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Differences in neutralizing antigenicity between laboratory and clinical isolates of HCoV-229E isolated in Japan in 2004–2008 depend on the S1 region sequence of the spike protein

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Human coronavirus (HCoV) is a causative agent of the common cold. Although HCoV is highly prevalent in the world, studies of the genomic and antigenic details of circulating HCoV strains have been limited. In this study, we compared four Japanese isolates with the standard HCoV-229E strain obtained from ATCC (ATCC-VR740) by focusing on the spike (S) protein, a major determinant of neutralizing antigen and pathogenicity. The isolates were found to have nucleotide deletions and a number of sequence differences in the S1 region of the S protein. We compared two of the Japanese isolates with the ATCC-VR740 strain by using virus-neutralizing assays consisting of infectious HCoV-229E particles and vesicular stomatitis virus (VSV)-pseudotyped virus carrying the HCoV-229E S protein. The two clinical isolates (Sendai-H/1121/04 and Niigata/01/08) did not react with antiserum to the ATCC-VR740 strain via the neutralizing test. We then constructed a pseudotype VSV-harboured chimeric S protein with the ATCC S1 and Sendai S2 regions or that with Sendai S1 and ATCC S2 regions and compared them by a neutralization test. The results revealed that the difference in the neutralizing antigenicity depends on the S1 region. This different antigenic phenotype was also confirmed by a neutralizing test with clinically isolated human sera. These results suggest that the HCoV-229E viruses prevalent in Japan are quite different from the laboratory strain ATCC-VR740 in terms of the S sequence and neutralization antigenicity, which is attributed to the difference in the S1 region.

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INTRODUCTION

The coronavirus (CoV) is an enveloped virus with a single-stranded, positive RNA genome of about 30 kb. Moreover, the virion is approximately 80–120 nm in diameter (Lai, 1990; Lai & Cavanagh, 1997) and is characterized in electron micrographs by its crown-like appearance of spikes protruding from the virion envelope (Lai, 1990; Lai & Cavanagh, 1997). A variety of virus species that infect domestic animals, as well as pets, are classified as CoVs. Some CoVs are highly pathogenic while others exhibit low virulence. Before severe acute respiratory syndrome (SARS)-CoV was reported in 2003 (Peiris *et al.*, 2004), only low-virulence human CoVs (HCoVs), such as 229E or OC43, were known to exist. After the SARS outbreak, more virulent HCoVs, such as HKU1 (Woo *et al.*, 2005) and NL63 (van der Hoek *et al.*, 2004), were isolated from humans with respiratory diseases.

HCoV-229E, which was first reported in 1966 (Hamre & Procknow, 1966), is a prototype of HCoV and belongs to the genus *Alphacoronavirus*, together with HCoV-NL63. Both cause respiratory diseases in human infants (van der Hoek *et al.*, 2004). Dijkman *et al.* (2008) reported that 75 and 65 % of the children between the age of 2.5 and 3.5 years were seropositive for NL63 and HCoV-229E, respectively, and most of the children seroconverted against both viruses by the age of six. This may mean that most people experience acute infection by these HCoVs during their childhood. However, while HCoV is highly

A supplementary figure is available with the online version of this paper.

prevalent worldwide, studies of the genomic and antigenic details of circulating HCoVs have been limited.

The CoV spike (S) protein is a class I fusion glycoprotein of approximately 180–200 kDa, and trimers of the S protein comprise the spikes on the virion. The S protein of some CoVs is cleaved by host-derived proteases into the N-terminal S1 and C-terminal membrane-anchored S2 subunits (Spaan *et al.*, 1988; Sturman *et al.*, 1985). The S proteins of some other CoVs, such as HCoV-229E or SARS-CoV, do not have obvious cleavage sites; however, these S proteins contain regions corresponding to the S1 and S2 subunits of the cleaved S protein (Kawase *et al.*, 2009; Matsuyama *et al.*, 2005; Qiu *et al.*, 2006; Simmons *et al.*, 2004; Yamada *et al.*, 1997; Yoshikura & Tejima, 1981). The S protein of CoVs shows a variety of biological functions: the S1 subunit binds to the receptor, while S2 is responsible for fusion activity (Kubo *et al.*, 1993; Spaan *et al.*, 1988; Sturman *et al.*, 1985). The S protein also contains most of the neutralizing antibody epitopes and is associated with pathogenicity (Holmes & Compton, 1995; Kubo *et al.*, 1993; Spaan *et al.*, 1988; Sayaka *et al.*, 1991). Therefore, variations in the S protein directly influence the biological characteristics of the virus, such as pathogenicity and antigenicity. However, the regions in the S protein affecting these certain characteristics have not yet been elucidated for HCoV-229E.

In our laboratory, we observed that the prototypic strain of HCoV-229E obtained from the ATCC was efficiently neutralized by the serum derived from a rabbit immunized with the same strain. However, the serum failed to neutralize some of the Japanese human isolates, which may mean that there are antigenic differences between the ATCC strain (ATCC-VR740) and the HCoV-229E viruses that cause the common cold in Japan. Chibo & Birch (2006) reported that clinical isolates of HCoV-229E isolated in Australia between 1979 and 2004 were divided into four clusters, depending on the sequence of S protein. In addition, the S proteins of these clinical isolates contain three amino acid deletions in the S1 subunit as compared with ATCC-VR740. In the present study, we compared the nucleotide sequences and antigenic relationship of ATCC-VR740 and four Japanese clinical isolates. We found, when compared with ATCC-VR740, nucleotide deletions and sequence diversity in the S1 region of the S gene. We further confirmed that the S1 region is critical for determining the neutralization antigenicity by using pseudotyped vesicular stomatitis virus (VSV) harbouring the chimeric S proteins generated with the S genes of ATCC-VR740 and the newly isolated Japanese isolate Sendai-H/1121/04.

