RESEARCH ARTICLE

Molecular detection and characterization of Canine distemper virus from domestic dogs (*Canis familiaris*) in Quezon City, Philippines

Czarina Kristine D Nobleza, DVM[1], **Maria Rosario S Racho,** DVM[1] **Dennis V Umali,** DVM, PhD[1,*]
and Hiromitsu Katoh, DVM, PhD[1,2]

[1]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of the Philippines, Los Baños, Laguna, 4031, Philippines.
[2]Poultry Products Quality Control (PPQC), 125-7 Daiwa Dakeonsen, Nihonmatsu, Fukushima, 964-0062, Japan.
*Correspondence author: Email: dvumali@up.edu.ph

Manuscript details:	**ABSTRACT**
  	Canine distemper (CD) is a highly infectious viral disease affecting all terrestrial carnivores worldwide. In spite of the availability of commercial modified live vaccines, several studies have reported the occurrence of CD in vaccinated animals. In this study, six dogs with clinical signs of CD were investigated. The age of the dogs ranged from 2 months to 8 years old, two of which were unvaccinated, while the others have outdated vaccination. General clinical signs observed were twitching, myoclonus, diarrhea, oculonasal discharge, and lethargy. Laboratory results revealed presence of anemia and lymphopenia. Oculonasal swabs were collected and nested reverse transcription polymerase chain reaction (nRT-PCR) was performed. Nucleotide sequencing of the hypervariable region of the hemagglutinin (H) gene showed that the field CDV strains belong to the Asia-4 lineage, a new CDV lineage first reported in Thailand in 2013. Nucleotide sequence similarity showed that the field CDVs were closely related (94%) to CDV strains from infected dogs in Thailand. Possible genetic evolution due to interspecies transmission and animal movement such as importation or international transportation of dogs and other susceptible carnivores are suspected. At present, this is the first study to molecularly characterize CDVs in the Philippines.

INTRODUCTION

Canine distemper virus (CDV) is a *Paramyxovirus* causing a highly infectious viral disease affecting all species of terrestrial carnivores worldwide, namely *Canidae* (fox, dingo, coyote, wolf, jackal); *Mustelidae* (ferret, mink, weasel, marten, skunk, badger, otter); *Procyonidae* (raccoon, kinkajou,

coati), *Felidae* (cheetah, lion, jaguar, margay, ocelot), *Ailuridae* (red panda), *Hyaenidae* (hyena), *Ursunidae* (bear), and *Viverridae* (palm civet). Infected carnivores manifest multisystemic signs affecting the gastrointestinal, respiratory and neurological systems (Namroodi *et al.,* 2013; Sykes, 2014). Given the extent of susceptible species and seriousness of its clinical signs, it is critical to gain more insight about the factors involved in its transmission and epidemiology in order to search for therapeutic strategies that will solve or prevent any future CD outbreak.

CD is presumptively diagnosed through clinical signs and routine laboratory procedures. The availability of specific laboratory tests limits the confirmation of CDV infection therefore treatment remains nonspecific, symptomatic, and supportive. The pathogenicity of the virus differs depending on the CDV strain. Clinical manifestations of the disease may then vary in extent and severity, thus affecting the treatment plan. Live attenuated CDV vaccines have been used to control the disease, however, there have been reports of CDV infection even on properly vaccinated dogs (Sarute *et al.,* 2013). Japan, Mexico, Argentina and the United States have reported recent cases of CD on immunized dogs suggesting re-emergence and increased activity of CDV worldwide (Wilkes, 2014).

