UNIVERSITI PUTRA MALAYSIA

MOLECULAR SCREENING OF FELINE MORBILLIVIRUS.

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MOLECULAR SCREENING OF FELINE MORBILLIVIRUS.

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It is hereby certified that we have read this project paper entitled "Molecular Screening of Feline Morbillivirus", by Hemadevy Manoraj 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 – Project.

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DEDICATIONS

"You don't choose your family.
They are God's gift to you, as you are to them"
-Desmond Tutu

To my dearest siblings,
Dr. Thiban Raj Manoraj
and
Shandini Devy Manoraj,
for being there whenever I needed you.

To my dear and lovely parents,
DSP Manoraj Appookutty
and
Mrs. Rajeswary Palanyappan,
for making me who I am today.

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celcius
AAFP	American Association of Feline Practitioners
AAHA	American Animal Hospital Association
cDNA	complementary DNA
CDV	Canine Distemper Virus
C.I	confidence interval
CRFK	Crandell-Rees feline kidney
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FmoPV	Feline morbillivirus
FPV	Fakulti Perubatan Veterinar
IACUC	Institutional Animal Care and Use Committee
ID	Identification
IFA	indirect immunofluorescence assay
IgG	Immunoglobulin G
kb	kilobase
n	number of cats sampled
RNA	Ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
TIN	tubulointerstitial nephritis
UPM	Universiti Putra Malaysia
USG	urine specific gravity
UVH	Universiti Veterinary Hospital

ABSTRAK

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

SARINGAN MOLEKULAR MORBILLIVIRUS FELIN.

Oleh

HEMADEVY MANORAJ

2015

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Morbillivirus felin (FmoPV) adalah virus negatif RNA yang tergolong dalam keluarga *Paramyxoviridae*. FmoPV telah dikesan baru-baru ini pada kucing di Hong Kong dan Jepun, dan dihubungkaitkan dengan nefritis tubulointerstisial. Dalam kajian ini, sampel darah dan air kencing telah diambil daripada 35 kucing milik pelanggan yang dibawa ke Hospital Veterinar Universiti- Universiti Putra Malaysia (UVH-UPM) dan klinik veterinar swasta yang turut menyertai kajian. Kriteria pemilihan untuk kajian ini adalah termasuk kucing yang sihat, dan mempunyai penyakit buah pinggang/sistem urinari. Analisis serum urea dan kreatinin serta graviti khusus air kencing telah dijalankan untuk menilai status kucing samaada berpenyakit buah pinggang/sistem urinari. Saringan FmoPV dijalankan dengan menggunakan sampel yang telah dikumpul dan ujian reaksi rantai polimerase transkriptase membalik (RT-PCR) dua langkah konvensional untuk membesarkan sebahagian daripada urutan N-gen virus ini. Daripada 25 kucing yang diuji bagi sampel-sampel darah, 1 kucing (4.0%) telah disahkan positif FmoPV. Di samping itu, 17 daripada 27 kucing (63.0%) yang diuji untuk sampel air kencing adalah positif untuk FmoPV. Selain itu, daripada 17 kucing yang diuji untuk kedua-dua sampel darah dan air kencing, 1 kucing (5.9%) telah disahkan positif FmoPV. Prevalens FmoPV di Malaysia adalah 48.6%. Analisis chi square menunjukkan tiada hubungan yang signifikan antara kucing yang berpenyakit buah pinggang/sistem urinari dan FmoPV (P=0.11).

Kata kunci: Morbillivirus felin, kucing, berpenyakit buah pinggang/sistem urinari, prevalens, ujian RT-PCR konvensional.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project.

MOLECULAR SCREENING OF FELINE MORBILLIVIRUS.

By

HEMADEVY MANORAJ

2015

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Feline morbillivirus (FmoPV) is a negative-sense, single stranded RNA virus that belongs to the family *Paramyxoviridae*. The FmoPV has been recently detected in cats in Hong Kong and Japan, and is associated with tubulointerstitial nephritis. In this study, urine and blood samples were collected from 35 client-owned cats presented to the Universiti Veterinary Hospital- Universiti Putra Malaysia (UVH-UPM) and participating private veterinary clinics. Healthy and renal/urinary-diseased cats were included in this study. Serum urea-creatinine and urine specific gravity analyses were performed to assess the renal or urinary disease status in these cats. FmoPV screening of the collected samples were conducted using two-steps conventional reverse-transcriptase polymerase chain (RT-PCR) assay amplifying a part of N-gene sequence of the virus. Of the 25 cats screened for the blood samples, 1 cat (4.0%) was tested positive for FmoPV. In addition, 17 out of 27 cats (63.0%) screened for urine samples were tested positive for FmoPV. From 17 cats screened for both blood and urine samples, 1 cat (5.9%) was tested positive for FmoPV. The prevalence of FmoPV in Malaysia is 48.6%. Chi square analysis revealed no significant association between renal/urinary diseased cats and FmoPV (P=0.11).

Key words: Feline morbillivirus, cats, renal/urinary-diseased, prevalence, conventional RT-PCR assay.

1.0 INTRODUCTION

Feline morbillivirus (FmoPV) is a negative-sense, single stranded RNA virus that belongs to the family *Paramyxoviridae*. Paramyxoviruses are divided into two subfamilies, which are *Paramyxovirinae* and *Pneumovirinae*. There are seven genera classified under the subfamily Paramyxovirinae besides the *Morbillivirus*, namely *Respirovirus*, *Rubulavirus*, *Henipavirus*, *Ferlavirus*, *Aquaparamyxovirus* and *Avulavirus* (Audsley& Moseley, 2013). FmoPV was first isolated and characterized in 2012 in Hong Kong (Woo *et al.*, 2012). The FmoPV was later detected in Japan with similar pattern of phylogenetic analysis that has been done previously in Hong Kong. The sequence from Japan isolates are similar to the Hong Kong isolates, with 92.0-94.0% nucleotide sequence identity (Furuya *et al*., 2013). According to Park *et al.*, (2014), the FmoPV genomes encodes eight types of structural and non-structural proteins, which are the N, P/V/C, M, F, H and L proteins. The L gene of the FmoPV was identified and sequenced in the study conducted in Hong Kong (Woo *et al.*, 2012).

FmoPV is related to the tubulointerstitial nephrits (TIN) in domestic cats which involves primary injury to the renal and eventually leads to renal failure. The virus is assumed to be involved in renal pathologic process as FmoPV was mostly detected in the urine samples (Woo *et al.*, 2012). However, a study that was conducted in Japan to isolate and characterize the FmoPV in domestic cats revealed that there was no association between the FmoPV infection and TIN (Sakaguchi *et al.*, 2014).

In Malaysia, no prevalence study has been conducted to detect the presence of FmoPV infection. In addition, it would be particularly interesting to determine the association between FmoPV and renal/urinary system diseases.

Thus, the objectives of this study include:

1. To detect the presence of feline morbillivirus in Malaysia.
2. To determine the prevalence of feline morbillivirus in Malaysia.
3. To determine the association between renal/urinary system diseases and feline morbillivirus.

The hypotheses for this study were:

1. Feline Morbillivirus is prevalent in cats in Malaysia.
2. There is a significant association between renal/urinary system diseases and feline morbillivirus.

