UNIVERSITI PUTRA MALAYSIA

BIOSURVEILLANCE OF CANINE DISTEMPER VIRUS (CDV) IN DOMESTIC DOGS AND SMALL WILD MAMMALS IN DUNGUN, TERENGGANU AND JERANTUT, PAHANG

ALIA YOHANIS BINTI MOHD AZAM

Ip
FPV 2022 95

BIOSURVEILLANCE OF CANINE DISTEMPER VIRUS (CDV) IN DOMESTIC DOGS AND SMALL WILD MAMMALS IN DUNGUN, TERENGGANU AND JERANTUT, PAHANG

ALIA YOHANIS BINTI MOHD AZAM

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia
Serdang, Selangor Darul Ehsan.

2023

It is hereby certified that we have read this project paper entitled "Biosurveillance of Canine Distemper Virus (CDV) in Domestic Dogs and Small Wild Mammals in Dungun, Terengganu and Jerantut, Pahang" by Alia Yohanis binti Mohd Azam and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

_____

DR. FARINA MUSTAFFA KAMAL
DVM (UPM), PHD (UC DAVIS)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

_____

DR. KHOR KUAN HUA
DVM (UPM), PHD (Queensland)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-supervisor)

_____

DR. TENGKU RINALFI PUTRA TENGKU AZIZAN
Baceelor of Applied Science (MUST), PHD (Canterbury)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-supervisor)

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the almighty God who had given me the chance to study and given me all the knowledge that has gotten me this far.

Next, I would like to express my gratitude to my supervisor, Dr Farina binti Mustaffa Kamal for the guidance, advice and moral support for the completion of this project paper. I would also like to acknowledge both my co-supervisors, Dr. Khor Kuan Hua and Dr. Tengku Rinalfi Putra Tengku Azizan for their guidance and patience throughout the sampling period.

Special thanks to Faris Sadali, the master student who had been very helpful and patient while spending his time to assist us during this project. Not to forget Encik Azman, for willing to spend time to provide technical assistance and guidance throughout the process of completing this project paper.

A big thanks also to my project partner, Nur Eizzah for helping, guiding and spending her time for this project. Lastly, I bid my gratitude to my fellow classmates and family members for the support, guidance and moral support given throughout the process of completing this project paper.

CONTENTS

		Page
TITLE		i
CERTIFICATION		ii
ACKNOWLEDGEMENTS		iii
CONTENTS		iv
LIST OF TABLES		vi
LIST OF FIGURES		vi
ABSTRAK		vii
ABSTRACT		ix
1.0	**INTRODUCTION**	1
2.0	**LITERATURE REVIEW**	3
	2.1 Morbillivirus genome organization and proteins	3
	2.2 Epidemiology of Canine Distemper Virus (CDV)	4
	2.2.1 Global perspective	4
	2.2.2 Canine Distemper Virus in Malaysia	7
	2.3 Molecular assay for the detection of Canine Distemper Virus	7
	2.4 Serological assay for the detection of Canine Distemper Virus	8
	2.5 Prevention methods	8
	2.5.1 Controlling of Canine Distemper Virus in dog population	8
	2.5.2 Prevention of spillover between species	9
	2.5.3 Controlling of Canine Distemper Virus in tigers	10
3.0	**MATERIALS AND METHODS**	12

3.1 Population and sampling method……. 12

3.2 Canine Distemper Virus Antigen Test Kit (RTK) ……... 12

3.3 Reverse-transcriptase Polymerase Chain Reaction (RT-PCR) test……... 13

3.3.1 RNA extraction and reverse-transcription (RT)… 13

3.3.2 Amplification, cloning and sequencing of H gene. 13

3.4 Commercial Immunochromatographic Assay (ICA)……... 14

4.0 RESULTS ……... 15

5.0 DISCUSSION…… 20

6.0 CONCLUSION……. 22

REFERENCES …….. 24

APPENDIX…… 27

LIST OF TABLES **Page**

Table 1 : Summary of sampling done ……..... 15

Table 2 : Immunochromatographic assay result …… 19

Table 3 : IgG titers in samples with positive ICA result…… 19

LIST OF FIGURES **Page**

Figure 1: RT-PCR result for the samples with the ID DG12 to DG16 taken from domestic dogs in Dungun,Terengganu…… 16

Figure 2: RT-PCR result for the samples with the ID JR1 to JR5 taken from domestic dogs in Jerantut, Pahang……..... 17

Figure 3: RT-PCR result for the samples with the ID JR6 to JR17 taken from domestic dogs in Jerantut, Pahang……... 18

Figure 4: RT-PCR result for the samples with the ID WDG5 to WDG12 taken from small wild mammals in Dungun, Terengganu…… 18

ABSTRAK

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

KAJIAN MENGENAI STATUS VIRUS *CANINE DISTEMPER* (CDV) DALAM ANJING DOMESTIK DAN MAMMALIA KECIL LIAR DI DUNGUN, TERENGGANU DAN JERANTUT, PAHANG

Oleh

Alia Yohanis binti Mohd Azam
2023

Penyelia: Dr. Farina Mustaffa Kamal

Virus *Canine Distemper* (CDV), sejenis *Morbillivirus*, umumnya menyebabkan masalah pernafasan, gastrousus dan saraf dalam kalangan anjing. Kajian terkini menunjukkan bahawa virus ini boleh menjangkiti bilangan hos yang ramai, terutamanya di dalam kalangan karnivor dan kucing besar. Seekor harimau Malaya ditemui mati selepas dijangkiti CDV di Terengganu pada Julai 2019 yang menimbulkan perhatian terhadap punca jangkitan tersebut di kalangan harimau. Kajian ini dijalankan untuk mengesan kewujudan antigen dan antibodi terhadap CDV sebagai bio-pengawasan di dalam kalangan anjing domestik dan mammalia kecil di sekeliling habitat harimau, iaitu di Dungun, Terengganu dan Jerantut, Pahang di mana tempat ini merupakan habitat biasa untuk harimau Malaya. Sampel diperoleh daripada 22 ekor anjing domestik dan 8 ekor mamalia kecil liar iaitu serum, calitan konjunktiva dan hidung. Serum diuji untuk antibodi terhadap CDV menggunakan imunoasai

kromatografi (ICA) dan menggunakan kit ujian segera CDV untuk pengesanan antigen. Sampel calita konjunktiva dan hidung diuji menggunakan transkripsi membalik reaksi rantai polimerase (RT-PCR) untuk pengesanan antigen. Adalah diperhatikan 100% sampel adalah negatif untuk pengesanan antigen. Namun, 64% (14/22) sampel serum anjing diuji positif terhadap antibodi IgG di mana di dalam kalangan sampel positif, 42.85% (6/14) mempunyai titer IgG rendah dan 57.15% (8/14) mempunyai titer IgG sederhana. Kajian ini menunjukkan ada kemungkinan bahawa anjing merupakan punca jangkitan CDV terhadap harimau, justeru meningkatkan risiko jangkitan kepada harimau pada masa hadapan. Namun, oleh kerana tiada sampel positif bagi RT-PCR untuk menentukan dinamik jangkitan, peranan mamalia kecil sebagai punca jangkitan CDV tidak boleh dikecualikan.