CDV is a pleomorphic, single stranded negative sense RNA virus. Its genome is consists of the following genes: Nucleoprotein (N), Phosphoprotein (P), Matrix (M), Fusion (F), Hemagglutinin (H) and Polymerase (L). Based on the analysis of the hypervariable region of the haemagglutinin (H) gene, there are at least seven established CDV lineages: Asia-1, Asia-2, America-1 (vaccines), America-2, Europe, Europe-wildlife, and Arctic-like (MacLachlan & Dubovi, 2011). Companion animal practice is a growing industry in the Philippines. A retrospective study by Matawaran (1997) revealed that 97.12% of the companion animal cases presented in the University of the Philippines Veterinary Teaching Hospital (UP-VTH) in Diliman, Quezon City from 1993-1996, were from the canine species. Canine distemper was among the two most predominant canine viral diseases diagnosed, second to canine parvovirus. Despite the implementation of vaccination programs for core vaccines, CD continues to occur among dog population in the Philippines. In order to better address the control and prevention of CD, a fundamental knowledge of the locally circulating strains in the Philippines is needed. In this study, field

cases of CDVs from Quezon City, Philippines were molecularly analyzed and compared to vaccine and reference strains from different parts of the world from different time periods to infer on potential epidemiological routes and mechanisms of transmission of CD in the Philippines. Knowing the prevalent genotypes, subgenotype and other phylogenetic characteristics of field CDVs in the Philippines will be useful in the formulation of more effective prevention and control strategies in the country.

MATERIALS AND METHODS

The use of animals in this study as described in the procedure below was approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños.

Sample Collection
Clinical specimens were obtained from the UP Veterinary Teaching Hospital in Diliman, Quezon City from October 2015 to March 2016. The owners' consents were obtained before collection of the samples. Six pooled oculonasal swab samples were collected from dogs presented with signs suggestive of Canine distemper, and confirmed through CDV antigen test kit (ImmunoRun®, Biogal Galed Labs). The specimens were transported at 4°C, then frozen at -20°C until further processing. Clinical profiles were recorded for each selected case which included age, sex, clinical signs exhibited, vaccination status, treatment done and the outcome.

Nucleic acid extraction
Viral RNA from ocular and nasal discharges were extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, West Sussex, UK) according to manufacturer's instructions.

RT-PCR amplification
The RNA extracted was transcribed to cDNA using reverse transcriptase (RT) and random primers. A specific CDV H gene primer was used to amplify the target H gene cDNA through polymerase chain reaction (PCR) as reported previously (Guo *et al.,* 2013). The following thermocycling condition were used: 5 minutes at 94°C (initial denaturation), 30 cycles of 30 seconds at 94°C, 30 sec at 51°C, 30 sec at

72°C, and a final extension of 10min at 72°C. The products were separated in a 1.5% agarose, 1X Tris-acetate-EDTA. They were then visualized using Gel Red™ nucleic acid stain (Biotium, Inc., California, USA) and LED light.

Nucleotide sequencing
Amplified products were excised and purified using QIAquick® Gel Extraction Kit (Qiagen, West Sussex, UK) according to manufacturer's instructions. Purified PCR products were submitted to the Philippine Genome Center for bidirectional sequencing. The oligonucleotide primers used in the RT-PCR were used to sequence the purified products.

Data analysis
Sequence assembly and editing were performed using CodonCode Aligner® (version 3.7.1, CodonCode Corporation, MA) and ClustalX® (version 2.1, Conway Institute UCD Dublin, Ireland). Deduced amino acid sequences were determined using Bioedit® software package version 7.1.3.0. Confirmation of identity and homology were performed using the Basic Local Alignment Search Tool (BLAST) of the National Center of Biotechnology Information, National Library of Medicine (http://www.blast.ncbi.nlm.nih.gov). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4. Phylogenetic trees were constructed by the neighbor-joining method with the maximum composite likelihood substitution model at 1000 bootstrap replicates.

RESULTS AND DISCUSSION

Signalment, vaccination history, clinical signs, laboratory results, treatment and outcome of the affected animals were analyzed (Table 1). The age of the dogs ranged from 2 months to 8 years old, two of which were unvaccinated, while the others have outdated vaccination. General clinical signs observed were twitching, myoclonus, diarrhea, oculonasal discharge, and lethargy. Laboratory results revealed presence of anemia and lymphopenia. Treatment protocols applied included the use of antibiotics for secondary bacterial infections, supplementation of vitamins and administration of expectorant especially for those manifesting respiratory signs. Follow-ups were conducted to determine the outcome of each cases, and three mortalities were noted (Table 1).

