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FULL PAPER *Virology*

Comparison of Molecular and Growth Properties for Two Different Canine Distemper Virus Clusters, Asia 1 and 2, in Japan

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ABSTRACT. To compare the molecular and growth properties of two newly isolated canine distemper virus strains in the Asia 1 and 2 groups with clinico-pathological findings in dogs, nucleotide and predicted amino acid sequence comparisons of genes H and P were performed together with comparative growth profiling. The predicted amino acid sequences of the H gene contained 12 cysteine residues that were conserved among the examined Asia 1 and Asia 2 viruses. The hydrophobic region in the H gene of the Asia 2 isolates was one amino acid longer than that of the Asia 1 group. The H gene of the Asia 1 group had nine putative asparagine (N)-linked glycosylation sites, while there were eight sites in the Asia 2 group. The titers of the cell-associated viruses for the Asia 1 strains were higher than those of the released viruses and were opposite to those of the Asia 2 strains in a previous study. The molecular and growth properties of the Asia 1 and Asia 2 groups seem to vary, although no significant differences were observed in the clinical signs and pathological findings between the two groups.

KEY WORDS: CDV, glycosylation site, growth profile, molecular character.

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Canine distemper virus (CDV) belongs to the *Morbillivirus* genus of the family Paramyxoviridae and is an enveloped single-stranded RNA virus that causes lethal systemic disease in dogs and other carnivores [1, 11]. Although canine distemper (CD) is generally controlled well with live attenuated vaccine, the number of typical CD cases has increased in European countries, America [4], and Asian countries such as Japan [6, 8, 16], Thailand [9], Cambodia, and Vietnam (data not shown). The CDVs in Japan and other Asian countries have been classified into two groups named Asia 1 and Asia 2 [7, 15] using a phylogenetic tree based on sequence analysis. These two groups differ from vaccine and known groups that comprise the phylogenetic tree of the only H gene.

Previous studies [13, 14] have examined the clinical signs and pathological findings of 6 CDV-infected dogs. Lan *et al.* [12–14] isolated new CDVs and confirmed the Asia 1 and 2 groups using the phylogenetic tree of the H and P genes. The growth profiles of three new CDV isolates, 007Lm, 009L and 011C, belonging to the Asia 2 group have also been reported [13]. However, no detailed or full analysis of the differences in molecular and growth characteristics between the two groups have been reported. Therefore, the objectives of the present study were to compare the molecular and growth properties of the CDV Asia 1 and Asia 2 groups and to clarify the correlation between the molecular and growth characteristics, clinical signs, and pathological findings caused by these CDV groups.

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MATERIALS AND METHODS

Viruses: The CDVs used included 007Lm, 009L, 011C, Ac96I, P94S, and S124C isolated from the lymph node, lung, cerebrum, large intestine, spleen, and cerebellum, respectively, of autopsied dogs showing evidence of CDV infection based on pathological findings and immunohistochemistry as described previously (Table 1) [13, 14].

Cells: Vero cells stably expressing dog SLAM (Vero-DST cells) as described previously [12], were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/m*l* of penicillin G, 100 μg/m*l* of streptomycin, and 0.4 mg of geneticin (G418) per m*l* and grown in a CO_2 incubator at 37°C. The virus was propagated in the Vero-DST cells and was stored in –80°C for subsequent steps.

Reverse transcription -PCR and sequencing: RNA extraction, reverse transcription (RT)-PCR, and the sequencing methods were as described previously [12]. Briefly, total RNA was extracted from CDV-infected Vero-DST cells with Trizol reagent (Invitrogen, California, U.S.A.) and RT-PCR was conducted by using a One Step RNA PCR Kit (AMV; Takara Bio Inc., Otsu, Shiga, Japan). A 429 bp fragment of the phosphoprotein (P) gene was amplified with universal primers upp1 and upp2 [2]. A 2100 bp length of the hemagglutinin (H) gene was amplified with the forward primer CDV-ff1 and reverse primer CDV-HS2. After PCR, the products of the P and H genes were purified using a QIAquick® PCR Purification Kit (Qiagen, Tokyo, Japan), and the purified DNA fragments were directly sequenced using a Big-Dye® Terminator Cycle Sequencing Kit V. 3.1 (Applied Biosystems Inc., Foster City, CA, U.S.A.). The internal H gene sequence primers

