Relationship Between Growth Behavior in Vero Cells and the Molecular Characteristics of Recent Isolated Classified in the Asia 1 and 2 Groups of Canine Distemper Virus

Nguyen Thi LAN^{1,4)}, Ryoji YAMAGUCHI^{1)*}, Takuya HIRAI¹⁾, Kazushige KAI²⁾ and Kazuhiro MORISHITA³⁾

¹⁾Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889–2192, ²⁾Department of Veterinary Microbiology, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753–8515, ³⁾Division of Tumor and Cellular Biochemistry, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki, 889–1692, Japan and ⁴⁾Department of Veterinary Pathology, Microbiology, and Infectious Diseases, Faculty of Veterinary Medicine, Hanoi University of Agriculture, Trau quy, Gia Lam, Hanoi, Vietnam

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ABSTRACT. Ten recent isolates of canine distemper virus (CDV) strains were classified according to the growth ability and development of syncytial cytopathic effects (CPE) in Vero cells. Strains P94S, Ac96I, S124C, MD231, MS232, MSA5 and 095Cr were classified as Type 1 and exhibited hardly and did not develop CPE in Vero cells. Strains 007Lm, 009L and 011C were classified as Type 2 as grew well but failed to develop a syncytial CPE in Vero cells. A comparison of the phylogenetic trees of the H and P genes showed that all Type 1 strains belonged to the Asia 1 group and all Type 2 strains belonged to the Asia 2 group. Our findings suggest that the recent Asia 2 isolated of CDV in Japan, but not Asia 1 may grow in Vero cells, and their growth ability may be related with their molecular characteristics.

KEY WORDS: CDV, classification, phylogenetic analysis, sequence, Vero cell.

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Canine distemper virus (CDV) is a genus morbillivirus of the family Paramyxoviridae. The CDV genome has 15,690 nucleotides and contains six non-overlapping genes encoding nucleocapsid, phosphoprotein (P), matrix, fusion (F), hemagglutinin (H) and large proteins [1, 19]. The P gene encodes C and V non-structural proteins [1]. The H and F proteins of CDV are major target antigens for the host immune system. The H protein has the highest antigenic variation [2] and is the most appropriate protein for differentiation of different morbillivirus strains, including CDV [17]. The P gene is most conserved within clades of a given CDV lineage [3].

Previously, we showed that strains MD77, Onderstepoort and KDK1 of CDV can grow and develop a syncytial cytopathic effect (CPE) in Vero cells expressing canine signaling lymphocyte activation molecule (Vero.Dog SLAM tag; Vero-DST cells) but that they behave differently against Vero cells without dog SLAM [10]. However, in our previous study, the growth profiles of only three CDV strains were tested in Vero cells. The sequences of CDV strain MD77 and the inferred relationship between the growth behavior in Vero cells and the molecular characteristics of recent CDV isolates have not been studied. Therefore, in the present study, to clarify and develop complete the idea that Vero cells are useful for classification of the various CDV strains [10], we investigated the sequences and growth properties in Vero cells of 10 recent CDV isolates in Japan.

We identified the relationships between the growth and molecular characteristics of CDV.

MATERIALS AND METHODS

Cells: Vero and Vero-dog SLAM tag (DST) cells were used as described previously [2].

Viruses: The ten CDV strains listed in Table 1 were isolated in Vero-DST cells and classified in Vero cells as described previously [10, 11, 12]. These CDV strains originated from different prefectures in Japan as shown in Table 1.

Immunohistochemistry: Cultured cells infected with CDV were immunolabelled as described previously [10].

Reverse transcription (RT)-PCR and sequence analysis: Reverse transcription (RT)-PCR and sequence analysis were performed as described previously [11, 12].

