroenteritis in Sweden [15]. Meanwhile, it has been reported that the incidence of central nervous system complications ranged between 2.5% and 3.7% of rotavirus gastroenteritis patients in the United States during the 1990's [18]. Although the complications could be determined based on the analysis of national and regional databases of hospitalization, more detailed information about the severe complications such as encephalitis/encephalopathy and sudden unexpected death of rotavirus gastroenteritis could be obtained by the questionnaire study. The present survey identified no remarkable sex difference in occurrence of rotavirus-associated encephalitis/encephalopathy and sudden unexpected death in Japan. Furthermore, marked predilection of age was not demonstrated in occurrence of the two severe complications. Additionally, similar to influenza virus encephalitis [19] and human herpesvirus 6 encephalitis [20], the majority of cerebrospinal fluid specimens collected from the rotavirus encephalitis/encephalopathy patients were normal. Surprisingly, the prognosis for rotavirus-associated encephalitis/encephalopathy was unexpectedly poor. Nearly one quarter of these patients had neurological sequelae after rotavirus-associated encephalitis/encephalopathy. As indicated in Table 2 many cases had severe neurological sequelae and 7 (12.1%) cases were fatal. Taken in combination with previous fatal case reports of rotavirus encephalitis/encephalopathy [21], the present data suggests that the prognosis of the disease is poor and we have to develop preventive measures to limit the complications. Universal vaccination has been shown to reduce rotavirus gastroenteritis in the several regions [22–24], and it is important to determine whether or not universal vaccination that will be introduced in the near future could reduce these severe complications in Japan.

Prediction of patient prognosis at the time of admission to the hospital is useful for deciding treatment strategy of the encephalitis/encephalopathy patient. The statistical difference was observed in the 9 of the 21 neuroimaging analysis and laboratory examinations between the patients with poor prognosis and those without sequelae. Finally, we found that combination of LDH (>500 IU/L) and pH (<7.15) was the best variables to predict patient's prognosis. The several biomarkers have been suggested to be important predictors for prognosis of childhood viral encephalitis [25–27]. Indeed, it is difficult to measure those biomarkers immediately in clinical setting. In contrast to the special biomarkers such as cytokines, as LDH and pH could be measured rapidly in general hospitals, these 2 predictors would be useful for clinicians. To our knowledge, this is the first study to identify the reliable predictors for prognosis of rotavirus-associated encephalitis/encephalopathy. Additionally present analysis demonstrated that the patients with neurological sequelae were significantly younger than those without the sequelae. The reason for the age difference in the presence of neurological sequelae is uncertain from this analysis. As it has been demonstrated that clinically mild encephalitis/encephalopathy with a reversible splenial lesion that demonstrates mild clinical course with good prognosis generally occurs in older children [28], clinical course of the patients might be different between the patients with and without neurological sequelae.

Although this study provides important information regarding rotavirus-associated encephalitis/encephalopathy and sudden unexpected death, it has several limitations because it is a questionnaire-based epidemiological study. It is important to note that the possibility of bias for the responding hospitals cannot be completely ruled

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out. In addition, most of the laboratory examinations were generally carried out at the time of admission to the hospital and were likely standard procedures; however, neuroimaging analysis may vary among the hospitals. Therefore, protocol for neuroimaging should be standardized to evaluate the reliability of the neuroimaging analyses in prediction of the patient's prognosis. In order to overcome these limitations, a nationwide reporting system for childhood encephalitis/encephalopathy and sudden unexpected death is necessary. Since the rapid test for rotavirus infection is highly sensitive and specific that is routinely used in Japan, we believe that when it is used in combination with a nationwide reporting system then a precise number of patients with severe complications could be elucidated.

Conflict of interest

The authors declare no conflict of interest.

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Case Report

A Case of Mild Encephalopathy with a Reversible Splenial Lesion Associated with G5P[6]Rotavirus Infection

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We report a case of mild encephalopathy with a reversible splenial lesion (MERS) associated with acute gastroenteritis caused by rotavirus (RV) infection. The patient (male, 4 years and 3 months old) was admitted to our hospital for diarrhea and afebrile seizures. Head MRI revealed a hyperintense signal in the splenium of the corpus callosum on DWI and a hypointense signal on the ADC-map. After awakening from sedation, the patient's disturbance of consciousness improved. On day 5 after admission of the illness, the patient was discharged from the hospital in a good condition. Electroencephalography on day 2 after admission was normal. On day 8 of admission, head MRI revealed that the splenial lesion had disappeared. RV antigen-positive stools suggested that RV had caused MERS. This RV genotype was considered to be G5P[6]; it may have spread to humans as a strain reassortment through substitution of porcine RV into human RV gene segments. This extremely rare genotype was detected first in Japan and is not covered by existing vaccines; this is the first sample isolated from encephalopathy patients. Few reports have investigated RV genotypes in encephalopathy; we believe that this case is valuable for studying the relationship between genotypes and clinical symptoms.

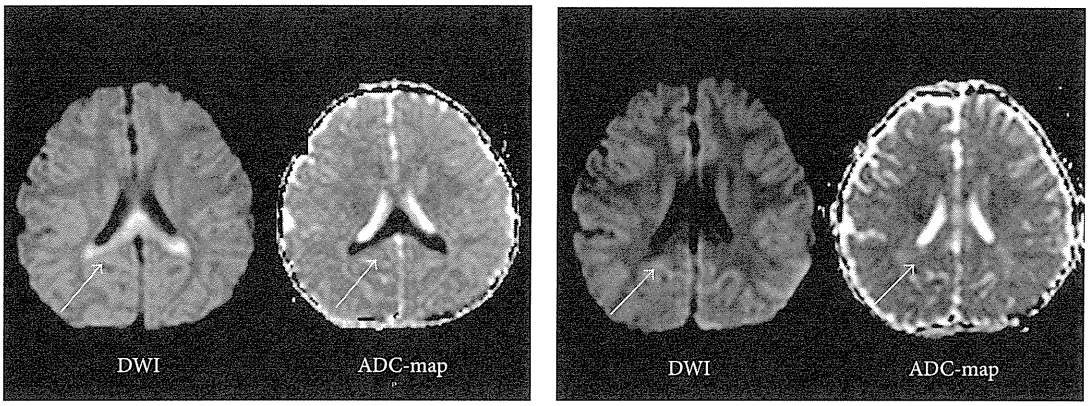
1. Introduction

Tada et al. (2004) reported a case involving clinically mild encephalopathy with a reversible splenial lesion (MERS) [1]. The clinical symptoms of this condition include delirium, disturbance of consciousness, seizures, and vomiting. The splenial lesions show hyperintense signals on diffusion-weighted imaging (DWI) and a low apparent diffusion coefficient (ADC). These lesions characteristically disappear within 1 week. The prognosis of MERS is generally good [2]. We report a case of MERS associated with rotavirus (RV) infection.

2. Case Presentation

A male patient aged 4 years and 3 months presented to the ER (emergency room) of our hospital with vomiting, diarrhea, and seizures. On day 1 of the illness, he had developed gastrointestinal symptoms, including vomiting and watery diarrhea that occurred 4-5 times per day. Four days later, bilateral generalized tonic-clonic seizures began, for which he was transported to our hospital. The seizures lasted up to 1 minute and subsided spontaneously. The patient was in a restless state of consciousness (level E3V4M5 according to the Glasgow Coma Scale and level II-10 according to the Japan Coma Scale). He stared, averted his gaze, continued to cry, and threw objects. This disturbance of consciousness persisted for 7 hours. The patient was sedated with midazolam, and MRI was performed. On awakening after 2 hours, the patient had regained a normal state of consciousness, after which there was no recurrence of disturbance of consciousness or seizures.

On admission, the patient's vital signs were as follows: temperature, 37.7°C; heart rate, 130 beats/minute; blood pressure, 100/60 mmHg; and SpO_2, 98% (room air). No



(a) (b)

FIGURE 1: (a) Head MRI on day of admission (day 4 of illness). MRI was performed on the day the seizures and disturbance of consciousness appeared. DWI reveals a hyperintense signal in the splenium of the corpus callosum and a hypointense signal on the ADC-map. (b) Head MRI performed 8 days after admission (day 12 of the illness). Head MRI (DWI, ADC-map) shows that the abnormal signal detected in the splenium of the corpus callosum had disappeared.

central nervous system abnormality, meningeal irritation, paralysis, or abnormalities in the thoracoabdominal region were observed. MRI revealed a hyperintense signal in the splenium of the corpus callosum on DWI and a hypointense signal in the ADC-map (Figure 1(a)). The patient's peripheral circulation was good.

The patient's medical history was unremarkable. He was born by spontaneous cephalic delivery after 39 weeks and 3 days of gestation (birth weight, 2664 g). No remarkable observations were noted during the pregnancy or perinatal period. The patient's growth and development were normal. The family medical history revealed that the father had febrile seizures.

The results of the patient's biochemical blood tests were as follows: WBCs, 3900/μL; Hb, 13.4 g/dL; Plt, 26.1 $\times$ 10^4/μL; BUN, 16 mg/dL; Cre, 0.3 mg/dL; Na, 132 mEq/L; K, 4.5 mEq/L; Cl, 101 mEq/L; AST, 41 IU/L; ALT, 21 IU/L; CRP, 2.59 mg/dL; Glu, 84 mg/dL; lactic acid, 7.8 mg/dL; pyruvic acid, 0.32 mg/dL; NH_3, 49 μg/dL; pH, 7.424; PCO_2, 39.6 mmHg; HCO_3, 25.5 mmol/L; and BE, 1.6 mmol/L. No abnormality was detected on blood amino acid analysis or tandem mass screening. Cerebrospinal fluid (CSF) examination revealed a cell count of 4 cells (all mononuclear), a glucose concentration of 95 mg/dL, and a total protein concentration of 16 mg/dL.