2.0 LITERATURE REVIEW

2.1 Paramyxoviruses

Paramyxoviruses are negative sense and single stranded RNA viruses of 13-19 kb in size. The family *Paramyxoviridae* are further divided into subfamilies, which are the *Paramyxovirinae* and *Pneumovirinae* (MacLachlan & Dubovi, 2011). There are seven genera classified into the subfamily *Paramyxovirinae*, namely *Respirovirus, Rubulavirus, Morbillivirus, Henipavirus, Ferlavirus, Aquaparamyxovirus* and *Avulavirus* (Audsley & Moseley, 2013). The genome of viruses in this subfamily encodes 9 to 12 types of protein; N, P, L, M, F and H that sometimes overlap within the phosphoprotein P. The paramyxovirus replicates in the cytoplasm of the infected cells and continuation of the replication is ensured with the presence of actinomycin D (MacLachlan & Dubovi, 2011).

2.2 Morbillivirus

Morbilliviruses has been associated with significant diseases in both wildlife and domesticated animals besides humans. Morbillivirus infection was reported affecting a wider range of animal host which includes the large felids, seals and dolphins (MacLachlan & Dubovi, 2011). Quigley *et al.*, (2010) documented the first case of morbillivirus infection in a wild free ranging Siberian Tiger. The examples of diseases listed under the genera *Morbillivirus* are measles in humans, rinderpest in small ruminants, and canine distemper in dogs and large felids (de Vries *et al.*, 2015). Canine distemper has been often diagnosed in dogs and animals in the family Canidae (Sakai *et al.*, 2013). Recently, the canine distemper virus has transmitted to other species of animals such as the ferrets, raccoons, seals and long-tailed macaque. Canine distemper is one of the causes of fatal disease in many species of carnivores (Barrett, 1999).

2.3 Diagnostic assay available for morbillivirus

Standard serological techniques, virus isolation, or antigen detection tests are usually used to diagnose morbillivirus infections (MacLachlan & Dubovi, 2011). Morbillivirus are made up of single strand of RNA, hence the viral RNA must be converted using reverse transcriptase into DNA before proceeding further to PCR assay. Therefore, two steps reaction that is known as reverse transcription/polymerase chain reaction (RT-PCR) should be conducted (Barrett, 1999). Furthermore, morbillivirus can be characterized at the genetic level by doing sequence analysis of the DNA product obtained from RT-PCR (Barrett, 1999). The sensitivity, specificity and rapidity of RT-PCR compared to other conventional methods makes this assay as a first choice diagnostic test (Elia *et al.*, 2006). The disadvantage of this method is it is not suitable for decomposed tissue samples. Histopathological examination and immunostaining on fresh tissue sample allows diagnoses of morbillivirus infections for different types of species (Kameo *et al.*, 2012; Woo *et al.*, 2012).

2.4 Feline Morbillivirus (FmoPV)

The absence of morbillivirus infection in domestic cats triggered a large screening study in Hong Kong to identify previously unrecognized morbillivirus in cats (Woo *et al.*, 2012). This lead to the first molecular epidemiology study and FmoPV was discovered and isolated in the stray cats' population in Hong Kong. Following this report, the FmoPV was then detected in Japan (Furuya *et al.*, 2013: Sakaguchi *et al.*, 2014).

The genome of FmoPV encodes eight structural and non-structural protein which includes N, P/V/C, M, F, H and L proteins. The complete genome size of FmoPV is the largest among the morbilliviruses, which is 16, 050 bases (Park *et al.*, 2014). The FmoPV was first detected using urine, rectal, oral swabs and blood samples from stray cats in Hong Kong. The viral RNA obtained from these samples were used to detect the FmoPV RT-PCR while viral culture of urine sample was done using the Crandell-Rees feline kidney (CRFK) and VeroE6 (African green monkey kidney) cells. Besides that, the antibodies against N protein of FmoPV was also detected using Western Blot analysis and histopathologic examination of necropsy kidney tissue that were FmoPV positive samples (Woo *et al.*, 2012).

2.5 Association between tubulointerstitial nephritis (TIN) and FmoPV

Woo *et al.*, (2012) discovered aggregates of inflammatory cells and tubular degeneration in kidneys in cats confirmed with FmoPV using histological examination. Cauxin expression was detected in the degenerated tubular epithelial cells. These findings are compatible to TIN. Furthermore, immunohistochemistry staining performed on the same organs using the guinea pig serum positive for anti-FmoPV N protein antibody revealed localization of FmoPV in renal tubular cells. Further investigation by Woo *et al.,* (2012) revealed that there were a positive association between TIN and FmoPV infection using RT-PCR assay on kidney, plasma and urine samples from 27 stray cats. Since FmoPV was detected mostly in urine samples, it was hypothesized that FmoPV is associated with renal pathologic process (Woo *et al.*, 2012).

On contrary, a similar study was conducted in Japan to isolate and characterize the FmoPV in a population of domestic cats. However, no clear relationship was found between FmoPV infection and nephritis (Sakaguchi *et al.*, 2014). Besides RT-PCR, this group of investigator isolated FmoPV from urine samples using the CRFK cells inoculation. In addition, indirect immunofluorescence assay (IFA) was also carried out to detect the viral antigens and immunoblot analysis to detect the antibodies against FmoPV. Their results from immunoblot analysis shown that some cats infected with FmoPV may or may not have detectable antibodies and it was presumed that each cats elicit antibodies against FmoPV in different ways.

Furthermore, based on the phylogenetic analyses by Sakaguchi *et al.*, (2014), it was proposed that there might be different antigenicity of each strain of FmoPV. Besides that, they also detected cross-reactivity between FmoPV and canine distemper virus (CDV) as anti-CDV dog sera responded to FmoPV antigens in their study. This may be due to the polyclonal antibodies used that was not specific and re-evaluation of anti-FmoPV antibodies was suggested for future studies. In addition, Sakaguchi *et al.,* (2014) suggested an epidemiological study which includes larger number of cats with experimental infection to make any significant conclusions on FmoPV infection besides determining the association between TIN and FmoPV.

2.6 Detection rate of FmoPV in Hong Kong and Japan using RT-PCR

FmoPV was detected in various samples from both Hong Kong and Japan. In Hong Kong, the RNA of FmoPV was detected in 53 urine samples, 4 rectal swabs and 1 blood sample out of 457 stray cats with the detection rate of

FmoPV using RT-PCR 12.0%, 0.88% and 0.22%, respectively. Two cats were positive for both urine and rectal swabs (Woo *et al.,* 2012).

In Japan, FmoPV RNA was detected in both blood and urine samples (Furuya *et al.,* 2013). Five out of 82 urine samples were tested positive for FmoPV while 1 out of 10 blood samples was tested for FmoPV with a detection rate 6.1% and 10.0%, respectively (Furuya *et al.,* 2013).

3.0 MATERIALS AND METHODS

3.1 Animals

Thirty-five cats presented to the Universiti Veterinary Hospital-Universiti Putra Malaysia (UVH-UPM), Serdang and participating private clinics were sampled in this study. Of the 35 cats, 22 healthy cats (63.0%) were presented for castration, ovariohysterectomy, regular check-up or vaccination, while 13 renal/urinary system diseased cats (37.0%) were presented for feline lower urinary tract disease or choric kidney disease. Institutional Animal Care and Use Committee (IACUC) approval was obtained before carrying out this project (FYP- 2014/FPV.011). In addition to that, client consent was also obtained before collecting samples from each cat (Appendix 8.1).

3.2 Inclusion criteria

Serum urea-creatinine analysis and urine specific gravity reading was obtained as sample inclusion criteria of this study. All the cats were further sub-grouped into 2 categories as followings; (i) healthy cats - cats with normal serum urea level (reference range, 3.0 – 10.0 mmol/L), normal serum creatinine level (reference range, 60 – 193 µmol/L) and normal urine specific gravity (USG) (reference range of >1.035) and (ii) renal/urinary system diseased cats with elevated serum urea >10.0 mmol/L, elevated serum creatinine >193 µmol/L and reduced urine specific gravity <1.035.