Kata kunci: *anjing domestik, mamalia kecil liar, virus Canine Distemper (CDV), RT-PCR, immunoassay kromatografi*

ABSTRACT

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

BIOSURVEILLANCE OF CANINE DISTEMPER VIRUS (CDV) IN DOMESTIC DOGS AND SMALL WILD MAMMALS IN DUNGUN, TERENGGANU AND JERANTUT, PAHANG

by

Alia Yohanis binti Mohd Azam

2023

Supervisor: Dr. Farina binti Mustaffa Kamal

Canine distemper virus (CDV), a *Morbillivirus*, generally causes respiratory, gastrointestinal and neurological disease in canids. Recent studies showed that CDV has an expanding range of hosts, especially carnivores and large felids. In July 2019, one Malayan tiger was found dead from CDV in Terengganu which raises the concern on the source of infections among these tigers. This study aims to detect the presence of antigen and antibody against CDV as a bio-surveillance in domestic dogs and small wild mammals in Dungun, Terengganu and Jerantut, Pahang where both of these places are common habitats for Malayan tiger. A total of 22 domestic dogs and 8 small wild mammals were sampled for serum, conjunctival and nasal swabs. The sera were tested for CDV antibodies using chromatographic immunoassay (ICA) and detection of antigen using CDV rapid test kit, while the swabs were tested for viral RNA antigen using reverse transcription polymerase

chain reaction (RT-PCR). It was observed that 100% of the samples tested negative for antigen detection. However, 64% (14/22) of the serum samples from domestic dogs were tested positive for IgG against CDV using chromatographic immunoassay (ICA). Among those tested positive, 42.85% (6/14) have low IgG titer and 57.15% (8/14) have medium IgG titer. This study revealed that there is a possibility of dogs serving as the main disease reservoir near tiger habitats suggesting high risk of infection to the tigers in the future. However, as there were no positive RT-PCR sample that could be further characterized to determine the transmission dynamic, the role of wild mammals as CDV reservoir could not be excluded.

Keywords: *domestic dogs, small wild mammal, canine distemper virus, RT-PCR, immunochromatographic assay*

1.0 INTRODUCTION

Canine Distemper Virus (CDV) is an enveloped, single-stranded RNA virus that belongs to the *Paramyxoviridae* family. It affects a large variety of mammals including the canidae, mustelidae, procyonidae, viveridae, ailuridae, ursidae, elephantidae, primates and large felidae with domestic dogs as the reservoir (Beineke, 2015). The virus spreads via aerosol droplet secretion from affected animals and replicates in the lymphatic tissue of respiratory tract, which will then cause viraemia. As a result, the virus affects various body systems, including the gastrointestinal, respiratory and central nervous system.

Recently, there have been two cases of CDV reported in Malayan Tigers (*Panthera tigris tigris jacksoni)* since July 2019 (Borah, 2021). However, the source of infection for these cases are still unknown to this date. There have also been a positive case reported among the three sightings of Malayan Tiger in Gua Musang recently. It is a possibility that it is spread by the domestic dogs living in the outskirts of the forest. Research also has shown that this virus can also be spread by other carnivores, thus making it possible to gain infection from other species of predators in the forest.

The reservoir for CDV is most likely to come from canine population near the tiger habitat such as the stray dogs and wild dogs that roams around at the edge of the jungle. However, there has been evidence on the transmission of CDV from other carnivores such as hyenas, jackals and foxes as seen in the outbreak in lions in the

Serengeti National Park (Roelke-Parker et.al 1996). This shows that dogs may not be the primary source of infection as other carnivores such as civets can also transmit this disease to the Malayan tiger population. Knowing the reservoirs for CDV are very important as the information can be used to manage the risk of this disease in tiger population. The aim for this study is to find the current status of CDV in dogs and wildlife surrounding the tiger habitat in order to distinguish the reservoir of CDV among Malayan tiger population in Malaysia.

Currently, there are not many research that have been done regarding the occurrence and prevalence of CDV in dog and other species of animals in Malaysia, therefore the exact number of cases are still unknown. Thus, the purpose of this research is as a surveillance of the current and previous infection of CDV among domestic dog population and small wild mammals living near tiger habitats. This will then encourage prevention measures such vaccination programme against CDV to be constructed and implemented in order to control the disease in the future. The hypothesis for this study is there is no detection of Canine Distemper Virus (CDV) antigen and antibody in domestic dogs and small wild mammals in Dungun, Terengganu and Jerantut, Pahang.

2.0 LITERATURE REVIEW

2.1 Morbillivirus genome organisation and proteins

Canine distemper virus comes from *Morbillivirus* genus under the family *Paramyxoviridae*. The genus *Morbillivirus* are extremely infectious virus in which they usually spread via respiratory route. Sourimant and Plemper (2016) state that there are many viruses that are closely related to each other under the genus *Morbillivirus* such as measles virus (MeV), canine distemper virus (CDV), peste de petits ruminant virus (PPRV), feline morbillivirus (FeMV), cetacean morbillivirus (CeMV) and phocine distemper virus (PDV).

Morbilliviruses are single stranded negative sense RNA that are found inside plasma membrane-derived lipid envelope. According to Sourimant and Plemper (2016), they lead to the expression of eight proteins, where there are two non-structural protein V and C and six structural proteins which are nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin (H) and large protein (L), all according to the genome organization from the inside to the surface of the virus. Among morbillivirus, canine distemper virus (CDV) contains two glycoproteins, H and F, in which both of them are the major target for the host immune system. Hemagglutinin (H) function is to attach the virus to the host cell while the fusion protein (F) mediates the fusion between virus and the host cell (Diallo, 1990). Canine distemper virus (CDV) is usually detected using the H or F gene fragments as they are different among the 14 genetic lineages of CDV, in which F protein shows 4% variability while H gene shows 10% variability as other strains shows different structural proteins. They are also different from other virus from the same family in which it is really useful in differentiating the CDV from other *Paramyxoviruses* (von Messling, 2001).