RESULTS AND DISCUSSION

Comparison of S protein sequences among HCoV-229E isolates

First, we successfully isolated four clinical isolates of HCoV-229E in Japan from pharyngeal swab specimens of outpatients. Three of them (Sendai-H/1121/04, AB691764; Sendai-H/826/04, AB691765 and Sendai-H/1948/04, AB691766) were isolated in Sendai city (Miyagi, Japan) in 2004 by using LLC-MK (No. 1121 and 1948) or human embryonic fibroblast (No. 826) cells. The fourth isolate Niigata/01/08 (AB691767) was isolated in Niigata city (Niigata, Japan) in 2008 by using CaCO2 cells that were cultured in the presence of trypsin (Hirokawa *et al.*, 2008). The replication kinetics in HeLa cells of the two clinical isolates Sendai-H/1121/04 and Niigata/01/08 was compared with that of ATCC-VR740. As shown in Fig. 1, the growth kinetics indicated that the titres of clinical isolates were about 1 $\log_{10}$ less than that of ATCC-VR740, though all isolates of HCoV-229E were propagated by using HeLa cells. Inefficient propagation of the Japanese isolates may be attributed to their non-adaptation to the cultured cells.

Because the S proteins of CoVs are reported to be associated with viral pathogenicity (Spaan *et al.*, 1988), the nucleotide sequences of the S genes were determined and the deduced amino acid sequences were compared. We found that the S gene of ATCC-VR740 maintained in our laboratory (AB691764) had four amino acid changes at positions 230 (C to F), 700 (L to I), 843 (S to L) and 848 (T to N) as compared with the sequence deposited in GenBank (ATCC-VR740; DQ243963). The former two changes were identical to the RW stocks of the ATCC strain (AF344186). We used our stock virus as the ATCC-VR740

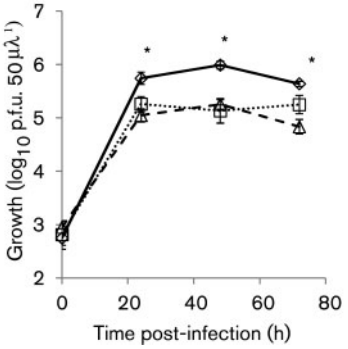

Fig. 1. The replication kinetics of clinical isolates in HeLa cells. Cells were infected with ATCC-VR740, Sendai-H/1121/04 and Niigata/01/08 at an m.o.i. of 1. After virus adsorption, cells were washed with PBS and incubated with Dulbecco's modified Eagle's medium (DMEM) containing 5% FCS. Cells were collected together with the culture medium at the indicated hours after infection and the titres were determined by a plaque assay on HeLa cells. The unpaired *t*-test was used to detect the statistical significance of the difference among the groups at each time point (n=6; *P<0.01). ◇, ATCC-VR740; □, Sendai-H/1121/04; △, Niigata/01/08. Error bars represent SD.

in this study. A sequence alignment comparison of amino acids was created by using ATCC-VR740, Sendai-H/1121/04 and Niigata/01/08 (Fig. S1, available in JGV Online). There were many changes, most of which were located in the S1 region and especially accumulated upstream of the receptor-binding region of the HCoV-229E strain (amino acid position 417–547) (Bonavia *et al.*, 2003). The sequence identity between ATCC-VR740 and Sendai-H/1121/04 was 89 % between amino acids 1–417 and 97 % in the remaining 751 amino acids. All clinical isolates had amino acid deletions at positions 228, 354 and 355 compared with ATCC-VR740, which is similar to the isolates in Australia reported by Chibo & Birch (2006). The amino acid homologies were about 99 % compared to those of the Australian isolates, while they were about 94 % homologous to ATCC-VR740, as shown in Table 1, which possibly means that our Japanese clinical isolates are closely related to the Australian isolates, but are different from the ATCC-VR740 strain.

Next, a phylogenetic analysis was performed by using the S protein sequence of the isolates and other sequences deposited in GenBank. Chibo & Birch (2006) deposited some full-length sequences of the S protein of their clinical isolates (DQ273964–DQ273986), while Dr K. V. Holmes' group directly deposited partial sequences of the S1 region of HCoV-229E (AY386379–AY386392). A phylogenetic tree was calculated with at least 1678 nt of the S1 region because the length of the shortest sequence thus far reported is 1678 bp (Fig. 2). Chibo & Birch (2006) reported that their HCoV-229E clinical isolates were clustered into four groups depending on the year of isolation. Some sequences (AY386379–AY386391) were clustered near ATCC-VR740, except for HCoV-229E 13-18 (AY386392). Our study showed that the Japanese isolates were clustered together with Australian strains isolated after 2001, indicating thereby that the S proteins of the four Japanese isolates are closely related to the Australian isolates in both nucleotide and amino acid sequences, rather than to ATCC-VR740.