Information from each cats recruited into the study were obtained from the patient's file retrieved. Information such as patient signalment and any previous history of renal/urinary system problems were filled into the patient information form (Appendix 8.2)

3.3 Sample collection

Blood samples of 1.0 mL were collected into each EDTA and plain tubes (Vacutest®, Italy) using 23G or 25G needles (B.Braun®, Germany) and 1 mL (B.Braun®, Germany), or 3 mL syringes (Terumo®, Phillipines) via jugular or saphenous venipuncture technique. Urine samples of 5.0 – 10.0 mL were collected into urine collection container via manual compression technique or cystocentesis. Blood and urine were sampled for serum urea creatinine and urine specific gravity reading respectively. The remaining blood and urine samples were then subjected for viral RNA extraction and two-step conventional RT-PCR. All samples were kept on ice before processing.

3.4 Serum urea-creatinine analysis and urine specific gravity

The blood collected in the plain tube was centrifuged using centrifuge machine (Hettich Zentrifugen, Germany) and the serum was obtained. The serum was then collected in a micro centrifuge tube and sent to the Haematology and Clinical Biochemistry Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) for the serum urea-creatinine analysis. The urine specific gravity reading (USG) was determined using a refractomer (Atago T2-NE Clinical, Japan).

3.5 Viral RNA extraction for blood and urine samples

The whole blood from the EDTA tube was used for the viral RNA extraction using QIAamp® RNA Blood Mini Kit (Germany) as instructed by the manufacturer (Appendix 8.3). The machines used throughout these process were vortex mixer (Snijder Tilburg, Holland), centrifuge machine (Hettich Zentrifugen, Germany).

The urine sample was first centrifuged (Hettich Zentrifugen, Germany) to obtain the supernatant. A 1mL RNAlater® was mixed to 5 mL supernatant and vortex mixed (Snijder Tilburg, Holland). The viral RNA extraction was then carried out using the QIAamp® Viral RNA Mini Kit (Germany) as instructed by the manufacturer (Appendix 8.4). Other machine used for these processes includes benchtop micro centrifuge (HettichMikro 20, Germany).

3.6 RNA Quantification

Viral RNA was later quantified using the Eppendorf BioSpectrometer® (Germany) and Eppendorf µCuvette (Germany) to obtain the concentration before subjected to the RT-PCR.

3.7 Two-steps conventional reverse transcriptase polymerase chain reaction (RT-PCR)

The first step of the RT-PCR was to convert the RNA to cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, United Kingdom) as instructed by the manufacturer (Appendix 8.5). The second step of the RT-PCR was to amplify the DNA using MyTaq™ Mix (Bioline, United Kingdom) as instructed by the manufacturer (Appendix 8.6). The machines used for both these steps were benchtop micro centrifuge (Eppendorf, Germany) and thermal cycler (C1000 Touch™ Thermal Cycler, California).

3.8 Agarose Gel Electrophoresis

The product from the second step of the RT-PCR was used to run the Agarose Gel Electrophoresis using 1.5% Agarose gel and Bio-Rad PowerPac 300 (USA). This step was done to separate the DNA according to the length of the base pairs The DNA fragments were then visualized using the Bio-Rad Gel Doc™ (USA).

3.9 Statistical Analysis

The statistical analysis was performed using the GraphPad Prism® software (GraphPad Software Inc, USA). The association between diseases of the renal/urinary system and detection of feline morbillivirus was analyzed using the Chi-square test, with 95% confidence interval (95% C.I)

4.0 RESULTS

4.1 Descriptive data of the sample size

4.1.1 Age

The cats sampled in this study were categorized into the age groups based on American Association of Feline Practitioners/American Animal Hospital (AFFP-AAHA) Feline Life Stage Guidelines (Appendix 8.7). Majority of the cats recruited are the junior cats (*n*=15), followed by the prime, senior, mature, kitten and geriatric categories. The age range of cats sampled in this study was between 6 months old to 18 years old with an average age of 4.7 years old as seen in Table 1.

Table 1: Age categories of the cats sampled

Categories	**Age**	**No. of cats sampled (*n*)**
Kitten	Birth to 6 months old	1
Junior	7 month to 2 years old	15
Prime	3 years to 6 years old	9
Mature	7 years to 10 years old	4
Senior	11 years to 14 years old	5
Geriatric	15 years old and above	1

4.1.2 Sex

The data of sex categories of this study was retrieved and tabulated in Table 2. Twenty-two male cats and 13 female cats were recruited. Out of the 22 male cats, 8 were castrated while 14 were intact male cats. Four out of the 13 females cats were spayed 9 were intact female cats.

Table 2: Sex categories of the cats sampled

Categories	No. of cats sampled (*n*)
Intact male	14
Castrated male	8
Intact female	9
Spayed female	4

4.1.3 Type of management

The data of the management of cats sampled in this study was categorized and tabulated in Table 3. Thirty cats were managed indoor while five were semi-roamers.

Table 3: Type of management of the cats sampled

Management	No. of cats sampled (*n*)
Indoor	30
Semi-roamer	5

4.2 Healthy and renal/urinary system diseased cats

Based on the inclusion criteria and sub-grouping set in this study, out of 35 cats, twenty-two (63.0%) cats were healthy and thirteen cats (37.0%) were with diseases related to the renal/urinary systems.

Table 4: Healthy and renal/urinary diseased cats

Categories	**No. of cats sampled (*n*)**	**Percentage (%)**
Healthy	22	63.0%
Renal/urinary diseased cats	13	37.0%

4.3 Detection rate of FmoPV in blood samples (*n*=25 cats)

The detection of FmoPV in blood samples in this study was found to be 4.0% whereby 1 cat out of 25 cats sampled was positive for FmoPV.

Table 5: Detection rate of FmoPV in blood samples by using RT-PCR

Categories	**No. of cats sampled (*n*)**	**Detection rate in blood samples (%)**
FmoPV positive	1	4.0%
FmoPV negative	24	96.0%

4.4 Detection rate of FmoPV in urine samples (*n*=27 cats)

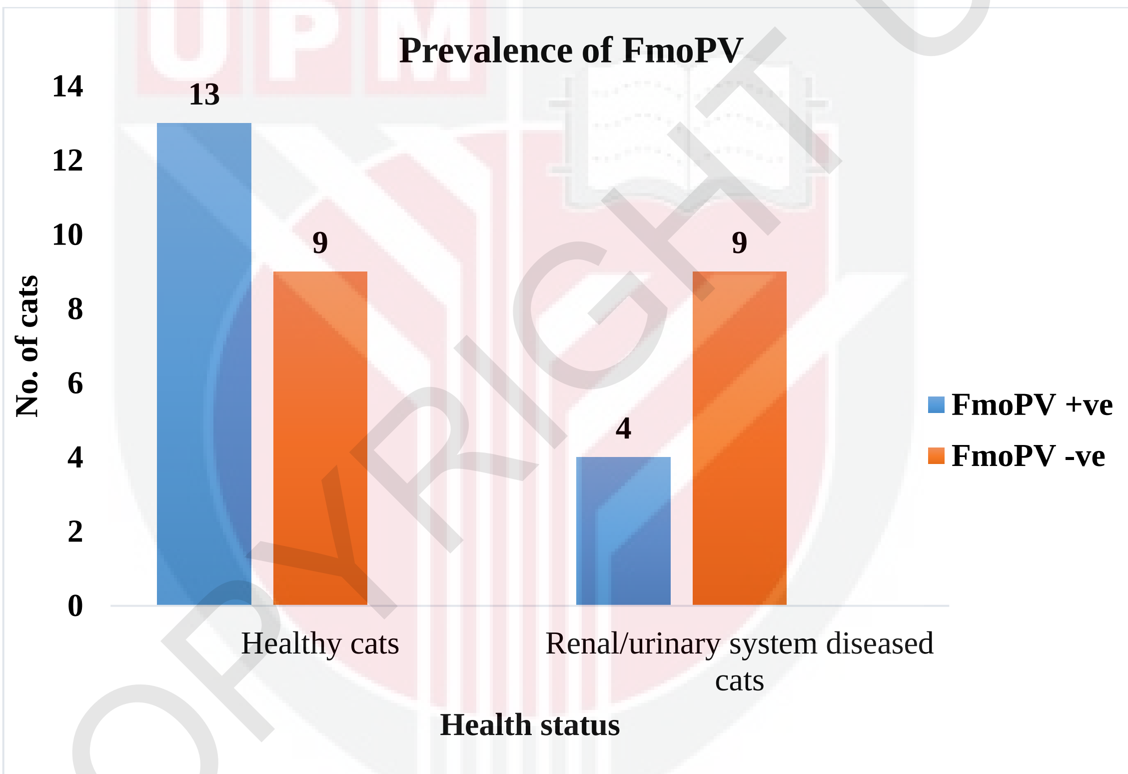
The detection of FmoPV in urine samples in this study was found to be 63.0% with 17 out of 27 cats sampled were positive for FmoPV.