Table 1. Clinical profile, vaccination history and laboratory results of dogs infected with CDV from Quezon City, Philippines.

Code, Sex, Breed	Age	Vaccination History	Clinical signs	Laboratory results	Outcome
CD1601, M LabRet	8yo	NU	N,G,R	+ CDV Ag	D
CD1602, M Golden Ret	3mos	NI	N,G,O,R	+CDV Ag, Anemia, Lymphopenia	D
CD1603, M Siberian Husky	2yo	NU	N	+ CDV Ag, Anemia	D
CD1604, F Mini Pinscher	2mos	NV	G,O,R	+ CDV Ag, + Ancylostoma	U
CD1605, M Mixed	4mos	NV	G,O,R	+CDV Ag, Anemia, Lymphopenia	R
CD1606, F Labrador Retriever	4 yo	NI	N,O,R	+CDV Ag, Anemia, Lymphopenia	U

NU – Not Updated, NV – Not Vaccinated, NI – No Information
N – Nervous, G – Gastrointestinal, O – Ocular, R - Respiratory
(+) CDV Ag – Positive CDV Antigen test kit
D – Died, R – Recovered, U – Unknown
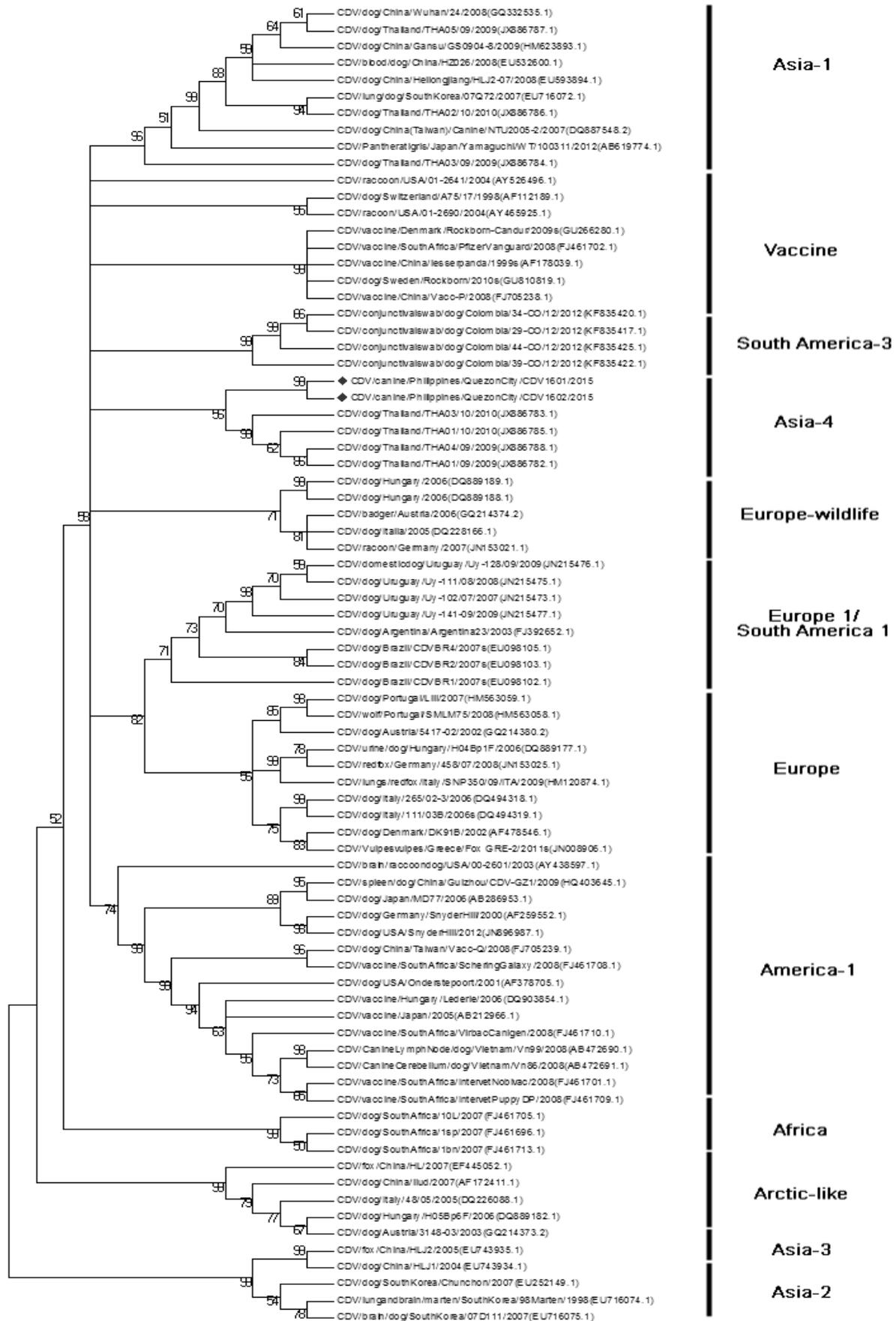
Figure 1. Phylogenetic relationships among CDV isolates based on nucleotide sequences of the H gene. Diamond marker (♦) indicates the two Philippine field strains analysed in this study.
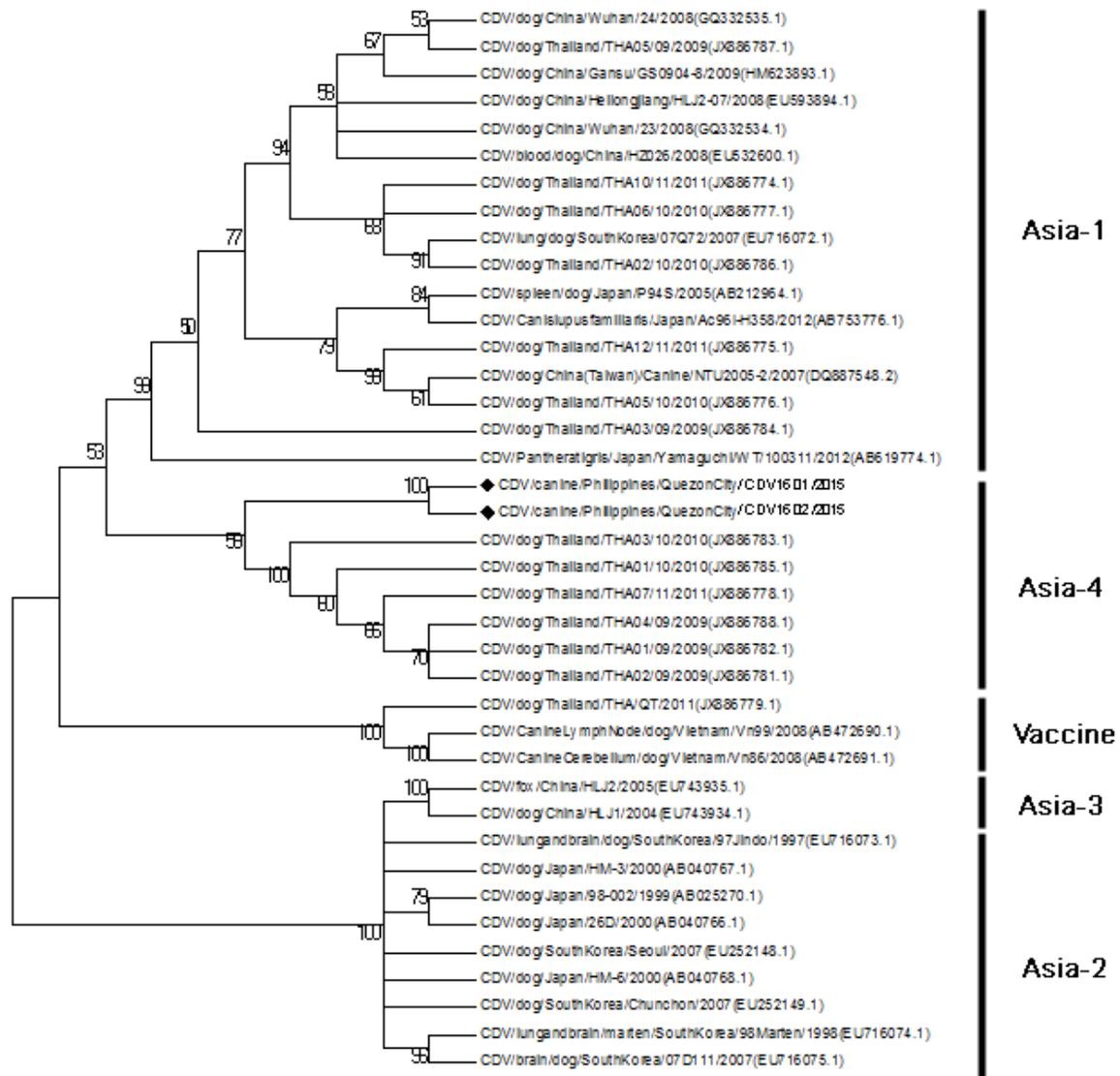
Figure 2. Phylogenetic relationships among CDV Asian strains based on the H-gene nucleotide sequences. Diamond marker (♦) indicates the two Philippine isolates analyzed in this study.