Nucleotide sequence accession numbers: The nucleotide sequence accession numbers in the GenBank database for the P gene sequences of the CDV strains used in the present study are AB286943 (MD231), AB286944 (MS232), AB286945 (MSA5), AB286946 (095Cr), AB212959 (Ac96I), AB212961 (S124C), AB212960 (P94S), AB212728 (007Lm), AB252714 (009L), AB252715 (011C), AB286947 (MD77), AB286948 (KDK1) and AF305419 (Onderstepoort, OND). The nucleotide sequence accession numbers in the GenBank database of H gene sequences of the CDV strains used in the present study are AB286949 (MD231), AB286950 (MS232), AB286951 (MSA5), AB286952 (095Cr), AB212963 (Ac96I), AB212965 (S124C), AB212964 (P94S), AB212730

*CORRESPONDENCE TO: YAMAGUCHI, R., Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889–2192, Japan.
e-mail ; a0d402u@cc.miyazaki-u.ac.jp

Table 1. The sources of the 10 recent isolates of CDV and growth ability in Vero cells

Virus strains	Organ isolated from	Place / year of isolation	References	Growth ability	Type in Vero cells	Genogroup
007Lm 009L 011C	Lymph node Lung Cerebellum	Saga/2004 Aichi/2004 Akita/2004	Lan <i>et al.</i> , 2005b	+	Type1	Asia 2
P94S Ac96I S124C	Spleen Intestine Cerebellum	Hyogo/2004 Aichi/2004 Saga/2004	Lan <i>et al.</i> , 2006	–	Type2	Asia 1
MD231 MS232 MSA5 095Cr	Lung Lung Anal swab Cerebrum	Kagoshima/2005 Kagoshima/2005 Aichi/2005 Aichi /2004	Present study	–	Type2	Asia 1

(007Lm), AB252718 (009L), AB252717 (011C), AB286953 (MD77), AB025271 (KDK1) and AF378705 (OND).

RESULTS

Growth properties of CDV in Vero cells: According to the classification of CDV phenotypes in Vero cells by Lan *et al.* [10], the type names of the viruses were as follows: Type 1, the virus hardly grows and show no syncytium formation; Type 2, the virus grows well but shows no syncytium formation or indistinguishable rounding CPE; and Type 3, the virus grows and shows a syncytial CPE. CDV strains 007Lm, 009L and 011C were classified as Type 2 and did not cause a syncytial CPE, but many CDV antigens were contained in the individual cells infected with those strains (Table 1; Fig. 1). CDV strains P94S, Ac96I, S124C, MD231, MS232, MSA5 and 095Cr failed to grow efficiently and develop a syncytial CPE in Vero cells and were classified as Type 1. No Type 3 strains were found among these strains. In contrast, all CDV isolates grew and developed a syncytial CPE in Vero-DST cells.

Nucleotide sequence and phylogenetic analyses: Phylogenetic analyses of the P and H genes of the 10 new isolates of CDV were performed using from the nucleotide sequence of a 390 bp fragment of the P gene (Fig. 2) and a sequence of 607 amino acids of the H gene (Fig. 3). The ten recent CDV isolates were divided into two groups, Asia 1 and Asia 2. Strains P94S, Ac96I, S124C, MD231, MS232, MSA5 and 095Cr were classified as Type 1 and joined the clade consisting of the Asia 1 group. Strains 007Lm, 009L and 011C were classified as Type 2 and belonged to the Asia 2 group. Sequence analysis of the H genes of the new Type 1 and 2 isolates showed that one potential N-glycosylation site at amino acid 584–586 was absent in the H gene in the Type 2 (Fig. 4).