Table 6: Detection rate of FmoPV in urine samples by using RT-PCR

Categories	**No. of cats sampled (*n*)**	**Detection rate in urine samples (%)**
FmoPV positive	17	63.0%
FmoPV negative	10	37.0%

4.5 Prevalence of FmoPV

The prevalence of FmoPV in this study was 48.6% with 17 out of 35 cats sampled detected positive for FmoPV as seen in Figure 1.



4.6 Association between renal/urinary system diseases and FmoPV

Chi-square analysis was done and the data was tabulated in Table 7. Based on the Chi-square analysis, there was no association (P=0.11) between presence of renal/urinary system disease with detection of morbillivirus in this cohort of cats.

Table 7: Data analysis to obtain the association between renal/urinary system diseases and FmoPV

Data Analyzed	**FmoPV positive**	**FmoPV negative**	**Total**	**P value**	**Odds Ratio**	**95% Confidence Interval of the Difference**	
						Lower	Upper
Healthy cats	13	9	22	0.11	3.250	0.7602	13.89
Renal/urinary system diseased cats	4	9	13				
Total	17	18	35				

5.0 DISCUSSION

5.1 Detection rate of feline morbillivirus

The detection rate of FmoPV was found to be 4.0% in the blood and 63.0% in urine sample. The result of our study is consistent with the study done by Woo and company in 2012 where detection of FmoPV is higher in urine samples compared to the blood samples (Woo *et al.*, 2012).

In contrast with study done by Furuya and co-researchers in 2013, the detection of FmoPV in blood samples was higher than the urine samples. However, the sample size (*n*=10) was smaller than the present study which would possibly give a higher detection rate in that study.

5.2 Prevalence rate of feline morbillivirus

The prevalence of feline morbillivirus in this study is 48.6%. In comparison with other reported study, the prevalence of FmoPV in Hong Kong was 12.3% (Woo *et al.*, 2012) and in Japan was 6.5% (Furuya *et al.*, 2013). Based on these results obtained in our study, the prevalence of FmoPV was higher most probably due to sample selection whereby the cats in this study was grouped into healthy and renal/urinary system diseases while random sampling was done in both Hong Kong and Japan. Besides that, the geographical location of our sampling area was restricted to one state/region while the sample collection of the other studies involved a much larger geographical location involving different regions (Sakaguchi *et al.*, 2014).

5.3 Association between renal/urinary system diseases and feline morbillivirus

In this study, it can be concluded that there was no significant association between presence renal/urinary system diseases and detection of morbillivirus in cats. However, the study conducted by Woo *et al.,* (2012) shown that there was significant association between tubulointerstitial nephritis and feline morbillivirus. This is based on ($P<0.05$) by Zar, (1999). Sakaguchi *et al.*, (2014) reported that no clear relationship was found between FmoPV infection and nephritis which was determined by detection of FmoPV RNA in urine and/or anti-FmoPV antibodies. The classification of cats with diseases related to the renal/urinary system in this cohort was based on both serum urea/creatinine and USG analysis which did not accurately reflect the current pathological state of a particular cat. Therefore, histopathological examination of kidneys of the FmoPV positive cats is recommended to determine the pathological state of a FmoPV positive cat and the association with tubulointerstitial nephritis. Furthermore, an epidemiological study with larger sample size from various locations besides Selangor is recommended in further study to understand the association between renal/urinary system diseases and FmoPV. Other cats such as the pet cats and the stray cats can be sampled to determine the actual prevalence of FmoPV in Malaysia.

In this study, the detection rate of FmoPV in healthy cats was higher compared to those with concurrent renal/urinary system-diseases. Since the epidemiology and the direct pathological consequences of this virus is unknown, further study should be carried out to determine the association between renal/urinary system diseases and FmoPV.

In 2011, MacLachlan & Dubovi stated that standard serological; antigen detection tests and virus isolation were usually done to detect morbillivirus infections. Test kits for common morbillivirus infection such as for the CDV are available in the market (Appendix 8.8). This test kit would decrease the time consumption and work load in detecting the virus unlike the standard serological or virus culture in the laboratory. Further study should be carried out to understand the pathogenesis and the antigen-antibodies which may help to produce test kits for detection of FmoPV antigen.

According to Buczkowski *et al.*, (2014) vaccines have been available for other diseases caused by morbillivirus such as the measles, rinderpest, canine distemper and peste des petits ruminats. Additional development of vaccines will aid the disruption of circulation of these morbilliviruses and even eradicate them in the near future as vaccination could be the best way to fight against morbilliviruses infection (de Vries *et al.*, 2015).

6.0 CONCLUSIONS AND RECOMMENDATIONS

The detection rate of feline morbillivirus in Malaysia is 4.0% in blood samples and 63.0% in urine samples. Since the sample size is quite small and do not include other factors in the inclusion criteria such as the type of management of the cats, a larger sampling size with more inclusion criteria could reflect a better detection rate in a larger cat population.

The prevalence of feline morbillivirus in Malaysia is 48.6%. This prevalence is higher compared to the other studies conducted. Sampling collection were from cats in a smaller region of the Klang Valley. Therefore, a larger sampling size and area will cover more cats with different backgrounds (i.e. pet and stray cats) to determine the true prevalence rate of feline morbillivirus in Malaysia.

Lastly, we could conclude from this study that there was no significant association between the renal/urinary system diseases and feline morbillivirus. Besides that, phylogenetic analysis is recommended to compare the local isolates with the isolates found in other studies to determine the origin and relationship of our local isolates. In addition, virus culture and isolation of our local FmoPV should be conducted to maintain the viral RNA stock found in our local cats.

7.0 REFERENCES

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8.0 APPENDICES

8.1 Client Consent Forms (English and Bahasa Melayu)

FAKULTI PERUBATAN VETERINAR
UNIVERSITI PUTRA MALAYSIA

Borang Persetujuan Pelanggan
Tajuk Kajian: Saringan Molekul 'Feline Morbillivirus'

Kami menjemput anda untuk mengambil bahagian dalam satu kajian untuk mengesan kehadiran Morbillivirus kucing; spesies virus yang telah baru-baru ini ditemui dan diasingkan pada kucing domestik di Hong Kong dan Jepun. Kajian ini juga menunjukkan bahawa terdapat hubungan yang signifikan antara morbillivirus kucing dan penyakit buah pinggang kronik (CKD). Kajian ini akan membantu kami untuk mengesan kehadiran morbillivirus kucing di kucing tempatan dan persatuan virus dengan CKD. Selain itu, kajian ini juga akan meningkatkan pemahaman kami tentang virus itu sendiri dan profil molekul dari kucing yang morbillivirus positif. Kajian ini diawasi oleh doktor haiwan kami sendiri, Dr. Farina Mustaffa Kamal dan Dr. Khor Kuan Hua.