2.2 Epidemiology of Canine Distemper Virus

2.2.1 Global perspective

2.2.1.1 CDV in dogs all over the world

Canine Distemper Virus (CDV) affects a wide range of animals especially carnivores. It has been known to the world as early as the 17th century. Bruyette (2020) states that canine distemper is a fatal disease of dogs with a worldwide distribution with a mortality of around 50 percent. Nowadays, the virus was detected in dogs in almost all regions of the world including American, Asian, African and European continent. The frequency of laboratory positive animals is found to be high in China, India, Brazil and Uganda (Costa VGD et. Al, 2019). A study in India shown that 86% of the tested dogs carries CDV antibodies showing a current or previous infection.

2.2.1.2 Symptoms in dogs

The symptoms that are usually shown are systemic signs such as transient fever in the early stage of the infection in which is followed by loss of appetite, depression, ocular and nasal discharge and tonsilitis after the virus had spread in the body (Martella, 2008). Martella also states that a weak immune response causes the virus to reach epithelial tissues and the central nervous system in which is the reason why the neurological signs such as circling and head tilt are delayed and hyperkeratosis can also be observed after all other clinical signs disappear.

2.2.1.2.1 Pathogenicity

Depending on the strain, age of animal and immunity status, the incubation period of CDV ranges between one to four week (Beineke, 2009). The manifestation of CDV also varies from no clinical signs to severe disease with 50% mortality. The virus enters the host primarily via inhalation of aerosol droplets containing airborne virus as the virus usually

sheds in the oro-nasal secretion. Then, it replicates in the tissues of the respiratory tract where they will trigger the reaction from macrophages in the respiratory epithelium and as a result, they will pick up the virus and propagate it. The first viraemic phase starts as the virus is disseminated systemically via the immune system. As a result, there will be a generalized infections in all lymphoid tissues giving rise to the first clinical signs, which are lethargy, dehydration, anorexia, weight loss and after 3-6 days, transient fever can also be observed.

The second viraemia will start days later as they attack the parenchyma and epithelial cells throughout the body such as in the respiratory, gastrointestinal, urinary, endocrine, lymphoid and central nervous system. During this time, high fever is usually observed as the clinical symptoms. Some infected animals may even have delayed progression of the disease where the disease started with a very subtle early clinical signs, but will show central nervous system (CNS) disturbances as a result of viral persistence in the CNS. Usually, dogs that shows nervous signs will die or have residual signs such as persistent myoclonus if recover.

2.2.1.2.2 Chronic neurological manifestation of CDV

In rare cases, CDV can cause chronic infection in which is characterized by progressive panencephalitis in mature dogs, or commonly known as Old Dog Encephalitis (Vandevelde, 1980). This neurological disease usually occurs in dogs that suffers from systemic CDV infection and eventually recovers from it. The neurological signs may also appear without other common clinical signs.

Canine distemper virus invades the central nervous system around 10 to 14 days post infection (Tripold, 1992) in

which they enter the brain parenchyma either via the cerebrospinal fluid pathway or across the blood-brain barrier. In the central nervous system, the virus replicates in the neurons and glial cells. As a result, this will cause demyelination of the grey and white matter at the predilection site of viral replication, such as the cerebellum, optic system and spinal cord. The chronic character of the disease may cause lack of immune response and inflammation during the chronic phase may cause more damage to the white matter (Vandevelde, 1980). The virulence strain and immunity status of the animal play a role in determining the outcome of the disease. Dogs who are able to response early during the infection will usually recover with little or no clinical sign, while dogs with delayed immune response are prone to develop chronic neurological disease.

2.2.1.3 Canine Distemper Virus (CDV) in other species

The first detection in wild animals were done as early as 1978 in which it affects silver-backed jackals and bat eared foxes according to Moehlman (1983) and in African wild dogs in 1991 according to Alexander (1994). However, the first major outbreaks among wildlife were recorded in 1994 where the virus affects the Serengeti lion population where 85% of all the lion population where positive for CDV antibodies in serological surveillance (Roelke-Parker et.al 1996). In 2013, CDV was known to cause at least 4 deaths among tiger population in India.

2.2.1.4 Symptoms in other species

According to Nagao (2011), tigers positive with CDV exhibit symptoms such as diarrhoea, vomiting, abdominal distress and respiratory distress. Some of them died soon after expressing the symptoms. Nagao (2011) also states that racoon dogs show symptoms such as pneumonia and gastroenteritis before many of them died from

the infection. Roelke-Parker (1996) states that the CDV in lions are fatal neurological disease characterized by grand mal seizures and myoclonus. Lions who had died from CDV also suffered from encephalitis and pneumonia.

2.2.2 Canine Distemper Virus in Malaysia

Since July 2019, one tiger was found dead from CDV in Terengganu, Malaysia (Borah, 2021). This is a concern to the tiger population in the wild as there are only less than 150 individuals left as of 2022 according to WWF. CDV is high likely to cause a more rapid decrease in the population of wild Malayan tiger in Malaysia as it has a high mortality rate. The question that needs answers are where does the infection on the tigers come from.

2.4 Molecular assay for the detection of Canine Distemper Virus

Samples that are usually taken from live animals for molecular detection of CDV are nasal and ocular swabs, serum or whole blood samples and cerebrospinal fluid (CSF). Tissue specimens can also be taken from the central nervous system (CNS), respiratory tract, spleen, urinary and gastrointestinal tract at necropsy. The concept for molecular assay is the detection of antigen in the body of animal showing current infection. The most common molecular assay done for the detection of CDV is reverse-transcription polymerase chain reaction (RT-PCR). Other molecular methods that have been used are ferret inoculation test, immunofluorescence test, immunocytochemistry and in-situ hybridization. However, majority of the tests listed are laborious, time-consuming and are not really useful for clinical specimens (Frisk, 1999). Even though immunohistochemistry is a highly sensitive and specific method for the detection of CDV, they are prone to give false-negative results compared to nested-PCR and RT-PCR especially in subacute or chronic form of infections (Jozwik & Frymus, 2005). According to Frisk (1999), RT-PCR is a fast, sensitive and specific method for early detection of CDV in ante-mortem diagnosis. Therefore, nested-PCR

and RT-PCR is considered to be the gold standard for molecular assay for the detection of CDV.

