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MOLECULAR AND SEROLOGICAL DETECTION OF CANINE DISTEMPER VIRUS IN DOMESTIC DOGS FROM ANIMAL SHELTERS ACROSS KLANG VALLEY

NUR EIZZAH BINTI AZMAN

MOLECULAR AND SEROLOGICAL DETECTION OF CANINE DISTEMPER VIRUS IN DOMESTIC DOGS FROM ANIMAL SHELTERS ACROSS KLANG VALLEY

by

NUR EIZZAH BINTI AZMAN

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Molecular And Serological Detection Of Canine Distemper Virus In Domestic Dogs From Animal Shelters Across Klang Valley", by Nur Eizzah binti Azman and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Final Year Project.

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Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek Ilmiah Tahun Akhir.

PENGESANAN MOLEKUL DAN SEROLOGI VIRUS DISTEMPER ANJING DALAM ANJING DOMESTIK DARI PUSAT PERLINDUNGAN HAIWAN DI SEKITAR LEMBAH KLANG

oleh

NUR EIZZAH BINTI AZMAN

2023

Penyelia: Dr. Farina Mustaffa Kamal

Penyelia bersama: Dr. Khor Kuan Hua, Dr. Intan Nurfatiha Shafie

Virus distemper anjing (CDV) adalah penyakit yang disebabkan oleh *Morbillivirus*, sejenis virus RNA yang sangat mudah merebak menyebabkan menyebabkan penyakit neurologi sistemik yang teruk. Bentuk jangkitan kronik penyakit ini digelar "*hard pad disease*". Anjing yang tidak divaksinasi terhadap CDV, dan anak anjing mempunyai risiko yang lebih tinggi untuk mendapat jangkitan. Pada masa ini, prevalens CDV di Malaysia masih tidak diketahui. Matlamat kajian ini adalah untuk menentukan prevalens

molekul dan serologi CDV dalam anjing domestik dari pusat perlindungan haiwan di sekitar Lembah Klang. Sejumlah lima pusat perlindungan haiwan dengan 10 anjing dari setiap pusat perlindungan haiwan telah dipilih. Sampel serum telah diuji untuk antibodi CDV dengan menggunakan imunoasai kromatografi (CI), manakala sampel konjunktiva dan swab hidung terkumpul diuji untuk RNA dengan penggunaan transkripsi membalik reaksi rantai polimerase (RT-PCR) di mana gen hemagglutinin (H) diamplifikasi. Sampel konjunktiva dan swab hidung terkumpul oleh RT-PCR menunjukkan 50/50 (100%) keputusan negatif bermaksud tiada jangkitan aktif. Keputusan CI menunjukkan 10/50 (20%) negatif, 22/50 (44%) titer tinggi, 17/ 50 (34%) titer sederhana, dan 1/50 (2%) titer antibodi IgG rendah menentang CDV. Kebanyakan anjing ini mempunyai antibodi terhadap CDV yang mungkin disebabkan oleh sejarah vaksinasi; walaupun vaksinasi tidak dilakukan secara berkala. Oleh itu, adalah dicadangkan bahawa protokol vaksinasi yang digunakan oleh pusat perlindungan ini membantu mengurangkan penularan penyakit dan risiko jangkitan dan memberikan beberapa darjah imuniti kelompok di dalam kalangan populasi anjing perlindungan.

Kata kunci: Virus distemper anjing; pengesanan molekul; pengesanan serologi; Malaysia; tempat perlindungan haiwan

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 - Final Year Project.

MOLECULAR AND SEROLOGICAL DETECTION OF CANINE DISTEMPER VIRUS IN DOMESTIC DOGS FROM ANIMAL SHELTERS ACROSS KLANG VALLEY

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Canine distemper virus (CDV) is a disease caused by *Morbillivirus,* a highly contagious RNA virus that attacks the immune system causing severe systemic neurological disease. Chronic form of infection also causes disease known as “hard pad disease”. Dogs that are not vaccinated against CDV, and puppies pose a higher risk of infection. Currently, the prevalence of CDV in Malaysia is still unknown. The goal of this study was to determine the molecular and serological prevalence of CDV in domestic dogs from animal shelters across Klang Valley. A total of five shelters with 10 dogs from each shelter was selected.

The serum sample was tested for CDV antibodies with chromatographic immunoassay (CI), while pooled conjunctival and nasal swab samples were tested for RNAs with the use of reverse transcription polymerase chain reaction (RT-PCR) where the hemagglutinin (H) gene was amplified. RT-PCR of pooled conjunctival and nasal swab samples showed 50/50 (100%) negative results indicative of no active infection. The CI results showed 10/50 (20%) negative, 22/50 (44%) high titer, 17/50 (34%) medium titer, and 1/50 (2%) low titer of IgG antibodies against CDV. Most of these dogs had antibodies against CDV which could be due to vaccination history; although the vaccination was not regularly performed. Hence, it is suggested that vaccination protocol employed by these shelters help to reduce disease transmission and risk of infection and provided some degree of herd immunity among the shelter dog population.