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Table 1. Clinical features and pathological findings of CDV- infected dogs

Group	Dog no. (isolated virus)	Breed (age in months)	Clinical history	Main gross findings	Histological findings: Brain	Lungs	Other sites
Asia 1	1 (P94S)	Papillon (3)	Purulent ocular and nasal discharge, cough, vomiting, hemorrhagic diarrhea Vaccination (+)	Multifocal pneumonia, Pleural effusion ascites	Demyelination and glial cell infiltration in the cerebrum, intracellular inclusion bodies	Suppurative bronchopneumonia and intracellular inclusion bodies	Depletion of lymphoid follicles in lymph nodes and the spleen. Gastroenteritis and intracellular inclusion bodies
	2 (Ac96I)	American cocker spaniel (3)	Purulent ocular and nasal discharge, cough, hemorrhagic diarrhea Vaccination (+)	Multifocal pneumonia, petechia on the ileum	Mild plexus choroiditis, neuronal necrosis, intracellular inclusion bodies	Interstitial pneumonia	Lymphoid depletion with lymphocytic necrosis in the spleen and lymph nodes. Catarrh enteritis and intracellular inclusion bodies
	3 (S124C)	Shiba (3)	Purulent nasal discharge, severe diarrhea Vaccination (+)	Purulent pneumonia	Chronic nonsuppurative encephalitis with intracellular inclusion bodies	Interstitial pneumonia with secondary infection, intracellular inclusion bodies	Lymphoid depletion of lymph nodes and white pulp in the spleen. Intracellular inclusion bodies in the epithelia of the stomach and intestine
Asia 2	4 (007Lm)	Labrador retriever (2)	Epileptic seizure, purulent ocular and nasal discharge Vaccination (+)	Multifocal pneumonia, swollen tonsil and lymph nodes	Nonsuppurative encephalitis	Multifocal pneumonia and intracellular inclusion bodies	Depletion of lymphoid follicles in lymph nodes and the spleen, intracellular inclusion bodies in epithelia of the stomach, intestine, and kidney
	5 (009L)	Weimaraner (2)	Ocular and nasal discharge, cough Vaccination (+)	Multifocal pneumonia, nasal discharge, purulent conjunctivitis	No notable lesions	Diffuse suppurative pneumonia, interstitial bronchopneumonia with inclusion bodies	Lymphocytic necrosis in the spleen, lymph nodes, and other sites
	6 (011C)	Cavalier (3)	Purulent nasal discharge, paralysis of hind limb Vaccination (+)	Multifocal pneumonia	Nonsuppurative encephalitis with inclusion bodies in the cerebrum	Severe suppurative bronchopneumonia	Lymphoid depletion of lymph nodes

were CDV-HS1, CDV-H for D, and CDV-Hr2. The P gene sequence primers were upp1 and upp2. A phylogenetic tree was constructed using the ClustalW program (DDBJ).

Nucleotide sequence accession numbers: The accession numbers of the P gene sequence of the new CDV isolates are as follows: AB212728 (007Lm), AB252714 (009L), AB252715 (011C), AB212959 (Ac96I), AB212960 (P94S), and AB212961 (S124C). The accession numbers of the H gene sequence of the new CDV isolates are as follows: AB212730 (007Lm), AB252718 (009L), AB252717 (011C), AB212963 (Ac96I), AB212964 (P94S), and AB212965 (S124C).

The accession numbers of the P gene sequences obtained from DNA databases are AB028914 (Yanaka), AB028916 (Jujo), AB028915 (Hamamatsu), AF305419 (Onderstepoort), AF181446 (Rockborn), AY286481 (Snyder Hill), AF259549 (Bulgarian dog), AY386315 (5804), AY286488 (01–2689), AY264266 (01–2690), AY288308 (01–2663), and AY288309 (01–2676). Accession numbers of H gene sequences from DNA databases are AF378705 (Onderstepoort), Z35493 (Convac), AF259552 (Snyder Hill), D85755 (Yanaka), D85753 (Ueno), D85754 (Hamamatsu), AB025271 (KDK1), AB016776 (Tanuki), AB025270 (Dog 98–002), AY297453 (Dog 5B), AB040767 (Dog HM-3), AB040766 (Dog 26D), AY297454 (Dog 5VD), AY378091 (Dog Taiwan), AY386315 (5804), AF478543 (Dog isolate ADen), AF478547 (Dog isolate CDen), Z47761 (Dog Denmark), Z47764 (US89), AY649446 (01–2689), AY498692 (01–2676), AY465925 (01–2690), AY443350 (00–2601), and AF164967 (A75–17).