2.5 Serological assay for the detection of Canine Distemper Virus

Serological assays are usually done for the detection of antibodies against canine distemper virus (CDV). Some of the most commonly used serological detection methods are enzyme-linked immunosorbent assay (ELISA) and viral neutralization test (VNT). ELISA is a relatively simple and rapid serological test and the type of ELISA commonly used is the indirect ELISA test for the detection of IgG antibody (Gemma, 1995). VNT is usually done for the diagnosis of CDV and the evaluation of vaccination efficacy. However, it is quite inefficient as it requires a reference virus, cell culture system and requires several days for the test. According to Gemma (1995), the sensitivity of ELISA test was higher than VNT. This makes ELISA a more preferable serological method compared to VNT other than the convenience of ELISA as the result can be read without an ELISA reader and the antigen-coated plates can easily be stored in -20 °C.

2.6 Prevention method

2.6.1 Controlling of Canine Distemper Virus on dog population

Vaccination is by far the best method to control the spread of CDV in dogs' population as they play important roles in reducing death rates, preventing clinical cases and controlling the spread of virus. It is recommended that a vaccine coverage of 95% of domesticated dogs is needed to control CDV in dogs' population. There are many types of vaccine available in the market and the most commonly found is Modified Live Virus (MLV) vaccine and Recombinant Canine Distemper Virus (rCDV) vaccine, both administered parenterally. These are included in the core vaccines that is highly suggested for every dogs. According to the WSAVA Canine Vaccination Guideline, the initial dose is to be given in a puppy starting from 6 to 8 weeks of age and then another dose every 2 to 4 weeks until 16 weeks or older. Adult vaccination is recommended to be done in two doses with 2 to 4 weeks interval according to the manufacturers, however, a single dose of

MLV or rCDV vaccine is considered to be protective. The booster is recommended at either 6 months or 1 year of age, and then not more often than every 3 years. The vaccines usually use hemagglutinin (H) and fusion (F) protein genes as they would induce serum neutralizing antibodies (Welter, 2000).

2.6.1.1 Immunity response of dogs on CDV vaccine

It is recommended to use MLV vaccines in a high-risk environment because of the rapid onset of immunity after vaccination (Larson & Schultz, 2006). According to Larson and Schultz (2006), rCDV vaccine provide almost a similar immunity as MLV vaccines therefore can be considered as a suitable alternative to MLV vaccine. A study was also done to compare the ability of rCDV and MLV vaccines to enhance the antibody response of a previously vaccinated dogs and the results are rCDV vaccine gives a more significant increase in antibody titer compared to MLV vaccines, thus, rCDV vaccine is a good choice to use as a booster vaccine.

However, there has been a reported case of vaccination-induced CDV infection in a fennec fox in 2020 according to Tamukai (2020). The fox was born at home, stayed indoors with no contact with other wildlife and carnivores and was vaccinated with multivalent MLV vaccines containing CDV. This shows that the vaccines' safety and efficacy among other species than dogs need to be well studied in the future.

2.6.2 Prevention of spill over between species

Canine distemper virus commonly affects dogs' population and also affects a wide range of carnivorous species such as the Canidae, Mustelidae, Procyonidae, Hyaenidae, Ursidae and Viveridae. Therefore, one of the methods of controlling the spread of CDV is by preventing the spill over of dogs near the carnivorous species in the wild. Currently, the best method for

breaking the circulation of CDV between susceptible wildlife population and domestic dogs is through regular vaccination and preventing them to roam freely and allowed to interact with unvaccinated dogs and wildlife (Kapil, 2011). The population of dogs continues to increase as the human population increase; therefore, they will cause more threat to the wildlife population as the human settlement is becoming closer to the wild. One of the methods is by controlling the number of feral dogs roaming around through trap-neuter and release programmes.

2.6.3 Controlling Canine Distemper Virus in tigers

Vaccination of endangered species that are susceptible to CDV, such as the Malayan tiger, is important to ensure their survivability of the species. However, the right vaccine to be used is still unknown to this date. In other wildlife species such as the black-footed ferret were once vaccinated with commercial Killed Vaccine (KV) and MLV vaccines, however, they are proven to be non-protective or fatal (Wimsatt, 2004). In the 1960s, KV vaccines were used to vaccinate wildlife and zoo animals, however, the VNT titer post-vaccination were generally quite low and several species had died from CDV outbreaks post-vaccination (Kapil, 2011). The use of MLV vaccines is often fatal to wildlife and zoo animals, thus, they have only been used in rare situations to control the disease in endangered species.

In the case of tigers, it is deemed worthy to try vaccinating the tiger population in order to protect it from CDV outbreak. However, it is hard to track down tigers in the wild, and it is almost impossible to capture every individual tiger for vaccination purpose as tigers are known for their aggressive behaviour. Oral bait vaccines are usually the choice of vaccination route for wild animals, however, oral vaccines against CDV are not yet available (Kapil, 2011). The two major issues in developing oral baits for CDV are achieving an adequate mucosal immune response in the gut and overcoming interference of maternal antibodies in infant animals. Studies shown that infant ferrets given vaccines intranasal and intramuscularly had 100% survival rates, but intraduodenal vaccines protected only 60% (Welter,

2000). It is also shown that parenteral vaccination in the presence of maternal antibodies did not protect the ferrets from CDV infections. Therefore, an efficacious CDV oral bait vaccine for wildlife and vigorous domestic dog vaccination programs will continue to be the primary means in controlling the disease.

3.0 MATERIALS AND METHODS

3.1 Population and sampling procedures

The samples were taken using simple random sampling method in two locations that have confirmed cases of CDV in Malayan tiger which were in Bukit Besi, Dungun, Terengganu and Jerantut, Pahang. The general population sample is consisting of domestic dogs and captured small wild mammals.

The wild mammals were captured using two sized traps, large and small, given to a villager in Dungun, Terengganu. The traps were placed in fruit orchards, forest fringes and in bushes near the forest. Among the eight wild mammals that were captured, two of them were palm civet, three squirrels and three rats.