Keywords: Canine distemper virus; molecular detection; serological detection; Malaysia; shelter

1.0 INTRODUCTION

Canine distemper virus (CDV) is a contagious disease caused by *Morbillivirus*. It is an enveloped virion that contains a single-stranded negative-sense RNA virus that originates from the *Paramyxoviridae* family. CDV affects domestic and wild dogs, including a wide variety of wild mammals such as members of the *Felidae*, *Mustelidae*, and *Procyonidae* families. It is known to affect the nervous, respiratory and gastrointestinal systems of the body. Animal will exhibit various common symptoms such as high fever, nasal discharge, coughing, lethargy, reduced appetite, and vomiting. Over time, it will induce systemic signs involving the nervous system and cause viral persistence in organs, including the central nervous system and lymphoid tissue (Bruyette, 2020). CDV is often fatal, with a mortality rate of 50% in adult dogs and 80% in puppies (Evermann & Kennedy, 2011). Transmission is airborne and mainly through coughing or sneezing. Dogs have been known to be carriers and can be a reservoir or source of transmission (Carvalho et al., 2012).

CDV is seen worldwide and many studies and research have been conducted in various countries, especially on its prevalence. However, in Malaysia, there is still limited information regarding CDV. Though there are many unpublished reported cases of the disease involving domestic dogs, it is typically in hospital settings. However, the status of this disease is unknown in the shelter population in Malaysia. Hence, the objective of this study is to determine the molecular and serological prevalence of CDV in domestic dogs from animal shelters across Klang Valley. The reasoning behind the choice of population is that in some shelters, the management has difficulty maintaining a proper

regular vaccination routine. So, it yields opportunities where the disease could be present, and cause high mortality.

2.0 LITERATURE REVIEW

2.1 Gene Organization of Morbillivirus

Morbillivirus genus belongs under the *Paramyxoviridae* family, which includes two other genera, which are the Paramyxovirus and Pneumovirus. This genus is a group of enveloped viruses with non-segmented, negative-strand RNA genomes that encode necessary protein for its survival. The genome contains 15,690 nucleotides throughout the length and encodes eight proteins. *Morbillivirus* has two types of protein on the basis of function: non-structural protein (NSP) and structural protein (SP). NSP is a protein that is encoded by the virus itself that helps in viral metabolism and interaction with the host's machinery, while SP makes up the structural component of the virus. In *Morbillivirus*, the NSPs consist of V and C proteins that are encoded within the P gene (Weston & Frieman, 2015). An overlapping open reading frame (ORF) and RNA editing by the insertion of a non-templated G residue during mRNA synthesis are being used by the P gene to encode the V and C proteins, respectively (Cattaneo, 1989). Next, there are six SPs that are encoded by their respective gene: two glycoproteins, which are the haemagglutinin (H gene) and fusion protein (F gene); two transcriptase-associated proteins which are the phosphoprotein (P gene) and large protein (L gene); nucleocapsid protein (N gene) that encapsulates the viral RNA, and matrix protein (M gene) (van Regenmortel, 2000). The

key protein for CDV is the H gene, which is used to attach itself to the host's receptors (Appel & Gillespie, 1972). Also, since the virus is a negative sense virus, the RNA is not readily detected by the host's ribosome; it is being treated as anti-sense RNA causing translation to not occur. Hence, the viral RNA polymerases will have to transcribe it into positive-sense RNA, which then will be translated by the host's ribosome to produce at least six mRNAs. In addition, due to the nature of the RNA polymerases, it is prone to copying error which affects the transcription causing mutation. Hence, it explains why the RNA is prone to mutation (Duffy, 2018).

2.2 Epidemiology of Canine Distemper Virus

2.2.1 Canine Distemper Virus Globally

Canine distemper virus (CDV) is seen worldwide, and the prevalence varies between countries. There are also many reported cases of CDV outbreaks involving wildlife. In Malaysia, there is limited information regarding CDV in domestic dogs, though there are many reported cases. This is due to the absence of concise research pertaining to CDV in Malaysia. This virus itself is easily transmissible via airborne exposure (e.g.: sneezing, coughing) making it possible for the number of cases to be high. The virus is also transmissible via vectors such as shared food and water bowls, as well as transplacentally. Furthermore, viral shedding can occur 60–90 days post-infection (Mahy & van Regenmortel, 2010). In other countries such as Bhutan, CDV is common due to the presence of a high population of feral dogs across the country, and there have been recurrent outbreaks resulting in mass mortality (Borah & Pasha, 2021). In another

study in Bhutan (Haa district), ELISA was used to test the samples for any CDV antibodies, and the seroprevalence was found to be 11.3% (95% CI 6.7–14.2) (Dorji et al., 2020). Though vaccination against CDV is done, it is typically in a limited number of pet dogs, while feral dogs remain unvaccinated, thus, making this group susceptible to infection. Also, in Wenzhou, China, a study had been done where 2406 dogs were tested for CDV, and 35.5% were positive (Luo et al., 2017).

2.2.2 Mechanism of Pathogenicity of Canine Distemper Virus

CDV causes severe systemic disease as it attacks the dog's immune system. The typical clinical signs that could be observed include discharge from the eye and nose, acute fever, lethargy, and respiratory and enteric signs. The virus can also cause the footpads and nose to thicken and harden (also known as "hard pad disease"). As the virus attacks the nervous system, dogs will show neurologic signs that include head tilt, walking in a circle, lack of coordination, seizure, convulsion with jaw-chewing movement, and partial/complete paralysis (AVMA, 2022).

Pertaining to its molecular pathogenesis, antigenic recognition and viral interaction rely on the hemagglutinin protein (H gene). It interacts with the host's two cellular receptors which are the signaling lymphocyte activation molecule (SLAM) in peripheral blood mononuclear cells; and nectin-4 (PVRL4) in the epithelial cells (Pratakpiriya, 2012). H protein is the key protein used at the first step of infection. Once it has attached, the F protein will cause the fusion of the cell membrane with the viral

envelope, and even membrane fusion between host cells, forming syncytia (Appel & Gillespie, 1972).

