宮崎大学学術情報リポジトリ
University of Miyazaki Academic Repository

Pathological and Molecular Biological Studies on Canine Distemper

メタデータ	言語: eng
	出版者:
	公開日: 2008-02-01
	キーワード (Ja):
	キーワード (En):
	作成者: Lan, Nguyen Thi
	メールアドレス:
	所属:
URL	http://hdl.handle.net/10458/1142

CHAPTER III

Comparative Analyses of Canine Distemper Viral Isolates From Clinical Cases of Canine Distemper in Vaccinated Dogs

Summary

Sequence and phylogenetic analyses of three isolates of canine distemper virus (CDV) isolated from three dogs with a vaccination history were compared with the same analyses of vaccine virus isolated from a vaccine used for dogs. The three dogs showed clinical signs of a recent major type of CD in Japan, including oculonasal discharge and diarrhea, and pathological findings including non-suppurative encephalitis, pneumonia, mild gastroenteritis, and lymphoid depletion. Inclusion bodies were in the stomach without inflammation and encephalitis was without clinical signs. One of the highest titers of CDV in different organs of the three dogs was commonly systemic lymphatic organs, including the spleen, lymph nodes and tonsils. New isolates of CDV joined to the clades of the Asia 1 group that is far from the vaccine group. These results surely indicate that wild strains of CDV from dogs with a vaccination history were not reversed vaccine virus, and that the dogs showed characteristics of recent CD in Japan.

Key words: CDV; pathological findings; phylogenetic analysis; sequence; vaccinated dog

Introduction

Canine distemper (CD) is an acute or subacute, highly contagious disease with signs of generalized infection, respiratory disease, foot pad hyperkeratosis, central nervous system disturbance or a combination of these (Krakowka *et al.*, 1985; Appel and Summers, 1995). Although CD has been generally controlled well throughout the world with live attenuated vaccine, the number of typical CD cases has increased in Japan and

European countries (Appel *et al.*, 1987; Blixenkrone *et al.*, 1993; Kai *et al.*, 1993; Shin *et al.*, 1995; Gemma *et al.*, 1996b). Many vaccinated dogs have been infected with CD virus (CDV) (Blixenkrone *et al.*, 1993). Measles epidemics have occurred among vaccinated young human adults in the USA (Rota *et al.*, 1992). Post-vaccinal inclusion body encephalitis and human parainfluenza virus have occurred in some cases after vaccination with modified live CDV vaccine (Ebata *et al.*, 1991; Hirayama *et al.*, 1991). Sometimes all dogs do not give a satisfactory response to the vaccine. The question remains in field studies, but not in animal experiments, of whether CDV infection in vaccinated dogs is the result of infection with wild strains or reversion to virulence of vaccine viruses. The virulence of attenuated CDV may reverse in vaccine (Appel *et al.*, 1978). Some studies have investigated molecular structures of new isolates of CDV and have compared them with those of the Onderstepoort strain from Genbank. However, these studies have unsatisfactorily answered the above question because the possibility can not be excluded that genes of the vaccine strain slightly changes in different batches of vaccine or the genes of CDV changed by passage in some different cell lines or by the level of passage, but this is unlikely. Therefore, to clarify if attenuated live vaccine is the source of CDV that causes disease in vaccinated dogs, we isolated viruses from the vaccine used for dogs. The H and P genes of the isolated vaccine strains were compared with these genes of new CDV strains isolated from vaccinated dogs. Because the H protein is the major determinant of tropism and cytopathogenicity (von Messling *et al.*, 2001), the highest antigenic variation is in the H protein (Blixenkrone-Moller *et al.*, 1992). The P gene is most conserved within clades of a given CDV lineage (Carpenter *et al.*, 1998). Genetic characterization of H and P genes is useful for phylogenetic analyses.

Other studies have analyzed the pathology of CD. Many vaccinated dogs infected with CDV showed typical clinical signs of CD (Iwatsuki *et al.*, 1997; Mochizuki *et al.*,

1999; Hashimoto *et al.*, 2001; Lednicky *et al.*, 2004). Recent widespread CD in Japan seemed to cause mainly enteritis and oculonasal discharge. In order to understand thoroughly and logically the full profiles of 3 cases of CD with a vaccination history, we described in detail the clinical signs and pathological findings of them.

Although some studies examined the distribution of the CDV antigen in tissues by immunohistochemistry (Machida *et al.*, 1993; Stanton *et al.*, 2003), the number of viruses in different organs of the field CD cases is unknown. Titers of virus in different organs have not been shown because convenient cells for titration are unavailable so far because of necessity of cell adaptation of the viruses. Lan *et al.* (2005b) showed the possibility of direct titration from fresh tissue. In this study, we tried to show virus titers of different organs of three recent CD cases of vaccinated dogs.

Materials and methods

Animals: Three 3-month-old dogs that showed clinical signs of CDV that included oculonasal discharge and enteritis were selected. All dogs, named 4, 5 and 6, were vaccinated at age 7 weeks and at 11 weeks with Duramune 8 that contained the original Onderstepoort CDV strain (Kyoritsu Pharmaceutical Co.Ltd).