On day 2 of admission, an electroencephalogram was obtained during sedated sleep. The spindle wave was appropriate for the patient's age. No bursts were observed. The patient's stool was positive for RV antigen. RT-PCR did not reveal RV RNA in the blood or CSF. RT-PCR analysis of RNA extracted from the stool revealed that the RV genotype was G5P[6].

On day 5 of admission, no recurrences were observed, and the patient was discharged. Eight days after admission, all abnormal signals had disappeared (Figure 1(b)). The clinical course was typical of MERS.

3. Discussion

RV is a dsRNA virus belonging to the Reoviridae family. Variability in the genotypes of RV in humans affects the expression of the outer shell proteins VP4 P (proteolytic cleavage protein, "P") and VP7 G (glycoprotein, "G") [4]. To date, 27 G genotypes and 35 P genotypes have been confirmed. Of these, 5 GP genotypes account for approximately 90% of human RV infections worldwide: G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [6].

In 2006, the administration of 2 types of live vaccines, namely, Rotarix and RotaTeq, was initiated worldwide. These vaccines are effective against the major genotypes of RV [7]. In our patient, the RV genotype isolated from the stool was G5P[6]. Because G5P[6] is extremely rare, the protective effects of existing vaccines have not been investigated.

According to a national survey by Hoshino et al. (2012), 40 (4.0%) of the 983 encephalopathy cases reported between 2007 and 2010 in Japan were caused by RV infection. RV-induced encephalopathy is not uncommon, ranking third after influenza virus (26.6%) and HHV-6 (17.0%). Furthermore, 18 of the encephalopathy cases (45%) caused by RV were MERS. Compared with the incidence of influenza virus (20.2%) and HHV-6 (1.8%), the incidence of MERS is high [3].

Few reports have investigated the genotypes of RV associated with encephalopathy. The genotypes of the 14 RV encephalopathy cases diagnosed between 2005 and 2010 in Japan were all group A RV, with 4 cases of G3, 3 cases of G1, 1 case of G2, and 6 cases untyped (detected from stool samples); all were highly prevalent human RV genotypes. To date, only five G5P[6] RV strains have been isolated worldwide, all of which were detected in human gastroenteritis patients [5, 8]. To the best of our knowledge, this is the first instance of G5P[6] RV detection in an encephalopathy patient.

It is unclear whether RV readily causes encephalopathy, and individual human factors may contribute to the pathogenesis.

This was the first G5P[6] RV sample isolated from an encephalopathy patient.

Moreover, G5P[6] is not covered by existing vaccines. Understanding RV genotypes in encephalopathy is important for defining this pathological condition and enabling the development of a suitable vaccine.

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Whole genomic analysis of a porcine-like human G5P[6] rotavirus strain isolated from a child with diarrhoea and encephalopathy in Japan

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An unusual rotavirus strain, Ryukyu-1120, with G5P[6] genotypes (RVA/Human-wt/JPN/Ryukyu-1120/2011/G5P[6]) was identified in a stool specimen from a hospitalized child aged 4 years who showed diarrhoea and encephalopathy. In this study, we sequenced and characterized the complete genome of strain Ryukyu-1120. On whole genomic analysis, this strain was found to have a unique genotype constellation: G5-P[6]-I5-R1-C1-M1-A8-N1-T1-E1-H1. The VP6 and NSP1 genotypes I5 and A8 are those commonly found in porcine strains. Furthermore, phylogenetic analysis indicated that each of the 11 genes of strain Ryukyu-1120 appeared to be of porcine origin. Thus, strain Ryukyu-1120 was found to have a porcine rotavirus genetic backbone and is likely to be of porcine origin. To our knowledge, this is the first report of whole-genome-based characterization of the emerging G5P[6] strains in Asian countries. Our observations will provide important insights into the origin of G5P[6] strains and the dynamic interactions between human and porcine rotavirus strains.

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Group A rotavirus (RVA), a member of the family *Reoviridae*, is the major cause of acute gastroenteritis in the young of humans and many animal species worldwide. In humans, RVA infections are associated with high morbidity and mortality, being responsible for an estimated 453 000 deaths annually (Tate *et al.*, 2012). RVA particles enclose an 11-segment genome of dsRNA (Estes & Kapikian, 2007). Because of the segmented nature of the genome, reassortment between/within human and animal strains is one of the major processes of genetic evolution of this virus.

RVA has two outer capsid proteins, VP7 and VP4, which are implicated independently in neutralization and define the G and P genotypes, respectively. To date, RVAs are classified into at least 27 G and 37 P genotypes (Matthijnssens *et al.*, 2011; Trojnar *et al.*, 2013). Among them, some specific G and P genotypes are dominant in individual animal species. In human RVAs, G genotypes G1–G4, G9 and G12, and P genotypes P[4], P[6] and P[8] are considered as major genotypes, whilst porcine RVAs are assigned mostly to G genotypes G3–G5, G9 and G11, and P genotypes P[6], P[7] and P[13] (Santos & Hoshino, 2005; Martella *et al.*, 2010 Matthijnssens *et al.*, 2010). Recently, a classification system based on the genetic characterization of all 11 gene segments has been proposed for RVAs with the aim of facilitating tracing of the origin and evolution of RVAs (Matthijnssens *et al.*, 2008).

G5 strains, originally detected in pigs, have also been identified in children with acute diarrhoea in combination with various P genotypes in Brazil (Gouvea *et al.*, 1994), Argentina (Bok *et al.*, 2001), Paraguay (Coluchi *et al.*, 2002), Cameroon (Esona *et al.*, 2004, 2009), the UK (Beards & Graham, 1995) and Bulgaria (Mladenova *et al.*, 2012). Recently, unusual G5P[6] strains have been detected in humans in Asian countries, such as China (Duan *et al.*, 2007; Li *et al.*, 2008), Vietnam (Ahmed *et al.*, 2007) and Taiwan (Hwang *et al.*, 2012). We also detected a human G5P[6] strain (RVA/Human-wt/JPN/Ryukyu-1120/2011/G5P[6]; short name, strain Ryukyu-1120) in a stool sample collected from a hospitalized child (4 years old) who showed acute diarrhoea and mild encephalopathy with a reversible splenial lesion (MERS) in Okinawa Prefecture, Japan (Matsuoka *et al.*, unpublished results, Yodoshi, T., Sugai,

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of VP1–4, VP6–7 and NSP1–5 of strain Ryukyu-1120 are AB741649–AB741659, respectively.

Two supplementary figures and two tables are available with the online version of this paper.

M., Hiyane, M., Matsuoka, T., Akeda, H., Ohfu, M., Komoto, S., Taniguchi, K., unpublished results). To our knowledge, this is the first detection of a human G5 strain in Japan.

Whole genomic analysis is a reliable method for obtaining conclusive data on the origin of an RVA strain and for tracing its evolutionary pattern (Matthijnssens *et al.*, 2008, 2011; Ghosh & Kobayashi, 2011). To date, all 11 gene segments of only one human G5P[6] strain, Bulgarian BG620, have been analysed, providing evidence of its porcine origin (Mladenova *et al.*, 2012). Partial genomic analyses of Asian G5P[6] strains (only three or four of the 11 segments) have also shown the presence of porcine-like gene segment(s) (Ahmed *et al.*, 2007; Duan *et al.*, 2007; Li *et al.*, 2008; Hwang *et al.*, 2012). However, the overall genomic constellation and the exact evolutionary patterns of Asian G5P[6] strains remain to be elucidated. Moreover, as strain BG620 was detected in Europe, whole genomic analysis of strain Ryukyu-1120 might be useful for gaining a proper understanding of the evolutionary patterns of emerging G5P[6] strains in Asian countries. In the present study, we analysed the whole genome of the first Japanese strain, Ryukyu-1120, with G5P[6] genotypes isolated from a diarrhoeic child with encephalopathy.