Rendon-Marin et al. (2019) also state that, since the SLAM receptor is located in the lymphoid cells, it directly causes immunosuppression, leading to the presence of systemic signs. The severity of it also depends on whether the dog develops a secondary bacterial infection or not, and whether the severity of it can develop neurological symptoms in its later stage — this is also known as chronic distemper encephalitis or also known as old dog encephalitis (ODE).

ODE is characterized by ataxia, compulsive movements (e.g. head pressing; continual pacing), and uncoordinated hypermetria. A dog with ODE typically does not have a history suggestive of systemic canine distemper infection. It may even go unnoticed up until the dog has reached old age. The inflammatory reaction in the central nervous system, which is associated with persistent canine distemper virus infection has been theorized to cause ODE, however, the exact mechanism is still unknown.

2.2.3 Diversity of Susceptible Host

It is well known that CDV is a highly prevalent viral infectious disease in dogs, and even for other carnivores, especially wild animals. Hence, it poses a conservation threat to endangered species worldwide. The exact mechanism by which wild animals can get infected remains to be fully understood (Seimon et al., 2013). A study has shown that even though there is a low level of infection, the virus can still circulate among wild animals, and it has been theorized that it is due to a complex reservoir system (Viana et

al., 2015). CDV cases have been reported occurring in non-canine hosts such as domestic cats, lions, tigers, elephants and primates such as rhesus macaques and cynomolgus monkeys (Martinez-Gutierrez & Ruiz-Saenz, 2016).

2.3 Protection against Canine Distemper Virus

2.3.1 Vaccination Regime

In order to combat the risk of getting CDV, one effective intervention strategy is vaccination. According to the World Small Animal Veterinary Association (WSAVA), core vaccines for dogs include those that protect against CDV, canine adenovirus (CAV), and the variants of canine parvovirus type 2 (CPV-2). In Malaysia, some animal shelters do not practice routine vaccination and this gives the virus a chance to infect the residents. Animal shelters are typically described as coming from a random source population with unknown vaccination history rendering them at higher risk of contracting an infectious disease (WSAVA, 2016). Any detection of the presence of CDV or its reservoir inside the shelter will help to control its transmission among the population.

The type of vaccine that is being used in Malaysia is the modified live vaccine (MLV), and recombinant virus (rCDV). Both types of vaccine have the same guidelines, as recommended by the WSAVA of which first initial vaccination at 6–8 weeks of age (this is to reduce the risk of maternal antibody interference), then every 2–4 weeks until 16 weeks of age or older. Then, the booster at either 6 months or 1 year of age, then not more often than every 3 years. There is a debate regarding the effectiveness of revaccination every 3 years instead of 1 year. Not to forget, since the H gene produces the

key protein for virus attachment, an adequate host immune response against the H protein may prevent CDV infection. It is also good to note that MLV has a risk of producing disease in immunosuppressed dogs. CDV vaccines provide prolonged immunity in a high percentage of dogs that receive one dose of MLV vaccine at or after 16 weeks of age. The efficacy of CDV vaccines approaches 99% and dogs develop protective immunity within a few days after vaccination (WSAVA, 2020).

2.4 Molecular Assay Detection of Canine Distemper Virus

There is differing opinions regarding the gold standard for diagnosing CDV. However, reverse transcription polymerase chain reaction (RT-PCR) is still preferable to many due to its rapidity, sensitivity, and specificity. RT-PCR uses RNA as a template, which is then transcribed into complementary DNA (cDNA) using reverse transcriptase. The whole procedure typically takes about 4 hours to complete with result visualization through gel imaging. As RNA is very unstable due to its single-stranded nature, it is vital to ensure that the handling and storage are being done meticulously as the factor that affects the success of RT-PCR is the quality of the RNA itself. Otherwise, a positive result is a good indicator of active infection. However, a negative result does not rule out CDV, especially when samples are obtained late in the course of the disease when the virus may no longer be shed. There is also reverse transcription loop-mediated isothermal amplification (RT-LAMP) that is used to detect the genomic RNA of CDV. In a study by Cho and Park (2005), four primers were designed from CDV genomic RNA that targets the nucleocapsid protein gene. The optimal reaction time and the temperature have been

determined, and the result pertaining to the relative sensitivity and specificity of RT-LAMP was found to be 100% and 93.3%, respectively. The other advantage that RT-LAMP has to offer is the duration of the procedure which only takes 1 hour to complete, and it can be visualized through the naked eye without the help of any machine imaging.

2.5 Serological Assay Detection of Canine Distemper Virus

The most common serological assay being done is enzyme-linked immunosorbent assay (ELISA). ELISA measures the antibody titer that is present in the dog's serum. Since antibody is being used, they can detect past infection. It is advisable to take serial titers on 2 serum samples as it is more accurate in detecting rising titers. But in the context of risk assessment pertaining to exposed dogs in a shelter, one serum sample is enough. In a study by Litster et al. (2012), a moderate to marked increase in serum antibody responses can help to differentiate resistant from susceptible animals. Hence, measurement of these antibody titers can be useful to determine the need for vaccination and risk assessment or population management related to shelter animals (Lechner et al., 2010). Other than ELISA, direct immunofluorescence assay (FA) can also be done. In a study by Athanasiou et al. (2017), the swab samples were stained with anti-CDV polyclonal antiserum conjugated to fluorescein isothiocyanate and imaged using a fluorescent microscope. A comparison between PCR and FA was done and out of 57 dogs tested, 19 were PCR positive versus 15 positive in direct FA. A good agreement was observed between the FA and PCR, with a k-value of 0.833 (95% CI: 0.678–0.989). It shows 100% specificity and a relatively sensitive assay.