3.1.1 Samples

The samples taken from the animals were serum, conjunctival and oronasal swabs. 1 ml of blood were taken via saphenous or cephalic vein for domestic dogs and via jugular, coccygeal, femoral, or intracardiac route for wild mammals depending on the size of the animal. The blood was stored and transported in a red plain blood tube for the collection of serum. The serum that was separated from the blood cells in the red blood tubes were collected and centrifuged to prevent blood cells from contaminating the serum. The conjunctival and oronasal swabs were taken using cotton swabs, which both were then stored in the same Viral Transport Media (VTM) as pooled sample and placed in an ice box immediately after collection.

3.2 Canine Distemper Virus Antigen Test Kit (RTK)

17 serum samples collected from domestic dogs in Jerantut,Pahang were used for the detection of antigen on site. The kit used was Anigen Rapid CDV Ag Test Kit (Bionote, South Korea), in which it is a chromatographic immunoassay for the qualitative detection of Canine Distemper Virus (CDV). The kit has a sensitivity of 98.8% and specificity of 97.7%. The procedure was done using the manual given in the test kit.

3.3 Reverse-transcriptase Polymerase Chain Reaction (RT-PCR) test

3.3.1 RNA extraction and reverse-transcription (RT)

The pooled samples in the viral transport media were used for RNA extraction for RT-PCR assay. The RNA extraction was done using NucleoSpin RNA Virus kit by Macherey-Nagel. The first step done was lysis of the viruses using the buffer given. After incubation, ethanol was added to adjust the binding conditions. The next step was to bind the viral RNA using the virus columns provided in the kit. Then, three wash techniques were done. The first wash was done to remove contaminants and PCR inhibitors, the second wash was when ethanolic buffer was added while the third wash was done to remove the ethanolic buffer completely. The viral RNA was then eluted using RNase-free water and 50 µl of the final product were obtained.

The reverse transcription was done using SensiFAST cDNA Synthesis Kit protocol where the whole procedure was done on ice. 10 µl of the RNA product was used and 4 µl of 5x TransAmp buffer, 1 µl reverse transcriptase and 5 µl DNase/RNase free-water was added into the mix. The primer annealing was done in 25 °C for 10 minutes, reverse transcription in 42 °C for 15 minutes and another 5 minutes in 85 °C for inactivation process. A total of 20 µl CDV cDNA product was obtained as the final product.

3.3.2 Amplification, cloning and sequencing of H gene

The CDV cDNA products were amplified with primer targeting H gene. The primer pair sequences were forward: 5'-TTC ATC CAA GCT GTC CTT AGT G -3'and reverse: 5'-GTG ATG TAC GGC CTC TGA TTT -3'. The procedure was done using Meridian Bioscience MyTaq Red Mix protocol. The PCR reaction was subjected to 35 cycles. Each cycle consists of denaturation at 94 °C for 1 minute, annealing at 49 °C for 1 minute and extension at 72 °C for 1 minutes. The amplified product was a 200- bp fragment. The positive control used was cDNA extracted from modified live vaccine and the negative control used was deionized water.

3.4 Commercial Immunochromatographic Assay (ICA) test

The serum samples were used for the detection of antibodies using the Vcheck CDV Ab Test Reagents (Bionote, Korea). The test kit is a chromatographic immunoassay as an in vitro diagnostic kit used for the quantitative detection of IgG to canine distemper virus (CDV) in serum or plasma samples. 5 µl of serum sample were taken and added into the assay diluent. The results were read using a V200 analyzer in which the test strips were placed inside the machine and 100 µl of the serum diluent were added into the sample hole. The result was displayed on the machine after 10 minutes. The result can be interpreted as negative if it is 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) or high titer 4-6 (above 1:64 as VN titer). However, this test was only done for serum samples collected from canine species as the test is specific for canine.

4.0 RESULT

The general sample were composed of 30 animals, 22 domestic dogs and 8 captured wild mammals. Samples from five dogs were taken in Dungun, Terengganu which were domesticated dogs owned by local villagers in Bukit Besi area. Another 17 samples taken from domesticated dogs owned by indigenous people in two villages, Kampung Sungai Retang and Kampung Sungai Tekai, where both of these villages were located near to Taman Negara, Pahang, where majority of Malayan tigers resided. All of the dogs sampled were owned domestically for protection and guarding purpose by indigenous people rather than being kept as pets.

Referring to table 1, a total of 30 samples was taken from 22 domestic dogs and 8 small wild mammals for CDV antigen rapid test kit, RT-PCR and chromatographic immunoassay (ICA). Out of 17 samples from domestic dogs in Jerantut, Pahang tested using CDV Ag RTK test, 100% of them were tested negative. The RT-PCR result for all 30 conjunctival and nasal swab samples were also negative for CDV antigen presence (refer to Figure 1-3).

Table 1: Summary of sampling done

Sampling location	***Species***	***No.of animals***	***Tests done***		
			RTK-antigen test	***ICA***	***RT-PCR***
Dungun, Terengganu	Canine	5		/	/
	Rat	3			/
	Squirrel	3			/
	Palm civet	2			/
Jerantut, Pahang	Canine	17	/	/	/
	Total sample	30	17	22	30

Figure 1: RT-PCR result for the samples with the ID DG12 to DG16 taken from 5 domestic dogs in Dungun,Terengganu targeting H gene with 200 bp product. All 5 samples were negative for CDV. Lane 1: DNA ladder (100bp ladder); lane 2: positive control; lane 3-7: samples; lane 8: negative control.

Figure 2: RT-PCR result for the samples with the ID JR1 to JR5 taken from 5 domestic dogs in Jerantut, Pahang targeting H gene with 200 bp product. All 5 samples were negative for CDV. Lane 1: DNA ladder (100bp ladder); lane 2: positive control; lane 3-7: samples; lane 8: negative control.