Viral dsRNAs were extracted from stool suspensions using TRI Reagent LS (Molecular Research Center). For G and P genotyping, a multiplex semi-nested reverse transcription (RT)-PCR-based genotyping method (Taniguchi *et al.*, 1992; Wu *et al.*, 1994) was used with some modifications. The primers used for G and P genotyping are listed in Table S1, available in JGV Online. For the first PCR step, the full-length VP7 gene (1062 bp) and the partial-length VP4 gene (1094 bp) were amplified with primers VP7/T31F and VP7/T32R and primers VP4/T52F and VP4/T74R, respectively. The PCR products were used as templates for the second multiplex PCR. For G genotyping, the second PCR was primarily carried out using a pool consisting of G1-, G2-, G3-, G4-, G8- and G9-specific sense primers that correspond to most of the G genotypes circulating in humans, and the conserved antisense VP7/T32R primer. However, we failed to amplify the VP7 gene of strain Ryukyu-1120 by the usual PCR method (Fig. 1a, left panel), and we modified the second PCR by employing new sense primers specific for G5, G6, G10, G11 and G12 genotypes that have been detected in humans at low frequencies. Through the multiplex PCR with the new primers, we successfully amplified the VP7 gene of strain Ryukyu-1120 as a fragment of 310 bp, the expected size for the G5 genotype (Fig. 1a, right panel). For P genotyping, the conserved sense VP4/T52F primer and a primer set consisting of P[4]-, P[6]-, P[8]- and P[9]-specific antisense primers were employed for the second PCR. Through this PCR, we successfully amplified a partial VP4 gene of strain Ryukyu-1120 as a fragment of 753 bp, the expected size for

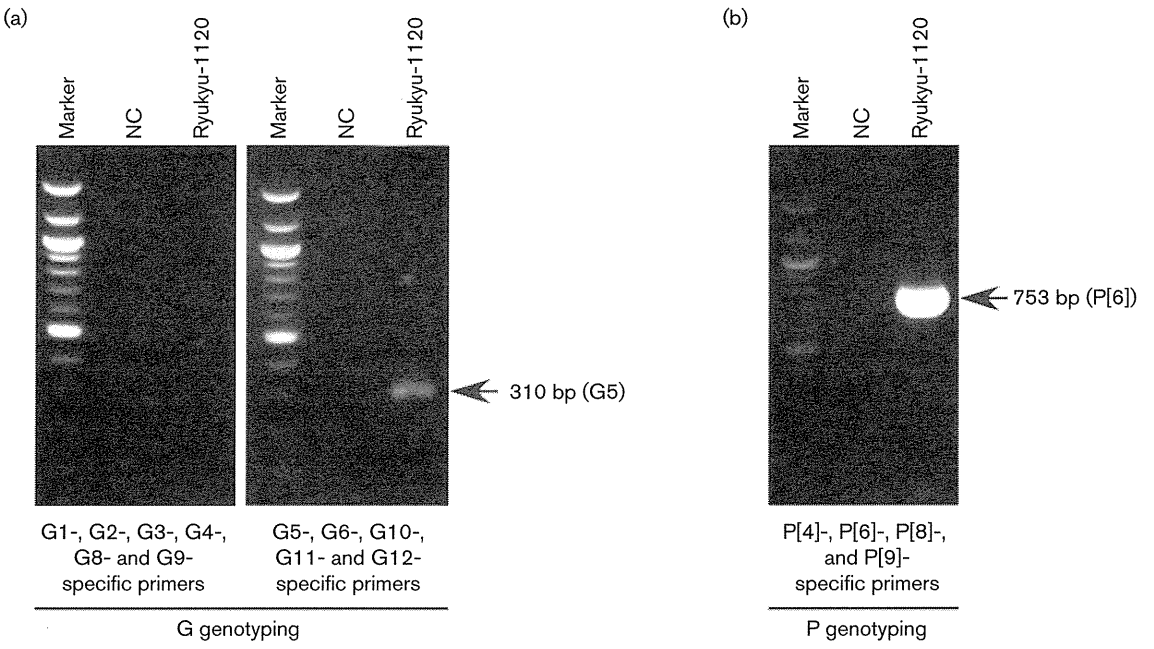


Fig. 1. PCR-based G and P genotyping of strain Ryukyu-1120. The full-length VP7 gene and the partial-length VP4 gene were amplified by the first PCR. The amplified cDNAs were used as templates for the second multiplex PCR, followed by separation in 1.5 % agarose gels. (a) For G genotyping, the second PCR was performed using a set of G1-, G2-, G3-, G4-, G8- and G9-specific primers (left panel), or a set of G5-, G6-, G10-, G11- and G12-specific primers (right panel). (b) For P genotyping, the second PCR was performed with a set of P[4]-, P[6]-, P[8]- and P[9]-specific primers. NC, negative control (viral dsRNA as the template was replaced with water). Marker, 100 bp ladder size markers.

Table 1. Genotypes of the 11 gene segments of strain Ryukyu-1120 compared with those of selected human and porcine strains

Italic indicates the gene segments with a genotype identical to that of strain Ryukyu-1120. The gene segments that are most similar to those of strain Ryukyu-1120 are highlighted in bold. –, No sequence data available in GenBank/EMBL/DDBJ.

Strain	Genotype										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Human-wt/JPN/Ryukyu-1120/2011/G5P[6]	*G5*	*P[6]*	*I5*	*R1*	*C1*	*M1*	*A8*	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/USA/Wa/1974/G1P[8]	G1	P[8]	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/JPN/KU/1974/G1P[8]	G1	P[8]	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/USA/DS-1/1976/G2P[4]	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/BEL/F01322/2009/G3P[6]	G3	*P[6]*	I2	R2	C2	M2	A2	N2	T2	E2	H2
RVA/Human-tc/USA/P/1974/G3P[8]	G3	P[8]	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/JPN/AU-1/1982/G3P[9]	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
RVA/Pig-tc/VEN/A131/1988/G3P[7]	G3	P[7]	*I5*	*R1*	C2	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/GBR/ST3/1975/G4P[6]	G4	*P[6]*	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Pig-tc/USA/Gottfried/1975/G4P[6]	G4	*P[6]*	I1	*R1*	*C1*	*M1*	***A8***	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/CHN/R479/2004/G4P[6]	G4	***P[6]***	*I5*	*R1*	*C1*	*M1*	A1	*N1*	T7	*E1*	*H1*
RVA/Pig-tc/USA/SB1A/xxxx/G4P[7]	G4	P[7]	–	–	–	–	–	*N1*	*T1*	*E1*	***H1***
RVA/Human-wt/CHN/LL3354/2000/G5P[6]	*G5*	*P[6]*	*I5*	–	–	–	–	–	–	*E1*	–
RVA/Human-wt/CHN/LL4260/2001/G5P[6]	*G5*	*P[6]*	–	–	–	–	–	–	–	*E1*	–
RVA/Human-wt/CHN/LL36755/2003/G5P[6]	*G5*	*P[6]*	–	–	–	–	–	–	–	*E1*	–
RVA/Human-wt/VNM/KH210/2004/G5P[6]	*G5*	*P[6]*	–	–	–	–	–	–	–	*E1*	–
RVA/Human-wt/TWN/03-98sP50/2009/G5P[6]*	*G5*	*P[6]*	*I5*	–	–	–	–	–	–	*E1*	–
RVA/Human-wt/BGR/BG620/2008/G5P[6]*	*G5*	*P[6]*	I1	*R1*	*C1*	*M1*	*A8*	*N1*	*T1*	*E1*	*H1*
RVA/Human-wt/CMR/6784/ARN/2000/G5P[7]	*G5*	P[7]	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/BRA/IAL28/1992/G5P[8]	*G5*	P[8]	*I5*	*R1*	*C1*	*M1*	A1	***N1***	*T1*	*E1*	*H1*
RVA/Pig-tc/USA/OSU/1975/G5P[7]	*G5*	P[7]	*I5*	***R1***	***C1***	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Pig-wt/JPN/TJ4-5/2010/G5P[13]/[22]	***G5***	P[13]/[22]	–	–	–	–	–	–	–	–	–
RVA/Human-wt/BEL/BE2001/2009/G9P[6]	G9	*P[6]*	*I5*	*R1*	*C1*	***M1***	*A8*	*N1*	T7	*E1*	*H1*
RVA/Pig-tc/KOR/PRG9121/2006/G9P[7]	G9	P[7]	*I5*	*R1*	*C1*	*M1*	*A8*	*N1*	***T1***	***E1***	*H1*
RVA/Human-tc/USA/WI61/1983/G9P[8]	G9	P[8]	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Human-wt/BEL/B3458/2003/G9P[8]	G9	P[8]	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/THA/Mc323/1989/G9P[19]	G9	P[19]	*I5*	*R1*	*C1*	*M1*	*A8*	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/THA/Mc345/1989/G9P[19]	G9	P[19]	*I5*	*R1*	*C1*	*M1*	*A8*	*N1*	*T1*	*E1*	*H1*
RVA/Human-tc/IND/mani-97/2006/G9P[19]	G9	P[19]	*I5*	*R1*	*C1*	*M1*	*A8*	*N1*	*T1*	*E1*	*H1*
RVA/Pig-tc/KOR/PRG942/2006/G9P[23]	G9	P[23]	***I5***	*R1*	***C1***	*M1*	*A8*	*N1*	***T1***	***E1***	*H1*
RVA/Pig-tc/KOR/PRG9235/2006/G9P[23]	G9	P[23]	***I5***	*R1*	***C1***	*M1*	*A8*	*N1*	***T1***	***E1***	*H1*
RVA/Pig-tc/MEX/YM/1983/G11P[7]	G11	P[7]	*I5*	*R1*	*C1*	*M1*	*A8*	*N1*	*T1*	*E1*	***H1***
RVA/Human-wt/BGD/Matlab13/2003/G12P[6]	G12	*P[6]*	I1	*R1*	*C1*	*M1*	A1	*N1*	T2	*E1*	*H1*
RVA/Human-wt/BEL/B4633/2003/G12P[8]	G12	P[8]	I1	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*
RVA/Pig-wt/IND/RU172/2002/G12P[7]	G12	P[7]	*I5*	*R1*	*C1*	*M1*	A1	*N1*	*T1*	*E1*	*H1*

*Genotype assignment based on reports by Hwang *et al.* (2012) (strain 03-98sP50) and Mladenova *et al.* (2012) (strain BG620). To our knowledge, to date, the nucleotide sequence accession numbers for strains 03-98sP50 and BG620 are not available in the GenBank/EMBL/DDBJ data libraries.

the P[6] genotype (Fig. 1b). Thus, PCR-based G and P genotyping indicated that strain Ryukyu-1120 was a novel RVA strain with G5P[6] genotypes.