For molecular confirmation of CD, nested RT-PCR was performed. A 611 base pair (bp) fragment of the hemagglutinin gene was detected using published H-gene primers. CDV RNA was detected in two out of six (33.3%) oculonasal swabs and these isolates were given the code CDV1601 and CDV1602. The antigen test kit has a sensitivity and specificity of 97.5% when oculonasal swabs are used for detecting CDV (Biogal Galed Labs, 2011). A positive result from the antigen test kit is already diagnostic of canine distemper. RT-PCR detects the viral RNA, and a positive result is a highly specific diagnosis indicative of infection (Greene and Appel, 2006). Due to the higher specificity of the RT-PCR, negative results could have been caused

by several factors including improper handling of the samples, low viral load from site of collection, degradation of the virus, storage conditions of the sample, and the duration from collection to the processing of the samples.

Nucleotide sequence analysis of the hypervariable region of the H gene showed that the field strains had 96% maximum identity to the Rockborn strain (GenBank accession no. GU266280), Brazil strain (JX912968) and China isolate from a lesser panda (AF178039). In addition, the Philippine strains were closely related to Thailand strains THA03/10 (94.6%), THA01/09 (94.5%), THA04/09 (94.5%), and

THA01/10 (94.3%). Commercial vaccines used in the Philippines, Nobivac® from Intervet (FJ461701.1), Canigen® from Virbac (FJ461710.1) and Vanguard® from Pfizer (JX886780) revealed 89.6%, 89.7% and 96% identity, respectively, which indicate that the field strains were not vaccine strains. A neighbour joining phylogenetic tree was generated using the field CDV strains and 78 representative strains from each genotype from different countries and time periods from Genbank. It was observed that CDV1601 and CDV1602 belonged to CDV group Asia-4 (Figure 1). Asia-4 is a newly discovered CDV genotype that separated from other established CDV Asian lineages (Asia-1 and Asia-2), and was first reported in Thailand in 2013 (Radtanakatikanon *et al.*, 2013). Subgenotypic analyses yielded same results confirming the earlier findings that the two Philippine strains belong to the Asia-4 CDV genotype (Figure 2).