Pathological examination and immunohistochemistry: After necropsy, samples were fixed with 10% formalin and were embedded in paraffin wax. Sections (4 *um*) were cut and stained with haematoxylin and eosin. Immunohistochemistry was done by using a mouse monoclonal antibody specific for CDV-nucleoprotein and an Envision polymer reagent (DAKO-Japan, Japan) as described previously (Kumagai *et al.*, 2004)

Reverse transcriptase-PCR and sequence: Total RNA was extracted from CDV-infected Vero-DST cells with Trizol reagent (Invitrogen, California, USA) and reverse transcriptase (RT)-PCR was done by using a one-step RNA PCR kit (AMV)

(Takara Bio Inc., Japan) as described previously (Lan et al., 2005b). Briefly, a 429bp fragment of the P gene was amplified with universal primers upp1 and upp2 (Barrett *et al.*, 1993). A 2100bp of the H gene was amplified with forward primer CDV-ff1 and reverse primer CDV-HS2. The RT-PCR was done in 50 μ l of a one-step PCR reaction mixture tube. The amplified PCR products were purified by using a QIA quick ® PCR purification kit (Qiagen, Japan) and were directly sequenced by using a Big Dye ® Terminator v.3.1 cycle sequencing kit (applied Biosystems Inc., CA). The internal H gene sequence primers were CDV-HS1, CDV-H for D and CDV-Hr2 (Table 4). The sequences were analysed by using the Clustal W program (DDJB). A phylogenetic tree was constructed by using the Phylip 95 software package that used the neighbor-joining method.

Results

Pathological findings: The main gross findings of the three dogs were pneumonia and sometimes mild gastroenteritis (Table 5). Congested trachea and bronchi contained much fluid or occasionally mucopurulent exudates in all dogs, and large areas of congestion and consolidation were in all lobes of the lungs. The gastrointestinal tract showed moderate congestion and catarrh or hemorrhage. Histological lesions consisted of broncho-interstitial pneumonia with some eosinophilic intracellular inclusion bodies in the epithelia of the bronchioli or alveoli (Fig 6a (a)); immunohistochemistry showed CDV antigens were positive at or around the bronchioles (Fig. 6a(b)). Lymphocytic depletion of lymphoid tissue was moderate to severe, and immunolabelled lymphocytes were diffuse (Fig 6a(c,d)). The white matter showed non-suppurative demyelinating encephalitis with mild to moderate demyelination and slight infiltration of inflammatory cells with strongly positive antigens (Fig. 6b (e,f). The stomach showed few necrotic

cells with lesional inclusion bodies (Fig.6b (g)), but epithelial cells immunolabelled for CDV were clearly shown and were broadly positive (Fig. 6b (h)). The intestines of all dogs showed catarrhal enteritis with lymphocytic and plasmacytic infiltration of the lamina propria. Eosinophilic cytoplasmic and nuclear inclusion bodies were detected in neuronal and glial cells of the central nervous system (Fig. 6c (i)) and in epithelial cells of intestinal crypts and gastric glands (Fig. 6c (j)).

Virus isolation and titers in different organs: The cytopathogenic effect (CPE) for each sample from all organs of the dogs inoculated into Vero-DST cells was recorded daily by using phase contrast microscopy and was confirmed by immunocytochemistry as described previously (Lan *et el.*, 2005a). Viruses from the vaccine were also isolated in Vero-DST cells and were confirmed by immunocytochemistry and RT-PCR.

At the same time, virus titers in each organ were calculated (Table 6). In dog 4, the tonsils and mesentery lymph node showed the highest titers of $2.3 \times 10^3 TCID_{50}$/ml. The organs showing the highest titers ($4 \times 10^4 TCID_{50}$/ml) of dog 5 were also the tonsils and mesentery lymph node. The highest virus titers in spleen of dog 3 were 5.9×10^3 $TCID_{50}$/ml. After isolation, viruses from the spleen of dog 4, large intestine of dog 5 and cerebellum of dog 6 were named P94S, Ac96I and S124C, respectively, and were used for molecular analyses.

Sequence and phylogenetic analyses: Sequence and phylogenetic analyses of the P and H genes of new isolates and viruses isolated from the vaccine used for dogs were done. Nucleotide sequence analyses of a 390bp fragment of the P gene indicated high homology amongst the 3 new isolates of CDV. The nucleotide identities between the vaccine strain and the Onderstepoort strain was 99.23%, and the nucleotide identities between the vaccine strain and P94S, Ac96I and S124C were 94.1%, 94.87% and 94.1%, respectively. However, the nucleotide identities between new isolates were higher from

97.44% to 98.72% (Fig. 7). A phylogenetic tree was constructed based on the nucleotide sequence of the 390bp fragment of the P gene (Fig.8). The vaccine strain isolated from Duramune vaccine belonged to the vaccine strain group. New isolates P94S, Ac96I and S124C joined to the Asia 1 group (Hashimoto *et al.*, 2001) that was far from the vaccine group.