Because G5 strains have been detected exclusively in pigs in Japan (Teodoroff *et al.*, 2005; Miyazaki *et al.*, 2011), the detection of a G5P[6] strain in the human population was suggestive of zoonotic transmission. In order to investigate this possibility, the full-length nucleotide sequences (exactly full-length sequences excluding the 5′- and 3′-end primer sequences) of all 11 segments of strain Ryukyu-1120 were determined and genotyped. The primers used for cDNA amplification of individual genes are listed in Table S2. The RT-PCR products obtained were cloned into the pMD20-T vector (Takara Bio). Three clones for each gene were sequenced using an ABI PRISM 3730 DNA Analyser (Life Technologies, Applied Biosystems). Sequencing was performed with the universal M13M4 and M13RV primers annealing to the pMD20-T vector. Primer-walking sequencing was performed to cover the complete sequences of the VP1, VP2, VP3 and VP4 genes. The genotype of each of the 11 gene segments was determined using the RotaC v2.0 automated genotyping tool (http://

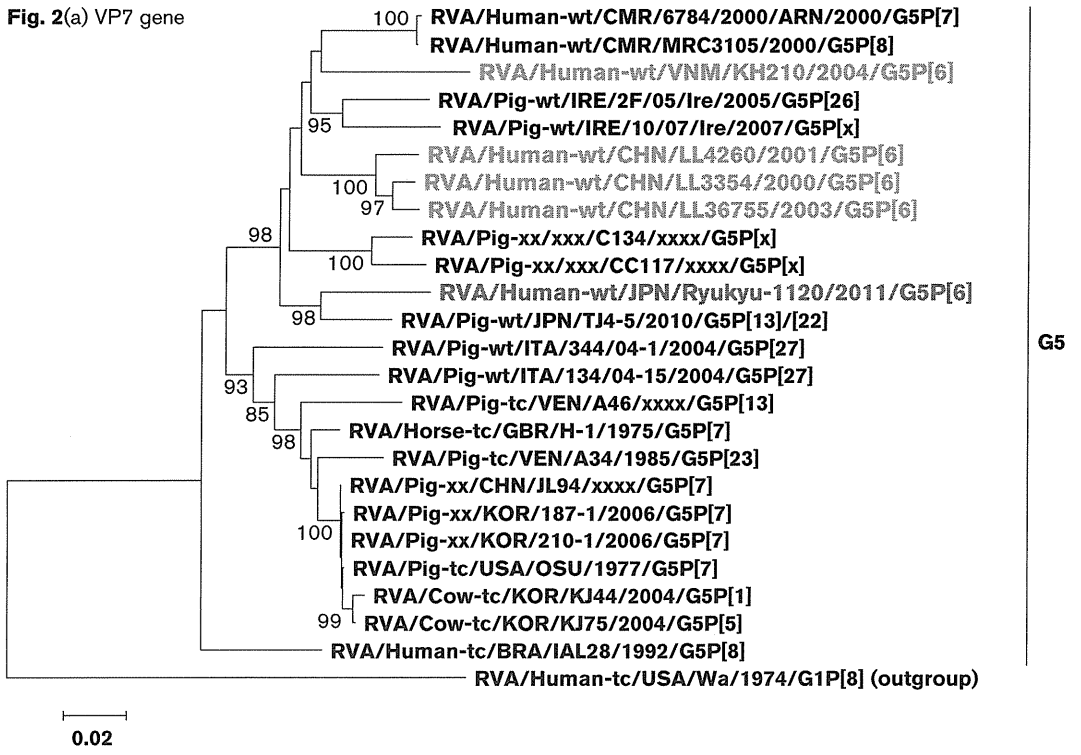


Fig. 2(a) VP7 gene

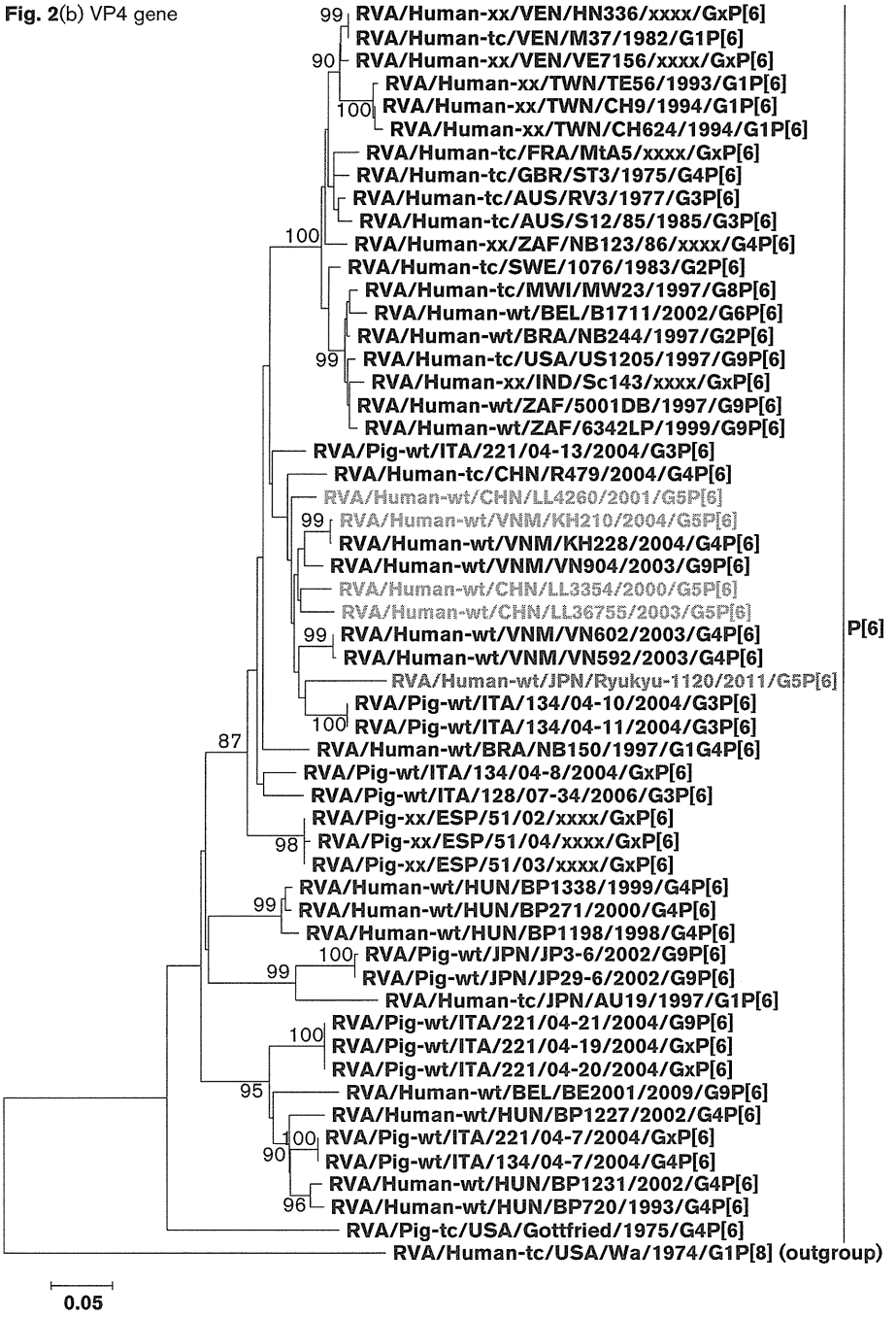
rotac.regatools.be/; Maes *et al.*, 2009) according to the guidelines proposed by the Rotavirus Classification Working Group. The VP7, VP4, VP6, VP1–3 and NSP1–5 genes were assigned to the G5, P[6], I5, R1, C1, M1, A8, N1, T1, E1 and H1 genotypes, respectively (Table 1). Strain Ryukyu-1120 was confirmed to have G5P[6] genotypes, as determined by PCR-based genotyping. Comparison of the complete genotype constellation of strain Ryukyu-1120 (G5-P[6]-I5-R1-C1-M1-A8-N1-T1-E1-H1) with those of other G5 and non-G5 strains is shown in Table 1. Although most segments (VP1–3 and NSP2–5) belonged to Wa-like genotypes, the VP6 and NSP1 genotypes (I5 and A8) are those commonly found in porcine strains (Zeller *et al.*, 2012). Strain Ryukyu-1120 was found to share the same constellation of VP6, VP1–3 and NSP1–5 genotypes (I5-R1-C1-M1-A8-N1-T1-E1-H1) with porcine strains {YM (G11P[7]), PRG9121 (G9P[7]), PRG942 (G9P[23]) and PRG9235 (G9P[23])} and porcine-like human strains {Mc323 (G9P[19]), Mc345 (G9P[19]) and mani-97 (G9P[19])}, although some porcine strains have been found to have other VP6 (I1 instead of I5) and NSP1 (A1 instead of A8) genotypes. Human strains Mc323, Mc345 and mani-97 have been shown to have porcine backbones and are likely to be of porcine origin through their full genomic analyses (Mukherjee *et al.*, 2011; Ghosh *et al.*, 2012). Thus, the genotype constellation of strain Ryukyu-1120 was mostly identical to that of porcine strains and porcine-like human strains.

We next constructed phylogenetic trees using the full-genome sequence for each of the 11 gene segments because phylogenetic analysis of RVA nucleotide sequences provides direct evidence of their relatedness to those of other strains, even within the same genotype (Matthijnssens *et al.*, 2008). Multiple alignment of each viral gene was performed using the CLUSTAL W program. Phylogenetic analysis was performed with MEGA5 (Tamura *et al.*, 2011) based on the neighbour-joining method. The reliability of the branching order was estimated from 1000 bootstrap replicates.