Antigenic differences between clinical isolates and ATCC-VR740

As described above, the S protein sequences of the Japanese clinical isolates contained sequence deletions and variations in the S1 region compared with those of the standard ATCC strain. The S protein of CoVs, in particular the S1 region, harbours various neutralizing epitopes (Holmes & Compton, 1995; Kubo *et al.*, 1993, 1994; Niesters *et al.*, 1987; Sayaka *et al.*, 1991). Therefore, the antigenic cross-reactivity was examined by a virus-neutralizing test with antiserum specific to ATCC-VR740 and to Sendai-H/1121/04 (Fig. 3a–c). ATCC-VR740 was completely neutralized by a 1 : 200 dilution of anti-ATCC-VR740 serum and the neutralizing efficiency decreased corresponding to the serum dilution. Anti-Sendai-H/1121/04 serum failed to neutralize ATCC-VR740 at a dilution of 1 : 100, although weak neutralization was observed following a 1 : 20 dilution of the serum (Fig. 3a). In contrast, Sendai-H/1121/04 and Niigata/01/08 were not neutralized by anti-ATCC-VR740 serum. Instead, both viruses were efficiently neutralized with 1 : 80 to 1:160 dilutions of anti-Sendai-H/1121/04 serum (Fig. 3b, c). All viruses were hardly neutralized by normal rabbit serum (control). These results show that the Japanese isolates can be differentiated from the ATCC-VR740 in terms of their neutralizing antigenicity.

To confirm that the S protein is responsible for the differences in neutralizing antigenicity, the VSV-pseudotyped particles that were carrying HCoV-229E S proteins (Kawase *et al.*, 2009) were used for virus neutralization assays (Fig. 3d, e). Production of the pseudotyped VSV with HCoV-229E S protein is described in Methods. Consistent with the results shown in Fig. 3(a–e), VSV pseudotyped with the S protein of ATCC-VR740 was almost completely neutralized by a 1 : 400 dilution of anti-ATCC-VR740 serum, while it was not fully neutralized by a 1 : 100 dilution of anti-Sendai-H/1121/04 serum (Fig. 3d). In contrast, VSV-pseudotyped particles carrying the S protein of Sendai-H/1121/04 were efficiently neutralized by a 1 : 100 dilution of anti-Sendai-H/1121/04 serum, but were

Table 1. Nucleotide and amino acid sequence homology among HCoV229E isolates

ATCC-VR740, DQ243963; ATCC (NIID stock), AB691763; 19/8/03, DQ243985; 27/8/01, DQ243978; Niigata/01/08, AB691767; Sendai-H/1121/04, AB691764; Sendai-H/826/04, AB691765; Sendai-H/1948/04, AB691766; were calculated using GENETYX software. Data were expressed as relative percentages and the nucleotide data are shown in the upper right and those of amino acids in the lower left.

	VR740	NIID	19/8/03	27/8/01	01/08	1121/04	826/04	1948/04
HCoV229E ATCC-VR740		99.9	96.4	96.7	96.4	96.5	96.4	96.3
HCoV229E ATCC (NIID)	99.7		96.3	96.5	96.3	96.3	96.3	96.2
HCoV229E 19/8/03	94.6	94.3		99.6	99.4	99.5	99.4	99.4
HCoV229E 27/8/01	94.8	94.5	99.6		99.5	99.7	99.5	99.5
HCoV229E Niigata/01/08	94.7	94.4	99.0	99.1		99.4	99.2	99.2
HCoV229E Sendai-H/1121/04	94.7	94.3	99.5	99.6	99.0		99.6	99.5
HCoV229E Sendai-H/826/04	94.3	93.9	99.1	99.2	98.6	99.3		99.5
HCoV229E Sendai-H/1948/4	94.1	93.8	99.0	99.1	98.5	99.1	98.8	