3.0 MATERIALS AND METHODS

3.1 Ethic statement

Before sampling, the study protocol was approved by the Institution of Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-R048/2022), and agreement for sampling from selected animal shelters was obtained.

3.2 Population and Sampling

Convenient sampling technique was used to select fifty (50) dogs of different sex, age, and breed, from multiple animal shelters across Klang Valley, and biological samples such as blood, nasal and conjunctival swabs were collected and screened for CDV. Any clinical signs related to CDV were assessed and recorded in the physical assessment form. About 1-2 mL blood sample was obtained via jugular, cephalic or saphenous venipuncture and it was stored in a plain tube, which was then centrifuged to obtain the serum sample. Nasal and conjunctival swabs were collected using viral transport media (VTM) tube as pooled samples, which were then stored in an ice box upon collection. The nasal and conjunctival swabs were then used for molecular assay and the serum was used for serology assay. The samples were labeled and the VTM tubes were kept inside an icebox to preserve the viability of the sample before transportation to the Virology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

3.3 Molecular Assay by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.3.1 Viral RNA Isolation

NucleoSpin© RNA Virus kit by Macherey-Nagel was used to extract the RNA virus from the pooled sample of nasal and conjunctival swabs. First, lysis of virus was done where 600 µL Buffer RAV1 containing carrier RNA was added into 150 µL of the sample. It was then mixed well and incubated at 70°C for 5 minutes. A total of 600 µL of ethanol was added to adjust the binding conditions and mixed well by vortexing. To bind the viral RNA, all the lysed sample was transferred into the NucleoSpin© RNA Virus Column in Collection Tubes, and then it was centrifuged for 1 minute at 8500 rpm. The next step was to remove contaminants and PCR inhibitors by washing and drying the silica membranes. The first wash which was the 500 µL of Buffer RAW was added into the virus column, and centrifuged for 1 minute at 8500 rpm of which the flowthrough was discarded. Then, the second wash of 600 µL of Buffer RAV3 was added into the virus column and centrifuged for 1 minute at 8500 rpm. Flowthrough with collection tube was discarded. Then, with a new collection tube, the 3rd wash was done where 200 µL of Buffer RAV3 was added and centrifuged for 2-5 minutes at 9900 rpm to remove ethanolic Buffer RAV3 completely. The last step is to elute the viral RNA. The virus columns were transferred into a sterile 1.5mL microcentrifuge tube where 50 µL RNase-free H_2O was added. It was then incubated for 1-2 minutes before being centrifuged at 1 minute for 9900 rpm.

3.3.2 cDNA Synthesis

SensiFAST™ cDNA Synthesis Kit by BioLine was used for first-strand cDNA synthesis. First, the master mix was prepared on ice. For one reaction, 4 µL of 5x TransAmp Buffer, 1 µL of Reverse Transcriptase, and 5 µL of RNase free-water were used. For this project, the optimum level of total RNA to be used is 10 µL. Next, 10 µL of the master mix was aliquoted into a microcentrifuge tube, and 10 µL of sample was then added, making the total amount of the final product to be 20 µL. To proceed, the thermocycler had been set as such: 25 °C for 10 minutes (primer annealing); 42 °C for 15 minutes (reverse transcription); 85 °C for 5 minutes (inactivation); and 4 °C hold.

3.3.3 Amplification

MyTaq™ Red Mix by BioLine was used for the PCR. The PCR set-up is as such (per one reaction): 4 µL of DNA template, 12.5 µL of MyTaq Red Mix, 0.5 µL forward (F) primer, 0.5 µL (R) reverse primer, and 7.5 µL deionized water. The primer pair was diluted into 10 µM, and the haemagglutinin (H) gene was targeted.

Table 1: Primer pair sequence used for PCR detection of *Morbillivirus* from a pooled sample of nasal and conjunctival swabs of dogs.

	Primer sequence	Target gene
Forward primer	5'-TTCATCCAAGCTGTCCTTAGTG-3'	H gene
Reverse primer	5'-AAATCAGAGGCCGTACATCAC-3'	H gene

First, the master mix was prepared on ice; the mix is as stated excluding the DNA template. Once it had been prepared, 21 µL of the mix was aliquoted into a microcentrifuge tube, where then 4 µL of DNA template was added. The total amount of the final product was 25 µL. For the positive control, DNA from an animal positive of having CDV was used, while for the negative control, deionized water was used. After this, the solution was ready for thermal cycling by using C1000 Touch™ Thermal Cycler (Bio-Rad, MyCycler USA). The PCR conditions are 95°C for 1 minute (initial denaturation); 94°C for 1 minute (denaturation); 49°C for 1 minute (annealing); 72°C for 1 minute (extension); 72°C for 3 minutes (final extension); and 12°C (hold). The denaturation, annealing, and extension would have to be repeated to a total of 35x.

3.3.4 Gel Electrophoresis and Gel Imaging

Gel electrophoresis was used for the qualitative result. A 1.5% agarose mixture consisted of agarose powder mixed with TAE buffer was used. Then, RedSafe™ by iNtRON Biotechnology was pipetted into the mixture to stain the nucleic acid. Once

ready, the electrophoresis machine parameters were set as stated: voltage (85V), current (180mA), and time (45mins). To visualize the DNA bands, GeneSnap software (version 7.12, Syngene, United Kingdom) was used. The gel was viewed under an ultraviolet transilluminator which showed the stained DNA as bright bands.