Virus growth profiling: Monolayers of Vero-DST cells in 48-well plates were infected with viruses at a multiplicity of infection of 0.001 and incubated at 37°C in 5% CO_2. The infected cells and supernatants were harvested at 12, 24, 36, 48, and 72 hr post inoculation (hpi). The titers of the released and cell-associated viruses at these times were identified using a 50% tissue culture infectious dose assay according to the method of Behrens-Karber [9], and the growth profiles of the viruses were recorded as described previously [13].

RESULTS

Sequence and phylogenetic analysis: Sequencing and phylogenetic analysis of the P and H genes of two newly isolated CDV strains, 009L and 011C, were conducted in the present study. Phylogenetic trees were constructed based on the nucleotide sequence of a 390 bp fragment of the P gene (Fig. 1). Sequences containing 607 amino acids of the H gene of CDV strains 009L and 011C (Fig. 2) were predicted for analysis of the phylogenic relationships among the CDVs. Both phylogenetic trees indicated that strains 009L and 011C belong to the Asia 2 group.

Comparison of the amino acid sequences of the H genes of the two CDV groups, Asia 1 and Asia 2: The results of our present and previous [14] studies indicate that new isolates, Ac96I, P94S and S124C belong to the Asia 1 group and that they are different from the 007Lm, 009L and 011C CDV strains, which belong to the Asia 2 group.

The H gene amino acids of all the new isolates in the Asia 1 and 2 groups were predicted to have 12 cysteine residues (Fig. 3). The major hydrophobic region was 19 amino acids long from amino acid positions 37 to 55 in the predicted H gene amino acids of Asia 1, while that of the Asia 2 group was 20 amino acids long. The H genes of strains P94S, Ac96I, and S124C in the Asia 1 group were predicted to contain nine potential N-linked glycosylation sites including amino acid positions 19–21, 149–151, 309–311, 391–393, 422–424, 456–458, 584–586, 587–589, and 603–605. The predicted H gene amino acids of Asia 2 group strains

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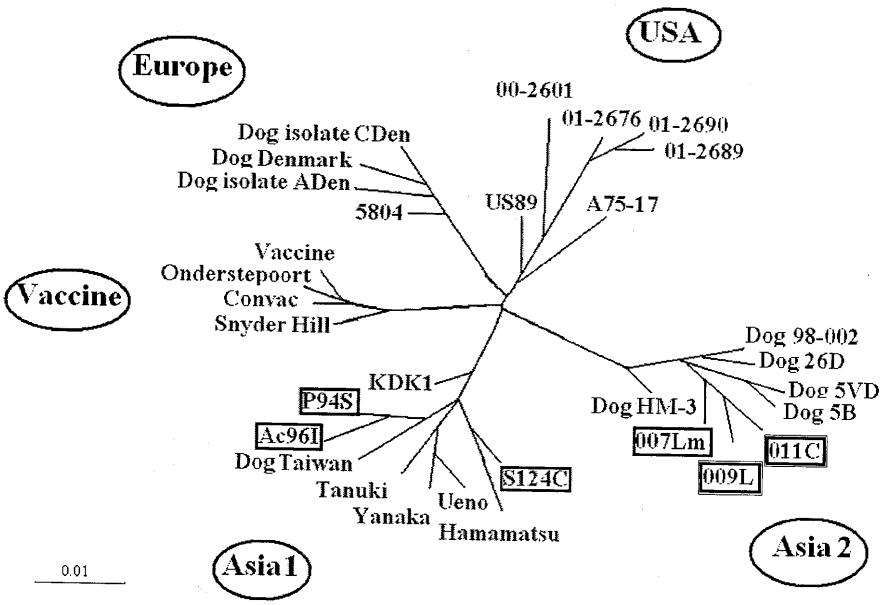

Fig. 1. Phylogenetic tree of the amino acid sequences of the coding regions of CDV H proteins. The new isolates are in the 009L and 011C strains.