DISCUSSION

Three *Morbilliviruses* measles virus (MV), CDV and rinderpest virus (RV), enter cells through SLAM (human, canine or bovine) [21]. Most wild-type MV strains prefer-

Fig. 1. CDV antigens were detected by immunocytochemistry in Vero cells infected with strains 007Lm (a), 009L (b) and 011C (c) at 24 hpi. Positive CDV antigens are shown in black (arrows: white). Vero cells were not infected with CDV as a negative control (d) at 24 hpi. These photographs were taken by light microscope ($\times 20$).

entially use the immune cell-specific protein SLAM as a receptor [6, 9, 16, 22], whereas MV Edmonston (vaccine strain of MV) enters cells more efficiently using the ubiquitous protein CD46 [5, 13, 15, 18]. MV enters cells either through the SLAM or CD46 protein. On the other hand, CDV, including the wild and vaccine strains, supposedly do not use CD46 according to the downregulation study of Gallbraith *et al.* [7]. After binding to a receptor, H protein supports fusion of the viral and cellular membranes by inducing a conformational change of trimeric fusion (F) protein [4, 24]. Vongpunsawad *et al.* [23] devised a strategy based on analysis of *Morbillivirus* H-protein sequences, iterative cycles of mutant protein production followed by receptor-based functional assays and a novel MV H three-dimensional model in order to identify the residues on the attachment H protein essential for fusion support through each receptor. Based on the binding sites of the MV-H gene and SLAM research, 9 amino acids, D505, D507, Y529,

Fig. 2. Phylogenetic tree based on the nucleotide sequences of 390bp gene fragments of the P genes of the CDV strains.

D530, R533, T552, Y553 and P554, for the MV-H binding sites of SLAM are comparable with D501, D503, Y525, D526, I529, R529, T548, Y549 and P551, which may be the CDV-H binding sites of dog SLAM (Fig. 4) [8, 14, 23]. Eight amino acids, D501, D503, Y525, D526, I527, R529, T548 and P550, in the $\beta 5$ sheet are constant in all strains in Asia 1 and Asia 2, although Y549 in the $\beta 5$ sheet is S549 for 095Cr and L549 for OND. Eight amino acids are conserved in all strains and would be essential sites for binding to dog SLAM.

The Type 1 CDV isolates in the Asia 1 group did not grow in Vero cells; however the Type 2 isolates in the Asia 2 group grew well in Vero cells. Sequence comparison of the gene between the isolates in the Asia 1 and Asia 2 groups of CDV showed that there were some clear differences between them at amino acid positions 61–77, 156–160, 313–341, 370–374, 530–531 and 542–543 (Fig. 4). These positions might be related to the different growth abilities of the two groups of CDV. However, to better understand this point, a recombinant virus should be used in future studies, as syncytium formation in CDV and MV virus requires the combined activities of H and F glycoproteins [4, 20, 24]. As previously suggested by Vongpun-sawad *et al.* [23], it is necessary to identify the residues on attachment protein H that are essential for fusion support through each receptor in both the Asia 1 and 2 of groups CDV.

The phylogenetic tree of strain MD77 and the new isolates showed that strain MD77 was of a lineage different from that of the Asia 2 group; however, its behavior in Vero

Fig. 3. Phylogenetic tree based on the amino acid sequences of the coding regions of the CDV H protein.

cells appeared to be similar to that of Asia 2 group. Strain MD77 might produce rounding CPE or probably produced no CPE.

In conclusion, there appears to be a relationship between the biological characteristics in Vero cells and results of molecular analysis of the Asia 1 and 2 groups of CDV. If the growth character of an isolated CDV is examined in Vero cells, the group to which the isolate belongs in the phylogenetic tree according to sequence analysis can be inferred; on the other hand, the growth properties in Vero cells of a CDV isolate can be surmised from its position in the phylogenetic tree.

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Fig. 4. Deduced H protein amino acid sequences of the 10 new isolates of CDV. Potential N-linked glycosylation sites are boxed with a straight line (—). An arrow shows a difference N-glycosylation site between Asia1 and Asia 2. Dots (.) indicate identity. The major hydrophobic region is overlined. Based on the MV-H sequences and binding sites of SLAM, comparable CDV binding sites (▼, 1~9) and estimated β sheets (dashed line box, ---) are shown.

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