3.4 Serological Assay

The Vcheck CDV Ab Test Kit by BioNote was the chromatographic immunoassay used for the detection of CDV antibodies inside the blood serum obtained. It was used to detect specific immunoglobulin G (IgG) against CDV antigens. The kit used highly selective antigens as both the capture and detector. Positive sample would be indicated by the purple test line while validity of the test was confirmed by the appearance of a control line.

First, 5 μL of serum was added into the assay diluent tube and mixed well by pipetting. Then, with the Vcheck Analyzer turned on and set to "Standard Test", the test device was then removed from the foil pouch, and inserted into the V200 analyzer once the display screen prompt showed up. With a 100 μL pipette, the sample with diluent was mixed again, and 100 μL of the mixed sample was added into the sample hole, and the start option was pressed to initiate testing. After 10 minutes, the result was displayed automatically, and the outcome was recorded. The result interpretations were as stated: negative will have below 1:4 as VN titer. A positive result can be interpreted as low titer 1-2 [1:8-1:16 as VN titer]; Medium titer 3-3.5 [1:32-1:48 as VN titer], indicative of a good

immune status; High titer 4-6 [above 1:64 as VN titer], indicative of a good immune status.

3.5 Statistical Analysis

The detection of CDV was calculated as the proportion of infected animals to the total population analyzed. Data were reported in percentages. Data were also tabulated into SPSS software. $P < 0.05$ will be considered significant.

4.0 RESULTS

From the 5 sampled shelter with a total of 50 dogs, RT-PCR results revealed 50 out of 50 (100%) sampled shelter dogs were negative for CDV (p=<0.001) (Figure 1-5).

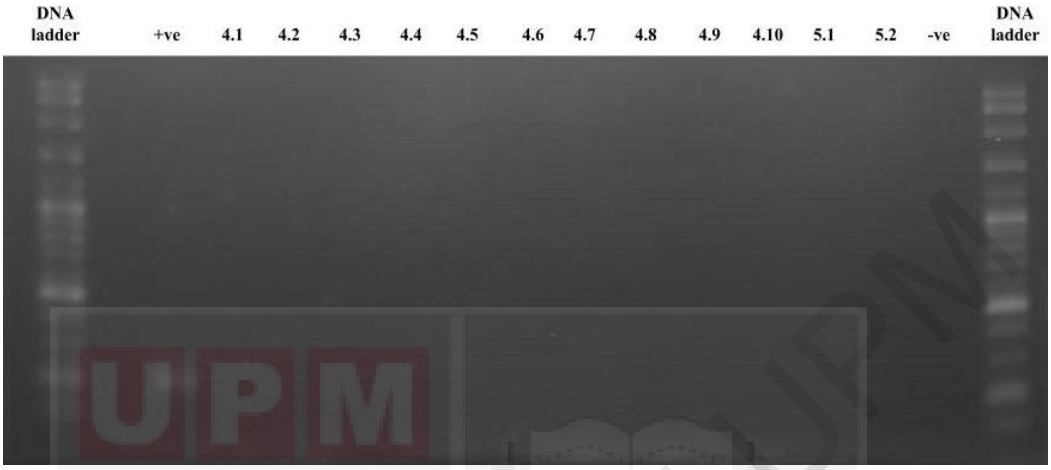

Figure 1: RT-PCR results of pooled nasal and conjunctival swabs of the first 12 dog samples (4.1-5.2) targeting H gene with 200 bp product. All 12 samples were negative for CDV. Lane 1: DNA ladder (100 bp ladder); lane 2: positive control (+ve); lane 3-14: samples; lane 15: negative control (-ve); final lane: DNA ladder (100 bp ladder).

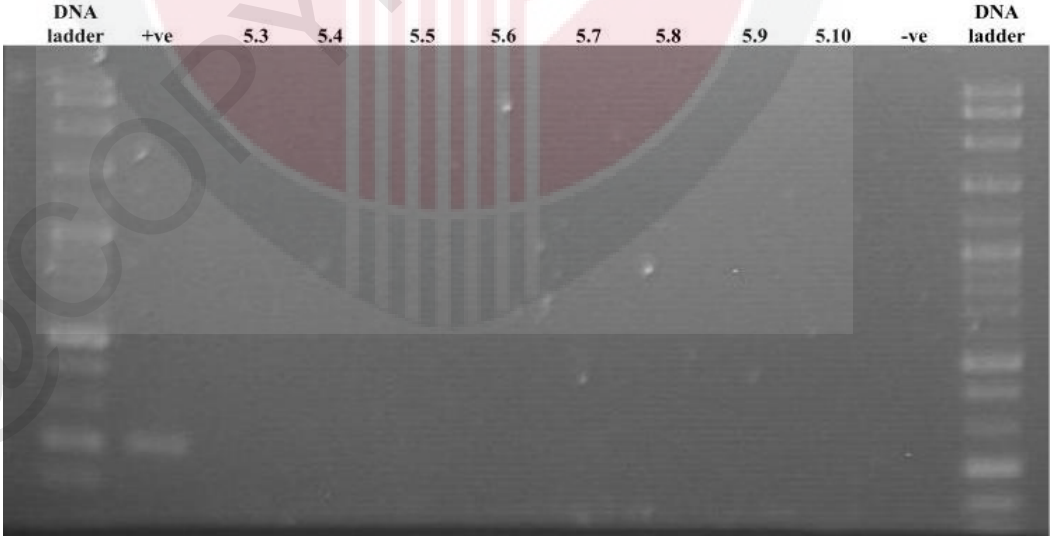

Figure 2: RT-PCR results of pooled nasal and conjunctival swabs of the next 8 samples (5.3-5.10) targeting H gene with 200 bp product. All 8 samples were negative for CDV. Lane 1: DNA ladder (100 bp ladder); lane 2: positive control (+ve); lane 3-10: samples; lane 11: negative control (-ve); final lane: DNA ladder (100 bp ladder).