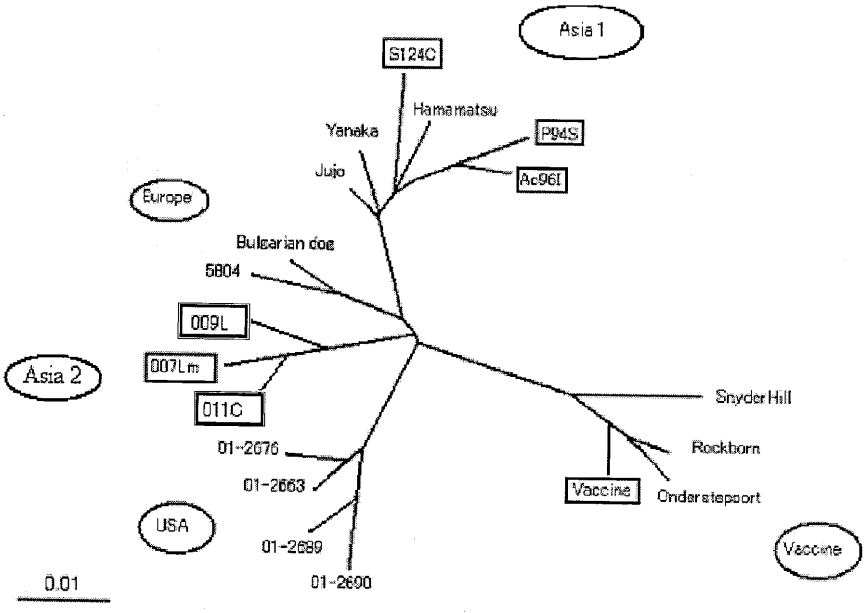

Fig. 2. Phylogenetic analyses of CDV strains based on a 390 bp nucleotide sequence. The new isolates are in the 009L and 011C strains.

007Lm, 009L, and 011C have eight N-linked glycosylation sites and lacked amino acid position 584–586.

Growth profiles: The growth kinetics of the Asia 1 strains showed that the titers of the cell-associated viruses were clearly higher than the titers of the released viruses (Fig. 4). Statistical evaluation showed that the titers of cell-associated viruses for both the S124C and Ac96I strains were significantly higher than those of the released viruses at 24, 36 and 48 hpi, respectively. The highest titers of the Asia 1 group strains coincided with observation of an extensive cytopathic effect (CPE; 90–95%).

DISCUSSION

In this study, we compared the sequences and phylogenetic trees of the H and P genes and their amino acid