Untuk menjalankan kajian ini, kami hanya memerlukan sejumlah kecil darah dan air kencing dari kucing anda. Semua sampel akan dikumpul oleh doktor haiwan berdaftar yang berpengalaman. Setiap langkah akan diambil untuk memastikan bahawa pengumpulan darah dan air kencing akan dilakukan dengan stress yang minima untuk haiwan kesayangan anda.

Jika anda membuat keputusan untuk mengambil bahagian dalam projek ini, kami menawarkan satu ujian serum kreatinin urea-analisis percuma, yang bernilai RM16.00.

Kami memastikan bahawa semua maklumat peribadi yang diperolehi adalah sulit.

Terima kasih atas penyertaan anda bagi menjayakan kajian ini.

Saya dengan ini memberi kebenaran untuk mengambil darah dan air kencing daripada haiwan kesayangan saya. Jika anda ingin dimaklumkan mengenai hasil kajian kami, sila berikan kami e-mel dan nombor telefon anda:

Nama Pelanggan: ____________________ Nama Haiwan: _______________ No. Kes: ______

Tandatangan: ___________
Alamat e-mel: _____________
Tarikh: _________

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8.1 Continue

FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA
Client Consent Form
Research Title: Molecular Screening of Feline Morbillivirus

We invite you to participate in a study to detect the presence of Feline Morbillivirus; a virus species that has been recently discovered and isolated in domestic cats in Hong Kong and Japan. The study also revealed that there was a significant association between feline morbillivirus and chronic kidney disease (CKD). This research will help us to detect the presence of the feline morbillivirus in our local cats and the association of the virus with CKD. Besides, this research will also increase our understanding of the virus itself and the molecular profile of feline morbillivirus-positive cats. This research is closely supervised by our very own veterinarians, Dr. Farina Mustaffa Kamal and Dr. Khor Kuan Hua.

In order to conduct this study, we only require a very small amount of blood and urine samples from your cat(s). All samples will be collected by an experienced registered veterinarian. Every measure will be taken to ensure that the process of blood and urine collection will be done as stress-free as possible for your cat(s).

If you decide to participate in this project, we are offering a free serum urea-creatinine analysis, which is worth RM16.00.

We ensure that all personal information obtained will be held confidential.

Thank you for your participation and helping us.I hereby give my consent for blood and urine collection from my cat(s). If you would like to be informed of the results of the study, please provide us your email and contact number.

Owner's Name: ______________________ Pet's name: ________________ Case No: ______

Signature: ____________
Email address: ______________________
Date: ___________

8.2 Patient information form

MOLECULAR SCREENING OF FELINE MORBILLIVIRUS

PATIENT INFORMATION FORM

CASE NO: LOCATION:

ID: AGE:

SEX: BREED:

DATE: WEIGHT:

VACCINATION: DEWORMING:

OWNER'S NAME:

OWNER'S CONTACT NO:

MANAGEMENT: INDOOR OUTDOOR SEMI ROAMER MULTICAT HOUSEHOLD OTHER: ___________

HEALTH STATUS: HEALTHY CKD OUTPATIENT CKD INPATIENT AKI/FLUTD

HISTORY AND CLINICAL SIGNS:

DIAGNOSIS:

RESULTS:

1) PCR (BLOOD) :
2) PCR (URINE) :
3) SERUM UREA-CREATININE :
4) USG:

8.3 Instructions for QIAamp® RNA Blood Mini Kit

Procedure

Blood

1. **Mix 1 volume of human whole blood with 5 volumes of Buffer EL in an appropriately sized tube (not provided).**

   For optimal results, the volume of the mixture (blood + Buffer EL) should not exceed 3/4 of the volume of the tube to allow efficient mixing. For example, add 5 ml of Buffer EL to 1 ml of whole blood, and mix in a tube which has a total volume of ≥8 ml.

   Note: Use an appropriate amount of whole blood. Up to 1.5 ml of healthy blood (typically 4000–7000 leukocytes per microliter) can be processed. Reduce amount appropriately if blood with elevated numbers of leukocytes is used. (In this case, also adjust amount of Buffer RLT in step 6.)

2. **Incubate for 10–15 min on ice. Mix by vortexing briefly 2 times during incubation.**

   The cloudy suspension becomes translucent during incubation, indicating lysis of erythrocytes. If necessary, incubation time can be extended to 20 min.

3. **Centrifuge at 400 x *g* for 10 min at 4°C, and completely remove and discard supernatant.**

   Leukocytes will form a pellet after centrifugation. Ensure supernatant is completely removed. Trace amounts of erythrocytes, which give the pellet a red tint, will be eliminated in the following wash step. See page 32 if larger amounts of erythrocytes remain.

4. **Add Buffer EL to the cell pellet (use 2 volumes of Buffer EL per volume of whole blood used in step 1). Resuspend cells by vortexing briefly.**

   For example, add 2 ml of Buffer EL per 1 ml of whole blood used in step 1.

5. **Centrifuge at 400 x *g* for 10 min at 4°C, and completely remove and discard supernatant.**

   Note: Incomplete removal of the supernatant will interfere with lysis and subsequent binding of RNA to the QIAamp spin column, resulting in lower yield.

6. **Add Buffer RLT to pelleted leukocytes according to the table below. Vortex or pipet to mix.**

   When not using healthy blood, refer to number of leukocytes to determine the volume of Buffer RLT required. Buffer RLT disrupts the cells. No cell clumps should be visible before you proceed to the homogenization step. Vortex or pipet further to remove any clumps.

Buffer RLT* (µl)	Healthy whole blood (ml)	No. of leukocytes
350	Up to 0.5	Up to 2×10^6
600	0.5 to 1.5	2×10^6 to 1×10^7

* Ensure β-ME is added to Buffer RLT (see "Things to do before starting").

8.3 Continue

Blood

7. **Pipet lysate directly into a QIAshredder spin column in a 2 ml collection tube (provided) and centrifuge for 2 min at maximum speed to homogenize. Discard QIAshredder spin column and save homogenized lysate.**

   To avoid aerosol formation, adjust pipet to ≥750 µl to ensure that the lysate can be added to the QIAshredder spin column in a single step.

   If too many cells have been used, after homogenization the lysate will be too viscous to pipet. If this is the case, please refer to page 32.

8. **Add 1 volume (350 µl or 600 µl) of 70% ethanol to the homogenized lysate and mix by pipetting. Do not centrifuge.**

   A precipitate may form after the addition of ethanol. This will not affect the QIAamp procedure.

9. **Carefully pipet sample, including any precipitate which may have formed, into a new QIAamp spin column in a 2 ml collection tube (provided) without moistening the rim. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Maximum loading volume is 700 µl. If the volume of the sample exceeds 700 µl, successively load aliquots onto the QIAamp spin column and centrifuge as above.**

   Discard flow-through* and collection tube.

   Optional: If performing optional on-column DNase digestion (see "DNase treatment", page 41), follow steps D1–D4 (page 42) after performing this step.

10. **Transfer the QIAamp spin column into a new 2 ml collection tube (provided). Apply 700 µl Buffer RW1 to the QIAamp spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash.**

    Discard flow-through* and collection tube.