The nucleotide and predicted amino acid sequences of the H gene of the new isolates were analyzed by comparing them with those of the vaccine strain (Fig.9). Three H genes of the new isolates consisted of 1821 nucleotides in one open reading frame encoding 607 amino acids, but consisted of only 604 amino acids in the H gene of the vaccine strain. Table 7 shows homologies of nucleotides and amino acids of the vaccine strain and new isolates. The nucleotide identities were 91.06% to 91.39% and the amino acid identities were 90.23% to 90.75% between the vaccine strain and new isolates. In contrast, within new isolates the nucleotide identities were higher at 98.58% to 98.63% and the amino acid identities were at 98.1% to 99.51%. The H gene of both vaccine strain and new isolates has 12 cystein residues that characterize the secondary structure of protein at an identical position (Fig.9). One major hydrophobic region (amino acids 37 to 55) was found in both vaccine strain and new isolates. Strains P94S, Ac96I and S124C contain nine potential asparagine (N)-linked glycosylation sites at the same position: amino acid 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 584-586, 587-589 and 603-605). Six of these sites were found in the vaccine strain (Fig .9)

Fig. 10 shows a phylogenetic tree of predicted amino acid sequences of CDV strains. Strains P94S, Ac96I and S124C belong to Asia 1 group that is far from the vaccine group that includes the vaccine strain isolated from the Duramune vaccine.

Discussion

Recently, different cell lines, such as B95a, Vero and Vero-DST cells, have been used to isolate new strains of CDV. However, the effects of cell type on CDV protein or gene expression should be considered. The H gene sequence of the CDV strain isolated in Vero-DST and in B95a cells differs by two amino acids (Seki *et al.*, 2003). After CDV adapts to the cell line, it quickly loses the ability to cause disease and the virulence of CDV is lost after passing into Vero cells (Hamburger *et al.*, 1991). Although vaccine strains have particular molecular markers and gene sequences, the possibility can not be excluded that genes of the vaccine strain slightly changes in different batches of vaccine or the genes of new isolated CDV changed by isolation in some different cell lines. For example, our vaccine strain and the Onderstepoort strain used as our reference had six potential glycosylation sites for N-linked glycosylation, but the vaccine Onderstepoort (AAG30920) of Iwatsuki *et al.*, (1997) and Hirama *et al.*, (2004) had only four N-linked glycosylation sites. To clarify this point beyond doubt, we compared the H and P genes of new isolates in Vero-DST cells with 1) viral RNA from fresh tissue and 2) the vaccine virus isolated from the Duramune vaccine in the same cell line, because no change was found in the H and P genes of CDV after isolation, in fresh tissue and in Vero-DST cells (Lan *et al.*, 2005c).

Strain 007Lm previously isolated from lymph nodes of necropsied dog belongs to the Asia 2 group that is different from the group that includes the new isolates of this study (Figs. 9, 10). The hydrophobic region containing 20 amino acids from amino acid positions 37 to 56 of strain 007Lm has one amino acid more than those of strains P94S, Ac96I, S124C and the vaccine strain. The H gene of strain 007Lm, new isolates (strains P94S, Ac96I and S124C) and the vaccine strain have eight, nine and six, respectively, N-linked glycosylation sites. Strain 98-002 of Asia 2 group has eight N-linked

glycosylation sites (Mochizuki *et al.*, 1999). Japanese CDV isolated in 1997 also had nine N-linked glycosylation sites (Iwatsuki *et al.*, 1997).

In this study, fresh tissue from different organs was titrated, and, interestingly, the titration showed the distribution of viruses in different organs. The number of viruses in different organs and the target of viruses differed according to the virus strain. The similarity of genes of viruses isolated from different organs in the same dog would be interesting to know.

The clinical history was oculonasal discharge and diarrhea without apparent neurological signs. Immunohistochemistry and virus recovery indicated non-suppurative encephalitis and evidence of virus existence. CDV has the potential to infect multi-organs. Recent widespread CD tended to have acute and persistent infection in the gastrointestine, lymphatic tissue and central nervous system.

In our experience in Japan, many vaccinated dogs are still infected with canine distemper virus. The possibility was considered that vaccine failed to have the intended result because of either poor quality or existing maternal immunity. Another possibility was the reversion to virulence of attenuated CDV in vaccine. In this study, the dogs were checked for negative or very low level anti-CDV IgG and IgM before vaccination. Therefore, the possibility of existing maternal immunity was eliminated. These three dogs were previously vaccinated at age 7 and 11 weeks by a large dog dealer where 700-1,000 dogs are vaccinated and sold every month and only a few dogs are infected with CDV, so the failure of vaccination because of poor technique and quality of vaccine was a low possibility. However, to know exactly if the dogs had immune response by vaccination but still infected with field CDV strains having a gene relationship far from the vaccine strain or dogs infected with new isolates before the development of immune response, we need detailed information of the antibodies of dogs after vaccination and

further research in neutralization activities amongst vaccine virus and new isolates and in experimental vaccinated dogs.

In conclusion, this study clarified that three dogs with a history of using vaccine infected with wild- type CDV had a genetic relationship far from the vaccine strain, and showed no evidence of reversion to virulence of vaccine virus.