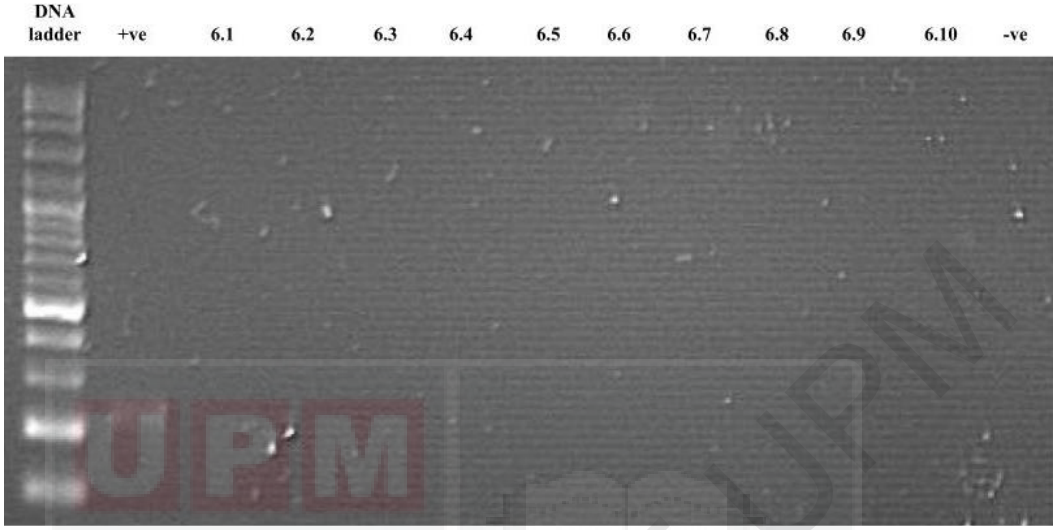

Figure 3: RT-PCR results of pooled nasal and conjunctival swabs of 10 dog samples (6.1-6.10) targeting H gene with 200 bp product. All 10 samples were negative for CDV. Lane 1: DNA ladder (100 bp product); lane 2: positive control (+ve); lane 3-12: samples; final lane: negative control (-ve).

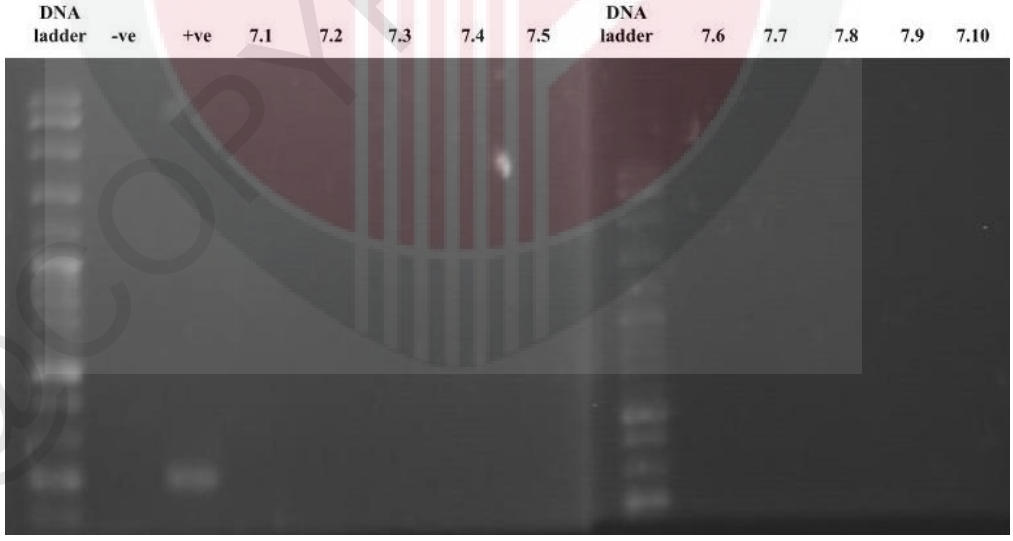

Figure 4: RT-PCR results of pooled nasal and conjunctival swabs of 10 dog samples (7.1-7.10) targeting H gene with 200 bp product. All 10 samples were negative for CDV. Lane 1: DNA ladder (100 bp product); lane 2: negative control (-ve); lane 3: positive control (+ve); lane 4-8: samples; lane 9: DNA ladder (100 bp product); lane 10-14: samples.

Figure 5: RT-PCR results of pooled nasal and conjunctival swabs of 10 dog samples (8.1-8.10) targeting H gene (200 bp product). All 10 samples were negative for CDV. Lane 1: DNA ladder (100 bp ladder); lane 2: positive control (+ve); lane 3-12: samples; final lane: negative control (-ve).

For the immunochromatographic assay (CI) as shown in Table 2, the majority of the shelter dogs were positive for the presence of specific IgG against CDV. Out of 50 dogs, 40/50 (80%) dogs have the IgG, while the other 10 (20%) dogs have zero antibodies (p=<0.001). As for the positive result, it is further divided into different antibody titers which were the low titer, medium titer, and high titer. A total of 10 out of 50 (20%) dogs was negative for the presence of IgG, indicative of having no protective levels of circulating IgG while 22/50 (44%) of the dog have high titers, 17/50 (34%) has medium titer and 1/50 (2%) has low titer.