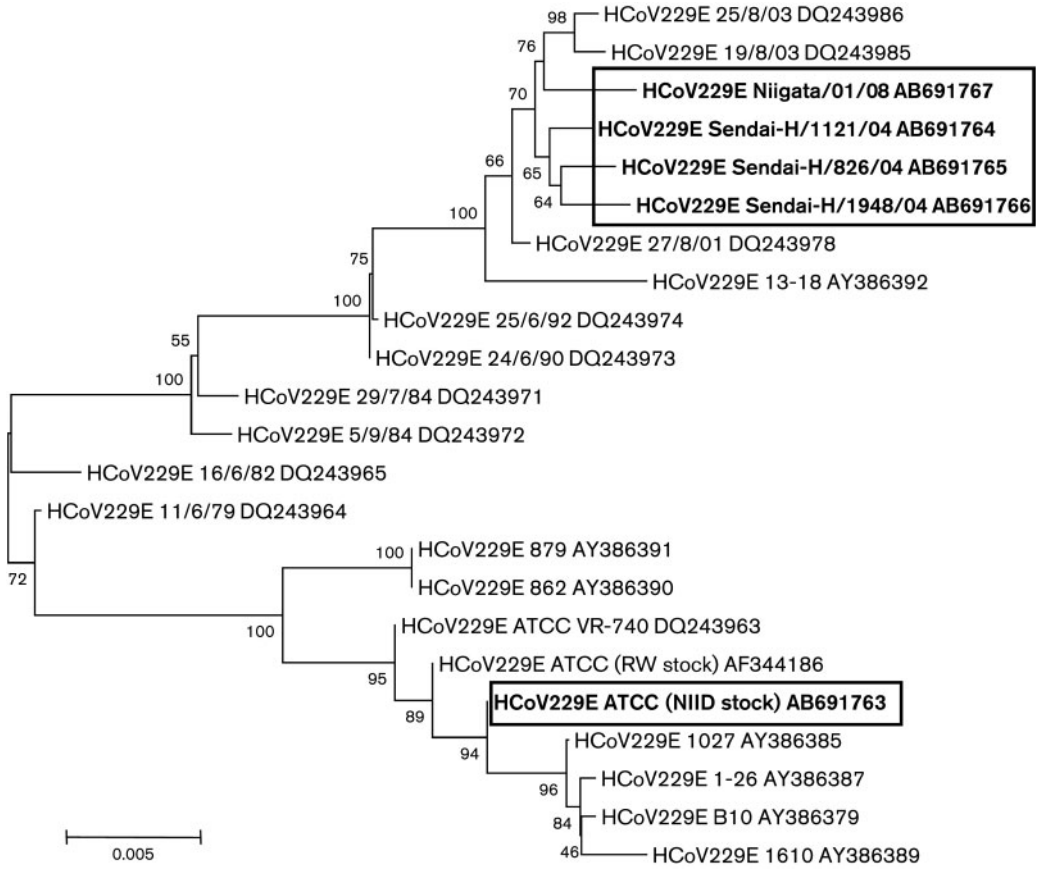

Fig. 2. Phylogenetic analysis of the S protein sequence of HCoV-229E isolates. Phylogenetic analysis was performed by using MEGA4 software which features a bootstrap test with the neighbour-joining method with the pairwise deletion option. The 1678 nt in the S1 region of available HCoV-229E sequences in GenBank was used to create the phylogenetic tree. Sequences of isolates discussed in the text are shown in bold. Bar, 0.005 amino acid substitutions per site.

not at all neutralized by the same dilution of anti-ATCC-VR740 serum (Fig. 3e). These results lead us to suggest that the neutralizing antigenicity of clinical isolates is different from that of ATCC-VR740 and the difference can be attributed to the S protein.

Next, the contribution of the S1 region to the neutralization of the isolates was determined by replacing the S1 and S2 region in ATCC-VR740 and Sendai-H/1121/04. As shown in Fig. 4(a), we generated plasmids which contained chimeric S proteins that cross over at an amino acid position between 690 and 691, presumably the cleavage site into S1 and S2 in the cleaved form of the S protein because coronaviral common sequences, which might be a fusion protein, existed at this position (Madu *et al.*, 2009). These were designated ^{S1}ATCC/^{S2}Sendai and ^{S1}Sendai/^{S2}ATCC. Then, the virus neutralization assay was performed with the VSV-pseudotyped particles carrying chimeric S proteins (Fig. 4b–f). VSV pseudotyped with ATCC-VR740 or Sendai-H/1121/04 was neutralized with each specific antiserum (Fig. 4b, c) and control VSV pseudotyped with VSV-G protein was not neutralized by either antiserum (Fig. 4d). The VSV-pseudotyped virus carrying the ^{S1}ATCC/^{S2}Sendai S protein was neutralized by anti-ATCC-VR740 serum but not by the anti-Sendai serum (Fig. 4e). In contrast, the titre of VSV pseudotyped with ^{S1}Sendai/^{S2}ATCC was neutralized efficiently by anti-Sendai serum but not by anti-ATCC-VR740 serum treatment (Fig. 4f). These results indicate that differences in terms of neutralization antigenicity found between the Japanese isolates and the ATCC standard strain result from the differences in the S1 region of the S protein.

The neutralizing experiments described above were performed with rabbit antiserum generated by immunization with each virus. To assess whether the antigenic differences can be recognized in natural infections of humans, the neutralizing test was performed with human serum specimens. We collected 112 serum specimens from healthy children and 16 serum specimens from healthy adult volunteers and performed virus neutralization assays using VSV-pseudotyped particles. The value of 50 % neutralization was used as the threshold and each experiment was performed three times for specimens from children and