The 611 base pairs fragment from both CDV1601 and CDV1602 were translated into a 203 amino acid long polypeptide of the H protein. They were then aligned with the amino acid sequences of representative vaccine strains and field strains from each lineage. BioEdit® software was used to examine the amino acid sequences for N-linked glycosylation sites. This is indicated by a three amino acid code N-X-S/T, where N stands for Asparagine, X stands for any amino acid, and S for Serine or T for Threonine. Guo *et al.* (2013) stated that N-glycosylation sites are found at amino acid positions 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 584-586, 587-589 and 603-605 of the complete sequence of H genes. It was suggested that N-glycosylation sites are involved in receptor recognition and attachment, and that reduction or deletion of these sites results into attenuation without affecting immunosuppression (Sawatsky and von Messling, 2010). Vaccine strains usually contain 4 (Onderstepoort) or 7 (CDV3, Lederle, and Convac) N-glycosylation sites, while field strains have 7 or 9. The partial fragment obtained from the hypervariable sequence of the field strains that started from the 310 amino acid position and ended at 513 showed four glycosylation sites at 309-311, 391-393, 422-424, 456-458 (Figure 3). Further analysis of the N-glycosylation sites cannot be done due to the inadequate length of the sequence.

Using Clustal Omega Percent Identity Matrix (PIM) (http://www.ebi.ac.uk), a high percent (100%) of amino acid identity was determined between CDV1601 and CDV1602. The four representative Thai strains belonging to the Asia-4 lineage shared a high amino acid sequence identity with the Philippine strains (94.09-94.58%). Vaccine amino acid sequences showed 87%-96% similarity. Based on the partial sequence analyzed, the high degree of identity in the H-gene of the isolates in this study and the Thailand CDV field strains suggests that they might have originated from common ancestors. The emergence of previously unidentified CDV lineages suggest possible genetic evolution due to interspecies transmission and animal movement such as importation or international transportation of dogs (Radtanakatikanon, 2013). However, due to the lack of genetic data on CDVs in the Philippines, it is quite early to conclude whether this genotype may have originated abroad or may have been an endemic strain that has been in existence in the Philippines for some time. It is highly recommended that further research on the epidemiological distribution of this genotype and the existence of other possible lineages of CDVs in the country be conducted.

The identity of the Philippine strains with commercial vaccines was also examined. Since the H gene is involved with the attachment of the virus to receptors, the animal's immune system should be able to recognize the antigen and elicit an adequate immune response. It is important to provide a vaccine with a high percent identity with that of the field strains present in a geographical area in order to provide a more efficient protection from CDV. Calderon *et al.* (2007) reported cases of vaccinated dogs infected with CDV. These were primarily attributed to presence of maternally derived antibodies and improper vaccination such as inappropriate handling or administration of vaccines and noncompliance to vaccination schedule. In this study, two dogs with outdated vaccinations showed manifestations of the disease. A lapse of more than one year from the next vaccination schedule would result into vaccinated animals having the same risk of CDV infection as the unvaccinated dogs (Latha *et al.,* 2007). This emphasizes the need for a regular annual vaccination for a more efficient protection against CDV.

Although there are other factors that could affect the animals' immunity to CDV, vaccine failure may also be a possibility. Most of the vaccines being used had been derived from America-1 strains, and were isolated in the 1950s. New and emerging strains of CDV may be

poorly neutralized by antibodies elicited by old, attenuated vaccine strains (Calderon *et al.*, 2007). It is therefore recommended that continued surveillance of CDVs in the country be conducted over multiple years so that a database of existing strains and emerging genotypes will be generated that may aid in the formulation of more effective prevention, treatment and control strategies.