Table 2: Proportion of antibody titers for 50 sampled dogs.

	POSITIVE			**NEGATIVE**
TITERS	**LOW**	**MEDIUM**	**HIGH**	**ZERO**
SHELTER A	0	2	3	5
SHELTER B	0	2	8	0
SHELTER C	0	4	4	2
SHELTER D	1	5	3	1
SHELTER E	0	4	4	2
TOTAL	1	17	22	10
PERCENTAGES	2%	34%	44%	20%

In addition, only two shelters achieved herd immunities which were shelter B and shelter D, with 100% and 90% respectively. While another two shelters (shelter C and E)

with 80% of its population had the presence of IgG against CDV. The lowest was shelter A with only 50% of its population were protected.

Table 3: The shelters and the proportion of dogs with the presence and absence of immunoglobulin G (IgG) that contributes to herd immunity.

	No. of dogs with presence of IgG	**%**	***No. of dogs without presence of IgG***	**%**
Shelter A	5	50	5	50
Shelter B	10	100	0	0
Shelter C	8	80	2	20
Shelter D	9	90	1	10
Shelter E	8	80	2	20

5.0 DISCUSSION

In Malaysia, the prevalence of canine distemper virus (CDV) is unknown and this study might be helpful to provide insight into the current status of CDV in Klang Valley, Malaysia especially in shelter settings where animals coming from different sources were mixed together within the same environment.

All these 5 shelters where the samples were collected had vaccination history; however, it was not regularly maintained. So, there is the knowledge that these animals had some sort of protection, though the degree of immunity was unknown at the time. In addition, during the time of sampling, there were no obvious clinical signs and all the animals appeared healthy. Hence, it further corroborates the RT-PCR results where all 50 dogs (100%) were negative, indicative of no current infection at the time of sampling. This could be contributed to the presence of antibody due to natural infection or vaccination which confer protective immunity to these dogs. It can also be contributed due to the absence of CDV reservoirs inside the shelter population itself.

Pertaining to the serological result, 20% of sampled dogs have negative antibody titers, and this can be interpreted based on age and prior vaccination history. In terms of age; puppies or young dogs with zero antibodies can be due to no previous natural infection, never being vaccinated, or due to failure of vaccination. According to Greene and Appel (2006), newborn pups, given the opportunity to ingest colostrum, will acquire an initial CDV serum neutralizing antibody (SN) titer of up to 77 percent of the serum titer of the dam. If the titer of passively acquired SN CDV antibodies in the pup is too

high, these can interfere with active immunization, whereas if the titer is too low, the pup could be susceptible to CDV infection (Pardo et al., 2007). Besides maternal-derived antibodies (MDA), other cause of vaccination failure could be due to genetic non-responder dogs (Decaro et al., 2020). Non-responder dogs are dogs that failed to develop antibody even after repeated vaccination. According to WSAVA (2016), the proportion of genetic non-responders in canine population for CDV is 1 in every 5,000 dogs. Other than that, factors such as improper storage and handling of vaccine can also cause vaccination failure. Vaccines typically have optimum storage temperature between 2-8°C, and any handling outside the said temperature, there should be continuation of the cold chain. Other than that, the usage of disinfectant before administering the vaccine can cause inactivation of MLV product.

Meanwhile, for negative test result in a protected dog, it could be due to declining antibody levels in a previously vaccinated adult dog that is not revaccinated (or naturally exposed), and it may fall to negative levels. This is why booster is vital in maintaining some degree of immunity. It is also where memory cells (B-lymphocytes) can be helpful as they can persist longer than antibodies (Palm & Henry, 2019). Hence, exposure to a field virus is expected to rapidly boost the dog's antibody response and protect it.

For 80% of the dogs with positive antibody titer, these dogs were likely a result of prior immunization combined with any natural exposure and suggest that these dogs had adequate immunologic memory; the mechanism that provides animals with protection from clinical disease during natural viral challenge. So, in unvaccinated healthy dogs, it

suggests recent infection, prior exposure to, and recovery from infection. Meanwhile, for the 22/50 (44%) dogs with high (>1:64 as VN titer) or the 17/50 (34%) dogs with medium (1:32~1:48 as VN titer) antibody titer, these ranges of titer suggested recent vaccination (Taguchi et al., 2011). McCaw et al. (1998), defined protective titers to CDV as ≥ 1:96, while Twark and Doods (2000), defined an adequate antibody response as a titer ≥ 1:32, as determined by serum neutralization testing. Hence, these high and medium antibody titers correlate well with protective immunity and can provide reassurance that there will be adequate antibody response to protect the dog. For 1/50 (2%) of dogs with low titer (1:8~1:16 as VN titer), there were some degree of protectiveness, but the dogs were due for next booster or revaccination as the level of circulating IgG was extremely low. This would ensure that adequate antibody response towards CDV could be achieved and to avoid the disease altogether via herd immunity. In addition to that, in a study by Larson and Schultz (2006), they stated that one dose of either MLV-CDV vaccine or rCDV vaccine that is given within minutes to hours would be to protects pups from death, and after 7 days, the vaccine provides complete protection against CDV.

To achieve herd immunity in a population, ≥90% of dogs must have the presence of circulating IgG against CDV (Rikula et al., 2007). In a study by Bergmann (2022), he stated that at least 90–95% of dogs should be immune to CDV in order to prevent outbreaks. In this study, only two shelters achieved herd immunities which were shelter B and shelter D, with 100% and 90% respectively. For the other remaining shelters, it is recommended for them to start again on the vaccination protocol as to further strengthen the protection against CDV, and to avoid outbreak.