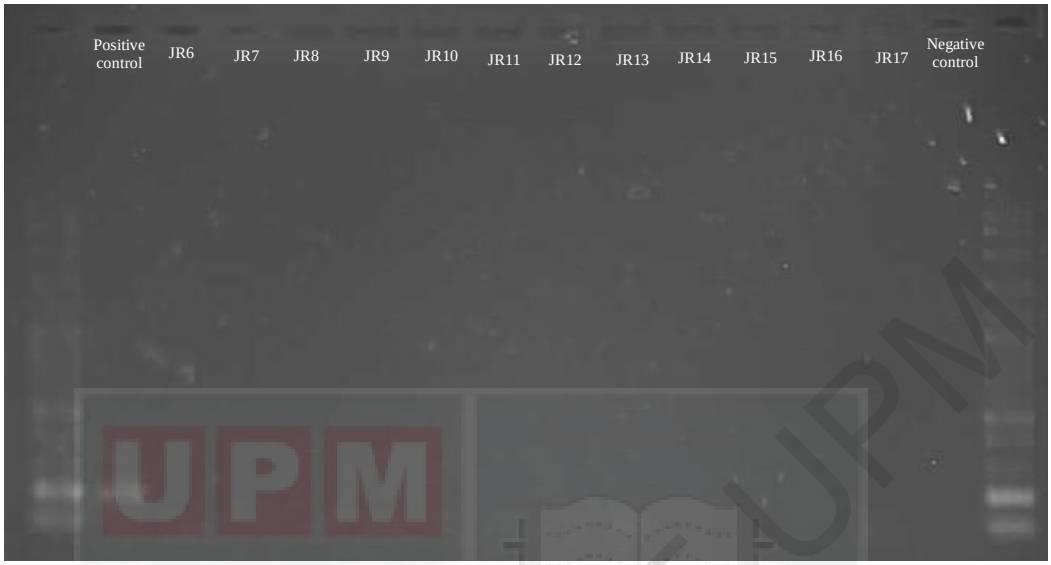

Figure 3: RT-PCR result for the samples with the ID JR6 to JR17 taken from 12 domestic dogs in Jerantut, Pahang targeting H gene with 200 bp product. All 12 samples were negative for CDV. Lane 1: DNA ladder (100bp ladder); lane 2: positive control; lane 3-14: samples; lane 15: negative control; final lane: DNA ladder (100bp ladder)

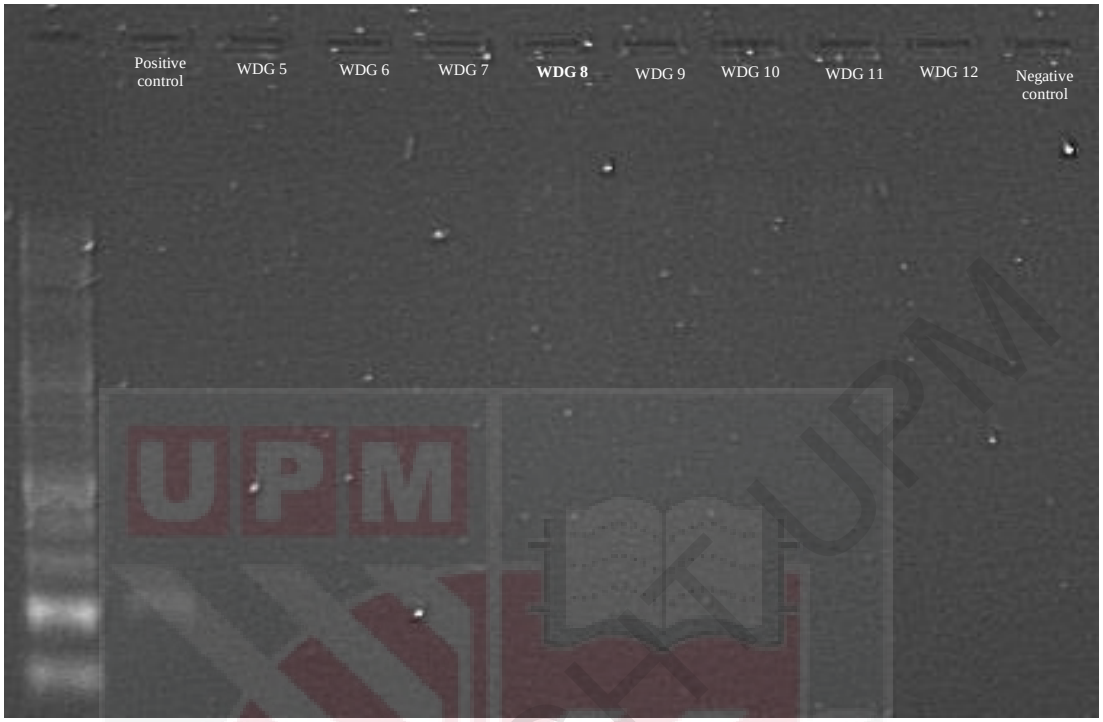

Figure 4: RT-PCR result for the samples with the ID WDG5 to WDG12 taken from 8 small wild mammals in Dungun, Terengganu targeting H gene with 200 bp product. All 8 samples were negative for CDV. Lane 1: DNA ladder (100bp ladder); lane 2: positive control; lane 3-10: samples; lane 11: negative control.

Based on table 2, out of 22 serum samples taken from the domestic dogs, 64% (14/22) of serum sample were tested positive while another 36% (8/22) were tested negative. Out of the 5 dogs sampled in Dungun, Terengganu, 60% (3/5) were tested positive while among the 17 dogs sampled in Jerantut, Pahang, 64.7% (11/17) were tested positive. Therefore, more than half of the population sampled were tested positive for both locations.

Table 2: Immunochromatographic assay result

Sampling location	*No. of dogs*	*Positive*	*Negative*
Dungun, Terengganu	5	3	2
Jerantut, Pahang	17	11	6
Total	22	14	8

Based on table 3, out of the 3 positive samples from Dungun, Terengganu, 2 were considered to have low IgG antibody titer and 1 had medium IgG antibody titer against CDV. Out of the 11 positive samples from Jerantut, Pahang, 4 of them has low IgG antibody titer and another 7 has medium IgG antibody titer against CDV.

Table 3: IgG titers in samples with positive ICA result

Sampling location	*No. of positive sample*	*Low titer 1-2*	*Medium Titer 3-3.5*	*High titer 4-6*
Dungun, Terengganu	3	2	1	-
Jerantut, Pahang	11	4	7	-
Total	14	6	8	0

Low titer = 1:8-1:16 VN titer; Medium titer = 1:32 – 1:48 VN titer; High titer = >1:64 VN titer

5.0 DISCUSSION

There were two tests done for the detection of antigen which were the CDV antigen rapid test kit and RT-PCR. Both of the tests resulted in 100% negative in all 30 samples, which gave the indication of current ongoing infection at the point of sampling for all animals sampled.