CONCLUSION

In conclusion, it was shown that the field CDVs namely CDV1601 and CDV1602 from Quezon City, Philippines that were obtained from dogs with clinical signs of the disease where confirmed to be CDVs from the Asia-4 lineage. This new CDV lineage was first identified in Thailand in 2013. The high degree of nucleotide sequence similarity (94%) between the Philippine and Thai strains may suggest common ancestry. Possible genetic evolution due to interspecies transmission and animal movement such as importation or international transportation of dogs and other susceptible carnivores are suspected. Future studies on the clinical and pathological characteristics of Asia-4 CDVs, and the utilization of field strains to develop immunotherapeutic compounds and diagnostic test kits for a more efficient treatment, prevention, and control of CDV in the Philippines are highly recommended. Further research and continued surveillance of CDV should be conducted over multiple years so that a database of existing strains and emerging genotypes will be generated.

Conflicts of interest: The authors stated that no conflicts of interest.

REFERENCES

Biogal-Galed Labs (2011) Canine Distemper Virus. *ImmunoRun Antigen Detection Kit Instruction Manual*. Retrieved on 29 September 2017 from http://www.biogal.co.il/component/phocadownload/category/41-ir-distemper

Calderon MG, Remorini P, Periolo O, Iglesias M, Mattion N and La Torre J (2007) Detection by RT-PCR and genetic characterization of canine distemper virus from vaccinated and non-vaccinated dogs in Argentina. *Vet Microbiol*. 125(3-4):341-9

Greene CE and Appel MJ (2006) *Infectious diseases of the dog and cat.* 3rd ed. Philadelphia: The W. B. Saunders Co., pp. 25-42.

Guo L, Yang SL, Wang CD, Hou R, Chen SJ, Yang XN, Liu J, Pan HB, Hao ZX, Zhang ML, Cao SJ and Yan QG (2013) Phylogenetic analysis of the haemagglutinin gene of canine distemper virus strains detected from giant panda and raccoon dogs in China. *Virol. J.* 10:109. doi:10.1186/1743-422X-10-109

Latha D, Srinivasan SR, Thirunavukkarasu PS, Gunaselan L, Ramadass P, and Narayanan RB (2007) Assessment of canine distemper virus infection in vaccinated and unvaccinated dogs. *Indian J. Biotechnol.* 6: 35-40.

MacLachlan NJ and Dubovi EJ (2011) *Fenner's Veterinary Virology*. 4th ed. London: Academic Press, pp 317-320.

Matawaran VA (1997) Retrospective study of cases presented and diagnosed at the University of the Philippines Veterinary Teaching Hospital from 1993-1996. *UP Endowment Fund Assistant Professorial Lecture*, College of Veterinary Medicine, University of the Philippines Los Baños, Laguna.

Namroodi S, Rostami A, Majidzadeh-Ardebili K, Ghalyanchi-Langroudi A and Morovvati A **(**2013) Molecular and serological detection of canine distemper virus (CDV) in rural dogs in Iran. *Iran J. Virol.* 7 (1 and 2): 37- 43.

Radtanakatikanon A, Keawcharoen J, Charoenvisal N, Poovorawan Y, Prompetchara E, Yamaguchi R and Techangamsuwan S (2013) Genotypic lineages and restriction fragment length polymorphism of canine distemper virus isolates in Thailand. *Vet. Microbiol.* 166:76-83.

Sarute N, Calderon MG, Perez R, La Torre J, Hernandez M, Francia L and Panzera Y (2013) The Fusion Protein Signal-Peptide-Coding Region of Canine Distemper Virus: A Useful Tool for Phylogenetic Reconstruction and Lineage Identification. *PLoS ONE* 8(5): e63595. doi:10.1371/journal.pone.0063595

Sawatsky B, and von Messling V (2010) Canine distemper virus expressing a hemagglutinin without N-glycans lose virulence but retain immunosuppression. *J. Virol.* 84(6): 2753-2761.

Sykes JE (2014) *Canine and Feline Infectious Diseases.* Missouri: Elsevier Saunders. pp 152-164

Wilkes RP, Tsai Y, Lee P, Lee F, Chang G and Wang T (2014) Rapid and sensitive detection of canine distemper virus by one-tube reverse transcription-insulated isothermal polymerase chain reaction. *BMC Vet. Res.* 10:213. doi:10.1186/s12917-014-0213-8