6.0 CONCLUSION

In conclusion, the study revealed that most of these individual shelter dogs were fairly protected due to the presence of IgG against CDV, though there was varying degree of immunity. Meanwhile, the other shelter dogs that do not have the IgG, they are dependent on the presence of herd immunity, which was when ≥ 90% of its population had circulating IgG. Lastly, from all the five shelters, there were no current circulating CDV infection.

7.0 RECOMMENDATION

As a recommendation for future study, it is encouraged to increase the sample size, with more dogs to be sampled from each shelter. Also, to do sampling from a wider coverage towards all parts of Klang Valley, Malaysia. Other than that, it is recommended to have accurate vaccination history for each dog to differentiate whether the presence of IgG against CDV is due to natural infection or vaccination; this is to produce more reliable results pertaining to its prevalence.

It is highly recommended for these shelters to have routine vaccination as vaccination protocol helps to reduce disease transmission, not only for CDV but for other viruses as well. Vaccination also provides herd immunity and directly contributes to lower mortality. Lastly, each shelter should have a well-managed system and environment, especially with a proper quarantine area. This is to ensure any new dogs that come in do not carry any diseases that could threaten the shelter dogs' population.

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9.0 APPENDICES

Table 4: Raw data of individual dogs.

SHELTER	PATIENT ID	BODY CONDITION	RESPIRATORY SYMPTOM	OCULAR ABNORMALITIES/ DISCHARGE	NEUROLOGY SYMPTOM	RT-PCR RESULT	SEROLOGY RESULT
A	4.1	Normal	No	No	No	Neg	High titer(4), VN 1:64
	4.2	Normal	No	No	No	Neg	High titer(6), VN >= 1:512
	4.3	Normal	No	No	No	Neg	High titer(5), VN 1:128
	4.4	Normal	No	Yes	No	Neg	Medium titer(3.5), VN 1:48
	4.5	Skinny	No	Yes (discharge)	No	Neg	Medium titer(3.5), VN 1:48
	4.6	Normal	No	Yes (discharge)	No	Neg	Negative(0), VN <= 1:4
	4.7	Normal	No	Yes (discharge)	No	Neg	Negative(0), VN <= 1:4
	4.8	Normal	No	No	No	Neg	Negative(0), VN <= 1:4
	4.9	Normal	No	No	No	Neg	Negative(0), VN <= 1:4
	4.1	Normal	No	No	No	Neg	Negative(0), VN <= 1:4
B	5.1	Skinny	No	Yes	No	Neg	High titer(6), VN >= 1:512
	5.2	Skinny	No	Yes	No	Neg	High titer(5.5), VN 1:256
	5.3	Skinny	No	Yes	No	Neg	High titer(5.5), VN 1:256
	5.4	Skinny	No	No	No	Neg	High titer(4), VN 1:64
	5.5	Normal	No	No	No	Neg	High titer(4), VN 1:64

	5.	Normal	No	No	No	Neg	High titer(4.5), VN 1:96
	5.7	Normal	No	No	No	Neg	High titer(4.5), VN 1:96
	5.8	Skinny	No	No	No	Neg	Medium titer(3.5), VN 1:48
	5.9	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	5.1	Normal	No	No	No	Neg	High titer(4.5), VN 1:96
	6.1	Normal	No	No	No	Neg	High titer(6), VN >= 1:512
	6.2	Normal	No	No	No	Neg	Medium titer(3), VN 1:32
	6.3	Normal	No	No	No	Neg	High titer(6), VN >= 1:512
	6.4	Normal	No	No	No	Neg	Negative(0), VN <= 1:4
C	6.5	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	6.6	Normal	No	No	No	Neg	Medium titer(3), VN 1:32
	6.7	Normal	No	Yes (discharge)	No	Neg	Negative(0), VN <= 1:4
	6.8	Normal	No	No	No	Neg	High titer(5.5), VN 1:256
	6.9	Skinny	No	No	No	Neg	Medium titer(3.5), VN 1:48
	6.1	Normal	No	No	No	Neg	High titer(4.5), VN 1:96
	7.1	Normal	No	No	No	Neg	Low titer(2), VN 1:16
D	7.2	Normal	No	No	No	Neg	High titer(5.5), VN 1:256
	7.3	Normal	No	No	No	Neg	Medium titer(3), VN 1:32

	7.4	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	7.5	Normal	No	No	No	Neg	High titer(6), VN >= 1:512
	7.6	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	7.7	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	7.8	Normal	No	No	No	Neg	High titer(4), VN 1:64
	7.9	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	7.1	Normal	No	No	No	Neg	Negative(0), VN <= 1:4
E	8.1	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	8.2	Normal	No	No	No	Neg	High titer(4), VN 1:64
	8.3	Obese	No	Yes	No	Neg	High titer(4), VN 1:64
	8.4	Normal	No	No	No	Neg	Medium titer(3), VN 1:32
	8.5	Normal	No	No	No	Neg	High titer(5), VN 1:128
	8.6	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	8.7	Normal	No	No	No	Neg	High titer(4), VN 1:64
	8.8	Normal	No	No	No	Neg	Medium titer(3.5), VN 1:48
	8.9	Normal	No	No	No	Neg	Negative(0), VN <= 1:4
	8.1	Normal	No	No	No	Neg	Negative(0), VN <= 1:4