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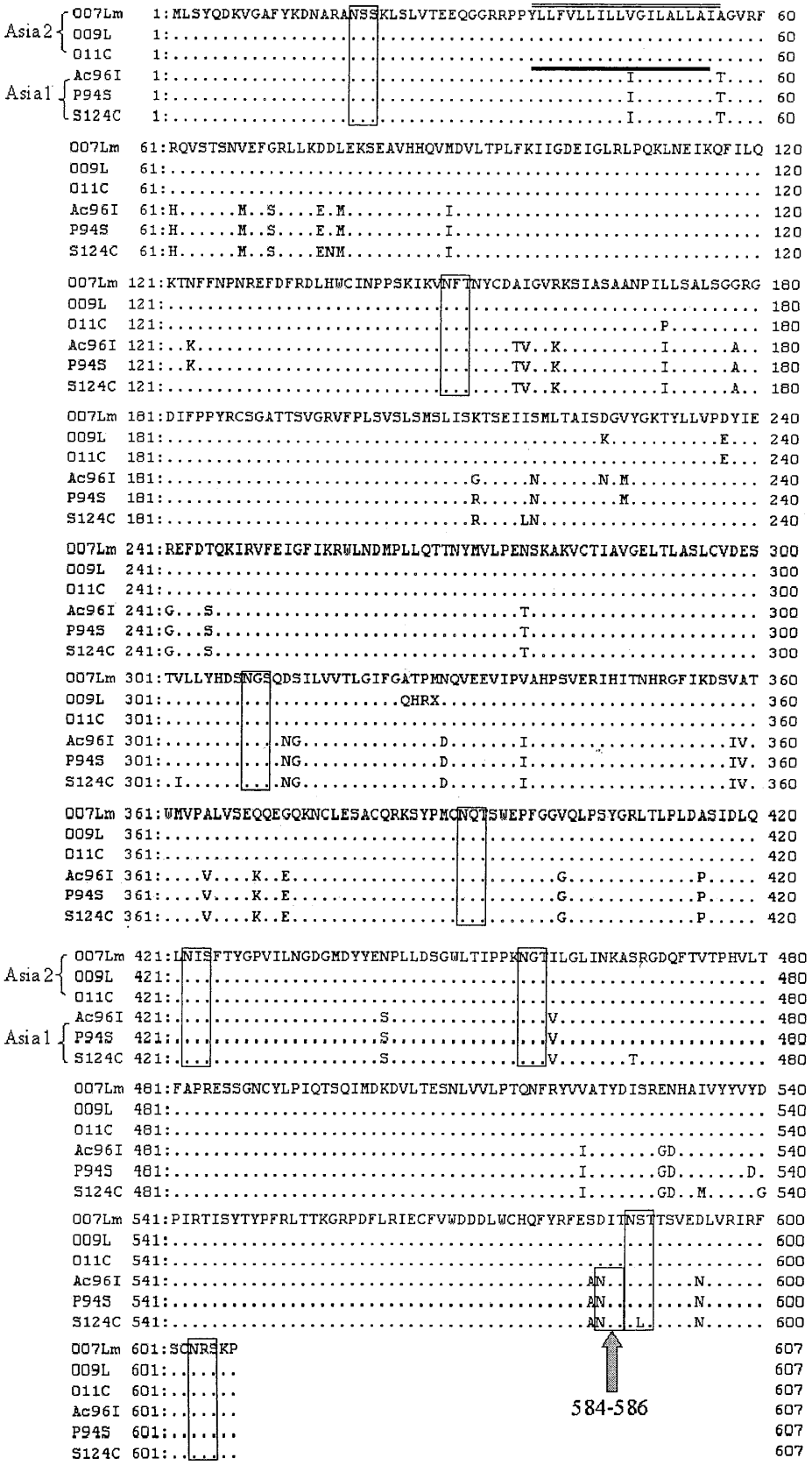

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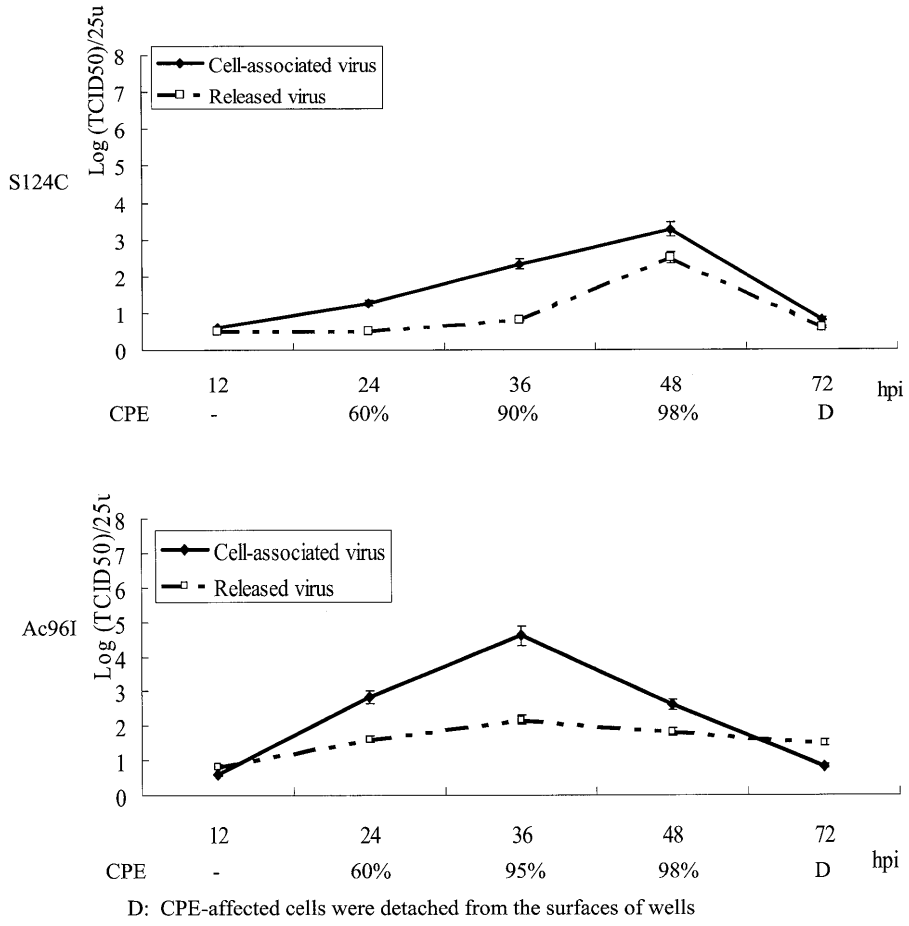