The VP7 gene of strain Ryukyu-1120 exhibited maximum nucleotide sequence identity (91.6 %) with that of Japanese porcine strain TJ4-5 (G5P[13]/[22]) (Miyazaki *et al.*, 2011) and lower similarities with the other porcine, porcine-like human and bovine strains (84.0–88.7 %). On phylogenetic analysis, both Japanese strains were closely related to each other, despite their isolation from different species (Fig. 2a).

The VP4 gene of strain Ryukyu-1120 showed highest nucleotide sequence similarity (92.9 %) with a Chinese porcine–bovine multi-reassortant human strain, R479 (G4P[6]) (Wang *et al.*, 2010), and comparable identity with Asian porcine-like human strains LL4260 (G5P[6]) (92.6 %) (Li *et al.*, 2008) and VN904/2003 (G9P[6]) (92.6 %) (Nguyen *et al.*, 2007). On phylogenetic analysis, strain Ryukyu-1120 was found to cluster with Italian porcine strains 134/04-10 (G3P[6]) and 134/04-11 (G3P[6]) sharing nucleotide sequence identity (91.7 %) (Martella *et al.*, 2006) in a common branch with several porcine and porcine-like human strains (Fig. 2b).

The VP6 gene of strain Ryukyu-1120 exhibited maximum nucleotide sequence similarity (94.5 %) with the Korean porcine strains PRG942 (G9P[23]) and PRG9235 (G9P[23]).



Phylogenetically, strain Ryukyu-1120 clustered with these porcine strains in a common branch with several porcine and porcine-like human strains within the porcine-like I5 genotype (Fig. 2c).

The VP1, VP2 and VP3 genes of strain Ryukyu-1120 showed maximum nucleotide sequence identities (87.5–93.0, 90.7–93.7, 89.5–90.1 and 90.2–91.9 %, respectively) with the cognate genes of reference porcine strains {OSU (G5P[7]), Gottfried (G4P[6]) and YM (G11P[7])} and the porcine-like human strain BE2001 (G9P[6]) (Zeller *et al.*, 2012). On phylogenetic analyses, strain Ryukyu-1120 was found to cluster near these porcine and porcine-like human strains (Fig. S1a–c).

The NSP1 gene of strain Ryukyu-1120 exhibited highest nucleotide sequence identity (92.7 %) with that of porcine strain Gottfried, and somewhat lower identities (87.7–90.5 %) with Indian porcine-like human strains {mani-97 (G9P[19]), mani-362 (G4P[6]), mani-253 (G4P[4]) and mcs-13 (G9P[6])} (Mukherjee *et al.*, 2009, 2010) and the Belgian porcine-like human strain BE2001 (G9P[6]). On phylogenetic analysis, strain Ryukyu-1120 clustered with strain BE2001 in a common branch with these porcine and

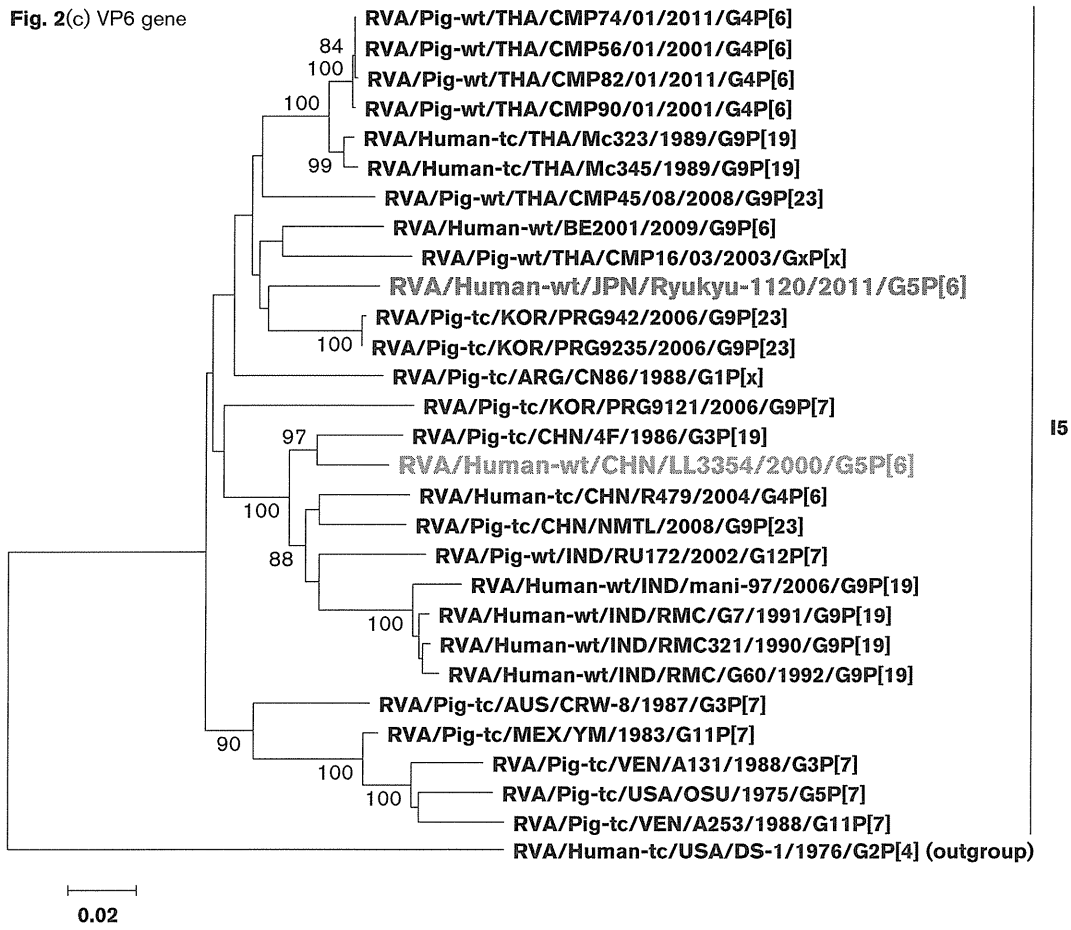


Fig. 2. Phylogenetic trees constructed from the nucleotide sequences of the VP7 (a), VP4 (b) and VP6 (c) genes of strain Ryukyu-1120 compared with those of other RVA strains. In all trees, the position of strain Ryukyu-1120 is shown in red, whilst the positions of the other G5P[6] human strains are shown in blue. Bootstrap values of <85 % are not shown. Bars, 0.02 (a, c) and 0.05 (b) substitutions per nucleotide.

porcine-like human strains within the porcine-like A8 genotype (Fig. S1d).

The NSP2 gene of strain Ryukyu-1120 exhibited maximum nucleotide sequence similarity (94.3 %) with that of a porcine-human reassortment strain, IAL28 (G5P[8]) (Timenetsky *et al.*, 1997; Heiman *et al.*, 2008), and somewhat lower similarities (91.2–92.4 %) with other porcine strains {PRG9121, PRG9235 and RU172 (G12P[7])} and a porcine–bovine multi-reassortant human strain (R479). On phylogenetic analysis, strain Ryukyu-1120 was found to be clustered with strain IAL28 in a common branch with these porcine and porcine-like human strains (Fig. S1e).

The NSP3 and NSP4 genes of strain Ryukyu-1120 showed highest nucleotide sequence similarities (95.1 and 95.3–95.5 %, respectively) with those of Korean porcine strains (PRG942, PRG9121 and PRG9235). On phylogenetic analysis, the NSP3 and NSP4 genes of strain Ryukyu-1120 were found to be closely related to those of these porcine strains (Fig. S1f, g).

The NSP5 gene of strain Ryukyu-1120 exhibited maximum nucleotide sequence identity (98.8 %) with porcine strains YM (G11P[7]) and SB1A (G4P[7]), and comparable genetic identities (98.6, 98.6 and 98.4 %, respectively) with porcine strains Gottfried, PRG9121 and RU172. On phylogenetic analysis, strain Ryukyu-1120 was found cluster with Indian porcine strain RU172 (G12P[7]) (Ghosh *et al.*, 2010) in a common branch with several porcine and porcine-like human strains (Fig. S1h).

Taken together, each of the 11 genes of strain Ryukyu-1120 was found to be closely related to porcine RVA genes. Therefore, strain Ryukyu-1120 has a porcine genetic backbone and is likely to be of porcine origin.

Because the ability to cultivate emerging G5P[6] strains in cell culture will undoubtedly enhance further studies on them, we then attempted to isolate strain Ryukyu-1120 using the MA104 cell line in roller culture (Urasawa *et al.*, 1981). Suspended stool samples containing strain Ryukyu-1120

were subjected to serial passages in MA104 cells. Although a significant cytopathic effect was not observed in the first and second passages, MA104 cells in the third passage showed an RVA-derived cytopathic effect, suggesting the isolation of cell culture-adapted strain Ryukyu-1120. Virion dsRNAs were extracted and analysed by PAGE followed by silver staining (Taniguchi *et al.*, 1994) to determine the genomic dsRNA profile. Fig. S2 shows the profiles of viral dsRNAs from human strain KU (G1P[8]) as a reference and strain Ryukyu-1120 extracted from stool samples and from cell culture. All strains showed a long electropherotype. The dsRNA migration pattern of cell culture-adapted strain Ryukyu-1120 was identical to that of the original strain Ryukyu-1120 present in stool samples. It was confirmed that the isolated strain Ryukyu-1120 retains G5P[6] genotypes by PCR-based genotyping (data not shown).