11. **Place QIAamp spin column in a new 2 ml collection tube (provided). Pipet 500 µl of Buffer RPE into the QIAamp spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm).**

    Discard flow-through* and collection tube.

    Note: Ensure ethanol is added to Buffer RPE (see "Things to do before starting").

12. **Carefully open the QIAamp spin column and add 500 µl of Buffer RPE. Close the cap and centrifuge at full speed (20,000 x *g*, 14,000 rpm) for 3 min.**

    Note: Some centrifuge rotors may distort slightly upon deceleration, resulting in flow-through, containing Buffer RPE, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

* Flow-through contains Buffer RW1 or RLT and is therefore incompatible with bleach. See page 6 for safety information.

8.3 Continue

13. Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

    This step helps to eliminate the chance of possible Buffer RPE carryover.

14. Transfer QIAamp spin column into a 1.5 ml microcentrifuge tube (provided) and pipet 30–50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute. Repeat if >0.5 ml whole blood (or >2 x 10^6 leukocytes) has been processed.

Blood

QIAamp RNA Blood Mini Handbook 04/2010 21

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8.4 Instructions for QIAamp® Viral RNA Mini Kit

Protocol: Purification of Viral RNA (Spin Protocol)

Spin Protocol

This protocol is for purification of viral RNA from 140 µl plasma, serum, urine, cell-culture media, or cell-free body fluids using a microcentrifuge. For automated purification of viral RNA using the QIAamp Viral RNA Mini Kit on the QIAcube, refer to the *QIAcube User Manual* and the relevant protocol sheet.

Larger starting volumes, up to 560 µl (in multiples of 140 µl), can be processed by increasing the initial volumes proportionally and loading the QIAamp Mini column multiple times, as described below in the protocol. Some samples with very low viral titers should be concentrated before the purification procedure; see "Protocol: Sample Concentration" (page 30).

Alternatively, larger sample volumes can be processed using one of the following kits, which provide simultaneous purification of viral DNA and RNA.

- QIAamp MinElute® Spin Kit* 200 µl
- QIAamp MinElute Vacuum Kit 500 µl
- QIAamp UltraSens® Virus Kit 1000 µl

Important points before starting

- Read "Important Notes" (pages 15–22) before starting the protocol.
- All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

- Equilibrate samples to room temperature (15–25°C).
- Equilibrate Buffer AVE to room temperature for elution in step 11.
- Check that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on page 17.
- Add carrier RNA reconstituted in Buffer AVE to Buffer AVL according to instructions on page 15.

Procedure

1. **Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.**

   If the sample volume is larger than 140 µl, increase the amount of Buffer AVL–carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL–carrier RNA) and use a larger tube.

* Fully automatable on the QIAcube. See www.qiagen.com/MyQIAcube for protocols.

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8.4 Continue

Spin Protocol

2. **Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.**

   To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3. **Incubate at room temperature (15–25°C) for 10 min.**

   Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA. Potentially infectious agents and RNases are inactivated in Buffer AVL.

4. **Briefly centrifuge the tube to remove drops from the inside of the lid.**

5. **Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.**

   Only ethanol should be used since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl of ethanol). In order to ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. **Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.**

   Close each spin column in order to avoid cross-contamination during centrifugation.

   Centrifugation is performed at 6000 x *g* (8000 rpm) in order to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. **Carefully open the QIAamp Mini column, and repeat step 6.**

   If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

8. **Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.**

   It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

8.4 Continue

9. **Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate any chance of possible Buffer AW2 carryover, perform step 10, and then continue with step 11.**

   Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing Buffer AW2, contacting the QIAamp Mini column. Removing the QIAamp Mini column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Mini column. In these cases, the optional step 10 should be performed.

10. **Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

11. **Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x *g* (8000 rpm) for 1 min.**

    A single elution with 60 µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double elution using 2 x 40 µl of Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate.

    Viral RNA is stable for up to one year when stored at –20°C or –70°C.

Spin Protocol

8.5 Instructions for SensiFAST™ cDNA Synthesis Kit

SensiFAST™ cDNA Synthesis Kit

Shipping: On dry/blue ice

Catalog numbers

BIO-65053: 50 reactions

Batch No.: See vial

BIO-65054: 250 reactions

BIOLINE

A Meridian Life Science® Company

Store at –20°C

Storage and stability:
SensiFAST cDNA Synthesis Kit is shipped on dry/blue ice and should be stored at -20°C upon receipt. When stored under optimum conditions, the reagents are stable for a minimum of one year from date of purchase.

Unit definitions:
Reverse Transcriptase: One unit catalyzes the incorporation of 1nmol of dTTP into acid-insoluble material in 10 minutes at 37°C in 50mM Tris-HCl, pH 8.6, 40mM KCl, 1mM $MnSO_4$, 1mM DTT, and 0.5mM [3H]TTP, using 200µM oligo(dT)$_{12-18}$-primed poly(A)$_n$ as template.

RNase Inhibitor: One unit inhibits 5ng of RNase A by 50%.

Safety precautions:
Harmful if swallowed. Irritating to eyes, respiratory system and skin. Please refer to the material safety data sheet for information regarding hazards and safe handling practice.

Signal word: WARNING

Description

SensiFAST cDNA Synthesis Kit provides a rapid and very sensitive method for first strand cDNA synthesis for use in real-time PCR studies. The 5x TransAmp™ Buffer provides highly optimized components for efficient reverse transcription, and includes a unique blend of anchored oligo dT and random hexamer primers to ensure unbiased 3' and 5' coverage for enhanced data accuracy. An extremely efficient reverse transcriptase delivers highly robust first strand synthesis and higher cDNA yields from a wide range of input RNA concentrations. SensiFAST cDNA Synthesis Kit offers enhanced sensitivity, efficiency and reproducibility for exceptional performance in subsequent real-time PCR experiments.

Components

Product Name	50 reactions	250 reactions
5x TransAmp Buffer	200µl	1ml
Reverse Transcriptase	50µl	250µl

SensiFAST cDNA Synthesis Mix Reaction Guidelines

Template Quality

- Intact, high-quality RNA is essential for the reverse transcription reaction
- All reagents for use with RNA must be prepared using nuclease-free, molecular biology grade water
- RiboSafe RNase Inhibitor is included in the SensiFAST cDNA Synthesis Kit to help reduce template degradation and increase yield of RT-qPCR product
- Low-copy-number genes may require an increase in starting material
- Use a suitable RNA extraction reagent e.g. TRIsure™ or ISOLATE™ II RNA Mini Kit

RNA Priming
A unique blend of random hexamer and anchored oligo dT primers in the SensiFAST cDNA Synthesis Mix provides optimal sensitivity and accuracy of first-strand cDNA synthesis. Anchored oligo dT primers anneal precisely to the junction of the poly-A tail (found on the 3' end of most eukaryotic mRNAs) and the gene of interest. This ensures that the coding 3' end of mRNAs are always represented. The reverse transcriptase can also prime from the random hexamers, to give broad coverage of all the regions of the RNA and thus a cDNA pool representative of the transcriptome. The combined benefits of both priming strategies offers enhanced data quality.

Reverse Transcription
Efficient reverse transcription can normally be achieved at 42°C for 15 minutes, as the TransAmp Buffer contains reverse transcriptase enhancers that reduce complex RNA secondary structure. For templates that have a high degree of structure, such as viral RNA and some plant RNA, we suggest using an additional 15 minute 48°C incubation step.

No RT Control
It is important to always include the appropriate 'no RT' or 'minus RT' control reactions in your experimental design. As the reverse transcriptase is a separate component of the SensiFAST cDNA Synthesis Kit, it is possible to include a formal cDNA synthesis control that includes all components except the reverse transcriptase.