Fig. 4. Growth curves of the CDV strains in the Asia 1 group (Ac96I and S124C). D: Cells showing a cytopathic effect (CPE) were detached from the surfaces of wells.

sequences. H protein is the major determinant of CDV tropism and cytopathogenicity [17] and has the highest antigenic variation [3]. On the other hand, the P gene is most conserved within the clades of a given CDV lineage [5]. Thus, genetic characterization of the H and P genes and their products is useful for phylogenetic analysis. Hashimoto *et al.* [7] showed that there were two groups of new CDVs in Japan and other Asian countries using phylogenetic trees of the H gene and direct RNA extraction from fresh tissue, but did not do so using phylogenetic trees of the P gene. We have previously only produced data for P gene of one strain (007Lm) from the Asia 2 group. In the present study, we clarified the phylogenetic relationships for other Asia 2 group isolates, including 007Lm, 009L and 011C. In addition, not only the H gene sequence but also some parts of the P gene sequence is useful for phylogentic grouping of CDV.

In the present study, we speculated that the growth profiles of the two groups of CDV isolates in Vero-DST would be different based on the number of N-linked glycosylation sites. Strains 007Lm, 009L, and 011C of the Asia 2 group grew well, and the titers of the released viruses were higher than those of the cell-associated viruses 24 hpi for strain 009L, 28 hpi for strain 011C, and 50 hpi for strain 007Lm [13]. On the other hand, the growth kinetics of the Asia 1 strains showed that the titers of the cell-associated viruses were clearly higher than the titers of the released viruses. This should be clarified by additional experiments including virus propagation under the conditions of glycosylation inhibition and artificially mutated virus construction to change the site of 584–586 in the H gene. We are currently starting to produce infectious clone viruses using reverse genetic technology.

In this study, we also compared clinical signs with the pathological findings of dogs 1, 2, and 3 with those of dogs

Fig. 3. Deduced H protein amino acid sequences of the CDV strains of the Asia 1 group (P94S, Ac96I, and S124C) compared with those of the Asia 2 group (007Lm, 009L, and 011C). Potential N-linked glycosylation sites are boxed. Dots (.) indicate identity. The major hydrophobic region of the Asia 1 group is overlined in bold. The major hydrophobic region of the Asia 2 group is double overlined.

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4, 5, and 6 in order to evaluate the possibility of virulence differences between the Asia 1 and Asia 2 groups (Table 1). Dogs 1, 2, and 3 seemed to show more severe clinical signs of diarrhea or bloody diarrhea than dogs 4, 5, and 6. Histopathological findings showed that all dogs in the Asia 1 group had enteritis and inclusion bodies in the epithelial cells of the gastrointestinal tract, and only dog 4 in the Asia 2 group had inclusion bodies in the epithelial cells of the stomach and intestine. Diarrhea was not observed in dog 4, however, the rest of the dogs experienced diarrhea when they were inoculated with a high dose of 007Lm isolated from dog 4 [12]. Viruses at different doses have different potential abilities to cause disease. Therefore, we could not conclude that the Asia 1 group was more virulent than the Asia 2 group despite the fact that all of the Asia 1 group dogs seemed to have enteritis and none of the Asia 2 group dogs seemed to have enteritis. To confirm the difference in virulence between the Asia 1 and Asia 2 groups, isolated viruses of the Asia 1 and 2 groups should be inoculated back into dogs under the same experimental conditions, such as age, breed, and virus doses, and the ability of the viruses to cause disease should be observed.

In conclusion, this study clarified that the two CDV groups isolated in Japan have different molecular characteristics and growth profiles; However, no clear the difference in virulence between the Asia 1 and 2 groups could be identified based on the clinical signs and pathological findings of the dogs.

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