In this study, there were no positive result for the detection of antigen using CDV rapid test kit and RT-PCR. However, in the case of positive result for CDV antigen in any of the samples, identification of CDV strain should be done in order to compare the strain found in the samples with the strain found in the tiger that was found dead in 2019. This step should be done as a confirmation that the disease was transmitted to the tigers and the animals sampled are the reservoir for CDV.

For the detection of antibody, the test done was commercial immunochromatographic assay (ICA) for the detection of immunoglobulin G (IgG) in canine species. According to Winters et al. in 1983, the detection of IgG indicated recovery from previous infection. IgG may persist in the body for up to 3 years and the titers will be high in early post infection and decline over time. The incubation period for CDV is around 3 to 6 days and it will reach the central nervous system after 10 to 14 days. The virus will stay in the circulation for up to 3 weeks post infection. Since more than half of the dog population sampled were positive with low to medium titers with no history of vaccination, this may be a sign of past infection. This suggests a previous outbreak of the disease in the area leading to a probable reservoir of CDV within the area. This is a risk to the tiger population surrounding the area as the dogs were used by indigenous people as a measure of security when they were exploring the jungle. The

dogs could be transmitting the disease to tigers or other mammals in the jungle when they were brought into the jungle with active infection. Therefore, dogs could serve as the potential reservoir for transmission of CDV in tigers in Malaysia. However, since we did not get any positive RT-PCR samples, the possibility of transmission between dogs to Malayan tigers or *vice versa* could not be proven.

In addition, the serology test that was used in this study could not be adopted for other than canine, therefore, the status for CDV among wildlife in Malaysia was only investigated by RT-PCR. Even though there is no current infection among the sample taken from the wild mammals in Dungun, Terengganu, we cannot confirm that there were no previous infection among the animals. Since the sampling technique used was opportunistic sampling, where we only sample any animals that we manage to capture, the size of population sampled were also very small for us to relate the result with the whole wild mammal population. The wild animals sampled were also limited to small wild mammal because we do not have the expertise and equipment needed to catch larger mammals. Thus, the animals sampled may not be the reservoir of CDV for tigers as they may not be a common diet for tigers and does not linger around tiger habitat. However, the possibility of these animals to act as intermediate reservoir to transmit the disease to other wild carnivores that might be near the tiger habitat could not be excluded.

6.0 CONCLUSION AND RECOMMENDATION

In conclusion, although there is no current infection among the animals sampled at the point of sampling, the presence of antibody in more than half of the population sample is highly suggestive of recovery from previous infection. There is a high chance that the dogs are the reservoir of CDV for tigers, but there is also a possibility for small wild mammals or other carnivores to be the reservoirs although it cannot be proven in this study. Thus, more studies with a higher number of population and tests specific to wildlife species need to be done in order to gain more fact and knowledge regarding the status of CDV among wildlife species in Malaysia.

As a recommendation, vaccination against CDV among dogs' population surrounding well-known tiger habitat should be done in order to avoid CDV outbreaks to happen near tiger population. More research and trials on wildlife vaccination, most preferably oral vaccination should be done in order to make a safer and more convenient vaccination method for wildlife in Malaysia. Next, the spill over between wildlife and domestic dog species could be reduced by limiting the contact either by creating a buffer zone or by controlling domestic dog population. Lastly, more research needs to be done on CDV in other areas especially areas with confirmed tiger sightings in order to retrieve a more accurate data and input on the status of CDV in domestic dogs and wildlife population in Malaysia.

REFERENCES

Beineke, A., Puff, C., Seehusen, F. and Baumgärtner, W. (2009). Pathogenesis and immunopathology of systemic and nervous canine distemper. 127(1-2), 0–18.

Borah, J. and Pasha, M.K.S. (2021). Canine Distemper Virus (CDV) -The COVID-19 of the Wild Cats. Academia Letters, Article 3114.

Bruyette D.S (2020) Canine Distemper. Clinical Small Animal Internal Medicine (80).

Calderon, M.G., 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., 125(3-4), 341–349.

Costa, V.G.D, Saivish, M.V., Rodrigues, R.L., Lima Silva, R.F., Moreli, M.L., Krüger, R.H. and Samy, A.M, (2019). Molecular and serological surveys of canine distemper virus: A meta-analysis of cross-sectional studies. PLOS ONE, 14(5), e0217594–.

Day, M.J., Horzinek M. C., Schultz R.D. and Squires R.A. (2016). Guidelines for the vaccination of dogs and cats. Journal of Small Animal Practice, 57, 17-19.

Diallo, A. (1990). Morbillivirus group: genome organisation and proteins. Veterinary Microbiology, 23(1-4), 155–163.

Frisk, A.L., König, M., Moritz, A. and Baumgärtner, W. (1999) Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. J Clin Microbiol. 37(11):3634-43.

Gemma, T., Miyashita, N., Shin, Y.S., Okita, M., Mori, T., Iwatsuki, K., Mikami, T. and Kai, C. (1995) Serological survey of canine distemper virus infection using enzyme-linked immunosorbent assay. J Vet Med Sci, 57(4):761-3.

Jóźwik, A. and Frymus, T. (2005). Comparison of the Immunofluorescence Assay with RT-PCR and Nested PCR in the Diagnosis of Canine Distemper. 29(4), 347–359.

Kapil, S. and Yeary, T.J. (2011). Canine Distemper Spillover in Domestic Dogs from Urban Wildlife. 41(6), 0–1086.

Larson, L.J. and Schultz, R.D. (2006) Effect of vaccination with recombinant canine distemper virus vaccine immediately before exposure under shelter-like conditions. Vet Ther, 7(2):113-8.

Larson, L.J., Hageny, T.L., Haase, C.J. and Schultz, R.D. (2006) Effect of recombinant canine distemper vaccine on antibody titers in previously vaccinated dogs. Vet Ther, 7(2):107-12.

Martella, V., Elia,G. and Buonavoglia, C. (2008). Canine Distemper Virus., 38(4), 0–797.

Moehlman, P. D. (1983) Recent Advances in the Study of Mammalian Behavior. In: American Society of Mammalogists. Lawrence, K.A. (Ed) Eisenberg, J. F. & Kleiman, D. G. pp.423–438

Nagao, Y., Nishio, Y., Shiomoda, H., Tamaru, S., Shimojima, M., Goto, M., Une, Y., Sato, A., Ikebe, Y. and Maeda, K. (2012) An outbreak of canine distemper virus in tigers (Panthera tigris): possible transmission from wild animals to zoo animals. J Vet Med Sci Jun;74(6):699-705.