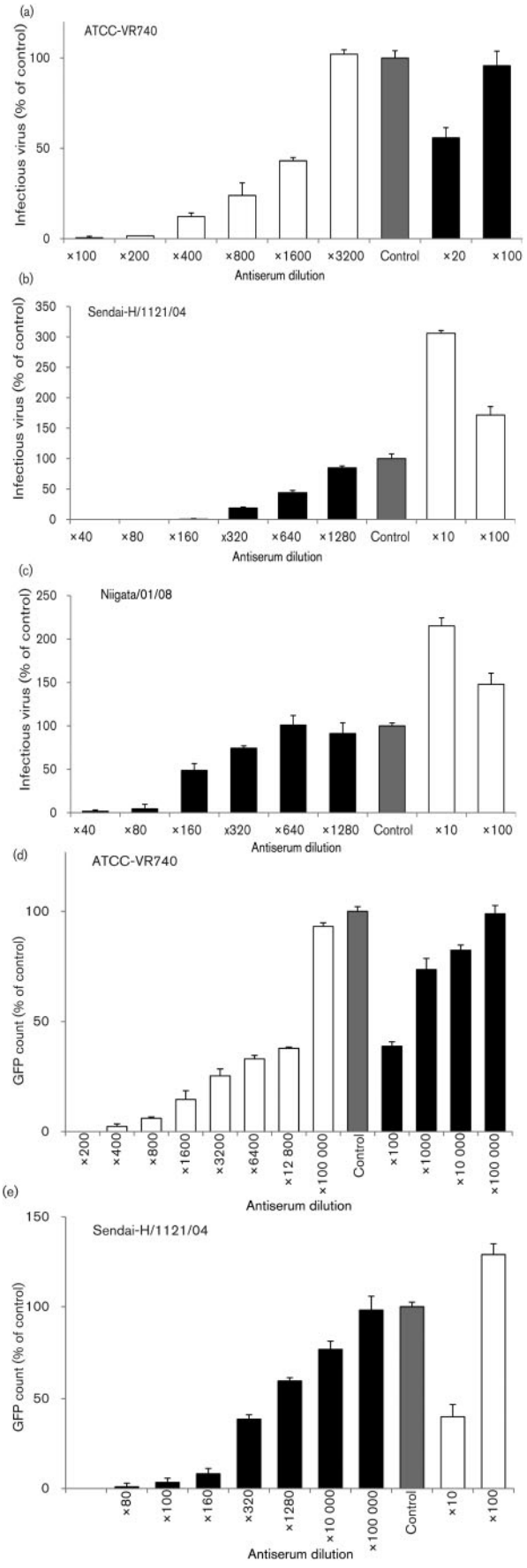

Fig. 3. (a–c) Antigenic cross-reactivity among HCoV-229E isolates as determined by virus-neutralizing assays. The ATCC-VR740 (a), Sendai-H/1121/04 (b) and Niigata/01/08 (c) isolates of HCoV-229E were neutralized by rabbit polyclonal antibody raised against ATCC-VR740 or Sendai-H/1121/04, as described in Methods. The data are represented as the percentage of infectious HCoV-229E particles relative to that of the control values ($n=3$). (d, e) Antigenic cross-reactivity of the S protein as determined by virus-neutralizing assays using VSV-pseudotyped particles carrying the S protein of HCoV-229E. VSV pseudotyped with the S protein of ATCC-VR740 (d) or Sendai-H/1121/04 (e) was neutralized by each specific anti-rabbit serum, as described in Methods. GFP fluorescence was captured and measured by using VH-H1A5 software. The data are represented as the percentage of the GFP count relative to the control value ($n=3$). White bars, anti-ATCC-VR740 serum; black bars, anti-Sendai-H/1121/04 serum and grey bars, normal rabbit serum (control). Error bars represent SD.

four times for specimens from adult volunteers. The results of the neutralizing test with specimens from children showed that 69 of 112 specimens (62 %) neutralized the VSV-pseudotyped particles carrying the Sendai-H/1121/04 S protein, while 20 of 112 specimens (18 %) could neutralize the ATCC-VR740 S protein-mediated infection. In contrast, the Sendai-H/1121/04 S protein was neutralized only in 4 of 16 specimens (25 %) obtained from adult volunteers, while the ATCC-VR740 S protein was neutralized in 7 of 16 adult specimens (44 %) (data not shown). These findings lead us to suggest that the Sendai-H/1121/04 S protein was neutralized more efficiently in specimens obtained from children than those from adult volunteers.

Neutralization assays with adult serum resulted in a variety of neutralizing patterns against ATCC-VR740 or Sendai-H/1121/04 S proteins (Fig. 5). Some sera (1–2, 5–7, 14–16) showed higher neutralizing activity against the ATCC-VR740 S protein than against the Sendai-H/1121/04 S protein, while four specimens (3, 9–11) showed the opposite neutralizing pattern (*$P<0.01$; **$P<0.05$). In contrast, serum sample 8 showed similar neutralizing activity against both S proteins but sample 13 did not neutralize either. These results may indicate that the human population could be infected with one type of HCoV-229E or infected, probably at a different time, with different antigenic viruses. It is also assumed that Japanese children have a greater chance of infection with the Sendai-type virus rather than with the ATCC-VR740 type.

We have also examined the neutralization of the VSV-G protein by human sera and each serum showed a variable response to this pseudotype virus (data not shown). This can be attributed to the unknown factor present in human serum to inactivate VSV, as described by DePolo *et al.* (2000), Mills *et al.* (1979) and Mills & Cooper (1978). Complement participation in this inactivation can be ruled out, since these sera were heat treated before examination. It is important to identify what substance in human serum is involved in the inactivation of VSV-G.