In the present study, we analysed the whole genome of the first Japanese strain, Ryukyu-1120, with G5P[6] genotypes (RVA/Human-wt/JPN/Ryukyu-1120/2011/G5P[6]) from a child with acute diarrhoea and MERS. Strain Ryukyu-1120 showed a unique genotype constellation: G5-P[6]-I5-R1-C1-M1-A8-N1-T1-E1-H1. The VP6 and NSP1 genotypes I5 and A8 are genotypes commonly found in porcine strains. Phylogenetic analysis revealed that each of the 11 genes of strain Ryukyu-1120 appeared to be of porcine origin. Therefore, strain Ryukyu-1120 was assumed to be the result of direct interspecies transmission from pigs to humans, or a reassortment event(s) between human and porcine strains.

Recently, unusual G5P[6] strains have been found in humans in Asia. Because it has not been examined whether or not two available RVA vaccines [Rotarix (GlaxoSmithKline) and RotaTeq (Merck)] are effective for prevention against G5P[6] strains, continuing RVA surveillance of this G/P combination in the human population may be required. Simultaneous monitoring of RVA strains in humans and animals is also essential for a better understanding of RVA ecology. Furthermore, it is necessary to survey the prevalence of G5P[6] strains in the Okinawa area of Japan where strain Ryukyu-1120 was detected.

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PLOS ONE

A New Method for the Detection of Neutralizing Antibodies against Mumps Virus

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Abstract

Neutralization test is the most reliable method of evaluating immunity against viral diseases but there is no standard procedure for mumps virus, with tests differing in the infectivity of the challenge virus, 50% plaque reduction or complete inhibition of cytopathic effects (CPE), and usage of complement. A reliable, easy, and simple neutralization test for mumps virus was developed in this study. A recombinant mumps virus expressing GFP was generated as a challenge virus. Complement was added to the neutralizing mixture at 1:200 when stocked serum samples were used. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution that did not exceed two-fold of FU values (GFP expression) of the cell control wells. A total of 1,452 serum samples were assayed by inhibition of GFP expression in comparison with those examined by conventional 100% inhibition of CPE. 1,367 (94.1%) showed similar neutralizing antibody titers when examined by both methods. The GFP expression inhibition assay, using a recombinant mumps virus expressing GFP, is a simple and time- saving method.

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Introduction

Mumps virus is a single-stranded negative sense RNA virus, belonging to the genus *Rubulavirus* of the family *Paramyxoviridae*. The mumps virus genome encodes seven major proteins in the following gene order: nucleocapsid (N), phospho (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) protein genes [1]. V and I proteins are also produced from the P gene. There are two envelope glycoproteins, F and HN. The HN protein is involved in the virus attachment to sialic acid receptors on the surface of host cells. This leads to a conformational change of HN which induces further conformational change of the F protein in the cascade reaction of cell fusion [1,2]. Thus, mumps virus infection is initiated by the F and HN proteins, and neutralizing epitopes are located on these proteins [3,4].

An acute infection of mumps virus is characterized by self-limiting demonstrable swelling of the parotid glands with tenderness and several complications have been reported following parotitis, including aseptic meningitis, deafness, orchitis, and pancreatitis [1,5]. Mumps virus circulates throughout the world, and genotype classification of the wild type is useful for identifying the pathway of transmission [6]. Recently, circulating mumps virus strains have been divided into 12 genotypes from A to N (excluding E and M) based upon the sequence diversity of the SH gene [7,8]. Currently circulating strains in Japan were divided into four genotypes, B, G, J, and L [9].

The isolation of mumps virus is essential for the diagnosis of patients and for monitoring the antigenicity of wild circulating strains. The efficiency of virus isolation depends mainly upon the infectious viral load in clinical samples and the sensitivity of the cells used for isolation. Vero cells have been used, but isolation is not always successful because of the low viral load, timing of sample taking, and transportation. Several serological tests have been employed for the diagnosis of mumps virus infections and, among them, the enzyme-linked immuno-assay (EIA) was used to detect IgM antibodies for diagnosis and IgG EIA to investigate immune status [10,11]. EIA antibodies did not reflect protective immunity and a neutralization test is the most sensitive way to predict protective immunity [12,13]. Neutralization tests take a long time to obtain results and involve several complicated procedures [14,15]. The sensitivity of neutralization test was enhanced when complement was added [15]. Recently, the addition of complement was found to lead to deposition on the surface of viral particles bound with antibodies and destroyed the structure of mumps virus during the neutralization reaction [16]. Thus, the presence of complement seems to be essential for neutralization testing against mumps virus. In this study, a recombinant mumps virus expressing green fluorescent protein (GFP) was generated and the requirement for complement was examined using fresh and stocked sera.

Materials and Methods

Mumps Virus Strain

The Hoshino vaccine seed strain KO3 was developed by attenuation through 22 passages in chick embryonic cells from a wild-type mumps virus isolated in 1972 [17]. Full-length cDNA was constructed from KO3 Hoshino. The GFP sequence was inserted between the P/V and M genes (Fig. 1). GFP Hoshino was recovered from 293 T cells transfected with N, P, and L expression plasmids, and full-length cDNA under the control of T7 RNA polymerase [18]. Monolayer of Vero cells was infected with GFP Hoshino at m.o.i = 0.01 and culture fluid was stocked for challenge virus.

Virus Infectivity

Vero cells were propagated in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). Infectivity was determined based on the $TCID_{50}$ in Vero cells. The virus culture fluid was serially diluted by 10-fold and a confluent monolayer of Vero cells was infected with 100 µl of each dilution in 96-well plates. The plates were incubated for 2 h at 37°C in 5% CO_2 and MEM supplemented with 2% FBS was added. Infectivity was determined after incubation for 7 days.

Serum Samples

Eight serum samples obtained from healthy adults aged 23 to 58 years during a routine health check were used for the experiments after obtaining verbal informed consent. The remaining portion of the sera was used for preliminary experiments or as in-house control serum. Stocked serum samples (n = 185) were obtained to assess immunity against measles, mumps, rubella, and chickenpox among new students of the nursing school of Ashikaga Red Cross Hospital, Tochigi prefecture. The serological study was approved by the ethics committee of the hospital and verbal informed consent was obtained. Fresh serum samples (n = 1,452) obtained to evaluate immunity against measles, mumps, rubella, and chickenpox among new students in primary, junior high, and high schools, were used for routine yearly immunological assessments of infection control and to advise regarding vaccination for antibody negative pupils. The serological study was approved by the Health Care Center of Keio University. The purpose of the study was explained and written informed consent was obtained from their guardians. Serum samples were anonymously transferred to our laboratory, labeled with simplified numbers.

Virus Neutralization Test

The fresh serum samples were divided into several aliquots and stocked at −20°C. The samples were kept at 56°C for 30 min to inactivate the complement, serially diluted by 2-fold starting from 1:4, and mixed with the same volume of GFP Hoshino containing 100 $TCID_{50}$ of infectious virus at 37°C for 90 min for neutralization. The mixture was placed in 96-well plates in duplicate for each dilution and 25,000 Vero cells were seeded in 0.1 ml. The plates were incubated for 7 days. In order to calculate the titers automatically, the plates were processed to detect fluorescence intensity (Fluoro-Units: FU) at an emission wavelength of 528 nm and excitation wavelength of 485 nm using a fluorescence reader, FLx800 (Bio-Tek Instruments, Vermont, USA), similar to a method used to detect measles neutralizing antibodies [19]. To evaluate the requirement of complement, various concentrations of guinea pig complement (Denka Seiken, Tokyo, Japan) were added to the neutralization mixture of serially

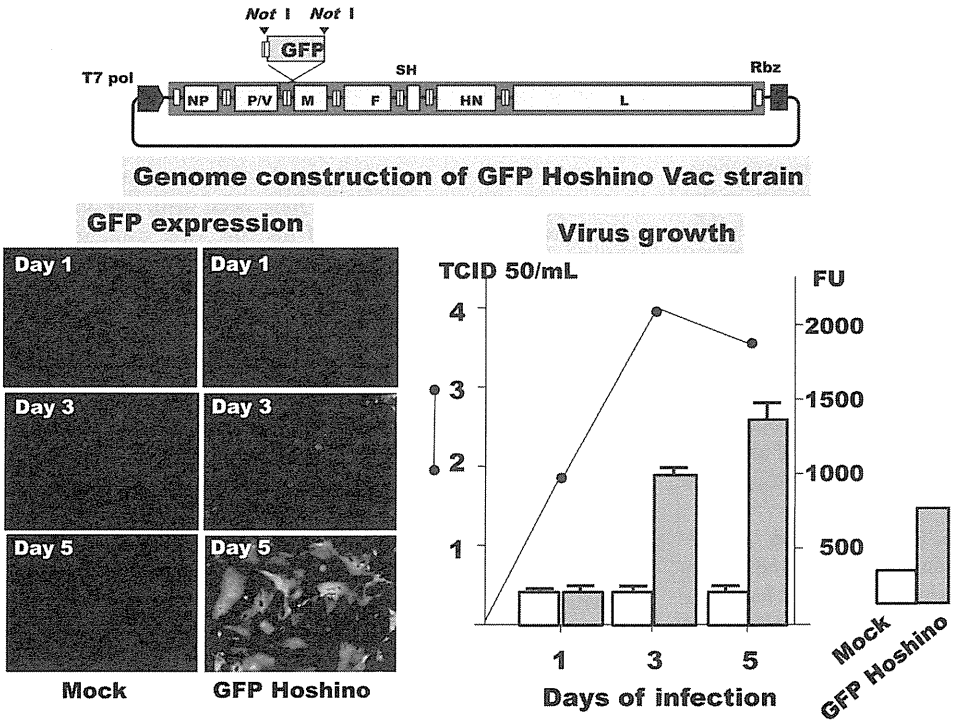

Figure 1. Genome construction of the recombinant mumps Hoshino strain expressing GFP and expression of GFP. Vero cells were infected with GFP Hoshino mumps strain at m.o.i. = 0.02 and subjected to experiments for GFP expression with fluoro EIA and microscopic examination on day 1, 3 and 5 of infection in comparison with mock-infection. Infectivity was assayed in culture supernatants on day 1, 3, and 5 of infection.
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diluted serum with challenge virus. Neutralizing antibody titers were determined as the reciprocal of the highest dilutions that did not exceed two-fold of FU values (GFP expression) of the cell control wells. Conventional neutralizing antibody titers were expressed as the reciprocal of the serum dilutions that showed 100% inhibition of CPE. Infectivity titer of the challenge virus was back-titrated in each assay, showing 50–120 $TCID_{50}$.