Roelke-Parker, M.E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S.J., Pospischil, A., Hofmann-Lehmann, R., Lutz, H., Mwamengele, G.L., Mgasa, M.N., Machange, G.A., Summers, B.A. and Appel, M.J. (1996) A canine distemper virus epidemic in Serengeti lions (Panthera leo). Nature, 1;379(6564):441-5.

Sourimant, J. and Plemper, R. (2016). Organization, Function, and Therapeutic Targeting of the Morbillivirus RNA-Dependent RNA Polymerase Complex. Viruses, 8(9), 251

Tamukai, K., Minami, S., Kurihara, R. et al. (2020) Molecular evidence for vaccine-induced canine distemper virus and canine adenovirus 2 coinfection in a fennec fox. Journal of Veterinary Diagnostic Investigation. 32(4):598-603.

Tipold, A., Vandevelde, M. and Jaggy, A. (1992), Neurological manifestations of canine distemper virus infection. Journal of Small Animal Practice, 33: 466-470

Vandevelde, M., Kristensen, B., Braund, K.G., Geene, C.E., Swango, L.J. and Hoerlein, B.F. (1980) Chronic canine distemper virus encephalitis in mature dogs. Vet Pathol, Jan;17(1):17-28

Von Messling, V., Zimmer, G., Herrler, G., Haas, L. and Cattaneo, R. (2001) The hemagglutinin of canine distemper virus determines tropism and cytopathogenicity. J Virol, Jul;75(14):6418-27

Welter, J., Taylor, J., Tartaglia, J., Paoletti, E. and Stephensen, C.B. (2000) Vaccination against canine distemper virus infection in infant ferrets with and without maternal antibody protection, using recombinant attenuated poxvirus vaccines. J Virol, 74(14):6358-67

Wimsatt, J., Biggins D.E. and Williams E.S. (2004) The quest for a safe and effective canine distemper virus vaccine for black-footed ferrets: Proceedings of the Symposium on the Status of the Black-footed Ferret and Its Habitat. In: Roelle J.E., editor. Recovery of the black-footed ferret: progress and continuing challenges. USGS; Fort Collins (CO): 2004. pp. 248–266.

APPENDIX

Domestic dog sample from Dungun, Terengganu and Jerantut, Pahang.

Sampling location	ID	Sex	Body Condition Score	Respiratory Symptom	Ocular abnormalities/discharge	Neurological symptoms
Kampung Besul, Bukit Besi, Dungun, Terengganu	DG12	M	3	N	N	N
	DG13	M	3	N	N	N
	DG14	M	3	N	N	N
	DG15	M	3	N	N	N
	DG16	M	3	N	N	N
Penempatan Orang Asli, Kampung Sungai Retang, Jerantut, Pahang	JR1	M	3	N	N	N
	JR2	M	3	N	N	N
	JR3	F	3	N	N	N
	JR4	M	3	N	N	N
	JR5	F	3	N	N	N
	JR6	F	3	N	N	N
	JR7	M	3	N	N	N
	JR8	M	3	N	N	N
	JR9	M	3	N	N	N
	JR10	M	3	N	N	N
	JR11	F	3	N	N	N

	JR12	F	3	N	N	N
	JR13	F	3	N	N	N
Penempatan Orang Asli, Kampung Sungai Tekai, Jerantut, Pahang	JR14	M	3	N	N	N
	JR15	F	3	N	N	N
	JR16	F	1.5	N	Y / discharge	N
	JR17	F	3	N	N	N

M=male, F=female, Y=yes, N=No

Wildlife sample from Dungun, Terengganu and Jerantut, Pahang

Sampling location	ID	Species	Sex	Weight (kg)	Body length (cm)
Kampung Besul, Bukit Besi, Dungun, Terengganu	WDG5	Rat	M		
	WDG6	Palm civet	M	2.9	
	WDG7	Palm civet	M	2.9	43
	WDG8	Rat	M		
	WDG9	Squirrel	M	0.4	4.4
	WDG10	Rat	M	0.1	31
	WDG11	Squirrel	M	0.4	42
	WDG12	Squirrel	F	0.4	43

CDV Antigen Rapid Test Kit (RTK) result on 17 dogs from Jerantut, Pahang

ID	Result
JR1	Negative
JR2	Negative
JR3	Negative
JR4	Negative
JR5	Negative
JR6	Negative
JR7	Negative
JR8	Negative
JR9	Negative
JR10	Negative
JR11	Negative
JR12	Negative
JR13	Negative
JR14	Negative
JR15	Negative
JR16	Negative
JR17	Negative

RT-PCR result on 30 dogs and wildlife samples from Dungun, Terengganu and Jerantut, Pahang

ID	Result
DG12	Negative
DG13	Negative
DG14	Negative
DG15	Negative
DG16	Negative
JR1	Negative
JR2	Negative
JR3	Negative
JR4	Negative
JR5	Negative
JR6	Negative
JR7	Negative
JR8	Negative
JR9	Negative
JR10	Negative
JR11	Negative
JR12	Negative
JR13	Negative
JR14	Negative
JR15	Negative
JR16	Negative
JR17	Negative
WDG5	Negative
WDG6	Negative
WDG7	Negative
WDG8	Negative
WDG9	Negative
WDG10	Negative
WDG11	Negative
WDG12	Negative

Immunochromatographic Assay (ICA) result on 22 domestic dogs from Dungun, Terengganu and Jerantut, Pahang

ID	Result	Titer	
DG12	Positive	Medium	3
DG13	Positive	Low	2
DG14	Negative		0
DG15	Positive	Low	1
DG16	Negative		0
JR1	Negative		0
JR2	Positive	Low	1
JR3	Negative		0
JR4	Positive	Medium	3
JR5	Positive	Medium	3
JR6	Negative		0
JR7	Negative		0
JR8	Positive	Medium	3.5
JR9	Positive	Medium	3
JR10	Negative		0
JR11	Positive	Medium	3
JR12	Positive	Low	1
JR13	Positive	Low	2
JR14	Negative		0
JR15	Positive	Low	1
JR16	Positive	Medium	3.5
JR17	Positive	Medium	3.5