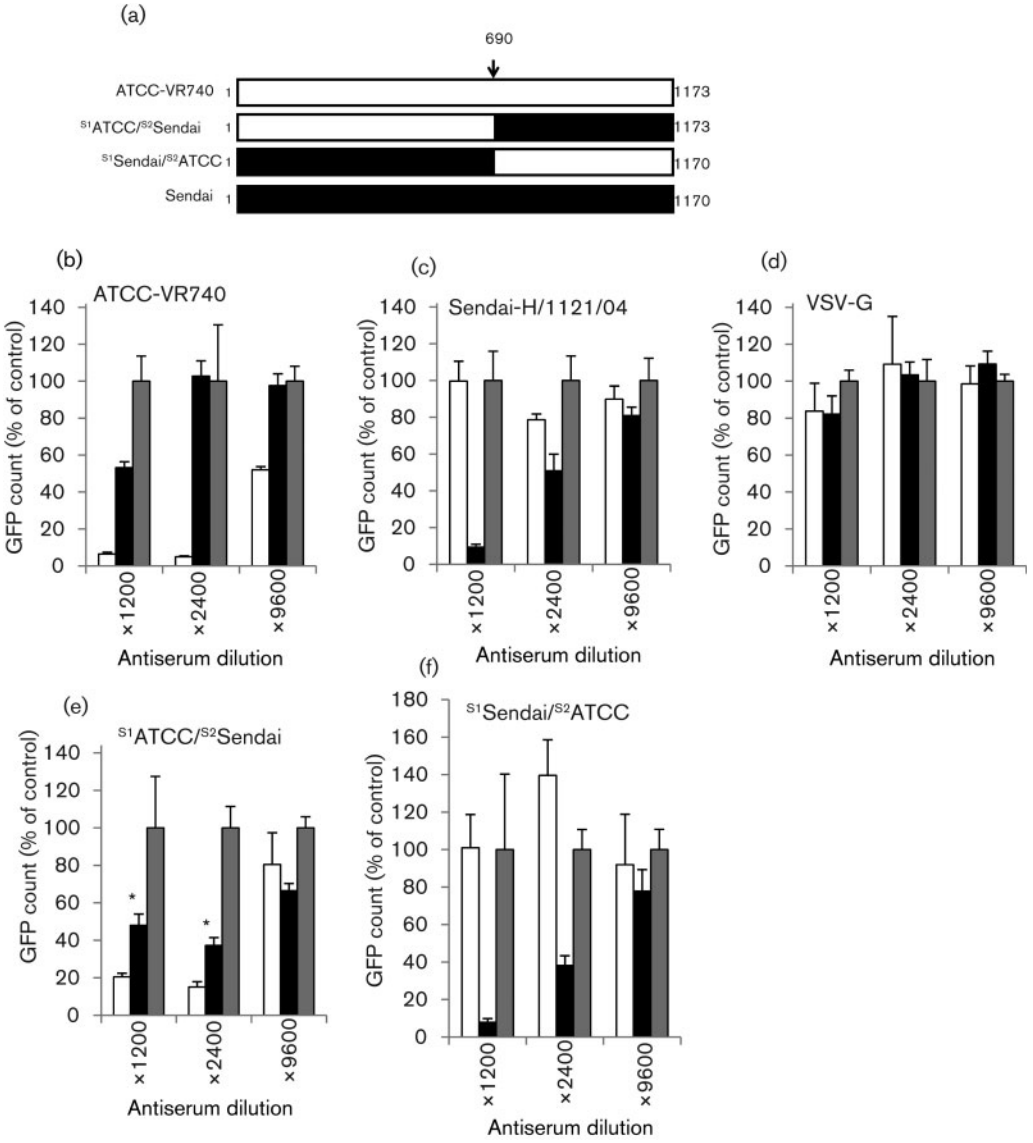

Fig. 4. Virus neutralization assays using VSV-pseudotyped viruses carrying the chimeric S protein of HCoV-229E. (a) A schematic presentation of constructed chimeric S proteins of ATCC-VR740 and Sendai-H/1121/04. White bars, ATCC-VR740; black bars, Sendai. (b–f) Neutralization assays were performed with VSV pseudotyped with the S protein of ATCC-VR740 (b), Sendai-H/1121/04 (c), VSV-G (d), ^{S1}ATCC/^{S2}Sendai chimera (e) and ^{S1}Sendai/^{S2}ATCC (f) chimera at the indicated dilution of each antiserum (×1200, ×2400 and ×9600). The neutralization was allowed for 30 min at 4 °C and samples were subsequently inoculated onto HeLa cells. The GFP fluorescence was measured after 24 h of infection and the data are represented as the percentage of the GFP count relative to the control value (n=4; *P<0.01). White bars, anti-ATCC-VR740 serum; black bars, anti-Sendai-H/1121/04 serum and grey bars, normal rabbit serum. Error bars represent SD.

As reported by Dijkman *et al.* (2008), most of the children seroconverted against HCoV-229E by the age of six. The standard ATCC-VR740 strain was identified in 1960–1970 and thus it is surmised that the positive rate against ATCC-VR740 is predominant in the older rather than the younger population. Actually, three of the four specimens within the forties and fifties age range showed neutralization against ATCC-VR740; however, one of the four specimens within the twenties age range also did. Thus, it is very important to perform a large-scale epidemiological study to show the age-specific seroconversion rate between the ATCC-VR740 and the Sendai type of HCoV-229E, which is at present in progress in our laboratory.

It was reported that neutralizing serum antibodies against viruses are sufficient to confer complete protection from lethal challenge by mouse hepatitis virus and SARS-CoV (Ishii *et al.*, 2009; Lamarre & Talbot, 1995). This selection

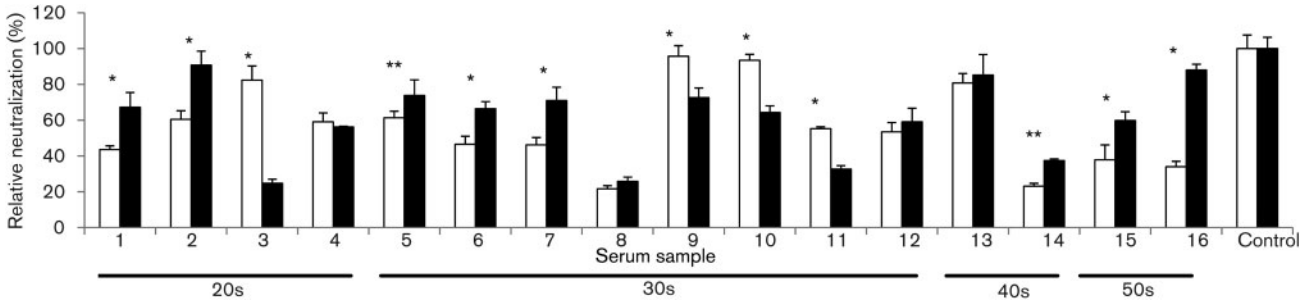

Fig. 5. Virus neutralization assays using serum specimens from adult humans. Neutralization assays were performed by using VSV-pseudotyped viruses (ATCC-VR740, Sendai-H/1121/04) with human adult serum (1:50 dilution). After neutralization at 4 °C for 30 min, viruses were inoculated on HeLa cells and incubated for 24 h. GFP-positive cells were counted, calculated and expressed as the percentage of the value relative to the non-serum treatment control. The data are represented in age-specific order, as shown by the decades. White bars, ATCC-VR740; black bars, Sendai-H/1121/04. (n=4; *P<0.01; **P<0.05.) Error bars represent SD.