Statistical Analysis

Statistical significance in the neutralizing antibody titers was examined between two groups by the Mann-Whitney test. A coefficient was used for the analysis of correlation between the NT and EIA titers.

Results

GFP Expression

GFP expression and the viral growth are shown in Fig. 1. Vero cells were infected with the GFP Hoshino strain in a 24-well plate, and culture fluids were obtained 1, 3, and 5 days later. A peak infective titer of 10^4 TCID 50/ml was obtained 3 days after infection. Mean GFP expression (FU) is shown with 1.0 standard deviation (SD) in four wells in comparison with mock-infected wells. Mock-infected wells showed approximately 300 FU during the experiment, and GFP expression in infected wells increased to peak (1,300 FU) on day 5 of infection. Together with FU in fluoro-ELISA, fluoro-microscopic findings of CPE expansion with GFP expression are also shown in Fig. 1. Few CPE were observed on day 3 of infection and extensive cell fusion was noted on day 5. The development of CPE was closely related to GFP expression.

Neutralizing Antibody Titers

The results of the neutralization tests are shown in Fig. 2. Serum samples were serially diluted 2-fold from 1:4 to 1:256, and mixed with the challenge virus. The NT assay was done in duplicate. The results for one serum sample are shown in Fig. 2. CPE were observed in one well at 1:32 and none at 1:16. The conventional neutralizing antibody titer was considered to be 1:16 for 100% inhibition of CPE. The mean FU of cell control wells (mock-infected wells) was 202 FU. The mean FU of serial dilutions from 1:4 to 1:256 was 252 FU, 239 FU, 234 FU, 450 FU, 543 FU, 581 FU, and 591 FU, respectively. GFP expression increased to 450 FU at 1:32 and thus the neutralizing antibody titer for the GFP expression assay was 1:16 for inhibition of the growth of GFP Hoshino. The infective titers of the challenge virus were back-titrated, showing 50–120 TCID 50. When CPE appeared in >20% of the wells, GFP expression was >500 FU.

To evaluate the consistency of neutralizing antibody titers assayed by 100% inhibition of the appearance of CPE or GFP expression, neutralization tests for both conventional and GFP expression methods were done in 1,452 fresh serum samples. Three cut-off levels for positive GFP expression were set: 1.5-, 2.0-, and 2.5-fold increase in FU compared to cell culture controls. Among the 1,452 samples, 1,287 (88.6%) showed the similar neutralizing antibody titers when assayed by both methods using the 1.5-fold cut-off, 1,367 (94.1%) with the 2.0-fold cut-off, and 1,058 (72.9%) with the 2.5-fold cut-off. Strong similarity was noted when the cut-off was defined as a 2.0-fold of FU value in FU of the control wells.

Effect of Heat Inactivation and Addition of Complement

Eight fresh serum samples (A–H) were obtained and stocked at −80°C. Neutralizing antibody titers were examined before freeze-thawing, and after three and five rounds of freeze-thawing. The results are shown in Fig. 3. For serum A, the neutralizing antibody titer was 1:256, 1:64, and 1:128, showing no significant difference within five rounds of freeze-thawing. It decreased to 1:8 or 1:16 after inactivation at 56°C for 30 min. The other serum showed similar results. Neutralizing antibody titers did not decrease but decreased after inactivation of the complement. Complement activity would be required for neutralization tests for mumps virus.

Five fresh serum samples (A–E) were inactivated at 56°C for 30 min. When inactivated sera were used, guinea pig complement was added to the neutralizing mixture at 1:200, 1:400, 1:800, and 1:1,600. Neutralizing antibody titers were examined and mean values for three independent assays are shown in Fig. 4. Guinea pig complement did not affect the assay system without any changes in Vero cell cultures and the addition of guinea pig complement in non-inactivated sera did not influence the neutralizing antibody titers. The titer was 1:32–1:128 and dropped to around 1:8 after inactivation. The reduced neutralizing antibody titers increased to levels similar to those before inactivation when the complement was added at 1:200 or 1:400. Therefore, complement was added at 1:200 to the neutralizing mixture in the subsequent experiments.

Effect of Complement

Twenty-one fresh serum samples were obtained and neutralizing antibody titers were examined for non-inactivated and inactivated sera supplemented with complement at 1:200 in the neutralizing mixture. The results are shown in Fig. 5. The peak distribution of neutralizing antibody titers for non-inactivated samples was 1:32 and shifted to 1:64, showing no significant change in those with addition of complement.

As for the 227 stocked sera, neutralization tests were performed before and after inactivation with the addition of complement. 74 serum samples showed negative and 70 became positive, when assayed after inactivation with the addition of complement. The peak distribution of neutralizing antibody titers markedly shifted from 1:4 for non-inactivated sera (98 sera) to 1:16 after inactivation supplemented with complement (75 sera). Stocked sera were considered to lose complement activity over long periods. Therefore, the addition of complement was required when the neutralizing antibody titer was examined for the stocked sera, probably because of decreased complement activity.

Discussion

There are several serological methods of detecting mumps antibodies. Complement fixation (CF) and hemagglutination inhibition (HI) tests are not sensitive and, in addition, HI antibodies are cross-reactive to parainfluenza virus [1,10]. EIA has high sensitivity and specificity and is a simple procedure, but is not related to protective activity [11]. Neutralizing antibodies are associated with protective activity but the neutralizing test involves several complicated steps. Neutralization of an infectious virus and the preparation of cell cultures are bothersome and most time-consuming is the very last step to determine the appearance of CPE in 96-well plates. For micro-neutralization assays, there are two methods; 50% plaque reduction and complete inhibition of CPE. There have been several reports on neutralizing tests, concerning the evaluation of plaque reduction or inhibition of the appearance of CPE, infectivity of a challenge virus, and requirement of complement for neutralizing tests [12,13,14,15].

Fujino et al. [19] reported the neutralization test for measles virus using a GFP-expressing recombinant measles virus to evaluate the neutralizing antibody titer by Fluorescent EIA reader. Here, a recombinant mumps Hoshino vaccine strain expressing

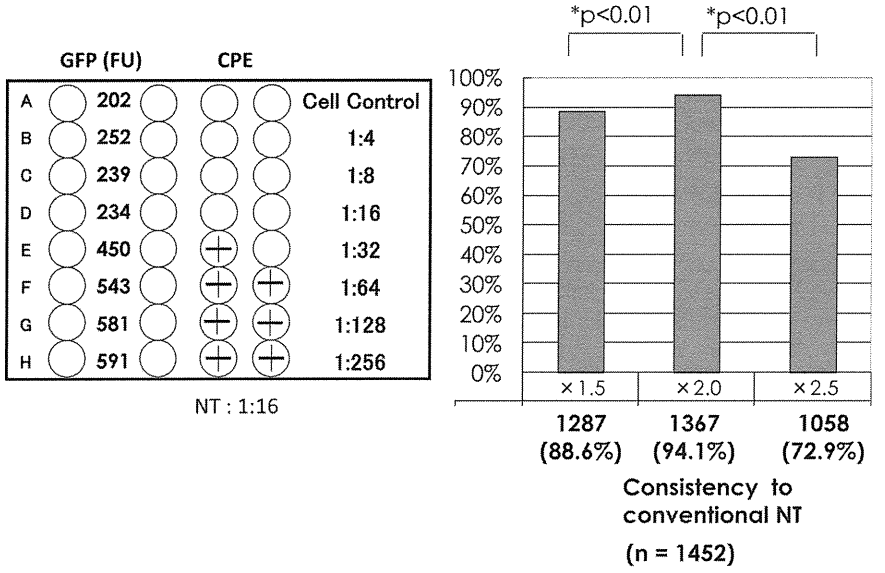

Figure 2. Relationship between the appearance of CPE and GFP expression. Serial two-fold dilutions from 1:4 to 1:256 were mixed with an equal volume of challenge virus. In the left panel, the schematic results of two neutralization methods are shown. CPE was observed in one of the two wells at 1:32, and the conventional neutralizing antibody titer was 1:16 by 100% inhibition of CPE. The mean FU value of the two cell control wells was 202 and that of the 1:32 dilution was 450, showing 1:16 of neutralizing antibody titer. Using 1,452 serum samples, the consistency of neutralizing antibody titers was compared based on different cut-off values for GFP expression: 1.5-fold, 2.0-fold, and 2.5- fold of FU values of the cell control wells.
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GFP was developed to check the expression of GFP instead of observing the appearance of CPE or plaque counting. GFP expression was examined by a fluorescent EIA reader as fluoro-units (FU). GFP expression increased as the virus genome was