DNase I digestion of total RNA
To eliminate any residual contaminating genomic DNA that can affect highly sensitive real-time PCR applications (e.g. probe-based quantification of a low abundant target), we recommend using a high quality RNase-free DNase I during or after RNA extraction protocols. DNase I removal by ethanol precipitation, or with a RNA clean-up kit e.g. ISOLATE II RNA Micro Clean-Up Kit is required before proceeding with first-strand cDNA synthesis.

SensiFAST cDNA Synthesis Kit Protocol

1. Prepare the mastermix on ice.
2. Vortex solutions and centrifuge briefly before use.

Total RNA or mRNA (up to 1µg)	nµl
5x TransAmp Buffer	4µl
Reverse Transcriptase	1µl
DNase/RNase free-water*	Up to 20µl

* Available separately (see Associated Products)

3. Mix gently by pipetting.
4. Set up the following program in a thermal cycler:
   - 25°C for 10 min (primer annealing)
   - 42°C for 15 min (reverse transcription)
   - *Optional Step: 48°C for 15 min (for highly-structured RNA)*
   - 85°C for 5 min (inactivation)
   - 4°C hold (or chill on ice)
5. Use 4µl (1/5th volume) of cDNA synthesis reaction product in a 20µl volume real-time PCR.
6. Alternatively, store reaction product at 4°C for 1 week or -20°C for long term storage. If desired, reaction product can be diluted in 10mM Tris-HCl (pH 8.0), 0.1mM EDTA and stored at -20°C.

This protocol is intended for use as a guide only; conditions will vary from reaction to reaction and may need optimization.

PI-50476 V4

Website: www.bioline.com/ email: info@bioline.com

8.6 Instructions for MyTaq™ Mix

MyTaq™ Mix

Shipping: On dry/blue ice

Catalog numbers:

BIO-25041: 200 x 50µl reactions: 4 x 1.25ml

Batch No.: See vial

BIO-25042: 1000 x 50µl reactions: 20 x 1.25ml

Concentration: 2x

Store at –20°C

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Storage and stability:
MyTaq Mix is shipped on dry/blue ice. On arrival store at -20°C for optimum stability. Repeated freeze/thaw cycles should be avoided.

Expiry:
When stored under the recommended conditions and handled correctly, full activity of the kit is retained until the expiry date on the outer box label.

Safety precautions:
Please refer to the material safety data sheet for further information.

Quality control specifications:
Bioline operates under ISO 9001 Management System. MyTaq Mix and its components are extensively tested for activity, processivity, efficiency, sensitivity, absence of nuclease contamination and absence of nucleic acid contamination prior to release.

Notes:
Research use only.

Description

MyTaq™ Mix is a ready-to-use 2x mix developed for fast and highly-specific PCR. The advanced formulation of MyTaq Mix exhibits more robust amplification than other commonly used polymerases, delivering a very high yield over a wide range of PCR templates, and making it the ideal choice for most routine assays. MyTaq Mix contains all the reagents (including stabilizers) necessary for trouble-free PCR set-up. The product is conveniently supplied all in one tube, reducing the number of pipetting steps and facilitating increased efficiency, throughput and reproducibility.

Components

	200 Reactions	1000 Reactions
MyTaq Mix, 2x	4 x 1.25ml	20 x 1.25ml

Standard MyTaq Mix Protocol

The following protocol is for a standard 50µl reaction and can be used as a starting point for reaction optimization.

PCR reaction set-up:
All reactions must be set-up on ice.

Template	200ng
Primers (20µM each)	1µl
MyTaq Mix, 2x	25µl
Water (ddH_2O)	up to 50µl

PCR cycling conditions
We suggest these conditions in the first instance.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	1min	1
Denaturation	95°C	15s	25-35
Annealing*	User determined	15s	
Extension*	72°C	10s	

* These parameters may require optimization, please refer to the Important Considerations and PCR Optimization section if needed.

Important Considerations and PCR Optimization

The optimal conditions will vary from reaction to reaction and are dependent on the template/primers used.

Primers: Forward and reverse primers are generally used at the final concentration of 0.2-0.6µM each. As a starting point we recommend using 0.4µM as a final concentration (*i.e.* 20pmol of each primer per 50µl reaction volume). Too high a primer concentration can reduce the specificity of priming, resulting in non-specific products.

When designing primers we recommend using primer-design software such as Primer3 (http://frodo.wi.mit.edu/primer3) or visual OMP™ (http://dnasoftware.com) with monovalent and divalent cation concentrations of 10mM and 3mM respectively. Primers should have a melting temperature (Tm) of approximately 60°C

Template: The amount of template in the reaction depends mainly on the type of DNA used. For templates with low structural complexity, such as plasmid DNA, we recommend using 50pg-10ng DNA per 50µl reaction volume. For eukaryotic genomic DNA, we recommend a starting amount of 200ng DNA per 50µl reaction, this can be varied between 5ng-500ng. It is important to avoid using template resuspended in EDTA-containing solutions (*e.g.* TE buffer) since EDTA chelates free Mg^{2+}.

Initial denaturation: An initial denaturation step of 1min at 95°C is recommended for non-complex templates such as plasmid DNA or cDNA. For more complex templates such as eukaryotic genomic DNA, longer initial denaturation times of up to 3mins are required in order to facilitate complete melting of the DNA.

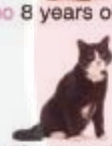
Denaturation: Our protocol recommends a 15s cycling denaturation step at 95°C which is also suited to GC-rich templates, however for low GC content (40-45%) templates, the denaturation time can be decreased to 5s.

Annealing temperature and time: The optimal annealing temperature is dependent upon the primer sequences and is usually 2-5°C below the lower Tm of the pair. We recommend running a temperature gradient to determine the optimal annealing temperature, alternatively 55°C can be used as a starting point. Depending on the reaction the annealing time can also be reduced to 5s.

PI-50155 V3

Website: www.bioline.com/ email: info@bioline.com

8.7 AFFP-AAHA Feline Life Stage Guidelines

	Life stage	Age of cat	Human equivalent
 Tigger 3 months old	Kitten birth to 6 months	0 – 1 month	0 – 1 year
		2 – 3 months	2 – 4 years
		4 months	6 – 8 years
		6 months	10 years
 Sugar 13 months old	Junior 7 months to 2 years	7 months	12 years
		12 months	15 years
		18 months	21 years
		2 years	24 years
 Rosie 3 years old	Prime 3 years to 6 years	3	28
		4	32
		5	36
		6	40
 Nemo 8 years old	Mature 7 years to 10 years	7	44
		8	48
		9	52
		10	56
 George 13 years old	Senior 11 years to 14 years	11	60
		12	64
		13	68
		14	72
 Chinarose 16 years old	Geriatric 15 years+	15	76
		16	80
		17	84
		18	88
		19	92
		20	96
		21	100
		22	104
		23	108
		24	112
		25	116

Source: American Animal Hospital Association (AAHA), 2010

8.8 Anigen Rapid CDV Ag Test Kit

One-step Canine Distemper virus Antigen Test

Anigen Rapid CDV Ag Test Kit

■ **Principles**

The Anigen Rapid CDV Ag Test Kit is a chromatographic immunoassay for the qualitative detection of Canine Distemper virus antigen in conjunctiva, urine, serum or plasma.

The Anigen Rapid Canine Distemper virus Ag Test Kit has a letter of "T" and "C" as test line and control line on the surface of the device. Both the test line and control line in result window are not visible before applying any samples. The control line is used for procedural control. Control line should be always appeared. If the test procedure is performed properly and the test reagents of control line are working. A purple test line will be visible in the result window if there is enough Canine Distemper virus antigens in the specimen.