pressure on the virus could result in a high frequency of escape mutations in the S gene in nature. The mutations in the S proteins especially occur in the S1 region, which is the main location for the neutralizing antibody epitopes, as described by Cavanagh *et al.* (1986), Kubo *et al.* (1994), Sui *et al.* (2004) and Sayaka *et al.* (1991). In addition, we have shown in the present study, by using a chimeric S protein, that S1 is responsible for the neutralizing antigenicity. Moreover, the antigenic difference between ATCC-VR740 and the clinical isolates could be shown by polyclonal rabbit antiserum and human serum in the neutralizing tests. These findings strengthen the idea that the S1 region of the S protein is a target of immunological pressure in infected humans.

As described above, the sequence diversities between ATCC-VR740 and the clinical isolates were high in the S1 region, especially upstream of the receptor-binding region (amino acids 417–547) (Bonavia *et al.*, 2003). Godet *et al.* (1994) reported that the regions recognized by neutralizing antibodies and receptor-binding determinants were distinct, since the S protein of the transmissible gastroenteritis virus that was bound to the receptor APN was recognized by neutralizing antibodies. On the other hand, it was reported that neutralizing epitopes exist within the receptor-binding region of the S protein of SARS-CoV (Rockx *et al.*, 2010). There are six amino acid differences in this receptor-binding region between ATCC-VR740 and Sendai-H/1121/04 and therefore, these could be responsible for the differences in the neutralization phenotype, as it was similar to that of SARS-CoV. At present, studies are in progress to see which of those amino acids are actually involved in the different levels of antigenicity found in the S1 of ATCC-VR740 and Sendai.

Since there is a clear difference in antigenicity between our isolates and the ATCC-VR740 strain, it is of great interest to see whether some differences exist in terms of pathogenicity or virulence. This study showed that virus propagation in HeLa cells resulted in lower titres for the Japanese clinical isolates when compared with the ATCC-VR740 strain. This may be due to the adaptation of ATCC-VR740 to the cultured cell and the replication kinetics could be different in tissues from the human respiratory tract. Viral virulence may be in parallel to the growth capability in epithelial cells in the upper respiratory tract. Unfortunately, as described by Yoshikawa *et al.* (2010), HCoV-229E could not replicate in Calu-3 cells, which is a widely used human bronchial epithelial cell line to mimic the natural topology of infection. Pyrc *et al.* (2010) reported that HCoV HKU-1 could infect and replicate in human ciliated airway epithelial cells that were reformed from the dissection of the respiratory tract. However, it is of great importance for the study of the virulence and pathogenicity of HCoV-229E to find an appropriate immortalized cell line derived from the human respiratory tract that permits the replication of these viruses.

In summary, we have studied four clinical HCoV-229E viral strains isolated in Japan. These isolates have three amino acid deletions and a number of amino acid mutations in the S1 region, which could result in a distinct neutralizing antigenicity as compared with that of the ATCC-VR740 strain. These findings may mean that clinical isolates circulating in the human population could be quite different from laboratory strains and accordingly, more comprehensive studies on a variety of HCoV-229E isolates should be undertaken to understand the antigenicity, pathogenicity and virulence in humans.

METHODS

Cells, viruses and specimens. HeLa cells (ATCC number CCL-2.1, HeLa 229) were maintained in DMEM (Nissui) containing 5 % FCS. BHK cells were obtained from the Health Science Research Resources Bank. The 293T/17 cells were obtained from ATCC (CRL-11268). BHK and 293T/17 cells were maintained in DMEM (D5796; Sigma-Aldrich) containing 5 % FCS. The ATCC strain of HCoV-229E

(ATCC-VR740), which was initially propagated in L132 cells and maintained with HeLa cells (Kawase *et al.*, 2009), was used as the ATCC-VR740 strain in this study [National Institute of Infectious Diseases (NIID) stock, GenBank accession no. AB691763]. We obtained four clinical isolates, three of which were isolated in Sendai city (Miyagi, Japan) in 2004 by using either LLC-MK2 (no. 1121 and 1948) or human embryonic fibroblast (no. 826) cells (Sendai-H/1121/04, AB691764; Sendai-H/826/04, AB691765 and Sendai-H/1948/04, AB691766). The Niigata/01/08 (AB691767) was isolated in Niigata city (Niigata, Japan) in 2008 by using CaCO2 cells in the presence of trypsin (Hirokawa *et al.*, 2008). All isolates were isolated from pharyngeal swab specimens of outpatients. All HCoV-229E isolates were propagated and titrated by using HeLa cells as described previously (Kawase *et al.*, 2009). The pseudotyped virus of VSV that expressed GFP instead of the G protein (VSVΔG) was kindly provided by M. A. Whitt, GTx, Inc., Memphis, TN, USA (Takada *et al.*, 1997). The seed virus stock of VSVΔG*G, which is a VSV G protein harboured VSVΔG, was prepared by using BHK cells, as previously described (Kawase *et al.*, 2009). The 112 serum specimens obtained from healthy children (1- to 4-years-old) from 1997 to 2003 were provided by the serum bank of our institute and the 14 adult serum specimens (in subjects aged from 22 to 57 years old) were collected from healthy volunteers in our branch under the approval of the Ethical Review Committee for the Study of Human Medical Science of our institute. All serum specimens were decomplemented by heat inactivation (56 °C for 30 min) before being used.