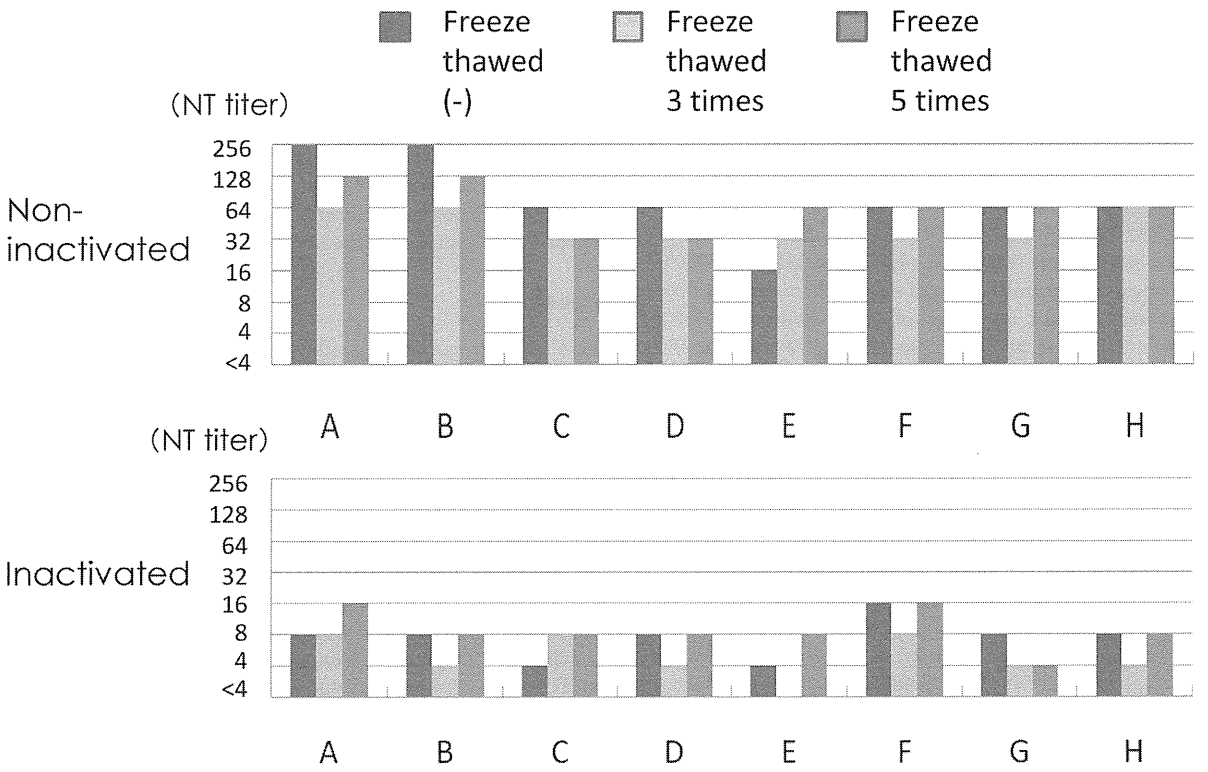

Figure 3. Effects of freeze-thawing and inactivation at 56°C for 30 min on neutralizing antibody titers. Upper panel shows the neutralizing antibody titers of eight fresh sera (A–H), without inactivation and after three or five rounds of freeze-thawing. Lower panel shows the results of neutralizing antibody titers after inactivation.
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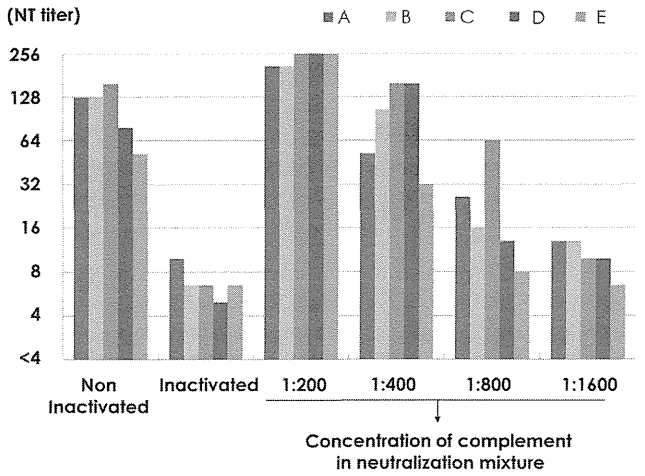

Figure 4. Neutralizing antibody titers of non-inactivated and inactivated sera with the addition of complement. Neutralizing antibody titers were examined in five sera (A–E) before and after inactivation. Complement was added at 1:200, 1:400, 1:800, and 1:1600 to the neutralizing mixture when inactivated sera were used. Each experiment was done in triplicate and mean titers were shown.
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transcribed after infection and was closely related to viral growth, as shown in Fig. 1. GFP expression in the cell control wells in a 96-well plate was approximately 200 FU. More than a two-fold increase in FU was considered positive for GFP expression (presence of CPE). Neutralizing antibody titers examined by GFP expression were similar to those by the conventional method for 100% inhibition of CPE (Fig. 2).

In several reports, the neutralizing step was performed without the addition of complement. Hishiyama et al. [15] reported that fresh guinea pig serum was required for neutralization tests for mumps virus. They used complement at 1:400 dilutions in the neutralizing mixture and the addition of complement increased the neutralizing antibodies titers. Complement has several important roles in immune responses and there are three main pathways, the classical, lectin, and alternative pathways. Complement is one of the first lines of host defense and is an important part of humoral immune responses. The complement system is immediately ready to target and eliminate viral particles and to interact with specific antibodies on the surface of a virus or infected cells [20]. Complement-dependent neutralizing antibody is reported to recognize the viral glycoproteins on the virus envelope, directly related to neutralization of Vesicular stomatitis virus [21,22], herpes simplex viruses [23,24], and West Nile virus [25]. Cooper et al. [26] reported that the deposition of antibody and complement on the surfaces of viral particles might physically interfere with infectivity in susceptible cells due to aggregation of the viral particles. However, Friedman et al. [23] suggested that complement inhibited the infection process of HSV, indicating that it affects viral replication: virus entry, uncoating, DNA transport to the nucleus, or immediate early gene expression, not requiring particle aggregation, viral lysis, or blocking of virus attachment. Johnson et al. [16] investigated the requirement of a complement system to neutralize three closely related paramyxoviruses, Simian virus 5 (SV5), mumps virus, and human parainfluenza virus type 2 (HPIV2). HPIV2 was neutralized in a complement-independent manner but neutralization of SV5 and mumps virus proceeded through alternative pathways. However, they were neutralized by different mechanisms; C3 deposition was observed on the surface of SV5 particles, resulting in aggregates. C3 deposition was also noted on the surface of mumps virus particles but they induced virion lysis through electron microscopic findings. In this sense, the presence of complement seemed to be essential for the neutralization tests for mumps virus. When fresh sera were examined for the detection of neutralizing antibodies against mumps virus, the addition of complement did not enhance the neutralizing antibody titers and the titers were stable for 5 rounds of freeze-thawing. But the complement activity was reduced after inactivation and during long-term preservation, and the addition of complement at 1:200 was required for neutralization tests against mumps virus.

EIA is simple and a large number of serum samples are handled without serial dilutions, which is suitable for surveillance but does not reflect protective immunity. A purified mumps virus antigen is used for the EIA antigen, and contains component proteins as well as viral particles. In our previous report, neutralizing antibodies assayed by the conventional method without complement showed a poor relationship to EIA titers. In the present study, there was again no significant relationship, with a low co-efficiency,

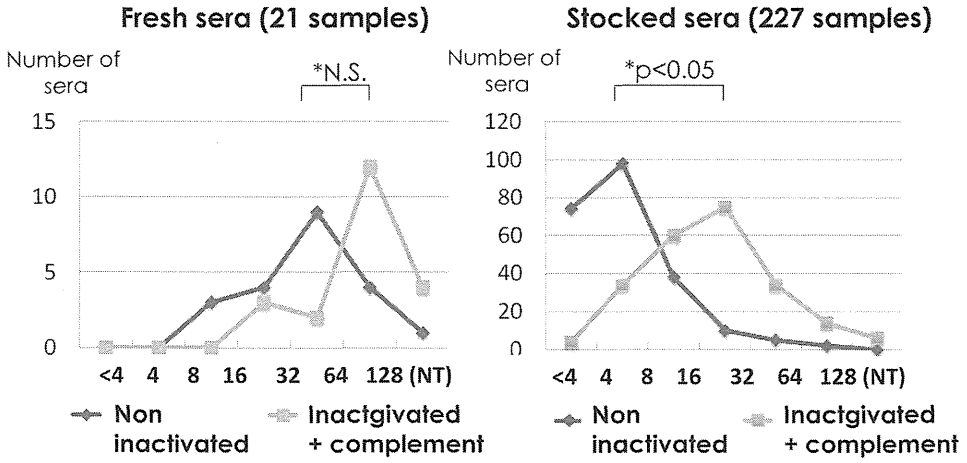

Figure 5. Effect of the addition of complement in 21 fresh and 227 stocked serum samples. Distribution of serum samples is shown for neutralizing antibody titers assayed without inactivation and for those assayed after inactivation with the addition of complement, using 21fresh serum samples (left panel). 227 stocked serum samples were assayed in a similar manner (right panel).
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