The specially selected Canine Distemper virus antibodies are used in test band as both capture and detector materials. These enable the Anigen Rapid CDV Ag Test Kit to identify Canine Distemper virus antigen in conjunctiva, urine, serum or plasma with a high degree of accuracy.

■ **Materials provided (10 tests/kit)**

1) Ten(10) Anigen Rapid CDV Ag Test Kit Devices
2) Ten(10) Specimen tubes containing assay diluent buffer
3) Ten(10) Sample collection swabs
4) Ten(10) Disposable droppers
5) One(1) Instruction for use

■ **Precautions**

1) For veterinary diagnostic use only.
2) For best results, strict adherence to there instructions is required.
3) All specimens should be handled as being potentially infectious.
4) Do not open or remove test kit from their individually sealed pouches until immediately before their use.
5) Do not use the test kit if the pouch is damaged or the seal is broken.
6) Do not reuse test kit.
7) All reagents must be at room temperature before running the assay.
8) Do not use reagents beyond the stated expiration date marked on the label.
9) Do not mix components from different lot numbers. The components in this kit have been quality control tested as standard batch unit.

■ **Storage and Stability**

The kit can be stored at room temperature(2~30℃) or refrigerated. The test kit is stable through the expiration date marked on the package label. DO NOT FREEZE. Do not store the test kit in direct sunlight.

■ **Specimen Collection and Preparation**

1) The test should be performed using the canine secretion of the conjunctiva, urine, serum or plasma.
2) After collecting the specimen using swab, the specimen should be immediately extracted and tested.
3) If specimens are not immediately tested, they should be refrigerated at 2~8℃, for storage not less than 48 hours, freeze the specimen at -20℃ or below.

■ **Procedure of the test**

1) Collect the samples from conjunctiva or urine using the sample collection swab pre-wetted with saline solution. In case of serum or plasma samples, you can use the dropper.
2) Insert the swab into the specimen tube containing 300ul of assay diluent.
3) In case of serum or plasma samples, add 2-3 drops of the serum or plasma into the specimen tube containing 300ul of assay diluent using the dropper.
4) Mix the swab samples with assay diluent to extract well.
5) Remove the test device from the foil pouch, and place it on a flat and dry surface.
6) Add four (4) drops of the mixed sample into the sample hole using the dropper, drop by drop and slowly
7) As the test begins to work, you will see purple color move across the result window in the center of the test device. If the migration has not appeared after 1 minute, add one more drop of the mixed sample to the sample well.
8) Interpret test results at 5-10 minutes.

[Figure for test procedures]

■ **Interpretation of the test**

A color band will appear in the left section of the result window to show that the test is working properly. This band is the control band. The right section of the result window indicates the test results. If another color band appears in the right section of the result window. This band is the test band.

1) Negative result

The presence of only one band within the result window indicates a negative result.

2) Positive result

The presence of two color bands ("T" and "C") within the result window, no matter which band appears first indicates a positive result.

3) Invalid Result

If the purple color band is not visible within the result window after performing the test, the result is considered invalid. The directions may not have been followed correctly or the test may have deteriorated. It is recommended that the specimen be re-tested.

■ **Limitations of the test**

Although the Anigen Rapid Canine Distemper virus Ag Test kit is very accurate in detecting Canine Distemper virus antigen, a low incidence of false results can occur. Other clinically available tests are required if questionable results are obtained. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the veterinarian after all clinical and laboratory findings have been evaluated.

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8.9 Patient Details

No.	Patient ID	Sex	Age	Management	USG	Serum Urea	Serum creatinine	FmoPV Blood	FmoPV Urine	Diagnosis
1.	Sugar	1yo	F	Indoor	-	6.6	156	Negative	-	Healthy
2.	Jade	6mo	F	Indoor	1.035	7.4	112	Negative	Positive	Healthy
3.	Oreo	1yo	M	Indoor	1.037	9.3	168	Negative	Negative	Obstructive FLUTD
4.	Labu	1yo	CM	Indoor	1.034	9.3	128	Negative	Negative	Obstructive FLUTD
5.	Crystal	13yo	CM	Indoor	1.037	9.4	141	Negative	Positive	Healthy
6.	Mamiko	8yo	F	Semi-roamer	-	4.8	90	Negative	-	Healthy
7.	Cumi	1yo	M	Indoor	1.028	11.6	140	-	Positive	Healthy
8.	Sasha	8mo	F	Indoor	1.055	7.0	124	-	Positive	Healthy
9.	Yulia	8mo	F	Indoor	1.046	7.5	141	Negative	Positive	Healthy
10.	Tarzan	7mo	M	Indoor	1.051	5.8	127	Negative	Positive	Healthy
11.	Baby	6yo	F	Indoor	1.012	90.9	1062	Negative	Positive	CKD Stage IV
12.	Ziggi	18yo	CM	Indoor	1.021	14.5	328	Negative	Positive	CKD Stage III
13.	Ortiz	2yo	M	Indoor	1.036	14.9	150	Negative	Positive	Non-obstructive FLUTD
14.	Bubu	2yo	M	Indoor	1.050	8.9	139	-	Positive	Healthy
15.	Snow	2yo	M	Indoor	1.020	6.1	91	-	Positive	Healthy
16.	Mummy	14yo	SF	Indoor	1.036	11.2	183	-	Positive	Healthy
17.	Lucky	11yo	SF	Indoor	1.034	13.2	118	Negative	Negative	Healthy
18.	Tommy	11yo	CM	Indoor	-	11.7	110	Negative	-	Healthy
19.	Chiki	7yo	F	Indoor	-	8.3	60	Negative	-	Healthy

20.	Teddy	2yo	CM	Indoor	1.030	-	-	-	Negative	Obstructive FLUTD
21.	Kuttiama	3yo	SF	Indoor	1.058	11.0	139	Negative	Negative	Healthy
22.	Simba	11mo	M	Semi-roamer	1.038	14.0	128	Negative	Positive	Healthy
23.	Adik	3yo	CM	Indoor	1.040	10.4	196	Positive	Positive	CKD Stage II
24.	Blur	7yo	M	Indoor	1.023	74.1	996	Negative	Negative	CKD Stage IV
25.	Qaseh	5yo	CM	Indoor	1.021	41.9	1219	Negative	Negative	Obstructive FLUTD
26.	Henry	3yo	M	Indoor	-	8.4	180	Negative	-	Healthy
27.	Bonnie	3yo	SF	Indoor	-	9.9	148	Negative	-	Healthy
28.	Garfield	2yo	M	Semi-roamer	1.018	56.4	887	Negative	Negative	Obstructive FLUTD
29.	Acing	7yo	M	Indoor	1.014	37.8	294	-	Negative	CKD Stage III
30.	Hanny	14yo	F	Indoor	-	13.2	277	Negative	-	CKD Stage III
31.	Melly	2yo	F	Indoor	-	8.0	115	Negative	-	Healthy
32.	Momo	4yo	M	Indoor	1.026	13.4	141	Negative	Positive	Healthy
33.	Gentle Giant	4yo	CM	Semi-roamer	1.051	7.0	101	Negative	Positive	Healthy
34.	Lulu	1yo	M	Indoor	1.024	-	-	-	Negative	Obstructive FLUTD
35.	Kuning	3yo	M	Semi-roamer	1.051	8.2	87	Negative	Positive	Healthy

mo: months old
yo: years old
M: Male
F: Female

CM: Castrated male
SF: Spayed female

USG: Urine Specific Gravity

FmoPV: Feline morbillivirus
CKD: Chronic Kidney Disease

FLUTD: Feline Lower Urinary Tract Disease