Virus infection. To determine the replication kinetics of HCoV-229E strains, ATCC-VR740, Sendai-H/1121/04 and Niigata/01/08 were inoculated onto HeLa cells at an m.o.i. of 1. After virus adsorption, cells were washed with PBS and incubated with DMEM containing 5 % FCS. Cells were collected with culture medium at the indicated hours of incubation and the titre was determined by plaque assay using HeLa cells in the presence of 5 μg trypsin ml^{-1}, as described previously (Kawase *et al.*, 2009).

Sequencing analysis. Viral RNA was extracted with TRIzol LS reagent (Invitrogen) following the manufacturer's instructions. First strand cDNA was synthesized using M-MLV reverse transcriptase (Takara-Bio) and oligo-$d(T)_{16}$ (Applied Biosystems). To determine the nucleotide sequence of the gene encoding the S protein, the viral sequence was amplified with platinum *Taq* high fidelity DNA polymerase (Invitrogen) and a specific primer set was designed, which was based on a deposited sequence in GenBank (NC_002645) [sense 5′-AATACGTCATCATCAGAAGCC-3′ (nt 20 254–20 274) and antisense 5′-AAGACAAGTTGACAGACTTCG-3′ (nt 24 231–24 212)]. The amplified DNA fragments were purified by using the Wizard SV Gel and PCR Clean-Up System (Promega) and then sequenced directly. All sequencing analyses were performed with BigDye terminator v3.1 and an ABI PRISM 3130xl (Applied Biosystems). The complementary rate was analysed by GENETYX software (Genetyx Corp.). The phylogenetic tree, constructed by using the neighbour-joining method with a pairwise deletion option, was created by MEGA4 software (Tamura *et al.*, 2007) and the bootstrap test was applied.

Generation of VSV pseudotyped with the HCoV-229E S protein. Generation of VSV pseudotyped with the HCoV-229E S protein was performed as described previously (Kawase *et al.*, 2009). Briefly, the plasmids which contained S proteins with a 19 aa truncation from the C-terminus were constructed by using the cDNAs of ATCC-VR740 and Sendai-H/1121/04. By using specifically designed primers and an in-fusion HD cloning kit following the manufacturer's protocol (Clontech), we constructed a plasmid containing the chimeric S protein with sequences from ATCC-VR740 and Sendai-H/1121/04 joined at the amino acid positions of 690 and 691, which is a tentative cleavage site of the S protein, with cleaved S1 and S2 being present on virions (Madu *et al.*, 2009). The S protein with the 19 aa deletion was reported to increase the efficiency of its incorporation into VSV particles (Fukushi *et al.*, 2005; Kawase *et al.*, 2009). The 293T/17 cells, prepared in collagen-coated, six-well plates, were transfected with these plasmids by using X-tremeGENE 9 reagent (Roche Applied Science) following the manufacturer's instructions. After 24–36 h of incubation at 37 °C, cells were infected with VSVΔG*G and incubated at 37 °C for 1 h. After adsorption, cells were carefully washed with PBS four times and incubated in DMEM containing 10 % FCS at 37 °C for 24 h. After cell incubation, the culture medium was collected and centrifuged at 1000 r.p.m. ($\sim 100\,\boldsymbol{g}$) for 5 min and the supernatant was stored at −80 °C until used.

Virus neutralizing tests. The specific neutralizing sera were obtained from rabbits immunized with purified whole virions of ATCC-VR740 or Sendai-H/1121/04 (Keari Corporation). Non-immunized rabbit serum was used as the negative control. The monolayer of HeLa cells was formed in 96-well plates (for VSV-pseudotyped virus) or in 24-well plates (for infectious HCoV-229E). For neutralization of the VSV-pseudotyped virus, approximately 500 GFP counts of viruses were mixed with DMEM containing 5 % tryptose phosphate broth (TPB) and the indicated dilution of rabbit sera or human specimens. For infectious HCoV-229E, 100 p.f.u. of virus was mixed with DMEM containing 5 % TPB, 1 % normal rabbit serum and a 1 : 20 dilution of clinical sample serum. Then, samples were incubated for 45 min at room temperature and inoculated onto the HeLa cell monolayers. Viruses were adsorbed for 1 h at 34 °C and cells were washed three times with DMEM and then DMEM containing either 5 % FCS (for VSV-pseudotyped virus) or 10 % TPB and 1.5 % carboxymethyl-cellulose (for infectious HCoV-229E) was added. For VSV-pseudotyped virus, the medium was replaced with fresh PBS after 24 h of incubation, cell images were captured and the level of GFP fluorescence analysed with VH-H1A5 software (KEYENCE). For infectious HCoV-229E, cells were fixed with 20 % formalin after 2 days of incubation, stained with crystal violet and then the plaques were counted. The neutralizing rates were represented as the relative percentage when compared with the mean number of positive controls. As described previously, VSV G protein was neutralized by human serum in a non-specific manner because of complements and other mechanisms (DePolo *et al.*, 2000; Mills *et al.*, 1979; Mills & Cooper, 1978). Therefore, mock treatment was used in the neutralizing test with human specimens as a control for calculation.

Statistical analysis. The unpaired *t*-test and chi-squared test were used to detect the statistical significance of the difference among the groups. A *P*-value of <0.05 was considered to be statistically significant.

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